Impact of White and Dark Red Kidney Beans on Intestinal and Metabolic Health in C57Bl/6 Male Mice

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Pulses (i.e. beans, lentils, and chickpeas) are enriched in plant-based proteins and non-digestible carbohydrates (e.g. fibre) that may beneficially impact intestinal and metabolic health. The objectives of this thesis were to determine if *i)* consumption of diet supplemented with kidney beans would improve biomarkers of intestinal and metabolic health, *ii)* dark-colored beans would provide additional health benefits compared to light-colored beans, and *iii)* background diet composition would impact the health effects of kidney bean consumption. Study 1: 5-week-old male C57Bl/6 mice were fed either a basal diet (BD; 20% casein, wt/wt), or isocaloric diets supplemented with 15% cooked white kidney beans or dark red kidney beans for 9 weeks (n=12/group); Study 2: 5-week-old male C57Bl/6 mice were fed either a high-fat diet (HF; 60% kcals from fat) or HF diet supplemented with 15% cooked white kidney beans or dark red kidney beans. In both studies, consumption of diets supplemented with beans improved microbiota community structure and activity, as indicated by increased abundance of short-chain fatty acid (SCFA) producing bacteria (*Prevotella* and *S24-7)* and intestinal SCFA concentrations, with dark kidney beans inducing the greatest effects. Furthermore, biomarkers of colonic inflammation, barrier integrity, and metabolic health were beneficially impacted by kidney bean consumption, however, the effects were greater in mice consuming low-fat vs. high-fat diets. In conclusion, this thesis demonstrated that while kidney bean consumption led to improvements in intestinal and metabolic health, the results were influenced by seed coat color and the nutrient composition of the background diet.

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> **From:** Jacob Albert Rodrigue
> **Sent:** Tuesday, November 13, 2012 9:47 PM
> **To:** Alexane Rodrigue
>
> Hey Little Dude,
>
> I hope all is well and that you are working hard and having fun. I hope also that things aren't too stressful either. Keep your chin up and remember that all you ever need to do, in life, is to do the most you can and try the hardest you can in any situation; the rest is, for the most part, totally outside your realm of control.
> …
> Getting back to my point, I just want you to be happy, and stay strong and just remember to work as hard as possible. We all love you no matter what. That is all. If you ever need anything, feel free to e-mail.
>
> Jacob,

To my late brother Jacob, I miss you everyday, and I wish you could have read this thesis, you would have been so proud. I want you to know that every time I wanted to give up, I thought about all the times you told me that I would be a brilliant mad scientist one day. Thank you for always believing in me and always being a great big brother.

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LIST OF ABBREVIATIONS:

BCFAs- branched chain fatty acids
BD - basal diet (control group)
BMI - body mass index
CLS - crown-like structures
DK - dark red kidney
DKB - basal diet supplemented with 15% dark red kidney bean powder
DKH - high-fat diet supplemented with 15% dark red kidney bean powder
DSS - dextran sodium sulphate
DW - dry weight
GAE - gallic acid equivalents
GIP - glucose-dependent insulinotropic polypeptide
GLP-1 - glucagon-like peptide 1
GOS - galacto-oligosaccharides
GPR - G-coupled protein receptors
HDL-C - high-density lipoprotein cholesterol
HF - high-fat basal diet (control group)
HFD - high-fat diet
IBD - Inflammatory Bowel Disease
IFN - interferon
IL - interleukin
LBP - lipopolysaccharide-binding protein
LDL-C - low-density lipoprotein cholesterol
LPS - lipopolysaccharide
MCP - monocyte chemoattractant protein
MUC - mucin
NF-κB - nuclear factor kappa-light-chain-enhancer of activated B cells
PCoA - principle coordinate analysis
PYY - peptide tyrosine tyrosine
SCFAs- short-chain fatty acids
TJ - tight-junction
TLR - Toll-like receptor
TNF- tumor necrosis factor
Treg - colonic regulatory T
WK - white kidney
WKB - basal diet supplemented with 15% white kidney bean powder
WKH - high-fat diet supplemented with 15% white kidney bean powder
ZO - Zonula-occludens

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Chapter 1: Introduction

Common beans (*Phaseolus vulgaris*) are members of the legume family most commonly referred to as pulses that are cultivated for their dried seeds[1]. Beans originate from two main ecogeographical gene pools, Andean and Mesoamerican, but they are grown in most regions around the world[2,3]. Beans are affordable, accessible, easy to transport and store as dried seeds, and the availability of canned beans in most grocery stores makes them an attractive plant-based protein food source[4,5]. However, while common bean consumption is elevated in Latin American regions[6], in general, pulse consumption is on the low end in North America[7,8], and most Canadians eat animal-based proteins as their primary source of protein[9,10]. Canada's New Food Guide recommends consuming more plant-based protein, and common beans are a good source of plant-based proteins[11,12]. Since beans are cheap and contain many nutritional and phytochemical components, they have the potential to improve human health. Studies have shown that common bean consumption has been associated with reduced risk of chronic diseases such as obesity and cardiovascular disease[13], diabetes[14], and colorectal cancer[15]. Furthermore, bean consumption can affect human health through improvements in glycemic response[14,16], increased satiety and changes in hormones involved in appetite regulation[17–19], and improved laxation through fecal bulking[20–22]. Importantly, the mechanisms of action responsible for these observed health effects are poorly defined. Therefore, to help enhance bean consumption in Canada and support the new Canada's Food Guide, the aim of this thesis project was to help fill knowledge gaps regarding the mechanisms through which common beans affect human health, in particular, intestinal and metabolic health.

Common beans are rich in non-digestible carbohydrates, such as insoluble and soluble fibres, galacto-oligosaccharides (GOS) and resistant starch[23], and come in a variety of seed coat

colours, which is attributable to their polyphenolic content[24]. Dietary fibres are known to affect intestinal health and the gut microbiota composition by increasing the number of beneficial microbes [25,26], and are associated with many health benefits, in particular, those related to metabolic health (e.g. obesity, type-2 diabetes, cardiovascular diseases)[27–32]. In addition, polyphenolic compounds have been shown to modulate aspects of intestinal[33–35] and metabolic health[36], therefore the health effects of common beans may also be attributed to their polyphenolic compound content. Importantly, the concentrations and profiles of polyphenolic compounds vary significantly across bean varieties with the highest and most diverse phenolic content found in darker coloured beans[24]. Thus, the impact of bean consumption on human intestinal and metabolic health may differ depending on the seed coat colour and consequently polyphenolic content/profile. Therefore, this thesis research explored the impact of kidney bean consumption in mice, on intestinal and metabolic health when consumed as part of a healthy or high-fat diet, as well as explored the impact of kidney bean seed coat colour (e.g. white and dark red) on intestinal and metabolic health outcomes.

Chapter 2: Literature Review

2.1 Intestinal Health

Intestinal health is an important area of study because impairments to the intestinal environment are associated with increased risk of infection by pathogens, inflammation, and metabolic and immune disorders[37,38]. The intestinal tract harbors a highly dense and diverse microbiome, and an array of microbial-derived and host-derived metabolites and factors, as well as an intact selectively permeable mucosal epithelial barrier, which act in a symbiotic manner to maintain human health and reduce risk of chronic disease[37,39]. The selectively permeable intestinal epithelium is composed of polarized entero- and colonocytes, with an apical membrane that faces the lumen, and a basolateral membrane that faces the serosa [40,41]. The integrity and permeability of the epithelium in controlled by adherens junction (e.g. E-cadherin and β-catenin) and tight junction (TJ) proteins, such as Occludin, claudins and Zonula-occludens (ZO), which aid in cell-cell adhesions and paracellular transport[41,42]. The absorption of luminal nutrients by the intestinal epithelium is also facilitated by microvilli, which form crypt and villi structures on the apical surface, that increase surface area and transport proteins for increased absorption capabilities[40]. The luminal surface of the epithelium is lined by a thick mucus layer formed by mucins secreted by goblet cells, which helps in the protection against pathogen invasion into systemic circulation[43,44]. Furthermore, the epithelium contains enteroendocrine cells, namely K and L cells, which secrete hormones and various peptides (e.g. ghrelin, glucagon-like peptides) in response to ingestion of certain dietary components, which participate in host metabolic homeostasis (e.g. satiety, insulin sensitivity, glucose tolerance) [45,46]. For example, glucose-dependent insulinotropic polypetide (GIP, formally gastric inhibitory polypeptide) is expressed by K cells in the intestinal epithelium and is involved in the regulation of glucose-induced

insulin secretion, and is also involved in fat storage[47,48]. Glucagon-like peptide 1 (GLP-1) is expressed by intestinal L cells and plays a role in stimulating insulin secretion for β-cells in the pancreas[49]. GLP-1 can supress the secretion of glucagon, which is involved in blood glucose upregulation, and is also involved in the inhibition of digestive enzymes being secreted into the lumen and can reduce gastrointestinal motility[50,51]. Both GIP and GLP-1 are incretins which are produced in response to carbohydrate and lipid ingestion[45,52,53]. Ghrelin, is secreted by ghrelinergic cells in the gastro-intestinal tract, and is a peptide hormone involved in appetite regulation by inducing hunger[54,55]. It can help reduce blood glucose levels, is a stimulator of growth hormone, but daily peripheral injections of ghrelin was shown to induce adiposity in rodents by reducing fat utilization [56–59]. Taken together, the response of the intestinal epithelium to ingested dietary components highlights the important role of a functional intestinal barrier in regulating systemic metabolic functions in the host.

The gastro-intestinal tract is also comprised of a microbiota (the community of microorganisms belonging to a specific environment) which serves many purposes to its host (**Figure 2-1**), such as regulating mucosal barrier components and immune system development to aid in the protection against pathogens, as well as the synthesis of vitamins and the digestion of food components that are not directly digestible by the host, such as plant polysaccharides[38,60]. The microbiota can also produce short-chain fatty acids (SCFAs) from the non-digestible polysaccharides which can be utilized by the host, as well as the production of phenolic secondary metabolites from plant polyphenolic compounds which can be more bioactive to the host than their parent compounds (**Figure 2-1**) [35,61,62]. A dysbiotic microbiota, one with abnormal composition, activity, and diversity, has been linked to certain chronic diseases such as obesity and metabolic syndrome [63–66]. The human intestinal microbiota structure is normally

comprised of two main phyla, *Firmicutes* and *Bacteroidetes*, which are gram-positive and gram-negative respectively[67]. These two phyla dominate the murine intestinal microbiota, as well as the intestinal microbiota of most vertebrates[63,68]. Other phyla common to the gut microbiota are *Proteobacteria*, *Actinobacteria* and *Verrucomicrobia*[67,69]. Certain genus of interest are *Prevotella* and *Bacteroides*, the first are associated with high-fibre plant-based diets while the latter are associated with animal protein diets[70]. Diet composition is one of the largest factors affecting the gut microbiota composition, and consequently activity, however, other factors such as genetics, medications, or environment can also affect the composition of the gut microbiota [69,71]. Previous studies have shown that perturbations to the microbiota based on diet composition remains steady after an average of 3.5 days on a diet[72]. These changes in microbiota structure can happen in as little as 24 hours after switching to a high-fat/low-fibre or low-fat/high-fibre diet[70].

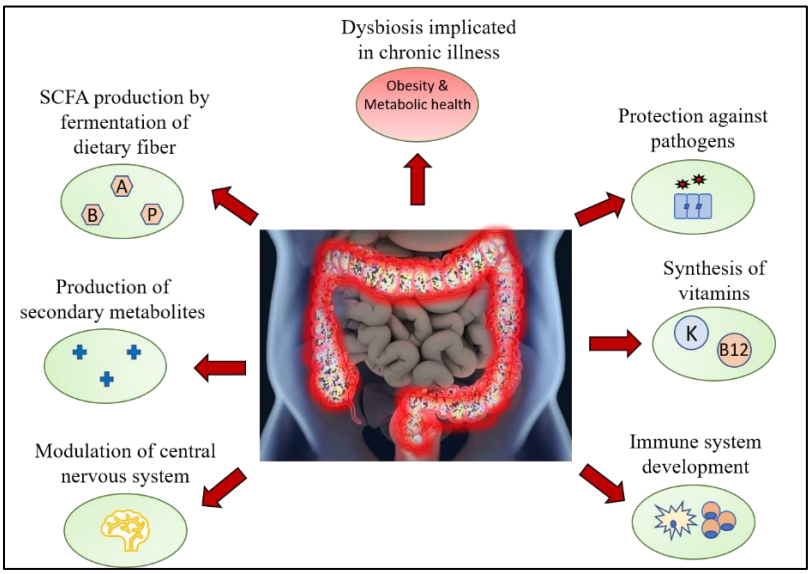

Figure 2-1: The Benefits of the intestinal microbiota and on human health.
Image adapted from Amon, P., et al. 2017[73].

The gut microbiota possess enzymes capable of digesting food that is not readily digested by host enzymes[60]. This symbiotic relationship between microbiota and host enables the production of microbial-derived essential vitamins and SCFAs that are utilized by the host,

which help maintain metabolic homeostasis[74–76]. SCFAs are mainly produced through the fermentation of non-digestible carbohydrates, where the microbiota transforms non-starch polysaccharides, oligosaccharides, and resistant starches into SCFAs[60,77,78]. Diets high in dietary fiber are associated with beneficial changes to the composition of the microbiota which is correlated to increased SCFA production[26]. SCFAs are made up of a carboxylic acid group attached to a hydrocarbon chain, consisting of 2, 3, and 4 carbons for acetic, propionic, and butyric acid respectively[79]. SCFAs are usually present in the molar ratio of 60:20:20 for acetic acid: propionic acid: butyric acid[61,80]. Germ-free mice and rats, rodents who have been raised without a microbiota, have low levels of SCFAs in their cecum content and serum as compared to conventional mice or rats, indicating the importance of microbial fermentation in the production of SCFAs[81].

SCFAs produced by the microbiota supply the colonic epithelial barrier with energy, and can account for approximately 10% of daily energy intake[82], which may promote intestinal barrier function by nourishing the intestinal epithelium. SCFAs are rapidly absorbed by the colonic epithelium; being absorbed either through non-ionic diffusion, or being actively transported into colonocytes by SCFA transporters [83–85]. While SCFAs are absorbed to a certain degree, butyrate is preferentially utilized by colonocytes as a respiratory substrate in oxidative metabolism[86–89]. This utilization of butyrate by colonocytes helps promote the growth of colonocytes through the production of ATP, resulting in cell proliferation[89,90]. On the contrary, in colon carcinoma cells butyrate can inhibit cell proliferation, and induce apoptosis leading to cell death[91–93]. Trials involving butyrate or mixed SCFAs administration resulted in improved disease activity index for patients and rodents with Inflammatory Bowel Disease (IBD) and colorectal cancer[92,94,95]SCFAs can also increase epithelial barrier function through facilitating ion transport

and improving water absorption[83,96]. Another function of SCFAs on the gastro-intestinal tract is their ability to promote epithelial barrier integrity, namely by enhancing TJ protein organization (ZO-1 and occludin) through the activation of AMP-protein kinase, and by increasing expression of TJ proteins (claudin-1)[97–99]. SCFAs can also promote epithelial barrier integrity by increasing mucin (MUC) gene expression in goblet cells, resulting in increased mucin production and improved mucosal barrier integrity[100,101].

