Endogenous and microbial modulators of inflammation

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For Zaida

On the mountains of truth, you can never climb in vain: either you will reach a point higher up today, or you will be training your powers so that you will be able to climb higher tomorrow. – Nietzsche

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ABSTRACT

Cardiometabolic disease is characterized by dysregulated, chronic inflammation, that may result from impaired resolution pathways or alterations in the gut microbiota. Thus, restoring specialized pro-resolving mediators (SPMs) or intestinal bacteria with immunomodulatory properties represent potential therapeutic strategies for patients.

First, we investigated the ability of the SPMs, lipoxins, to modulate neutrophils from individuals with atherosclerosis. Treatment of neutrophils from patients with atherosclerosis with lipoxins attenuated elevated reactive oxygen species production and expression of the high-affinity conformation of CD11b/18 integrin, and enhanced lymphatic migration. The potential therapeutic effect of lipoxins in atherosclerosis was demonstrated, along with the need to tailor treatment to the requirements of the individual.

Second, for the development of a next-generation probiotic supplementation, we co-isolated *Faecalibacterium prausnitzii* with *Desulfovibrio piger* and demonstrated a cross-feeding mechanism that enhanced growth and butyrate production. *F. prausnitzii* is a highly prevalent and abundant human gut bacteria with immunomodulatory properties and associations with health. For development into a next-generation probiotic, *F. prausnitzii* was adapted to tolerate exposure to oxygen. *F. prausnitzii* and *D. piger* formulation was well tolerated by mice and humans. We demonstrated a method by which strict anaerobic bacteria can be adapted to tolerate oxygen without impacting potential beneficial properties.

Finally, *D. piger* has been found to be both ubiquitous among individuals but has also been associated with disease. To identify if the discrepancies lie in inter-strain variation, we isolated a *D. piger* strain with genomic similarity to FI11049, previously isolated from a patient with colitis, to compare with the strain co-isolated with *F. prausnitzii*. Anti-inflammatory properties and phenotypic differences were found between the strains. Further studies are required to investigate inter-strain variation to understand disease associations.

Keywords: Inflammation, resolution, specialized pro-resolving mediators, lipoxins, neutrophils, cardiometabolic disease, next-generation probiotic, oxygen-tolerance, *Faecalibacterium prausnitzii*, *Desulfovibrio piger*, inter-strain variation

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Sammanfattning på Svenska

Inflammation hjälper kroppen att bekämpa infektioner och reparera skadad vävnad, och är därför viktigt för vår överlevnad. När en individ drabbas av en skada utlöses en inflammatorisk kaskad av molekylära signaler av de skadade cellerna. Signalerna gör att immunceller, som bidrar till kroppens försvar mot mikrober, toxiner och skadade celler, rekryteras från blodcirkulationen. Vid inflammation vidgas blodkärlen som svar på proinflammatoriska molekyler, vilket möjliggör ett ökat flöde av syrerikt blod till det skadade området. Infiltration av immunceller och ökad vaskulär permeabilitet leder till vätskeansamling i vävnaden, vilket resulterar i svullnad. De första immuncellerna som kommer till det inflammerade området är neutrofiler. Neutrofilerna bekämpar mikrober med en bred arsenal av vapen och rensar upp skadade celler. När bakterierna eller de skadade cellerna är borta påbörjas en process där ämnen som dämpar inflammationen frisätts. Dessa ämnen signalerar att vävnaden ska repareras och att neutrofilerna ska ätas upp av andra immunceller eller föras bort från platsen via lymfkärlen. Lymfkärlen transporterar celler, molekyler och vätska från de perifera vävnaderna tillbaka till blodcirkulationen. Vid kroniska sjukdomar, som ateroskleros eller diabetes, är de inflammatoriska processerna kroniskt aktiverade. Detta leder till förhöjda nivåer av proinflammatoriska ämnen i blodet och aktivering av immunceller, vilket i sin tur leder till skador på frisk vävnad. Lipoxiner är en grupp naturligt förekommande lipider som dämpar inflammatoriska processer. Vi visar att neutrofiler från individer med ateroskleros är mer aktiverade än neutrofiler från friska kontrollpersoner och att de svarar på lipoxinbehandling genom att sänka sin proinflammatoriska aktivitet.

Tarmens mikrobiota bidrar till att forma vårt immunförsvar genom flera olika mekanismer. Förändringar i tarmfloran förekommer hos personer med kardiometaboliska sjukdomar, t.ex. ateroskleros och typ 2-diabetes. *Faecalibacterium prausnitzii* är en av de vanligaste bakterierna i våra tarmar. Den har immunmodulerande egenskaper och man har visat att personer med kardiometabola sjukdomar har låga *F. prausnitzii* nivåer. Detta indikerar att behandling med *F. prausnitzii* kan ha terapeutisk potential. Vi isolerade *F. prausnitzii* från en frisk individ tillsammans med en stam av

bakterien *Desulfovibrio piger*. Vi visade att de båda bakterierna bidrar till varandras tillväxt och till ökad produktion av den immunmodulerande metaboliten butyrat. *F. prausnitzii* är extremt syrekänslig och dör snabbt när den utsätts för syre. För att göra det möjligt att använda levande *F. prausnitzii* i kosttillskott skapade vi, genom stegvis adaptation, en ny stam med ökad syretolerans. Vi visade att odling av den nya stammen tillsammans med *D. piger* ger hög bakterietäthet, vilket möjliggör produktion i industriell skala. Genom studier i möss och människor visade vi att konsumtion av de två bakterierna är säkert.

D. piger förekommer allmänt bland friska individer, men korrelerar också positivt med vissa sjukdomar, och det råder oenighet om *D. piger* är bra eller dålig för hälsan. För att förstå bakgrunden till dessa motsättningar isolerade vi en *D. piger* stam som är nära besläktad med en stam som tidigare isolerats från en kolitpatient. Vi jämförde den nya stammen med den *D. piger* stam vi tidigare isolerat tillsammans med *F. prausnitzii*. Våra resultat visar att stammarnas metabola egenskaper skiljer sig åt. Detta indikerar att de olikheter som rapporterats i tidigare studier kan bero på att man inte tagit hänsyn till stamvariation hos *D. piger*.

Lay Summary

Inflammation is essential to our survival for fighting infections and repairing damaged tissues. When an individual sustains an injury, an inflammatory cascade of molecular signals is launched by the damaged cells. Immune cells are recruited from the blood circulation to defend or provide protection against microbes, toxins, or damaged cells. The first immune cells to arrive on site are known as neutrophils. They have a broad arsenal of weapons to combat microbes and protect from damaged cells. As the infection or injured cells are cleared, another class of molecules are released to promote the resolution of inflammation. These molecules send signals to repair the tissue and for neutrophils to either be eaten up by other immune cells, or to be cleared from the site through the lymphatics.

The cornerstone of chronic diseases is dysregulation of inflammatory pathways. This may be due to impaired molecular signals for promoting inflammatory resolution or an altered gut microbiota that may be promoting inflammation. This can lead to continuous immune cell activation, resulting in damage to healthy tissues.

One potential method of treatment is to promote the resolution of inflammation with specialized molecules known as lipoxins. We show that the front-line defense immune cells, neutrophils, from individuals with atherosclerosis are more activated than healthy controls. They also respond to lipoxin treatment by lowering their pro-inflammatory activity.

Another potential mechanism to target chronic inflammation is to try to restore gut bacteria that have immunomodulatory properties through supplementation. *Faecalibacterium prausnitzii* is one of the most abundant bacteria in our guts, has immune modulating properties and has been found to be lowered in individuals with cardiometabolic disease. Thus, it may provide therapeutic benefits if it can be developed into a supplement. We isolated *F. prausnitzii* from a healthy individual along with another intestinal bacteria known as *Desulfovibrio piger*. They can cross-feed each other to enhance growth and production of the immunomodulatory metabolite, butyrate. *F. prausnitzii* is extremely oxygen sensitive. To develop *F. prausnitzii* into a supplement to be given to people, we adapted it to

tolerate oxygen. Growing the adapted *F. prausnitzii* with *D. piger* yielded enough bacteria to enable production on an industrial level scale for human consumption. We demonstrate that supplementation of these two bacteria is safe for human consumption.

D. piger has been shown to be ubiquitous among healthy adults, but it has also been correlated with certain diseases. The jury is still out on whether this bacterium is detrimental to human health. To identify the reason for contradictory findings, we isolated a strain of *D. piger* that was genomically similar to a strain that was previously isolated from a patient with colitis. We aimed to compare it to the strain that we co-isolated with *F. prausnitzii* and found that the strains differed in growth and metabolism, suggesting that the discrepancies in previous findings may be due to *D. piger* inter-strain variation.

List of papers

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Kraft JD, Blomgran R, Bergström I, Soták M, Clark M, Rani A, Rohini Rajan M, Dalli J, Nyström S, Quiding-Järbrink M, Bromberg J, Skoog P, Börgeson E. Lipoxins modulate neutrophil oxidative burst, integrin expression and lymphatic transmigration differentially in human health and atherosclerosis. *The FASEB Journal* 2022; **36** (3) e22173.

II. Khan MT, Dwibedi C, Sundh D, Pradhan M, Kraft JD, Caesar R, Tremaroli V, Lorentzon M, Bäckhed F. Synergy and oxygen adaptation for development of next-generation probiotics. *Nature* 2023; **620**, 381-385.

III. Kraft JD, Dwibedi C, Makki K, Florén A, Hempenstall E, Bäckhed F, Khan MT, Tremaroli V, Caesar R. Phenotypic variation of novel *Desulfovibrio piger* stains isolated from human faeces. *Manuscript*

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ABBREVIATIONS

15S-H(p)ETE	15S-hydroperoxyeicosatetraenoic acid
15S-HETE	15S-hydroxyeicosatetraenoic acid
AA	Arachidonic acid
APC	Antigen presenting cell
API	Analytical profile index
CFU	Colony forming units
CRP	C-reactive protein
DAMPs	Damage-associated molecular patterns
DHA	Docosahexanoic acid
EPA	Eicosapentaenoic acid
fMLP	N-formylmethionine-leucyl-phenylalanine
FPR	Formyl peptide receptor
FSC	Forward scatter
GF	Germ-free
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPCR	G-protein coupled receptor
IBD	Inflammatory bowel disease
ICAM-1	Intracellular adhesion molecule-1
IL	Interleukin
LPS	Lipopolysaccharide
LTA_4	Leukotriene A_4
LTB_4	Leukotriene B4
LXA_4	Lipoxin A_4

LXB_4	Lipoxin B_4
LYVE-1	Lymphatic vessel endothelial hyaluronan receptor 1
MAGs	Metagenome-assembled genomes
MAMP	Microbial-associated molecular pattern
MPO	Myeoloperoxidase
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NAFLD	Non-alcoholic fatty liver disease
NET	Neutrophil extracellular trap
NF-ϰB	Nuclear factor kappa B
PAF	Platelet activating factor
PCR	Polymerase chain reaction
PRR	Pattern recognition receptors
ROS	Reactive oxygen species
SCFA	Short-chain fatty acid
SPM	Specialized pro-resolving lipid mediator
SRB	Sulfate-reducing bacteria
SSC	Side scatter
TLR	Toll-like receptor
TNBS	Trinitrobenzene sulphonic acid
TNF	Tumour necrosis factor
ZO	Zonula occludens

1 INTRODUCTION

From the beginning of life on earth, organisms have been required to adapt and evolve according to their environment to survive. The first signs of life, in the form of unicellular organisms, date back approximately 3.8 billion years, only 750 million years after the earth was formed. The earliest fossil evidence found of presumed multicellularity dates back approximately 2.1 billion years [1]. Over the course of Earth's history, our ancient single-celled predecessors independently gave rise to multicellular organisms on multiple occasions [2]. This leap is theorized to occur out of necessity to adapt to the ever-changing environment [3].

As multicellular organisms evolved, they carried their unicellular counterparts along. Multicellular organisms leveraged the genetic and metabolic diversity of the accompanying unicellular organisms to adapt to their environment without requiring substantial alterations to their own genetic makeup. The unicellular organisms were partitioned in sections according to spatial organization on the multicellular organism, forming what we now call a "holobiont" [4].

The human holobiont, an amalgamation of microbial life and mammalian cells, requires an immune system to regulate what and who can be carried along. The immune system is an elaborate network of processes, cells and chemicals essential to protect the host from infection and repair tissue damage to maintain homeostasis [5, 6]. The microbes on humans have adapted to this environmental niche. Appropriate immune training, selection and adaptation to the environment leads to a symbiotic relationship where both uni- and multicellular organisms can mutually benefit [4]. This complex inter-kingdom relationship occurs within every individual.

The body's immune system responds instantaneously to assaults whether microbial or sterile [6, 7]. Temporally restricted bouts of inflammation are orchestrated by activated immune and non-immune cells to combat infections, toxins and physical injury. Upon eradication of the threat, these cells also promote tissue repair [7]. However, left unchecked, unresolved inflammation can wreak havoc systemically, leading to a cycle of continuous immune activation, and is the underlying feature of many chronic

human morbidities [6-9]. Thus, striving to understand factors involved in the onset of inflammation and promoting resolution is essential to breaking the vicious cycle. The aim of this PhD project was to explore the role of both endogenous and microbial modulators of inflammation.

1.1 Immune system

The immune system is conventionally classified into innate and adaptive immunity. The innate immune system is the primary line of defense in all multicellular organisms. It instantaneously responds to injury or infection with no immunologic "memory" of the antigen that initiated the response [5]. Activation is initiated by pattern recognition receptors (PRRs) on innate immune cells identifying microbial-associated molecular patterns (MAMPs) or damage-associated molecular patterns (DAMPs) [5]. The rapid responses include pro-inflammatory molecule production, cell recruitment, phagocytosis (engulfment of microbes or cell debris) and killing microbes [5, 10]. It is estimated that around 500 million years ago, the adaptive immune system developed in vertebrates [10]. Complementary to the innate immune system, the adaptive immune system is essential when the innate immune system is unable to combat infection. It responds specifically to an antigen with a slight lag time between recognition to maximal response [5]. The adaptive immune system consists of B cells and T cells that recognize antigens as "self" or "non-self" and generate a pathogen-specific immunological memory of pathways that killed the specific pathogen. Thus, this "memory" allows for a faster and more effective response upon subsequent exposure to the antigen [5]. T cells of the adaptive immune system are presented with antigens from antigen presenting cells (APCs), such as dendritic cells, macrophages or B cells [5]. This promotes proliferation and differentiation of T cells with the receptor to recognize the specific antigen presented to destroy infected cells infected. Upon clearance of infection, most of the T cells die and are cleared through phagocytosis, but a few remain as memory cells to launch a rapid response upon later exposure to the same antigen [5]. B cells can directly recognize and produce antibodies against foreign antigens [5]. The innate and adaptive immune systems work in tandem to defend the host.

