# The Effects of Probiotics and the Low-Carbohydrate High-Protein Diet in Modulating Atherosclerosis

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# ABSTRACT

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Antoine St-Amant

Atherosclerosis, a disease characterized by the accumulation of lipid-rich plaques in medium to large arteries, represents the most common cause of death in the world. Mitochondrial dysfunction contributes to many steps of the disease, through the production of reactive oxygen species (ROS) and by disrupting cell signalling pathways. Importantly, ROS induce the dedifferentiation of vascular smooth muscle cells, after which they migrate to the plaque region and proliferate, rendering the disease irreversible.

The western diet (W-DIET), rich in unhealthy fats and sugars, contributes to the epidemic of obesity and metabolic diseases, leading many individuals to alternative dietary patterns, including the low-carbohydrate high-protein diet (L-DIET). However, emerging evidence, both in humans and in animals, show that the L-DIET could worsen the progression of atherosclerosis, although the pathways through which the plaque buildup occurs remains largely unknown. In parallel, poor dietary patterns induce gut dysbiosis, which represents a major driver of diseases and strongly correlates with the development of cardiovascular pathologies. Hence, by restoring the integrity of the gut microbiota, probiotics supplements could prevent the buildup of atherosclerotic plaques.

Using a meta-analysis, high-resolution respirometry, immunoblotting, and polymerase chain reaction sequencing, this thesis explores the role of (1) supplements of probiotics and (2) the L-DIET in modulating oxidative stress, the mitochondria, and the gut bacterial composition. The data reported here present evidence that the L-DIET could induce mitochondrial dysfunction and gut-dysbiosis. The results also support that by improving mitochondrial integrity and biomarkers of oxidative stress, probiotics represent an affordable, low-risk alternative treatment in the prevention of atherosclerosis.

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**Acronyms**

AFLD: alcoholic fatty liver disease
Akt: protein kinase B
AMPK: 5 AMP-activated protein kinase
ApoB: apolipoprotein B
ApoE: apolipoprotein E
AS: antioxidant status
BA: bile acid
BMI: body mass index
CLD: chronic liver disease
CVD: cardiovascular disease
Cyt C: cytochrome C
EC: endothelial cell
ECM: extracellular matrix
EPC: endothelial progenitor cell
ETC: electron transport chain
FA: fatty acid
$FADH_2$: flavin adenine dinucleotide
FCCP: carbonyl cyanide-p-trifluoromethoxy phenylhydrazone
FFA: free fatty acid
GSH: glutathione
$H_2O_2$: hydrogen peroxide
HDAC: histone deacetylase
HDL: high-density lipoprotein
IL: interleukin
IR: insulin resistance
KD: ketogenic diet
LDL: low-density lipoprotein
LDLr: low-density lipoprotein receptor
LPS: lipopolysaccharides
LSEC: liver sinusoidal cell
MDA: malondialdehyde
mTOR: mammalian target of rapamycin
NADH: nicotinamide adenine dinucleotide
NAFL: hepatic steatosis
NAFLD: non-alcoholic fatty liver disease
NASH: hepatic steatohepatitis
NEFA: non-esterified fatty acid
Nf-kβ: nuclear factor Kappa B
NO: nitric oxide
$O_2^-$: superoxide anion radical
OS: oxidative stress
OxLDL: oxidized low-density lipoprotein
RCT: randomized controlled trial
ROS: reactive oxygen species
RS: reactive species

SCFA: short-chain fatty acid
SHH: sonic hedgehog ligand
SOD: superoxide dismutase
TAC: total antioxidant capacity
TC: total cholesterol
TCA: tricarboxylic acid
TG: triglyceride
TLR: toll-like receptor
TMA: trimethylamine
TMAO: trimethylamine N-oxide
TNF-α: tumor necrosis factor alpha
UCP: uncoupling protein
VDAC: voltage dependent anion channel
VSMC: vascular smooth muscle cell

# Chapter 1

Review of the Literature

## 1.1.Theorical Context

According to the *World Health Organization*, cardiovascular disease (CVD) represents the most common cause of mortality worldwide (1). The most prescribed medication to prevent CVD are statins, or 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase inhibitors, that target cholesterol synthesis pathways and act in large part by reducing low-density lipoproteins (LDL). However, besides its important side effects, statin therapy only lowers the risk of clinical events by 20–40%, highlighting the need for alternative treatments (2).

The cardiovascular system is composed of the heart, blood, and blood vessels and provides oxygen, nutrients, and hormones to the body. Arteries transport the oxygenated blood from the heart towards peripheral tissues, while veins transport deoxygenated blood in the opposite direction. The arterial wall contains 3 layers, the intima, media, and adventitia, with varying structures and functions. The intima, the innermost layer of the artery, contains connective tissues and a monolayer of endothelial cells (EC) that separates the vessel from the lumen. The media contains vascular smooth muscle cells (VSMC), which dilate/constrict to control the blood pressure and the flow exerted on the vessel. Finally, the adventitia connects the artery with vascular nerves.

## 1.2. Atherosclerosis

Atherosclerosis is a disease characterized by the formation of lipid-rich plaques in the intima of medium to large arteries. As the plaque develops, it reduces the luminal space of the blood vessel and can lead to stenosis, form a thrombus, or detach from the arterial wall and form an embolism that enters circulation. Atherosclerosis represents the root cause of most CVD events, as the rupture of the plaque triggers most myocardial infarctions and accounts for 80% of strokes (3,4). Until the 1980s, atherosclerosis was portrayed as a lipid disorder, a hypothesis supported by the fact that early plaques contain high amounts of LDLs. However, pivotal experiments found that the same plaques also contain high numbers of macrophages, B and T cells, which highlighted the importance of inflammation in the pathogenesis of this disease (5). Since then, innate and adaptative immune cells and molecules have been observed at every stage of the disease (3,6–8).

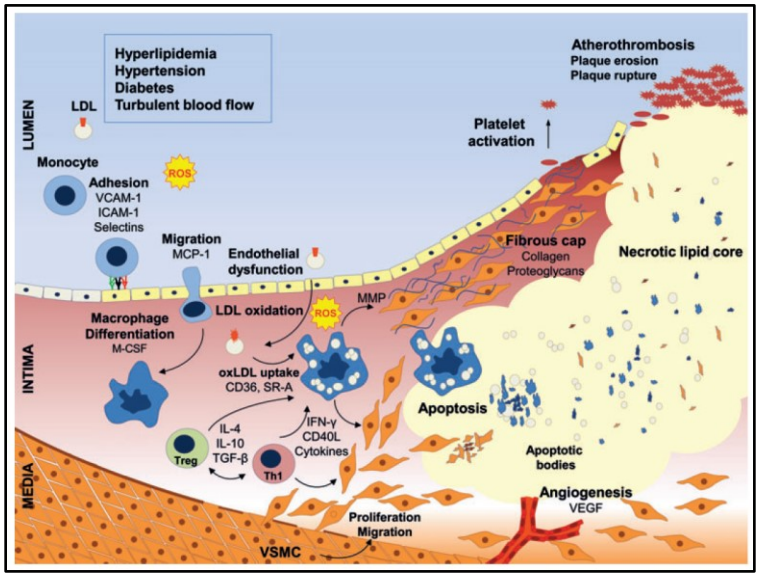


*Figure 1.1.* The Key Events Involved in the Pathogenesis of Atherosclerosis (9).

## 1.3. The Early Atherosclerotic Lesion

### *1.31. The Endothelial Dysfunction Hypothesis*

In the 1970s, the *response to injury theory* stated that atherosclerosis occurs following an injury to the endothelium separating the intima from the lumen, leading to its desquamation. This theory has developed into the current hypothesis, the *endothelial dysfunction theory*, which stipulates that the initial stage of the disease occurs following a shift in the function of the arterial wall, which modulates the nutrient exchange between the lumen and the intima (7). A dysfunctional endothelium allows for unwelcomed cells and molecules to penetrate inside the intima to initiate the accumulation of lipids (10). Many risk factors correlate with a higher incidence of endothelial dysfunction, such as smoking, aging, hypercholesterolemia, hypertension, obesity, but also liver disease, diabetes and other chronic inflammatory conditions such as rheumatoid arthritis (7,10,11).

### *1.32. The Formation of a Fatty Streak*

In a normal cellular environment, LDLs transport lipids from the liver and towards peripheral tissues, while high-density lipoproteins (HDLs) collect lipids from the periphery and towards the liver for storage (12). When the endothelial wall of the artery becomes dysfunctional, circulating LDLs penetrate inside the intima through junctions, and their apolipoprotein-B (ApoB) complex binds to proteoglycans of the extracellular matrix. Trapped LDLs accumulate and undergo a multistep process of oxidation involving reactive species (RS) but also nitric oxide (NO), enzymes, local cytokines and metal ions, turning them into oxidized low-density lipoproteins (oxLDLs) (13–15). The oxidation of LDLs activates the innate branch of the immune system, promoting the chemotaxis of monocytes from both the bone marrow and the spleen, and towards the injury site, in an attempt to clear oxLDLs (8). To infiltrate the intima, monocytes attach to endothelial surface binding receptors, primarily vascular cell adhesion protein 1, intercellular adhesion molecule 1 and selectins (8,13,16). Once inside, the monocytes can differentiate into macrophages and dendritic cells, as part of the innate immune response, a process primarily mediated by macrophage colony-stimulating factor, a pro-inflammatory cytokine present in the local environment (16). Macrophages have two main phenotypes, M1 or M2, with pro and anti-inflammatory effects, respectively, and individuals with CVD have increased circulating monocyte and a higher fraction of the M1 phenotype. Also, oxLDLs promote an epigenetic reprogramming of macrophages, inhibiting the conversion from M1 to M2 (17). In parallel to the innate immunity, the ApoB at the surface of oxLDLs acts as antigen for the adaptative inflammatory response that cause the secretion of inflammatory cytokines by B and T-cells (8). Similarly to macrophages, the role of cytokines is nuanced, as they can have both a protective and atherogenic effects, depending on the molecule and the stage of the disease (8,18).

Macrophages contribute to the normal physiological response to injury by ingesting oxLDLs by phagocytosis via their scavenger receptors, including cluster of differentiation 36, and oxidized low-density lipoprotein receptor 1. However, when the concentration of oxLDLs surpasses the clearance capacity of the immune cells, they prolong their stay in the intima and later turn into foam cells (13,15,19). The aggregation of foam cells leads to the formation of the fatty streak, the earliest type of lesion, observable as early as in infants and children (13,20).

## 1.4. The Intermediate Atherosclerotic Plaque

Over time, fatty streaks can progress to an atherosclerotic plaque or disappear (3). The atherosclerotic plaque comprises two parts, including a lipid-rich core composed of foam cells, and a fibrous cap containing vascular smooth muscle cells (VSMCs) and collagen (2,7,13).

*1.41. The Different Phenotypes of Vascular Smooth Muscle Cells*

The VSMCs are usually found inside the media of the artery and play a role in its vasoconstriction and vasodilatation (21). Skeletal and cardiac muscles are terminally differentiated, meaning that they cannot change into other cell types, but in comparison, VSMCs can shift from a contractile to synthetic, a process called dedifferentiation. In contrast to terminally differentiated neurons, skeletal muscles, cardiomyocytes and adipocytes, the VSMCs can transdifferentiate into other cell types, including macrophages and chondrocytes, in response to changes in the cellular environment (22,23). The phenotypes of VSMCs have distinct morphologies and functions. The contractile VSMCs are elongated, spindle-shaped and have a high concentration of α-actin and calponin. This phenotype contains the necessary material to allow the binding of the myosin head on the actin filament in response to calcium influx. In parallel, synthetic VSMCs cannot contract, are less elongated, are rhomboid shaped and have a higher concentration of the organelles involved in producing extracellular matrix (ECM) (21–24).

*1.42. The Transition from the Contractile to Synthetic Phenotype*

During the pathogenesis of atherosclerosis, VSMCs dedifferentiate from contractile to synthetic, migrate from the media to the intima, and proliferate to form the fibrous cap. The loss of myocardin mediates this process, which serves to stabilize the foamy core (3,7,13,22). Many risk factors promote the loss of myocardin on VSMCs, including changes in mechanical forces and stresses applied to the cell, but also changes in its chemical environment, primarily in ROS, growth factors but also cytokines, inflammatory cells, LDLs and oxLDLs (24,25). Once VSMCs enter the intima, they migrate towards the endothelial wall, proliferate, and form a fibrous cap by secreting ECM to stabilize the injured region. In addition, VSMCs in the intima can become unstable, deteriorate or transdifferentiate to macrophage-like cells or foam cells (22). After a prolonged stay in the intima, most VSMCs become apoptotic or senescent as shown by shortened telomeres, which marks the point after which the disease becomes irreversible (22,26). The senescent cells are unique, as they stop replicating, resist apoptosis and autophagy, and accumulate to produce pro-inflammatory molecules. The accumulation of such cells in tissues represents a major hallmark for cardiovascular diseases, and the primary driver of chronic inflammation (27–29).

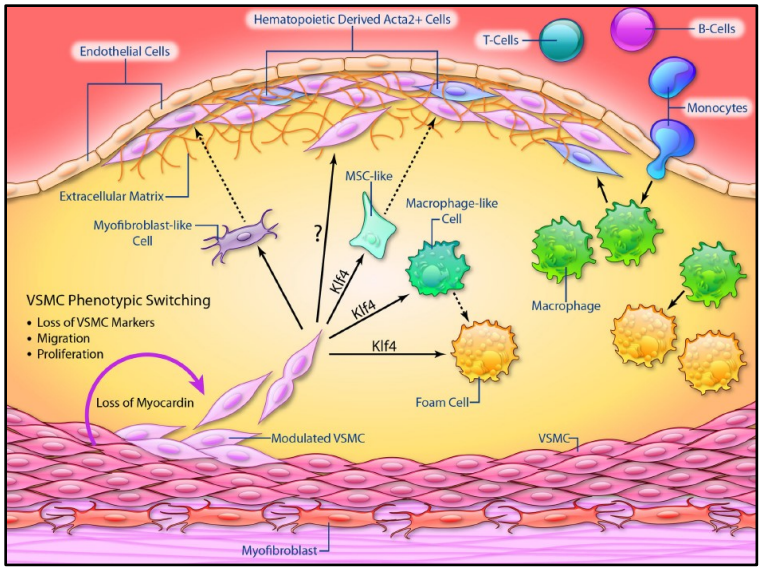


*Figure 1.2.* The Migration and Proliferation of Vascular Smooth Muscle Cells to Form a Fibrous Cap (22).

## 1.5. The Late-Stage Atherosclerotic Plaque

*1.51. The Events Leading to the Rupture of the Fibrous Cap*

Atherosclerotic plaques susceptible to rupture exhibit high levels of immune cell infiltration paired with an abnormally thin fibrous cap, deprived in VSMCs and collagen (4). Also, the necrotic core contains high amounts of dead foam cells and increased neovascularization (4). The increased blood flow contributes to the delivery of nutrients to the plaque, but it allows the infiltration of leukocytes that accumulate and release pro-inflammatory mediators and matrix metalloproteinases, powerful proteolytic enzymes that render the plaque unstable by degrading the collagen, elastin and other components of the ECM (13,30). In the long term, multiple atherosclerotic plaques can form in the same vessel, further reducing the luminal space in the artery (2).

*1.52. The Formation of the Thrombus*

A thrombus occurs when the fibrous cap ruptures and releases the content of the plaque into the lumen, and about 60% of plaque ruptures occur at the shoulder region (31). Once the plaque's content reaches the lumen, platelets aggregate and increase the size of the thrombus by binding to glycoproteins on the collagen intertwined with the necrotic core material (4). After platelet binding, they release their granule content into circulation, which further increases platelet recruitment (4). A thrombus can obstruct blood vessels and reduce the oxygen supplied to the heart, the brain, or other organs. Rarely, the thrombus detaches from the arterial wall and becomes an embolism that enters systemic circulation (4,13,31). Similarly to a thrombus, an embolism can lead to life-threatening complications such as myocardial infarctions and strokes (2,4,7,13).

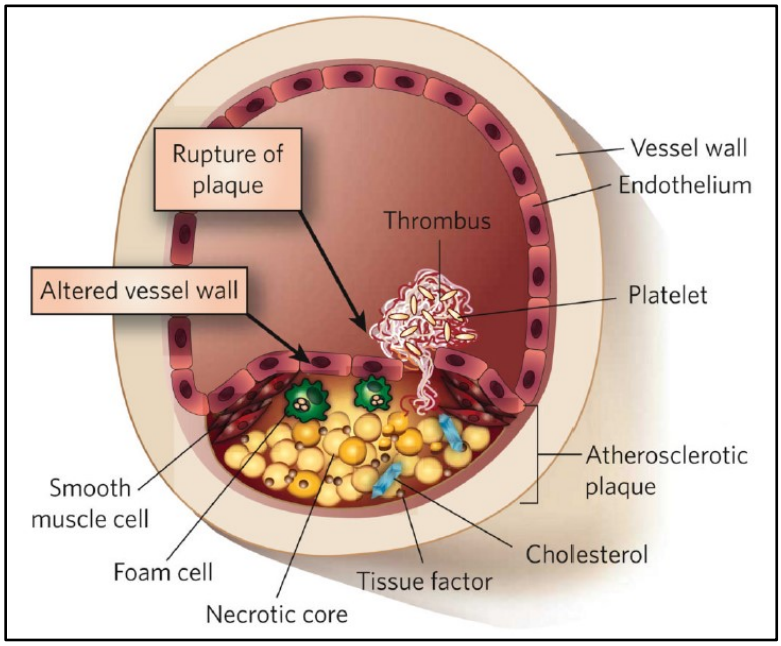


*Figure 1.3.* The Formation of a Thrombus (4).

*1.53.Mice Models for the Study of Atherosclerosis*

Animal research receives about 10% of R&D spending and remains an ethical dilemma. While 96% of general practitioners agree on its contribution, 82% also believe that animal data is

misleading, as shown by a survey in 2004. Other avenues are being explored, to complement/replace animal research, including cell cultures and micro dosing in humans, although their applications are very limited (32). Hence, mice represent the most commonly used specie for the study of atherosclerosis, with a median translational success rate of up to 75% (33). The most frequent genetically modified mice to study CVD are apolipoprotein E knockout (ApoE-/-) and low-density lipoprotein receptor knockout (LRLr-/-) mice, which both rapidly develop lipid-rich plaques when fed a high-fat diet. ApoE is synthesized by the liver and macrophages and serves as a ligand for the LDLr, allowing the clearance of very low-density lipoproteins, intermediate density lipoproteins and chylomicron remnants from circulation (34,35). Animals that lack the ApoE accumulate 5x more cholesterol and develop more foam cells as compared to controls after 3 months. Furthermore, after 8 months, the foam cells induce severe occlusions of the arteries (36).