SCFAs also play a large role in modulating the intestinal immune system. SCFAs bind to different receptors, namely G-coupled protein receptors (GPR); GPR41, GPR43, and GPR 109a (formally named free-fatty acid receptors 2 and 3, and hydrocarboxylic acid receptor 2) which are widely expressed in intestinal cells[102–104]. GPR41 is expressed in a variety of tissues such as bone marrow, lymph nodes, blood mononuclear cells, pancreas, spleen, and adipose tissue[105,106]. GPR43 is found in immune cells, heart, skeletal muscle, spleen and adipose tissue [105]. GPR109a is expressed in adipose tissue, spleen, skin cells, as well as a variety of immune cells[103,107,108] Butyric and propionic acid have the highest affinity for GPR41, and butyrate also binds to GPR109a with low affinity, while acetate has a higher affinity for GPR43[103,109]. Once bound to these receptors, they can activate the immune system by exerting their effects on a variety of immune cells. For example, SCFAs administered to conventional mice led to increases in colonic regulatory T (Treg) cells, which are involved in limiting intestinal inflammation, and increases in anti-inflammatory interleukin (IL)-10 production, and these results were dependant on GPR43 expression in Treg cells[110]. These findings were supported in another study which demonstrated that supplementation with SCFAs can increase levels of IL-10 and promote intestinal homeostasis in mice models and human subjects with IBD[111]. Furthermore, butyric acid has been demonstrated to reduce intestinal inflammation, by down-regulating the function

of macrophages through the inhibition of histone deacetylase resulting in a reduction in pro-inflammatory cytokines secreted from macrophages, such as IL-6 [112,113]. Butyrate can also prevent the maturation of dendritic cells stimulated by microbial-derived lipopolysaccharide (LPS), and reduces the production of IL-12 (inflammatory cytokine) from dendritic cells[114]. Further improvements in inflammation have been seen with butyrate bound to GPR109a, which has been demonstrated to reduce inflammation in healthy and cancerous colon cells by suppressing activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)[103].

SCFAs can also exert effects on peripheral tissues and can have metabolic actions. Propionate is primarily metabolized by the liver, and is involved in gluconeogenesis, blood pressure regulation, and lipid metabolism [115,116]. Propionate has been demonstrated to reduce cholesterol levels, inhibit fatty acid synthesis, and affect the release of gut hormones such as GLP-1 (which can reduce blood glucose levels) and peptide tyrosine tyrosine (PYY) (which can reduce appetite)[117–120]. Meanwhile, acetate primarily enters systemic circulation and is metabolized by peripheral tissues such as skeletal and adipose tissues, and has been shown to increase cholesterol synthesis and reduce blood pressure[121–123]. Acetate can also exert metabolic effects by increasing the release of GLP-1 and PYY into systemic circulation[124].

2.2 Obesity and Metabolic Health: role of the intestinal microenvironment

An increase in a sedentary lifestyle combined with an increase in consumption of a Western diet, typically high in saturated fats, refined sugars, and low in fiber, are amongst the contributors that increase the risk of obesity and its associated co-morbidities (diabetes, cardiovascular disease, colon and other cancers, and mental illness)[125–127]. Obesity and its

comorbidities are increasing, and the costs to treat these conditions puts a substantial load on healthcare systems[125]. A hallmark of obesity is chronic low-grade inflammation and adipose tissue dysfunction. Since adipose tissue is modulated by energy-balance, it is sensitive to over-nutrition, meaning an excess in energy can severely disrupt adipose tissue function[128,129]. Over-nutrition may lead to lasting adipose tissue dysfunction, as removal of high fat feeding has been shown to normalize body weight in obese mice, but only to body fat levels 40-50% higher than lean controls[130]. Obesity is characterized by an increase in chronic systemic inflammation driven in-part by dysfunctional adipose tissue, leading to unregulated circulation of pro-inflammatory mediators, combined with a decrease in the production of anti-inflammatory mediators[131,132].

Adipose tissue presents itself as white adipose tissue, which is largely distributed over the body and serves as an energy reserve, or as brown adipose tissue, which is stored in smaller factions and serves as a fat metabolizer by generating heat and increasing metabolism[133–136]. Normal adipose tissue functions by storing excess energy, and can activate the immune system by the release of cytokines, however in obesity, adipose tissue becomes dysfunctional and contributes to chronic low-grade inflammation[131,137]. During adipose tissue dysfunction, adipocytes expand, and immune cells such as macrophages[138], infiltrate causing an increased release of pro-inflammatory mediators[139]. This inflammatory mechanism is intensified in the obese phenotype, where excess adipose tissue accumulation is seen[140,141], causing macrophages to congregate around dead adipocytes to form crown-like structures (CLS) in both mice and humans[139,142]. Adipose tissue distribution and dysfunction are important contributing factors to obesity co-morbidity disease progression[140,143], with increased central adiposity being associated with higher mortality rates[144]. The formation of CLS occurs more frequently in visceral obesity, and CLS and adipocyte hypertrophy are related to insulin resistance[131,139,140,142]. Once

adipocytes are infiltrated, secretion of pro-inflammatory adipokines such as IL-1, IL-6, tumor necrosis factor (TNF)-α, interferon (IFN)-γ and resistin are increased, whereas adiponectin and IL-10 (anti-inflammatory adipokines) are decreased[65,128,138,145–147], which contributes to chronic low-grade systemic and tissue inflammation.

In addition to a dysfunctional adipose tissue, the intestinal microenvironment also contributes to chronic low-grade inflammation associated with metabolic diseases such as obesity[148,149]. Prolonged regular consumption of high-fat diets negatively influences the intestinal microbiota, and barrier integrity and function in a multitude of ways[150–153]. High fat feeding can cause drastic changes in the intestinal microenvironment, such that increases in the proportion of fat in diet can alter the spatial distribution, composition, activity, and diversity of the intestinal microbiota[63,64]. Furthermore, bacterial species diversity is decreased within obese individuals, where decreases in beneficial species such as members of the *Bifidobacterium* genus and *Ruminococcus flavefaciens* are observed, which may in part be due to competition causing an increase in species abundance (e.g. *Firmicutes spp.*) that can better harvest energy from high calorie diet intake[63,76,154,155]. This change in the types and numbers of microbial species present is referred to as dysbiosis[66]. The impact of high-fat diet (HFD) on the intestinal microenvironment results in a dysbiotic microbiome that is capable of extracting more energy from non-digestible dietary components compared to a healthy microbiome, as indicated by an increase in SCFA concentrations in subjects with obesity vs. lean subjects [76,156].

For example, one study found that when female resistin-like molecule beta (RELMβ) knock-out (a phenotype that remains lean on a HFD) and wild-type C57Bl/6 mice were switched from a standard chow diet to a high fat diet for 21 weeks, alterations in the microbiota were seen, mainly decreases in *Bacteroidetes*, and an increase in *Firmicutes* [157]. These shifts in microbial

populations were independent of the obese state, as similar shifts in *Bacteroidetes and Firmicutes* were reported for the wild-type and RELMβ knock-out mice, even though body weight was significantly lower in the RELMβ knock-out mice[157]. These shifts in the microbiota were accompanied by increases in bacterial genes involved in active transport of nutrients such as sugars, lipids, peptides, and genes involved in sugar utilization[157]. Several other studies have also observed reduced bacterial diversity, as well as increases in *Firmicutes* phyla abundance and a decrease in the *Bacteroidetes* phylum in subjects living with obesity[38,63,158,159].

The role of the gut microbiome in the onset of obesity has been substantiated by Bäckhed and colleagues, who demonstrated that a high fat diet alone could not cause obesity in germ free (gnotobiotic) mice[75]. Moreover, they found that when germ-free mice underwent conventionalization, a process where the germ-free mice were inoculated with the microbiota of obese mice, they developed obesity, demonstrated by increased body fat and weight, and dysregulation of metabolic homeostasis (e.g. reduced leptin (regulates food intake and body weight[160]) and insulin sensitivity[75]. Another effect of high-fat feeding on the intestinal health is that it can impair cholesterol metabolism and bile acid synthesis, notably driven by a dysbiotic microbiota[161,162]. This can lead to increased cholesterol levels and increased risk of cardiovascular disease and fat storage[161].

Microbial dysbiosis is defined as a change in the types and numbers of microbes present, and are associated with certain diseases or conditions[66]. One potential consequence of microbial dysbiosis in obesity is endotoxemia. Metabolic endotoxemia is defined as an increase in circulating plasma LPS, caused by alterations to the intestinal barrier which allows microbial-derived LPS into systemic circulation, leading to chronic low-grade inflammation[65,163,164]. Although LPS is increased during metabolic endotoxemia, it is present in considerably lower

quantities than those found during sepsis or other infection[163,165]. It is theorized that a dysbiotic microbial community is a source of reduced intestinal barrier integrity, which allows an increase in inflammatory microbial-derived LPS to cross the intestinal barrier and enter the host (endotoxemia)[65,163]. LPS is a component of gram-negative bacterial cell walls, making up the major glycolipids of the bacterial outer membrane[166]. LPS will bind to LPS-binding protein (LBP), where it can then facilitate the transfer to CD14 and Toll-like receptor(TLR)-4[167,168]. TLR4 is transmembrane protein, and a pattern recognition receptor that binds to Lipid A moiety found on LPS[167]. LPS must bind to TLR4 for it to activate the release of pro-inflammatory cytokines, by activating NF-κB dependent pathway causing a release of pro-inflammatory mediators [166,169]. Metabolic endotoxemia is associated with increased gut permeability, impairment of mucosal barrier permeability and function, adipose tissue dysfunction and an increase in the secretion of pro-inflammatory cytokines IL-1, IL-6, & TNF-α[163,166,170,171].

LPS may cause systemic inflammation by entering the bloodstream through a mechanism termed “leaky gut”, where LPS crosses the intestinal epithelium due to loss of barrier integrity caused by prolonged high fat feeding [65,163]. Infusions of LPS has been demonstrated to cause an inflammatory cascade which leads to obesity and insulin resistance in mice [163]. Another study has found that the microbiota in mice fed a high fat diet promote intestinal inflammation, as seen by an increase by NF-κB activation and TNF-α, which also correlated with obesity and insulin resistance [40]. Certain inflammatory cytokines such as TNF- α, IFN- γ, and some IL’s have been identified to increase intestinal barrier permeability by regulating TJ protein expression and organization[172,173]. This induction of intestinal permeability by inflammatory cytokines has been found to precede obesity and may lead to the development of metabolic issues such as insulin resistance and dyslipidemia, commonly found in obesity[163,174].

Collectively, the findings from the studies reviewed above highlight that several components of the intestinal microenvironment play a role in human health and disease. In the healthy state, the microbiota and intestinal epithelium work in a symbiotic manner that contributes to the health of the host (**Figure 2-1**) (e.g. nutrient digestion, vitamin production, immune homeostasis, metabolic homeostasis)[61,73,113,118]. On the other hand, the microbiota, in particular when exposed to prolonged high fat feeding[72,151], can negatively impact the host by impairing the integrity of the barrier and contributing to endotoxemia and chronic low-grade inflammation, which is associated with metabolic diseases (**Figure 2-1**), such as obesity[65,131]. Therefore, strategies to improve and/or maintain intestinal health may be considered an effective means to prevent and/or treat human chronic disease. One such strategy may be through improved dietary patterns, such as increasing the consumption of plant-based foods such as common beans.

2.3 Common bean effects on intestinal and metabolic health

Common beans are a class of legume, which are harvested for the dried seeds, and come in a wide variety of shapes and seed coat colours. Common beans are grown in most regions of the world and are very nutritious as they contain high levels of protein and fibre, while containing low levels of fat and sugar. Certain components of common beans such as resistant starch, GOS, and polyphenols have been demonstrated to improve the intestinal barrier and microbiota community structure and are therefore an ideal candidate for improving intestinal health[33,175–177]. Consumption of common beans also exert metabolic health benefits such as being negatively associated with obesity, cardiovascular disease, and type 2 diabetes[13,14].

2.3.1 Common beans and intestinal health

The impact of common bean consumption on intestinal health has been assessed in *in vitro* fermentation models[178,179], *in vivo* animal models[170,180–184] , and in human subjects [185,186]. Studies have explored the differences between different coloured beans of the same variety on intestinal health and disease. One study utilizing C57Bl/6 male mice fed 20% cooked navy and black beans supplemented diet, found that bean consumption for 3 weeks led to improvements in intestinal health[170]. Both bean diets influenced the gut microbiota, as demonstrated by increased microbial diversity, and increased SCFA production, however only the black bean diets had significantly increased microbial richness[170]. Both bean diets significantly increased the genus *Prevotella* and an unassigned genus in the family *S24-7*, members of *Bacteroidetes* phyla that are involved in carbohydrate production and metabolism[77,187]. Furthermore, the black bean diets, which have higher levels of polyphenolic compounds than navy beans, led to improved aspects of the intestinal barrier compared to the navy bean and basal diet, as indicated by enhanced epithelial crypt height, goblet cell numbers, and mucus content[170].

In addition to improving intestinal health, studies have also shown that consumption of common beans can attenuate inflammation and reduce the symptoms and severity of colitis. In a study utilizing the same navy and black bean dietary intervention as described above except after 3 weeks of bean diet consumption mice were exposed to an inflammatory challenge (2% dextran sodium sulphate, DSS), found that bean supplementation attenuated colonic inflammation (e.g. increased anti-inflammatory cytokine IL-10 mRNA expression, and decreased pro-inflammatory cytokines TNF-α, IFN-γ, and IL-6)[188]. Additionally, only black beans reduced colonic pro-inflammatory cytokine IL-17a mRNA expression, suggesting an enhanced effect of darker beans on reducing intestinal inflammation[188]. In a similar study, white kidney (WK) and dark red

kidney (DK) beans were supplemented into a basal diet, at 20% supplementation, for 3 weeks prior to DSS inflammatory challenge in male C57Bl/6 mice[184]. Kidney bean supplementation led to decreased disease activity index, decreased colon/length ratio, and increased colonic mucus production, irrespective of bean seed coat colour[184]. Other improvements in inflammation were found, such as decreased colonic mRNA gene expression of IL-1β, IL-6, and increases in IL-10, and these were correlated with their respective colon cytokine protein concentrations[184]. Interestingly, mice pre-fed WK beans had decreased colonic TNF-α mRNA gene expression compared to controls, while only mice pre-fed DK beans had decreased colonic gene expression of monocyte chemoattractant protein (MCP-1) compared to controls, suggesting that differences exist in the health effects induced by bean varieties with different seed coat colours within the same gene pool. Another mouse model of colitis using darkening and non-darkening cranberry beans found that supplementation with 20% cranberry beans for 3 weeks prior to an inflammatory challenge (2% DSS, in drinking water for 7 days) improved colitis disease activity index[183]. Furthermore, cranberry bean consumption was associated with improved intestinal barrier integrity as demonstrated by increased crypt height, goblet cell number, and mucin content, as well as increased colonic mRNA gene expression of IL-10, MUC1 and MUC3, and decreased expression of TNF-α and IL-6 in mice following an inflammatory challenge[183]. Additionally, the bean diets significantly altered the intestinal microbiota composition as demonstrated by increases in *Prevotella* spp., and *S24-7* (a member of *Bacteroidetes*), and altered microbial activity by increasing SCFAs[183]. The darker coloured cranberry beans also provided enhanced benefits compared to the non-darkening variety, as colon/length ratio and histological damage score (indicators of intestinal inflammation and crypt damage), were significantly decreased compared to colitis controls[183]. These results suggest that darker coloured

beans of the same gene pool may provide additional benefits to intestinal and metabolic health by improving the gut microbiota composition and epithelial barrier function and integrity.

