THE IMPACT OF GUT MICROBIOTA AND ARONIA MELANOCARPA ON INFLAMMATION AFTER A HIGH-FAT DIET IN HUMANIZED MOUSE MODELS

by

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GLOSSARY

ARO - Aronia Juice
cDNA - Complementary DNA
CON - Control Juice
CKD - Chronic Kidney Disease
CRP - C-Reactive Protein
CT - Threshold Cycle
CVD - Cardiovascular Disease
DEPC - Diethyl Pyrocarbonate
DNA - Deoxyribonucleic Acid
EtOH - Ethanol
FFA - Free Fatty Acid
FMT - Fecal Microbiota Transplant
GLUT4 - Glucose Transporter Type 4
GPx - Glutathione Peroxidase
HFD - High Fat Diet
HI - High Inflammatory Phenotype
IL - Interleukin
IR - Insulin Resistance
LFD - Low Fat Diet
LO - Low Inflammatory Phenotype
LPS - Lipopolysaccharide
MAPK - Mitogen-activated protein kinase
MetS - Metabolic Syndrome
NAFLD - Non-alcoholic Fatty Liver Disease
NASH - Non-alcoholic Steatohepatitis
NF-kB - Nuclear Factor kappa-light-chain-enhancer of activated B cells
NO - Nitric Oxide
qPCR - Quantitative PCR
RNA - Ribonucleic Acid
ROS - Reactive Oxygen Species
SCFA - Short Chain Fatty Acid
SOD - Superoxide Dismutase
T2D - Type 2 Diabetes
TGF-β - Tumor Growth Factor Beta
TLR - Toll-like Receptor
TNF-α - Tumor Necrosis Factor Alpha

ABSTRACT

Introduction: Chronic low-grade inflammation is exacerbated by high-fat diets (HFDs) and significantly increases the risk of metabolic disorders. Understanding how the gut microbiome influences and counteracts HFD-induced inflammation can inform targeted interventions to alleviate metabolic dysfunction. Aronia melanocarpa is a polyphenolic berry known for its antioxidant and anti-inflammatory impacts. This pilot study investigated how donor inflammation status impacts inflammation in metabolically active tissues in humanized mice after a HFD.

Methods: C57BL/6 mice received fecal transplants from human donors with similar metabolic profiles but varying levels of systemic inflammation (low, high). Over eight weeks, mice were given either *Aronia melanocarpa* or a sugar-matched drink alongside a 6-week HFD rich in sugars and saturated fats. Liver and muscle tissues were collected post-sacrifice for RNA extraction and qPCR, measuring gene expression of C-reactive protein (CRP), interleukin (IL)-10, IL-1ß, IL-6, tumor necrosis factor (TNF)-α, and tumor growth factor (TGF)-b. Given the small pilot sample size, the fold change of observed differences of high and low donor phenotypes within aronia and control mice and the fold change of control and aronia supplementation within high and low phenotypes were used, given the small pilot sample size (n =13).

Results: Results showed higher inflammatory gene expression in the liver (6/6 markers) and muscle (3/6 markers) of control mice with the HI inflammation donor than the LO inflammation donor. Inflammatory gene expression was greater in the liver (1/6) and muscle (2/6) with the HI inflammation donor than the LO inflammation donor in aronia mice.

Conclusion: These findings suggest that the inflammatory phenotype may be transferred with the gut microbiota, and low-inflammatory phenotypes may confer protection against HFD-induced inflammation. This highlights potential strategies for managing inflammation associated with HFD and its complications. Further research could uncover novel avenues for inflammation management in metabolic disorders.

CHAPTER ONE

INTRODUCTION

Development of the Problem

Inflammation-induced by high-fat diets (HFDs) is a critical driver in the development of inflammation and obesity-related metabolic disorders, including insulin resistance (IR), type 2 diabetes (T2D), cardiovascular diseases (CVD), and non-alcoholic fatty liver disease (NAFLD). HFDs trigger chronic low-grade inflammation in various tissues like the liver, muscle, and immune system through multiple pathways, exacerbating metabolic dysfunction (1). The detrimental effects of chronic inflammation, such as insulin resistance and oxidative stress, contribute to metabolic syndrome and associated complications. Inflammation is crucial for tissue repair, but its prolonged presence can be related to early disease development (2,3). Fecal microbiota transplants (FMT) have been shown to effectively transmit the inflammatory state of donors to recipient mice, suggesting a direct relationship between gut microbiota composition and inflammation (4,5). *Aronia melanocarpa* has shown promising results in modulating gut microbiome composition, reducing inflammatory stimuli, and improving health outcomes in mice and humans (6). Understanding the influences of gut microbiota composition on inflammatory status may provide insight into underlying metabolic disorders and chronic disease progression. Therefore, developing strategies to mitigate inflammation is crucial for improving public health outcomes and preventing the progression of obesity-related health conditions.

The gut microbiota resides in the large intestine of the gastrointestinal tract and is comprised of a diverse conglomerate of organisms. It is essential in digestion, metabolism,

immune regulation, and nutrient synthesis, but disruptions in composition and equilibrium can influence overall health and contribute to various diseases (7–10). The interdependent relationship between host and intestinal microbiota significantly impacts metabolism and the inflammatory response linked to obesity (11). Gut microbial populations may be active in chronic disease pathogenesis, and gene and species-level variation can define an increased risk of inflammation-related metabolic disorders (12). Microbial phenotypes may provide interpretable insights into the host–microbiome mechanisms of disease (13). Humanizing mice has become a gold standard for studying the impact of microbial phenotypes and human immunity on health.

Mouse humanization typically involves an FMT from a human donor into a mouse model recipient. Studies have shown that FMT procedures can successfully transfer 85% of genera after seven days (14,15). Commonly in research, humanizing mice aims to create mouse models with a gut microbiota composition that resembles humans' and replicate disease phenotypes seen in humans (16). This opens avenues to study how specific human gut microbes or microbial communities interact with the host and influence aspects of health and disease.

Research in humans and animals suggests that alterations in the gut microbiome induced by HFDs can promote obesity by enhancing energy harvest and fostering inflammation in various tissues (11,12,17,18). Humanized mice fed HFDs mimic disease conditions seen in humans, and current knowledge of gut microbiota functions results from germ-free mouse models. They are also used to study the microbiome's pathogenic and therapeutic potential within various disease states. As more research links HFDs to gut microbiome alterations and inflammation, the interplay between dietary factors and gut health becomes paramount. Supplementing humanized mice with *Aronia melanocarpa*, an antioxidant berry, enables the

assessment of its potential anti-inflammatory effects and metabolic benefits under high-fat dietary conditions. Inflammatory markers, including interleukin (IL)-6, tumor necrosis factor-alpha (TNF-α), IL-1β, and C-reactive protein (CRP), can be evaluated to gauge the impact of both the FMT and acute antioxidant supplementation on host inflammation levels.

Aronia melanocarpa, black chokeberry, is recognized for its antioxidant capacity and contains high levels of polyphenols, particularly anthocyanins, procyanidins, and hydroxycinnamic acids, which have demonstrated anti-inflammatory properties in clinical settings (19,20). Clinical trials indicate that dietary interventions incorporating aronia can induce changes in gut microbiome composition, reduce inflammatory stimuli, and improve health outcomes, even with acute dietary shifts (21,22). This functional food and many others like it may be an effective strategy for mitigating inflammation and reducing the risk of developing chronic diseases (23). Experimental approaches provide valuable insights into the therapeutic potential of dietary antioxidants for mitigating inflammation and improving metabolic health in the context of obesity-related disorders.

The present study was undertaken to further explore the findings of a previous study from our research group and is follow-up work completed by Wilson et al. (24), which investigated the metabolic impact of polyphenol-rich aronia fruit juice on humanized mouse models with varying gut microbiome inflammation statuses. Two individuals from a large cohort study (n=40) were assessed for resting and post-prandial inflammation response to a high-fat meal challenge, generating the HI and LO inflammation donors for humanized mouse models. Wilson et al. found that aronia juice increased alpha diversity in mice with a low-inflammation microbiota and that it protected against high-fat diet-induced loss of alpha diversity in low-inflammation mice.

High inflammation mice showed a decline in alpha diversity with aronia juice, like control groups. Additionally, over 1,000 metabolites were identified in each sample using LCMS analysis, and Trimethylamine-N-oxide (TMAO) concentrations were measured in mouse serum. This research sheds light on how aronia fruit juice impacts metabolism and gut microbiome inflammation, highlighting potential benefits for human health and nutrition. Dysbiosis in the gut microbiota is linked with conditions with high systemic inflammation; a less diverse microbiota may be found in individuals with high levels of inflammation (25). Additionally, introducing polyphenols to the diet in individuals with high levels of inflammation at rest and post-prandially may provide a protective effect against inflammation. A high-fat diet-induced inflammatory responses in HI and LO inflammation in humanized mice (n=13), and aronia juice was supplemented to elucidate anti-inflammatory effects.

Purpose and Hypothesis

The purpose of this study was to determine 1) whether the inflammation phenotype of the human FMT donor transferred to the humanized mice with the gut microbiome and 2) the impact of *Aronia melanocarpa* supplementation on inflammation of liver, muscle, and perirenal fat after a 6-week HFD. Through the analysis of tissue samples via ribonucleic acid (RNA) extraction and quantitative polymerase chain reaction (qPCR), this research aims to elucidate the potential anti-inflammatory properties of *Aronia melanocarpa* supplementation and provide valuable insights into its therapeutic potential for mitigating inflammation associated with high-fat diet in critical organs related to metabolic health. It is hypothesized that mice that received high (HI) inflammatory human characteristics will have larger inflammation responses to the high-fat diet than mice that received low (LO) inflammatory human characteristics. It is also hypothesized

that though HI inflammation mice will have a larger inflammation response to the high-fat diet, the HI mice that were supplemented with *Aronia melanocarpa* juice will have a lesser response than their control-matched juice counterparts.

Implications

This research will provide additional information about the relationship between gut microbiota, polyphenols, and inflammation induced by a HFD. The extent of *Aronia melanocarpa*'s polyphenolic effects has yet to be thoroughly studied, but it has come into the spotlight as a potent antioxidant. This study provides insight into the anti-inflammatory capacity of *Aronia melanocarpa* through humanized mouse models. However, further human studies on *Aronia melanocarpa* are needed to understand the physiologic changes it may derive in humans and its impact on health.

Limitations and Gap in Knowledge

While this study holds promise for understanding the effects of polyphenolic supplementation on inflammatory cytokine expression through the use of humanized mice, it is critical to acknowledge its limitations. First, findings from humanized mouse models may not directly translate to humans because humans are incredibly complex. Additionally, the 8-week study period may not capture the long-term effects of *Aronia melanocarpa* or chronic conditions that could develop over an extended period of exposure to a HFD.

This study's qPCR analysis only assessed a specific subset, six in total, of inflammatory cytokines. The analysis might not include other relevant cytokines or inflammatory markers, potentially missing crucial information. Since this study employed a small number of mouse

subjects (n=13), it may not be generalized to all individuals with HI or LO inflammation characteristics. Despite these limitations, this research is essential for understanding the potential effects of polyphenols on inflammatory cytokine expression in a controlled experimental setting. It provides a foundation for further studies and possible insights into dietary interventions for managing inflammation.