Immune training is initiated *in utero* [11]. The innate immune system responds and/or tolerates foreign substances from the maternal circulation, including microbial metabolites and antigens, as well as autologous molecules [11, 12]. It consists of various cell types including both immune cells (neutrophils, dendritic cells, monocytes and macrophages) and non-immune cells (epithelial and endothelial cells) [13]. Throughout fetal development, these cell types mature at different stages [14]. All cell types in early development appear to have "impaired" innate signaling pathways with reduced responses to inflammatory stimuli when compared to mature adult cells [14]. This allows the fetus to tolerate maternal antigens, adapt to continuous development and upon birth, to avoid an exaggerated immune response following exposure to the abundance of novel antigens from the outside world [11, 14]. Postpartum, the immature immune system of a newborn infant is exposed to a plethora of microbes, environmental molecules, gases, toxins and particulates, to which it must learn to either tolerate or defend against [14]. As the newborn grows, the immune system rapidly adapts and evolves identifying safe and potential life-threatening substances [14]. The exposure to microbes early in life is important for adaptive immunity and has a lasting impact on the developing immune system [11]. Upon reaching teenage years to early adulthood, the immune system is considered to be fully matured [14].

1.1.1 Inflammation

In response to tissue injury, infection or foreign substance recognition, an inflammatory cascade is initiated by both immune and non-immune cells to provide protection to the host [6-8]. Depending on the stimulus and the location in the body, a tightly orchestrated course of events unravels. Resident cells at the site of injury or infection, including epithelial cells, endothelial cells, macrophages and dendritic cells, recognize antigens and release signals. These signals consist of cytokines, lipid mediators, gases and other molecules and are sent to both surrounding and distant cells, leading to the recruitment of immune cells from the circulation [7, 13, 15].

1.1.2 Frontline troops

Neutrophils are the first cells among the innate immune system to be recruited from the circulation to the site of infection or tissue damage. They are the most abundant leukocyte in circulation, representing 50-70% of the total pool. Greater than 10^{11} neutrophils are produced in the bone marrow each day [9, 16, 17]. "Fresh" neutrophils enter the circulation and become subjected to phenotypic drift as they age and develop distinct properties over the course of approximately 12 hours [16, 18]. The circadian aging process contributes to the heterogeneity of distinct neutrophil phenotypes and function [18, 19].

Equipped with a broad range of receptors, neutrophils can recognize and respond appropriately to signals upon tissue infiltration [17]. They have a tightly regulated munition of molecular weapons ready to attack foreign invaders in response to inflammatory signals [16]. Depending on the stimulus, activated neutrophils have the potential to rapidly release a variety of antimicrobial agents including reactive oxygen species (ROS) or packaged granules containing myeloperoxidase (MPO), lactoferrin, defensins, proteases, antimicrobial peptides or enzymes [16]. In addition to the release of antimicrobial agents, neutrophils can ingest damaged tissue or foreign particles via phagocytosis or release a web of chromatin with cytosolic and granular proteins, known as neutrophil extracellular traps (NETs), to capture microbes [16]. Because of the strong weaponry carried by neutrophils, dysregulated deployment can result in collateral damage to surrounding tissue and in a chronic activated form, can have destructive systemic effects [9].

1.1.2.1 Neutrophil recruitment & activation

There are numerous mechanisms that balance the recruitment and response of neutrophils. The adhesion of neutrophils to vascular endothelial cells depends on the surface expression of adhesion proteins, such as integrins [20]. In healthy individuals, circulating neutrophils have low levels of adhesion proteins, including the glycoprotein receptors CD11b and CD66a, in a resting state. During a steady state, only a small subset, approximately 10%, of neutrophils express these adhesion receptors and are primed (discussed more further down) for rapid recruitment in case of injury [20, 21]. Through pro-inflammatory signals or

through interactions with endothelial integrins upregulated during inflammation, neutrophils become primed to enter tissue [20]. Neutrophil extravasation is initiated with the binding of their glycoprotein receptors to endothelial integrins, followed by rolling along the endothelium and transmigrating into the tissue towards a chemoattractant gradient, known as diapedesis [20, 22]. Chemoattractants are released by cells at the site of damage or infection, such as the pro-inflammatory molecules interleukin-8 (IL-8), platelet activating factor (PAF), complement fraction C5a or N-formylmethionine-leucyl-phenylalanine (fMLP) [22]. fMLP is an *N*-formyl peptide which is both a byproduct of bacterial metabolism and associated with damaged mitochondria [23, 24]. It binds the G-protein coupled receptors (GPCR), formyl peptide receptor (FPR) 1, 2, and 3, and is a potent chemoattractant and activator for neutrophils [17, 24-26]. When fMLP binds neutrophil FRPs, leukotriene B_4 (LTB_4), a pro-inflammatory lipid mediator, is released causing the recruitment of exponentially more neutrophils. This leads to "neutrophil swarming," a type of coordinated neutrophil movement in response to acute inflammation [27].

Once in the tissue, PPRs, such as Toll-like receptors (TLRs), on neutrophils recognize chemokines or MAMPs. Neutrophils phagocytose pathogens into a phagosome [22]. Within the phagosome, proteases, myeloperoxidase, antibacterial peptides, and superoxide anions (O_2-) are released, triggered by the activation of the multi-protein membrane-bound nicotinamide adenine dinucleotide phosphate-oxidase (NADPH, also known as NOX2) oxidase complex [22]. This successively triggers the release of intra-phagosomal ROS that further aids in killing the microbe [22]. ROS production, including hydroxyl radical, hydrogen peroxide and hypochlorous acid, is a powerful anti-microbial weapon in the neutrophil arsenal that can be released within the phagosome or extracellularly [28]. Stringent regulatory measures are required to keep production in check, as absence of ROS results in severe microbial infections and continuous release can cause excess inflammation leading to severe damage [28].

1.1.2.2 Neutrophil ROS priming

Neutrophil priming is essential to have a rapid innate immune response [29]. Neutrophils can be primed to produce ROS upon binding of GPCRs, TLRs or cytokines receptors [28]. This primed state refers to the assembly

of NADPH oxidase from four segregated cytosolic proteins to two transmembrane proteins [22]. This preparatory stage allows for a more immediate ROS response [22]. For example, pre-treating neutrophils with tumour necrosis factor (TNF)-α or granulocyte-macrophage colony-stimulating factor (GM-CSF) led to an elevated fMLP-induced ROS production [22]. Neutrophil chemoattractants such as PAF, IL-8, C5a, LTB_4, and fMLP also have a priming effect. At low concentrations, fMLP has a priming effect while high concentrations can induce ROS production [22]. TLR binding agonists, including microbial lipopolysaccharide (LPS), flagellin, triacylated lipopeptides or zymosan, can also prime neutrophils [22]. High circulating levels of chemoattractants, cytokines or TLR agonists can lead to excessive neutrophil priming, resulting in uncontrolled ROS production and subsequent tissue damage seen in chronic diseases such as atherosclerosis. Thus, utilizing inflammatory resolution pathways to prevent or reduce priming of neutrophil ROS production may be a promising therapeutic strategy in chronic inflammatory diseases.

1.1.3 Resolution of inflammation

Acute inflammation in response to tissue damage or pathogens is inherently self-restricted. Once the threat has been mitigated, inflammation is resolved through a series of synchronous signaling events and tissue homeostasis is restored [7]. The temporal response is modulated by a complex symphony of pro-resolving molecules that actively promote resolution of inflammation [8, 30-34]. These molecules reduce inflammatory signal production, halt immune cell recruitment, stimulate apoptosis of neutrophils and promote a pro-resolving macrophage phenotype, stimulating efferocytosis [8, 33, 35]. Efferocytosis, meaning 'to carry to the grave' in Greek, is the process by which macrophages clear cells and tissue debris by phagocytosis of apoptotic cells [36]. In addition, infiltrated immune cells begin to clear by either egressing through the lymphatics or migrating back into systemic circulation, leading to restoration of tissue homeostasis [35]. The conductors of this symphony of events are pro-resolving mediators including peptides, gaseous mediators, and lipids [8, 33-35, 37].

1.1.3.1 Specialized pro-resolving lipid mediators

Specialized pro-resolving mediators (SPMs) are a class of endogenously produced lipid mediators that have a major role in the resolution of inflammation [35]. They can be derived from either omega-3 (eicosapentaenoic acid (EPA) or docosahexanoic acid (DHA)) or omega-6 fatty acids (arachidonic acid (AA)) through lipoxygenase [35]. AA-derived SPMs include lipoxin A_4 (LXA_4) and lipoxin B_4 (LXB_4). SPMs have a wide range of targets with downstream immunomodulatory pro-resolving effects.

Halting neutrophil recruitment is one of the major processes of resolution, driven by SPMs. SPMs, such as LXA_4 or DHA-derived resolvin D1 and protectin D1, inhibit neutrophil tissue infiltration by preventing neutrophil-endothelial cell interactions or via modulation of cytokine expression [38-43]. In addition, macrophage efferocytosis of neutrophils has been demonstrated to be enhanced by LXA_4 and protectin D1 treatment [32, 44].

The first family of SPMs identified were lipoxins [45]. Interestingly, the production and release of AA-derived lipid mediators occurs at the onset of inflammatory stimuli. Initially, AA is converted into pro-inflammatory eicosanoids such as prostaglandins and leukotrienes, leading to the recruitment of neutrophils, as mentioned above. However, during the acute inflammatory phase, there is a "switch" whereby AA is converted into pro-resolving lipoxins, this is known as lipid-class switching [46].

1.1.3.2 Lipoxin synthesis

Lipoxins can be synthesized through various mechanisms (Figure 1) [35]. In monocytes, eosinophils or airway epithelial cells, 15-lipoxygenase inserts a molecular oxygen into AA yielding 15S-hydroperoxyeicosatetraenoic acid (15S-H(p)ETE) or 15S-hydroxyeicosatetraenoic acid (15S-HETE) [47]. These molecules are shuttled to either neutrophils or monocytes where they are converted to lipoxins by 5-lipoxygenase [45]. The 5-lipoxygenase in neutrophils also produces the pro-inflammatory leukotriene A_4 (LTA_4), which can then be converted to lipoxins by 12-lipoxygenase in platelets through neutrophil-platelet interaction (Figure 1) [45]. In addition, a more stable and potent lipoxin can be synthesized with aspirin treatment of endothelial cells and neutrophils, known as aspirin-triggered lipoxins, 15-epi-lipoxins [48-50].

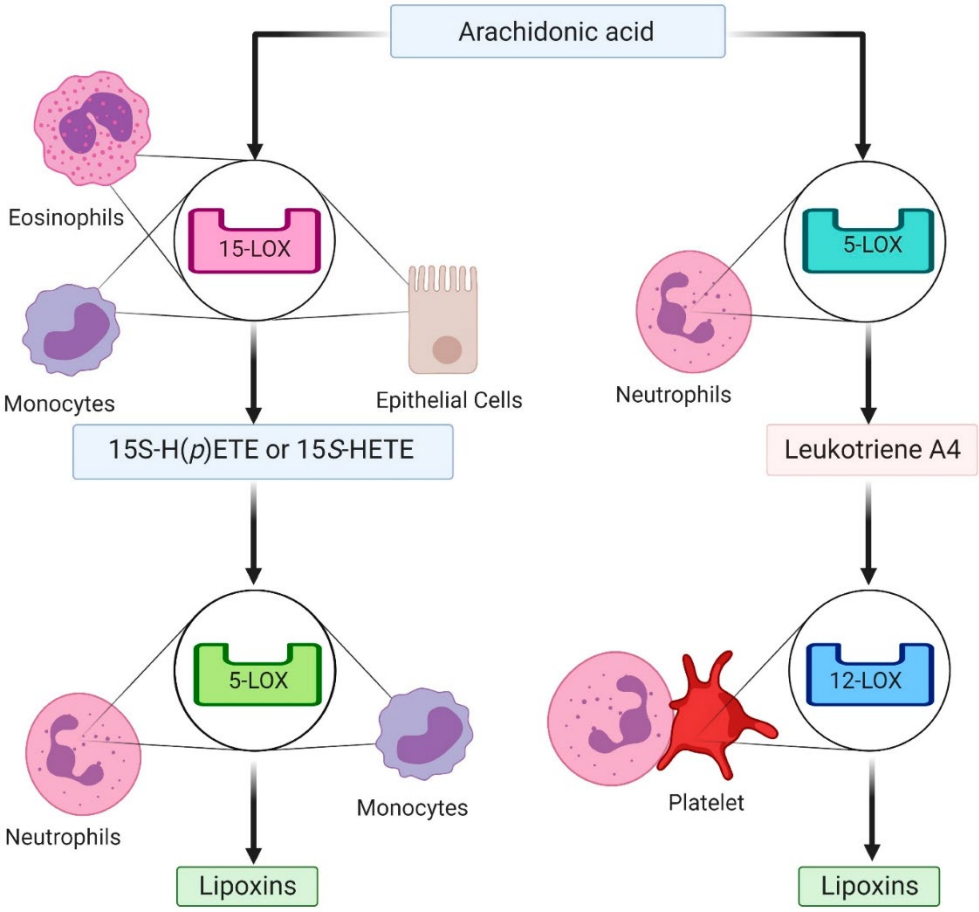


Figure 1. Routes of lipoxin synthesis *from review by Kraft et al. 2021. Specialized pro-resolving mediators and the lymphatic system. International Journal of Molecular Sciences 22(5), 2570.*

1.1.3.3 Lipoxins mode of action

Lipoxins act on numerous processes to promote resolution. They alter leukocyte trafficking [39, 40, 51-53], endothelial adhesion [39, 41], chemokine and cytokine expression, action and secretion [53, 54]. Neutrophils are essential for clearance of inflammatory stimuli, but excess activation and numbers can cause severe damage. As such, an important target of lipoxins is the reduction of neutrophils to the site of the injury [8]. Specifically, in neutrophils, LXA_4 treatment also reduces superoxide formation [53], downregulates CD11/18 expression [55, 56], reduces ROS and NET production [54], promotes neutrophil apoptosis and increases phagocytosis of apoptotic neutrophils by macrophages [54, 57] (Figure 2). Thus, lipoxins may represent a potent therapeutic to target neutrophils in chronic inflammatory diseases.