The improved intestinal microbiota structure and increases in SCFAs seen in mice or humans consuming common beans may be due to the high levels of non-digestible carbohydrates (fibre)[170,182–184,188]. The recommended daily fibre intake for men is 38 grams/day and for women 25grams/day, with diets rich in fruits and vegetables providing upwards of 60 grams of fibre per day, while the typical western diet contains around 20 grams of fibre per day[189–191]. Non-digestible carbohydrates are resistant to complete digestion in the upper gut and are fermented by the microbiota more distally in the gastro-intestinal tract[60]. There are many types of non-digestible carbohydrates which will be discussed further below, that can be classified based on the number of sugar subunits (also known as degree of polymerization) (**Table 1-1**)[192]. Common beans contain various levels of dietary fibre, with ranges of 12-16 gram insoluble fibre, 6-10 grams soluble fibre, 3.5-5 grams GOS, for a combined total of approximately 24-27 grams total dietary fibre per 100 grams dry weight (DW)[193,23].

Table 2-1: The Major Dietary Carbohydrates

Class (Degree of polymerization*):	Subgroup:	Principle Examples:	Principle Sources:
Sugars (1-2)	Monosaccharides	Glucose, fructose, galactose	Fruits
	Disaccharides	Sucrose, lactose, maltose,	Sugar cane, beets
	Polyols (sugar alcohols)	Sorbitol, lactitol, mannitol, xylitol, maltitol	Synthetic
Oligosaccharides (3-9)	Malto-oligosaccharides (α-glucans)	Maltodextrins	Glucose syrups
	Non- α-glucan oligosaccharides	Raffinose, stachyose, fructo- and galacto-oligosaccharides, inulin, polydextrose	Beans, Peas, onion, chicory root
Polysaccharides (≥10)	Starch (α-glucans)	Pectin, amylopectin, amylose	Potatoes, apples

	Non-starch polysaccharides	Cellulose, hemicellulose, arabinoxylans, β-glucan, plant gums	Fruits, vegetables

*Degree of polymerization refers to the number of single sugar subunits. Table adapted from Cummings, J.H., et al. 2007[192], and Livingston, K. A., et al. 2016[194].

Health Canada defines dietary fibre as carbohydrates that are not digested in the small intestine, and are found in plant foods with a degree of polymerization (the number of single sugar subunits) of 3 or higher[195]. Novel fibres, also included in the definition, are synthesized from either plant sources or synthetically manufactured, and must confer a physiological health benefit to the host and also have a degree of polymerization of 3 or more[195]. Known physiological health benefits attributed to fibre recognized by Health Canada include: improved laxation and fecal bulking, reduced blood total and low-density lipoprotein (LDL-C) cholesterol levels, reduced post-prandial blood insulin and glucose levels, increasing insulin sensitivity, and enhancing the production of metabolites by fermentation[195]. In general, fibre contains an energy value of 2 kcal (8 kJ)/g. Other physiologic health benefits of fibre include, but are not limited to increased satiety, modulation of the microbiota composition, and high fibre intake is inversely associated with colorectal cancer and obesity[154,196,189,197–199]. Dietary fibres can be further classified by their solubility into soluble or insoluble fibres, with soluble fractions being solubilized in water, whereas insoluble fibres do not dissociated easily in water and therefore are involved in fecal bulking and decreased transit time[200]. Not all common beans are expected to have same effect on intestinal health due to the different types and concentrations of dietary fibres present. Pulses contain high levels of starch (~30-45%) and consequently resistant starch (~4% DW), with amylose being the most prevalent type of starch, accounting for 30-40% of the starch in common beans[23]. Amylose (a linear nonbranched polymer of glucose units) is more difficult to digest than other types of starch and may account for the high levels of resistant starch in pulses[14]. Starch may also be resistant to digestion by physical transformations that can

occur during the cooking process of starchy foods[23]. Resistant starch can increase viscosity and decrease the glycemic index of foods, which may provide enhanced benefits on glucose homeostasis, and can act as prebiotics by increasing intestinal levels of *Bifidobacterium* and *Lactobacilli* [201,202]. A study conducted on obesity prone rats found that increased amounts of resistant starch in diet decreased energy intake, weight gain, visceral fat, plasma triglyceride, cholesterol concentration, and increased total SCFAs[175]. Other studies have found that resistant starch can also reduce the risk of colon cancer by increasing colonic butyrate concentrations[202]. Beans also contain GOS, which are non α-glucan oligosaccharides and are not easily digested in the upper intestinal tract and are therefore fermented by the colonic microbiota[203]. Pulses contain around 1-4% GOS, with stachyose being the most prevalent[23,178]. GOS has prebiotic effects as it is known to increase the number of beneficial bacteria in the gut, such as *Bifidobacterium* and *Lactobacilli* [204,205].

On top of being excellent sources of fibre, common beans contain very high-levels of plant-based protein, usually around 20-25% crude protein, making them an attractive food as they are a cheap alternative to animal-based proteins[206]. While rich in protein, common beans contain sub-optimal concentrations of the essential amino acids cysteine and methionine[206]. Protein digestibility and content in beans increases when cooked and proteins that reach the colon undigested are metabolized by the microbiota into branched chain fatty acids (BCFAs), while other by-products such as sulphuric compounds and ammonia are produced as protein-fermentation by-products[178,206–208]. BFCAs were reported to be decreased in mice consuming navy and black bean diets compared to controls whose diets sole source of protein is from casein[170]. Plant-based proteins can also affect the gut microbiota composition, namely by increasing the abundance of Bacteroidetes when compared to animal-based proteins[209]. Plant-

based protein consumption is associated with decreased LDL and total cholesterol, when compared to animal-based protein consumption in individuals with high cholesterol[210]. Taken together, these studies suggest there may be an added benefit to consuming plant-based proteins over animal-based proteins when it comes to intestinal and metabolic health.

The beneficial effects of common beans on intestinal health (described above) may also be due to the presence of polyphenolic compounds in beans [183,184,188]. Polyphenols are compounds containing a phenolic ring and carboxylic acid moiety, and can be distinguished into hydrobenzoic acids (gallic acid, protocatechuic acid, vanillic acid, syringic acid) which are derived from benzoic acid, and hydroxycinnamic acids (p-coumaric acid, caffeic acid, ferulic acid, sinapic acid) which are derived from cinnamic acid[211,212]. Polyphenols are not well absorbed in the small intestine and the majority of polyphenols reach the colon and are therefore available for biotransformation into more bioactive secondary phenolic metabolites by the colonic microbiota[213,214]. Polyphenolic compounds may also interfere with carbohydrate digestion, thereby facilitating their passage to the colon where they may be fermented by the microbiota[212,215]. Common beans vary in their phenolic compound profiles (phenolic acids, flavonoids, and anthocyanins), and these profiles contribute to the multitude of common bean seed coat colors[170,183,188,216]. Darker coloured bean varieties are known to contain anthocyanins, which give beans their purple/red hue[217]. Accordingly, darker coloured variety beans contain higher and more diverse levels of phenolic compounds compared to their light-coloured counterparts (**Table 2-2**)[188,218].

Polyphenolic compounds are known to affect many aspects of intestinal health such as reducing the risk of cancer, enhancing intestinal epithelial barrier integrity, can modulate the intestinal microbiota community structure, and affect SCFA production[219–221]. Polyphenolic

compounds and their metabolites can increase TJ protein expression, thereby increasing colonic barrier integrity and decreasing permeability[222]. Polyphenols have been demonstrated to improve inflammation by reducing levels of IL-1β, IL-6, and TNF-α, and can supress NF-κB activation, and reduce oxidative stress[220,222–224].

Table 2-2: Total phenolic content in beans

Bean Class	Extraction & Analysis Method:	Phenolic Concentration	Citation:
Cranberry Regular Darkening	70% MeOH containing 1% HCl (v/v), TPC assay	2.82 mg GAE/g whole raw bean	Chen, P.X., et al. 2015. (216)[218]
Cranberry Regular Non-darkening	70% MeOH containing 1% HCl (v/v), TPC assay	0.67 mg GAE/g whole raw bean	Chen, P.X., et al. 2015. (216)[218]
Light Red Kidney Bean	50% EtOH in H_20, TPC assay	9.53 mg GAE/g cooked cotyledon, 56.52 mg GAE/g cooked hull	Sutivisedsak, N., et al. 2010. (217)[225]
Dark Red Kidney Bean	50% EtOH in H_20, TPC assay	10.92 mg GAE/g cooked cotyledon, 63.77 mg GAE/g cooked hull	Sutivisedsak, N., et al. 2010. (217)[225]
Navy (white bean)	50% EtOH in H_20, TPC assay	8.67 mg GAE/g cooked cotyledon, 8.34 mg GAE/g cooked hull	Sutivisedsak, N., et al. 2010. (217)[225]
Black Bean	50% EtOH in H_20, TPC assay	8.46 mg GAE/g cooked cotyledon, 60.77 mg GAE/g cooked hull	Sutivisedsak, N., et al. 2010. (217)[225]

Polyphenolic compounds can also affect metabolic health in a multitude of ways, for instance it has been reported that polyphenols can modify lipid metabolism, and lower blood lipid levels[226–228]. Polyphenol consumption has also been associated with improved glucose homeostasis and insulin sensitivity, as well as improved cardiovascular health[229,230]. Pulses, rich in polyphenolic and flavonoid compounds, have been attributed to decreasing pro-inflammatory cytokines, increasing anti-inflammatory cytokines, improving cholesterol levels, as well as improving intestinal barrier function and mucosal layer thickness[182–185,231]. Phenolic compounds, as previously mentioned, can have anti-oxidant and anti-inflammatory capabilities, therefore it

can be inferred that bean varieties high in phenolic compounds may exert more beneficial health effects compared to those with lower levels of phenolic compounds.

Common beans and their bioactive components have been known to improve many aspects of intestinal health, such as improvements in microbiota composition, reduced intestinal inflammation, improved epithelial barrier integrity and function under inflammatory challenges, and increased microbial activity[170,180,182–184,188]. However, gaps in knowledge exists, as the previous studies described were of short-feeding duration and featured small sample sizes. Studies with longer feeding durations and with adequate sample size are required to fully comprehend the impact of chronic consumption of different bean varieties at physiologically relevant levels on the intestinal microbiota and epithelial barrier function and integrity.

2.3.2 Common bean effects in obesity and metabolic health: is the intestinal microenvironment a target for intervention?

Not only have beans been demonstrated to improve multiple aspects of the intestinal epithelial barrier and microbiota, but they also exert many beneficial effects on metabolic health. As reviewed in Chapter 2.2, obesity is considered an intestinal-health associated disease wherein intestinal dysbiosis and dysfunction can contribute to chronic low-grade inflammation and metabolic dysfunctions, which are common attributes of the obese phenotype. Therefore, it may be hypothesized, based on the beneficial impact of common beans on intestinal health described above, that common beans may also improve aspects of the obese phenotype through modulation of intestinal health. Pulse consumption has been associated with reduced parameters associated with obesity and metabolic health, such as satiety and regulation of appetite hormones, insulin resistance, body weight, blood pressure, lipid metabolism, reduced body mass index (BMI), body

fat, triglycerides, and thickness of adipose tissue[232,233,203,234,235]. Common beans also improved blood glucose homeostasis and insulin resistance which may be attributed to their low-glycemic index and their increased viscosity allow for slower absorption of nutrients into the body[14,16,23]. Furthermore, the reduction of carbohydrate digestion allows for increased resistant starch to reach the colon and be metabolized by the microbiota into SCFAs which can exert metabolic effects such as improved lipid metabolism[116,233]. Thus, one mechanism through which common bean consumption can improve intestinal health to reduce metabolic dysfunctions in obesity is through increased production of SCFAs.

This has been exemplified in a human study which compared the effects of consuming a ½ cup of cooked pinto beans versus an isocaloric soup daily for 12 weeks to determine changes in serum lipids in pre-metabolic syndrome or healthy adults, as well as fecal bacterial populations and SCFA production which was assessed in fecal samples using an *in vitro* fermentation model [185]. Feces collected from both healthy and pre-metabolic syndrome adults, demonstrated that subjects fed pinto beans had increased *in vitro* production of propionic acid and total SCFA suggesting similar capability of the microbiota to ferment non-digestible carbohydrates, despite the health status [185]. Furthermore, of the 6 fecal bacterial species analyzed, only *Eubacterium limosum* was decreased in bean-fed subjects irrespective of whether they were healthy or pre-metabolic subjects. Pinto bean consumption also lead to a decrease in total cholesterol, high-density lipoprotein cholesterol (HDL-C), and LDL-C in both healthy or pre-metabolic syndrome adults, suggesting an improvement in serum lipid profiles[185]. Although this study highlights minor changes in the intestinal microbiota following common bean consumption, the methodology used to assess the microbiota community structure was limited. The use of more advanced gene sequencing technologies may provide a more comprehensive

assessment of bean-induced changes to the structure and function of the microbiota following bean consumption by human subjects.

In a more recent study, Monk *et al* utilized a mouse model of HFD induced obesity, in which C57Bl/6 male mice were fed cooked navy beans incorporated into a high fat diet at a supplementation level of 15% (wt/wt) for 12 weeks. Their results revealed that improvements to intestinal health occurred through increased fecal microbiota community diversity, increased key "anti-obesity" bacterial species [e.g. 20-fold increase in *A. muciniphila*[236]]; increased SCFA production and the abundance of SCFA-producing microbiota (e.g. *Prevotella spp.*). Furthermore, navy bean consumption lead to an increased intestinal barrier integrity and function, as demonstrated by increased expression of epithelial TJ proteins (ZO-1 and occludin)[182], as well as reduced intestinal permeability. In this study, the microbiota community was assessed by 16S rRNA gene sequencing which allowed the researchers to comprehensively assess the impact of navy bean consumption on the obese fecal microbial community. These positive changes in microbiota composition, epithelial barrier, and SCFA production were accompanied by beneficial metabolic health effects as demonstrated by increased serum adiponectin and decreased insulin resistance in the high-fat bean diet group compared to control[182]. Further, improvements in adipose tissue dysfunction were seen, as demonstrated by reduced adipocyte size and the presence of CLS, caused by infiltrating macrophages around dead adipocytes[142], in the high-fat bean diet group compared to the high-fat control, which was accompanied by lower pro-inflammatory adipokines. Overall, this was the first paper to comprehensively examine the impact of common bean (i.e. navy bean) consumption on the obese intestinal environment and its role and potential link to improving metabolic health.