### 1.6. Mitochondrial Dysfunction in Atherosclerosis

The mitochondria are double-membrane cytoplasmic organelles with their own genetic material that depending on the organ and cellular environment, produce most of the cellular energy. During aerobic metabolism, macromolecules including glucose, fatty acids (FAs), and amino acids convert to acetyl coenzyme A (acetyl-CoA) and enter the tricarboxylic acid (TCA) cycle inside the mitochondria. This process produces guanosine-5-triphosphate, $CO_2$, as well as the cofactors nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) (37). The electron transport chain (ETC) on the inner membrane of the organelle comprises 5 complexes, arranged together in a dynamic supercomplex, in which complex I accept electrons from NADH while complex II accepts electrons from $FADH_2$ (38). Then, electrons travel to complex III via the ubiquinone shuttle, and to complex IV via the cytochrome C (Cyt-C) protein, where they bind to oxygen to produce a water molecule, the final electron acceptor (39). This process of electron transfer along the ETC allow complexes I, III and IV to enter a reduced state and pump protons from the matrix and towards the inter-membrane space. This electrochemical gradient activates the ATP synthase, or complex V, which converts ADP and phosphate into ATP. Besides returning to the matrix through the ATP synthase, protons pass the inner membrane by other passive and active pathways. The term basal proton leak refers to the unregulated passing by passive diffusion, depends on the composition and function of the inner membrane and accounts for about 5% of the total leak. In comparison, regulated proton leak corresponds to the return of protons through tissue specific uncoupling proteins (UCP) 1–5, dissipating the proton-motive force on the ETC and potentially, the ROS production (40).

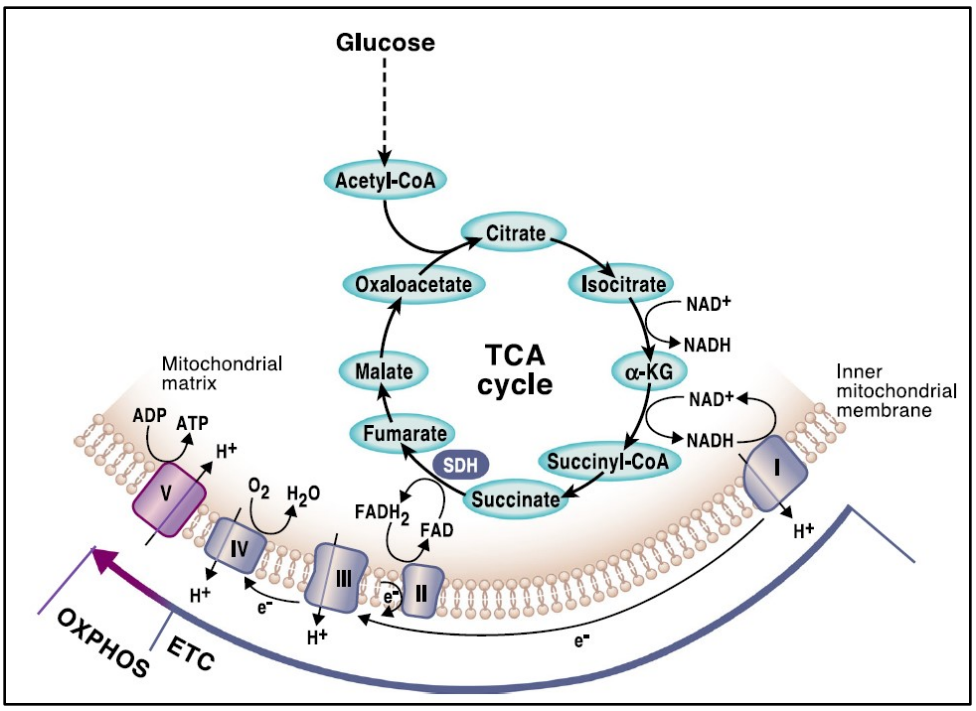


*Figure 1.4.* The Tricarboxylic Acid Cycle Provides Cofactors to the Electron Transport Chain (39).

*1.61. The Mitochondrial Dysfunction and Free Radical Theory of Disease*

In the last decade, major hallmarks were identified as the primary contributors to the loss of physiological integrity underlying all human pathologies (27). The *mitochondrial dysfunction theory of disease* stipulates that diseases occur following a deterioration of mitochondrial structures, including the membrane, the ETC and mitochondrial DNA. Changes in the form, the rate of fission/fusion and in the function of the organelle lead to an energy crisis, but also alter communication pathways with the cytosol, involved in cell growth, differentiation, cell cycle control and apoptosis. One major communication pathway between the organelle and the cell involves the mitochondrial release of Cyt C, which initiates cellular apoptosis (41,42). In addition, mitochondrial fusion refers to the physical merging of both the outer and inner membranes from two distinct mitochondria while fission corresponds to the fragmentation of a mitochondrion into two distinct organelles (42). This is important, as changes in the balance between fission/fusion towards greater fragmentation promotes autophagy, but acutely impairs the oxygen uptake and electrochemical potential of the ETC (42). The cytosol also communicates with the mitochondria, primarily through the release of calcium from endoplasmic reticulum reservoirs. The calcium enters the outer membrane through voltage dependent anion channels (VDACs) and the inner membrane through the mitochondrial calcium uniporter. Once inside the mitochondrial matrix, the ion modulates protein synthesis, oxidative phosphorylation, the production of ROS, and activates TCA-cycle enzymes (42). During the pathogenesis of atherosclerosis, the dedifferentiation of VSMCs from contractile to synthetic correlates with altered mitochondrial bioenergetics, from a glucose-centric metabolization to rely on fatty acids, paired with an increase in leak, fission, as well as decreased density and protein content (42–44).

Besides mitochondrial dysfunction, the *free radical theory of diseases* describes that tissue and cells deteriorate following oxidative damage. Many pathways produce oxidants, including the immune response to intruders, where the nicotinamide adenine dinucleotide phosphate oxidase on neutrophils release large volumes of ROS to non-specifically breakdown invaders, a process called the "oxygen burst" (45). Furthermore, the mitochondria represent the primary intracellular producer of ROS, as in normal physiological conditions, about 1–3% of all electron passing through the ETC leak before reaching complex IV, primarily from complex I and III (45). During this process, the electrons that slip transfer to circulating oxygen molecules to create a superoxide anion radical ($O_2^-$), the most biologically relevant ROS. Then, the superoxide can combine with two hydrogen molecules, to form a hydrogen peroxide ($H_2O_2$), another potent oxidant heavily involved in cell signalling (40,45). In low concentrations, ROS and other oxidants promote vascular health by contributing to stem cell proliferation and cellular differentiation. Also, many antioxidant systems capture free electrons to prevent oxidation, primarily the superoxide dismutase (SOD), that catalyses the dismutation of the superoxides into oxygen or hydrogen peroxide, and the glutathione (GSH), that reduces the hydrogen peroxide into water (46). However, a chronic depletion of antioxidant reserves causes oxidative damage, characterized by the carbonylation of the DNA, lipids, and protein structures of the cell. This state of oxidative stress (OS) correlates with the pathogenesis of many metabolic conditions including cancer, diabetes, liver disease as well as atherosclerosis (25,45,47,48).

*1.62. Mitochondrial Bioenergetics During Atherosclerosis*

Altered mitochondrial bioenergetics has been reported in a variety of metabolic conditions (44,49). During atherosclerosis, the transition from the contractile to the synthetic VSMCs correlates with major modifications in the mitochondrial profile. Namely, in 2016, Scheede-Bergdahl *& al*., observed that synthetic VSMCs have a reduced mitochondrial complex expression and density, paired with an increased leak and maximal respiration, as compared to the contractile phenotype (44).

## 1.7. The Implications of the Diet in Cardiovascular Disease

According to the *Public Health Agency of Canada*, 1 out of 4 of Canadians are obese, characterized by a BMI over 30 $kg/m^2$. Worryingly, rates of obesity have doubled between 1978 and 2004, and projections show that they could further increase over the next two decades (50). Many factors contribute to this trend, including economic growth, industrialization, mechanized transports, urbanization, increases in sedentary lifestyle, and the emergence of processed foods. While genetics contribute to obesity, estimates show that individuals who score high on genetic prevalence of weight gain display a difference of 2.7 $kg/m^2$ when compared with low-scoring individuals (51). Therefore, obesity and its associated metabolic diseases are heavily influenced by lifestyle and environmental factors. Importantly, the western-style diet (W-DIET), rich in unhealthy fats and sugars, represents a major driving force for obesity and metabolic disease (52,53).

The *calorie-based model of obesity*, which maps the underlying processes involved in the pathogenesis of obesity, stipulates that weight gain occurs following an imbalance in the calories consumed as compared to the calories utilized. Furthermore, a positive energy balance leads to the storage of excess energy in adipose tissues (54,55). While this model might appear simplistic, it underlies a very complex biopsychosocial interaction between systems, heavily involving the brain, gastrointestinal system, adipose and endocrine tissues (55). Furthermore, while humans are

≈99% effective at matching energy intake and expenditure, as low as a 0.24% increase in calorie balance results in a 0.5 kg gain per year (54).

In contrast to the calorie-based model, the *carbohydrate-insulin model of obesity* stipulates that the high availability of processed, high glycemic-index foods is at the root of the current obesity epidemic (56). After a meal, the breakdown of carbohydrates follows a multistep process, ultimately increasing circulating glucose. This state of hyperglycemia signals to the pancreas to release calcium inside the islets of Langerhans, where it stimulates the secretion of insulin-containing vesicles into the portal vein (57). The hormone insulin exerts anabolic effects by binding to receptors on muscles, adipose tissues, and on the liver. Insulin promotes [1] the synthesis of glycogen from circulating glucose in muscles and the liver, [2] the storage of free fatty acids (FFAs) in adipose tissues, as triglycerides (TGs), and [3] the storage of amino acids in muscle tissues (58,59). In contrast, during a fasted state, the hypoglycemia leads to the secretion of the hormone glucagon by α-cells from the islets. Glucagon exerts catabolic effect and partially counteracts insulin by binding to its receptors, primarily on adipose tissues and the liver. Glucagon promotes [1] the breakdown of stored glycogen in the liver into the systemic circulation to serve as fuel for other organs, [2] the release of FFAs from adipose tissues and towards the liver, as well as [3] their conversion to ketone bodies, through ketogenesis (12,60).

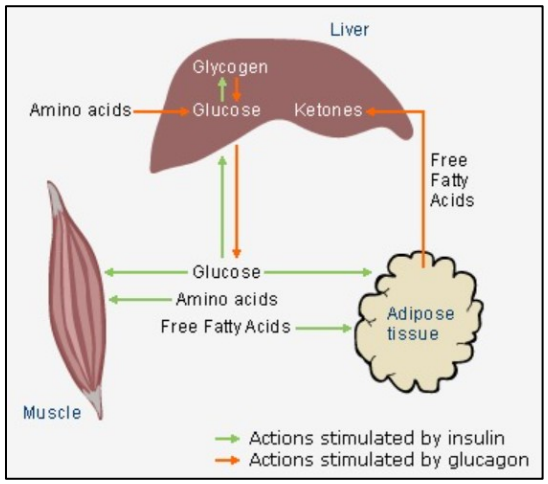


*Figure 1.5.* The Effects of Insulin and Glucagon (58).

In line with the *carbohydrate-insulin model*, diets with a low glycemic load have gained enormous popularity. Such diets, including the ketogenic diet (KD) and the low-carbohydrate high-protein (L-DIET), are now commonly utilized as tools to facilitate weight loss and improve metabolic health (61–63). According to Robert Atkins, the founder of the Atkins diet, individuals consuming a L-DIET would naturally reduce their calorie intake by 400–600 kcal/day. This theory stems from the observation that that lowering insulin could redirect calories towards metabolic active tissues such as the heart, muscle, and liver, instead of adipose tissues, thus decreasing hunger and fatigue (56,64). To support this, large-scale human trials show that the L-DIET effectively stimulates weight loss (61,62). However, well-designed trials also show no variation in weight, especially when guidelines for cardiovascular health are met (63,65).

*1.71.The Low-Carbohydrate High-Protein Diet Causes Atherosclerosis*

The interest on the relationship between the L-DIET and CVD stems from a pivotal study by Foo *& al*, who observed that mice fed the diet develop more and bigger plaques in their arteries as

compared to animals fed the unhealthy western diet (W-DIET), without changing biomarkers associated with CVD (66). Two distinct components should be accounted for when assessing the clinical significance of the L-DIET, including the reduced carbohydrates, and the increased protein intake. Regarding carbohydrates, in 2018, a meta-analysis concluded that consuming between 50 and 55% of calories from carbohydrates caused the greatest reduction in CVD risk. The same study observed that the association between the intake of carbohydrates and the risk of death followed a U-shaped relationship (67). Similarly, a prospective cohort study following over 40,000 middle-aged Swedish women for 15 years observed that each decrease in carbohydrate intake by 20 g/day increased the risk of developing CVD by about 5% (68). Interestingly, both studies noted that replacing carbohydrates with quality proteins or fats could counter the detrimental effects of such changes (67,68). As for proteins, a large-scale trial comparing over 25 diets with over 850 mice found that animals consuming diets with a high protein to carbohydrate ratio had a decreased lifespan (69). However, the diets were extreme, as about 65% of the calories came from proteins, which would equate to over 400 g for a 2500 kcal diet. Furthermore, in a more realistic setting, a pan-European human randomized controlled trial (RCT) observed that subjects on a high-protein diet (25% proteins) gained less weight, had lower inflammation, blood pressure and lipids, as well as higher satiety signals as compared to their low-protein counterparts (70). It is therefore plausible that high and extreme protein intakes have opposite cardiovascular affects.

Many factors could explain the detrimental impact of the L-DIET on blood vessels. Diets that severely restrict a macronutrient could impair metabolic flexibility, defined as the ability of a system to switch metabolic pathways following changes in the cellular environment. Metabolic inflexibility correlates with a variety of chronic diseases (71,72). Moreover, Foo and colleagues found that the L-DIET down-regulates the expression of the protein kinase B (Akt) in endothelial progenitor cells, which reduces the repair capacity of the blood vessel following a vascular injury. The Akt serves as a stress response mechanism by translocating into the mitochondria to preserve its integrity and match the acute increase in ATP demand (73). Furthermore, high-protein diets, specifically the essential branched chain amino acid leucine, upregulate the activity of the nutrient-sensing mammalian target of rapamycin (mTOR), which promotes growth and proliferation but blunts autophagy and mitophagy pathways (69,74). Therefore, the damaging effects of the L-DIET potentially occur through reductions in mitochondrial quality control signalling.

### 1.8. The Gut Microbiota's Implications in Atherosclerosis

The term nutraceutical refers to supplements isolated from food sources such as herbs, cereals, fruits, or dairy products, that are said to confer health benefits. Many nutraceuticals target the gut microbiota by acting as pre, pro or post-biotics.

The gut (gastrointestinal or digestive tract) refers to the muscular tube, open at both ends that contains the mouth, pharynx, oesophagus, stomach as well as the small and large intestine (75). The gut microbiota corresponds to the sum of all the microorganisms along the gut, while the gut microbiome represents the genome these cells harbour (76). Most of such microorganisms reside in the intestines. Specifically, the acidic environment inside the duodenum, jejunum and ileum of the small intestine, rich in oxygen and antimicrobials, represents a hospitable environment for few aerobic organisms that reside in its epithelial and mucus lining (77). In comparison, a dense and diverse population of facultatively anaerobic and anaerobic bacteria reside in the oxygen reduced caecum, colon, and the rectum of the large intestine, based on their oxygen sensitivity (77).

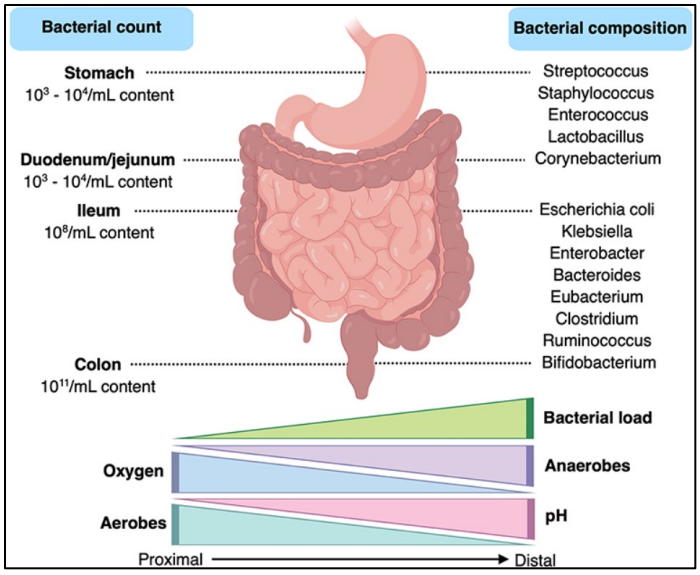


*Figure 1.6.* The Distribution and Composition of Bacteria in the Stomach and Intestines (78).

A few decades ago, the function of gut microbiota was thought to be limited to nutrient filtration, absorption and digestion, but it is now evident that this complex system modulates health and disease (77). The 300–500 bacterial species play an especially critical function in modulating CVD, as individuals with a low bacterial count and diversity tend to have more adipose tissues, paired with increased inflammation, insulin resistance (IR) and dyslipidemia (79). On the contrary, healthy individuals have a high concentration of the genus *Lactobacillus* and *Bifidobacterium*, but also *Butyrivibrio*, *Alistipes, Akkermansia*, *Coprococcus* and *Methanobrevibacter* (79,80). The gut microbiota communicates with other organs by producing metabolites, neuroactive substances, and hormones (81). Gut-derived molecules modulate the activity of neurons from the sympathetic, parasympathetic, and enteric nervous systems. Furthermore, the bacteria also signal to systemic organs by translocating compounds into the portal vein (12).

*1.81. The Gut Microbiota Can Promote a State of Disease*

Gut dysbiosis refers to disturbances in microbial homeostasis and causes the secretion of endotoxins such as lipopolysaccharides (LPSs), derived from the outer membrane of gram-negative bacteria. When bacterial LPS leak and enter entero-hepatic circulation, they bind to toll-like receptors (TLRs) or myeloid differentiation receptors on innate immune cells in a broad range of organs, primarily the liver. The activation of such receptors starts a downstream event that phosphorylates the nuclear factor kappa B (Nf-kβ), a transcription factor that switches hundreds of genes involved in immunity. The Nf-kβ increases the protein expression of pro-inflammatory cytokines and chemokines, such as interleukin (IL)-1 α, -1 β, -6, -8, 12 as well as tumor necrosis factor alpha (TNF-α) (82). Besides LPS, other endotoxins from the gut can also disrupt homeostasis, including trimethylamine (TMA) (83). Red meat, fish, eggs, and other animal products contain choline, phosphatidylcholine, and L-carnitine, that gut bacteria metabolize into TMA, which then gets oxidized by the liver into trimethylamine N-oxide (TMAO), by the flavin monooxygenase 3 enzyme. TMAO likely contributes to atherosclerosis through innate immunity, by reducing reverse cholesterol transport, and by disrupting platelet formation (84–86). In addition to the production of endotoxins, gut bacteria convert primary bile acid (BA), released by the liver and into the small intestine, into secondary BA, such as deoxycholic acid and lithocholic acid. The

colon reabsorbs some secondary BA, but in excess, they target folate receptors and BA membrane receptors, involved in inflammation and blood pressure regulation (87). Lastly, pathogenic bacterial species can directly leak from the gut, as such bacteria have been observed in atherosclerotic plaques (85,86).