Despite research in chronic inflammation, gut microbiome composition, and dietary interventions concerning metabolic health, several knowledge gaps still need to be addressed within this topic. These gaps include determining optimal nutritional strategies for modulating gut microbiome composition and mitigating inflammation, elucidating the underlying mechanistic pathways at a molecular level, investigating the long-term effects of interventions on chronic disease progression, addressing population variability in response to interventions, and translating preclinical and observational findings into clinical practice through randomized controlled trials. Bridging these gaps through further research will provide deeper insights into the complex mechanisms underlying metabolic health and facilitate the development of personalized strategies for preventing and managing metabolic disorders.

CHAPTER TWO

LITERATURE REVIEW

Introduction

Understanding the complex interactions between chronic inflammation, gut microbiome composition, dietary interventions, and their collective impact on metabolic health is essential for chronic disease progression. Persistent, elevated levels of proinflammatory cytokines characterize chronic low-grade inflammation and are a hallmark of various metabolic disorders, including metabolic syndrome (MetS), NAFLD, T2D, and CVD (26–28). Central to understanding these conditions is the role of the gut microbiome. The gut microbiome is a complex ecosystem of microorganisms that reside in the gastrointestinal tract, which plays vital roles in digestion, metabolism, and immune regulation (29). Disruptions in gut microbiome composition have been implicated in the pathogenesis of metabolic disorders, influencing inflammation, nutrient metabolism, and host immune responses (30). Here, the potential of dietary interventions, particularly those incorporating functional foods rich in polyphenols like *Aronia melanocarpa*, is explored to modulate gut microbiome composition, mitigate inflammation, and improve metabolic health outcomes. By investigating the intricate connections between chronic inflammation, gut microbiota dynamics, and dietary interventions, this study aims to elucidate novel strategies for the early prevention and management of chronic diseases associated with metabolic dysfunction and inflammation.

Inflammation and Chronic Disease

Inflammation is the body's response to injuries and infections. While it is essential for tissue repair, prolonged inflammation can have negative consequences and is likely involved in early disease development (27). Chronic low-grade inflammation is a persistent, mild inflammatory state in the body that persists over an extended period, often without noticeable symptoms (26). Unlike acute inflammation, a temporary and localized response to injury or infection, chronic low-grade inflammation is systemic and may involve various tissues and organs throughout the body (31,32). It is characterized by elevated levels of inflammatory markers, such as cytokines, in the bloodstream and tissues (33). For example, in response to exercise, individuals with chronic kidney disease (CKD) had higher plasma concentrations of IL-6 (2.8-fold higher), IL-10 (1.2-fold higher), and TNF-α (1.3-fold higher) and increased expression of intramuscular TNF-α (8.7-fold increase) compared to non-CKD participants indicating higher levels of systemic and tissue inflammation (34). While the inflammatory response is a natural defense mechanism, chronic low-grade inflammation can harm health over time and is associated with obesity, T2D, CVD, and other chronic diseases.

The accumulation of macrophages is believed to be a significant factor in chronic low-grade inflammation and leads to an unfavorable change in glucose and lipid metabolism (28,35). A macrophage is a white blood cell derived from monocytes and part of the innate immune system (36,37). They are responsible for detecting and destroying pathogens and removing dead or damaged cells and cellular debris from the body (38). HFDs in mouse models have been shown to increase macrophage numbers in the liver compared to mice treated with an inflammatory mediator (39). Macrophages are found throughout the body and are highly

versatile cells capable of adapting their functions based on the signals they receive from their environment and are imperative to the immune response.

Critical characteristics of many major chronic diseases, in addition to inflammation, include altered glucose metabolism, lipid metabolism, and hypertension (40,41). Intervention strategies are needed to counteract the inflammatory stimuli, metabolic dysfunction, and chronic low-grade inflammation in metabolically at-risk populations, as those contribute to the early onset of metabolic syndrome and chronic disease progression. Understanding how gut microbiota composition and diet influence chronic inflammation provides a comprehensive framework for elucidating the complex pathways underlying metabolic disorders and chronic disease progression.

Inflammation is complex in metabolic dysfunction, particularly in conditions like obesity, T2D, and MetS, and is characterized by increased secretion of pro-inflammatory cytokines such as TNF-α, IL-6, and CRP (42). This chronic low-grade inflammation interferes with insulin signaling pathways in various tissues, including muscle, liver, and adipose tissue (38). TNF-α and IL-6 activate serine kinases, phosphorylating insulin receptor substrate proteins, leading to insulin resistance in T2D patients, and infusion of TNF-α and IL-6 in healthy humans leads to IR (43). When cells become resistant to insulin, their glucose uptake through GLUT4 is impaired, which contributes to elevated blood sugar levels, another hallmark of metabolic dysfunction (44). Additionally, adipose tissue secretes various hormones and signaling molecules called adipokines, and inflammation can disrupt the balance of adipokines, leading to an abnormal secretion pattern (45,46). For example, adiponectin, an anti-inflammatory adipokine that enhances insulin sensitivity, was reduced in obese and metabolically at-risk individuals

(45,47,48). Conversely, pro-inflammatory adipokines like leptin and resistin are elevated in mouse models after HFDs, further promoting insulin resistance and metabolic dysfunction (45,47). Insulin sensitivity and reduced glucose uptake are essential factors of metabolic disease exacerbated by chronic inflammation. However, understanding the impact of inflammation at the tissue level may provide better insights into disease development.

Chronic liver, muscle, and adipose inflammation are associated with obesity and insulin resistance (49,50). Hepatic inflammation-induced insulin resistance leads to increased gluconeogenesis and glycogenolysis, leading to elevated blood glucose levels and a vicious cycle of inflammation and insulin resistance, leading to obesity and other metabolic diseases (51–53). Additionally, through diet or metabolic dysfunction, excessive lipid accumulation within hepatocytes triggers various inflammatory responses in the liver (49,51). Immune cell recruitment of neutrophils, monocytes, and lymphocytes are increased to the liver in NASH compared to non-NASH participants ($p=0.02$) (54). These infiltrating immune cells produce pro-inflammatory mediators and promote tissue damage by releasing reactive oxygen species (ROS) and proteases (55). Immune cell infiltration into tissue contributes to further localized inflammation and may lead to cell mitochondrial dysfunction (56,57). Impaired mitochondrial function can produce an enhanced quantity of ROS involved in inflammatory pathways and cause cellular damage (58,59). Chronic systemic inflammation can also disrupt the balance of gut microbiota, leading to dysbiosis. A leaky gut (increased gut permeability) caused by inflammation allows lipopolysaccharide (LPS) and other bacterial products to enter the bloodstream, further exacerbating systemic inflammation and metabolic dysfunction (60). Overall, inflammation is a crucial driver of metabolic dysfunction through its effects on insulin

sensitivity, adipose tissue function, hepatic lipid metabolism, systemic inflammation, and gut microbiota.

High Fat Diet

A high-fat diet is generally defined as one in which a significant portion of the total caloric intake comes from fats (61). In research and clinical studies, a HFD is often characterized by a percentage of total caloric intake derived from fats (61,62). In HFDs, fat commonly exceeds 35% of total caloric intake (63). The Western diet tends to be relatively high in carbohydrates (30-40%), low in protein (15-25%), and often high in saturated and trans fats, contributing around 30% to 40% of total caloric intake (63). Foods within the Western diet have corresponded to a high rate of obesity, inflammation, and metabolic diseases (64). HFDs, especially those rich in saturated fats, can activate Toll-like receptors (TLRs), particularly TLR4 (65). TLRs are involved in recognizing pathogens, but they can also identify particular components of fatty acids and trigger the release of proinflammatory mediators (66). In a 12-week study, rats were fed a HFD to determine whether changes in gut epithelial function, inflammation, and microbiota were diet; an increase in TLR4-MD2 complex was detected due to the increase in saturated fat from the HFD and LPS activating TLR4 (67). These signaling molecules are important in the normal postprandial inflammatory response of a HFD. HFDs, compared to low-fat diets (LFD), contribute to fat accumulation in adipose tissue, triggering adipocytes to release adipokines and further attract immune cells (68). In addition to adipose, HFDs can trigger an inflammatory response in liver and muscle tissues, contributing to a heightened risk of metabolic disorder.

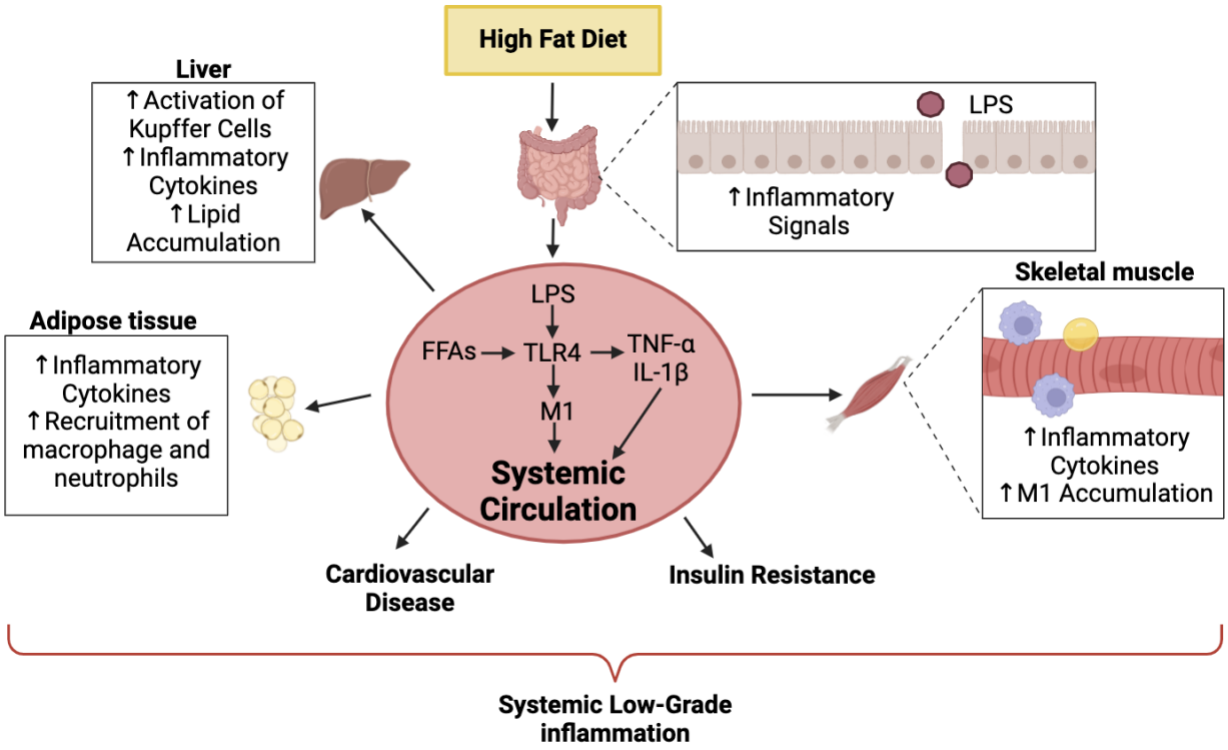

Figure 1. Overview of HFD stimuli in liver, skeletal muscle, and adipose tissue. LPS is a primary stimulator of the gut inflammatory response. It stimulates TLR4 activation, which triggers downstream systemic circulation of inflammatory stimuli. Chronic systemic circulation can lead to cardiovascular disease, insulin resistance, and systemic low-grade inflammation.