Chapter 3: Rationale, Hypotheses, and Objectives

3.1 Rationale

Health Canada recently launched a new Canada's food guide which recommends consuming more plant-based foods, in particular those that are rich in protein such as legumes. Dietary pulses are a class of legumes and include common beans, lentils, chickpea and peas. In general, common beans are rich sources of intestinal health promoting components (non-digestible carbohydrates and phenolic compounds), and their consumption has been shown to beneficially alter intestinal and metabolic health and attenuate the severity of gut-associated diseases such as IBD, colon cancer, and obesity. On the other hand, there are ~400 edible common bean varieties arising from two main ecogeographical gene pools (Andean and Mesoamerican)[2,3], which range in size, seed coat color, and nutrient composition; therefore, it should not be assumed that all bean varieties will similarly induce intestinal and metabolic health benefits. **Further research is therefore required to determine the extent to which different common bean varieties impact intestinal and metabolic health, which may influence consumers when selecting bean varieties**. Studies comparing the effects of different bean varieties within the same gene-pool on intestinal and metabolic health are limited. However short-term intervention trials (2-3 weeks) in mice have suggested that consumption of dark-colored bean varieties (i.e. black beans) may provide additional health benefits compared to light-colored bean varieties (i.e. navy beans), perhaps due to the additional polyphenolic compounds present in dark colored beans. **Therefore, further studies are required to determine if bean-induced changes in intestinal and metabolic health differ depending on the bean variety (seed coat color) consumed.**

Finally, it may be important to consider the composition of the background diet when evaluating the host response to bean supplementation. While the majority of the research conducted thus far investigated the role of bean supplementation into a healthy control diet, it is well-known that high calorie or high fat diets which contribute to metabolic dysfunction and obesity development, induce adverse effects on the intestinal microenvironment. The diversity in the intestinal microbiota, as well as intestinal barrier physiology and function are altered by high-fat feeding, therefore, it is important to understand the role of background diet when evaluating the effects of long-term bean consumption. **Thus, the effects of bean consumption may differ depending on the nutritient composition of the background diet.**

3.2 Hypotheses

It is hypothesized that:

1) Supplementation of cooked white and dark red kidney beans at physiologically relevant doses in a murine model, will improve aspects of the intestinal and metabolic health

2) Since white and dark red kidney beans differ dramatically in their seed coat color and thus phenolic compound content and profile, dark red kidney beans will have additional beneficial effects in improving the intestinal and metabolic health in mice compared to white kidney bean.

3) Since high fat diets negatively impact intestinal and metabolic homeostasis, the effects of kidney beans on intestinal and metabolic health will differ in mice consuming a high fat diet compared to mice consuming a (low-fat) basal diet.

3.3 Objectives

My thesis objectives were to determine if:

1. long-term kidney bean supplementation will improve intestinal and metabolic health by measuring bean-induced changes in microbiota community structure and activity, and integrity and function of the intestinal mucosal barrier, as well as body composition, HOMA-IR, serum hormones and cytokines.

2. seed coat colour will affect measured outcomes of intestinal and metabolic health by comparing the effects induced by white and dark-red kidney beans.

3. the effects of white and dark red kidney bean supplementation on intestinal and metabolic health are similarly impacted by background diet nutrient composition (low or high-fat) by supplementing beans into a regular or a high-fat (obesogenic) diet

Chapter 4: Bean Cooking Methods, Nutrient Composition, and Animal Diet

4.1 Methods

4.1.1 Bean Varieties

Two dried kidney bean varieties (*Phaseolus vulagris*) were generously donated by the University of Guelph Bean Breeding Program (Guelph, ON). Bean varieties were Yeti (A35/871710W; white kidney (WK) bean), and Dynasty (17/076D1-D1; dark red kidney (DK) bean) (Gentec, Idaho) (**Figure 4-1A**).

4.1.2 Bean Preparation

Kidney beans are not consumed raw since dried seeds contain anti-nutrients such as trypsin inhibitors and lectins[231]. Pulses can be cooked in a variety of ways, however soaking and boiling dry beans is the most common methods of consumption in North America, other than canned beans. Soaked, cooked and dried bean powders retain much of their nutritive compounds during the preparation process[62], while eliminating many anti-nutritive compounds such as tannins and lectins[231] Soaking and cooking dry beans can reduce these anti-nutrients while also affecting the digestibility of other components such as starch and proteins [12,23,208,237].

WK and DK beans were prepared following recommendations from Pulse Canada[238], and a visual walkthrough of the cooking procedure is found in Figure 4-1. In brief. beans were sorted, and any cracked, shrivelled, or discolored beans were discarded (**Figure 4-1A**). 200 g of dry beans were rinsed thoroughly in cold tap water, then soaked in 750 mL tap water for 24 hours to allow beans to be evenly soaked (**Figure 4-1B**). Soaked beans were rinsed then boiled in 1250 mL of fresh tap water for 10 minutes at heat setting 7 on an induction ceramic cooktop

(Bertazonni, Model # PRO304INSX), then simmered on heat setting 4 until cooked (**Figure 4-1C**). WK beans simmered for 21 minutes, and DK beans simmered for 13 minutes to be fully cooked to the same tenderness before beans started to split, as determined by taste test. Cooked beans were then pureed in a blender (Waring, Model # WF2212114) with 355 mL of cook water for 15 seconds (**Figure 4-1D**). Blended bean purees were poured into a tinfoil pan, placed in Turbofan® convection oven (Moffat Ltd., Model # E32D5) set at 105°C, and stirred every 15 minutes to allow even moisture evaporation. After 120 minutes, the bean purees were spread out in a thin layer on parchment paper placed on a metal tray and placed back in the oven for another 60 minutes (**Figure 4-1E**), after which the dried beans were blended. The blended powders were placed in a container covered with aluminum foil with holes on top and placed back in the oven for 20-40 minutes until moisture content was below 5% (**Figure 4-1F**).

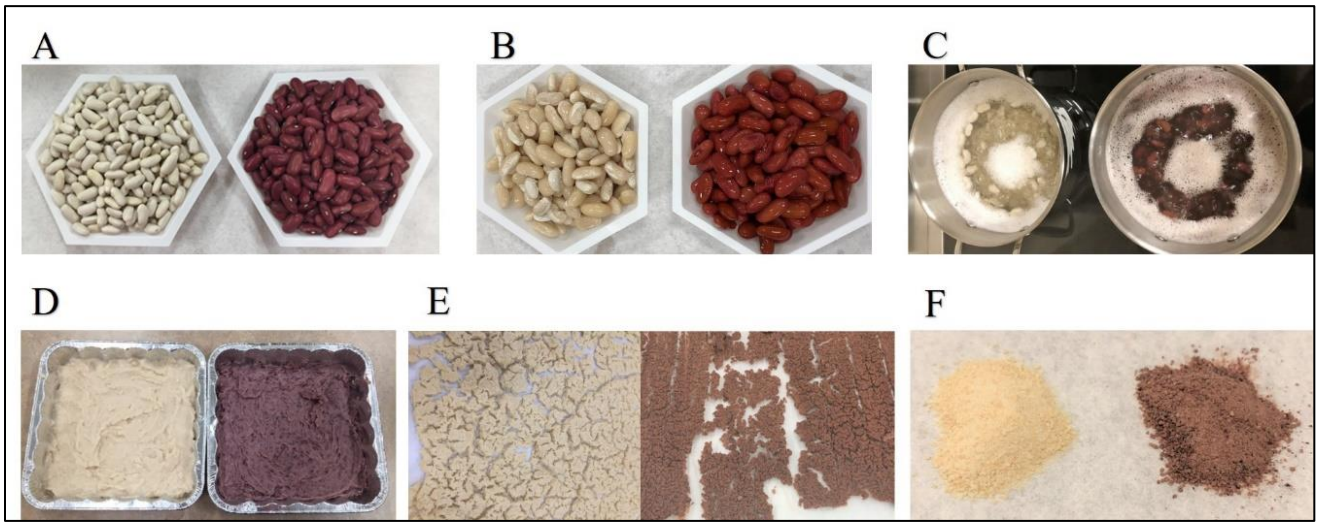

Figure 4-1: Representative Images of the Stages of the Bean Cooking Protocol.
A: Images of dry white and dark red kidney beans (200g/weigh boat). Before cooking, beans were sorted, and cracked and shriveled seeds were removed. **B:** Images of kidney beans after an overnight soak. **C:** Images of beans while boiling on the stovetop. **D:** Images of blended bean purees before drying. **E:** Images of the bean powder after drying. **F:** Images of dried bean powder after passing through the mesh sieve to ensure equal particle size.

Moisture content was determined using a moisture analyzer (Mettler Toledo, Model # HE53). Briefly, 1g of bean powder was placed on a metal tray and placed into the analyzer. The

initial weight was recorded, and the sample was heated to 160°C until the rate of moisture loss was less than 1 mg/30 seconds. Once the bean powder was less than 6% moisture, it was passed through an 850 µm mesh sieve (VWR, Cat. #57334-562), to ensure even particle size, before being stored at -20°C until further analysis of nutritional composition and incorporation into animal diets.

4.1.3 Bean Powder Macronutrient and Fibre Content

Cooked WK and DK bean powders were analyzed for macronutrient composition, and soluble and total fibre content by Maxxam Analytics (Mississauga, ON) using standard methods highlighted in **Table 4-1**. The kidney beans used in this study were determined to contain 22-23% protein (wt/wt), ~1.4% fat (wt/wt), ~39% available carbohydrates (wt/wt), while the fibre content of the beans was determined to be 6-7% insoluble fibre (wt/wt), and ~21-22% insoluble fibre (wt/wt). The macronutrient composition of the kidney beans used in this study are typical for kidney beans as demonstrated in previous studies[184].

Table 4-1: Cooked white and dark red kidney bean powder macronutrient composition

	WK powder (g/100g DW)	DK powder (g/100g DW)	Method
Protein	23.22	22.49	AOAC 992.15
Fat	1.38	1.41	AOAC 922.06, 933.05
Carbohydrate	66.0	67.8	Calculated by difference
Available Carbohydrate	39.1	38.8	Calculated by difference: carbohydrate -total fibre
Total Fibre	28.7	27.2	AOAC 991.43, 985.29
Soluble Fibre	7.2	6.2	AOAC 991.43(mod)
Insoluble Fibre	21.5	21	Calculated by difference: total fibre-soluble fibre
Ash	4.2	4.3	AOAC 923.03
Moisture	5.2	4.0	AOAC methodology
Energy (kcal)	311.9	319.6	Calculated

4.1.4 Starch Content

Starch content of bean powders were determined using the Total Starch Assay kit (Megazyme, Cat. # K-TSTA-100A) following the manufacturers recommendations with minor modifications for use with a microplate. In brief, bean powder samples (100 mg) were placed in a 50 mL Polystyrene conical tube (Corning®, Cat. #CA21008-951) with 5 mL of EtOH (80% v/v), and the tubes were mixed by vortex and incubated in a water bath at 85°C for 5 minutes. Another 5 mL of EtOH (80% v/v) was added to the tubes and was briefly mixed by vortex before being centrifuged at 1,800x g for 10 minutes. The supernatant was discarded, and the pellet was resuspended in 10 mL of EtOH (80% v/v) and mixed by vortex briefly. The tubes were centrifuged at 1,800x g for 10 minutes and supernatant discarded. A magnetic stir bar (VWR®, Cat. # 58948-397) was added to the tubes and 2 mL of 2M KOH was added to resuspend the pellets, the tubes were incubated in an ice-water bath over a magnetic stirrer for 20 minutes. Over the magnetic stirrer, 8 mL of 1.2M sodium acetate buffer (pH 3.8) was added, immediately followed by 100 μL each of thermostable α-amylase and amyloglucosidase buffers, and the tubes were mixed well before being incubated in 50°C water bath for 30 minutes with intermittent mixing of samples by vortex. Contents were transferred to a 100 mL volumetric flask and the volume was adjusted to 100 mL with distilled H_20. A 150 μL aliquot was placed in a microcentrifuge tube and centrifuged at 1,800x g for 10 minutes. Next, 100 μL aliquots (in triplicate) were placed in 15 mL Polystyrene conical tube (Corning®, Cat. # 352099), and 3 mL of GOPOD Reagent was added to each tube. Then, 100 μL of the provided D-Glucose solution (1mg/mL) was mixed with 3 mL of GOPOD Reagent and used as control, while the blank solution consisted of 100 μL distilled H_2O and 3 mL of GOPOD reagent. The samples, control, and blank solutions were incubated in a water bath at 50°C for 20 minutes before 100 μL of each was transferred to a microplate. The plate was read at λ=510 nm using a microplate reader and

the absorbance, minus the blank, was recorded. Starch content was calculated using Megazyme Mega-Calc™, with the following equation: % Starch = (ΔA) x (F) x (FV/0.1) x (1/1000) x (100/W) x (162/180), where ΔA is the absorbance read-blank, F is 100 μg D-glucose/absorbance for 100 μg of glucose, FV is the final volume, W is weight of sample analyzed in mg. Starch is presented as g/100g (DW).

4.1.5 Experimental Diet Composition

The average level of reported pulse intake in Canadian adults is ~112g/day (equivalent ¾ cup /day)[239] while the highest level of intake is ~300g/day (less than 2 cups/day or 400kcals/day)[7]. At the highest level of intake, pulses can account for ~16% of the daily caloric intake (assuming 2500kcal/day diet), therefore beans incorporated into an experimental diet up to a maximum of 16% kcals is considered physiologically attainable. To achieve a physiological relevant supplementation level, experimental diets were supplemented with 15% (wt/wt) cooked bean powder which contributed ~10% of the daily kcals consumed (based on Maxxam proximate analysis **Table 4-1**).

Six experimental diets were prepared by Envigo™ (Wisconsin, USA) for the mouse feeding trials. 1) Basal Diet (BD): A modified AIN-93G control diet; 2) White kidney bean basal diet (WKB): BD supplemented with 15% white kidney bean powder; 3) Dark red kidney bean basal (DKB): BD supplemented with 15% dark red kidney bean powder; 4) A high-fat control diet (HF) in which fat contributed 60% of the diets' caloric density; 5) White kidney bean high fat diet (WKH): HF supplemented with 15% white kidney bean powder; and 6) Dark red kidney bean high fat diet (DKH): HF supplemented with 15% dark red kidney bean powder. The nutritional composition of the experimental diets is shown in **Table 4-2**. The diets were prepared

in accordance with the AIN-93G diet formulation, however modified to contain 7% fibre (instead of 5%) to account for the higher fibre content of the bean powders. Furthermore, the fibre profile was modified from the original AIN-93G formulation which included insoluble fibre (cellulose) (5% wt/wt) as the only source of dietary fibre. This modification to the original AIN-93G diet (which contains only 5% cellulose as sole source of fibre), will permit for more accurate results that are driven by bean consumption rather than from the lack of soluble fibre in the control diets. The fibre profile was adjusted to include 5% cellulose and 2% pectin (wt/wt), which more accurately simulates the 3:1 ratio of insoluble to soluble fibre typically found in legumes[240].

Table 4-2: Experimental diet composition of BD, WKB, DKB, HF, WKH, DKH

Nutrient (g/kg)	BD TD.180554	WKB TD.180555	DKB TD.180556	HF TD.180557	WKH TD.180558	DKH TD.180559
Casein	200.0	160.0	161.3	265.0	225.0	226.22
L-Cystine	3.0	3.0	3.0	4.0	4.0	4.0
Corn Starch	377.486	310.606	308.716	0.0	0.0	0.0
Maltodextrin	132.0	132.0	132.0	160.0	94.72	91.3072
Sucrose	100.0	100.0	100.00	92.586	92.586	92.586
Lard	0.0	0.0	0.0	310.0	310.0	310.0
Soybean Oil	70.0	69.93	68.27	30.0	28.33	28.2728
Cellulose	50.0	17.75	18.5	50.0	17.75	18.5
Pectin	20.0	9.2	10.7	20.0	9.2	10.7
Mineral Mix, AIN-93G-MX (94046)	35.0	35.0	35.0	48.0	48.0	48.0
Calcium Phosphate, dibasic	0.0	0.0	0.0	3.4	3.4	3.4
Vitamin Mix, AIN-93-VX (94047)	10.0	10.0	10.0	14.0	14.0	14.0
Choline Bitartrate	2.5	2.5	2.5	3.0	3.0	3.0
TBHQ, antioxidant	0.014	0.014	0.014	0.014	0.014	0.014
White Kidney Bean Powder	0.0	150.0	0.0	0.0	150.0	0.0
Dark Red Kidney Bean Powder	0.0	0.0	150.0	0.0	0.0	150.0
Contribution of total calories (% kcal) from each macronutrient						
Protein	19.2	19.2	19.2	18.4	18.5	18.5
Carbohydrate	63.2	62.8	63.2	21.1	20.7	20.8
Fat	17.6	18.0	17.6	60.5	60.8	60.7
Energy Density (kcal/g)	3.7	3.7	3.7	5.1	5.1	5.1

The protein, available carbohydrates, fat, soluble and insoluble fiber content of the bean supplemented diets were adjusted to account for these macronutrients and fibers found in the added bean powders (as measured by Maxxam Analytics; **Table 4-1**) in order to create experimental bean diets that were isocaloric to their respective low fat (BD) or high fat (HF) bean-free control diets.