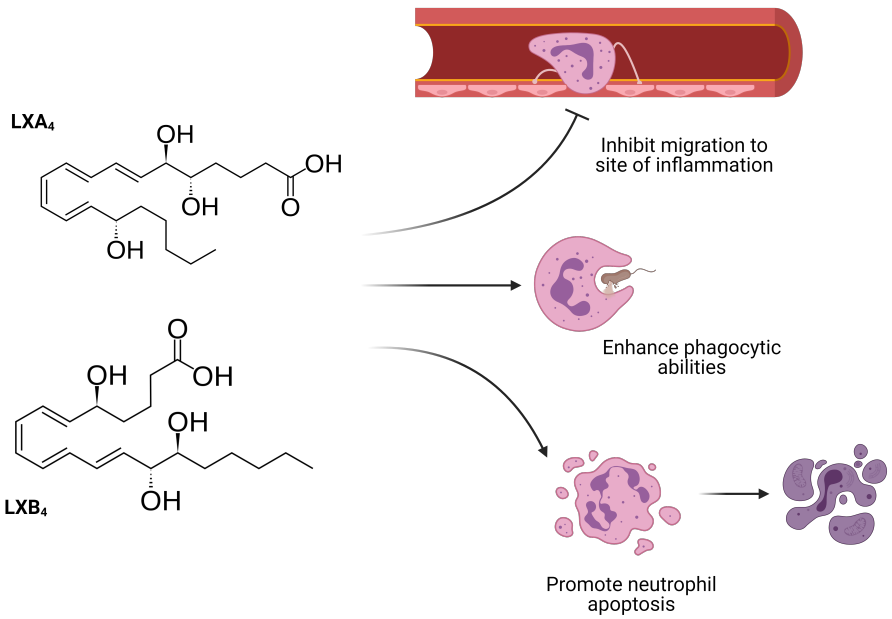


Figure 2. Known lipoxin mediated effects on neutrophils. *Lipoxin treatment of neutrophils can prevent tissue infiltration, enhance phagocytosis and promote apoptosis. (Created with Biorender)*

1.1.4 Lymphatics

"White blood" was first described by Hippocrates (460–377 B.C.). He was referring to lymph which consists of immune cells, antigens, nutrients, macromolecules, and excess plasma from blood [58, 59]. Lymphatic vessels are responsible for draining lymph from interstitial tissue space [58, 59]. The lymphatic vasculature is a vast network that maintains tissue homeostasis and modulates inflammation as well as other physiological processes, such as transporting lipids from the intestines [58]. The lymphatic vasculature runs through almost all tissues and is unidirectional. Initial lymphatic vessels in peripheral tissues consist of a single layer of lymphatic endothelial cells which have loose button-like tight junctions that allow the passage of solutes and guide them to the collecting lymphatic vessels through a pressure gradient. Collecting vessels have zipper-like tight junctions that prevent leakage of the lymph as it is directed to the lymph nodes [58, 59]. The adaptive immune response is initiated in the lymph nodes. Once filtered through the lymph nodes, the lymph then proceeds to the subclavian vein and back into systemic circulation [58-61]. The lymphatics act as an immune surveillance network by providing continuous information from peripheral tissues to the lymph nodes [59].

Unlike the blood vasculature, research on the lymphatics had been limited due to the lack of lymphatic endothelial cell markers preventing visualization. However, since the 1990s. lymphatic research has exploded with the identification of both markers for distinguishing lymphatics, such as lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1, Figure 3), and the development of *in vitro* models [58].

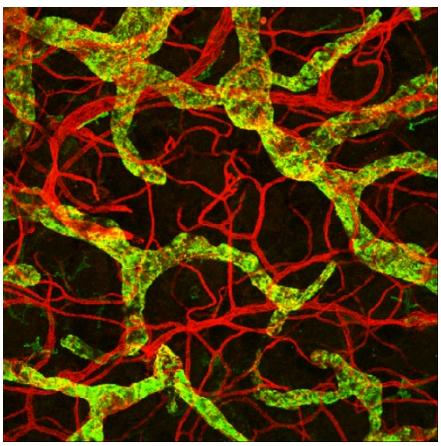


Figure 3. Immunofluorescent image of the lymphatic and blood vessels in mouse ear. *Lymphatic endothelial vessels were stained with LYVE-1 antibody (green) and blood vessels were stained with CD31 (red).*

Proper functioning of the lymphatic system is intricately associated with numerous physiological and pathological processes. Perturbed lymphatic function leads to accumulation of pro-inflammatory cells and signaling molecules that results in a chronic feedback loop fueling inflammation. Lymphatic impairment and irregular morphology are found in chronic diseases, such as cardiovascular disease [60]. In atherosclerosis, the lymphatics have an important role in macromolecule clearance, immune cell egression and reverse cholesterol transport [59, 62]. Thus, therapeutics promoting lymphatic drainage to clear inflammatory cells and signaling is essential for maintaining tissue homeostasis in disease.

1.1.5 Cardiometabolic disease

When the immune system is continuously activated or resolution is impaired, a state of chronic low-grade inflammation occurs leading to a surfeit of morbidities, such as cardiometabolic disease [8, 63, 64]. As the global prevalence of obesity is surging to pandemic proportions, so are the associated cardiometabolic diseases, including cardiovascular disease and type 2 diabetes [65, 66]. Metabolic syndrome is characterized by hypertension, central adiposity, dyslipidaemia and insulin resistance [64]. Due to sustained levels of inflammation, individuals with obesity have a higher frequency of cardiovascular disease and diabetes than those with a healthy weight [67]. The increased visceral fat drives vascular and systemic inflammation through shifts in adipokine production and additional secretion of pro-inflammatory mediators, which are correlated with the onset of insulin resistance and diabetes [68, 69]. In addition, skeletal muscle inflammation in individuals with obesity contributes to insulin resistance. This occurs due to increased pro-inflammatory macrophages in the muscles resulting in a shift in the production of myokines [68]. Individuals with diabetes have higher levels of innate immune acute-phase proteins, such as plasminogen activator inhibitor, haptoglobin, fibrinogen, sialic acid, serum amyloid A and C-reactive protein (CRP), as well as pro-inflammatory cytokines [69, 70]. Consequently, elevated levels of certain pro-inflammatory markers, such as CRP and serum amyloid A, are strong predictors of cardiovascular disease and are associated with increased risk of adverse cardiovascular events [69, 71-74]. The trinity of these comorbidities, diabetes, obesity, and cardiovascular disease, is underscored by chronic-low grade systemic inflammation [64, 72].

1.1.5.1 Cardiovascular disease

Cardiovascular disease remains the leading cause of chronic morbidity and mortality worldwide [63]. It is a group of disorders of the heart and blood vasculature that start asymptomatically in early adulthood and progress throughout the aging process [75]. Specifically, atherosclerosis, vascular plaque accumulation in the artery, is mainly driven by lifestyle choices including cigarette smoking, exposure to toxins, lack of movement, continuous stress, poor diet and sleep quality that increase inflammation [9].

Atherosclerosis is a progressive thickening and hardening of arterial walls as plaque builds up [75]. As the artery narrows, there is a reduced supply of blood that can lead to other cardiovascular diseases including stroke, peripheral arterial disease, and coronary artery disease [76]. The onset of atherosclerosis is initiated with a lesion in the arterial wall causing damage to the endothelial cell lining and/or the accumulation of low-density lipoproteins in the wall [76]. The oxidation-prone low-density lipoproteins along with the endothelial damage in the lesion launches an inflammatory response by both the innate and adaptive immune system. The response results in activation of platelets, oxidative stress, and upregulation of endothelial cell adhesion molecules, including selectin, vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 (ICAM-1) [76, 77] (Figure 4). Circulating monocytes bind these receptors and migrate into the endothelium between the endothelial cells and the vascular smooth muscle cells [76, 77]. Infiltrating monocytes differentiate into macrophages triggered by macrophage colony-stimulating factor [76]. Macrophages accumulate oxidized low-density lipoproteins through scavenger receptors and form foam cells, leading to the development of a fatty streak [76, 77]. The low-density lipoproteins also bind TLRs and lead to the secretion of additional cytokines and chemokines, further exacerbating inflammation by causing the recruitment of more immune cells, such as neutrophils. Migration of leukocytes mediated by chemokine signaling is a key step in the formation and progression of atherosclerotic lesions [78]. The increased inflammation as a result of neutrophils in the atherosclerotic lesion, in turn, leads to the recruitment of additional monocytes and a vicious pro-inflammatory cycle.

As more immune cells are recruited and activated, the fatty streak plaque continues to accumulate and fill with foam cells until they are oxygen deprived and begin to die. This results in a lipid-rich necrotic core which is the next stage of atherosclerosis, the fibrous plaque [77]. As the plaque worsens to an advanced stage, the surrounding cells produce a fibrous cap to stabilize the plaque. The chronic inflammatory state of the plaque wall can cause it to become a "vulnerable" plaque, characterized by a necrotic core and a thin fibrous cap. These plaques can rupture and are the most common cause of cardiovascular death [68, 74, 79, 80].

1.1.5.2 Neutrophils in atherosclerosis

Notably, elevated levels of neutrophils infiltrating in plaques are associated with acute coronary events [81] and elevated circulating neutrophils in individuals with peripheral artery disease are a predictor of future cardiovascular events [82]. Circulating neutrophils are increased under conditions of hypercholesterolaemia and hyperglycaemia, which are strong risk factors for cardiovascular disease [19, 83, 84]. Moreover, neutrophils have been implicated in accelerating atherosclerotic plaque development, decreasing stability, and associated with plaque rupture [78, 85, 86]. Studies have demonstrated that either depleting neutrophils or preventing neutrophilia in a mouse model of atherosclerosis attenuated plaque formation [85, 87]. In the plaque, neutrophils secrete pro-inflammatory molecules and NETs, and are associated with increased intimal apoptosis [85, 88] (Figure 4). In a hyperglycaemic environment, neutrophils produce S100A8 and S100A9, which are alarmins that drive myelopoiesis, thrombocytosis, and increase platelet numbers, promoting atherogenesis [19]. Further, circulating neutrophils interact with platelets in chronic inflammatory conditions leading to vascular adhesion and formation of NETs, exacerbating platelet activation [89] (Figure 4). Another study has demonstrated that elevated levels of neutrophil-derived membrane vesicles are found in mice fed with high-fat diets [90]. In an atherosclerosis mouse model fed a high fat diet, neutrophil membrane vesicles increased vascular inflammation, enhanced macrophage accumulation in the plaque and exacerbated atherosclerosis progression [90]. As the front-line troops, the role of neutrophils in atherosclerosis is now well recognized, thus they represent a promising cell to target to promote inflammatory resolution to reduce their collateral damage.

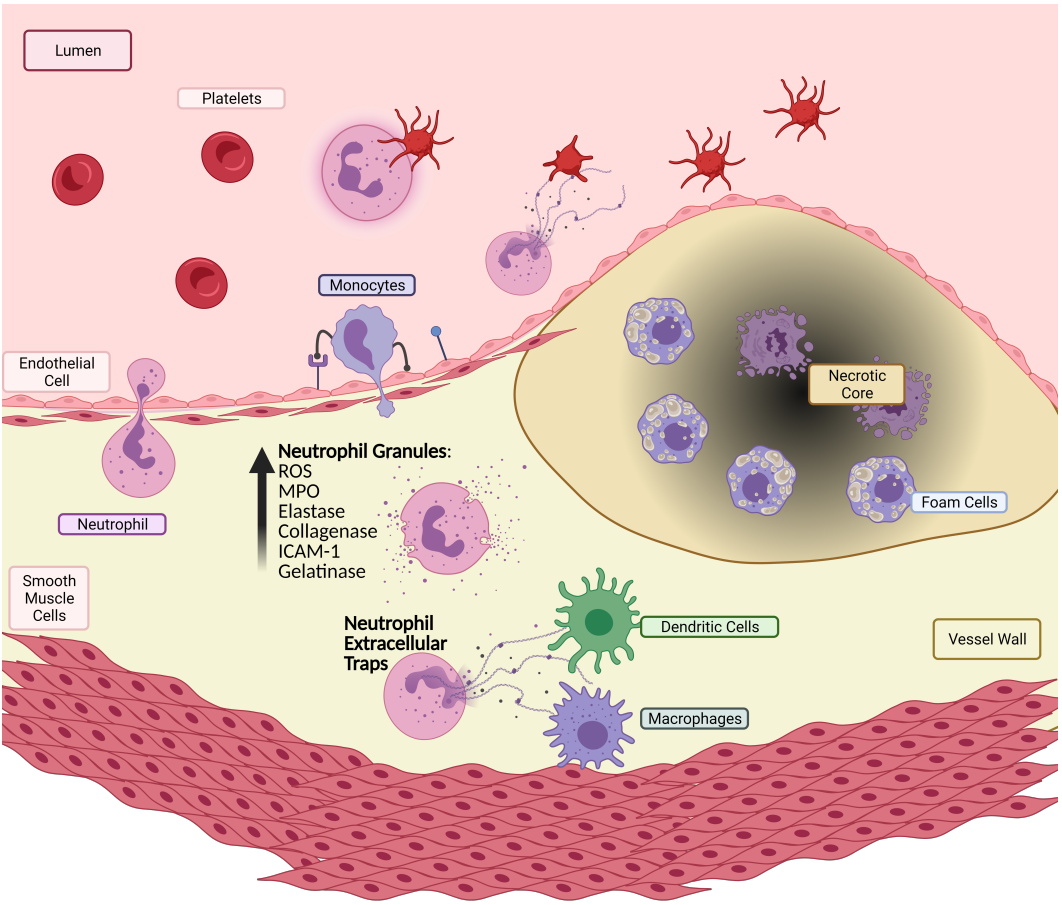


Figure 4. Role of neutrophils in atherosclerosis. *Neutrophils are recruited across vascular endothelium, into the atherosclerotic plaque and become activated and degranulate. They release ROS, granules containing myeloperoxidase (MPO) and NETs that increase integrin expression, such as ICAM-1, leading to additional immune cell recruitment. Further, they interact and activate platelets in circulation. Continuous activation of the neutrophil arsenal of weapons leads to an increased necrotic core and can lead to plaque destabilization. (Created with Biorender)*

1.1.5.3 Therapeutics targeting inflammatory resolution

Instead of trying to halt a natural response to an inflammatory stimulus, enhancing resolving capacity of the body is of great interest when treating chronic inflammatory diseases. There are an increasing number of trials supplementing omega-3 polyunsaturated fatty acids to individuals with cardiometabolic disease in an attempt to increase SPMs to enhance inflammatory resolution [91-96]. For example, trials supplementing omega-3 fatty acids or fish oil to patients with cardiovascular disease found increases in SPMs in plasma [91, 94]. While there are fewer human studies on drugs targeting inflammatory resolution compared to those which inhibit inflammation in cardiometabolic diseases, there are studies that assess the effects of pro-resolving drugs in other inflammatory-related pathologies. For example, an injection of a cocktail of SPMs into a site of inflammation (blister) reduced the number of granulocytes, indicating an enhanced resolution [97].