HFDs can induce low-grade inflammation in the liver and muscle by causing the infiltration of immune cells, such as macrophages and T cells, into the liver and muscle tissues (Figure 1) (69). The increased intake of dietary fats, especially saturated fats, can produce pro-inflammatory IL-6, TNF-α, and IL-1β (70). Hepatic inflammation contributes to the progression of NAFLD to more severe forms (NASH). HFDs activate Kupffer cells, resident immune cells in the liver, and activated Kupffer cells produce inflammatory mediators and ROS, contributing to liver inflammation and damage (71). In addition to Kupffer cells, HFDs, compared to a control diet, recruit more circulating immune cells to the liver in mouse models (71). Unrelenting tissue and systemic inflammation can contribute to insulin resistance and glucose metabolism

dysfunction through glucose transporter type 4 (GLUT4) translocation inhibition, further amplifying the inflammatory response and contributing to tissue damage and chronic disease development.

ROS can be generated as a byproduct when the body metabolizes fats, particularly saturated fats. During beta-oxidation, which primarily occurs in mitochondria, fatty acids are broken down to produce energy (72). The electron transport chain in mitochondria is a primary site of ROS generation during cellular respiration, and ROS generated from lipid metabolism can trigger inflammatory responses in cells and tissues (72,73). ROS and other inflammatory factors activate signaling pathways involved in inflammation and metabolic dysfunction, such as nuclear factor-kappa B (NF-κB), producing pro-inflammatory cytokines and enhancing low-grade inflammation (74). In a 12-week trial, abdominal NF-κB activity was elevated 2-fold in HFD-fed mice compared to LFD, and whole-body NF-κB activity increased 3.5-fold in HFD-fed mice compared to their baseline (75). When NF-κB is activated, it can enhance the expression of suppressor of cytokine signaling (SOCS), which impairs insulin signal transduction and therefore promotes IR (76). While fats are essential macronutrients for energy production and various physiological functions, the excessive intake of high-fat diets, especially those rich in saturated fats, can contribute to oxidative stress and the production of free radicals (72). This oxidative stress can further contribute to the development and progression of chronic diseases.

Gut Microbiome

The gut microbiota resides in the large intestine of the gastrointestinal tract and is comprised of diverse microorganisms, including bacteria, viruses, fungi, and other microbes (77). It plays vital roles in digestion, metabolism, immune regulation, and nutrient harvest (78).

Disruptions in the composition and equilibrium of the gut microbiome can influence overall health and contribute to various diseases (79). The symbiotic relationship between the host and the intestinal microbiota can significantly impact metabolism and the inflammatory response linked to metabolic disease (80,81). Genetic predisposition, environmental influences, and dietary habits shape the composition and abundance of gut microorganisms (82,83). For example, in a study investigating the alterations of the gut microbiome in obesity, researchers found that chow-fed rats had a more diverse microbiota than HF-fed rats (84). Particular gut microbial populations may be active in chronic disease pathogenesis, and variation in the gut microbiome in as little as 6 weeks at the species levels can define an increased risk population of inflammation-related metabolic disorders (85). In a study treating human tuberculosis, microbiota composition predicted peripheral inflammatory state. The researchers observed that the antibiotic treatment groups had increased expression of tuberculosis-associated inflammatory genes through the depletion of Clostridia species in the gut, indicating that microbiome composition may set the tone of systemic inflammation (86).

Though the microbiome can drive disease pathogenesis, 24 weeks of probiotic supplementation in obese patients with T2D shifted alpha diversity from a disease state to a healthy (87). Identifying microbial phenotypes that may promote inflammation may provide interpretable insights into the host–microbiome mechanisms of disease (88,89). A healthy microbiota community can be categorized by high taxonomic diversity, high microbial gene richness, and stable core microbiota. At the same time, perturbations in composition and function through diet may represent an unhealthy gut microbiota (90,91). Categorizing microbial

phenotypes is case-dependent and poorly studied but may provide insights into disease progression and prevention.

Research in humans and animals suggests that alterations in the gut microbiome, mainly induced by HFDs, can promote obesity by enhancing energy harvest and fostering inflammation in various tissues (92,93). HFDs, in as little as six weeks, can induce large changes in the composition and function of the gut microbiome, leading to dysbiosis and altered physiological and biochemical outcomes (94,95). Studies have observed an increase in potentially harmful bacteria with HFDs, such as *Proteobacteria* and certain species of *Firmicutes*, associated with inflammation and metabolic dysfunction (96,97). The gut microbiome interacts closely with the intestinal epithelial cells, which form a barrier between the gut's contents and the underlying tissues, and this barrier prevents the entry of harmful pathogens and toxins while allowing the absorption of nutrients (98). Disruption of the intestinal barrier through HFDs can translocate bacteria and microbial products across the intestinal lining, triggering inflammation in the underlying tissues, which is transported through the blood to the systemic circulation (79). The sensitivity of mice to LPS was increased in the HFD group compared to the control, and after only a 2mg/kg dose of LPS, none of the C57BL/6 mice survived after 5 days, indicating that the HFD may allow for more LPS translocation and TLR4 activation (99). Additionally, inflammatory cytokines IL-6 and INF- γ were significantly greater than in LPS-treated HFD mice over 12 hours (99). These findings may indicate that the HFD may cause an exaggerated systemic response to LPS and a hyper-responsive systemic immune response to the endotoxin.

After a HFD, the gut microbiota can contribute to macrophage-mediated inflammation in adipose tissue (76,81). The gut microbiome is crucial in regulating the immune system as gut-

resident immune cells constantly sample the microbial products in the gut lumen, helping to distinguish between harmless commensal bacteria and pathogenic invaders. Dysbiosis, or alterations in the composition of the gut microbiome, can lead to an inappropriate immune response, resulting in chronic and potentially systemic inflammation.

HFDs, by altering bacterial species, can change microbial metabolic activity, leading to shifts in the production of short-chain fatty acids (SCFAs), bile acids, and LPS (100,101). Total SCFA concentrations were significantly decreased in the high-fat group, compared with other diet groups ($p<0.001$) (95). These metabolites significantly influence host metabolism, immune function, and inflammation (100). Certain beneficial bacteria in the gut microbiome ferment dietary fiber to SCFAs, such as acetate, propionate, and butyrate. Compared to a high-fat diet, SCFA production in low-fat diet mice improved anti-inflammatory properties, which maintained the integrity of the intestinal barrier and regulated immune responses by promoting the differentiation of regulatory T cells (Tregs), suppressing inflammatory signals (81,102). HFDs can alter the composition of the gut microbiome, which may lead to an underproduction of SCFAs, reducing the anti-inflammatory properties of the gut microbiome (103,104). Additionally, saturated fats introduced to cell cultures promoted the translocation of LPS, which may lead to increased intestinal permeability, systemic inflammation, metabolic dysfunction, and insulin resistance in animal models (60,105). LPS and other microbial products can interact with immune cells and trigger inflammatory responses when present in excess or when the gut microbiome composition is altered.

Dysbiosis of the gut microbiome has been implicated in the pathogenesis of several inflammatory diseases. In these conditions, alterations in the gut microbiome composition and

function contribute to the development and perpetuation of chronic inflammation. Dietary interventions have the potential to mitigate inflammation, and research linking high-fat diets to gut microbiome alterations and inflammation underscores the importance of understanding the interplay between nutritional factors and gut health.

Aronia Melanocarpa

Incorporating functional foods into the diets of metabolically at-risk populations can effectively mitigate inflammation, change gut microbiota composition, and reduce the risk of developing chronic diseases when consumed regularly (23,106). Functional foods often contain bioactive compounds with specific physiological effects like polyphenols. Polyphenols are antioxidants that counter cellular damage caused by free radicals, modulate inflammatory gene expression, and undergo bacterial degradation to form simpler phenolic metabolites (107). These compounds can enhance gut barrier integrity, potentially mitigating downstream pro-inflammatory processes and modulating the composition of intestinal microbes (108). For example, a polyphenol in pomegranates, urolithin, has been shown to significantly improve tight junctions, decrease intestinal permeability, and reduce systemic inflammation by interacting with Nrf2 gene expression pathways in mice (109). *Aronia melanocarpa*, Black chokeberry, is recognized for its antioxidant capacity and contains high levels of polyphenols, particularly anthocyanins, procyanidins, and hydroxycinnamic acids, which have demonstrated anti-inflammatory properties (19,20). Studies in humans and mice indicate that dietary interventions incorporating aronia can induce changes in gut microbiome composition, reduce inflammatory stimuli, and improve health outcomes, even in shorter dietary interventions (21,22).

The antioxidant and anti-inflammatory effects of chokeberry are attributed to its rich content of bioactive compounds (110). These compounds act through various mechanisms to reduce oxidative stress, inflammation, and cellular damage (111). Polyphenols, particularly flavonoids and anthocyanins, present in aronia berries act as potent antioxidants by scavenging free radicals and ROS in the body (110). Antioxidants are compounds that neutralize free radicals produced during normal metabolic processes (112). Unchecked and active free radicals can damage cells and tissues, leading to inflammation, oxidative stress, and chronic diseases (112). Antioxidants donate electrons to unstable radicals, neutralizing their harmful effects and preventing oxidative damage to lipids, proteins, and DNA (112). Aronia's polyphenols can inhibit ROS production by modulating the body's enzymatic and non-enzymatic antioxidant defense systems (113,114). For example, they enhance the activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx), which play crucial roles in neutralizing ROS and protecting cells from oxidative damage (115). Under healthy conditions, there exists a delicate balance between ROS and antioxidants, maintaining homeostasis. The influx of inflammatory stimuli commonly resulting from chronic inflammation can be effectively counteracted by antioxidants and polyphenols from the diet, facilitating a normal immune response (116). Disruptions in lipid or glucose metabolism, coupled with impaired antioxidant function, can lead to a dysregulation of this balance, resulting in chronic inflammation (117). This shift marks a critical point where the body's inflammatory response becomes prolonged and dysregulated, contributing to the pathogenesis of various metabolic disorders and chronic diseases.

Aronia possesses anti-inflammatory properties through the polyphenolic inhibition of the production and activity of pro-inflammatory mediators and the suppression of the activation of inflammatory signaling pathways mitogen-activated protein kinases (MAPKs) and NF-κB, which regulate the expression of inflammatory genes (118). In cell cultures and mouse models, different concentrations (4, 8, or 16 μg/mL) of a combination of wheatgrass and aronia (TAAR) showed that expression levels of cytokines (IL-1β and IL-6) in LPS-stimulated cells were suppressed by TAAR treatment and was concentration-dependent (119). Aronia polyphenols can also regulate the activity of macrophages, dendritic cells, and T cells by modulating cytokine production, antigen presentation, and immune cell activation (120). Additionally, aronia has been shown to protect endothelial cells lining blood vessels from oxidative stress and inflammation, thereby preserving endothelial function and vascular health (121). Polyphenols enhance nitric oxide (NO) production, reduce endothelial dysfunction, and inhibit the adhesion of inflammatory cells to endothelial surfaces, improving vascular function (121). Chokeberry can also modulate gene expression patterns associated with antioxidant defense, inflammation, and cellular stress responses by regulating the expression of genes involved in antioxidant enzyme activity, exerting protective effects against oxidative and inflammatory damage (122,123).