4.1.6 Phenolic Extraction of Kidney Bean Powders and Experimental Diets

Kidney beans vary in their polyphenolic compound profiles (phenolic acids, flavonoids, and anthocyanins), and these profiles are related to seed coat color [170,183,188,216]. Kidney beans comes in a variety of colours ranging from white, to light-red to dark-red, with the darker coloured bean varieties containing anthocyanins, which give darker kidney beans their purple and red hue[217,241]. As a result, darker coloured variety beans contain higher levels of polyphenolic compounds compared to their lighter-coloured counterparts[241]. Kidney bean phenolic profiles have been previously reported, however levels of plant phenolics can change over different growing seasons and is affected by growing conditions, storage methods, and means of processing[24,242,243]. It was therefore imperative to determine if the dark red kidney beans used in this study contained higher levels of phenolic compounds than their white kidney bean counterparts.

Free phenolic acids were extracted according to Luthria and Pastor-Corrales[24] and Zhang et al.[244], with slight modifications. Briefly, 0.5 g of samples (WK and DK bean powders and all experimental diets) were weighed, finely ground and placed in an aluminum foil wrapped 15 mL screw-capped tube (Corning®, Cat. #352099) with 7 ml of 70% methanol containing 0.1% HCl (v/v). Free phenolic acids were extracted at room temperature on a microplate shaker (VWR®,

model # 12620-926) set at 400 rpm for 18 hours. Samples were centrifuged at 3000 g for 10 min at 21°C, supernatants were collected, filtered through a 0.22 μm PVDF filter (Millex®, Cat. #SLGVX13NL) and stored at -20°C until further analysis.

4.1.7 Total Phenolic Content

Total phenolic content of the phenolic extracts (described in Chapter 4.2.6) was determined using the Folin-Ciocalteu colorimetric assay, as described by Singleton and Rossi[245] and adapted for a microplate with some modifications[246]. Briefly, 75 μL distilled H_20 was added to each well, followed by 25 μL of either the blank (70% MeOH, 1% HCl (v/v)), standard (gallic acid), or phenolic extracts to appropriate wells. 25 μL of freshly prepared Folin-Ciocalteu reagent (diluted 1:10 in distilled H_20) was added to each well using a multi-channel pipette. The plate was sealed and briefly mixed and incubated at room temperature for 6 minutes. After incubation, 100 μL of 7.5% Na_2CO_3 was added to each well using a multichannel pipette.

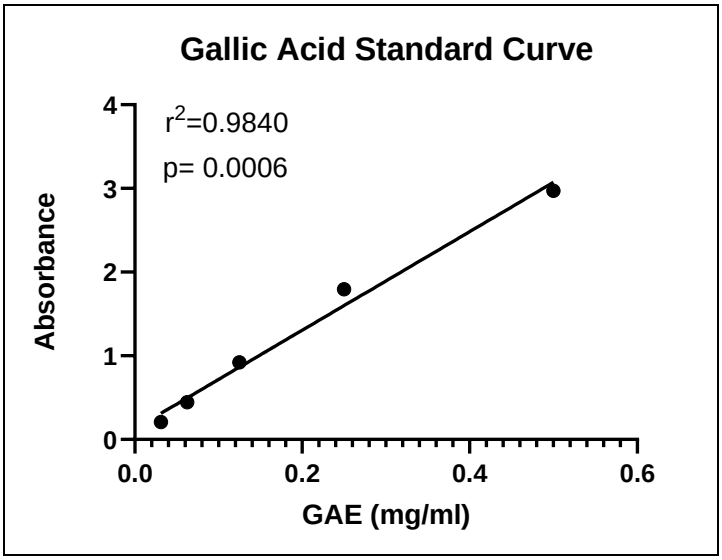

Figure 4-2: Gallic Acid Standard Curve
Absorbance was measured at at λ= 765 nm and is plotted on the Y axis, and gallic acid concentration (mg/mL) is plotted on the X axis. The goodness of fit was measured at r^2=0.9840.

The plate was covered and incubated in the dark for 90 minutes, briefly mixed then absorbance was measured at λ= 765 nm using a fluorescence microplate reader (Tecan, Spark® Multimode Microplate Reader). Phenolic content was determined using a gallic acid standard curve (0.03125 mg/ml, 0.0625 mg/ml, 0.125 mg/ml, 0.25 mg/ml, 0.5 mg/ml) (**Figure 4-4**) and expressed as μg gallic acid equivalents (GAE) per gram dry weight (DW).

4.1.8 Statistical analysis

Data were analyzed by unpaired two-tailed student's t-test when comparing 2 groups, and One-way ANOVA followed by Tukey's Post-hoc test when comparing three or more groups. Data with p-value < 0.05 was considered significant.

4.2 Results and Discussion

4.2.1 Starch Content

Total starch was measured using Megazyme Total Starch Assay kit and is presented in g/100g dry weight basis (**Figure 4-3**). The starch content of the kidney bean powders was ~40g/100g and there were no significant differences between the WK and DK bean powders ($p>0.05$). Total starch content was measured in the bean powders to confirm the macronutrient composition measured by Maxxam Analytics in Chapter 4.1.3, since total dietary carbohydrates were calculated by difference, and not measured directly. These results are in agreement with the calculated available carbohydrate content of the bean powders (Total carbohydrate – total dietary fibre) obtained from results provided by Maxxam Analytics (**Table 4-2**). The starch content in

the kidney beans used in this study are also representative of the total starch found in beans as previously demonstrated[23,184].

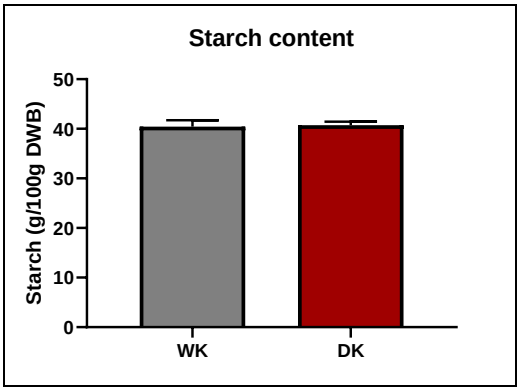

Figure 4-3: Starch content in white kidney (WK) and dark red kidney (DK) bean powder.
Starch content was measured in WK and DK bean powders, using Megazyme Total Starch Assay kit. Data was analyzed using a two-tailed unpaired student's t-test and is presented as g starch/100g DW ± SEM (n=3 per group).

4.2.2 Total Phenolic Content

Total phenolic content (TPC) was measured in both bean powders (WK & DK) and in all experimental diets (BD, WKB, DKB, HF, WKH, DKH) using the Folin-Ciocalteu colorimetric assay, and is presented in gallic acid equivalents (mg GAE/g DW). The TPC of the WK bean powder was 1.24 mg GAE/g DW, while that of the DK powder was 2.02 mg GAE/g DW, with DK powder having significantly more free phenolic acid content than WK ($p<0.05$) (**Figure 4-4A**). This confirms that the DK beans used in this study contained significantly more polyphenolic compounds than the WK beans, with the DK bean powder having twice as much TPC compared to WK bean powder.

In the experimental diets, both control diets without supplemented bean powders, BD and HF, had similar phenolic content (~0.25 mg GAE/g diet) ($p>0.05$), which was most probably attributed to background assay absorbance from the added vitamins, antioxidants, and free sugar in the rodent diets [247,248]. Within the low-fat diets (BD, WKB, DKB), DKB had the highest phenolic content, followed by WKB and BD, and all diets were significantly different from each

other ($p<0.05$). Within the high-fat diets (HF, WKH, DKH), DKH had the highest TPC, followed by WKH and HF, and all diets were significantly different from each other ($p<0.05$). There were significant differences in TPC between the low, WKB and DKB, and high-fat, WKH and DKH, bean diets, with the high-fat bean diets being significantly lower than the low-fat bean diets ($p<0.05$) (**Figure 4-4B**). While the supplementation level of kidney bean powder in both low-fat

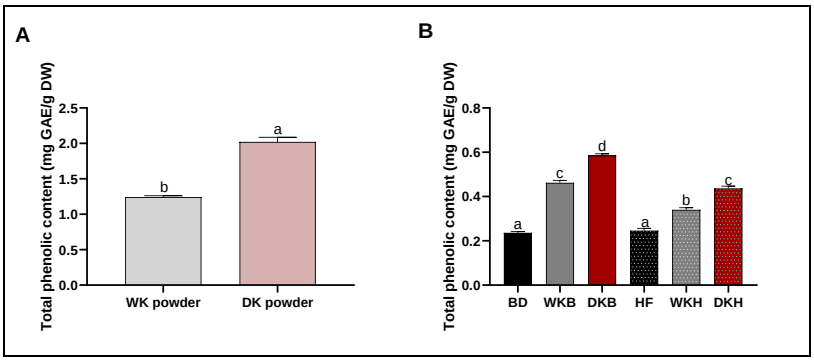

Figure 4-4: Total phenolic content in kidney bean powder and experimental diets.
A: Total phenolic content in white and dark red kidney bean powder. **B:** Total phenolic content in BD, WKB, DKB, HF, WKH, DKH diets. Bean powder phenolic content was analyzed by two-tailed unpaired student's t-test, and phenolic content of experimental diets was analyzed by one-way ANOVA followed by Tukey's Post-hoc test and is presented as GAE (mg/g DWB) ± SEM (n=3 per group). Data not sharing a lowercase letter are significantly different ($p<0.05$). BD=Basal Diet, WKB=White kidney bean supplemented basal diet, DKB= Dark red kidney supplemented basal diet, HF=High-fat basal diet, WKH= white kidney bean supplemented high-fat diet, DKH= Dark red kidney bean supplemented high-fat diet.

and high-fat diets was the same (15g bean powder/100g diet, 15%), the high-fat bean diets (WKH and DKH) had significantly lower TPC, this was likely due to differences in phenolic extraction efficiencies between the low and high-fat diets. The high-fat diets were more difficult to blend into a fine particle size as a result of the high-fat content causing particles to clump together, thus, the extraction efficiency may have been compromised from the high-fat diets compared to the low-fat diets, resulting in higher TPC in low fat bean diets vs high fat bean diets[249]. Another explanation for the differences in phenolic content between the low-fat and high-fat bean diets are that the bioavailability of phenolic compounds depends on the food matrix, and certain components such as lipids, proteins and carbohydrates can bind to

polyphenols and interfere with the availability of free phenolic acids[212,250]. The low and high-fat diets had different levels of lipids, proteins and carbohydrates (**Table 4.2**), therefore it could be expected that these differences resulted in different absorbances. Furthermore, sugars and vitamins can also interfere with the TPC assay, since the high-fat diets contained more vitamin mix it is plausible that this caused interference with the absorbance of free phenolic acids leading to reduced TPC in the high-fat bean diets compared to the low-fat bean diets[247].

Chapter 5: The effects of cooked kidney bean supplementation into a low-fat diet on mouse intestinal and metabolic health

5.1 Introduction

Most Canadians primary source of protein comes from animal sources[9,10], but it has been recently recommended by Canada's New Food Guide to increase protein consumption from plant-based sources. Kidney beans are high in protein[208], as well as non-digestible carbohydrates[23], which are known to increase the abundance of beneficial bacteria in the intestinal tract[25,26]. Dietary fibres may also improve aspects of metabolic health that are impaired in metabolic illness such as obesity, cardiovascular disease and type 2 diabetes[27–32]. Furthermore, beans contain polyphenolic compounds which are known to modulate intestinal[33–35] and metabolic health[36], with darker coloured bean varieties containing more polyphenolic compounds[24].

Studies have shown that common bean consumption has been associated with changes in fecal microbiota composition and activity, leading to increases in SCFAs[170,183]. SCFAs are utilized by the colonic epithelium as energy substrates and are involved in the growth of colonocytes, facilitating ion transport, and enhancing epithelial barrier integrity by enhancing the expression and organization of TJ proteins and mucin genes which promote mucosal barrier integrity[83,89,90,96–101,251]. SCFAs have also been demonstrated to reduce colonic inflammation by increasing IL-10 production, while also decreasing production of pro-inflammatory cytokines IL-6 and IL-12[110–114]. SCFAs can also have metabolic actions as they can reach peripheral tissues and are involved in functions such as lipid metabolism and fatty acid synthesis, blood pressure

regulation, gluconeogenesis, and can affect the production of gut hormones such as GLP-1 and PYY [115–124].

Polyphenolic profiles also vary between bean varieties, with darker coloured beans containing anthocyanins[241]. Previous studies on beans with different seed coat colours have shown that darker coloured beans may provide additional intestinal and metabolic health effects due to the higher presence of polyphenolic compounds[170,183,184,188]. Furthermore, polyphenolic compounds may interact with starch thereby increasing the gelatinization and decreasing the digestion of starch in the upper intestines and increasing their presence in the colon where they can be fermented into SCFAs[212].

Therefore, the objectives of this study were to determine if *i)* chronic white or dark red kidney bean consumption affects intestinal and metabolic health in healthy mice consuming a diet representative of a healthy balanced diet, and *ii)* if intestinal and metabolic health outcomes were differently affected by kidney bean seed coat colour.

5.2 Methods

5.2.1 Animals

5-week old male C57Bl/6 mice were purchased from Charles River, (Kingston, USA), and were acclimatized for 1 week with free access to water and BD. Mice were individually caged under controlled light cycles (12 light cycle/12 dark cycle), temperature (23°C), and humidity (~40%). Mice had free access to food and water and were kept on cob bedding. All experimental conditions were reviewed and approved by the uOttawa Animal Care Committee (ACC) (Animal Use Protocol: HS-2857).

5.2.2 Experimental Procedures

Thirty-six 6-week old mice were assigned to the three experimental diet groups (BD, WKB, DKB) (n=12 mice/group) such that there were no differences in mean body weight (BW) between groups. BW was measured twice weekly using a digital scale, diet intake (DI) was measured twice weekly using the following equation: DI=diet in (g) – diet out (g)/2 days and presented as daily DI (g/mouse/day), and energy intake (EI, kcal/mouse/day) was measured by multiplying DI (g/mouse/day) by the energy density of the diets (3.7 kcal/g).

Whole-body composition (lean and fat mass) was measured at weeks 2 and 6 using nuclear magnetic resonance technology (EchoMRI). Mice were weighed, then placed in a restraining tube which was inserted into the EchoMRI. Data is expressed as lean and fat mass (g) and as % BW.