The therapeutic potential of lipoxins in cardiovascular disease has been assessed in experimental *ex vivo* or rodent models. For instance, *ex vivo* LXA_4 treatment in human whole blood reduced neutrophil-platelet aggregation by modulating neutrophil integrin activation [98]. Further, treatment of *ex vivo* human atherosclerotic plaques with LXA_4 promoted transcription of pro-resolving microRNAs [99] or attenuated inflammation through inhibition of pro-inflammatory cytokine release [100]. Similarly, in mice, lipoxin treatment attenuated atherosclerotic plaque development or significantly reduced atherosclerotic lesions [100-102]. These studies demonstrate the efficacy in animal and *ex vivo* models of lipoxin treatment in reducing inflammation and potentially attenuating cardiovascular disease development.

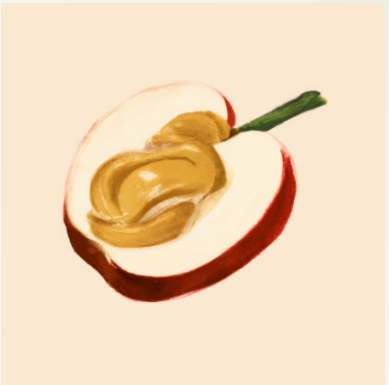
1.1.6 Summary

In summary, individuals with cardiometabolic disease have dysregulated chronic levels of inflammation. Neutrophils are now well recognized as major contributors to atherosclerotic plaque development and rupture. Further, impaired lymphatic function is also a key feature of cardiovascular disease. Thus, targeting the body's endogenous inflammatory resolution pathways may be a promising therapeutic strategy for treating individuals with atherosclerosis. Exogenous lipoxin treatment has been shown to modulate neutrophil function which is further explored in **Paper I**.

1.2 Gastrointestinal tract

The human digestive tract can be seen as a tube-like structure, from mouth to anus, that is incessantly exposed to foreign substances and environmental factors [103]. Thus, the approximately 8–9-meter long gastrointestinal tract is equipped with the largest portion of the immune system [103, 104]. It begins in the mouth, followed by the pharynx (throat) to the esophagus, stomach, small intestine (duodenum, jejunum, ileum), large intestine, rectum and ending in the anus [103]. We have a complex digestive system responsible for harvesting energy from organic molecules we consume from the environment (Box 1).

BOX 1.



When an individual takes a bite of an apple slice covered in peanut butter, the digestion process begins. With a jaw force between 100 to 400 N, the chewing motion initiates salivation and breaks down the apple into smaller pieces [103]. The saliva contains amylases and lingual lipases that enzymatically degrade the starches in the apple into disaccharides and begin to metabolize lipids in the peanut butter [103]. The chewed up semi-broken down apple bits with the peanut butter and saliva form a bolus and are then swallowed down the esophagus by the pharynx, pushed through by peristalsis across the cardia to the acidic environment of the fasted stomach (pH 1-3) [103]. An empty stomach forms wrinkles and folds, called rugae, and has a volume of 25-50 mL. After an average meal it expands to 1-1.5 L. When fully stretched, the stomach volume can reach 4 L [103]. In the stomach, the bolus undergoes further degradation through both mechanical and chemical mechanisms. Enzymes are secreted by specialized cells lining the stomach wall to further break down proteins and lipids [103]. In addition, the acidity level of the stomach acts as a filter for the microbes coated on the apple [105]. The muscles surrounding the stomach contract to grind, mix and push the digestive juices and food particles (now called chyme), after 1 to 3 hours, across the pylorus and into the duodenum. This triggers a cascade of hormone, bile and pancreatic digestive enzyme secretion [103]. The liver produces bile which is stored in the gall bladder and is released into the duodenum to emulsify lipids permitting absorption. Most of the nutrients from food, such as the fructose from the apple and the fats from the peanut butter, will be absorbed in the 6-7-meter-long small intestine. The folds and the villi of the small intestine create a surface area of approximately 4500 m^2 for the absorption of monosaccharides, amino acids, phosphates, minerals and vitamins over the course of 1-5 hours [103]. The microvilli at the border secrete a variety of enzymes that further degrade digestive products. Anything left unabsorbed makes it through the ileocecal valve to the large intestine where the undigested substrates reach the largest population of microbes in our bodies [103].

1.2.1 Intestine

In the intestine, there is a single layer of epithelial cells that separates the immune cells harbored in the lamina propria from the teeming microbial life in the lumen through both chemical and physical barriers [104, 106]. Maintenance of these barriers preserves homeostatic conditions and prevents unnecessary inflammatory responses to commensal microbes or safe foreign substances [104, 106]. There are various types of intestinal epithelial cells, such as absorptive epithelial cells, Paneth cells, microfold cells, enteroendocrine cells, tuft cells, and goblet cells [107]. All differentiate from stem cells at the base of the crypts and have important roles in modulating the host response to the intestinal environment [107].

Absorptive intestinal epithelial cells are joined by tight junction proteins, such as tetraspan transmembrane claudins and occludins and the intracellular peripheral membrane protein zonula occludens (ZO) [107]. These tight junction proteins are able to size- or charge-exclude solutes from paracellular passage [108]. The tight junction proteins are at the base of the microvilli, followed by adherens junction, linked to actin-based microfilaments, and lower down, desmosomes, associated with cytokeratin-based intermediate filaments, forming the apical junction complex [108].

On the luminal surface, intestinal epithelial cells secrete a protective layer of transmembrane glycoproteins, known as the glycocalyx [109]. The glycocalyx is a network of glycolipids or glycoproteins acting as sites for bacterial adhesion. This interaction promotes the attachment of mutualistic microbes thus hindering the colonization of pathogens [109]. The mucus is an additional protective layer secreted by goblet cells. It is enriched in mucins, an intricate matrix of glycoproteins, that are produced and secreted in response to the crosstalk between microbes or their metabolites and the host [107]. The mucus layer in the colon is much thicker than in the small intestine due to an increased number of goblet cells, forming both an inner and looser outer layer to separate the densely populated microbial community [107]. In a healthy individual, the colonic inner mucus is free of microbes due to the secretion or transport of antimicrobial molecules, such as β-defensins, by the intestinal epithelial cells [107]. Whereas the outer colonic mucosal layer contains microbes adapted to this niche that

utilize mucins as their energy source [107]. In the small intestine, the mucus layer is thin to facilitate absorption of nutrients. Specialized secretory epithelial cells, known as Paneth cells, secrete an array of antimicrobial molecules, such as the peptide α-defensin, limiting the proliferation of microbes in this environment [107, 110]. Thus, the intestinal epithelial cells modulate the microbial community contained within which, in turn, has a profound impact on the immune system and modulation of inflammation.

1.2.2 Gut microbiota

From training our immune systems, to protecting us from pathogens to harvesting energy, the microbes in our gut have been invaluable for our evolution, fitness and overall health [111]. A wide array of organisms, including bacteria, archaea, viruses, phages and fungi have established residence in our intestinal environment [112]. It is estimated that the gut microbiome contains greater than 500 times more genes than our human genome [113]. Through multiple trophic interactions, the microbes in our gut extract energy from food, metabolize drugs, degrade toxins, and produce bioactive molecules that can signal to various organs throughout the body [4, 111, 112, 114, 115]. The gut microbiota produces or modifies bioactive molecules such as vitamins, amino acids, lipids, and bile acids which are utilized or recognized by host cells and induce a response [4, 64, 111, 112, 114-116]. A mutualistic relationship occurs within the human holobiont dependent on continuous crosstalk between the host and the microbiota in health.

High microbial gene richness, diversity and a relatively stable composition are all key features of what is considered to be a "healthy" microbiota [112]. Our gut microbiota is influenced by a host of factors, including, but not limited to, birth mode, breastfeeding, medication, environmental exposure, exercise frequency, lifestyle, diet, age, medication and host genetics [114, 115, 117-130]. The microbiota composition is unique among individuals but also varies within an individual depending on time of day, previous meals, stress levels, social interaction, hormones, and topography (the environment within the gut) [131-137]. For example, the small intestine has a high flow rate, bile concentration and antimicrobial peptides, which all limit bacterial growth and create an environmental niche. Therefore, the bacterial density is relatively low [138, 139]. Whereas, the colon has a

slower flow, a more neutral pH and less antimicrobial molecules which permits increased microbial density within communities [139]. As there is a decreasing oxygen gradient from the mucosa to the lumen, the colonic microbiota consists of more anaerobic microbes [118, 139]. The colon is estimated to be the most densely populated community of microbes on earth [140]. Thus, maintaining a diverse and balanced intestinal ecosystem can contribute to host health.

1.2.3 Gut microbiota and disease

Alterations in gut microbiota composition have been studied in relation to human diseases, including cardiometabolic disease [70, 141-144]. However, a meta-analysis found that several of the studies exploring compositional changes in the gut microbiota of obese individuals were underpowered [145]. There is not a typical microbial signature that defines individuals with obesity or diabetes. However, their microbiota is frequently associated with low gene richness and decrease of commensals, such as butyrate-producing bacteria [64, 146-148]. Individuals who were overweight or obese with low gene richness had worse metabolic parameters with increased fasting serum triglycerides, insulin resistance, higher LDL cholesterol and inflammation compared to those who were obese with higher gene richness [147]. Increasing gene richness through energy-restricted dietary intervention decreased adiposity, circulating cholesterol and highly sensitive CRP (a marker of inflammation). Thus, this indicates that restoring gene richness can be associated with improvements in metabolic parameters [147].

Other studies have found an inverse association with body mass index and *Christensenellaceae*, which has been shown to be enriched in lean individuals [129, 149]. Whereas *Ruminococcus gnavus* has been associated with increased body fat percentage, even with adjustment for confounding variables [148]. Further, multiple studies have found that individuals with type 2 diabetes or prediabetes have a decreased abundance of commensals such as *Akkermansia muciniphila* and several butyrate-producers, such as *Alistipes* spp., *Clostridium* spp., *Roseburia* spp., *Oscillibacter*, and *Faecalibacterium prausnitzii* [142, 150-154]. Butyrate-producing bacteria play an important role in intestinal ecology with several beneficial effects that modulate host physiology, discussed in more detail in section 1.2.4.1. Importantly, when

investigating the role of the gut microbiota in obesity and metabolic disease, it is important to note that associations are partial to diet-microbe interactions because microbiota composition is strongly influenced by diet [64, 70, 155]. In summary, these findings indicate some association between metabolic disease, gene richness and altered gut microbiota.

1.2.3.1 Causal role of the microbiota

To explore the causal role of the microbiota in health and disease pathophysiology, germ-free (GF) mice models have been used as the gold standard. When GF and conventionally-raised mice were fed a chow diet, the GF mice ate more, but had a significantly lower body fat percentage and adiposity [156]. GF mice colonized with microbiota of conventionally-raised mice lowered their food intake but had a significant increase in body fat percentage [156]. Another study demonstrated that human microbiota transferred from a lean twin or their obese counterpart to GF mice transmitted the phenotype, i.e., the mice that received the microbiota from the obese twin gained significantly more body mass and adiposity compared to the mice that received the lean twin's microbiota [157]. Notably, when the mice with the obese microbiota were cohoused with the mice with the lean microbiota, they did not develop increased adiposity when fed a low-fat, high fiber diet, however, this attenuation was muted if the cohoused mice were fed a high saturated fat diet that was low in fiber [157]. These studies highlight the importance of the gut microbiota in developing an obese phenotype. Further, adipose inflammation, which is associated with glucose metabolism, is lower in GF mice [158, 159]. Individuals with type 2 diabetes have increased levels of circulating LPS, known as metabolic endotoxemia [160]. LPS is the major component of the outer membrane of Gram-negative bacteria. Increased levels of LPS contribute to insulin resistance by activating the immune system and upregulating inflammatory cytokines [160, 161]. Continuous infusion of LPS in mice increased body weight, adipose tissue, and liver weight as well as fasted glycemia and insulinemia [161]. Taken together, these studies demonstrate a causal role for the microbiota in modulating fat storage and metabolism in mice.

1.2.4 Microbial metabolites

Dietary interventions have demonstrated that microbiota-derived metabolites can alter metabolic pathways [64, 114, 116]. Short chain fatty acids (SCFAs) are produced by microbial fermentations of non-digestible dietary fibers, as well as peptides. They have wide ranging effects that include regulating hormone production, appetite, pancreatic function, and insulin release [115, 162]. SCFAs, including acetate, propionate and butyrate, can modulate metabolic pathways by binding to GPCRs, such as free fatty acid receptor 2 (FFAR2), also known as GPR-43, expressed on intestinal epithelial cells, as well as adipocytes and immune cells [163]. Mice deficient of FFAR2 became obese despite consuming a regular diet. Conversely, even on a high-fat diet, mice overexpressing FFAR2 remained lean [164]. Further, in these mouse models, if they were raised germ-free or treated with antibiotics, they maintained a lean phenotype, highlighting the importance of the microbiota and their metabolites in metabolic pathways [164].

Further, microbially produced metabolites derived from food can also result in harmful consequences. For example, imidazole propionate is a microbially produced metabolite from the diet-derived amino acid, histidine, and is elevated in individuals with type 2 diabetes [165]. Mice treated with imidazole propionate had impaired insulin signaling and glucose tolerance [166]. A predictive risk factor of cardiovascular is the phosphatidylcholine or choline-derived microbial metabolite, trimethylamine, that is rapidly oxidized to trimethylamine-*N*-oxide (TMAO) in the liver [167, 168]. TMAO treatment exacerbates atherosclerotic development in mice [168]. Further, the gut microbiome's increased potential to produce branched-chain amino acids (BCAAs) correlates with insulin resistance, characterized by elevated serum BCAAs [169]. BCAAs are derived from either food or from the gut microbiota. Supplementation with a species driving BCAA biosynthesis, *Prevotella copri*, led to an increase in serum BCAAs, insulin resistance and worsened glucose tolerance in mice [169]. These brief examples underscore the significant role microbially produced metabolites play in metabolism, shaping outcomes in potentially beneficial or detrimental ways.