Studies have investigated the effective dosage of chokeberry and its potential health benefits. However, the optimal dosage can vary depending on the specific health condition targeted, the form of aronia extract or supplement used, and individual characteristics such as age, weight, and overall health status. Additionally, while aronia is generally considered safe for most people when consumed in dietary amounts, excessive intake may lead to potential adverse effects. Research on the effective dosage of aronia extract or supplements is ongoing, and no

universally agreed-upon standard dosage exists. Studies have used a wide range of doses, typically 100 mg to 1,000 mg or more daily, depending on the specific study objectives and measured outcomes (124,125). These studies have shown promising results in various health conditions, such as CVD, MetS, T2D, and cancer prevention (122,124–127). While Aronia is generally safe for consumption in moderate amounts as part of the diet, high doses of aronia supplements may cause gastrointestinal discomfort, such as stomach upset, diarrhea, or constipation, in some individuals (128).

The exploration of dietary approaches for the early prevention of chronic diseases is of heightened interest, focusing on enhancing antioxidant intake through polyphenol-rich foods. These strategies target gut microbiota composition and inflammatory stimuli, influencing host responses via bioactive compound production. We transplanted stools from donors with varying inflammation levels to gnotobiotic mice to mimic genetic and microbiota diversity. By administering aronia juice alongside a HFD, we assessed its potential protective effects against inflammation using qPCR. This study aims to elucidate the interplay between dietary interventions, gut microbiome alterations, and systemic inflammation within critical metabolic tissues in humanized mouse models, including the liver, muscle, and perirenal fat.

CHAPTER THREE

THE IMPACT OF GUT MICROBIOTA AND ARONIA MELANOCARPA ON INFLAMMATION AFTER A HIGH-FAT DIET IN HUMANIZED MOUSE MODELS

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Abstract

Introduction: Chronic low-grade inflammation is exacerbated by high-fat diets (HFDs) and significantly increases the risk of metabolic disorders. Understanding how the gut microbiome influences and counteracts HFD-induced inflammation can inform targeted interventions to alleviate metabolic dysfunction. Aronia melanocarpa is a polyphenolic berry known for its antioxidant and anti-inflammatory impacts. This pilot study investigated how donor inflammation status impacts inflammation in metabolically active tissues in humanized mice after a HFD.

Methods: C57BL/6 mice received fecal transplants from human donors with similar metabolic profiles but varying levels of systemic inflammation (low, high). Over eight weeks, mice were given either *Aronia melanocarpa* or a sugar-matched drink alongside a 6-week HFD rich in sugars and saturated fats. Liver and muscle tissues were collected post-sacrifice for RNA extraction and qPCR, measuring gene expression of C-reactive protein (CRP), interleukin (IL)-10, IL-1ß, IL-6, tumor necrosis factor (TNF)-α, and tumor growth factor (TGF)-b. Given the small pilot sample size, the fold change of observed differences of high and low donor phenotypes within aronia and control mice and the fold change of control and aronia supplementation within high and low phenotypes were used, given the small pilot sample size (n =13).

Results: Results showed higher inflammatory gene expression in the liver (6/6 markers) and muscle (3/6 markers) of control mice with the HI inflammation donor than the LO inflammation donor. Inflammatory gene expression was greater in the liver (1/6) and muscle (2/6) with the HI inflammation donor than the LO inflammation donor in aronia mice.

Conclusion: These findings suggest that the inflammatory phenotype may be transferred with the gut microbiota, and low-inflammatory phenotypes may confer protection against HFD-induced inflammation. This highlights potential strategies for managing inflammation associated with HFD and its complications. Further research could uncover novel avenues for inflammation management in metabolic disorders.

Introduction

Chronic low-grade inflammation is a hallmark feature of chronic diseases, including metabolic syndrome, non-alcoholic fatty liver disease (NAFLD), type 2 diabetes (T2D), and cardiovascular disease (CVD) (26–28). While inflammation is crucial for tissue repair, its prolonged presence can lead to detrimental effects and is associated with early disease development (2,3). Metabolic disorders are marked by altered glucose and lipid metabolism, hypertension, and increased tissue infiltration and activation of immune cells (40,42).

Intervention strategies are crucial to counteract the inflammatory stimuli in at-risk populations. The gut microbiota has been shown to impact metabolism and the inflammatory response (129,130). Fecal microbiota transplants (FMT) have been shown to effectively transmit the inflammatory state of donors to recipient mice, suggesting a direct relationship between gut microbiota composition and inflammation (4).

Moreover, gut microbiome alterations induced by high-fat diets can exacerbate inflammation and contribute to metabolic dysfunction. *Aronia melanocarpa* supplementation has shown promising results in modulating gut microbiome composition, reducing inflammatory stimuli, and improving health outcomes. Understanding how chronic inflammation influences and is influenced by gut microbiota composition provides pathways for understanding underlying metabolic disorders and chronic disease progression.

Comprised of diverse microorganisms, including bacteria, viruses, fungi, and other microbes, the gut microbiota resides in the large intestine of the gastrointestinal tract. It plays vital roles in digestion, metabolism, immune regulation, and nutrient synthesis (78). Disruptions in the composition and equilibrium of the gut microbiome can influence overall health and contribute to various diseases (7–10). The symbiotic relationship between the host and the intestinal microbiota significantly impacts metabolism and the inflammatory response linked to obesity(11). Genetic predisposition, environmental influences, and dietary habits shape the composition and abundance of gut microorganisms (82). Gut microbial populations may be active in chronic disease pathogenesis, and variation in the gut microbiome at gene and species levels can define an increased risk population of obesity-related metabolic disorders (12). Microbial phenotypes may provide interpretable insights into the host–microbiome mechanisms

of disease (13). Research with FMT on mice has demonstrated the effective transmission of the donor's inflammatory state. This suggests that the FMT process may successfully transfer the inflammatory profile from donor to recipient, highlighting the importance of gut microbiota composition on inflammatory status (4). A healthy microbiota community can be categorized by high taxonomic diversity, high microbial gene richness, and stable core microbiota, while perturbations in composition and function represent an unhealthy gut microbiota (17). Though categorizing microbial phenotypes is case-dependent and poorly studied, it may provide insights into disease progression and protection against HFD-induced inflammation.

Research in humans and animals suggests that alterations in the gut microbiome, mainly induced by HFDs, can promote obesity by enhancing energy harvest and fostering inflammation in various tissues (11,12,17,18). Moreover, compromised gut barrier integrity, mucus bilayer composition, and chronic exposure to dietary fat can fuel inflammatory responses and intensify gut barrier dysfunction (131,132). Current knowledge of gut microbiota functions results from germ-free mouse models and is used to study microbiota's pathogenic and therapeutic potential with various diseases (reword, include FMT). As dietary interventions incorporating functional foods gain traction for their potential to mitigate inflammation, research linking high-fat diets to gut microbiome alterations and inflammation underscores the importance of understanding the interplay between dietary factors and gut health.

Incorporating functional foods into the diets of metabolically at-risk populations can be an effective strategy for mitigating inflammation and reducing the risk of developing chronic diseases when consumed regularly (23). These foods often contain bioactive compounds with specific physiological effects. Polyphenols in these foods are antioxidants that counter cellular

damage caused by free radicals, modulate inflammatory gene expression, and undergo bacterial degradation to form simpler phenolic metabolites (107). These compounds can enhance gut barrier integrity, potentially mitigating downstream proinflammatory processes and modulating the composition of intestinal microbes (108). In turn, gut microbes further catabolize polyphenols to release additional bioactive metabolites (6). *Aronia melanocarpa*, Black chokeberry, is recognized for its antioxidant capacity and contains high levels of polyphenols, particularly anthocyanins, procyanidins, and hydroxycinnamic acids, which have demonstrated anti-inflammatory properties (19,20). Studies in humans and mice indicate that dietary interventions incorporating aronia can induce changes in gut microbiome composition, reduce inflammatory stimuli, and improve health outcomes, even in acute dietary shifts (21,22).

The exploration of dietary approaches for the early prevention of chronic diseases is of heightened interest, focusing on enhancing antioxidant intake through polyphenol-rich foods. These strategies target gut microbiota composition and inflammatory stimuli, influencing host responses via bioactive compound production. We transplanted stool from donors with varying inflammation levels to gnotobiotic, germ-free mice to mimic human donors' genetic and microbiota diversity in a mouse model. By administering aronia juice alongside a HFD, we assessed its potential protective effects against inflammation using quantitative polymerase chain reaction (qPCR). This study aims to elucidate the interplay between dietary interventions, gut microbiome alterations, and systemic inflammation within critical metabolic tissues in humanized mouse models, including the liver, muscle, and perirenal fat. The purpose of this study was to determine 1) whether the inflammation phenotype of the human donor transferred to the humanized mice with the gut microbiome and 2) the impact of *Aronia melanocarpa*

supplementation on inflammatory response after a 6-week HFD. It is hypothesized that the inflammation phenotype of the human donor was successfully transferred to the humanized mice with the gut microbiome and that *Aronia melanocarpa* supplementation will have reduced expression of tissue inflammation compared to the sugar-matched control.

Methods

Research Design

Human donors (add in high-level overview) were chosen from a larger group of participants (n=40) based on their metabolic profiles and responses to a high-fat meal challenge, assessing both resting and postprandial inflammation. Fecal microbiota transplantation (FMT) was conducted from two human donors, one with high inflammation and one with low inflammation, to four mice, aiming to replicate the gut microbiome of the donors. The offspring of these initial mice completed an eight-week study comprising two weeks of either aronia juice or a sugar-matched control, followed by six weeks of a high-fat diet with juice or control (Figure 1). Tissue samples from the liver, muscle, and perirenal fat were collected from the mice at sacrifice. RNA extractions were performed, followed by quantitative polymerase chain reactions (qPCR) to assess the gene expression levels of specific inflammatory cytokines.