At weeks 3 and 7, mice were fasted for 6 hours by removing food from the cages at 8:00 am (at 10 min intervals to account for time required for fasting blood collection). Fasted blood was collected (~50ul) from snipped tail tips at 2:00 pm using capillary Microvette® CB300 tubes

(Sarstedt, Cat. # 16.440.100). Fasted blood glucose was measured with a Contour® Next EZ blood Glucose Meter (Bayer, model 7268) and is presented in mmol/L. Fasted blood was allowed to clot at room temperature for 30 minutes, then incubated on ice for 15 minutes, followed by centrifugation at 10,000 g for 30 minutes to obtain fasted serum. Fasted serum was collected by pipetting the supernatant and was stored at -20°C for 1 week, then at -80°C until further analysis.

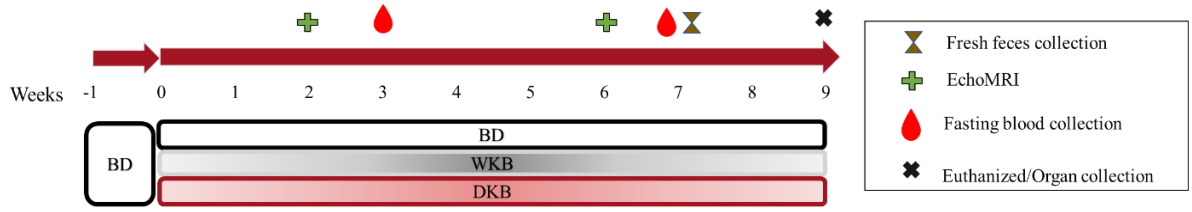

Figure 5-1: Experimental Design
Mice were acclimatized on BD for 1 week, after being assigned to either BD, WKB, DKB groups, and fed their diets for 9 weeks. Body composition was assessed at weeks 2 and 7 by EchoMRI. Fasted blood was taken at weeks 3 and 7. Fresh feces were collected at week 7, and tissues were collected upon study termination at week 9.

5.2.3 BMI and Organ Weights

Upon study termination, final BW (g) was measured and body length (cm) was recorded by taking the lengths from the tip of the nose to the base of the tail with the mouse flat on its stomach. BMI (g/cm^2) was calculated using the equation[252]: BMI= Body Weight (g) / Body Length $(cm)^2$. After excision, the cecum and cecum content, colon, liver, perinephric adipose tissue (PAT), epididymal adipose tissue (EAT), and brown adipose tissue (BAT) were weighed and expressed in milligrams. Colon length was also measured by ruler and expressed in cm.

5.2.4 Histology

Upon termination, 1cm sections of proximal colon were placed in histology cassettes and immediately fixed in 10% buffered formalin phosphate (Fisher chemical, Cat. # SF100-4) for 24 hours and then switched to 70% ethanol and stored at 4°C before being paraffin-embedded and

sectioning by the University of Ottawa Histology Core (Roger-Guidon Hall, Ottawa). Proximal colon cross sections (4 μm) were stained with hematoxylin and eosin stain (H&E) to asses colon crypt length using a Zeiss microscope equipped with Axiocam 506 mono. Crypt length was determined by taking the mean length (μm) of 15 crypts/mouse, starting from the basolateral base of the crypt towards the luminal end of the crypt, using ImageJ software (**Figure 5-2**). Proximal colon cross sections stained with alcian blue/nuclear fast red were assessed for mucus content by measuring the intensity of the stained mucus using imageJ software (**Figure 5-3**). Briefly, a line was drawn around the perimeter of the intestinal epithelium, cropping out the luminal content, muscle layer, and submucosa to determine crypt area (**Figure 5-3B)**. Red, blue, and green colour channels were split, and the blue was selected for analysis, which represents the total crypt area staining positive for alcian blue (**Figure 5-3C**). Alcian blue stained crypt area density was divided by total crypt area to determine mucus content and is presented as mucus content/μm^2.

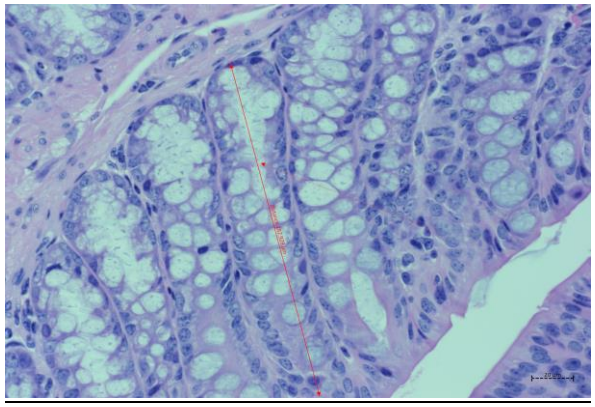

Figure 5-2: Proximal colon cross-section stained with H&E.
Representative image of a cross-section from the proximal colon epithelium stained with H&E. Crypt lenght was determined by measuring the average length (from the base of the cypt to the lumen = red line) of 15 crypts/mouse using image J software; 40x magnification.

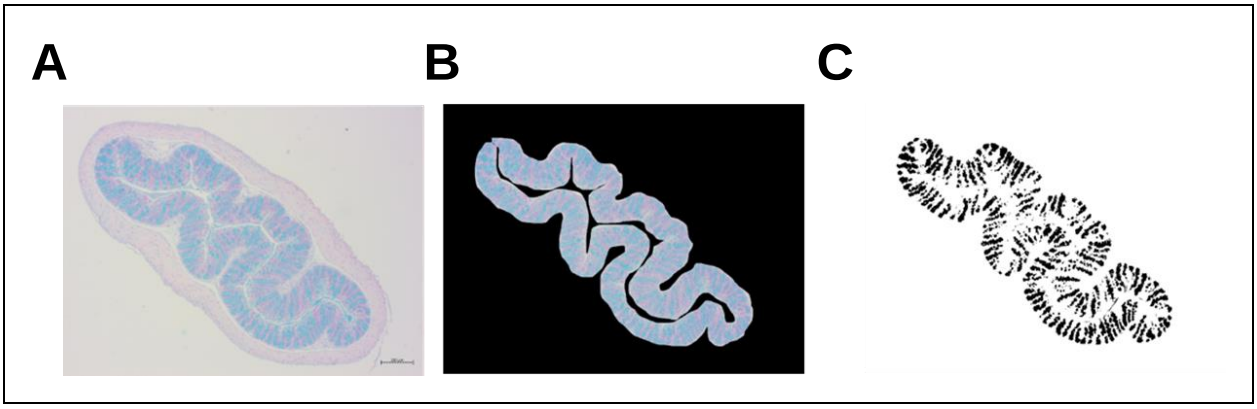

Figure 5-3: Representative images illustrating the method used to assess epithelium mucus content in proximal colon cross-sections.
A: Photo of a proximal colon cross-section stained with Alcian-Blue/Nuclear Fast Red highlighting the mucus content in turquoise color. **B:** Photo demonstrating the selection of the epithelium with the rest of image is removed in order to measure epithelium area by image J. **C:** Using imageJ software, the red, blue, green colour channels were split, and blue channel (showing staining positive for mucus) was selected and the staining intensity quantified; 10x magnification.

5.2.5 Microbial Community Structure

5.2.5.1 Fecal DNA Extraction

Freshly expelled fecal pellets were collected from each mouse at week 7 and were stored in 2 mL polypropylene cryogenic vials (Corning Inc., Cat. #430659) at -80°C. Fecal DNA was extracted using QIAamp® Fast DNA Stool Mini Kit (Qiagen, Cat. #51604, Germany) following manufacturers recommendations. Briefly, ~50 mg of fecal samples were placed in 2 mL tough tubes with caps (VWR®, Cat. # 10158-556) containing 5 ceramic beads (VWR®, Cat # 10158-554). 1 mL of InhibitEX® buffer was added to the tough tubes, and caps were shut tightly before tubes were placed in Bead Mill 24 (Fisherbrand™, model 15340163). Samples were homogenized at 4000x g for 2.5 minutes, then the supernatant was transferred to microcentrifuge tubes and incubated in a water bath at 95°C for 6 minutes. The samples were vortexed for 15 seconds, and centrifuged for 1 minute at 21,694x g. The supernatant (600 µL) was transferred to

2 ml flat top microcentrifuge G-tubes® (Bio Plas Inc., Cat. # 4050) containing 25 µL Proteinase K, then vortexed briefly. Buffer AL (600 µL) was added to G-tubes® and tubes were vortexed for 15 seconds, then submerged in 70°C water bath for 10 minutes. Next, 550 µL of 100% EtOH was added to the G tubes, and vortexed for 30 seconds and 600 µL of lysate was loaded with a pipette onto QIAamp® Mini spin columns without touching the column membrane. Columns were centrifuged for 1 minute at 21,694x g and the filtrate was discarded. This was repeated until all lysate was passed through column. Next, 500 µL Buffer AW1 was added to column, and centrifuged for 1 minute at 21,694x g, and filtrate was discarded. Buffer AW2 (500 µL) was added to column, and centrifuged for 3 minutes at 21,694x g, and filtrate was discarded. Columns were placed in new collection tubes and centrifuged for 3 minutes at 21,694x g. Columns were transferred to labelled 1.7 mL Safe Seal microcentrifuge tube (Cat #11700, Sorenson Bioscience Inc.) and 30 µL 10mM TRIS-Cl (pH 8.5) was added to the spin column and incubated at room temperature for 1 minutes before being centrifuged for 1 minute at 21,694x g to elute DNA. DNA concentration was measured by NanoDrop™ One (Thermo Scientific™, model ND-ONE-W).

5.2.5.2 16S Library Preparation

The 16S ribosomal RNA (16S) V3-V4 regions were amplified in the fecal DNA samples previously extracted (described in 5.1.5.1) following Illumina guidelines[253]. Briefly, 12.5 ng of microbial fecal DNA was mixed with 5 µL of Amplicon PCR Forward Primer (1 µM) and 5 µL Reverse Primer (1 µM) (**Table 5-1**), and 12.5 µL 2x KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Cat. # KK2601) in a 96-well 0.2 ml PCR plate (Bio-Rad, Cat. # MSP-9601). The plate was sealed with Microseal B (Bio-Rad, Cat #MSB-1001) and placed in thermal cycler with the following conditions: 95°C for 3 minutes, followed by 25 cycles of 95°C for 30 seconds,

55°C for 30 seconds, and 72°C for 30 seconds, then finalized with 5 minutes at 72°C. PCR product was then verified on a 1.5% agarose gel (described in 5.2.7.3).

Table 5-1: 16S amplicon primer sequence

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
16S amplicon	TCGTCGGCAGCGTCAGATGTGTATAAGA GACAGCCTACGGGNGGCWGCAG	GTCTCGTGGGCTCGGAGATGTGTATAAG AGACAGGACTACHVGGGTATCTAATCC

The 16S V3-V4 amplicon was purified by adding 20 µL of AMPure XP beads (Beckman Coulter Genomics, Cat # A63881) to each well, and mixed by pipetting. The plate was incubated at room temperature for 5 minutes, then the plate was placed on magnetic stand (Life Technologies, Cat. # AM10027) for 2 minutes, until supernatant was clear. Supernatant was removed with a pipette and discarded. The beads were then washed by adding 200 µL of 80% EtOH to each well and incubated on the magnetic stand for 30 seconds. The supernatant was removed, discarded, and the wash was repeated. The beads were then allowed to air dry for 10 minutes, then 52.5 µL of 10 mM Tris (pH 8.5) was added to each well. The beads were resuspended by mixing the well contents by pipette. The plate was then incubated at room temperature for 2 minutes, then placed on magnetic stand for 2 minutes, until supernatant was clear.

Dual indices and Illumina sequencing adapters were attached to PCR product by adding 5 µL of the supernatant to a new 96 well PCR plate, 5 µL of Nextera XT Index Primer 1 (FC-131-1001, N7XX) and 5 µL of Nextera XT Index Primer 2 (S5XX) was added to each well, followed by 25 µL of 2x KAPA Hifi HotStart ReadyMix and 10 µL of PCR grade water. The contents of each well were mixed by pipetting and the plate was sealed. The plate was then centrifuged at 1,000 g for 1 minute at 20°C, then placed in a thermal cycler with the following conditions: 95°C

for 3 minutes, followed by 8 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds, followed by a final 5 minutes at 72°C. The PCR product was purified using AMPure XP beads and 2 EtOH washes as described above. The beads were air dried for 10 minutes, the plate was then removed for the magnetic stand and 27.5 µL of 10 mM Tris (pH 8.5) was added to each well. The contents were mixed by pipetting, then incubated at room temperature for 2 minutes. The plate was placed on the magnetic stand for 2 minutes, until supernatant cleared.

Next, 25 µL of supernatant was transferred to new PCR plate, and DNA was quantified using Qubit using methods described by the manufacturer (Life Technologies™, MAN0002325|MP32850 Revision: A.0). Briefly, 198 µL Qubit solution and 2 µL DNA from the PCR plate were mixed in 500ul Qubit tubes, then read via Qubit. The final amplicon library was diluted to 4 nM in 10 mM Tris (pH 8.5) and 5 µL of diluted DNA from each well was pooled in a microcentrifuge tube and mixed by pipetting. DNA was denatured by adding 5 µL of freshly prepared 0.2 N NaOH to the microcentrifuge tube, vortexed briefly, then centrifuged at 280 g for 1 minute at 20°C. The denatured DNA library was the incubated at room temperature for 5 minutes. Pre-chilled Illumina Hybridization buffer (HT1) (990 µL) of was added to the tube, to form a 20 nM denatured DNA library in 1 mM NaOH. The denatured DNA was then diluted to 4 nM by adding 120 µL of 20 nM denatured DNA and 480 µL of pre-chilled HT1 to a new microcentrifuge tube and was mixed by inverting the tube several times then vortexed briefly. This was kept on ice until further use.

A PhiX library was diluted to 4 nM by adding 2 µL of 10 nM PhiX (Illumina, Cat. # FC-110-3001) and 3 µL of 10 nM Tris (pH 8.5). Then 5 µL of 4 nM PhiX solution was added to new centrifuge tube with 5 µL of 0.2 NaOH and vortexed briefly, to form 2 nM PhiX library solution. This was incubated at room temperature for 5 minutes, to denature the PhiX library. Next, 990

μL of pre-chilled HT1 was added to the denatured PhiX library and vortexed briefly to form 20 nM PhiX denatured library. The PhiX library was then diluted by adding 90 μL of PhiX and 510 μL of pre-chilled HT1 to form a 15% PhiX control solution. 30 μL of PhiX control solution was added to 570 μL of the previously denatured and diluted amplicon library, then incubated on a 96°C heat block for 2 minutes. The tube was inverted several times to mix, then placed in ice-water bath for 5 minutes. The samples were then loaded onto the Miseq reagent kit V3 600 cycle cartridge (Illumina®, Cat. # MS-102-3003) and placed in the Illumina Miseq (Illumina®, Miseq) for sequencing.