1.2.4.1 Butyrate

Butyrate is a microbially produced SCFA that plays an important role in maintaining gastrointestinal health [170]. Concentrations of butyrate in the intestinal lumen ranges between 10 to 20 mM and approximately 95% is absorbed by colonic epithelial cells [171]. The effect of butyrate on epithelial cells is multifaceted and produces distinct and sometimes opposing outcomes dependent on the target and concentration. One of its main functions is as a primary energy provider for colonocytes through β-oxidation and the tricarboxylic acid cycle [171]. In addition, butyrate improves intestinal barrier function and plays an immunomodulatory role [172, 173]. For instance, butyrate treatment *in vitro* can induce transcription of tight-junction proteins and can increase or maintain transepithelial resistance [174-178]. It can also inhibit histone deacetylases involved in nuclear factor kappa B (NF-κB)-induced pro-inflammatory cytokine production [172] and increase the expression of the anti-inflammatory cytokine IL-10 [176]. Interestingly, based on functional annotation analyses, individuals with type 2 diabetes have decreased butyrate-producing potential [179]. Thus butyrate-producers represent promising next-generation probiotics for the treatment of metabolic diseases.

1.2.4.2 Hydrogen sulfide

Gasotransmitters are small gas molecules which can be produced both endogenously and enzymatically in a regulated fashion and by microbes. They are permeable to membranes, have well-defined and specific functions, cellular or molecular targets and can induce physiological changes [180]. The trinity of gasotransmitters consists of nitric oxide, carbon monoxide and hydrogen sulfide [180].

Hydrogen sulfide is produced in every organ system in the body and over the past 20 years has been emerging as an important modulator of a wide range of physiological processes. In host cells, it is endogenously produced through cystathionine γ-lyase, cystathionine β-synthase, and 3-mercapto-pyruvate sulfurtransferase [181]. It is also produced through anaerobic respiration of sulfur-containing substrates by intestinal microbes [182]. At low levels, hydrogen sulfide serves as a pro-resolving mediator that promotes inflammatory resolution through inhibiting leukocyte infiltration, inducing neutrophil apoptosis, reducing pro-inflammatory

signaling and promoting tissue repair [183-186]. In the gut it exerts beneficial effects through enhancing mucus production, defense, healing and inflammatory resolution [184]. For instance, hydrogen sulfide treatment protected the intestinal epithelial cell model, Caco-2, from inflammation-induced increases in permeability [187]. In addition, hydrogen sulfide prevents pro-inflammatory cytokine production through inhibiting the activation of the NF-κB-pathway [187]. Hydrogen sulfide also affects leukocyte migration by downregulating adhesion molecules [188, 189] and promotes inflammatory resolution in rat intestinal models of colitis [186]. It also acts as a vasodilator and can increase gastric mucosal blood flow and protect from nonsteroidal anti-inflammatory drug-induced gastric injury [188]. These highlighted instances provide a glimpse into the potent beneficial regulatory effects of the ideal concentration of hydrogen sulfide in the gastrointestinal tract.

However, hydrogen sulfide is a Jekyll and Hyde molecule that exhibits complex duality within the human body. While it is an essential gaso-transmitter regulating multiple physiological and cellular functions, excessive amounts can result in detrimental consequences [180, 182, 185, 187, 190-193]. For example, elevated levels of hydrogen sulfide in the gut can cause damage to the intestinal mucus and prevent intestinal epithelial cells from oxidizing butyrate [194, 195]. Hydrogen sulfide can affect cell cycles by reducing intestinal epithelial cell proliferation and inducing acute hypoxia [196, 197]. In addition, increased levels of hydrogen sulfide have been correlated with colon cancer and inflammatory bowel disease (IBD) [194, 198, 199]. These mechanistic studies taken together with clinical observations demonstrate the necessity of maintaining appropriate concentrations of hydrogen sulfide in the intestine.

1.2.5 *Faecalibacterium prausnitzii*

One of the most abundant bacteria in healthy human adults is *Faecalibacterium prausnitzii* [200]. *F. prausnitzii* is a non-motile, gram-positive, non-spore forming bacteria part of the Firmicutes phylum, Clostridia class and *Ruminococcaceae* family [201]. The reference strain A2-165 was the first complete genome of *F. prausnitzii* and was sequenced during the 'Human Microbiome Project' [201]. This glucose-fermenting strict anaerobe plays an important role in intestinal ecology by producing formate, lactate and ample amounts of butyrate [201]. In addition to butyrate production, *F. prausnitzii* releases other molecules which have anti-inflammatory effects both *in vitro* and *in vivo* [200-205]. In Caco-2 cells, *F. prausnitzii* supernatant dampened IL-8 secretion in response to IL-1β stimulation by inhibiting NF-κB activation [203]. In addition, in mononuclear cells, *F. prausnitzii* treatment induced production of the anti-inflammatory cytokine IL-10 and lowered production of pro-inflammatory IFN-γ and IL-12 [203]. In a mouse model of colitis induced by 2,4,6-trinitrobenzenesulphonic acid (TNBS), both the bacteria and the cultured media protected against the damaging effects of TNBS [203]. Decreased levels of *F. prausnitzii* have been associated with diseases such as IBD, atherosclerosis, diabetes and obesity [142, 179, 203, 206]. Due to its anti-inflammatory properties, butyrate-production capacity and associations with healthy microbiota, development of *F. prausnitzii* as a next-generation probiotic may be a promising supplement for individuals with cardiometabolic disease.

1.2.6 Sulfate-reducing bacteria

Sulfate-reducing bacteria (SRB) influence microbial community composition in the earth's ecosystem and may have a similar role in the gastrointestinal tract [207, 208]. They can metabolize both organic and inorganic sources of sulfate, either from the diet, the host, or other gut microbes through dissimilatory sulfate reduction to yield hydrogen sulfide [209, 210]. In the gut, SRB, such as species of *Desulfovibrio*, use sulfate as an electron acceptor and utilize the fermentation by-products, hydrogen and lactate, as electron donors, yielding acetate [209, 210] (Figure 5).

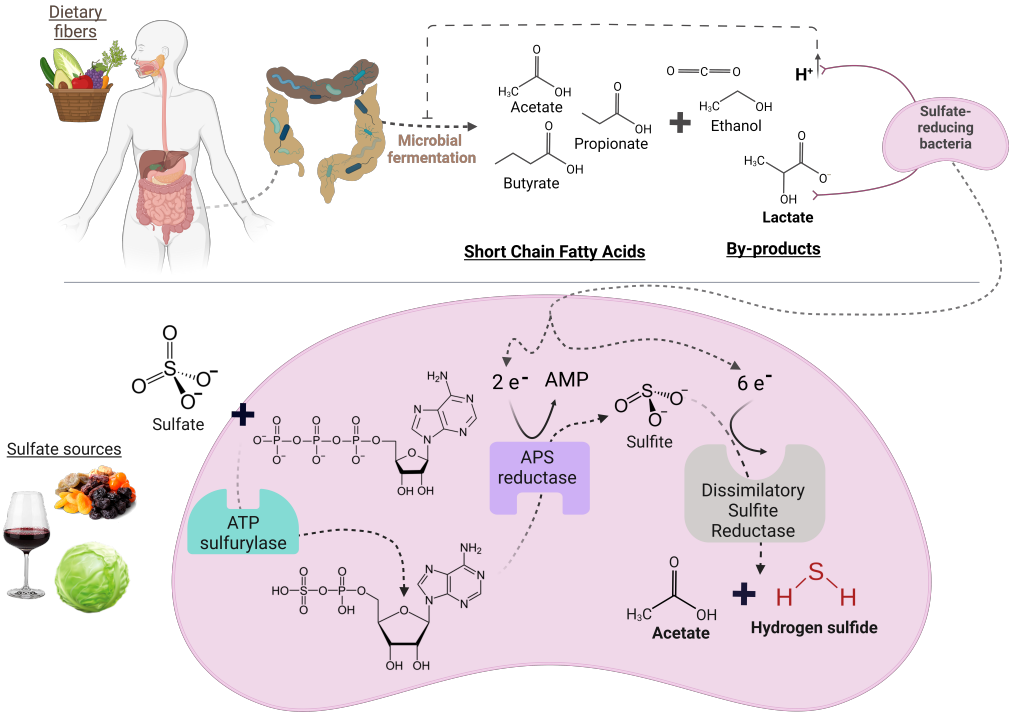


Figure 5. Role of sulfate-reducing bacteria (SRB) in the gut. *In the colon, fermenting gut bacteria metabolize dietary fibers, proteins and mucus components yielding SCFAs, as well as byproducts, such as lactate and gases, hydrogen (H_2), carbon dioxide (CO_2) or methane (CH_4)* [211-213]. *To regenerate nicotinamide adenine dinucleotide (NAD+), multiple fermenters utilize H+ to accept electrons, producing H_2* [211]. *In all living cells, NAD+ is required for metabolism of the cell as a co-substrate, an enzyme and redox cofactor* [214, 215]. *Accumulation of H_2 in the colon prevents primary fermenters from renewing NAD+, inhibiting their ability to ferment and grow* [212]. *Thus, timely removal of H_2 by microbes including acetogens, methanogens and SRB is necessary to maintain intestinal homeostasis* [211, 212, 216]. *SRB use sulfate as an electron acceptor and can use hydrogen or lactate as electron donors. Sulfate is reduced through a series of steps with adenosine triphosphate (ATP) sulfurylase, adenosine-'5'-phosphosulfate (APS) reductase and dissimilatory sulfite reductase to yield acetate and hydrogen sulfide. These trophic interactions have a potential to promote synergistic interactions in gut microbial communities.* *(Created with Biorender)*

In the human gut, sulfate sources include diet, intestinal mucins, and amino acids, such as cysteine or taurine [209, 210]. Diet is the main source of sulfate and sulfur absorption is important for amino acid formation. Foods high in sulfate include breads, dried fruit and brassica vegetables, or beverages such as beer and wine [217]. Hence, intestinal sulfate levels vary between individuals, and dietary intake is a strong mediator of SRB abundance in the intestine. SRB abundance and composition impacts the amount of hydrogen sulfide produced in the intestine [209].

The most well studied intestinal species are *Desulfovibrio*, *Desulfobacter*, *Desulfobulbus* and *Desulfotomaculum* [216]. The genus, *Desulfovibrio*, accounts for 67-91% of SRBs present in the human gut [194, 218]. *Desulfovibrio* are Gram-negative bacilli that are facultative anaerobic, non-fermenting and characterized by the presence of the pigment desulfoviridin [219-221].

1.2.6.1 Sulfate-reducing bacteria and disease

SRB presence and abundance has traditionally been associated with diseases and inflammation [190, 222-228]. Multiple case reports have isolated SRB *Desulfovibrio* species, mainly *D. desulfuricans* and *D. fairfieldensis,* from blood cultures of individuals with bacteremia or abscesses and have suggested these species as causative agents [229-235]. For example, SRB isolated from faeces of individuals with colitis induced apoptosis in an *in vitro* intestinal epithelial cell model, however, interestingly, SRB from healthy controls had no effect [223]. Furthermore, intestinal *Desulfovibrio* has been associated with Parkinson's disease [225, 226, 228] and found to be enriched in individuals with IBD and ulcerative colitis [227].

However, due to low-resolution and limited data availability, studies have yet to establish a correlation between intestinal *Desulfovibrio* and disease. For example, one study identified similar levels of *Desulfovibrio* in faecal samples and rectal biopsies from both individuals with ulcerative colitis and healthy controls, regardless of age [236]. Further, Scanlan *et al.* quantified *Desulfovibrio sp.* with polymerase chain reaction (PCR) from individuals with colorectal cancer, polypectomized patients, and two healthy control groups of elderly and young adults. In all samples, *Desulfovibrio* was detected and there was a significant reduction in *Desulfovibrio* counts in the colorectal cancer group compared to

polypectomized patients and young healthy adults. Specifically, *Desulfovibrio piger* predominance was a feature of both healthy and diseased groups [237]. Similarly, another study found no difference in the counts of SRB isolated from individuals with ulcerative colitis compared to controls [238]. These studies indicate that *Desulfovibrio* spp. are ubiquitous in the intestinal tract of humans, but SRB distribution and activity in relation to human diseases remains poorly understood.

With the rise of metagenomic sequencing and technical advances, high resolution identification of subspecies and strains enable high throughput analyses of complex environments [239]. Traditional methods were limited in that they were low-resolution and relied on culturing bacterial isolates of microbial communities. We now recognize that there is substantial variability not only between different species, but also between strains [239]. For example, *Escherichia coli* inter-strain variability demonstrates that one species can have either environmental, commensal or pathogenic strains [240]. Studies referencing only the genera in the title of an article fail to adequately encompass the comprehensive insights into the effects of a specific SRB species or strain associated with disease [190, 218, 222, 224-228, 241, 242]. This practice has led to review articles making sweeping assumptions about associations of SRB or *Desulfovibrio* with disease. Whereas other articles unambiguously referenced the species *D. desulfuricans* [229, 243-255] or *D. fairfieldensis* [231, 256, 257] in the title which benefits the readers by specifying the species in relation to disease. The increased accessibility of cost-effective sequencing and advancements of metagenomic tools has made it feasible to decipher species and strains. Thus, research investigating species and strain level variations of SRB will provide further insight into their role in disease.

1.2.6.2 *Desulfovibrio piger*

The first reported strain of *D. piger*, previously known as *Desulfomonas pigra*, was isolated from human faeces [258]. *D. piger* has yet to be isolated outside of the gut, indicating a strong likelihood that this species is adapted to a specific ecological niche within this habitat [220, 259]. In faecal samples from healthy individuals, *D. piger* has been identified as the most abundant of the *Desulfovibrio* species [212, 260].

Associations of *D. piger* with health-related parameters and disease states are contradictory, perhaps due to the lack of strain resolution mentioned above. For example, *D. piger* was enriched in faeces of children with obesity and non-alcoholic fatty liver disease (NAFLD) compared to obese controls without NAFLD [261]. Additionally, individuals hospitalized for IBD had significantly higher *D. piger* prevalence compared to healthy controls measured through PCR [218]. On the contrary, Chen *et al.* [260] found that *D. piger* was positively associated with certain characteristics of a "healthy" microbiota, such as community diversity and abundance of commensal genera, including *Akkermansia, Faecalibacterium, Roseburia, Bacteroides, Coprococcus* and *Oscillospira*. In addition, *Desulfovibrio* was negatively correlated with genera that are generally considered deleterious to health, such as *Escherichia, Klebsiella, Ralstonia* and *Clostridium* [260]. Further, they demonstrated that a strain which clusters in the *D. piger* clade, based on a phylogenetic tree, was negatively correlated with BMI, waist circumference, uric acid, and triglycerides, while having a positive correlation with systolic blood pressure, high-density lipoproteins, and sleep time [260]. These opposing findings may indicate that high metagenomic resolution to the strain level is required to distinguish between disease and health-related parameter associations with *D. piger*.