*1.82. The Gut Microbiota Can Promote Health*

While gut dysbiosis promotes a state of disease, a healthy microbiota contributes to the production of key vitamins (K and B2), hormones (serotonin), appetite mediators, including glucagon-like peptide-1 and peptide YY, and other important metabolites (88). The SCFAs (acetate, butyrate and propionate) are fatty acids with fewer than 6 carbon atoms derived from the bacterial fermentation of dietary fibres in the large intestine, and are of interest in CVD (88). Butyrate represents the substrate of choice for colonocytes, and helps regulate the tight-junction proteins connecting the enterocytes, therefore improving the integrity of the gut lining (88). By binding to G-protein coupled receptors, SCFAs also downregulate the Nf-kβ and histone deacetylase (HDAC) pathways, and switches on the 5 AMP-activated protein kinase (AMPk) pathway (88,89). HDACs are nucleus enzymes that serve as epigenetic modulators, by altering the tightness with which histones bind to the DNA backbone, hence inhibiting the expression of gene regions. In atherosclerosis, HDACs contribute to the early endothelial dysfunction, in part by regulating the production of NO, ROS as well as angiogenesis (90). The AMPK are energy sensing enzymes in the cytosol and nucleus of liver, muscle, and brain cells, that regulates genes involved in the restoration of the ATP supply following a cellular stress (88,88,91,92).

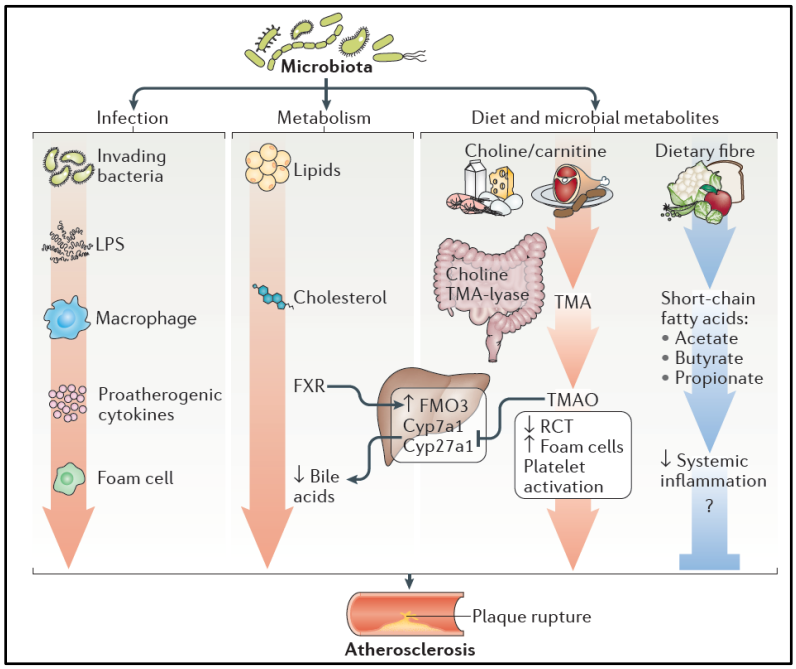


*Figure 1.7.* The Pathways by which the Gut Microbiome Modulates Atherosclerosis (86).

## 1.9. The Gut-Liver Axis and Its Implications in Atherosclerosis

The liver, the largest human organ located in the right upper quadrant of the abdominal cavity, receives 25% of the cardiac output, the most of any body part. About 30% of its blood flux originates from the hepatic artery, while the remaining 70% comes from the portal vein. Both pathways converge into hepatic sinusoids that release in the hepatic venous system (93). Through

the portal vein, the organ receives blood from the stomach, spleen, pancreas, and intestines to breakdown, detoxify and activate nutrients, drugs, and toxins before their release into the systemic circulation (12). Furthermore, the liver regulates the blood chemistry and the synthesis of BAs (94). Hepatocytes represent the parenchymal cells in the liver, accounting for 70–85% of its mass. Other important non-parenchymal cells types include stellate cells, involved in vitamin A and lipid storage, Kupffer cells, the resident macrophages as well as liver sinusoidal endothelial cells (LSECs), which separate hepatocytes from the sinusoidal lumen (95,96). Hepatocytes are organized in hexagonal 15-layer lobules, with a single central vein. As the blood flows from the periphery (zone 1) to the centre (zone 3) of the lobule, the available nutrient and oxygen concentration changes, leading to a graded shift in the gene expression of cells, altering their metabolic activity. The microenvironment renders the peripheral hepatocytes more susceptible to hepatotoxicity, while central hepatocytes are more vulnerable to lipotoxicity (97).

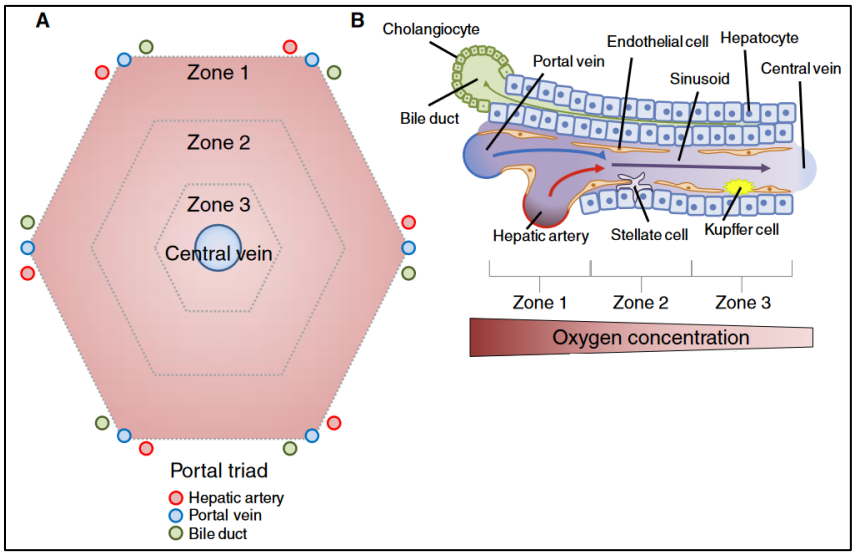


*Figure 1.8.* The Anatomy and Physiology of the Liver (98).

*1.91.Non-Alcoholic Fatty Liver Disease and Atherosclerosis*

Epidemiological evidence supports the correlation between the incidence of chronic liver disease (CLD) and of CVD, as non-alcoholic fatty liver disease (NAFLD) leads to a 1.68-fold increase in stroke chance (99). Also, most deaths from NAFLD occur due to cardiovascular complications, as shown by impaired vasodilatation as well as increased arterial stiffness, calcification, inflammation and intimal-medial thickness (99–102). NAFLD likely induces atherosclerosis through the release of hepatokines, coagulation factors and TG rich lipoproteins. Hepatic derived compounds sustain a state of chronic inflammation, IR and dyslipidemia, all major risk factors for the development of atherosclerotic plaques (100,103).

*1.92. The Pathogenesis of Non-Alcoholic Fatty Liver Disease*

In 2017, 1.6 billion individuals suffered from a form of chronic liver disease (CLD), including NAFLD, alcoholic fatty liver disease (AFLD), chronic viral hepatitis B and C, as well as hemochromatosis (104). NAFLD accounts for 60% of the cases of CLD and corresponds to the accumulation of fat in the liver, without secondary causes, such as alcohol abuse or medications (105). Important risk factors for NAFLD include the W-DIET, a sedentary lifestyle, obesity and IR (106,107). NAFLD can take the form of hepatic steatosis (NAFL), steatohepatitis (NASH), and

progress to fibrosis, and cirrhosis. The t*wo-hit-hypothesis* proposed by Day and James in 1998 stipulates that IR, paired with an accumulation of fatty acids (FAs) in hepatocytes promote the early NAFL (first hit), while oxidative stress, lipid peroxidation, and mitochondrial dysfunction contribute to the progression to NASH (second hit) (108). This view has evolved, and recent evidence favours the *multi parallel hit model,* which describes the pathology of NAFLD as a first hit induced by IR, followed by multiple hits involving a complex parallel interaction between the liver, the gut and adipose tissues (107,109–111).

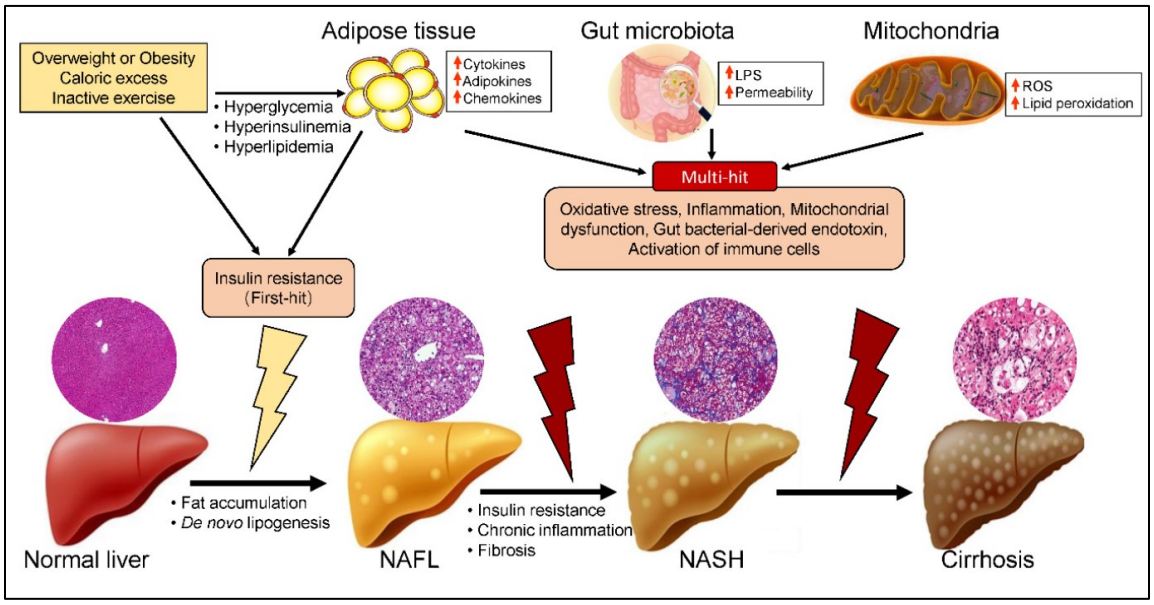


*Figure 1.9.* The Pathophysiology of Non-Alcoholic Fatty Liver Disease (112).

*1.93. The First Hit in Non-Alcoholic Fatty Liver Disease*

NAFL, characterized by an intrahepatic fat accumulation of at least 5% of liver weight, without inflammation or markers of injury, corresponds to the earliest and most benign form of NAFLD (110,113). The condition occurs following an increased influx of free fatty acids (FFAs) from adipocytes, as well as from the intestines (chylomicrons) and from *de novo* lipogenesis (113), leading to the intrahepatic accumulation of FAs and cholesterol (107,109). Dysfunctional and hypertrophied adipose release chemoattractants that recruit and promote the infiltration of bone marrow-derived monocytes. Once macrophages accumulate in adipocytes, they polarize into the M1 phenotype and release pro-inflammatory cytokines and adipokines into the systemic circulation (107,114). The adipocyte-induced IR represents the primary driver of NAFL, by deregulating pathways involved in adipose tissue lipolysis, as well as hepatic *de novo* lipogenesis and β-oxidation.

*1.94. The Multiple Hits in Non-Alcoholic Fatty Liver Disease*

NASH refers to the recruitment of Kupffer cells, bone marrow-derived macrophages, stellate cells and LSECs, in an effort to restore or replace or repair apoptotic or dysfunctional hepatocytes, and is characterized by the presence of inflammation (115). As FAs accumulate, they increase the demand placed on hepatic mitochondria, the proton motive force in the ETC and the production of ROS. FAs also deplete the antioxidant resources of hepatocytes, which altogether causes a state of oxidative damage (115,116). Furthermore, the chronic exposure to FAs damages the mitochondrial membrane and reduce the β-oxidation capacity, leading to the accumulation of lipids in the cytosol,

which induces lipotoxicity. Not all intrahepatic lipids are lipotoxic, as TGs do not contribute to the development of NAFLD. However, saturated FAs, cholesterol and other lipids induce cellular damage (117). In parallel, intrahepatic FAs and bacterial endotoxins derived from the gut also interact with innate cells by binding to TLRs, which activate Kupffer cells and non-specifically damages hepatocytes (111,115,118). To recruit neighbouring cells, dysfunctional hepatocytes secrete the sonic hedgehog ligand (SHH), either directly into the ECM, or through exosomes, small signalling vesicles. The SHH communicates with other hepatocytes, as well as local non-parenchymal cells to elicit a repair response (115).

Liver fibrosis corresponds to the phenotype switching of stellate cells in the space of Disse, from a dormant to a proliferative, migratory, and contractile myofibroblasts lining that produces collagen I and III into the ECM (98,119). Many factors contribute to the change in the expression of stellate cells, including the secretion of fibrogenic mediators by dysfunctional hepatocytes, as well as inflammatory cytokines released by Kupffer cells, including platelet-derived growth factor, transforming growth factor beta and cytokines (TNF-α and IL-1) (120). Lastly, although the etiology varies between conditions, cirrhosis represents the end-stage of CLD, and corresponds to the degeneration, senescence and necrosis of hepatocytes, their replacement by fibrotic tissues, and ultimately, the loss of liver function (120). During cirrhosis, myofibroblasts also induce the defenestration and capillarization of LSECs, leading to an impaired nutrient exchange and portal hypertension (119).

*1.95.The Potential of Probiotic Supplements to Prevent Mitochondrial Dysfunction*

By remodelling the gut microbiota, probiotics could prevent mitochondrial dysfunction. A healthy gut produces more SCFAs, improve the mitochondrial quality in a variety of organs by upregulating the AMPK cellular repair signalling pathway (88). For instance, Fan and associates reported that AFLD mice fed a cocktail containing blueberry juice and probiotics have a marked reduction in histological measures of hepatic mitochondrial injury, swelling and necrosis. Furthermore, the same animals had marked improvements in hepatic mitochondrial function, as shown by an increase in the protein expression of complex II and IV, biomarkers of OS (GSH, SOD, ROS and MDA) as well as decreased leak, coupled respiration and a higher respiratory control ratio (121).

*1.96. The Potential of Probiotic Supplements to Prevent Atherosclerosis*

The term probiotic refers to the bacteria in the gut which confer health benefits, and evidence supports its potential as a nutraceutical to prevent CVD (122). In 2015, Shimizu and colleagues performed a meta-analysis of 11 human RCTs in which they reported that probiotics improve the blood lipid profile of hypercholesterolemic participants, as shown by significant reductions in total cholesterol (TC) and LDLs (123). Other meta-analyses support that cocktails containing probiotics improve risk factors of CVD, including inflammation, glycemia and OS (124–126). Similarly, Mencarelli and colleagues conducted an animal trial and found that the VSL#3 cocktail, a combination of 8 probiotic strains, restores the intestinal lining integrity, leading to improvements in systemic inflammation and IR (127). Using a similar setting, Chan *& al* discovered that the same cocktail prevents the detrimental effects of a high-fat diet on the formation of atherosclerotic plaques, risk factors for endothelial dysfunction and inflammation comparably to an anti-hypertension medication (128).

## 2. Rationale and Objectives

With all the research linking the gut-liver axis to CVD, probiotics show potential as a nutraceutical intervention to prevent a diet-induced atherosclerosis through its modulatory effects on the mitochondria. However, many strains of probiotics lack data regarding their efficacy. *In vitro*, animal and human experiments show benefits for the *Lactobacillus Helveticus* (129–131) and *Bifidobacterium Bifidum* (132,133) strains, for a wide range of health outcomes, including the prevention of hypercholesterolemia, but also systemic inflammation and vascular health. However, no study has ever investigated the modulatory effects of said probiotic strains in the function of mitochondria, both in the liver and the arteries. In parallel, it is possible that the L-DIET promotes weight loss, but recent evidence points to the detrimental effects of such a dietary pattern in the development of atherosclerosis.

**(1)** The first manuscript of this thesis, a meta-analysis, investigates the potential of probiotic supplements to improve biomarkers of OS, a major risk factor for atherosclerosis. The mitochondrial dysfunction represents the primary intracellular producer of oxidants, and therefore, **(2)** the second manuscript assesses the effects of probiotic supplements and the L-DIET on the function of mitochondria in the liver. Due to its anatomical proximity to the gut and its role in systemic detoxification and metabolism, the liver is especially susceptible to damage associated with bacterial-derived endotoxins or a poor diet. However, this thesis is framed in the context of atherosclerosis, but whether changes in the hepatic mitochondrial profile translate into dysfunctional blood vessels remains to be proven. Hence, the **(3)** third manuscript assesses the effects of probiotics and L-DIET on aortic mitochondria, to quantify the shift from a contractile to a pro-atherogenic synthetic VSMC phenotype. This manuscript also maps the changes that occur in the gut bacterial composition in response to the diet-probiotic conditions.

## 3. Significance

CVD accounts for the death of >50,000 Canadians each year, and treatments primarily targeting blood lipids are only partly effective and cause important side effects (127,128). The diet is a major contributor in the development of atherosclerosis, and in the recent years, studies have highlighted the potential cardiovascular risks associated with the L-DIET. In parallel, with all the research linking the gut microbiota to atherosclerosis, the challenge remains to find ways to remodel this complex bacterial ecosystem to promote a state of health. This thesis is important as it clarifies some of the potential mechanisms associated with the L-DIET, but investigates of potential of probiotics supplements, an affordable and low-risk alternative treatment in the prevention atherosclerosis.

# Chapter 2

## A Systematic Review and Meta-Analysis of Randomized Controlled Trials Investigating the Effects of Probiotics on Oxidative Stress in Healthy Adults.

Antoine St-Amant, Andreas Bergdahl

**Contribution of Authors**

*Antoine St-Amant*: data collection, statistical analysis, preparation of manuscript.

*Andreas Bergdahl*: data collection, editing of manuscript.

# A Systematic Review and Meta-Analysis of Randomized Controlled Trials Investigating the Effects of Probiotics on Oxidative Stress in Healthy Adults.

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**Summary**: the oxidative stress (OS) theory of disease stipulates that a chronic imbalance in the ratio of oxidants to antioxidants in the cellular environment leads to a variety of debilitating conditions, including type 2 diabetes, cardiovascular and liver diseases. Through its communication with major organs, the gut microbiome contributes to health and disease, and many randomized controlled trials (RCTs) have investigated the potential of probiotics as a nutraceutical intervention to improve parameters of OS. Therefore, the objective was to gather relevant human RCTs exploring the potential of probiotic supplements to prevent OS in metabolically healthy individuals. This systematic review and meta-analysis was registered on PROSPERO (CRD42021297210). The *PubMed* database was searched using keywords related to probiotics and OS. In total, 652 studies were screened, 9 of which matched the inclusion criteria. The results show that probiotics improve some markers associated with the antioxidant status (AS), including total antioxidant capacity (TAC) (SMD: 0.83 mmol/L, 95% CI: 0.25-1.40, $p$ = 0.005) and glutathione (GSH) (SMD: 0.45, 95% CI: 0.13-0.77, $p$ = 0.006). However, probiotics do not significantly alter superoxide dismutase (SOD) (SMD: 0.33, 95% CI: -0.27-0.93, $p$ = 0.28). Also, probiotics significantly decrease the plasma concentrations of the OS biomarker malondialdehyde (MDA) (SMD: -0.55, 95% CI: -1.11–0.00, $p$ = 0.05). In conclusion, probiotics improve biomarkers associated with AS and OS in metabolically healthy individuals. However, more studies are needed to address the moderate to high heterogeneity.