5.2.5.3 Miseq Reporter Metagenomics

The subsequent FASTQ files comprising 300-basepair dual-index paired-end reads were demultiplexed and analyzed with QIIME version 1.9.1 [254,255]. The reads were aligned by overlapping sequences with FASTQ-join. Sequences with a quality score less than 3 were trimmed, and sequences that were <75% of their original length or containing 1 or more ambiguous bases were removed as described[182], and remaining reads were demultiplexed. Representative sequences were then aligned using PyNAST algorithm against GreenGenes core set[256]. The remaining reads were clustered at 97% sequence similarity, and operational taxonomic units (OTUs) were clustered using Usearch version 11[257]. Phylogenetic relationships were determined using FastTree[258]. α-diversity metrics were calculated at a sequence read depth of 20,000 sequences per sample, and β-diversity distance metrics were based on unweighted UniFrac distance matrices[259], which were used for principle coordinate analysis (PCoA) as previously described [182]. Differences between groups in α-diversity metrics were analyzed using one-way ANOVA followed by Tukey's post-hoc test, while β-diversity distance metrics were analyzed by PERMANOVA in Qiime (Version 1.9.1). Taxa relative abundance at the phylum,

genus, and species level were analyzed by Kruskal-Wallis test followed by Dunn's multiple comparison test, and p-values were adjusted by Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli; $p<0.05$; False Discovery Rate 0.05; n=9 per group.

5.2.6 Microbial Activity

Fecal (collected at week 7) and cecal (collected at week 9) short chain fatty acid (SCFA) concentrations (acetic acid, butyric acid, propionic acid, and valeric acid) and branched chain fatty acids (BCFAs) (isobutyric and isovaleric acid), were measured by gas chromatography (GC) [182]. Cecal and fecal samples were weighed (approximately 50 mg), and freeze-dried for 24 hours (Labconco, Freezone 12 bulk tray dryer), then subsequently weighed to obtain dry weight and moisture content (Moisture content= (Wet weight – Dry weight)/ Wet weight *100). Fecal samples were then incubated for 2 hours in MilliQ water to form a solution containing 10% feces (20% for cecal samples) by dry weight. The samples were vortexed for 10 seconds every 30 minutes during incubation to form a homogenous solution. After incubation, the samples were centrifuged at 14,000 g for 30 minutes at 4°C to form a pellet. The supernatant was collected, and placed in new microcentrifuge tubes, and vortexed again at 14,000 g for 30 minutes at 4°C, and pH was measured (Thermo Scientific™ Orion Star™ A111 pH Benchtop Meter, Thermo Scientific ROSS MICRO PH ELECTRODE, Canada). Samples were spiked with 10 µL internal standard comprised of 5.5mmol/L 2-ethylbutyric acid (Sigma-Aldrich, Cat. #109959-100ML) in 100% formic acid, and pH was measured. Samples (pH 3.5) were centrifuged at 14,000 g for 30 minutes at 4°C and supernatant was transferred into GC tubes containing glass insets (Mandel, Cat. #SZ-220-91521-03). 1 µL supernatant was injected into the GC (Nexis GC-2030), equipped with a 0.25µm column (Restek, Cat. # RSK-10226), column flow rate was set at 1.5 mL/minute,

at a 20:1 split ratio. Helium was used as the carrier gas. Initial oven temperature was 100°C which was increased to 200°C at a rate of 10°C /minute; injector temperature was set at 200°C and detector temperature was set at 300°C. Samples were ran in triplicate, and run time was 20 minutes per sample, and SCFA concentrations were determined based retention time peaks compared with those from Volatile Free Fatty Acid Mix (SUPELCO, Sigma, #46975-U), which was serially diluted in distilled H_20 to form a standard curve to make the following concentrations: 0.1 mM, 0.25 mM, 0.5 mM, 1 mM, 5 mM, and 10 mM. Fecal and cecal SCFA and BCFA concentrations are presented as μmol/g of dry fecal or cecal weight.

5.2.7 Proximal colon mRNA gene expression

5.2.7.1 RNA Extraction

Proximal colon RNA was extracted using RNA/Protein Purification Plus Kit (Norgen, Cat. 48200). Tissues pieces (~ 30mg) were placed in 2 mL tough tubes with caps (VWR®, Cat. # 10158-556) containing 5 ceramic beads (VWR®, Cat # 10158-554). SK buffer (300 μL) containing β-mercaptoethanol (0.01% v/v) was added to samples and then placed in a Bead Mill 24 (Fisherbrand™, model 15340163) set at 2500 RPM for 25 seconds. Supernatants were collected and placed in RNAse free microcentrifuge tubes and centrifuged at 2000 RPM for 2 minutes. Genomic DNA was removed by placing the supernatant in Genomic DNA Removal Columns and centrifuged at 8000 RPM for 2 minutes. 100% EtOH was added to the flow-through and RNA was isolated by transferring flow-through into RNA/Protein Purification Column tubes and centrifuged at 6000 RPM for 1 minute. The bound RNA was washed from impurities using Wash solution A, which was added to the column and centrifuged at 6000 RPM for 1 minute, this was repeated three times. Purified RNA was eluted using 42 μL RNAse free

H_20 (Invitrogen™, Cat # 10977-015). RNA quality was assessed with 2 µL of each sample on NanoDrop™ One (Thermo Scientific™, model ND-ONE-W).

5.2.7.2 cDNA Preparation

Total RNA (1 µg) was transcribed to complimentary DNA (cDNA) using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Cat # 4368814). Briefly 10X RT Buffer, 25X dNTP Mix (100 mM), 10X RT Random Primer, Multiscribe™ Reverse Transcriptase, and Nuclease-free H_20 were mixed and added to 0.2 mL PCR tubes (Eppendorf™, Cat # 951010006). RNA (1 µg) was added to PCR tubes, and cDNA was synthesized in a T100™ Thermal Cycler (Bio-Rad, model 1861096) with the following conditions: 10 minutes at 25°C, 120 minutes at 37°C, and 5 minutes at 85°C. Samples were stored at -20°C until further use.

5.2.7.3 Primer Validation

Reference gene primers (RPLP0 and EEF2) were validated to ensure they are stable under this specific experimental treatment (kidney bean consumption)[260], all other primers have been previously validated for use in mouse colon tissue, but their specificity was confirmed by running a melt curve following the thermal gradient[184]. A pooled representative sample

Table 5-2: Primer Sequences

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
RPLP0	ACTGGTCTAGGACCCGAGAAG	TCCCACCTTGTCTCCAGTCT
EEF2	TGTCAGTCATCGCCCATGTG	CATCCTTGCGAGTGTCAGTGA
GPR 41	GTGACCATGGGGACAAGCTTC	CCCTGGCTGTAGGTTGCATT
GPR 43	GGCTTCTACAGCAGCATCTA	AAGCACACCAGGAAATTAAG
GPR 109a	ATGGCGAGGCATATCTGTGTAGCA	TCCTGCCTGAGCAGAACAAGATGA
Occludin	GTCCGTGAGGCCTTTTGA	GGTGCATAATGATTGGGTTTG
ZO-1	ACTCCCACTTCCCCAAAAAC	CCACAGCTGAAGGACTCACA
TNFα	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC
IL-6	AACGATGATGCACTTGCAGA	GAGCATTGGAAATTGGGGTA
IL-10	GGTTGCCAAGCCTTATCGGA	ACCTGCTCCACTGCCTTGCT
IL-1β	AGTTGACGGACCCCAAAAG	AGCTGGATGCTCTCATCAGG
TLR2	GGGGCTTCACTTCTCTGCTT	AGCATCCTCTGAGATTTGACG
TLR4	AGAAAATGCCAGGATGATGC	CTGATCCATGCATTGGTAGGT

(n=3/dietary group) of cDNA was diluted 1:10 in RNAse free H_20, and tested by quantitative real-time Polymerase chain reaction (qPCR) using a master mix consisting of PowerUp™ SYBR™ Green (Applied Biosystems, Cat. # A25742) as the detection reporter dye and gene specific primers (IDT™) (**Table 5-2**). Samples were ran in duplicate, and reaction volume was 10 µL, consisting of 4 µL pooled cDNA sample, and 6 µL master mix. The following cycle conditions were carried out on CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, model C1000 Touch™): 2 minutes at 50°C, 2 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C, thermal gradient (63°C in row A to 51°C in row H) for 30 seconds, followed by 1 minute at 72°C. The thermal gradient during the annealing stage of the PCR cycle was to determine the optimal primer annealing temperature, which was the temperature with the lowest quantitative threshold (C_q). A melt curve was then run from 65°C to 95°C in 0.5°C increments for 5 seconds, and primer specificity was confirmed by the presence of melt peaks at a single temperature (**Figure 5-4**).

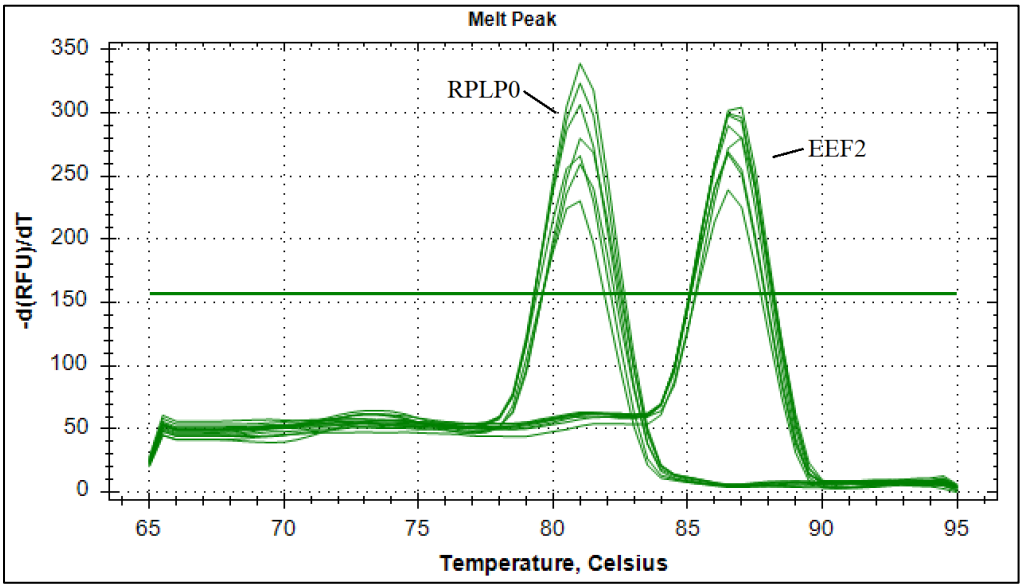

Figure 5-4: Melt Curve for RPLP0 and EEF2.
A melt curve was run on the pooled cDNA for primers RPLP0 and EEF2 (other primers not shown) to determine primer specificity. Melt curve was run from 65°C to 95°C in 0.5°C increments for 5 seconds. The melt peak was at 81°C for RPLP0 and at 87°C for EEF2.

To validate the specificity of the primers, a single PCR product was visualized following separation on an agarose gel. In short, 0.75 g agarose (Bio Basic, Cat. #D0012) was added to a conical flask with 50 mL of 1X TAE buffer (Bio Basic, Cat. #A0023). The solution was stirred then microwaved for 2 minutes with occasional stirring until all agarose was dissolved and the gel solution was clear. The gel solution was cooled for 5 minutes before the addition of 5 µL Ethidium Bromide (Bio Basic, Cat. #D0197). The gel solution was poured in a loading dock containing a comb and allowed to set for 50 minutes. The comb was removed, and the gel was covered entirely with 1X TAE buffer. A DNA (100bp-3000bp) ladder (Bio Basic, Cat. #GM347) (3 µL) was added to the first well, and 1.5 µL loading buffer (Cat#K945-5mL) was mixed with 5 µL PCR product which was added to respective wells. The gel ran for 50 minutes at 100 V, before being viewed using (Bio-Rad®, VersaDoc™ Imaging System, model #4000 MP) (**Figure 5-5**)

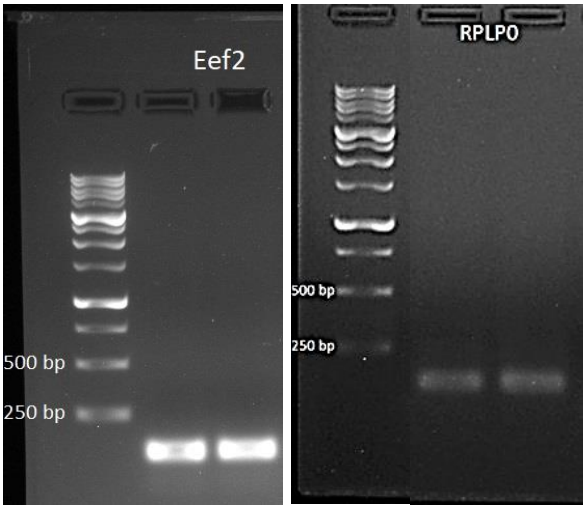

Figure 5-5: Gel Electrophoresis of PCR product of pooled cDNA with primers EEF2 and RPLP0. The presence of one PCR product of the correct size was determined by gel electrophoresis for primers EEF2 (left) and RPLP0 (right).

To determine amplification/reaction efficiency, an 8-point standard curve was produced by diluting pooled cDNA in 1:2 series starting from undiluted cDNA (**Figure 5-6**). qPCR was

carried out using CFX96 Touch™ (BIO-RAD, model#CFX96™ Real-Time System), using SYBR Green, 10mM Primers, and RNAse free H_20 as the master mix, of which 6 µL was plated with 4 µL previously diluted (1:2 series) standard curve sample cDNA. Reaction efficiency (E) was calculated by: $E = -1+10^{(-1/slope)}$. cDNA samples for analysis were diluted in RNAse free H_20 to the midpoint of the standard curve (1:10).

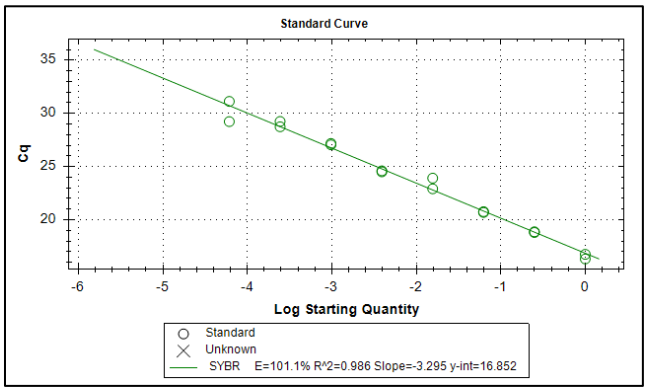

Figure 5-6: RPLP0 standard curve validation
An 8-point standard curve in 1:2 series for primer RPLP0. Reaction efficiency is calculated by: $E = -1+10^{(-1/slope)}$. Reaction efficiency was determined to be 101.1%.

5.2.7.4 Proximal Colon Gene Expression Analysis

qPCR was carried out using CFX96 Touch™ (BIO-RAD, model#CFX96™ Real-Time System), using SYBR Green, 10mM Primers, and RNAse free H_20 as the master mix, of which 6 µL was plated with 4 µL previously diluted (1:10) sample cDNA. Data were analyzed using CFX Maestro Software, and relative gene expression was calculated using the $\Delta\Delta C_q$ method, expression levels of each gene were normalized to that of the reference genes (Eef2 & RPLP0). Data is expressed as normalized expression relative to zero.