Mouse studies of *D. piger* have been performed to elucidate its role in the intestine. High-fat diet-induced obese mice gavage-fed with *D. piger* type strain, ATCC 29098, for 4 weeks, had increased liver steatosis and fibrosis. Surprisingly, they had improved insulin sensitivity compared to the control obese mice [261]. Interestingly, after longer term supplementation with *D. piger* ATCC 29098, the mice gained significantly less body weight compared to the high-fat diet fed controls and had less hepatic fat accumulation but increased fibrosis [261]. *D. piger* supplementation caused exacerbated intestinal damage induced by the high-fat diet as measured by villus length, crypt depth, ZO-1 expression and intestinal permeability [261]. Another study on GF mice colonized with a consortium of microbes, including *D. piger* strain GOR1, demonstrated that a diet with high fat/high sucrose increased levels of *D. piger* compared to a low fat/high plant polysaccharide diet [212]. *D. piger* growth was enhanced through trophic interactions with bacteria, such as *B. thetaiotaomicron*, that released sulfates from host sulfated glycans [212]. On the contrary, another study demonstrated that *D. piger*

abundance increased by supplementation with chondroitin sulfate in mice improved oral glucose tolerance, reduced food consumption and enhanced glucagon-like peptode-1 and insulin secretion [262]. Taken together, these mouse studies remain inconclusive on the effects of intestinal *D. piger*, and this may be due to the use of different strains within the studies.

1.2.7 Next-generation probiotics

As the intestinal microbiota is inextricably linked with host health, developing microbiota-targeted treatments represents an emerging therapeutic strategy for diseases [263, 264]. The advances in metagenomic research have led to the discovery of a wide range of microbes that have the potential to be developed into next-generation probiotics. Traditional probiotics are live strains of well-studied bacteria, including *Lactobacillus* spp. and *Bifidobacterium* spp., that are typically used as supplements for improving digestive health [264]. They come in a variety of forms, including fermented foods, tablets, capsules, liquids, or powders. Whereas next-generation probiotics consist of a broader range of microbes that are newly discovered or less common in the literature and are targeted to treat or cure specific health conditions [264]. The ultimate goal of next-generation probiotics is to be able to customize therapy depending on an individual's unique intestinal ecological needs, taking a step towards personalized medicine.

Approximately 70% of species detected in human microbiota metagenomic data remain uncultured, offering vast untapped potential for identifying strains that may benefit human health [265, 266]. The development and research into next-generation probiotics is well underway. For example, supplementation with *Anareobutyricum soehngenii* improved insulin sensitivity in individuals with metabolic syndrome [267]. Another study demonstrated that a probiotic formulation containing inulin, *Akkermansia muciniphila, Clostridium beijerinckii, Clostridium butyricum, Bifidobacterium infantis* and *Anaerobutyricum hallii*, supplemented to individuals diagnosed with type 2 diabetes improved postprandial glucose control [268]. We are at the precipice of a surge of discoveries that have the potential to transform human health through next-generation probiotic supplementation once we overcome key challenges in the field.

A few prominent hurdles remain in developing next-generation probiotics. First, most intestinal microbes are specifically adapted to the intestinal environment making them extremely oxygen sensitive. Hence, developing methods to adapt candidate microbes to tolerate oxygen would facilitate the growth of sufficient quantities for large-scale production and distribution of viable next-generation probiotics to treat individuals. Second, the intestinal environment of each individual represents its own unique ecological microcosm with specific requirements for trophic interactions, conditions and energy sources. Hence, pinpointing microbes for next-generation probiotics that can successfully benefit the host may necessitate a symbiotic consortium of microbes.

1.2.8 Summary

To summarize, for the advancement in the field of next-generation probiotics, mechanistic insights into the trophic interactions between microbes could facilitate development of an effective probiotic-based formulation. Several metagenomic studies have revealed *F. prausnitzii* as one of the potential candidates for a next-generation probiotic, as it is regarded as one of the most abundant butyrate-producing intestinal microbes with anti-inflammatory properties. Butyrate-producing bacteria have been found to be reduced in obesity, type 2 diabetes, and atherosclerosis. Thus, an *F. prausnitzii* based next-generation probiotic could be beneficial to these individuals by modulating intestinal butyrate production. Exploiting the synergy between *F. prausnitzii* and *D. piger*, via lactate-acetate cross-feeding, may allow for growth and altered intestinal community structure that could benefit the host. Further, this trophic interaction may enable sufficient growth for industrial production for human consumption, which is explored in **Paper II**. Moreover, we aimed to delineate the strain level differences which could explain discrepancies found in the literature that associate *D. piger* with disease, investigated in **Paper III**.

2 Aims

2.1 Paper I

Determine the effect of lipoxins on neutrophils from patients with atherosclerosis

SPMs represent a potential therapeutic strategy utilizing the body's endogenous mechanisms to resolve the chronic inflammatory state of individuals with atherosclerosis. The aim of this project was to assess the effects of lipoxins on neutrophils from patients with atherosclerosis and compare to a control population.

1. Determine ROS production capacity of neutrophils at baseline and in response to lipoxin treatment
2. Measure the expression of receptors on neutrophils at baseline and when treated with lipoxins
3. Quantify neutrophil transmigration across lymphatic vasculature in response to lipoxin treatment

The results of this study demonstrate that lipoxins can have a pro-resolving effect on neutrophils from patients that have diagnosed atherosclerosis. However, we also demonstrate the ability of lipoxins to have opposing effects on neutrophils depending on whether they were from patients with atherosclerosis or controls.

2.2 Paper II

Identify and develop obligate anaerobes into next-generation probiotics

With the anaerobic environment of the intestine, the majority of microbes are obligate anaerobes. The aim of this project was to identify potential strains that can be developed into next-generation probiotics for human consumption by increasing oxygen tolerance and biomass of extremely oxygen sensitive microbes.

1. Identify strains of *F. prausnitzii* to be developed as next-generation probiotics
2. Adapt *F. prausnitzii* to become oxygen tolerant and compare properties of adapted *F. prausnitzii* to the parent strain
3. Determine the cross-feeding mechanism between *F. prausnitzii* and a co-isolated *D. piger* strain
4. Assess safety and tolerability in mice and humans of the next-generation probiotic formulation containing the adapted *F. prausnitzii* and *D. piger*

In this study we developed a method for increasing oxygen tolerance of the extremely oxygen sensitive *F. prausnitzii*. Further, we identified trophic interactions between *F. prausnitzii* and *D. piger* which were found to be safe for human consumption.

2.3 Paper III

Demonstrate inter-strain variation in *D. piger*

Characterization of the complex communities in the intestinal microbiota has often relied on 16S ribosomal RNA (rRNA) gene sequencing. This method does not have sufficient resolution to elucidate variations in the genome or in the functionality of species or strains. Hence, important variations between strains may occur unnoticed. Due to the lack of high-resolution data, the role of *D. piger* in disease remains controversial. The aim of this paper was to study *D. piger* inter-strain variation on a phenotypic level.

1. Obtain isolates of bacteria genomically related to *D. piger* FI11049 to compare inter-strain variation
3. Characterize inter-strain variation in terms of growth, metabolism, stress response, motility and morphology
4. Measure the inflammatory modulation capacity of strains on intestinal epithelial cells

The results from this study demonstrated that there are phenotypic variations between strains. Elucidating how these phenotypic differences can translate to effects on intestinal microbiota may provide insight into distinguishing associations of *D. piger* with disease.

3 METHODOLOGICAL CONSIDERATIONS

For a detailed description of methods and patient characteristics please refer to the methods section for each paper.

3.1 Human cohorts

In **Paper I**, an *ex vivo* and *in vitro* approach is used by taking the blood from patients scheduled for vascular surgery for revascularization and from a control group of individuals that were not diagnosed or treated for diabetes, hypertension, or hyperlipidemia. By using patient samples compared to control groups, we were able to identify variations in response of neutrophils. This study was limited in the population size as only ten were enrolled for each group and should thus be considered a pilot study. It is important to have sufficient power in human studies as there is much more heterogeneity compared to research on animal models or *in vitro*.

In **Paper II**, we used a cohort of humans to assess the safety and tolerability of *F. prausnitzii* and *D. piger*. In a randomized placebo-controlled study, a low (1×10^8–5×10^8 colony forming units (CFU) per capsule) or high (1×10^9–5×10^9 CFU per capsule) dose was given to 50 healthy men and women between the ages of 20-40. A placebo arm was added to control for non-specific effects that may influence the outcome of the study, such as potential side effects. However, as we found that *D. piger* DSM 32187 and the parental *F. prausnitzii* DSM 32186 were highly prevalent in healthy individuals at baseline (43/43 and 35/43, respectively), there was likely a very small risk for healthy individuals.

Regulatory agencies usually require robust safety data from healthy volunteers in phase 1 clinical trials prior to testing in patients. Testing in healthy volunteers reduces effects of confounding variables, such as medications or disease status, and provides insight into safe dosage levels or potential side effects. It allows for the measurement of a "normal" physiological response that would allow for further understanding of potentially "abnormal" reactions in later phases of drug/supplement

development when testing on patients. Further, testing in a healthy cohort may provide insights into the mechanisms and potential therapeutic applications.

3.2 Mouse models

Prior to human clinical trials, animal models are used to assess safety of any potential therapeutics. In **Paper II** Swiss Webster mice were used to assess the safety of *F. prausnitzii* DSM 32379 and *D. piger* DSM 32187. In the last century, major strides in biomedical research have been made due to the use of mouse models. Humans and mice share over 90% similarity in their genes, allowing for discovery of genes involved in certain pathways [269]. Mice have a short generation time, are small and easy to handle and maintain, which are all important advantages in using them as models to study disease [269]. Moreover, manipulating microbiota composition or creating gene knockouts permits functional and mechanistic studies in host-symbiont interactions [270].

Despite substantial homology in host genes between mice and humans, there are distinct physiological and genetic differences that limit the use of mice as research models. Numerous therapies that have been proven effective in mouse models of disease ended up failing in humans [269]. In gut microbiota research, mouse models are widely used to disentangle causation and correlation, but it cannot be excluded that bacterial species maybe unable to colonize in mouse models due to host specificity [271].

There are several differences between mice and humans that can affect the microbiome. Most humans have a complete emptying of their gastric contents prior to the next meal, whereas mice eat more consistently with very short time delays between meals, altering transit time [272, 273]. Mice also harbour a distinct bile acid profile compared to humans which can select for microbes [274]. Nutrition, exercise, and environmental exposure all evidently impact the gut microbiota. Even if humans and mice gut communities share 90% phyla and 89% genera [275], there is still colonization resistance of host-specific microbes [276, 277]. Further, there are distinctions in the immune system [269]. Differences in mouse microbiota, diet, environmental exposure, and genes, can limit translational applicability when using these models for probiotic development.

3.3 *In vitro* cell models

3.3.1 Lymphatic endothelial cells

Lymphatic endothelial cells used in research are isolated from either the skin tissue of adults undergoing panniculectomy or from neonatal foreskins [58]. For **Paper I**, human dermal lymphatic microvascular endothelial cells from skin tissue of adults were used. Adult lymphatic endothelial cells are considered a better model when studying adult immune cell migration since the neonatal immune system is immature [278, 279]. However, the model is limited in that most donors suffer from metabolic disease which can impact lymphatic function [61]. To mimic lymphatic drainage of neutrophils from peripheral tissues, transwells were flipped and the bottom was coated with 0.1% gelatin as a basement membrane prior to adding lymphatic endothelial cells, allowing a monolayer to form after 3 days of growth. Prior to experiments, the transwell was flipped right-side up and the apical well simulates the tissue, and the basal well the lumen. This model is used to study interstitial flow from tissue to the lumen through the lymphatics.

3.3.2 Neutrophils

In **Paper I**, circulating neutrophils were isolated from plasma by using negative bead isolation. Negative bead isolation utilizes antibodies to bind receptors that are expressed on circulating immune cells which are not neutrophils. We optimized negative bead isolation for neutrophils by combining the EasySep Direct Human Neutrophil Isolation Kit (STEMCELL Technologies) with the EasySep Pan-Granulocyte Iso-lation Cocktail (STEMCELL Technologies) to achieve a purer population of neutrophils as characterized by 98-99% of $CD45^+$ $CD16^+$ $CD66b^+$ neutrophils. Positive bead isolation is another method which can be used to obtain a pure population by binding directly to neutrophil receptors. However, positive bead isolation has its limitations when using the cells for downstream experiments as it can result in activation of neutrophils.

3.3.3 Intestinal epithelial cells

To explore the effects of various molecules, whether diet-derived or from drugs, Caco-2 cells are one of the most widely used intestinal models. They are derived from a colon carcinoma and upon confluency, spontaneously differentiate by developing characteristics that mimic absorptive enterocytes [280]. They form tight junctions between adjacent cells, acquire an apical brush border with microvilli, and express enzymatic activities and transporter proteins similar to enterocytes [280]. Caco-2 cells are easy to propagate and maintain. The ease of this cell line combined with its enterocyte-like properties make it the ideal high-throughput model to study transport, drug effects, interactions with the gut microbiota, associated metabolites and bioactive food components. However, its use as a gut epithelial model has several limitations. It lacks a mucus layer, and the intestine contains several cell types. Further, the uptake or transport of substances can be modulated by other molecules in the intestine, such as bile acids and phospholipids, which are missing in this model [280]. Caco-2 cells have been propagated in multiple laboratories across the world resulting in numerous lines, including HTB-37. Additionally, a notable limitation is that these cells were derived from a colon carcinoma originally used to study cancer development [280]. Nevertheless, they are now widely used as human intestinal epithelial cell models [281]. Although they have features that resemble enterocytes, these limitations are important to consider when extrapolating conclusions.

In **Paper II** and **Paper III**, Caco-2 HTB-37 cells are used to explore the inflammatory effects of bacterial spent growth media. Caco-2 HTB37 cells exposed to IL-1β have a robust pro-inflammatory response. One of the roles of IL-8 is as a chemoattractant for neutrophils, secreted by intestinal epithelial cells in response to inflammation. Thus, measurement of IL-8 secreted from Caco-2 cells in response to stimuli is one readout of inflammatory modulation.

3.4 Flow cytometry

Flow cytometry is a specialized and powerful technique which allows detection and measurement of different chemical and physical properties of cell populations or particles. Characteristics of cells, such as size, granularity, surface/intracellular receptors, gene expression and DNA/RNA content can be measured [282]. Using a microfluidic system, a single cell/particle passes a laser which produces scattered and fluorescent light to a detector. The acquired data gives information on size (forward scatter (FSC)), granularity (side scatter (SSC)) and fluorescence. Cells can be labelled with fluorescent dyes or antibody combinations which can indicate cell type, cell cycle status, expression of proteins or protein post-translational modification [282].