**Key Words:** Probiotic, oxidative stress, oxidant, antioxidant, meta-analysis.

## Introduction

The term reactive species (RS) describes both free radicals and reactive oxygen species (ROS), small oxidizing molecules involved in many important cell signalling pathways (1–3). Furthermore, endogenous, and exogenous factors, including genetics and poor lifestyle habits can lead to deregulation in the balance of oxidants/antioxidants, and cause a state known as oxidative stress (OS). Chronically, OS damages DNA, lipids, and protein structures of the cell (1,3), represents a major hallmark of aging (4), and correlates with debilitating conditions such as diabetes (5), liver (6,7) and cardiovascular diseases (CVD) (2,8,9). Hence, reducing OS represents a major therapeutic target to improve metabolic health and lifespan.

Probiotics are bacteria in the gut known to confer health benefits (10). Probiotic supplements are commonly prescribed to treat intestinal disorders (11), mounting evidence shows that they could improve other parameters of health. Cross-sectional data demonstrate that subjects with a low total microbial count and a less diverse bacterial profile have more adipose tissue, chronic low-grade inflammation, insulin resistance and dyslipidemia (12). Also, these individuals develop fatty liver and obesity

(13). Probiotic supplements show promise for many health outcomes in animal studies (14–20). In humans, meta-analyses show that probiotics improve blood lipids in mildly hypercholesterolemic individuals (21), as well as fasting glucose and insulinaemia in diabetics (22). Meta-analyses report improvements in the oxidant/antioxidant profile following supplementation with probiotics (22–27). However, key methodological features reduce their applicability, as such studies include participants suffering from a major metabolic condition, or consider trials combining probiotics with other nutraceuticals, such as prebiotics and synbiotics. Therefore, the aim of this systematic review and meta-analysis was to congregate randomized controlled trials (RCTs) investigating the effects of probiotic supplements, in the prevention of OS in metabolically healthy individuals.

## Methods

This systematic review and meta-analysis was registered on *PROSPERO* (CRD42021297210) and followed the guidelines of the *Cochrane Handbook for Systematic Reviews of Interventions* (28) as well as the *PRISMA 2020 Updated Guideline for Reporting Systematic Reviews* (29).

### *Search Strategy*

All relevant published trials were identified by searching *PubMed* up to December 2021 and by scanning the citation lists of meta-analysis and review papers (23–26,30,31). The keywords consisted of (probiotic OR *Lactobacillus* OR *Bifidobacterium* OR symbiotic OR synbiotic) AND (oxidative Stress OR metabolic profile OR free radical OR malondialdehyde OR MDA OR nitric oxide OR NO or superoxide OR reactive oxygen species OR ROS OR antioxidant OR total antioxidant capacity OR TAC OR glutathione OR GSH) AND (clinical trial OR randomized clinical trial OR randomized controlled trial OR randomized OR RCT). The search was not restricted to any language or year of publication.

### *Study Selection and Eligibility Criteria*

All RCTs that investigated the effects of probiotics or foods supplemented with probiotics on biomarkers of antioxidant status (AS) (TAC, SOD, GSH) and OS (MDA, NO and ROS) were included. Studies had to meet the following inclusion criteria: [1] All participants were adults (>18 years old). [2] The experimental design included an intervention and a placebo group. [3] The duration of the treatment was at least one week. [4] The study provided information on the concentration of oxidant/antioxidant at baseline and at the end of the trial.

The exclusion criteria were as follows: [1] The study administered probiotics paired with another nutraceutical (prebiotics, postbiotics), or provided through food only in the intervention group, not in the control group. [2] The study was a non-RCT trial, review, meta-analysis, or any clinical trial without a control group. [3]: the study was conducted in animals or *in vitro*. [4] The population consisted of participants diagnosed with a major metabolic disease, including but not limited to diabetes, cancer, and CVD. The two authors screened all relevant articles independently using the *Rayyan* software (32), and settled disagreements by discussion at the end of the process.

### *Data Extraction*

The selected articles were scanned to extract the data, including [1] the first author's name, [2] the year of publication, [3] the country, [4] the study design, [5] the duration of the

treatment, [6] the number of participants in each group, [7] the type of participants, [8] the body mass index (BMI), [9] the age, [10] the sex, [11] the dose of probiotics, [12] the strain of bacteria and [13] the influence of probiotics, pre-and post intervention on the concentration of oxidants or antioxidants.

*Statistical Analysis*

The statistical analyses were performed with *SPSS Statistics 26* and *RevMan 5.4.1*. The effect of probiotics was converted to the standardized mean difference (SMD) (28), standard deviation (SD) and 95% confidence interval (CI), using a random effects model (Dersimonian-Liard). In studies where the $SD_{change}$ was not mentioned, the value was calculated with the formula from section 6.5.2.8 of the *Cochrane Handbook for Systematic Reviews and Interventions*, assuming a correlation coefficient (Corr) of 0.5, a conservative value (28,33):

$$SD_{change} = \text{square root } [(SD_{baseline})^2 + (SD_{post\text{-}treatment})^2 - (2\times \text{Corr x } SD_{baseline} \times SD_{post\text{-}treatment})]$$

In the studies where only CI was provided, the SD was calculated based on the number of participants in each group (n) (28):

$$SD = \text{square root (n) x (upper limit—lower limit)}/3.92$$

The heterogeneity was assessed using the I-square statistic ($I^2$). An $I^2$ values of 25%, 50% and 75% were considered low, moderate and high, respectively (34). For the effect size (SMD), a value of 0.2, 0.5 and 0.8 were considered as small, medium and large, respectively (35). A sensitivity analysis was conducted to establish the effect of individual study on the biomarker of interest.

*Risk of Bias Assessment*

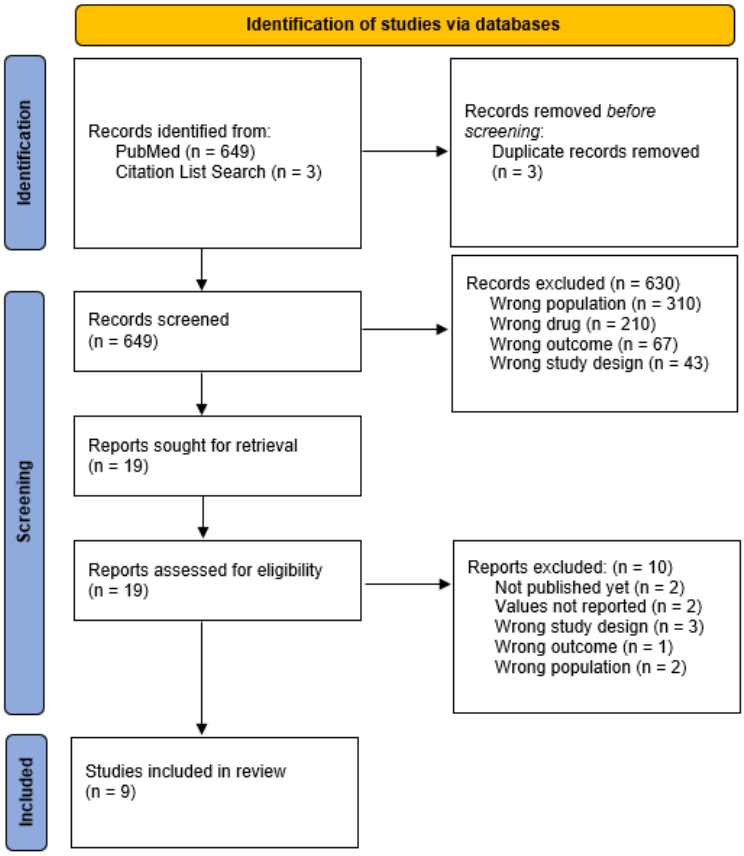
The *Cochrane Collaboration Tool* was used to assess risk of bias in the study designs (28,36). The following domains were included: [1] Selection bias (random sequence generation and allocation concealment), [2] performance bias (blinding of participants and personnel), [3] detection bias (blinding of outcome assessment), [4] attribution bias (incomplete outcome data), [5] reporting bias (selective reporting) and [6] other sources of biases.

**Results**

*Literature Search*

A total of 652 articles were considered, including 649 identified from the *PubMed* database, in addition to 3 studies manually added from relevant literature, and 3 duplicates were removed. After screening the titles and abstracts, 630 studies not matching the criteria were removed. The trials were excluded based on "wrong population" (n=310), "wrong drug" (n=210), "wrong outcome" (n=67) and "wrong study design" (n=43). Eventually, 19 articles were assessed for eligibility. After retrieval, 2 trials were excluded as they were not published yet, in addition to 3 studies that did not report the values of interest, 2 for "wrong study design", 1 for "wrong outcome" and 2 for "wrong population". At the end, 9 studies were included in the analysis, with a total of 430 subjects, comprised of 219 participants in the intervention and 211 participants in the placebo group (37–45) (Figure 1).

**Figure 2.1**. PRISMA Flow Diagram of the Study Selection Process.




*Summary of Study Characteristics*

The RCTs ranged from 2012 to 2021 and were conducted in Iran (37,38,41,42), Taiwan (39), Brazil (40), Poland (43), Czech Republic (44) and Spain (45). The trials included between 22 and 70 subjects ranging from 18 and 70 years old and had a duration of 6–14 weeks. The average BMI of study participants ranged between 22 and 33. The studies included patients with a depressive disorder (37), pregnant women (38,42), men diagnosed with infertility (41), overweight or obese women (40,43) and athletes (44,45). The dose of probiotics administered varied from $1 \times 10^7$ to $2 \times 10^{11}$ colony forming units (CFU) per day. In total, 14 different strains of probiotics were administered, including [1] *L. acidophilus* [2] *L. casei* [3] *B. bifidum* [4] *B. lactis* [5] *L. paracasei* [6] *Lactococcus lactis* [7] *L. rhamnosus* [8] *L. bulgaricus* [9] *B. breve* [10] *B. longum* [11] *S. thermophiles* [12] *L. brevis* [13] *L. salivarius* [14] *L. Helveticus*. The most common probiotic strains were *L. acidophilus* and *L. casei*, both administered in 6 of the 9 trials, followed by the *B. Bifidum* strain, present in 4 of the studies (Table 1).

**Table 2.1.** Characteristics of the Randomized Clinical Trials Included in this Systematic Review and Meta-Analysis.

| First Author | Year of Publication | Country | Study Design | Duration (weeks) | Number of Participants | | Participant | BMI (kg/m$^2$) | | Age | Sex | | Dose of Probiotics (total) | Strain of Probiotics |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Intervention | Control | | Intervention | Control | | M | F | | |
| 1. Akkasheh | 2015 | Iran | Randomized, double-blind, placebo-controlled clinical trial | 8 | 20 | 20 | Diagnosed with major depressive disorder | 27.6 ± 6.0 | 26.3 ± 4.1 | 20-55 | 34 | 6 | $6 \times 10^9$ CFU/day | *L. acidophilus, L. casei, B. bifidum* |
| 2. Asemi | 2012 | Iran | Randomized single-blind controlled clinical trial | 9 | 37 | 33 | Pregnant women | 27.5 ± 4.1 | 27 ± 4.1 | 18-30 | 0 | 70 | $1 \times 10^7$ CFU/day | *L. acidophilus, B. lactis* |
| 3. Chen | 2020 | Taiwan | Randomized, double-blind, placebo-controlled, adaptive-design trial | 4 | 27 | 25 | Healthy volunteers | 21.9 ± 2.2 | 21.6 ± 2.6 | 20-65 | 17 | 35 | $1 \times 10^{10}$ CFU/day | *L. paracasei* |
| 4. Gomes | 2017 | Brazil | Double-blind, randomized, placebo-controlled study | 8 | 21 | 22 | Overweight or obese women | 33.34 ± 4.69 | 31.70 ± 3.90 | 20-59 | 0 | 43 | $2 \times 10^{10}$ CFU/day | *L. acidophilus, L. casei, Lactococcus. lactis, B. bifidum, B. Lactis* |
| 5. Helli | 2020 | Iran | Randomized, double-blind controlled clinical trial | 10 | 25 | 25 | Infertile men | 27.23 ± 3.07 | 26.3 ± 2.11 | 20-45 | 56 | 0 | $2 \times 10^{11}$ CFU/day | *L. casei, L. rhamnosus, L. bulgaricus, L. acidophilus, B. breve, B. longum, S. thermophiles* |
| 6. Jamilian | 2016 | Iran | Randomized clinical trial | 12 | 30 | 30 | Pregnant women | 25.6 ± 4.2 | 25.5 ± 4.1 | 18-37 | 0 | 60 | $6 \times 10^9$ CFU/day | *L. acidophilus, L. casei, B. bifidum* |
| 7. Majewska | 2020 | Poland | Randomized double-blind placebo-controlled trial | 12 | 25 | 25 | Obese women | 36.6 ± 6.0 | 36.1 ± 4.4 | 45-70 | 0 | 50 | $1 \times 10^{10}$ CFU/day | *B. bifidum, B. lactis, L. acidophilus, L. brevis, L. casei, L. salivarius, Lactococcus lactis* |
| 8. Michalickova | 2018 | Czech Republic | Randomized, double-blind, placebo-controlled study | 14 | 12 | 10 | Elite athletes | 23.0 ± 1.2 | 22 ± 0.5 | 20-24 | 22 | 0 | $2 \times 10^{10}$ CFU/day | *L. Helveticus* |
| 9. Sánchez | 2021 | Spain | Randomized double-blind and controlled single-center clinical trial | 6 | 22 | 21 | Healthy athletes | 23.6 ± 2.6 | 23.6 ± 2.6 | 18-45 | 43 | 0 | $1 \times 10^9$ CFU/day | *B. longum, L. casei, L. rhamnosus* |

*Risk of Bias*

The categories blinding of participants and blinding of the outcome had the highest risk of bias, with 6 of the 9 studies showing an unclear/high risk. Also, 3 of trials had an unclear risk of bias for random sequence generation, 3 revealed an unclear/high risk for allocation concealment and 3 had an unclear risk for the category incomplete outcome data. Two trials had a low risk of bias for all categories (37,40) (Table 2).

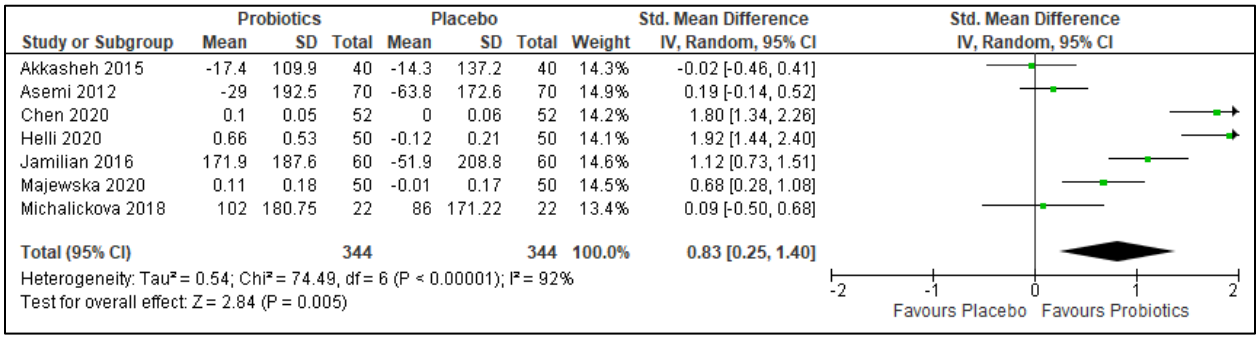
**Table 2.2.** Risk of Bias for the Included Studies, Assessed According to the Cochrane Risk of Bias Tool. The risk of bias is expressed as low (L), uncertain (U) or high (H).

| Author (year) | Random Sequence Generation | Allocation Concealment | Blinding of Participants and Personnel | Blinding of Outcome Assessment | Incomplete Outcome Data | Selective Reporting | Other Bias |
|---|---|---|---|---|---|---|---|
| Akkasheh (2015) | L | L | L | L | L | L | L |
| Asemi (2012) | U | H | H | H | L | L | L |
| Chen (2020) | L | L | U | U | L | L | L |
| Gomes (2017) | L | L | L | L | L | L | L |
| Helli (2020) | L | L | U | U | L | L | L |
| Jamilian (2016) | L | L | L | L | L | L | L |
| Majewska (2020) | U | U | U | U | U | L | L |
| Michalickova (2018) | U | U | U | U | U | L | L |
| Sánchez (2021) | L | L | U | U | L | L | L |

*Total Antioxidant Capacity*

To investigate the total antioxidant capacity (TAC), 7 studies were identified, with 344 participants, including 176 subjects in the intervention and 168 subjects in the control group (37–39,41–44). The results show that probiotics significantly increase the plasma concentration of TAC (SMD: 0.83, 95% CI: 0.25-1.40, $p = 0.005$). The studies had a high level of heterogeneity ($I^2 = 92\%$) (Figure 2).

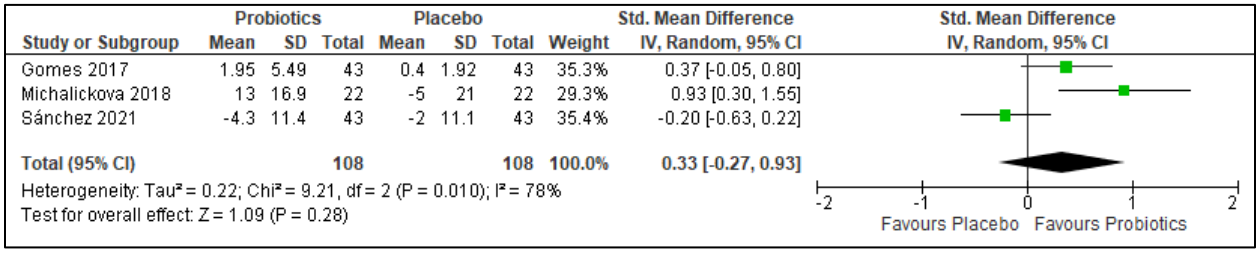
**Figure 2.2.** The Effects of Probiotic Supplements on Changes in Total Antioxidant Capacity (TAC), Presented as Standardized Mean Difference (SMD) and 95% Confidence Interval (CI).




*Superoxide Dismutase*

To explore the effects of probiotics on the superoxide dismutase (SOD), 3 studies were included with a total of 108 participants, containing 55 cases and 53 controls (40,44,45). The results show that probiotics do not significantly increase the plasma concentrations of SOD as compared to the placebo group (SMD: 0.33, CI: -0.27-0.93, $p$ = 0.28) (40,44,45). The level of heterogeneity was high ($I^2$ = 78%) (Figure 3).

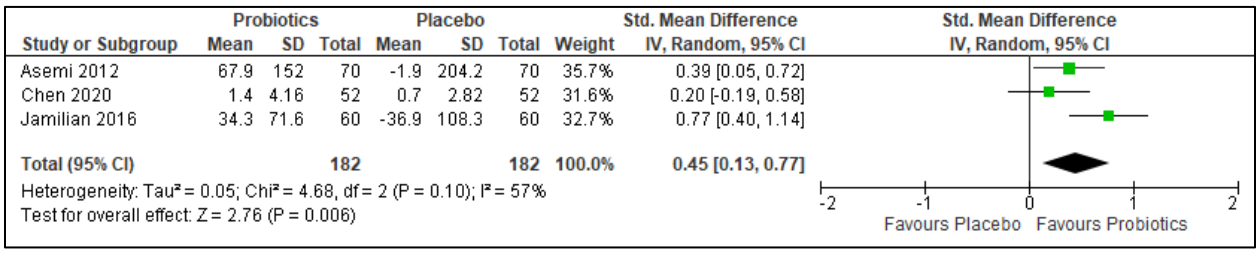
**Figure 2.3.** The Effects of Probiotic Supplements on Changes in Superoxide Dismutase (SOD) Presented as Standardized Mean Difference (SMD) and 95% Confidence interval (CI).