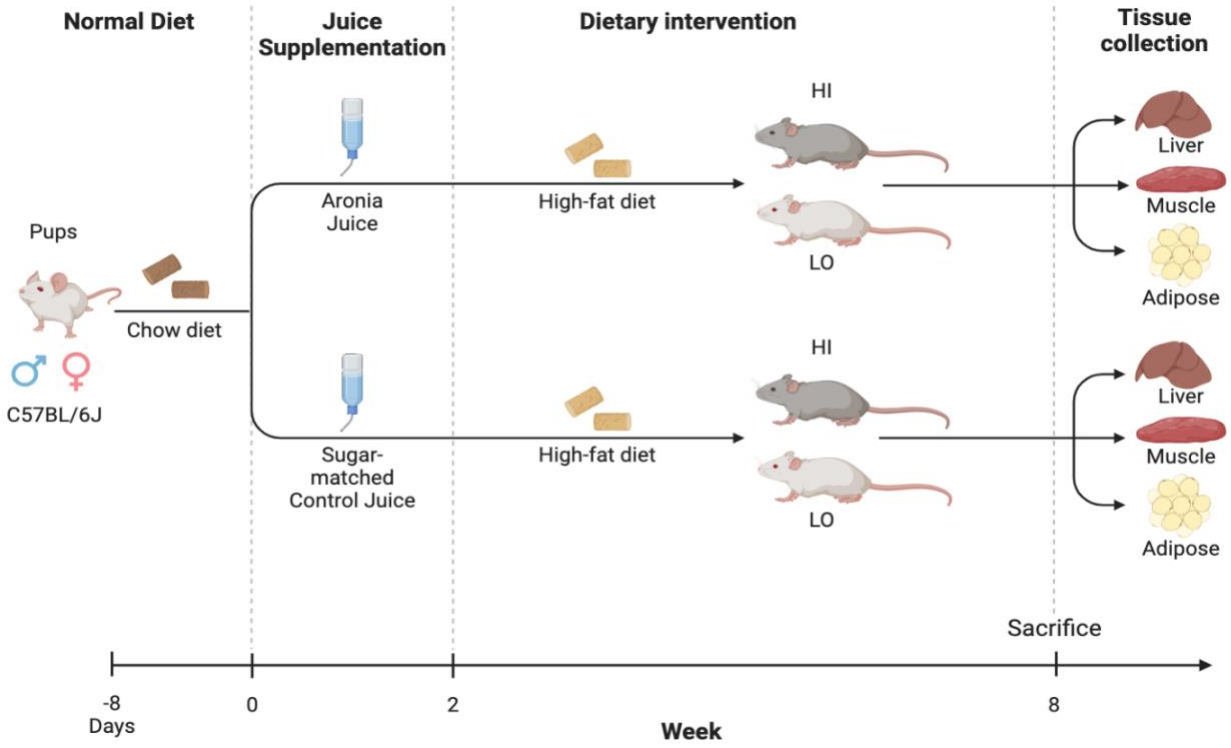

Figure 2. Mouse Experimental Timeline. Mice began the experiment 8 days after birth with either the aronia or control juice. They were fed juice for two weeks, then placed on a high-fat diet (HFD) and the juice for six weeks (a total of 8 weeks).

Characteristics of Microbiome Donors and Animal Models

As Wilson et al. (21) described, the human subject testing for this study occurred at the Nutrition Research Laboratory at Montana State University, spanning from March 2016 to June 2018. Subjects were recruited through phone screening to determine eligibility, with inclusion criteria set between ages 18 and 55 and a body mass index (BMI) ranging from 27 to 36 kg/m^2. Exclusion criteria encompassed recent antibiotic use within 90 days before enrollment, regular intake of anti-inflammatory medications, reliance on estrogen-only contraceptives, allergies or intolerances to wheat and dairy, pregnancy, or any pre-existing musculoskeletal, cardiovascular, gastrointestinal, or immunological conditions that could impact the study. The Institutional

Review Board at Montana State University approved the protocol involving human subjects, and written informed consent was obtained from all participants before their involvement. The study was retrospectively registered on ClinicalTrials.gov (NCT04128839) in October 2019. Forty overweight and obese individuals, both men and women, were enrolled to undergo anthropometric measurements and assessments of serum metabolic and inflammatory markers during a high-fat meal challenge involving a 50 g oral fat load and stool collection for analysis (21).

Subjects were stratified based on their fasting inflammation levels, with individuals classified as having high fasting inflammation if their baseline levels exceeded the group median in at least four out of six cytokines. Conversely, subjects with low fasting inflammation were identified if their baseline levels surpassed the group median in two or fewer cytokines. Those exceeding the group median in three cytokines were neither high nor low. Confirmation of fasting phenotype separation was obtained through a two-sample t-test for each cytokine measure. Furthermore, participants were subjected to k-means analysis to group them based on postprandial cytokine concentrations following an oral fat tolerance test. This analysis revealed three distinct inflammation groups within the cohort: low-, mid-, and high-responders. To investigate the gut microbiome's influence on inflammation, stool donors for the gnotobiotic mouse experiments were selected based on their fasting and postprandial inflammation levels, ensuring one donor represented LO and the other HI. Both donors were matched as closely as possible for other factors influencing inflammation, including sex (both female), body fatness, and waist circumference. Additionally, the selected stool donors were aged 52 and 34, identified as non-Hispanic Caucasian, and did not meet the criteria for metabolic syndrome (21) (additional

information about donors is found in Table 1). Two participants were selected from a pool after inflammation and metabolic status assessment—this pool was used as stool donors to humanize mice for the germ-free mouse experiments.

Table 1. Human stool donor characteristics

	Donors (n=2)	
	Low	High
Women/Men	1/0	1/0
Age (Years)	52	34
Body Mass Index (kg/m2)	27.7	35.9
Fat Mass (%)	35.1	48.1
	Metabolic Syndrome	
Presence/Absence	0/1	0/1
Fasting glucose (mmol/L)	5.4	5.2
Fasting triglyceride (mmol/L)	2.4	2.6
Fasting cholesterol (mmol/L)	5.9	5.5
Fasting high-density lipoprotein (mmol/L)	1.6	2.0
Waist circumference (cm)	92.3	90.3
Systolic blood pressure (mmHg)	128	114
Diastolic blood pressure (mmHg)	78	83

Two human stool donors, categorized as HI or LO donors, were selected based on their inflammation profile before gut microbial community profiling. HI and LO donors' FMT was transplanted into four female gnotobiotic C57BL/6 (Jackson Laboratory in Bar Harbor, ME) mice, two females for each donor. Three male and three female pups were born to LO mice, and five male and three female pups were born to HI mice. The HI donor stool was transplanted into two female mice, and the LO donor stool was transplanted into two female mice. Germ-free male mice were introduced to each female cage to produce pups. Mouse pups were housed by sex and treatment (4 cages), and the dietary intervention experiment was started eight days after birth. Mice were sacrificed via rapid CO2 asphyxiation, and tissue samples were snap-frozen and stored at -80 C° after eight weeks of experimentation.

Aronia Intervention and Diet Administration

At baseline (T0), regular drinking water was replaced with ARO or CON juice to begin a two-week familiarization period with the juice. ARO mice received an unpasteurized blend of aronia juice, and CON mice received the sugar-matched beverage containing water, sorbitol, glucose, and fructose. The mice were housed in cages based on sex and treatment (4 cages) with free access to their respective juice. During the familiarization period, all mice received standard chow. After the two-week familiarization period (T2), mice began a 6-week high-fat diet, delivered *ad libitum* concomitant with juice consumption. The HFD (Teklad TD. 96132) was chosen to induce obesity and present inflammatory stimulus. The HFD mimics a Western-style diet and consists of 40.6% fat, 40.7% carbohydrates, and 18.7% protein. It is particularly rich in sugars and trans-fatty acids. All chow provided was sterilized via autoclaving or irradiation. A total of 150mL of juice was provided per cage each week and was refilled three times each week (50mL per refill). Mice were sacrificed via rapid CO_2 asphyxiation, and liver, muscle, and perirenal fat were harvested, snap-frozen, and then frozen for long-term storage at -80 C°.

Table 2. Aronia juice carbohydrate and polyphenol concentrations.

Compound	*Concentration*
	Carbohydrate (mM)
Sorbitol	672.74
Fructose	433.40
D-glucose	435.89
	Polyphenol (µM)
Neochlorogenic acid	8987.59
Chlorogenic acid	8598.77
Cyanidin 3-arabinoside	1224.29
Cyanidin 3-galactoside	589.40
Quercitin 3-glucoside	127.45
Cyanidin 3-glucoside	66.92
Cyanidin 3-xyloside	42.57
Quercitin 3-rutinoside	33.47
Quercitin 3-galactoside	19.36

RNA Isolation and Analysis of mRNA Levels

Mice were sacrificed via rapid CO_2 asphyxiation, and harvested tissue samples were snap-frozen and then stored at -80 C° until RNA extraction procedures. A homogenate was prepared from the frozen liver, perirenal fat, and muscle tissues in a biological safety cabinet. An autoclaved scalpel was prepared to cut approximately 100 mg of the tissues and was cleaned with 70% ethanol between each tissue sample. A handheld tissue homogenizer (Cole-Parmer HO-600 Digital Handheld Tissue Homogenizer) was cleaned with RNase and ethanol in a fume hood before homogenization. Each of the 100 mg tissue samples was added to the side of a 50 mL conical tube, with 1 mL of TRIzol added per 100 mg of the sample to each conical. TRIzol reagent was used under a fume hood. The tissue lyser was set to 18000 RPM, ensuring the tissue was between the tube's wall and the teeth of the lyser. After the initial plunge into TRIzol, a circular motion was made to ensure the tissue was fully homogenized. A tissue/TRIzol slurry was made, and no large tissue chunks were seen. The tissue lyser was rinsed in a 100-proof ethanol 4-

tube dilution between samples. All the TRIzol slurry samples were transferred to Eppendorf tubes, and 160 µL of chloroform per 1 mL of TRIzol was added. The samples were vortexed for about 15 seconds or until the samples were bright pink. The samples were incubated at room temperature for three minutes, then centrifuged at 12,000 RPM for 15 minutes at 4 C°. The samples were separated into three distinct layers (colorless, white, and red). The colorless phase contained the RNA of interest, the white interphase contained fats and carbohydrates, and the red phase contained DNA and protein. The colorless phase was transferred to a new Eppendorf tube, and 500 µL of 2-isopropanol was added to each sample. Samples were stored at -20 C° overnight. Isolation was continued immediately the following morning.

After overnight storage, the samples were incubated at room temperature for 10 minutes. Then, the samples were centrifuged at 12,000 RPM for 15 minutes at 4 C°. The supernatant was discarded by slowly dumping the liquid without tapping the tube; each sample was washed with 1 mL of 75% Ethanol (EtOH). The RNA pellet occasionally shifted during supernatant discard. Samples were centrifuged at 12,000 RPM for 15 minutes at 4 C°. The supernatant was discarded again by slowly turning the tubes. The samples were incubated at room temperature for 10 minutes to evaporate any remaining EtOH. The RNA pellet was dissolved with 50 µL of DEPC water and was manually broken down with the pipette tip. A nanodrop machine measured each sample's ng/µL of RNA. Then, the sample concentrations were normalized to 1000 ng/µL. Some RNA concentrations were not able to be normalized to 1000ng/µL. The samples were then frozen at -20 C° until cDNA synthesis.

cDNA Synthesis

The master mix was created using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermofisher). 10 µL of master mix and 10 µL of normalized RNA sample were added to each cDNA tube. The thermocycler was programmed to run a cDNA synthesis cycle, as listed in Table 3. After the thermocycler cycle, the samples were stored at -20 C° until the qPCR protocol.

Table 3. Thermocycler settings for cDNA production.

Settings	***Step 1***	***Step 2***	***Step 3***	***Step 4***
Temperature	25 C°	37 C°	85 C°	4 C°
Time	10 minutes	120 minutes	5 minutes	Hold

Quantitative Polymerase Chain Reaction (qPCR) Protocol

An ice bucket was prepared for qPCR to hold the Forward and Reverse Primers and SYBR green. cDNA samples were taken from the freezer and set in the hood to thaw. The primer master mixes were prepared under a PCR hood for each cytokine of interest by creating 50 reagent reactions to ensure enough volume for 9 µL of master mix in each well. Once the master mix for the primers of interest was created, 9 µL was added to the desired wells, and then 1 µL of cDNA was added to the desired wells. After loading the plate, it was sealed with a plate cover and was centrifuged for 3 minutes at 1500 RPM. The plate was placed in the Quant 5 machine to run the qPCR protocol. The Quant 5 machine was set to run Comparative CT cycles.