3.4.1 ROS production

In **Paper I**, flow cytometry was used to measure intracellular neutrophil ROS production using a PhagoBurst assay where dihydrorhodamine 123 is oxidized to fluorescent rhodamine 123 by intracellular ROS. From whole blood, the neutrophil populations were gated based on $CD16^+$, FSC and SSC. Neutrophils producing ROS were identified, and the amount of ROS produced per cell quantified using the rhodamine 123 fluorochrome (**Paper I**, Supplementary Figure S1A, B).

3.4.2 Integrin expression

In **Paper I**, the baseline circulating neutrophil integrin expression and in response to lipoxin treatment in whole blood were measured using flow cytometry. In addition to the gating strategy for neutrophils outlined above, cells were labelled with antibodies against the LXA_4 receptor, FPR2/ALX, and high affinity CD11b (**Paper I**, Supplementary Figure S1C, D).

3.5 Molecular methods in gut microbiota research

Over the last two decades, research on the intestinal microbiota has undergone remarkable expansion and the gut microbiota is now recognized as our "last organ" [283]. Numerous factors must be taken into consideration when studying the intestinal microbiota and health. To begin with, there is no consensus on what a "healthy" microbiota is, particularly in terms of species/genomic composition. Each individual has unique microbiota that can be equated to a fingerprint that has been shaped by genetics, environmental exposure, diet and lifestyle [284, 285].

3.5.1 Use of faeces

The majority of intestinal microbial communities in humans have been characterized using DNA extracted from faecal samples. While specific microbes exist along the intestinal tract, invasive sampling from different sections of the intestine remains a challenge that may result in the oversight of certain bacteria associated with the mucosa [285, 286]. Thus, when extrapolating conclusions on faecal microbiota composition, we must acknowledge that these samples are not representative for microbes close to the mucosal surface or in the small intestine. In **Paper II**, we identified potential next-generation probiotics isolated from faeces. Whole genome sequencing has mostly been performed on DNA from faecal samples, thus, health associations have been made with microbes that were detected.

3.5.2 Isolation of specific bacteria

To isolate specific microbes such as SRB, we used selective media that contains nutrients and metabolites specific for their growth. Postgate's media (PGM) contains a high amount of lactate (as an electron donor) and sulfate (as an electron acceptor) [287]. Further, when sulfate is reduced to hydrogen sulfide, it interacts with the iron in the media forming a dark precipitate of iron sulfides that allows for identification of SRB. In **Paper II** and **Paper III**, SRB were isolated using this technique through a series of subculturing steps. By selecting colonies that turned black, we were able

to select hydrogen sulfide producing bacteria. Potential SRB *D. piger* candidates for **Paper II** were assessed by negative Gram-staining (Figure 6).

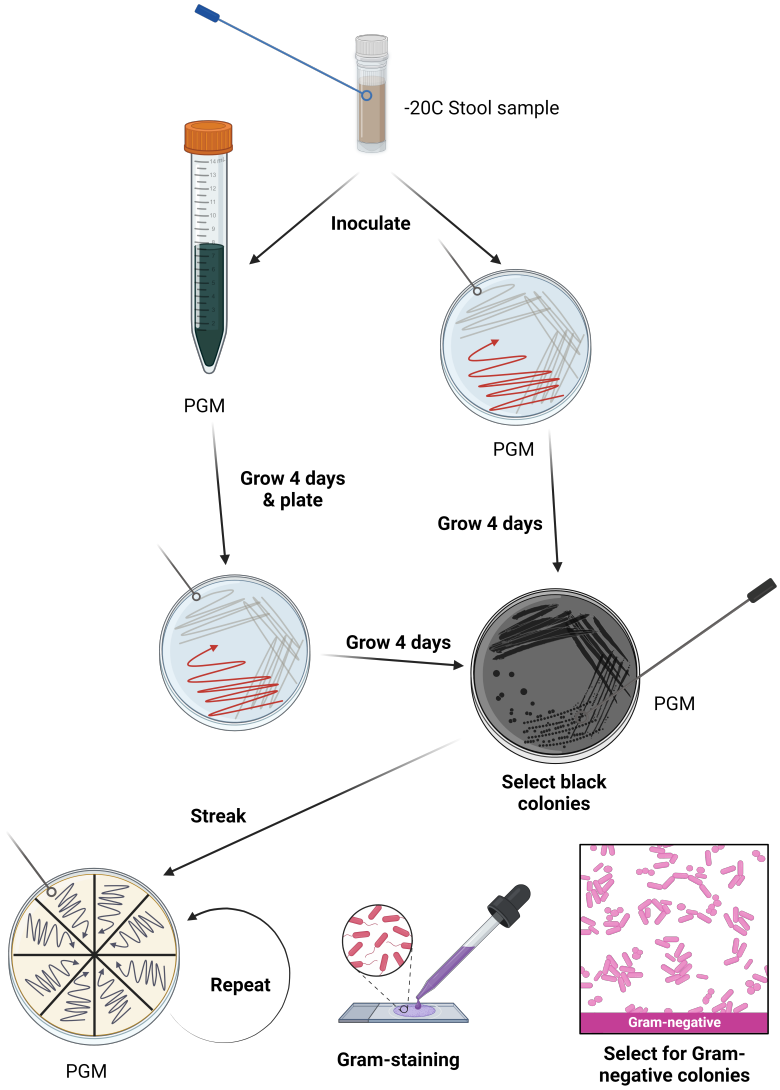


Figure 6. Isolation of sulfate-reducing bacteria, Desulfovibrio piger, in Postgate's media (PGM) from faecal samples. *Faecal samples were inoculated into PGM broth or agar. They were sub-cultured onto agar plates, and black colonies were selected based on the formation of black iron sulfides which form due to the production of hydrogen sulfide and subsequent reaction with iron in the media. They were sub-cultured until pure colonies were obtained based on gram-staining. Gram-negative isolates were selected for further sequencing to identify D. piger isolates. (Created with Biorender)*

3.5.3 Bacterial lab adaptations

When isolating bacteria from faecal samples, it is important to note that the bacteria begin to adapt to laboratory conditions even after overnight growth [288]. Mutations appear that can increase the fitness under the conditions of the media or that are rare in the original stock [288, 289]. Therefore, in **Paper III**, when evaluating *D. piger* characteristics, growth, and metabolism, it is important to consider that it is likely not mimicking what is happening in the intestinal environment.

3.5.4 Characterization of bacteria

There are numerous tools to characterize microbes *in vitro*. Growth curves provide insight into cell physiology and growth kinetics in different conditions. Growth curves can be determined using optical density (OD), protein concentration, qPCR, or CFU counts. All methods have their benefits and limitations. In **Paper II**, as CFU counts reflect the viability of microbial cells, this measurement of bacterial cell counts was performed for next-generation probiotic development. To calculate CFU count, serial dilutions of bacterial suspension were streaked on an agar plate and incubated for a specified time (corresponding to the stationary growth, to allow for the detection of the maximum number of viable cells present in the dilution). Colonies were then counted on the plate which grew between 30-300 colonies to calculate CFU/mL [290]. To perform the growth curve in **Paper III**, protein concentration was measured using a bicinchoninic acid assay. This method allows for the detection of the total microbial biomass produced, independent of cell viability, and it is therefore a sufficient method to estimate growth yields as a function of nutrient sources. For a rapid and simplified measurement to assess *D. piger* growth in different conditions after 24 hours, OD measurements were performed. OD measures the turbidity of the solution which can depend on multiple factors, including cell size, number of cells, metabolites produced and length of the light path [291]. In **Paper III**, an aliquot of bacterial suspension was put into a microtiter plate and read on a spectrophotometer at a wavelength of OD 620 nm.

In **Paper III**, we used analytical profile index (API) kits to investigate microbial metabolism of different substrates. Traditionally, API kits were

created for identification of medically relevant bacteria, so they are only capable of distinguishing known species. The kits contain dehydrated substrates to assess enzymatic activity. Bacteria are suspended in a specialized media and left for a set number of hours before observing changes in colour, either spontaneously, or after adding revealing agents. If the wells change colour to that described in the manual of the kit, this can indicate enzymatic activity for the specific substrate.

In **Paper III**, we also explored the ability of *D. piger* to use different electron acceptors or electron donors *in vitro*. We either replaced the lactate or the sulfate in PGM to see if *D. piger* could still grow after 24 hours. It is important to note that this only demonstrates the ability of *D. piger* to utilize different electron donors or acceptors under the specific conditions in PGM for a limited time. It is possible that *D. piger* has the ability to use different sources of electrons or to donate electrons to other sources, but it may take more time or additional co-factors.

3.5.5 Genomic analyses

In **Paper III**, we explored the role of inter-strain variation in *D. piger*. A strain is defined as a "set of genetically similar descendants of a single colony or cell" and a clade is defined as a "group of taxonomic entities composed of one ancestor and all of its evolutionary descendants" [239]. In **Paper III** we sought to isolate a strain of *D. piger* that clusters in the same clade as the previously sequenced genome of the strain FI11049 to compare it with the *D. piger* strain DSM 32187 from **Paper II** and the type strain, DSM 749. DNA was extracted from *D. piger* strains, MTK1492.2 and DSM 749, which had not been sequenced previously, to try to generate a complete genome. It remains a challenge to get sufficient good quality DNA from *D. piger*. We were able to obtain sufficient high-quality DNA to sequence both long and short reads for *D. piger* DSM 32187 in **Paper II**. However, when repeating this for DSM 749 and MTK1492.2, we were unsuccessful at the given time. Thus, in **Paper III**, *in silico* analysis to create a phylogenetic tree to group *D. piger* strains into different clades was performed on the draft genomes. The draft genomes were assembled based on overlapping areas of the short reads, forming what is known as contigs. Contigs may not overlap, resulting in gaps in the genome where there is no nucleotide information. Combining long reads and short reads can allow

for the formation of a complete genome as the long reads can bridge the gaps between the contigs. Draft genomes can result in a loss of genetic information, high rates of error, potential contaminants, and a decreased ability to distinguish additional plasmids [292]. Metagenome-assembled genomes (MAGs) are generated through a computational process that assembles microbial genomes from metagenome data. MAGs are a useful tool to have an overview of the metabolic potential of the intestinal microbiota, however, when zooming in to investigate inter-strain variation, they are insufficient due to the possible loss of genetic information. However, culturing and isolating individual strains allows us to generate complete genomes. In **Paper III** we isolated the *D. piger* strain MTK1492.2 and sequenced it along with DSM 749. We will be generating complete genomes of these two isolates to perform downstream analyses to compare genomic inter-strain variation.

4 RESULTS AND DISCUSSION

4.1 Paper I

4.1.1 ROS production in neutrophils

Intermittent regulated bouts of neutrophil ROS production play an essential role in the innate immune response [29]. Activated neutrophils in circulation migrate across the endothelium to the site of inflammation in response to chemoattractants, where they engulf pathogens into a vacuole. Intracellular ROS is produced to kill the trapped pathogens inside the vacuole. This respiratory burst is regulated by NADPH oxidase [22]. Upon activation, the separated transmembrane proteins and soluble proteins making up NADPH oxidase assemble to become activated. However, continuous activation can be detrimental to the surrounding healthy tissues and result in a chronic low-grade inflammation.

We demonstrated that neutrophils derived from the circulation of patients with diagnosed atherosclerosis have a higher baseline production per cell of ROS. In addition, in response to the inflammatory stimuli, fMLP or *E. coli,* the percentage of neutrophils producing ROS was higher from patients with atherosclerosis compared to controls. The amount of ROS produced per cell was also higher in neutrophils from patients with atherosclerosis upon fMLP stimulation. Upon exposure to PMA, the neutrophils from both groups produced the maximum amount of ROS, as expected. In healthy individuals, circulating neutrophils in a resting state maintain a separation between the NADPH oxidase proteins, as described above. Certain stimuli, such as pro-inflammatory cytokines, like TNF-α, fMLP or GM-CSF, can result in 'priming' of neutrophils where they do not necessarily induce activation but result in assembly of NADPH oxidase. This prepares the neutrophils for a more rapid ROS production response [22]. Thus, increased ROS production at baseline and in response to fMLP and *E. coli* stimuli in patients with atherosclerosis indicates previous priming of circulating neutrophils. Increased circulatory levels of the inflammatory marker CRP in patients with atherosclerosis further validates

that neutrophils from these individuals were surrounded by an inflammatory environment in the circulation leading to neutrophil priming.

The known LXA_4 receptor, FPR2/ALX, also binds fMLP and depending on the ligand can induce a pro-inflammatory or pro-resolving response [293, 294]. We demonstrated that the percentage of neutrophils expressing FPR2/ALX decreased in both patient and controls in response to fMLP stimulation, however, the amount expressed per cell did not differ. FPR2/ALX expressed on neutrophils can recognize fMLP and result in activation or priming [295]. CD11b is constitutively expressed on all neutrophils. Upon activation in response to fMLP treatment, CD11b expression increased on neutrophils from both controls and patients with atherosclerosis, similar to what has previously been shown [296]. Increased CD11b expression on activated neutrophils enhances their ability to adhere to the vascular endothelium [296].

In patients with atherosclerosis, lymphatic clearance is impacted [60]. Both neutrophils isolated from the blood of patients or controls transmigrated across an *in vitro* monolayer of lymphatic endothelial cells towards IL-8. To summarize, neutrophils from patients with atherosclerosis are more primed to produce ROS as compared to controls, however, their receptor expression of CD11b and FPR2/ALX, and their ability to migrate across lymphatic endothelial cells does not differ at baseline.

4.1.2 Lipoxin effects on neutrophils

Treating whole blood from patients with atherosclerosis with LXA_4 or LXB_4 reduced the number of ROS-producing neutrophils and the amount of ROS produced per cell in response to subsequent stimuli with fMLP. In contrast, treating whole blood from healthy controls with LXA_4 increased *E. coli*-induced ROS production per cell, while LXB_4 increased the number of ROS producing neutrophils and the amount of ROS produced per cell in response to both fMLP and *E. coli*. This indicates that lipoxins may prime neutrophils from healthy controls to produce ROS, while having a pro-resolving effect on neutrophils from patients with atherosclerosis. Lipoxin treatment did not impact the expression of FPR2/ALX. However, lipoxin treatment downregulated the amount of CD11b high affinity conformation expression per neutrophil from patients with

atherosclerosis. This emphasizes the pro-resolving capabilities of lipoxins in neutrophils from patients with atherosclerosis as CD11b mediates platelet-leukocyte aggregation and transendothelial migration [98]. Upon treatment with LXB_4, neutrophils from patients with atherosclerosis transmigrated at a higher rate across lymphatic endothelial cells. This further demonstrates that LXB_4 has pro-resolving potential, but only in neutrophils from patients with atherosclerosis. These findings highlight the importance of considering treatment options for patients depending on their inflammatory status.