*Glutathione*

In total, 3 studies were included to explore the effects of probiotics on plasma levels of glutathione (GSH), including 94 cases and 88 controls, for a total of 182 participants. (38,39,42). The results show that participants administered the probiotic treatment displays a significant increase in GSH as compared to controls (SMD: 0.45, CI: 0.13-0.77, $p$ = 0.006), with a moderate level of heterogeneity ($I^2$ = 57%) (Figure 4).

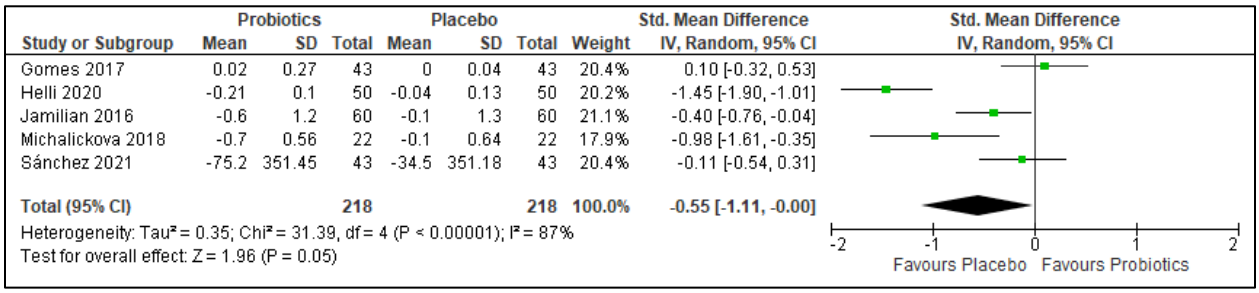
**Figure 2.4.** The Effects of Probiotic Supplements on Changes in Glutathione (GSH), Presented as Standardized Mean Difference (SMD) and 95% Confidence Interval (CI)




*Malondialdehyde*

The analysis for malondialdehyde (MDA) contained 5 studies, with 218 participants, including 110 cases and 108 controls (40–42,44,45). The results show that probiotics significantly decrease the levels of MDA when compared to controls (SMD:-0.55, CI : -1.11–0.00, $p = 0.05$). The studies had a high level of heterogeneity ($I^2 = 87\%$) (Figure 5).

**Figure 2.5.** The Effects of Probiotic Supplements on Changes in Malondialdehyde (MDA), Presented as Standardized Mean Difference (SMD) and 95% Confidence Interval (CI)




*Other Biomarkers*

Only one study reported values for changes in nitric oxide (NO), showing that probiotics significantly increase the plasma concentration of NO (SMD: 1.35, CI: 0.79-1.92, $p < 0{,}00001$) (42). No studies covered changes in ROS.

**Discussion**

The objective of this systematic review and meta-analysis was to investigate the potential of probiotic supplements to improve biomarkers associated with OS in disease-free individuals. High levels of OS correlate with the development of diseases (2,4,5), and therefore, therapies that ameliorate the profile of oxidants/antioxidants should translate into improvements to other, underlying health parameters. The results support that probiotic supplements improve some biomarkers associated with AS, as demonstrated by a significant increase in TAC and GSH, but not SOD. Furthermore, probiotics reduce OS, as demonstrated by a significant reduction in MDA. The findings show that even in metabolically healthy individuals, probiotics prevent the OS associated cellular and organ damage.

Similar meta-analyses have been conducted in recent years, but over half the studies incorporated patients with a form of diabetes.

This is important, as diseases likely disrupt pathways involved in the regulation of OS (2,46). It is also plausible that diseases have a unique signature in the gut, and that probiotics could translate into greater benefits for particular microbiotas or pathologies (47). Still, in 2019, Roshan and colleagues observed that probiotics significantly improve GSH levels, but only in nondiabetic subjects (23). Also, Zamani and associates reported that in diabetics, probiotic supplements increase TAC and reduce MDA, but similarly to Roshan *& al*, do not alter the concentrations of GSH (26). GSH is a tripeptide composed of cysteine, glutamate and glycine, and reports show that diabetics with microvascular complications have lower GSH concentrations, potentially caused by substrate deficiencies or deregulation in the two major enzymatic steps involved in its synthesis (48,49). Many studies have investigated the potential of nutraceutical cocktails containing probiotics to prevent OS. However, as the interventions mostly combined probiotics with prebiotics and symbiotics, it is impossible to isolate the effects of probiotics alone. In 2020, Pourrajab and associates conducted a meta-analysis and found that similarly to here, a cocktail containing probiotics improves serum TAC, GSH and NO, while reducing MDA in adults (25). Heshmati and colleagues arrived at similar conclusions, as they found an amelioration in the plasma concentrations of TAC, GSH, SOD, NO and MDA (24). All around, the results show that supplements containing probiotics improve AS and OS in a variety of populations.

Some limitations should be accounted for when interpreting the results. The small number of RCTs prevented a subgroup analysis (28). Hence, no conclusions could be drawn as to the source of the moderate to high heterogeneity between studies. Also, over half of the experiments originated from Iran and Taiwan, and therefore, the results are more generalizable to those populations and their specific dietary and lifestyle habits. This is important, as the gut bacterial profile can vary greatly, even within regions of a country (51). Finally, while populations with a major metabolic disease were excluded, such as cancer, diabetes, cardiovascular and liver disease, some studies involved pregnant women, individuals suffering from depression as well as generally healthy obese patients. These subgroups might have influenced the results and their transferability.

In conclusion, the results show that probiotic supplements improve the plasma concentrations of biomarkers associated with OS and AS in generally healthy individuals and could therefore offset the development of diseases, as chronic OS correlates with cellular ageing and many pathologies. However, the conclusion should be interpreted carefully and should not serve for the development of clinical guidelines, as all the analyses had few studies and moderate to high heterogeneity. While promising, the heterogeneity, the limitations, and the lack of information regarding the best dosage, strain and treatment duration highlights the clear need for more quality trials before clinical recommendations can be made.

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# Chapter 3

## Probiotic Supplements and the Low-Carbohydrate High-Protein Diet Alter Hepatic Mitochondrial Function in Apolipoprotein-E Knockout Mice.

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**Contribution of Authors**

*Antoine St-Amant*: animal handling, surgeries, respiration measurements, statistical analysis, preparation of manuscript.

*Andreas Bergdahl*: animal handling, surgeries, respiration measurements, immunoblotting, editing of manuscript.

# Probiotic Supplements and the Low-Carbohydrate High-Protein Diet Alter Hepatic Mitochondrial Function in Apolipoprotein-E Knockout Mice.

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**Summary:** In 2017, 1.6 billion individuals suffered from a form of chronic liver disease (CLD), 60% of which from non-alcoholic fatty-liver disease (NAFLD). The mitochondrial dysfunction in hepatic cells induced by a western-style diet (W-DIET) represents a major contributor to the pathogenesis of CLD. Furthermore, more and more individuals turn to a low-carbohydrate high-protein diet (L-DIET) to lose weight, but its effects on the mitochondria remain largely unknown. In parallel, the gut microbiome modulates health and disease, and probiotic supplements could restore mitochondrial integrity by remodeling the composition of the bacteria residing in the gut. Therefore, the objective of this study was to investigate the potential of probiotic nutraceuticals to prevent a diet-induced mitochondrial dysfunction in hepatocytes. Apolipoprotein-E knockout (ApoE-/-) mice were randomly assigned to 3 groups, including a control diet (C-DIET), W-DIET, and L-DIET. Each group was further divided based on the dose of the probiotic, including a control, low and high probiotic dose. The cocktail administered contained a 1:1 ratio of *Lactobacillus Helveticus* and *Bifidobacterium Bifidum*. The bodyweight and water intake were monitored weekly. At the end of the 6-week period, the liver was removed by necropsy for high-resolution respirometry and immunoblotting for voltage dependent anion channels (VDACs) and mitochondrial complexes (I-V). The results show that the L-DIET increases mitochondrial density and leak. Also, both the L-DIET and probiotics reduced parameters of normalized mitochondrial respiration, although two opposing mechanisms of action likely explain the similar outcome observed in both conditions. In conclusion, probiotics show promise as a nutraceutical treatment to restore hepatic mitochondrial function induced by a poor diet.

**Keywords:** non-alcoholic fatty liver disease, gut microbiota, probiotics, low-carbohydrate high-protein diet, hepatocyte, mitochondria.

## Introduction

The liver plays many important functions, being involved in digestion, metabolism, the production of hormones and in systemic detoxification (1). The hepatocytes contain large volumes of mitochondria to support their important ATP demands (1,2). Besides inducing an energetic crisis, dysfunctional mitochondria foster a state of oxidative stress (OS), damaging lipids, proteins, and DNA structures of the cells, and deregulate pathways associated with growth, differentiation, inflammation, apoptosis and necrosis (1,3,4). Hence, impairments in mitochondrial integrity represent a major contributor to the pathogenesis of non-alcoholic fatty liver disease (NAFLD) (1).

The western-style diet (W-DIET), rich in unhealthy carbohydrates and fats, strongly promotes obesity and NAFLD (5). To prevent such detrimental effects, more and more individuals turn to a low-carbohydrate high-protein diet (L-DIET), with the primary aim to lose bodyweight (6). However, the effects of the L-DIET on hepatic mitochondria and the pathogenesis of NAFLD remain unclear. Foo and colleagues reported that in ApoE-/- mice, the L-DIET promotes as much atherosclerotic plaque buildup in the aorta as the W-DIET (7).

Furthermore, Solon-Biet *& al* showed that high protein diets decrease lifespan in mice (8). Such findings point to the necessity to clarify the effects of the L-DIET in respect NAFLD.

Gut dysbiosis promotes a variety disease (9,10). The liver is especially vulnerable to the endotoxins derived from bacterial metabolism, that translocate into the portal vein through the gut-liver axis (11). Therefore, by remodeling the bacterial ecosystem residing inside the gut, probiotics administered as a nutraceutical treatment could relieve liver toxicity and prevent NAFLD. Epidemiological trials in humans suggest that supplementing with probiotics improves biomarkers associated with lipidaemia, glycemic control and OS (12–15). Animal studies report benefits of probiotics for body composition, systemic inflammation, intestinal barrier integrity and OS, in a variety of organs (16–22). Two strains of probiotics have shown promise for a wide range of therapeutic targets, including the *L. Helveticus* (21,23) and *B. Bifidum* (22,24), for many outcomes, including for the prevention of hypercholesterolemia, systemic inflammation and vascular health. For example, Cavallini and associates reported that a soy product fermented with *L. Helveticus* and *Enterococcus Faecum* decreases the atherosclerotic lesion size in the aorta and improves the blood lipid profile (21). Similar benefits were reported by Rerksuppaphol and colleagues for a cocktail containing *L. acidophilus* and *B. Bifidum* in hypercholesterolemic individuals (22). However, no study has investigated the potential of these two strains to prevent mitochondrial dysfunction in hepatocytes. Therefore, the objective of this study was to explore the effects of a L-DIET and of probiotic supplements in the prevention of mitochondrial dysfunction.

## Methods

### *Animals*

The Animal Ethics Committee at *Concordia University* approved this project (protocol ID: #30000259) and follows the guidelines of the *Canadian Council on Animal Care*. ApoE-/- mice, genetically modified to develop dyslipidemia (25,26) were selected from the breeding colony at *Concordia University* (originally purchased from *Jackson Laboratories*, Maine). The rodents were weaned and separated based on sex at 21–28 days and males were placed individually in a thermo-neutral environment at 22◦C with a 12:12h photoperiod.

### *Diet and Probiotic Specifications*

The animals were randomly assigned to 1 of 3 diets, with varying percentages of carbohydrates (C), fats (F) and proteins (P), including a control (C-DIET) (63 % C, 14% F, 24% P) (*5075 Charles River Autoclavable Rodent Diet*, Wilmington, United-States), a western-style (W-DIET) (42.2% C, 42,1% F, 15.7% P) (*Harlan Laboratories, TD. 110229,* Indianapolis, United-States), and a low-carbohydrate high-protein (L-DIET) (11% C, 43.2% F, 45.8% P) (*Harlan Laboratories, TD. 04524,* Indianapolis, United-States). Animals had access to food and waters *ad libitum*, and the C-DIET and L-DIET were isocaloric. The W-DIET and L-DIET had a similar cholesterol content (1.5 g/kg). The mice were further divided in 3 subgroups, based on the dose of probiotics, including a control (C-PROB), low (L-PROB) and high-dose (H-PROB), for a total of 9 groups (n = 11–12 animals/group). The probiotics (*Lallemand Health Solutions, Lafty L10,* Montreal, Canada*)* contained a 1:1 ratio of *Lactobacillus Helveticus* and *Bifidobacterium Bifidum* ($4.94 \times 10^9$ colony forming units (CFU) for a 5 B dose and $4.94 \times 10^8$ CFU for a 0.5 B dose). The bacteria

were administered through the drinking water.

*Experimental Protocol*

The animals were assigned to the diets and probiotics for 6 weeks, and the water intake and bodyweight were monitored weekly. At the end of the 6-week period, necropsy was performed to remove the left lateral lobe of the liver. The tissue was placed in an ice-cold physiological solution (BIOPS) containing (in mM) CaK2EGTA 2.77, K2EGTA 7.23, Na2ATP 5.77, MgCl2·6H2 O 6.56, taurine 20, Na2Phosphocreatine 15, imidazole 20, dithiothreitol 0.5, MES 50, and pH 7.1. After surgery, a fresh piece of the liver tissue was cut with sharp forceps to measure mitochondrial respiration. The remaining tissue was stored in a -80◦C freezer for immunoblotting.

*Mitochondrial Respiration Measurements*

A sequential substrate addition protocol was conducted, using a polarographic sensor (*Oxygraph-2k*, *Oroboros Instruments*, Innsbruck, Austria) (27). Between 2 and 3 mg of liver tissue was placed in either chamber of the Oxygraph in a hyper-oxygenated environment, in mitochondrial respiration medium (MiR05) at 37◦C. The oxygen flux was registered using the *DatLab* software. The liver tissue was added to the chamber in an environment deprived in ADP and substrates, followed by the sequential addition of malate (4 mM), octanoyl-carnitine (10 µM), ADP (10 mM), glutamate (5 mM), pyruvate (5 mM), cytochrome C (Cyt C) (5 µM), succinate (20 mM) and cyanide p-trifluorome thoxyphenylhydrazon (FCCP) (1 µM).

*Protein Extraction and Immunoblotting*

Immunoblotting was performed on the liver to quantify the voltage-dependent anion channels (VDACs) and the mitochondrial complexes expression (I-V) (28). The tissue was pulverized/homogenized using liquid nitrogen combined with ~70 µL lysis buffer containing 250 mM NaCl, 50 mM HEPES (pH 7.5), 10% glycerol, 1% triton X-100, 1,5 mM MgCl2 1 mM EGTA, 10 mM Na4P2O7 NaF, 800 µM Na3VO4. After 1 hour on ice, the sample was centrifuged at 12,000 × *g* for 10 minutes to collect the supernatant. Then, 10 µL of the supernatant was combined with 2 µl DTT and 2 µl buffer on a 10% acrylamide-SDS gel for separation, which was transferred on a 0.45 µm nitrocellulose membrane (*165-0115 Bio-Rad*, Hercules, United-States), in 10 mM of sodium tetraborate buffer. The even loading and transfer of proteins was assessed using Ponceau S staining. Afterwards, the membrane was blocked with 3% bovine serum albumin (BSA), in 0.1% tween 20 in tris-buffered saline (TBS) (10 mM Tris-HCl, pH 7.5, 150 mm NaCl), for 1 hour at room temperature, to prevent non-specific binding of antibodies.

*Voltage Dependant Anion Channels*

The membrane was incubated overnight at 4◦C with the primary VDAC antibody (Anti-VDAC1/porin antibody, *ab14734 Abcam*, Cambridge, United-Kingdom), followed by a wash and incubation with the secondary antibodies (1:15000, anti-mouse, *ab6728 Abcam*, Cambridge, United-Kingdom). The membrane was exposed to ECL chemiluminescence (*Immun-Star Chemiluminescent; 1705070; Bio-Rad,* Hercules, United-States) to detect the VDACs using the *ImageJ Software*.

*Mitochondrial Complex Subunit Expression*

The membrane was incubated overnight at 4◦C with the primary antibodies (total OXPHOS rodent WB antibody cocktail, *MitoSciences,* Eugene, United-States), followed by a wash and incubation with the secondary antibodies (1:15000, anti-mouse, *ab6728 Abcam*, Cambridge, United

Kingdom). The membrane was exposed to ECL chemiluminescence (*Immun-Star Chemiluminescent; 1705070; Bio-Rad*, Hercules, United-States) to detect the VDACs using the *ImageJ Software*.

*Statistical Analysis*

The data are presented as means and standard error of the mean (SEM) for all values (29). The interquartile range test was used to identify outliers, which were excluded from the analysis. A two-way ANOVA paired with a Tukey's honestly significant difference (HSD) post-hoc test was conducted to test for significance for all measures. A *p*-value of $< 0.05$ was considered as statistically significant. For the graphs, the letters correspond to significant differences as compared to (a) the C-DIET, (b) the W-DIET and (c) the L-DIET. Also, numbers correspond to significant differences as compared to (1) the C-PROB, (2) the L-PROB and (3) the H-PROB conditions. All statistical analysis was performed using the *IBM SPSS Statistics 26* software.

## Results

**Figure 3.1.** The Effects of the Diets and Probiotics on the Mitochondrial Density, Bodyweight and Water Intake. **(1)** After the 6-week period, the mitochondrial density of the liver was assessed using immunoblotting (VDACs). In addition, **(2)** the bodyweight and **(3)** the average water intake were measured weekly. Lowercase letters (a, b, c) represent significant differences ($P<0.05$) between diet conditions, as compared to (a) the C-DIET, (b) the W-DIET and (c) the L-DIET. Numbers (1, 2, 3) represent significant differences ($P<0.05$) between probiotic conditions, as compared to the (1) C-PROB, (2) L-PROB and (3) H-PROB conditions. The statistical significance was assessed using a two-way ANOVA paired with a Tukey's (HSD) post-hoc test. C: C-DIET; W: W-DIET; L: L-DIET; P: L-PROB; PP: H-PROB. Values are presented as means ± the SEM (n=11-12/group).