Table 4. Gene of Interest (GOI) forward and reverse primer sequences used for qPCR protocol.

Primer Name	***Sequence***
RPLP0-F	AGATTCGGGATATGCTGTTGGC
RPLP0-R	TCGGGTCCTAGACCAGTGTTC
CRP-F	ATGGAGAAGCTACTCTGGTGC
CRP-R	ACACACAGTAAAGGTGTTCAGTG
mTNF-A-F	CCCTCACACTCAGATCATCTTCT
mTNF-A-R	GCTACGACGTGGGCTACAG
TGF-B-F	CTTCGACGTGACAGACGCT
TGF-B-R	GCAGGGGCAGTGTAAACTTATT
m_IL-1-B-F	GCAACTGTTCCTGAACTCAACT
m_IL-1-B-R	ATCTTTTGGGGTCCGTCAACT
IL-10-F	CTTACTGACTGGCATGAGGATCA
IL-10-R	GCAGCTCTAGGAGCATGTGG
IL-6-F	CTGCAAGAGACTTCCATCCAG
IL-6-R	AGTGGTATAGACAGGTCTGTTGG

Table 5. Quantity of reagents needed for qPCR.

Reagent	***1 Reaction (µL)***	***50 Reactions (µL)***
SYBR Green	5	250
Forward Primer	0.5	25
Reverse Primer	0.5	25
DEPC Water	3	150

Data Reduction and Analysis

The fold change of observed differences was used for experimental comparisons given the small pilot sample size (n=13 to 3 or 4 per group). The data processing and statistical procedures were conducted using R Studio (v 4.3.1). To assess fold changes, the means of each group were calculated, and subsequent comparisons were made by dividing the high inflammation (HI) group by the low inflammation (LO) group of the CON and ARO groups and the control (CON) group by the aronia (ARO) group of the HI and LO groups. This allowed for determining fold changes relative to the respective baseline groups. Data visualization was

performed using R Studio. Fold change values of key inflammatory markers, including tumor necrosis factor-alpha (TNF-α), transforming growth factor-beta (TGF-β), C-reactive protein (CRP), interleukin-6 (IL-6), interleukin-10 (IL-10), and interleukin-1 beta (IL-1β), were analyzed across different conditions, including HI vs. LO in the CON treatment condition, HI vs. LO in the ARO treatment condition, CON vs. ARO in the LO inflammatory phenotype group, and CON vs. ARO in the HI inflammatory phenotype group.

Results

HI vs. LO Gut Microbiome Mice in the Control Treatment Group

Inflammatory gene expression was greater for six out of the six inflammatory markers for the liver and three out of the six for muscle in mice with the HI inflammation donor compared to the LO inflammation donor (Table 6).

HI vs. LO Gut Microbiome Mice in the Aronia Treatment Group

Inflammatory gene expression was greater for one out of the six inflammatory markers for the liver and two out of the six for muscle in mice with the HI inflammation donor than the LO inflammation donor (Table 6).

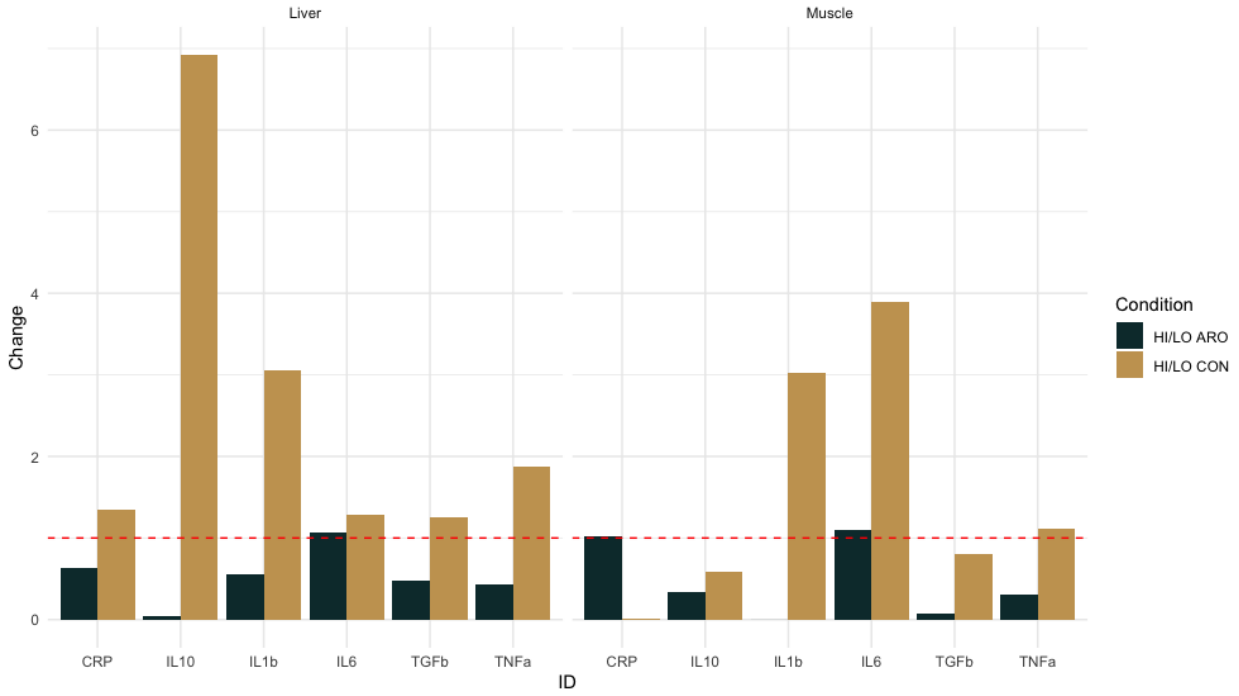

Figure 3. This bar chart represents the fold change seen for each GOI. Blue bars represent the aronia treatment, while gold represents the control treatment. Bars above the red line indicate high gene expression in the reference group versus the comparison group; bars below the red line, lower gene expression.

Table 6. Raw fold change values. Values >1 indicate high gene expression in the reference group versus the comparison group; <1, lower gene expression.

	HI V LO (CON)		**HI V LO (ARO)**	
	Liver	*Muscle*	*Liver*	*Muscle*
CRP	**1.34**	**0.0037**	**0.63**	**1.02**
IL10	**6.92**	**0.58**	**0.039**	**0.34**
IL1B	**3.05**	**3.03**	**0.56**	**0.001**
IL6	**1.29**	**3.9**	**1.07**	**1.10**
TGFb	**1.25**	**0.80**	**0.47**	**0.07**
TNFa	**1.87**	**1.12**	**0.43**	**0.31**

Discussion and Conclusion

Using gnotobiotic mice, we investigated the impact of the inflammation status of the gut microbiome donors and the effects of aronia juice supplementation on inflammatory cytokines following a high-fat diet. We achieved the transfer of the donor microbiome to subsequent

generations of mice. Notably, mice in the control treatment characterized by a high-inflammatory (HI) phenotype displayed heightened expression of inflammatory cytokines in the liver compared to those with low-inflammatory (LO) phenotypes (Table 6). This indicates the successful transmission of the inflammatory profile from human donors to mice, suggesting a potential protective effect conferred by an LO gut phenotype against high-fat diet-induced inflammation. Aronia supplementation did demonstrate the anticipated protective effects against high-fat diet-induced inflammation across different inflammatory phenotypes (Table 6). This finding suggests that the expected modulation of inflammatory phenotypes by aronia was observed in our experiment. Though we were able to determine the effect of aronia, there is still a necessity for further investigation to unravel the underlying mechanisms and optimize therapeutic interventions for managing inflammatory conditions. Importantly, this observation has broad implications for the use of animals, the effects of aronia juice supplementation, and models in studying inflammation.

The donor phenotype, characterized by either HI or LO status, influenced inflammatory cytokine expression in liver and muscle in the humanized mouse models. The successful transmission of the donor phenotype to subsequent generations of mice highlights the role of the gut microbiome in mediating inflammatory expression. Mice that inherited the HI phenotype exhibited heightened expression of inflammatory cytokines in critical metabolic tissues compared to those with the LO phenotype, indicating a direct correlation between donor inflammation status and inflammatory outcomes in the mouse models. Analysis of tissue samples revealed distinct inflammatory responses between mice with HI and LO donor phenotypes, emphasizing donor characteristics' influence on the recipient mice's inflammatory profile. The

differential expression of inflammatory markers in response to a HFD underscores the importance of considering donor inflammation status in understanding the variability of inflammatory outcomes in metabolic disorders. The findings suggest that the donor phenotype influences baseline inflammatory status and modulates the response to dietary interventions like aronia supplementation. Mice inheriting the HI phenotype may exhibit a more pronounced inflammatory response to HFD consumption, highlighting the importance of personalized approaches to inflammation management based on donor characteristics and gut microbiota composition. In a study treating tuberculosis, researchers observed that antibiotic treatment groups had increased expression of tuberculosis-associated inflammatory genes through the depletion of *Clostridia* species in the gut, attributing the microbiota composition to the observed peripheral inflammatory state (86). These findings suggest that the microbiome composition may set the tone of systemic inflammation.

Supplementation with aronia juice did demonstrate protective effects against HFD-induced inflammation in mice within LO and HI inflammatory phenotypes, as evidenced by decreased fold changes of cytokine expression levels in aronia compared to control mice, suggesting that the polyphenolic content of the juice did exert anti-inflammatory effects. Because the fold changes of cytokine gene expression in the liver of the aronia treatment group were consistently lower than those of the control, we can infer that aronia had some effect on the inflammatory status, no matter the inflammatory status provided through the FMT. Most fold changes in the liver of the aronia group were less than 1 while the fold changes for the control group were above 1, suggesting that aronia ameliorated the inflammatory response seen in the HI group. To support our findings, in a 2023 study comparing the effects of soluble and whole

polyphenols on acute colitis in mice, the relative expressions of IL-6, IL-8, and TNF-α mRNA in colon tissues of polyphenol-treated groups had decreased mRNA expression compared to mice in the control group (133). This provides evidence that polyphenols may down-regulate the gene expression of some inflammatory cytokines and alleviate inflammation associated with chronic disease. The present study corroborates the findings of Wilson et al. (24) found that an introduction of aronia fruit juice led to a shift in beta-diversity that was donor-independent, suggesting a consistent effect of the juice on the gut microbiome, and these findings contribute to our understanding of how the consumption of aronia fruit juice, in the context of different gut microbiome inflammatory statuses, can influence the metabolic profile and diversity of the gut microbiome in a humanized mouse model.

While this study holds promise for understanding the effects of polyphenolic supplementation on inflammatory cytokine expression and the impact of FMT donor inflammation status after a HFD, it is critical to acknowledge its limitations. Findings from humanized mouse models may not directly translate to human responses. Studies have shown that an 8-week intervention induces inflammation and obesity in C57BL/J6 mice (134,135), but the 8-week study period may not capture the long-term effects of *Aronia melanocarpa* as human participants underwent 24 weeks of a low daily aronia dose that exerted a protective effect against ROS (136). Notable differences in tissue quality, particularly in the perirenal fat, were observed between the aronia and control groups. Due to its poor quality, perirenal fat did not precipitate sufficient RNA for downstream qPCR and data analysis.