4.2 Paper II

4.2.1 Identification of bacterial trophic interactions

We demonstrated that trophic microbial interactions can be important for the development of next-generation probiotics. By plating faecal samples from a healthy individual directly onto PGM selective media, we found that a strain of *F. prausnitzii*, DSM 32186, was able to grow as a co-culture with a strain of *D. piger*, DSM 32187. Had the faecal samples been inoculated into selective liquid broth media and diluted as normal praxis, detection of this potential symbiotic relationship might not have been possible given the difficulty in discerning the interaction between two colonies in liquid, potentially leading to oversight. Further, it is likely that individual *F. prausnitzii* cells would not have been able to grow in this broth medium due to the absence of glucose and other nutrients required by this species.

The complete genomes of *F. prausnitzii* DSM 32186 and *D. piger* DSM 32187 were sequenced. *D. piger* DSM 32187 clustered with previously sequenced *D. piger* strains, and *F. prausnitzii* DSM 32186 clustered with *F. prausnitzii* phylogroup II. As both genomes formed their own clades in the tree, we hypothesized that they represent previously uncharacterized strains. By adding glucose to PGM, we demonstrated that *F. prausnitzii* DSM 32186 grew significantly more when in co-culture with *D. piger* than alone. Further, after 24 hours, SCFAs were measured in the mono- and co-cultures. In mono-culture, there was little metabolic activity of *F. prausnitzii*. *D. piger* consumed lactate and produced acetate. However, when the two were in the co-culture, a significant amount of butyrate and lactate was produced. This represents a cross-feeding mechanism whereby *D. piger* consumes lactate (from the media and *F. prausnitzii*) to produce acetate, without consuming glucose, and *F. prausnitzii* uses glucose in the media and the *D. piger* produced-acetate to produce lactate and butyrate. In the symbiotic relationship, *D. piger* acts as an electron sink by consuming lactate to yield acetate for butyrate production by *F. prausnitzii*.

4.2.2 Adapting bacteria to become oxygen tolerant

Two major hurdles in next-generation probiotic research are to be able to adapt intestinal microbes to tolerate oxygen along with ensuring sufficient biomass to be able to develop a product to be given to humans [264]. We developed a method to adapt the extremely oxygen sensitive *F. prausnitzii* to tolerate oxygen. We gradually exposed *F. prausnitzii* DSM 32186 to oxidizing conditions that permitted adaptation to develop oxygen tolerance. The adapted strain, DSM 32379, was isolated after 10 generations. DSM 32379 still produced butyrate and had synergistic growth with *D. piger* DSM 32187, which was already oxygen tolerant. The adapted *F. prausnitzii* DSM 32379 also maintained anti-inflammatory effects. This was assessed by the reduction of IL-8 secretion from IL-1β-stimulated Caco-2 cells due to protection from the spent media of *F. prausnitzii* strains.

The oxygen tolerant strain retained ant-inflammatory properties, and we were able to demonstrate that it could yield sufficient biomass in co-culture with DSM 32187, adequate for production of formulations for human consumption. Further, based on stability criteria, we demonstrated that freeze-dried DSM 32379 could be stored at -20 °C for 2 weeks. Thus, *D. piger* DSM 32187 and *F. prausnitzii* DSM 32379 could be suitable as a next-generation probiotic for human consumption.

4.2.3 Next-generation probiotic formulation safety

Safety studies in mice and humans were performed. Bacterial suspensions of *F. prausnitzii* DSM 32379 and *D. piger* DSM 32187 were gavage-fed to Swiss Webster mice 5 times during the first week, and twice a week for the following 3 weeks and were well tolerated. There was no change in the caecal levels of either strain as quantified by qPCR. This may be due to the mode and frequency of administration, human host-specificity of the strains, colonization resistance from the mouse microbiota, as well as colonization in other parts of the gastrointestinal tract.

The bacteria capsules of low (1×10^8–5×10^8 CFU per capsule) and high dose (1×10^9–5×10^9 CFU per capsule) were well tolerated when administered to a group of healthy men and women aged between 20 and 40 years in a randomized placebo-controlled study for 8 weeks. There were no changes in the blood biochemistry, faecal SCFAs or hydrogen sulfide from

baseline to 8 weeks after administration in either group. Whole-genome metagenomic sequencing revealed no differences between groups or within each group in overall composition between the baseline or after administration However, the group receiving the high dose had increased reads for *D. piger* at the species level after 8 weeks, while the *F. prausnitzii* levels remained unchanged. This may be because the parental strain, DSM 32186, of the oxygen-tolerant *F. prausnitzii,* DSM 32379 strain, was already present in all participants at baseline (43/43), saturating the niche. The percent abundance of *F. prausnitzii* was between 3.4% and 25.9%, which is within the range that has been previously found in healthy cohorts. Using genome capture to quantify the specific strain *D. piger* DSM 32187, we found that it was increased in the high-dose group, with a noticeable increase in individuals with a low baseline relative abundance, indicating that the niche may not have been saturated. Terminal genes for butyrate production were significantly positively correlated with the increase in *D. piger* DSM 32187 after 8 weeks. This suggests that *D. piger* DSM 32187 may support overall butyrate production of the human gut microbiota.

4.3 Paper III

We isolated a D. *piger* strain in **Paper II** that had promoted the growth and butyrate production of *F. prausnitzii*. In the literature, reports on *D. piger* have opposing findings for associations with health parameters [260, 266] or disease [211, 218, 259, 297]. Thus, we hypothesized that the discrepancies may be due to a lack of resolution in metagenomics analyses, and that inter-strain variation may explain differences in the associations.

4.3.1 Isolation of *D. piger* strains

Previous reports on *D. piger* have been represented in metagenomic studies largely by the complete genome of FI11049, isolated from a patient with colitis [211]. As this strain was not available, we sought to isolate it from human faecal samples. We isolated *D. piger* strain MTK1492.2 using modified PGM containing peptone. Phylogenetic analyses of the draft genomes demonstrated that MTK1492.2 clustered with FI11049, separately from the *D. piger* strain from **Paper II**, DSM 32187 and the type strain DSM 749. Although these analyses were performed on draft genomes, to confirm these findings, closed genomes will be necessary.

4.3.2 Phenotypic characteristics of *D. piger* strains

Phenotypic characteristics of MTK1492.2 and DSM 32187 were compared to the type strain DSM 749 to explore inter-strain variation. Grown anaerobically in PGM, MTK1492.2 grew slower than the other two strains, while DSM 32187 converted lactate to acetate the fastest. Motility also differed between the strains, as all three could swim, however, only MTK1492.2 could not swarm. All three strains had the ability to use thiosulfate, but not nitrate or sulfite, as an electron acceptor, instead of sulfate. Further, they could also use pyruvate as an electron donor. DSM 749 and MTK1492.2 could grow in 1% NaCl, but DSM 32187 could not. The variations between the strains may provide insight into the contradicting associations of *D. piger* prevalence in different populations.

4.3.3 Anti-inflammatory effect of *D. piger* strains

Studies have found increased *D. piger* prevalence in individuals with IBD compared to healthy controls [218, 298]. Interestingly, we demonstrated

that the spent media from *D. piger* strains did not induce an inflammatory response as measured by IL-8 secretion from the intestinal epithelial cell model, Caco-2. On the contrary, treatment with IL-1β induced IL-8 secretion from Caco-2 cells and *D. piger* spent media diluted 1:4 and 1:10, but not 1:100, reduced IL-8 secretion in this experimental setup. In four independent experiments, we found that when the spent media was diluted 1:4 or 1:10, DSM 32187 and DSM 749 lowered the IL-8 secretion more than MTK1492.2. Further, we also demonstrated that this was not due to the cells dying in response to the *D. piger* spent media. The anti-inflammatory effect may be due to the presence of hydrogen sulfide as previous research has demonstrated its ability to reduce inflammatory response [182].

Taken together, these findings demonstrate that there are inter-strain variations in phenotypic characteristics and potentially the anti-inflammatory properties within the *D. piger* species. However, biological triplicates will be performed to statistically validate the potential anti-inflammatory differences we observed.

5 CONCLUSION

- When developing therapeutic strategies with SPMs, special caution should be taken to evaluate the inflammatory status of the patient receiving treatment. Lipoxin treatment had opposing effects on neutrophils depending on whether they were isolated from a healthy control or a patient diagnosed with atherosclerosis.

- For the development of next-generation probiotics, trophic cross-feeding interactions can be considered for achieving cost-effective production for human consumption. Further, oxygen tolerance can be acquired by extremely oxygen sensitive bacteria that allows for easier production and longer shelf life. Further, the oxygen adapted strain of *F. prausnitzii* retains its beneficial anti-inflammatory properties.

- When evaluating associations of a particular species with disease, it is essential to look at differences within the strains. While this is well known for classical probiotics (e.g., *Lactobacillus* and *Bifidobacterium* strains), less attention to strain heterogeneity has been given for potential next-generation probiotic candidates, likely due to the more recent characterizations. Phenotypic analyses of *D. piger* demonstrated inter-strain variation that provides insight into the potential rational for the contradictory associations of *D. piger* with disease in the literature.

6 LIMITATIONS AND FUTURE PERSPECTIVES

Intermittent, controlled bouts of inflammation in response to injury and infections are essential for our survival. However, as modern-day lifestyle factors continue to promote a chronic state of systemic inflammation, the rise of non-communicable diseases is a significant burden, and our "healthcare" system is turning into a "disease-care" system. Thus, understanding pathways to promote resolution and/or restoring intestinal gut microbes with immunomodulating properties was the aim of this thesis.

Promoting inflammatory resolution in individuals with atherosclerosis represents a promising therapeutic strategy to break the vicious loop of inflammation. **Paper I** sets the foundation to explore the role of lipoxins in modulating neutrophils from patients with atherosclerosis. One of the major limitations of this study was the sample size. Future studies would benefit from increased participants as well as a balance in males and females. To increase robustness, it would also be beneficial to have age-matched controls to account for the possibility of "inflammaging" [299]. Further, elucidating the mechanisms by which lipoxins modulate neutrophil ROS production, integrin expression and lymphatic migration could identify novel potential pathways for additional drug targets to promote resolution. Characterizing neutrophils from lipoxin treated or omega-6 supplemented mouse models of atherosclerosis, such as *Apo* -/- [300], could be the next step to validate our *in vitro* findings *in vivo*.

Interestingly, in **Paper I**, we demonstrated that LXB_4 can enhance neutrophil ROS production from "healthy" controls. To our knowledge, lipoxins, particularly LXB_4, have not been shown to enhance inflammation by priming neutrophils to produce ROS. Identifying the mechanism by which LXB_4 can prime neutrophils would provide more insight into understanding the fine-tuned balance of lipid-class switching and the role of this SPM. Regardless of FPR2/ALX expression, lipoxins were able to decrease ROS production, indicating that there are other receptors by

which they may be acting. Future studies on identifying additional lipoxin receptors and their downstream pathways are of great interest.

As lymphatic dysregulation plays a key role in atherosclerosis, identifying the mechanism by which LXB_4 can enhance migration of neutrophils would also be of interest. To explore whether enhanced migration is due to the ability of LXB_4 treatment to downregulate CD11b, making the neutrophils less "sticky", one could pre-treat the neutrophils, then wash and subsequently add to the transwell to measure migration. CD11b antibodies could also be used to try to block the binding capacity to the lymphatic endothelium. These experiments would determine if the reason for enhanced lymphatic migration upon LXB_4 treatment is due to downregulation of CD11b expression.

When looking to develop new therapies for treating the ever-increasing number of chronic inflammatory diseases, utilizing the body's endogenous mechanisms for resolving inflammation is another approach by which we can tackle the chronic inflammatory loop.

In **Paper II**, we were able to adapt *F. prausnitzii* to become oxygen-tolerant, but we were unable to describe the molecular mechanism responsible for the development of oxygen tolerance. Future studies could use genome-wide transposon mutagenesis known as INsertion Sequencing (INSeq) to potentially identify the mechanism [301]. If the mechanism was identified, the snippet of the genome could be introduced to other extremely oxygen sensitive strains to allow them to be developed into oxygen-tolerant next-generation probiotics.

In **Paper II**, *D. piger* and *F. prausnitzii* supplementation did not alter the microbiome, SCFAs, hydrogen sulfide or levels of *F. prausnitzii* in healthy individuals, likely due to the participants being healthy volunteers with, what we would expect, a stable and resilient microbiota. Thus, exploring whether supplementation could impact the microbiota of individuals with low levels of *F. prausnitzii*, such as individuals with prediabetes, diabetes, other metabolic and/or inflammatory diseases such as atherosclerosis, would be the next step. In addition, it would be of interest to understand the impact of *F. prausnitzii* or *D. piger* on the microbiota and intestinal inflammation.

To properly illustrate inter-strain variation in **Paper III**, the genome of MTK1492.2 and DSM 749 must be completed with long reads. To make the phylogenetic trees, the MASH method used relies on first filtering genomes based on similarity of 16S rRNA gene reference strains and then the next step relies on having a database of genomes. By using MASH on draft genomes, 16S rRNA gene-based reference strains can give inaccurate distances as it does not capture the full genomic diversity. In addition, if a strain or species is not represented in the database, this may lead to inaccurate results. Further, the Genome BLAST Distance Phylogeny algorithm and the distances based on the bitscore depend on the quality of the input sequences and selected reference genomes. The accuracy of the results may be impacted by variations in sequencing or assembly. For future endeavours, high quality closed genomes are necessary to compare inter-strain variation. Further, tools that compare cluster of orthologous genes, average nucleotide identity and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analyses on the complete genomes would provide further insight inter-strain variation.

To build upon **Paper III**, additional experiments are needed to investigate the competition between strains. Bioreactors of faecal slurries could be set-up and inoculated with two strains of *D. piger* to determine which will out-compete the other depending on the growth substrates, salinity and/or growth media that resembles different diets. Further, to understand the mechanism of the IL-8 attenuating effect of *D. piger* spent media on Caco-2 cells, numerous experiments should be performed to determine if there is a specific molecule or metabolite that modulates the anti-inflammatory response. For example, future experiments could measure the effect of proteins, lipids, outer membrane vesicles, and hydrogen sulfide produced by *D. piger* on the immunomodulation of Caco-2 cells. In addition, heat-killed *D. piger* could be added to the Caco-2 cells to determine if the bacteria itself has any impact on inflammation. We could also explore the prevalence of different strains in human populations though age, geography and lifestyle. This could allow us to determine the effects of diet or environment on the intestinal environment that could create a niche for different *D. piger* isolates.

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