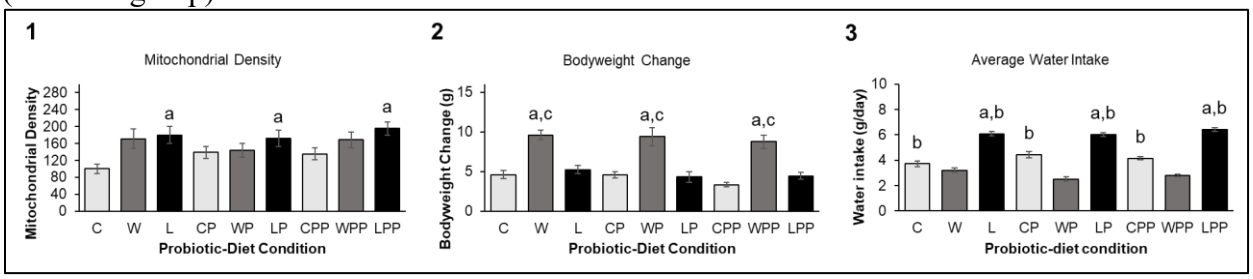


**Figure 3.2.** The Effects of the Diets and Probiotics on Parameters of Mitochondrial Respiration. After the 6-week period, the mitochondrial respiration of the liver was assessed using high-resolution respirometry, following the sequential addition of **(1)** malate, **(2)** octanoyl carnitine, **(3)** ADP, **(4)** glutamate, **(5)** pyruvate, **(6)** Cyt C, **(7)** succinate, and **(8)** FCCP. In addition, **(9)** the lipid coupling ratio (L/P Ratio) assessing the mitochondrial lipid oxidation efficiency, was calculated (Oct/ADP). **(10)** The substrate control ratio (SCR), assessing the mitochondrial substrate oxidation efficiency, was also measured (Glutamate/Succinate). **(11)** Lastly, the mitochondrial leak was measured (malate + octanoyl carnitine). Lowercase letters (a, b, c) represent significant differences ($P$<0.05) between diet conditions, as compared to (a) the C-DIET, (b) the W-DIET and (c) the L-DIET. Numbers (1, 2, 3) represent significant differences ($P$<0.05) between probiotic conditions, as compared to the (1) C-PROB, (2) L-PROB and (3) H-PROB conditions. The statistical significance was assessed using a two-way ANOVA paired with a Tukey's (HSD) post-hoc test. C: C-DIET; W: W-DIET; L: L-DIET; P: L-PROB; PP: H-PROB. Values are presented as means ± the SEM (n=11-12/group).

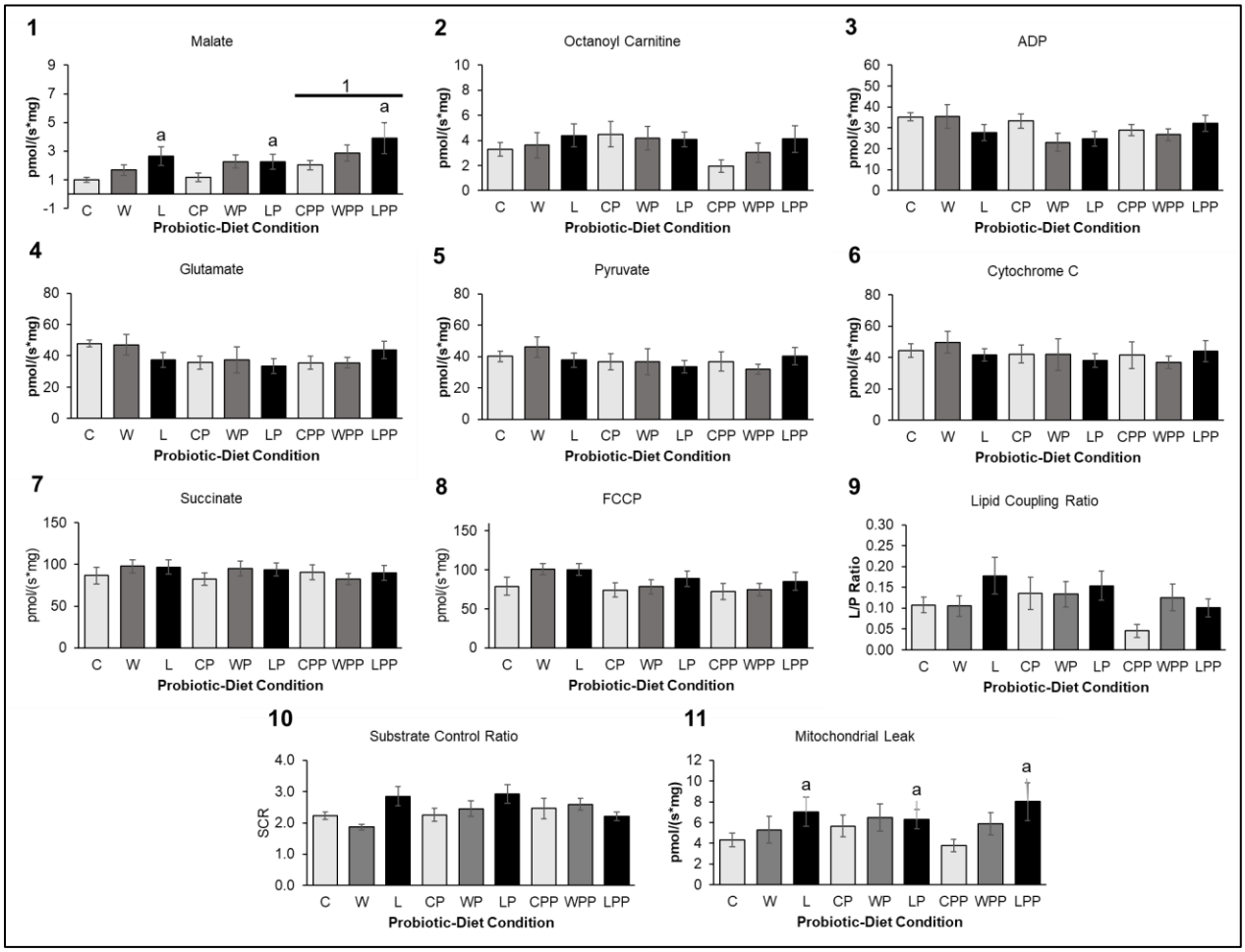
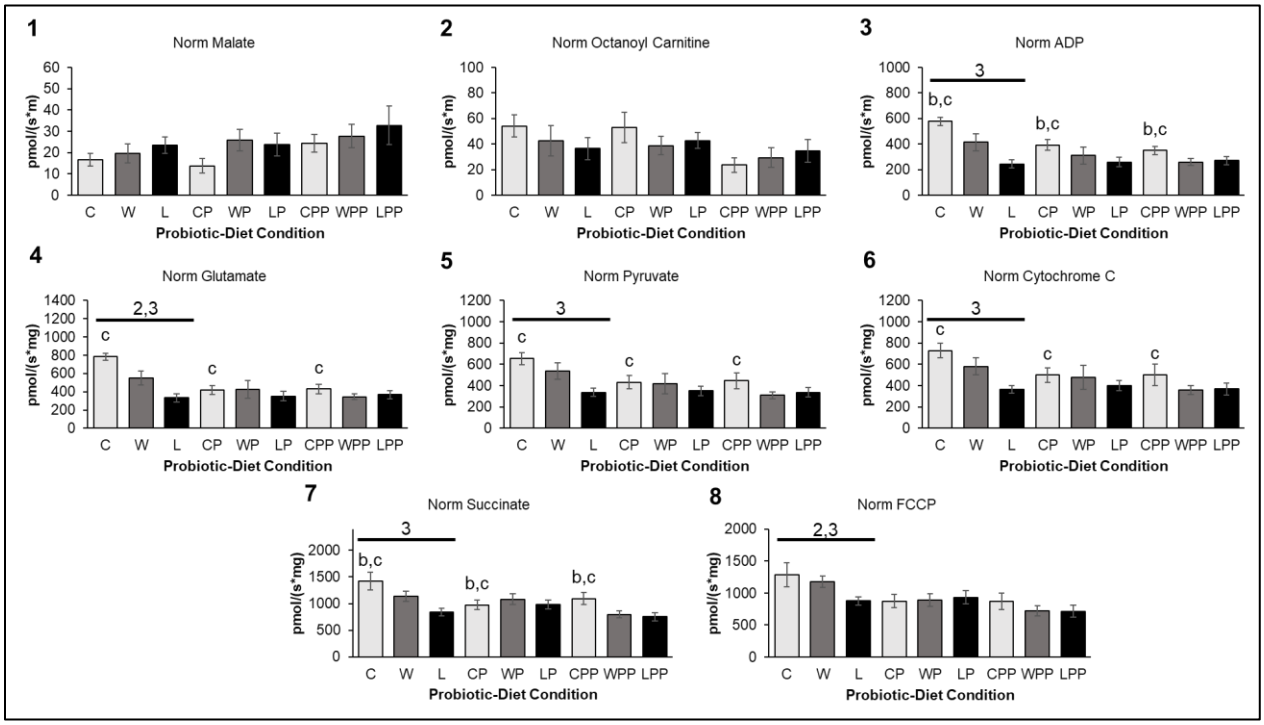


**Figure 3.3.** The Effects of the Diets and Probiotics on Parameters of Normalized Mitochondrial Respiration. The liver mitochondrial respiration measurements assessed by high-resolution respirometry (Figure 2) were normalized based on the mitochondrial density (VDAC) values (Figure 1). Lowercase letters (a, b, c) represent significant differences ($P$<0.05) between diet conditions, as compared to (a) the C-DIET, (b) the W-DIET and (c) the L-DIET. Numbers (1, 2, 3) represent significant differences ($P$<0.05) between probiotic conditions, as compared to the (1) C-PROB, (2) L-PROB and (3) H-PROB conditions. The statistical significance was assessed using a two-way ANOVA paired with a Tukey's (HSD) post-hoc test. C: C-DIET; W: W-DIET; L: L-DIET; P: L-PROB; PP: H-PROB. Values are presented as means ± the SEM (n=11-12/group).




*Mitochondrial Density*

The mitochondrial density was 30% higher in the L-DIET as compared to the C-DIET (mean difference (MD): 52.99, CI : 23.50-82.48, $p < 0.001$) (Figure 1).

*Bodyweight*

Animals on the W-DIET gained 54% more weight over 6-week period as compared to the C-DIET (MD: 5.00, CI : 3.74-6.27, $p < 0.001$) and 50% more weight as compared to the L-DIET (MD: 4.58, CI : 3.34-5.83, $p < 0.001$) (Figure 1).

*Mitochondrial Leak*

The H-PROB had 39% more complex I leak, as shown by malate, when compared to the C-PROB (MD: 1.15, CI : 0.011-2.28, $p = 0.047$). Furthermore, the L-DIET had 36% more total mitochondrial leak as compared to the C-DIET (MD: 2.55, CI : 0.10-4.99, $p = 0.038$) (Figure 2).

*Normalized Mitochondrial Respiration*

After normalizing with VDACs, the C-DIET groups had the highest coupled respiration as shown by a 41% higher response when compared to the L-DIET (MD: 181.68, CI : 92.44-270.91, $p < 0.001$). Similarly, the C-DIET had a 36% higher response to

glutamate (MD: 198.02, CI : 83.37-312.67, $p < 0.001$), 33% to pyruvate (MD: 171.63, CI : 54.83-288.43, $p = 0.002$), and 27% higher response to succinate (MD: 319.69, CI : 128.98-510.40, $p < 0.001$) when compared to the L-DIET. As for probiotics, the C-PROB condition had both the highest coupled and uncoupled respiration, as shown by a 27% higher response to ADP (MD: 108.07, CI : 24.12-192.02, $p = 0.008$), 30% to glutamate (MD: 158.01, CI : 47.49-268.53, $p = 0.003$), 28% to pyruvate (MD: 140.47, CI : 26.08-254.85, $p = 0.012$), 22% to succinate (MD: 247.86, CI : 63.02-432.70, $p = 0.005$), and a 31% higher response to FCCP (MD: 341.60, CI : 132.47-550.73, $p = 0.001$) (Figure 3).

**Discussion**

The objective of this study was to investigate the potential of the *L. Helveticus* and *B. Bifidum* probiotics to prevent a diet-induced mitochondrial dysfunction in the liver of ApoE-/- mice. The results show that the L-DIET significantly increases the mitochondrial density and mitochondrial leak, while reducing the normalized coupled respiration as compared to both the C-DIET the W-DIET. In parallel, probiotics increase complex I leak and decrease the normalized mitochondrial coupled and uncoupled respiration.

*The Low-Carbohydrate High-Protein Diet Increases Mitochondrial Density and Reduces the Normalized Mitochondrial Respiration*

The mitochondria adapt to match the ATP demands, and interpretations of respiration data should be confined in the context of the tissue and its stressors. Hepatocytes contribute to digestion, metabolism, and systemic detoxification, and Parry and colleagues found that a ketogenic diet (KD) improves the lifespan of rats by 20%, while also increasing the mitochondrial density, both in skeletal muscles and in the liver. Also, Luukkonen and colleagues reported that despite an increase in the plasma non-esterified fatty acids (NEFA) concentration of 35%, a KD could reverse symptoms of NAFLD, as shown by decreased intrahepatic triglycerides by 31% in a short 6-day period (30). However, the KD used in such studies controlled for calories and had a much lower protein content, of 23% proteins in the Parry *& al* study, and 28% in the Luukkonen *& al* trial, as compared to the 45% protein administered here (31). This is important, as Solon-Biet and associates found that very high-protein diets increase mortality rates in mice, potentially through its stimulating effects on the mTOR pathway (8).

For the L-DIET, disruptions in mitophagy signalling or an incapacity to match the ATP demand in response to physiological stressors could explain the increase in mitochondrial density. In fact, obese individuals with NASH have a higher mitochondrial density as compared to their healthy counterparts (32). Furthermore, Foo and colleagues showed that ApoE-/- mice fed a L-DIET develop more and bigger plaques as compared to W-DIET animals, potentially by downregulating the activity of the protein kinase B (Akt) in endothelial progenitor cells (EPCs) and therefore, reducing neovascularization following a vascular injury (7). In addition to its function in ECPs, Akt translocate into the mitochondria following a metabolic stress, such as an ischemia-reperfusion (I/R) injury and modulates glucose metabolism, apoptosis, ATP production as well as mitochondrial swelling, integrity and OS in cardiomyocytes (33). Findings from arteries and cardiomyocytes do not always translate to the liver, as Rocha *& al* show that the L-DIET increases coupled respiration in cardiomyocytes, opposingly to findings reported here (28). Still, it is possible that the increased mitochondrial density paired with a reduction in respiration parameters in the L-

DIET animals occurred through alterations in stress response pathways in the liver.

*Probiotics Decrease Normalized Mitochondrial Respiration without Altering Mitochondrial Density in the Liver*

By reducing the endotoxin translocation towards the liver, probiotics likely lowers the ATP demand and protect the liver. To support this, Mencarelli and colleagues reported that a VSL#3 cocktail containing 8 probiotic strains improves the integrity of the gut lining, while also decreasing biomarkers of liver injury in mice with dextran sodium sulfate induced colitis (18). In addition, Chan *& al* found that the same VSL#3 cocktail improves the gut microbial composition and reverses the systemic low-grade inflammation in mice fed a high-fat unhealthy diet (17). Lastly, Fan and associates showed that in an alcoholic-fatty liver disease (AFLD) mouse model, a combination of probiotics (*B. infantis*, *B. animalis*, and *L. acidophilus*) and blueberry juice reduces hepatic mitochondrial injury, swelling and necrosis. Fan & al also found that in mice receiving the cocktail, the hepatic mitochondria had an increased content (complex II and IV), improved efficiency (SCR), biomarkers of OS and importantly, a reduction in coupled respiration (19). Therefore, the outcomes reported here likely correspond to improvements in the integrity and a lower energetic demand placed on hepatocytes, but future studies should validate the safety and efficacy of using the *L. Helveticus* and *B. Bifidum* strains for the treatment of NAFLD.

*Both the Low-Carbohydrate High-Protein Diet and Probiotics Increase Mitochondrial Leak*

The leak of electrons from the electron transport chain (ETC) represents the primary intracellular producer of ROS, which functions as a signalling molecule in vascular homeostasis (34). However, a chronic production of ROS depletes the antioxidant stores and potentially, damages DNA, protein and lipid structures of the cell (35). Here, the results show that a high dose of probiotics increases the complex I leak, as shown by malate. In comparison, the L-DIET increases both complex I and total mitochondrial leak. However, it is unclear to what extent such findings translate to a physiological loss of function (36), as Staňková *& al* showed that mice fed an unhealthy W-DIET produce fewer ROS and have no biomarkers of OS after 30 weeks, although the same animals had more tissue hypoxia, inflammation, fibrosis and steatosis as compared to control animals (37). Therefore, whether the increased leak translates to oxidative stress and tissue damage remains to be proven and should be assessed by histological measurements.

*The Low-Carbohydrate High-Protein Diet Does Not Promote Weight Loss After 6 Weeks.*

The W-DIET, rich in fats and sucrose, promotes obesity, mitochondrial dysfunction and NAFLD (5,37,38). Here, the animals fed a W-DIET gained more weight over the 6-week period as compared to the C-DIET and L-DIET groups. Interestingly, while the L-DIET is becoming increasingly popular for its potential to promote weight loss (39–41), there was no difference in the weight gained as compared to the C-DIET.

*Probiotic Supplements Do Not Promote Weight Loss After 6 Weeks.*

The *L. Helveticus* and *B. Bifidum* probiotic supplements did not promote weight loss over the course of the experiment. Chan and associates reported similar findings, as no difference in bodyweight was reported between mice after 12 weeks of supplementation with a VSL#3 probiotic cocktail (17). However, such findings differ

from Fåk and colleagues, who reported that a 12-week supplementation with *Lactobacillus Reuteri* could prevent a diet-induced obesity in mice (16). Therefore, it is possible that the ability of probiotics to induce weight loss is strain specific.

*Conclusion*

Both the diet and probiotics impacted parameters of mitochondrial function. When framed in the context of the literature, the L-DIET appears to impair the function of hepatic mitochondria, while probiotics prevent a diet-induced mitochondrial dysfunction. However, such findings should serve as hypothesis generating, and future studies should validate and explore the safety and efficacy of both a L-DIET and the *L. Helveticus* and *B. Bifidum* probiotic strains in the development CLD and its complications.

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# Chapter 4

## Pilot Study Assessing the Potential of Lactobacillus Helveticus and Bifidobacterium Bifidum and the Low-Carbohydrate High-Protein Diet to Prevent Mitochondrial Dysfunction in the Aorta of Apolipoprotein-E Knockout Mice.

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*Antoine St-Amant*: animal handling, surgeries, respiration measurements, polymerase chain reaction sequencing, statistical analysis, preparation of manuscript.

*Lise Cougnaud*: polymerase chain reaction sequencing, editing of manuscript.

*Olivier Mathieu*: polymerase chain reaction sequencing, editing of manuscript.

*Jeremie Auger*: machine learning, editing of manuscript.

*Amanda Piano*: polymerase chain reaction sequencing.

*Andreas Bergdahl*: animal handling, surgeries, respiration measurements, editing of manuscript.

# Pilot Study Assessing the Potential of *Lactobacillus Helveticus and Bifidobacterium Bifidum* and the Low-Carbohydrate High-Protein Diet to Prevent Mitochondrial Dysfunction in the Aorta of Apolipoprotein-E Knockout Mice.