Additionally, though gnotobiotic mice are considered a gold standard method of studying the effect of gut microbiomes on health, replicating these studies in animals and humans is

critical. Humans have a wide range of physiological responses due to stimuli. They are unique organisms, so understanding the effect of the gut microbiome in human hosts is crucial for better understanding the relationship between the gut microbiome and health. qPCR analysis in this study only assessed a specific subset of inflammatory cytokines. The analysis might not include other relevant cytokines or inflammatory markers, potentially missing crucial information. Since this study employed a small number of mouse subjects (n=13), it may not be generalized to all individuals with HI or LO inflammation characteristics. Despite these limitations, this research is essential for understanding the potential effects of polyphenols on inflammatory cytokine expression in a controlled experimental setting. It provides a foundation for further studies and possible insights into dietary interventions for managing inflammation.

In conclusion, the study highlights the interplay between gut microbiome composition, donor inflammation status, dietary interventions, and systemic inflammation in humanized mouse models subjected to a HFD. The unexpected findings regarding aronia supplementation underscore the complexity of modulating inflammatory responses through nutritional interventions and emphasize further research to unravel underlying mechanisms and optimize therapeutic strategies for managing inflammation and metabolic disorders.

The study aimed to investigate the impact of donor inflammation status on inflammation in humanized mouse models after a High-Fat Diet (HFD). By exploring how the gut microbiome and *Aronia melanocarpa* supplementation influence inflammatory responses, the research sought to provide insights into potential strategies for managing inflammation associated with HFD and its related complications. We hypothesized that the inflammation phenotype of human donors would be transferred to humanized mice with the gut microbiome and that mice with high

inflammatory characteristics would exhibit larger inflammation responses to the HFD compared to mice with low inflammatory characteristics. Additionally, it was hypothesized that mice with high inflammatory characteristics supplemented with *Aronia melanocarpa* juice would show a lesser inflammation response to the HFD compared to their control-matched counterparts.

The study successfully demonstrated the transfer of the inflammation phenotype from human donors to mice through the gut microbiome, indicating a direct influence of donor characteristics on inflammatory responses in the mouse models. Mice with high-inflammatory phenotypes exhibited heightened expression of inflammatory cytokines in critical metabolic tissues compared to those with low-inflammatory phenotypes, highlighting the impact of donor inflammation status on systemic inflammation. Consistent with expectations, aronia supplementation did demonstrate the anticipated protective effects against HFD-induced inflammation across different inflammatory phenotypes, suggesting further investigation to optimize therapeutic interventions for managing inflammatory conditions. The findings underscore the personalized nature of inflammatory responses and the importance of considering donor characteristics and gut microbiome composition in developing tailored strategies for mitigating inflammation and improving metabolic health in obesity-related disorders. Understanding the impact of the donor phenotype on inflammation provides valuable insights into the personalized nature of inflammatory responses and metabolic health outcomes. Further research in this area could lead to the development of tailored therapeutic interventions that consider individual inflammatory profiles and gut microbiome composition to optimize strategies for mitigating inflammation and improving metabolic health in the context of obesity-related disorders.

CHAPTER FOUR

CONCLUSION

Chronic inflammation is central in developing and progressing metabolic disorders such as MetS, T2D, NAFLD, and CVD (137,138). The intricate interplay between chronic inflammation, gut microbiome composition, and dietary interventions underscores the complexity of metabolic health and disease. Disruptions in the gut microbiome composition through diet have been implicated in the pathogenesis of metabolic disorders, influencing inflammation, nutrient metabolism, and host immune responses (139). Dietary interventions incorporating functional foods rich in polyphenols, such as *Aronia melanocarpa*, hold promise in modulating gut microbiome composition, mitigating inflammation, and improving metabolic health outcomes.

In addition to chronic inflammation and dietary interventions, the gut microbiome plays a pivotal role in shaping metabolic health and disease. The gut microbiome, a complex ecosystem of microorganisms residing in the gastrointestinal tract, influences various aspects of host physiology, including digestion, metabolism, immune regulation, and nutrient synthesis. Dysbiosis has been implicated in the pathogenesis of metabolic disorders, contributing to inflammation, insulin resistance, and obesity (140). The symbiotic relationship between the host and the intestinal microbiota has been shown to positively impact metabolism and reduce the inflammatory response associated with chronic disease (141,142). Research in humans and animals suggests that alterations in the gut microbiome induced by dietary habits, environmental influences, and genetic predisposition can promote obesity and chronic low-grade inflammation by enhancing energy harvest and promoting inflammatory pathways in various tissues (143,144).

Therefore, understanding the interplay between chronic inflammation, gut microbiota dynamics, and dietary interventions is essential for elucidating the complex pathways underlying metabolic disorders and chronic disease progression, ultimately leading to the development of targeted interventions for improving metabolic health.

Donor inflammatory phenotype within the gut microbiome is critical in understanding the interplay between chronic inflammation, gut microbiome composition, dietary interventions, and metabolic health (145). By categorizing microbial phenotypes and identifying variations in gut microbiome composition at gene and species levels, this research may highlight the significance of the gut phenotype in defining the risk of inflammation-related metabolic disorders. Characterizing healthy and unhealthy gut microbiota communities based on taxonomic diversity, microbial gene richness, and inflammatory state may provide valuable insights into disease mechanisms and prevention strategies (146–148). Along with the fasting microbiome inflammatory state, the impact of long-term dietary patterns may provide insights into gut microbial phenotype changes (149). Understanding the effects of the gut phenotype on metabolism, inflammation, and disease progression is essential for developing targeted interventions and personalized approaches to improve metabolic health outcomes.

Aronia melanocarpa exerts its antioxidant and anti-inflammatory effects through its rich content of bioactive compounds, including polyphenols, flavonoids, and anthocyanins (150). These compounds are highly effective in scavenging free radicals, inhibiting oxidative stress, modulating inflammatory gene expression, and regulating immune responses (151). By enhancing antioxidant capacity, suppressing pro-inflammatory signaling pathways, and protecting endothelial function, aronia contributes to preventing and managing chronic diseases

associated with metabolic dysfunction and inflammation. While research on the effective dosage of aronia extract or supplements is ongoing, studies have shown promising results of polyphenols on various health conditions (152,153). However, individual factors such as age, weight, and overall health status should be considered when determining the optimal dosage and can provide a more personal approach to dietary supplementation. Additionally, while aronia is generally safe for consumption in moderate amounts, excessive intake may lead to potential adverse effects (154). Therefore, further research is needed to establish aronia supplementation's optimal dosage and long-term safety profile.

This research on the impact of gut microbiome and *Aronia melanocarpa* on inflammation after a HFD in humanized mouse models has provided valuable insights into the complex interplay between dietary interventions, gut health, and inflammatory responses. This research has shed light on the potential implications for managing inflammation associated with high-fat diets and metabolic disorders. It is important to replicate these types of studies in larger sample sizes and diverse human populations to enhance the applicability of the research to real-world scenarios. By including a broader representation of individuals with varying characteristics, the study's generalizability is strengthened, offering a more comprehensive understanding of personalized responses to interventions in managing inflammation. By exploring how different populations respond to dietary interventions, genetic, environmental, and lifestyle factors influencing individual responses can be better understood, paving the way for tailored and targeted approaches to managing inflammation and metabolic disorders associated with high-fat diets.

Furthermore, the study highlights the significance of personalized approaches and strategies based on individuals' unique characteristics and needs. Understanding individual variations in treatment outcomes is essential for developing targeted interventions that are effective and feasible for a wide range of individuals, ultimately improving the relevance of the research to practical healthcare settings.

Future research informed by this study could delve into exploring the chronic effects of gut microbiome modulation and *Aronia melanocarpa* supplementation on chronic disease progression, addressing population variability in response to interventions, and translating preclinical findings into clinical practice through randomized controlled trials. By investigating the underlying mechanisms of how these interventions impact metabolic health and inflammation over time, future studies could provide deeper insights into personalized strategies for preventing and managing metabolic disorders. Additionally, exploring optimal dosages, durations, and potential synergistic effects of combining interventions could offer new avenues for enhancing the effectiveness of dietary strategies in managing inflammation associated with high-fat diets.

In conclusion, this research contributes to the growing knowledge of the role of gut microbiome modulation and dietary interventions in inflammation and metabolic health. The study's implications extend beyond the laboratory setting, emphasizing the importance of personalized strategies, real-world applicability, and the need for further research in larger and more diverse populations to optimize interventions for managing inflammation and improving metabolic health in the context of high-fat diets and related disorders.

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APPENDIX: ADDITIONAL FIGURES AND TABLES

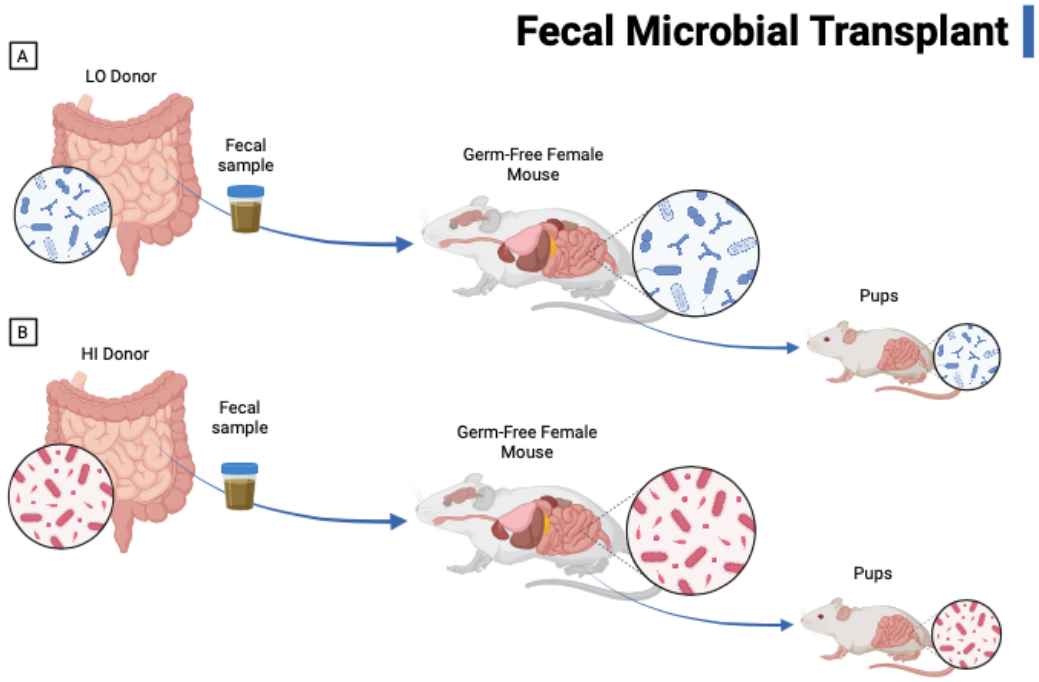

Figure 4. Fecal Microbiota Transplant completed to humanize experimental mice.