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**Summary:** Atherosclerosis represents the primary cause of death in the world and is highly preventable through lifestyle interventions. The mitochondrial dysfunction induces many stages of the disease, as it promotes oxidative damage and deregulates important signalling pathways. The western diet (W-DIET), rich in unhealthy fats and sugars, represents a potent driver of CVD, which leads many individuals to alternative dietary patterns, such as the low-carbohydrate high-protein diet (L-DIET). However, recent evidence, both in animals and in humans, show that such diets accentuate the progression of atherosclerosis. In parallel, probiotic supplements have been investigated as a potential nutraceutical intervention for many conditions, but its effects on the mitochondria remain largely unknown. Therefore, the objective of this study was to assess the effects of probiotic supplements containing *Lactobacillus Helveticus* and the *Bifidobacterium Bifidum* strains, and the L-DIET on the function of mitochondria in the aorta. To do so, apolipoprotein E knockout (ApoE-/-) mice were randomly assigned to 3 diet groups, including a control diet (C-DIET), a W-DIET, and a L-DIET. Then, each group was subdivided in either a control or high dose probiotic group. The body weight and water intake were monitored weekly, and at the end of the 6-week period, high-resolution respirometry was performed on the aorta to assess different parameters of mitochondrial respiration. Also, PCR analysis was conducted on the caecum to assess changes in the gut bacterial composition. The results show that although the W-DIET rapidly induces obesity, neither the probiotics nor the diet significantly alter parameters of mitochondrial respiration in the aorta after a 6-week period. However, the PCR analyses show that the gut microbiota of the L-DIET and the W-DIET cluster together. Such findings are worrying, and future experiments should explore the effects of the L-DIET and probiotics in maintaining a healthy bacterial profile and on the mitochondria.

**Keywords:** Atherosclerosis, gut microbiota, probiotics, low-carbohydrate high-protein diet, vascular smooth muscle cells, mitochondria.

## Introduction

Atherosclerosis, a disease characterized by the buildup of lipid-rich plaques in medium to large arteries, represents the most common cause of death in the world, and accounts for over 80% of cardiovascular diseases (CVD) (1). The mitochondria represent the primary ATP supplier in blood vessels (2–4). Also, the organelle contributes to many steps of the disease, through the production of reactive oxygen species (ROS), but also by deregulating complex signalling pathways, ultimately leading to an energy crisis and chronically, cellular apoptosis or senescence (2,5). Importantly, mitochondrial

dysfunction promotes the migration, dedifferentiation, and proliferation of vascular smooth muscle cells (VSMCs) from the media and towards the intima, a key event after which the disease progression becomes irreversible (6,7).

Maintaining a healthy lifestyle is fundamental in the prevention of atherosclerosis, as plaques develop over decades. Yet, westerners are sedentary, obese and the western diet (W-DIET), high in unhealthy sugars and fats, causes mitochondrial dysfunction (8,9) and gut dysbiosis (10,11), two major risk factors for atherosclerosis (12–14). To prevent such outcomes, more and more individuals turn to low-carbohydrate high-protein diet (L-DIET). However, emerging studies, both in humans and in animals, show that the L-DIET worsens the accumulation of plaques in blood vessels (15,16).

Many trials show that probiotics prevent atherosclerosis in animals (17–19). For example, the *Lactobacillus Helveticus* and *Bifidobacterium Bifidum* strains reduce hypercholesterolemia, oxidative stress and maintain vascular health, both in mice and *in vitro* (20–23). However, whether the plaque regression occurs through improvements in mitochondrial integrity remains to be proven. Therefore, the objective of this pilot experiment was to assess the potential of probiotic supplements and of the L-DIET on the function of aortic mitochondria and on the gut microbiota.

## Methods

*Animals and Diets*

The animal ethics committee at Concordia approved this project (protocol ID: #30000259) and follows the guidelines of the Canadian Council on Animal Care. Apolipoprotein-E knockout (ApoE-/-) mice, genetically modified to develop dyslipidemia, a major risk factor for atherosclerosis, were selected from the breeding colony at *Concordia* (originally purchased from *Jackson Laboratories*, Maine, United States) (24,25). The rodents were weaned and separated based on sex at 3–4 weeks, after which males were placed individually in a thermo-neutral environment at 22◦C with a 12:12h photoperiod. The mice had access to water and the diets *ad libitum*. For the duration of 6 weeks, the animals were randomly assigned to one of 3 diets, including a normal, healthy control diet (C-DIET), an unhealthy western-style diet (W-DIET), and a low-carbohydrate high-protein diet (L-DIET) (table 1). The C-DIET and L-DIET were isocaloric. The W-DIET and the L-DIET had a similar cholesterol content (1.5 g/kg). Also, all diet groups were divided in two subgroups, based on the dose of probiotics administered, including a control (C-PROB), and a high-dose group (H-PROB), for a total of 6 groups (n=12 animals/group). In colony forming units (CFU), the probiotics (Lafty L10, *Lallemand Health Solutions,* Montreal, Canada) contained a 1:1 ratio of *Bifidobacterium Bifidum* ($5.04 \times 10^9$ CFU/dose) and *Lactobacillus Helveticus* ($4.94 \times 10^9$ CFU/dose). Probiotics were administered through the drinking water. The water intake and bodyweight were monitored weekly, and at the end of the 6-week period, necropsy was performed to collect the aorta and the caecum.

**Table 4.1.** Macronutrient Content of the Diets. The table highlights the percentage (%) of carbohydrates, fats and proteins in the C-DIET, W-DIET, and the L-DIET.

| | **C-DIET** *5075 Charles River Autoclavable Rodent Diet* (Wilmington, United States) | **W-DIET** *Harlan Laboratories (TD. 110229)* (Indianapolis, United-States) | **L-DIET** *Harlan Laboratories (TD. 04524)* (Indianapolis, United-States) |
|---|---|---|---|
| Carbohydrates | 63 | 42.2 | 11 |
| Fats | 14 | 42.1 | 43.2 |
| Proteins | 24 | 15.7 | 45.8 |

*Respiration Measurements*

Following necropsy, the aorta was immediately placed in an ice-cold physiological solution (BIOPS), containing (in mM) CaK2EGTA 2.77, K2EGTA 7.23, Na2ATP 5.77, MgCl2·6H2 O 6.56, taurine 20, Na2Phosphocreatine 15, imidazole 20, dithiothreitol 0.5, MES 50, pH 7.1. Then, the aorta was freshly cut open using sharp forceps, incubated in 3 ml of BIOPS buffer containing 50 µg/ml saponin for 30 minutes and washed in mitochondrial respiration medium (MiR05) containing (in mM): EGTA 0.5, MgCl2·6H2 O 3.0, K-lactonionate 60, Taurine 20, KH2PO4 10, HEPES 20, Sucrose 110, BSA 1g/L, pH 7.1, for 2 × 10 minutes. After the wash, 2-2.5 mg of aortic tissue was immediately added to the Oxygraph chamber in 2 ml of MiR05 for high-resolution respirometry. The mitochondrial respiration was assessed using a sequential substrate addition protocol, using a polarographic sensor (*Oxygraph-2k*, *Oroboros Instruments,* Innsbruck, Austria), following the protocol proposed by Dall *& al* (26). The measurements of oxygen consumption were performed in MiR05 at 37◦C, and the oxygen flux was registered using the *DatLab* software. The aorta was added to the Oxygraph chamber in an environment deprived in ADP and substrates, but hyper-oxygenated. Then, malate (4 mM) and octanol-carnitine (10 µM) were added to assess mitochondrial leak. Afterwards, the maximal coupled respiration was attained through the sequential addition of ADP (10 mM), glutamate (5 mM) pyruvate (5 mM) cytochrome C (5 µM) and succinate (20 mM). Lastly, the maximal uncoupled respiration was reached with cyanide p-trifluorome thoxyphenylhydrazon (FCCP) (1 mM).

*Statistical Analysis*

The respiration data are presented as means and standard error of the mean (SEM) for all values. Outliers were identified and eliminated, using the interquartile range (IQR) statistical test, and a two-way ANOVA paired with a Tukey's honestly significant difference (HSD) post-hoc test was used to assess for statistical significance. Results were considered as statistically significant if the $p$ value was $< 0.05$. For respiration measurements, the statistical analysis was performed using the *IBM SPSS Statistics 26* software.

*Sample Preparation and DNA Extraction for PCR Analysis*

At the 6-week time point, the caecum was collected and frozen at -80 °C. The caecal content was squished out into a lysis tube, with a mass ranging from 50 to 90 mg. In total, 67 caecum samples were weighted (n = 10–12/group). The DNA was extracted

(*ZymoBIOMICS 96 MagBead DNA kit*, Irvine, United States). Samples were then homogenized using a bead beater (*MP Biomedicals Fastprep-24 5G instrument*, Solon, United States) for 5 cycles of 1 min at 6.5 m/s with a 1 min break between cycles. Samples were centrifuged at 12,200 rpm for 1 min, followed by 7,700 rpm for 5 mins. Then, 200 µL of the supernatant, 600 µL of β-Mercaptoethanol (*Sigma Aldrich*, St-Louis, United States) and 25 µL of MagBinding beads were added to a 96-well plate. An automated DNA extraction was performed using the *KingFisher Flex (ThermoFisher Scientific,* Waltham, United States). Samples free of the magnetic bead were then transferred to a new 96-well reaction plate, and the DNA concentration was measured using a spectrophotometer (*NanoDropTM 8000, ThermoFisher Scientific,* Waltham, United States). Plates were sealed using an adhesive sealing sheet and stored at -20 °C until normalization.

*DNA Normalization*

The concentration of each sample was normalized using a liquid handling robot (*Eppendorf Epmotion 5075,* Hamburg, Germany). Using the nanodrop measurement, each sample was diluted in PCR water to a DNA concentration of 20 ng/µL. Plates were sealed and put in the freezer for the next step.

*Amplicon PCR*

The normalized plates were thawed at room temperature. To prevent contamination, all pipettes were cleaned using 20% bleach solution and UV-ed for 20 minutes prior to the amplicon PCR. To increase sequencing diversity, each plate within the same run used different pairs of primers (#0, 1, 2 or 3) (*Integrated DNA Technologies*, Iowa, United States). In a new 96 well PCR plate, 22.5 µL of master mix and 5 µL of normalized DNA were added in each well. The master mix contained 5 µL of each forward and reverse primers from the same pair, at 1 µM, and 12.5 µL of 2x KAPA TAQ polymerase HIFI Hot start ready mix (*Roche Diagnostics, cat # KK 2802*, Basel, Switzerland). Negative and positive controls were added on each plate. The negative control contained the master mix without any DNA. The positive control contained commercial Mock Community with DNA from known bacterial strains with specific quantities and was purchased from the *American Type Culture Collection*. The plate was sealed with aluminum, and the PCR was performed using the following parameters: 95 °C for 3 min, 30 cycles (95 °C for 30 sec, 55 °C for 30 sec, 72 °C for 30 sec), 72 °C for 5 mins (*5333 Mastercycler Thermal Cycler, Eppendorf,* Hamburg, Germany). Following the amplification, 5 µL of each reaction was mixed with 5 µL of homemade loading dye, and the amplicon presence was determined using 2% agarose 96-well gel, (*Invitrogen,* Waltham, United States), pre stained with *SYBR* safe. The gel was then run for 10 mins on the gel stand and visualized using a *Bio-Rad Image labTM* (Hercules, United States).

*Amplicon PCR Cleanup*

After amplification, the DNA was cleaned to remove the unincorporated primers, and the incompletely amplified shorter DNA fragments. The entire PCR reaction was transferred into a 96-well MIDI plate, and 25 µL of the *Beckman AMPure XP beads* (Brea, California) was added to each well. The DNA and beads were mixed using a thermoshaker at 1800 rpm for 2 mins. The mix was then incubated at room temperature for 5 mins to allow the DNA to bind the beads and then placed on a magnetic stand for 2 mins. The supernatant was discarded, and the beads were washed twice with 200 µL of freshly prepared 80% ethanol. Then, the beads were air-dried for 10 min, and 52.5 µL of 10 mM Tris buffer (pH 8.5) (*Fisher Bioreagents BP1758-100*, Hampton, United

States) was added to each well to release the DNA from the beads. The plate was shaken (1800 rpm for 2 min) and incubated at room temperature for 2 min, followed by 2 more minutes on a magnetic stand. The supernatants were transferred to a new 96 PCR plate, which was sealed and put in the freezer for the next step.

*Index PCR*

The second PCR attaches dual indexes and sequencing adapters in preparation for the *MiSeq* analysis. These indexes serve as unique molecular tags that are attached to the amplicons to help identify each sample in a multiplex pool of samples (*Nextera XT Index Kit V2, Illumina*, San Diego, United States). Firstly, 2.5 µL of reverse and forward primers was added in each well of a new 96-well PCR plate, followed by 17.5 µL of master mix (2x KAPA HIFI Hot Start ready mix) and 5 µL of cleaned amplicon PCR template DNA. The plate was covered with an aluminum seal, and the index PCR was performed with the following cycling conditions: 95 °C for 3 min, 8 cycles (95 °C for 30 sec, 55 °C for 30 sec, 72 °C for 30 sec), 72 °C for 5 mins. After the amplification, the DNA was cleaned, using the same protocol as after the amplicon PCR.

*Quantification of DNA Concentration by PicoGreen*

A PicoGreen analysis was performed to precisely measure the DNA concentration in each sample (*dsDNA Assay Kits and Reagents, Invitrogen P7589,* Waltham, United States). Briefly, 50 µL of 1x TE was added in each well of the row A of a black 96 well plate (except A12). This row corresponds to a serial dilution of Lambda DNA standards. For the standard curve dilutions, 100 µL of the 2 µg/mL Lambda solution was added to the well A12. Then, 50 µL was transferred to A11, while mixing with several ups and downs with the pipet tips (changed after every dilution transfer), and so on until A3. In the other rows, 49 µL of 1x TE and 1 µL of cleaned Index PCR reaction DNA were added. Finally, 50 µL of 1X PicoGreen was added in each well. Plates were incubated in the dark for 10 min and read using a fluorescence plate reader at 480 nm (excitation) and 520 nm (emission) wavelengths (*VarioSkan Lux Multimode microplate reader, ThermoFisher,* Waltham, United States*)*. Each sample within the plate was normalized by adding volumes corresponding to a DNA quantity of 200 ng using a liquid handling robot (*EpMotion 5075*, *Eppendorf,* Hamburg, Germany).

*QuBit Analysis*

The pool was quantified with QuBit Broad Range (*BR, Invitrogen,* Waltham, United States) working solution (2 µL pooled DNA and 198 µL working solution), and the fluorometer quantification system (*Qubit4, ThermoFisher,* Waltham, United States) relative to 2 nucleic acid standards (containing 190 µL working solution and 10 µL standard 1 and 2). This pool was further diluted in PCR water, to a concentration of 1 ng/µl, prior to a third Qubit run in triplicate (10 µL pool DNA with 190 µL BR working solution), to obtain with precision the DNA concentration for subsequent molarity calculations.

*Library Quality Control with TapeStation Analysis*

To ensure the sequencing success, the pool was assayed on the TapeStation analysis (*4200 TapeStation System, Agilent,* Santa Clara, United States) for amplicon size determination as well as amplification specificity. First, the *Agilent D1000 TapeStation* reagents were brought up to room temperature for 30 minutes. Each quantified well of a PCR tube strip was loaded with 2 µL buffer. Then, the first well of the PCR tube strip was loaded with 2 µL

ladder, the second with 2 µL of the diluted pool (1 ng/µL), and the last with 2 µL of a positive control(already processed good amplicon library). The amplicon size in base pairs (bp) obtained with the TapeStation was used to calculate the molarity (nM).

*MiSeq Loading*

The *Illumina MiSeq reagent kit V3* (San Diego, United States) was used to load the MiSeq sequencer at 12 pM. Before loading the machine, a power cycle was done and a maintenance wash consisting of three 0.5% Tween 20 cleanups were performed. The flow cell was thoroughly cleaned using HPLC H2O and dried completely with Kim wipes. The final library was denatured by mixing 5 µL of the pool DNA (1 ng/µL) with an equal volume of 0.2 N NaOH solution and incubated at room temperature for 5 minutes. After, the solution was diluted in the HT1 buffer to a final concentration of 12 pM. In parallel, the PhiX (10 nM) sequencing quality control solution was prepared by mixing 2 µL PhiX with 2 µL 0.2 N NaOH and incubated at room temperature similarly to the final library sample. HT1 buffer (996 µL) was added to reach a final volume of 1 ml [20 pM]. A 600 µL sample volume of final library combined with 5% PhiX were for 2 minutes at 96◦C followed by 5 minutes on ice. The entire volume was loaded in the MiSeq cartridge. The *Illumina MiSeq System* was loaded with the cartridge and started for 2 x 300 paired-end sequencing cycles (600 total cycles).

*Machine Learning Analysis*

The 16S amplicon sequencing results were exported in *fastq* format from the MiSeq and imported into *Qiime2*, which allows the tracking of every parameter used in the various modules and integrated software. After import, the reads were inspected for quality, then the forward reads were trimmed both at 280 base pairs and based on q-scores. The selected reads were clustered into ASV abundance tables using the *denoiser module deblur* and the ASV tables were used for the principal coordinates analysis (PCoA), visualized using *Emperor*. Other figures were viewed using the *Qiime2* server online (https://view.qiime2.org/). Each data of the PCoA represents a caecal sample and the distance matrix between them based on the diet and probiotics. The *ASV* tables were converted to genus-level taxonomic abundance tables using the *Qiime* feature classifier trained on the *GreenGenes* 16S database. These genus-level abundance tables were used to determine the microbiome profiles associated with group characteristics, specifically the diet and probiotic dose, using the *Qiime* sample-classifier tool and the Machine Learning algorithm *Extra Trees Classifier*. For the receiver operating characteristic (ROC) curve, an area under the curve (AUC) value of 0.9-1 was considered excellent, 0.8-0.9 very good, 0.7-0.8 good, 0.6-0.7 sufficient, 0.5-0.6 bad and any value under 0.5 rendered the test not useful (27).

## Results

**Figure 4.1.** The Effects of the Diets and Probiotics on the Bodyweight, Water Intake and Parameters of Mitochondrial Respiration. **(1)** The bodyweight and **(2)** water intake were monitored over the 6-week period. In addition, after the 6-week period, the mitochondrial respiration of the aorta was measured using high-resolution respirometry, following the sequential addition of **(3)** malate, **(4)** octanoyl carnitine, **(5)** ADP, **(6)** glutamate, **(7)** pyruvate, **(8)** Cyt C, **(9)** succinate, and **(10)** FCCP. From the parameters of mitochondrial respiration, **(11)** the lipid coupling ratio (L/P Ratio) assessing the mitochondrial lipid oxidation efficiency was calculated (Oct/ADP). **(12)** The substrate control ratio (SCR), assessing the mitochondrial substrate oxidation efficiency was also measured (Glutamate/Succinate). **(13)** Lastly, the mitochondrial leak was measured (malate + octanoyl carnitine). Lowercase letters (a, b, c) represent significant differences ($P$<0.05) between diet conditions, as compared to (a) the C-DIET, (b) the W-DIET and (c) the L-DIET. The statistical significance was assessed using a two-way ANOVA paired with a Tukey's (HSD) post-hoc test. C: C-DIET; W: W-DIET; L: L-DIET; PP: H-PROB. Values are presented as means ± the SEM (n=12/group).

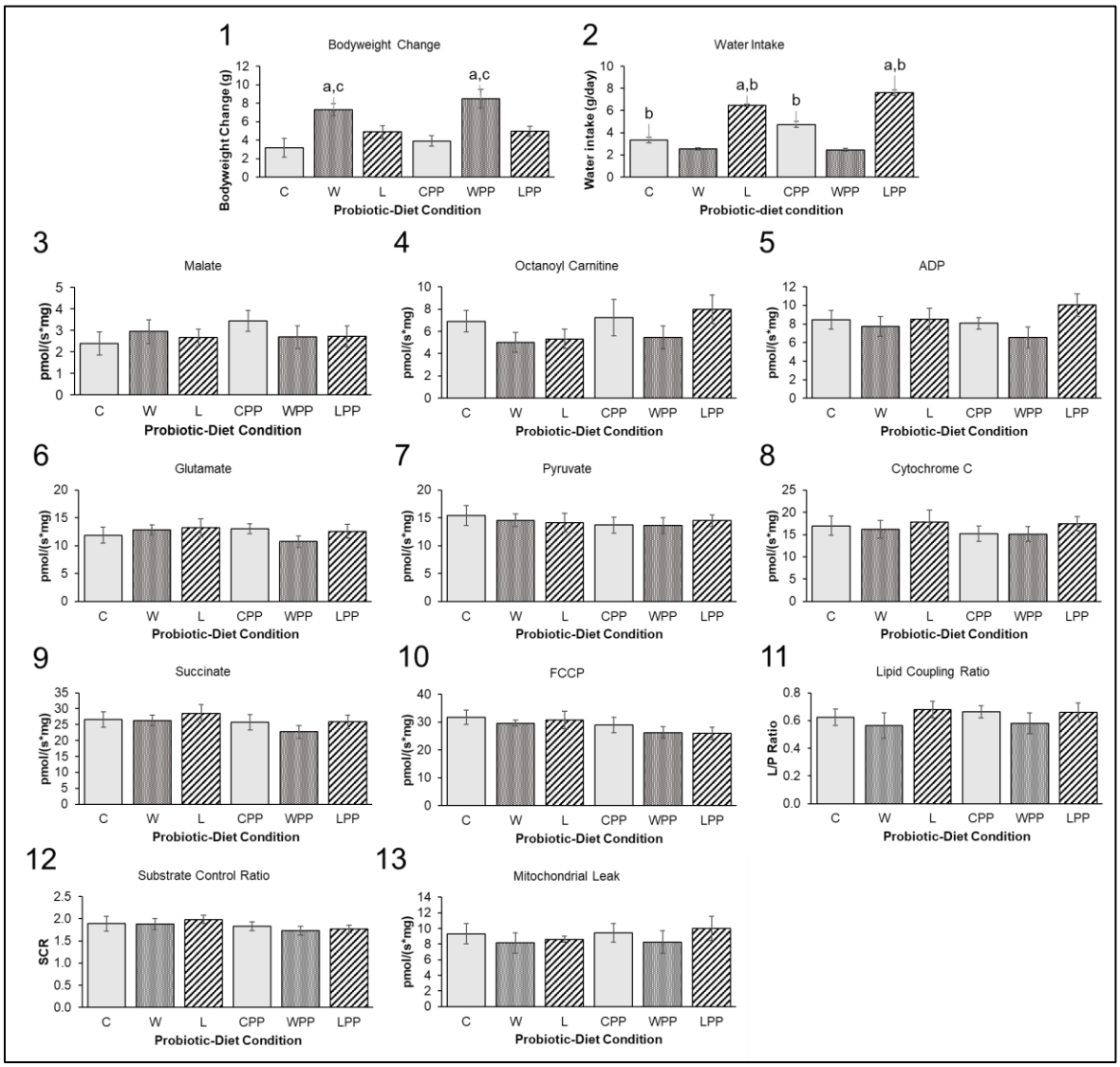


**Figure 4.2.** The Effects of Probiotics and Diets on the Gut Microbiota. After the 6-week period, the microbiome content of the caecum was squished in a tube and assessed using 16S rRNA PCR sequencing. The beta diversity was quantified using **(1a)** unweighted and **(1b)** weighted UniFrac PCoA, based on the diet Administered. The beta diversity was also analysed by **(2a)** unweighted and **(2b)** weighted UniFrac PCoA based on the dose of probiotics administered. Each point represents a caecal sample and the distance matrix between them based on the relative abundance (weighted) or the presence/absence (unweighted) of species/taxa. CD: D-DIET; WD: W-DIET; LD: L-Diet; None: C-PROB; High: H-PROB (n=12/group).

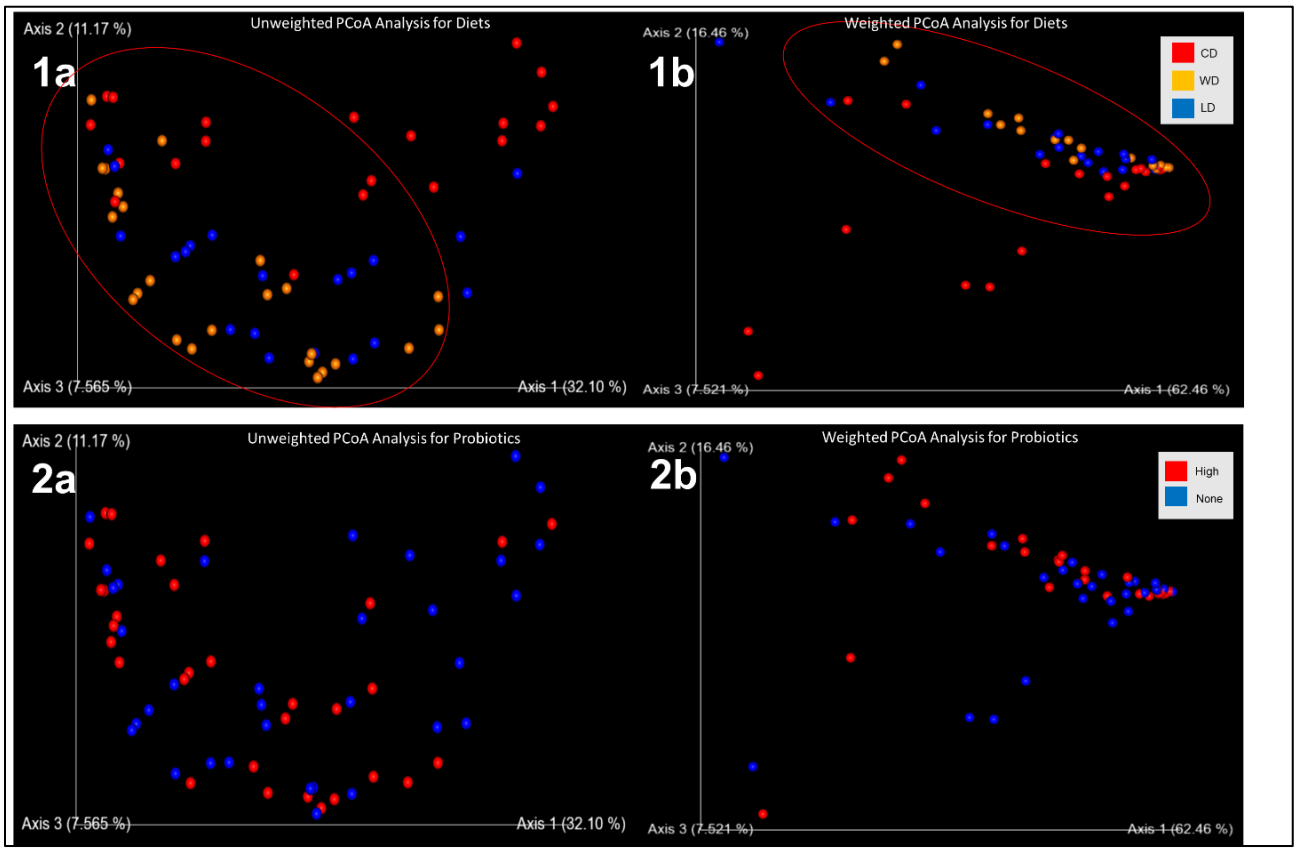
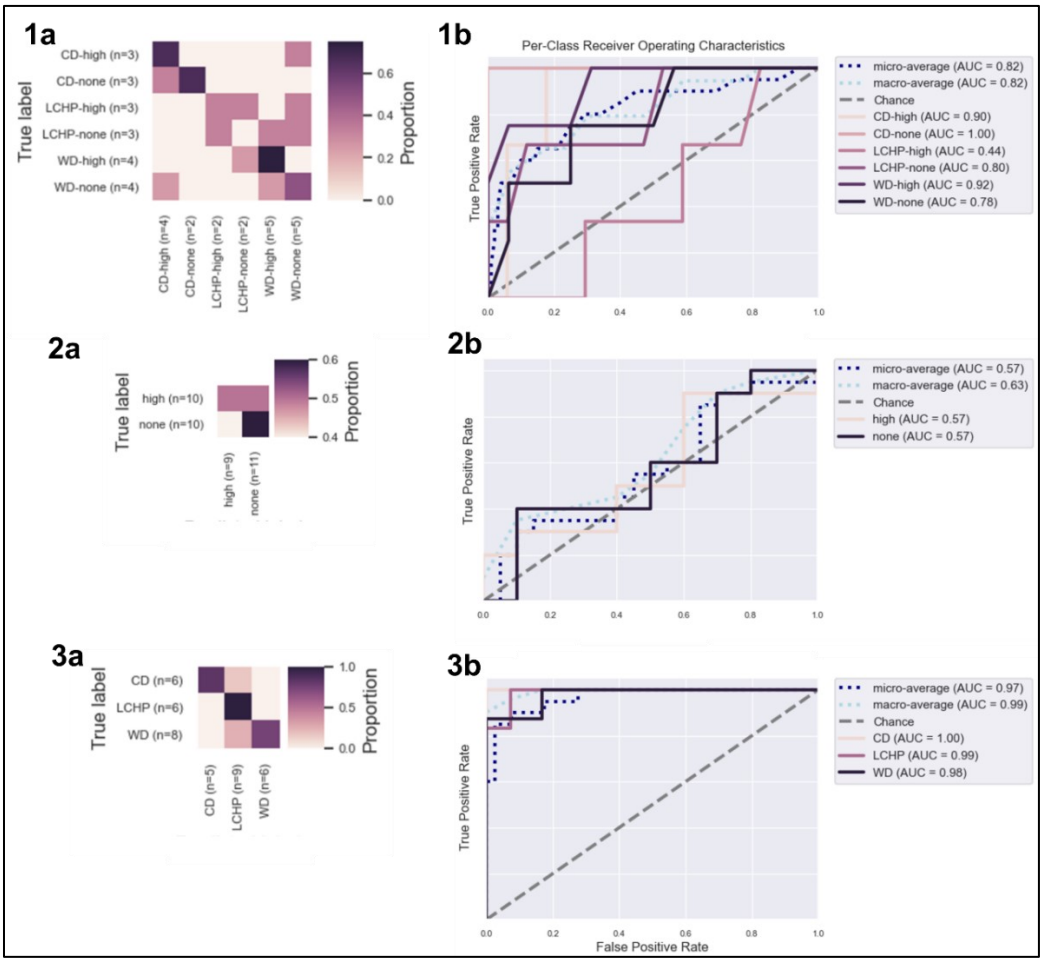


**Figure 4.3.** The Performance of the Machine Learning Classification Model. The gut microbiota quantified by 16S rRNA PCR sequencing were exposed to a machine learning algorithm to decipher between conditions based on the bacterial content in the gut. The model accuracy and ROC curve, measuring the performance of the machine learning classification model at deciphering between groups, were conducted based on **(1a-b)** the diet-probiotic conditions, **(2a-b)** the probiotic conditions only, and **(3a-b)** the diet conditions only. CD: C-DIET; WD: W-Diet; LCHP: L-DIET; None: C-PROB. High: H-PROB (n = 12/group).




*Bodyweight*

During the 6-week period, animals fed the W-DIET gained 55% more weight as compared to the C-DIET (mean difference (MD): 4.35, CI : 2.54-6.17, $p < 0.001$) and 50% more weight as compared to the L-DIET (MD: 2.96, CI : 1.13-4.80, $p < 0.001$).

*Respiration Measurements*

Also, the diets did not alter any parameter of mitochondrial respiration. However, a 30% higher response to ADP was observed in mice fed the L-DIET as compared to the W-DIET (MD: 2.14, CI : -0.40-4.68, $p = 0.115$). Similarly, while no significant difference was seen between probiotic groups, the C-PROB condition had a 9% higher response to succinate (MD: 2.38, CI : -0.33-6.09, $p = 0.204$), a 12% higher response to FCCP (MD: 3.65, CI : -0.246-7.54 $p = 0.066$), and a 7% higher SCR as compared to the H-PROB group (MD: 0.140, CI : -0.53-3.33, $p = 0.152$) (figure 1).

*Gut Microbiota*

For the microbial data, both in the weighted and unweighted caecal microbiome data sets, the W-DIET and L-DIET animals cluster visually, as compared to the C-DIET. However, no clear clustering appears based on the dose of probiotics (figure 2). Furthermore, while the overall accuracy ratio (AR) was good (AR = 2.75), the model was better at differentiating between animals based on the diet (AR = 2.13) as compared probiotics to (AR = 1.10). Besides, when pairing the probiotic and diet conditions, the AUC values varied from poor to excellent. However, when isolating the diet and probiotic variables, the algorithm was excellent at distinguishing the bacterial profiles based on the diet (C-DIET: AUC = 1.00; W-DIET: AUC = 0.99; L-DIET: AUC = 0.98), but bad at deciphering between probiotic groups (H-PROB: AUC = 0.57; C-PROB: AUC = 0.57) (figure 3).

**Discussion**

The objective of this pilot study was to assess the effects of the *Lactobacillus Helveticus* and *Bifidobacterium Bifidum* probiotics, as well as the L-DIET, on the function of mitochondria in the aorta and the gut bacterial composition. Here, while non-significant, the L-DIET condition led to a greater respiration as compared to the obese W-DIET animals. Such findings imply a greater level of dedifferentiation in the L-DIET. Also, the L-DIET appears to induce gut dysbiosis, as the bacterial profile of L-DIET animals closely clustered with that of the unhealthy W-DIET animals (figures 2 and 3). In 2009, Foo *& al*, who reported that after 12 weeks, ApoE-/- mice fed a L-DIET develop more atherosclerotic plaques as compared to a W-DIET, without altering biomarkers associated with lipidaemia, inflammation or oxidative stress. Therefore, the L-DIET might promote atherosclerosis through its effects on the mitochondria. During the pathogenesis of atherosclerosis, mitochondrial bioenergetics is heavily altered during the shift from a contractile to a synthetic VSMC phenotype (6). Specifically, studies have reported that synthetic VSCMs have a higher coupled respiration, more leak and increased reliance on fatty acids as compared to glucose (28,29).

In parallel, probiotics could reverse the mitochondrial dysfunction induced by a poor diet (figure 1). Here, while no significant difference was observed for probiotics, the trends show that the H-PROB condition reduces parameters of both coupled and uncoupled respiration. Such results point to a reduction in the switching from a contractile to a synthetic phenotype. However, such findings could be attributed to luck, as the bacterial profile does not appear to cluster in response to the dose of probiotics alone (figures 2 and 3). Still, the findings reported here do not encapsulate the complexity of the bacterial ecosystem, and changes might have occurred at the micro level (bacterial strains).

*The Diet Does Not Alter Mitochondrial Bioenergetic in the Aorta*

Surprisingly, no change was observed for mitochondrial respiration based on the diet. Many trials have reported that a W-DIET impairs mitochondrial metabolism in many tissues, including skeletal muscles (8), hepatocytes (9) and cardiomyocytes (30). For example, Rocha and associates found that after 6 weeks, the L-DIET increases coupled respiration, leak and reduces mitochondrial efficiency in the cardiomyocytes of ApoE-/- mice (30). However, cardiac muscles have the highest mitochondrial density of all tissues, accounting for 35% of its volume, as compared to 3–5% in VSMCs, and therefore might be more sensitive to changes in substrate availability (31). Therefore, a 6-week period might not be enough to induce significant changes in VSMCs, and longer periods might translate into larger effects. Namely, many trials show that after 12 weeks

on a W-DIET, mice develop atherosclerotic lesions in the aorta (17–19).

*The Gut Microbiota of Low-Carbohydrate High-Protein Diet and Western-Style Diet Mice Cluster Together*

The gut microbiota modulates atherosclerosis (12–14,32), and many trials highlight the efficacy of the W-DIET at disrupting the gut microbial profile (10,11,33). In 2018, Liu and colleagues found that W-DIET animals develop more atherosclerotic lesions, paired and increased ratio of *Firmicutes* to *Bacteroidetes*, which correlates with CVD (11,14). Also, Hasegawa and colleagues found that after 15 months on a W-DIET, mice become obese, and secrete more endotoxins, including TMA and TMAO (10). It is therefore worrying that here, the machine learning model clusters the L-DIET with the W-DIET (figure 2).

*Probiotic Supplements Do Not Alter Mitochondrial Bioenergetic in the Aorta*

Similarly, to the diet, probiotic supplements did not induce changes in mitochondrial respiration. This is also unexpected, as Fan and colleagues found that a cocktail containing probiotics and blueberry juice reverses hepatic mitochondrial injuries and improves many respiration parameters in an alcoholic fatty liver disease mouse mode after only 10 days (34). The ability of probiotics to modulate mitochondrial function could be strain specific. However, the metabolic demand placed on the liver differs from that of VSMCs, and future experiments should assess the potential of the *L. Helveticus* and *B. Bifidum* to prevent mitochondrial dysfunction after longer periods.

*Conclusion*

The objective of the trial was to assess changes in aortic mitochondrial respiration and in the gut microbiota following the administration of an L-DIET and probiotic supplements (*L. Helveticus* and *B. Bifidum*). While nonsignificant, the L-DIET causes a trend towards a greater VSMC dedifferentiation, paired with changes in the gut bacterial profile resembling that of W-DIET animals. In parallel, it remains unclear whether probiotics protect the blood vessel against the formation of plaques, and future studies should assess [1] changes that occur in bacterial strains, such as in the ratio of *Firmicutes* to *Bacteroidetes*, [2] alterations in mitochondrial density, and [3] functional differences in the contractile capacity of the aorta.

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# Chapter 5

Concluding Remarks

## Concluding Remarks

The objective of this thesis was to assess the potential of probiotics and the effects of the L-DIET on the pathogenesis of atherosclerosis, primarily through the lens of mitochondrial dysfunction. The data presented here should serve as hypothesis generating, and not for the development of clinical guidelines. Still, the results show that both probiotics and the L-DIET alter cellular and biochemical mechanisms that warrant further investigation. The first manuscript, a meta-analysis shows that probiotics improve a variety of biomarkers associated with both the antioxidants status and oxidative stress and could therefore prevent oxidative damage. The second manuscript highlights some interesting mitochondrial changes following the administration of probiotics in the liver, which was hypothesized to counteract the detrimental effects of the L-DIET. The last manuscript displays some worrying preliminary data, as the L-DIET appears to promote changes in the gut microbiota that resemble that of a W-DIET. Still, in the light of this thesis, future work should **(1)** pair mitochondrial respiration data with histologic measurements to assess for tissue damage. Also, studies should **(2)** measure whether the mitochondrial leak observed in the L-DIET translates to oxidative stress, primarily in hepatocytes, which due to their anatomical location and complex physiology, appear to be highly susceptible to nutrient changes and endotoxins as compared to blood vessels. Lastly, **(3)** trials should explore specific bacterial changes that occur in response to probiotics and the L-DIET, including the ratio of *Firmicutes* to *Bacteroidetes*.

# Chapter 6

Chapter 1 References

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