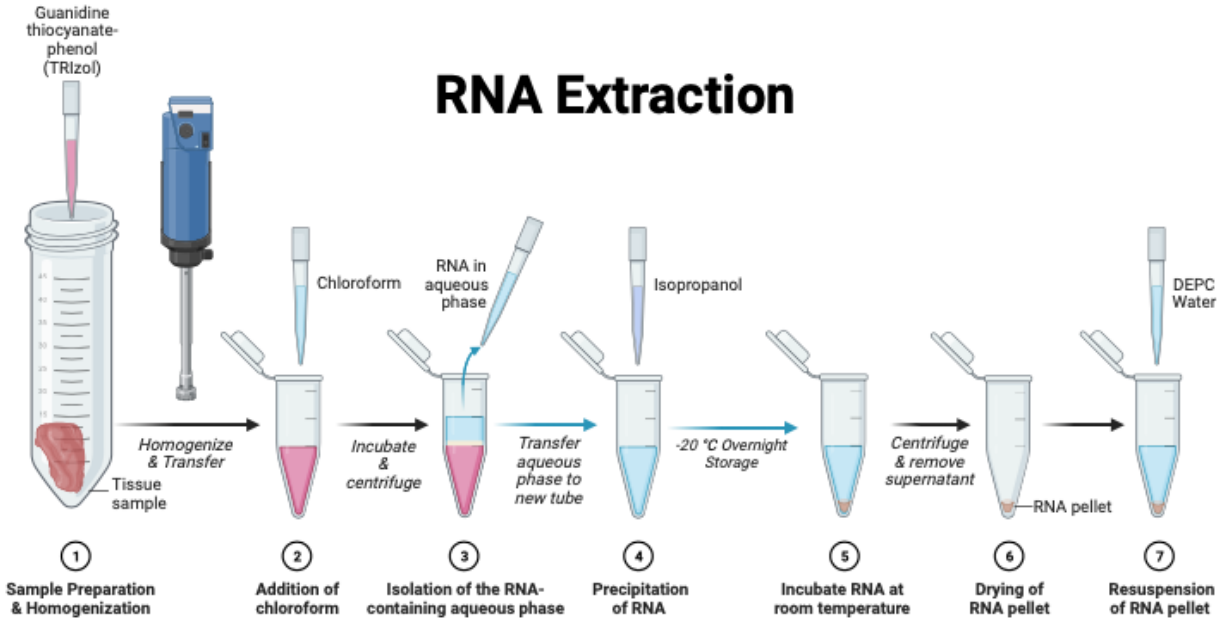

Figure 5. RNA extraction protocol used for tissue samples.

Table 7. CRP Raw CT Values

Tissue	*Treatment*	*Inflammation Phenotype*	*Δ* CT	$2^{-\Delta CT}$
Liver	Berry	LO	0.866301	0.548552
	Berry	LO	1.785957	0.289983
	Berry	LO	-0.00769	1.005344
	Berry	HI	0.477001	0.718469
	Berry	HI	0.983259	0.505836
	Berry	HI	-1.75556	3.376582
	Berry	HI	3.061543	0.11978
	Control	LO	1.461123	0.36321
	Control	LO	0.901762	0.535233
	Control	LO	0.693119	0.618515
	Control	HI	-0.28988	1.222538
	Control	HI	1.376241	0.385221
	Control	HI	1.248392	0.420917
Muscle	Berry	LO	2.454256	0.182472
	Berry	LO	1.310268	0.403246
	Berry	HI	1.441553	0.368171
	Berry	HI	1.937506	0.261067
	Berry	HI	0.822475	0.565471
	Control	LO	1.018309	0.493695
	Control	LO	0.758842	0.59097
	Control	HI	8.962524	0.002005
Perirenal Fat	Berry	LO	2.674059	0.156685
	Berry	LO	0.844816	0.556782
	Berry	HI	1.272152	0.414042
	Berry	HI	7.25292	0.006556
	Berry	HI	5.684778	0.019441
	Control	LO	1.293978	0.407825
	Control	HI	11.63288	0.000315

Table 8. IL10 Raw CT Values

Tissue	*Treatment*	*Inflammation Phenotype*	*Δ* CT	$2^{-\Delta CT}$
Liver	Berry	LO	3.31048732	0.10079617
	Berry	LO	0.48325911	0.71535977
	Berry	LO	4.37152666	0.04831026
	Berry	HI	1.12846844	0.45740104
	Berry	HI	7.4489584	0.00572322
	Berry	HI	8.48649792	0.00278811
	Berry	HI	-1.1386593	2.20176316
	Control	LO	7.92783643	0.00410661
	Control	LO	7.85292047	0.00432549
	Control	LO	5.31866573	0.0250566
	Control	HI	2.94152518	0.13017053
	Control	HI	5.35808658	0.02438121
	Control	HI	0.99662794	0.50117003
Muscle	Berry	LO	0.18742897	0.87816931
	Berry	LO	7.51424783	0.00546998
	Berry	HI	4.22766507	0.053376
	Berry	HI	1.48340762	0.35764307
	Berry	HI	-2.0758105	4.21581177
	Control	LO	1.97264792	0.25478497
	Control	LO	4.59458411	0.04138971
	Control	HI	6.3320392	0.0124127
	Control	HI	2.64357385	0.16003132
Perirenal Fat	Berry	LO	12.204579	0.00021186
	Berry	LO	12.2723433	0.00020214
	Berry	HI	7.92807568	0.00410593
	Berry	HI	9.63762943	0.00125541
	Berry	HI	4.69371254	0.0386413
	Control	LO	12.6656419	0.00015391
	Control	HI	12.204579	0.00021186

Table 9. IL6 Raw CT Values

Tissue	*Treatment*	*Inflammation Phenotype*	*Δ* CT	$2^{-\Delta CT}$
Liver	Berry	HI	3.91294289	0.06638758
	Berry	LO	3.30941582	0.10087106
	Berry	LO	2.52713585	0.17348275
	Berry	LO	7.78848839	0.00452305
	Berry	HI	-7.4602699	176.102298
	Berry	HI	10.5337944	0.00067455
	Berry	HI	4.6995182	0.03848611
	Control	HI	4.99370956	0.03138655
	Control	HI	9.1786232	0.00172568
	Control	HI	1.58125687	0.33419062
	Control	LO	3.17648315	0.11060717
	Control	LO	2.99141312	0.12574622
	Control	LO	4.0024147	0.06239548
Muscle	Berry	HI	-0.7846394	1.72266164
	Berry	HI	5.53781605	0.0215254
	Berry	LO	3.82435036	0.07059206
	Berry	LO	1.95837212	0.25731864
	Berry	HI	3.16579056	0.11142999
	Control	HI	2.65056419	0.15925779
	Control	HI	1.59052086	0.33205155
	Control	HI	-0.7005043	1.62507275
	Control	LO	2.49881935	0.17692142
	Control	LO	2.43564796	0.1848404
Perirenal Fat	Berry	HI	7.13636971	0.00710786
	Berry	HI	7.96193504	0.00401069
	Berry	LO	2.47258377	0.18016819
	Berry	LO	1.67453957	0.31326607
	Berry	HI	3.10040092	0.11659672
	Control	HI	8.83211708	0.00219416
	Control	LO	0.41377831	0.75065489

Table 10. TNFa Raw CT Values

Tissue	*Treatment*	*Inflammation Phenotype*	*Δ* CT	$2^{-\Delta CT}$
Liver	Berry	HI	9.46524605	0.00141474
	Berry	LO	12.094865	0.0002286
	Berry	LO	7.88737187	0.00422342
	Berry	LO	8.48022641	0.00280025
	Berry	HI	3.46619702	0.09048378
	Berry	HI	9.91269537	0.00103748
	Berry	HI	10.6237302	0.00063378
	Control	HI	9.05033734	0.00188615
	Control	HI	8.87306715	0.00213275
	Control	HI	9.05441713	0.00188083
	Control	LO	13.7727772	7.1446E-05
	Control	LO	8.38264532	0.00299621
	Control	LO	13.6814543	7.6115E-05
Muscle	Berry	HI	14.7654042	3.5906E-05
	Berry	HI	11.6545404	0.00031019
	Berry	LO	9.5842722	0.00130271
	Berry	HI	10.1787435	0.00086277
	Berry	HI	10.3232331	0.00078054
	Control	HI	12.1041212	0.00022714
	Control	HI	11.5554342	0.00033225
	Control	HI	13.779513	7.1114E-05
	Control	LO	10.4249768	0.00072739
	Control	LO	9.9795433	0.00099051
Perirenal Fat	Berry	HI	9.72934534	0.00117808
	Berry	LO	6.39816005	0.01185665
	Berry	HI	16.8426738	8.5084E-06
	Control	HI	10.0171361	0.00096503
	Control	LO	6.87364401	0.0085276

Table 11. TGFb Raw CT Values

Tissue	*Treatment*	*Inflammation Phenotype*	*Δ* CT	$2^{-\Delta CT}$
Liver	Berry	HI	5.36385155	0.02428398
	Berry	LO	9.77723503	0.00113962
	Berry	LO	4.6339798	0.04027477
	Berry	LO	3.48644829	0.08922252
	Berry	HI	-0.4169521	1.33510401
	Berry	HI	4.96833229	0.03194353
	Berry	HI	4.75419807	0.03705474
	Control	HI	4.76496696	0.03677918
	Control	HI	4.66973877	0.03928878
	Control	HI	9.04177666	0.00189738
	Control	LO	13.0157604	0.00012074
	Control	LO	4.0055542	0.06225985
	Control	LO	12.4649754	0.00017688
Muscle	Berry	HI	10.4724598	0.00070384
	Berry	HI	7.02743721	0.00766533
	Berry	LO	4.35676193	0.04880721
	Berry	HI	7.32194519	0.00624993
	Control	HI	7.28463936	0.00641365
	Control	HI	10.2968006	0.00079498
	Control	HI	9.89401245	0.00105101
	Control	LO	14.5604172	4.1388E-05
	Control	LO	7.19306374	0.00683395
Perirenal Fat	Berry	HI	5.98784637	0.01575718
	Berry	HI	4.2574482	0.05228539
	Berry	LO	4.94073868	0.03256038
	Berry	LO	3.91927147	0.066097
	Berry	HI	12.1156349	0.00022534
	Control	HI	5.12000275	0.02875581
	Control	LO	7.0798912	0.00739163

Table 12. IL1b Raw CT Values

Tissue	*Treatment*	*Inflammation*	*Δ* CT	$2^{-\Delta CT}$
Liver	Berry	HI	8.90272331	0.00208936
	Berry	LO	13.1608028	0.0001092
	Berry	LO	8.77435303	0.00228379
	Berry	LO	6.3582859	0.01218892
	Berry	HI	-2.0658302	4.18674842
	Berry	HI	5.47090149	0.0225473
	Berry	HI	5.06380081	0.02989813
	Control	HI	10.4179916	0.00073092
	Control	HI	5.65167999	0.01989183
	Control	HI	7.7564621	0.00462458
	Control	LO	13.9521427	6.3094E-05
	Control	LO	6.93009567	0.00820037
	Control	LO	16.3429794	1.203E-05
Muscle	Berry	HI	13.4898367	8.6927E-05
	Berry	LO	12.4320526	0.00018096
	Berry	LO	3.97519493	0.06358389
	Berry	HI	13.9046516	6.5205E-05
	Control	HI	14.5179672	4.2624E-05
	Control	HI	12.8605652	0.00013446
	Control	HI	12.0935802	0.00022881
	Control	LO	14.4494247	4.4698E-05
Perirenal Fat	Berry	HI	11.03759	0.00047572
	Berry	LO	1.99054718	0.25164343
	Control	HI	11.6023102	0.00032163