5.2.8 Serum Biomarkers

Fasted serum collected at week 7 was thawed on ice for 15 minutes. Serum biomarkers (ghrelin, GIP, GLP-1, glucagon, insulin, leptin, PAI-1, resistin) were analyzed using Bio-Plex

Pro™ Diabetes Panel 8-Plex (Bio-Rad, Cat. #171-F7001M, USA) following manufacturers recommendations. Briefly, lyophilised standards were reconstituted in 500 µL of Standard Diluent, vortexed and incubated on ice for 30 minutes and 128 µL of reconstituted standards were diluted in 72 µL standard diluent, vortexed, then serially diluted (1:4) in standard diluent to form an 8-point standard curve. Serum samples were diluted fourfold in sample diluent and equilibrated to room temperature before use. Coupled beads were vortexed for 30 seconds at medium speed, diluted to 1X in assay buffer and vortexed again. 50 µL diluted coupled beads were added to each well using a multi-channel pipette. The plate was washed twice using the following wash procedure: 100 µL of wash buffer is added to each well with a multichannel pipette, the plate is held on a magnetic stand and buffer discarded, then plate is removed from magnetic stand. Previously diluted standards, samples and blanks (50 µL) were added to the appropriate wells, the plate was sealed, covered in foil, and incubated on orbital shaker at 850 rpm for 1 hour. 10 minutes before use, detection antibodies were prepared by adding 150 µL of 20X detection antibody stock to 2850 µL of detection antibody diluent, then vortexed. After incubation, the plate seal was removed, and the plate was washed three times with 100 µL wash buffer following the wash procedure. Diluted detection antibodies were vortexed briefly, then 25 µL was added to each well using a multi-channel pipette. The plate was sealed, covered in aluminum foil, and incubated at room temperature on an orbital shaker at 850 rpm for 30 minutes. 10 minutes before use, the streptavidin-PE (SA-PE) solution was prepared by adding 60 µL of 100X SA-PE to 5490 µL assay buffer, then vortexed. After incubation, the plate seal was removed, and the plate was washed three times with 100 µL wash buffer following the wash procedure. Diluted SA-PE was vortexed briefly, then 50 µL 1X SA-PE was added to each well using a multichannel pipette. The plate was sealed, covered in aluminum foil, and incubated at

room temperature on an orbital shaker at 850 rpm for 10 minutes. After incubation, the plate seal was removed, and the plate was washed three times with 100 µL wash buffer following the wash procedure. The beads were then resuspended in 125 µL of assay buffer. The plate was sealed and placed on orbital shaker at 850 rpm for 30 seconds to mix the suspension. The plate was placed in Magpix reader (Bio-Plex® MAGPIX™ Multiplex Reader), and data was acquired with Bio-plex manager software (Software #171015001) and is presented as concentration (pg/mL). Serum adiponectin was measured using Bio-Plex Pro™ Diabetes Adiponectin Assay (Bio-Rad™, Cat. #171-F7002M) which follows the above protocol with the following modifications; serum samples are diluted 1:1600 in serum-based diluent.

Homeostatic Model Assessment of Insulin Resistance (HOMA-IR), a measure of β-cell function and insulin sensitivity[261], was calculated using the formula[262]: (glucose (mmol/L)/ insulin (mU/L) x 22.5) using the fasting blood glucose and fasting insulin measurements.

5.2.9 Statistical Analysis

Data were analyzed using GraphPad Prism (version 8), using two-way repeated measures ANOVA for data recorded over time and for single time-point measurements, one-way ANOVA followed by Tukey's post-hoc test was utilized. Data with p-value < 0.05 was considered significant. Mice of C57Bl/6 genetic background are known to contain over responders to diet-induced obesity, (e.g. becoming obese on low-fat diets). Therefore, outliers were detected using the final body weight data with a box/whisker plot, with the upper 10% as a cut off (obesity prone on low-fat diet). Once outliers (n=3) were detected they were removed from all data analysis.

5.3 Results:

5.3.1 Mice body weight (BW), diet intake (DI), energy intake (EI)

BW was measured twice-weekly over the 9-week feeding trial and there were no significant differences between BD, WKD, and DKD groups throughout the study ($p>0.05$) (**Figure 5-7A**). Consequently, there were also no differences in final BW between dietary groups ($p>0.05$) (**Figure 5-7B**). DI and EI were measured twice weekly and there were no significant differences between any groups at any time ($p>0.05$) (**Figure 5-7C & D**).

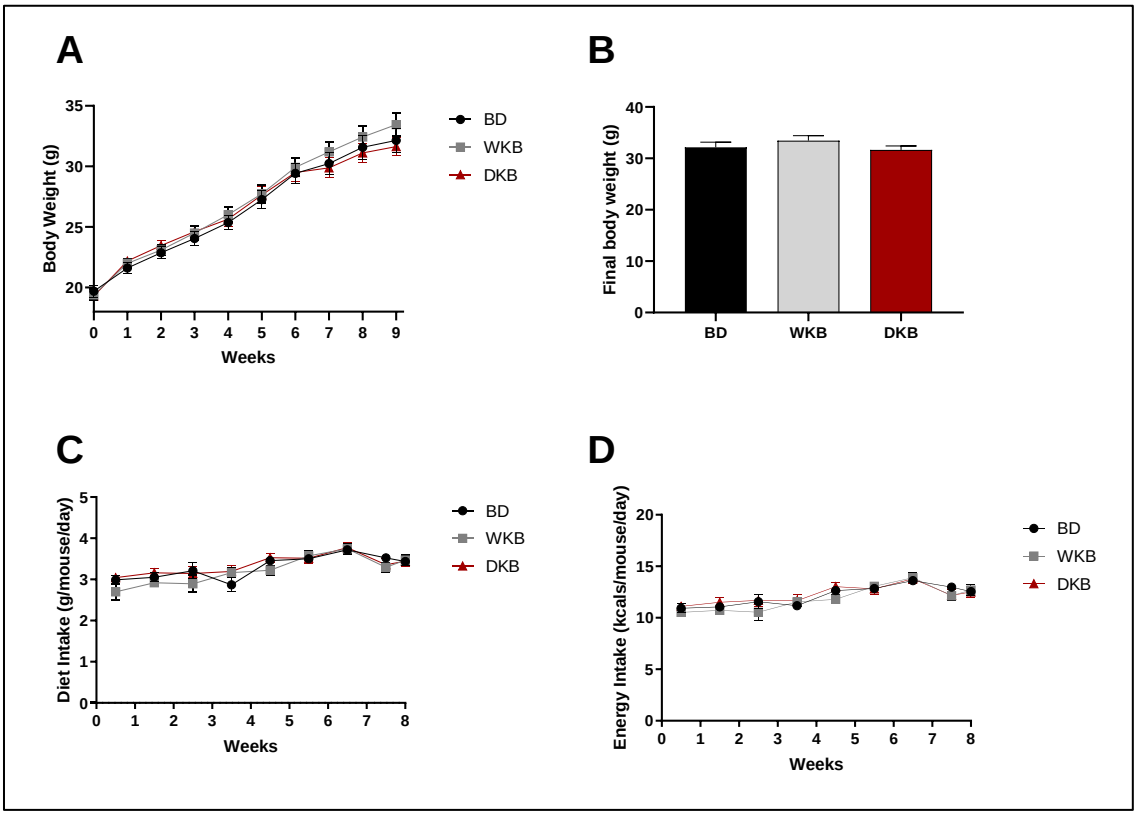

Figure 5-7: The effects of bean supplementation into a BD on mouse body weight (DW) and diet (DI) and energy intake (EI)
A: BW (g) over time (weeks) in BD, WKD, and DKD fed mice. **B**: Final BW (g). **C:** DI (g/day/group) over time (weeks). **D:** Energy intake (kcal/day/group) over time (weeks). DI, EI, and BW were analyzed by mixed-effects two-way ANOVA followed by Tukey post hoc test (n=11/group). Final BW was analyzed by one-way ANOVA followed by Tukey post hoc test (n=11/group). Data points or bars not sharing a lower-case letter are significantly different ($p<0.05$). BD=Basal Diet, WKB=White kidney bean supplemented basal diet, DKB= Dark red kidney supplemented basal diet.

5.3.2 BMI and body composition

BMI (g/cm^2) was calculated using the formula[252]: BMI= Body Weight (g) / Body Length (cm)^2. There were no significant differences in BMI between the dietary groups ($p>0.05$) (**Figure 5-8**), supporting the final body weight data (**Figure 5-7B**).

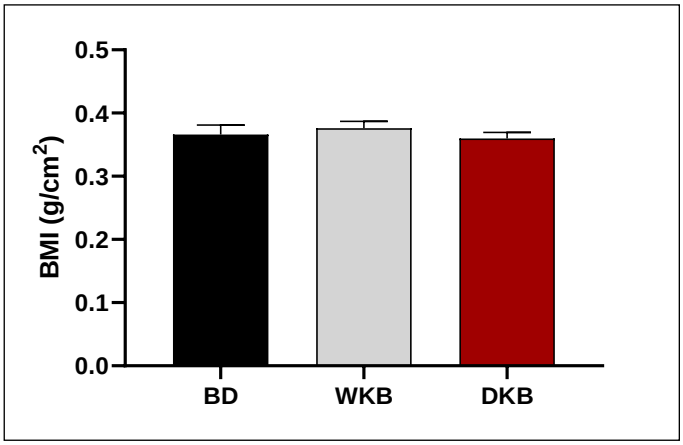

Figure 5-8: BMI in BD, WKB, and DKB fed mice.
BMI (g/cm^2) calculated using the formula: BMI= Body Weight (g) / Body Length (cm)^2 at week 9. Data was analyzed by one-way ANOVA followed by Tukey post hoc test (n=11/group). Bars not sharing a lower-case letter are significantly different ($p<0.05$). BD=Basal Diet, WKB=White kidney bean supplemented basal diet, DKB= Dark red kidney supplemented basal diet.

Body composition was assessed by EchoMRI at weeks 2 and 6, and lean mass and fat mass are expressed in grams and as a percentage of body weight (% BW). As shown in **Figure 5-9A-D**, there were no significant differences between groups in lean mass or fat mass at weeks 2 and 6 ($p>0.05$). Lean mass (g) and fat mass (g) were significantly increased over time in all dietary groups ($p<0.05$), however when measured as a %BW, lean mass significantly decreased over time ($p<0.05$), while fat mass significantly increased over time in all dietary groups ($p<0.05$) (**Figure 5-9C&D**).

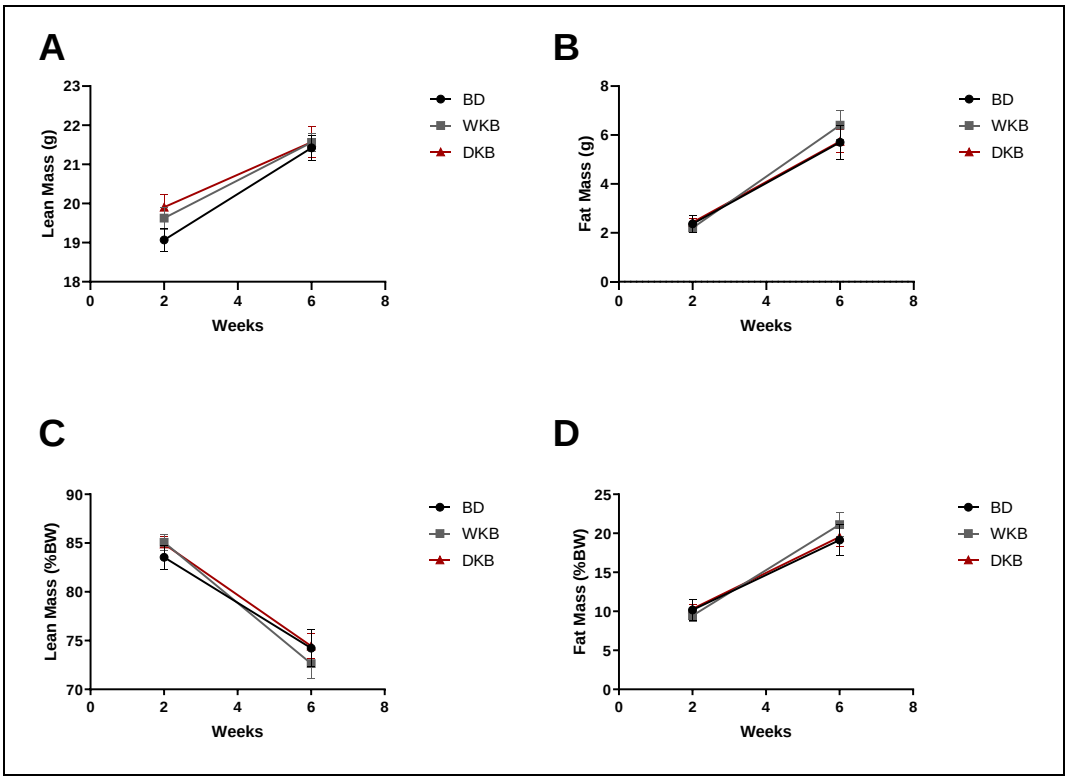

Figure 5-9: Body Composition assessed by EchoMRI in BD, WKB, and DKB fed mice.
A: Lean mass (g) in mice at weeks 2 and 6. **B:** Fat mass (g) in mice at weeks 2 and 6. **C:** Lean mass (% BW) in mice at weeks 2 and 6. **D:** Fat mass (% BW) in mice at weeks 2 and 6. Data was analyzed by mixed-effect two-way ANOVA followed by Tukey's post-hoc test, (n=11/group). Data points not sharing a lower-case letter are significantly different ($p<0.05$). BD=Basal Diet, WKB=White kidney bean supplemented basal diet, DKB= Dark red kidney supplemented basal diet.

5.3.3 Organ and fat pad weights

After the 9-week feeding trial, mice were euthanized, and organs (cecum, colon, and liver) and fat pads (epididymal (EAT), perinephric (PAT), and brown adipose tissue (BAT)) were excised, weighed and are presented in g and % BW ± SEM. There were no significant differences in fat pad weights (g or % BW) between groups, with BD, WKB, and DKB having similar weights for EAT, PAT, and BAT ($p>0.05$) (**Table 5-3**).

Table 5-3. Mean fat pad weight in BD, WKB, and DKB fed mice.

	BD	WKB	DKB
Epididymal (g ± SEM)	1.112 ± 0.109	1.372 ± 0.109	1.173 ± 0.092
Epididymal (% BW ± SEM)	3.395 ± 0.244	4.045 ± 0.226	3.675 ± 0.221
Perinephric (g ± SEM)	0.6248 ± 0.062	0.7258 ± 0.068	0.5926 ± 0.047

Perinephric (% BW ± SEM)	1.906 ± 0.140	2.135 ± 0.153	1.859 ± 0.113
Brown (g ± SEM)	0.2006 ± 0.017	0.2129 ± 0.013	0.1972 ± 0.011
Brown (% BW ± SEM)	0.6156 ± 0.037	0.6331 ± 0.025	0.6207 ± 0.025

Data was analyzed by one-way ANOVA followed by Tukey's post-hoc test (n=10-11/group). Data not sharing a lower-case letter are significantly different ($p<0.05$). BD=Basal Diet, WKB=White kidney bean supplemented basal diet, DKB= Dark red kidney supplemented basal diet.

Cecum, cecum content, colon and liver were weighed and are presented in g. Cecum content was significantly higher in WKB group, compared to both BD and DKB groups ($p<0.05$), and there were no significant differences between BD and DKB ($p>0.05$) (**Figure 5-10A**). Cecum weight showed similar results, with BD and DKB groups being significantly lower than WKB ($p<0.05$), while BD and DKD were of similar weight ($p>0.05$) (**Figure 5-10B**). Colon weight demonstrated a similar pattern to cecum weight, however there were no significant differences between BD, WKB, and DKB ($p>0.05$) (**Figure 5-10C**). Liver weights were similar in all mice irrespective of diet ($p>0.05$) (**Figure 5-10D**).

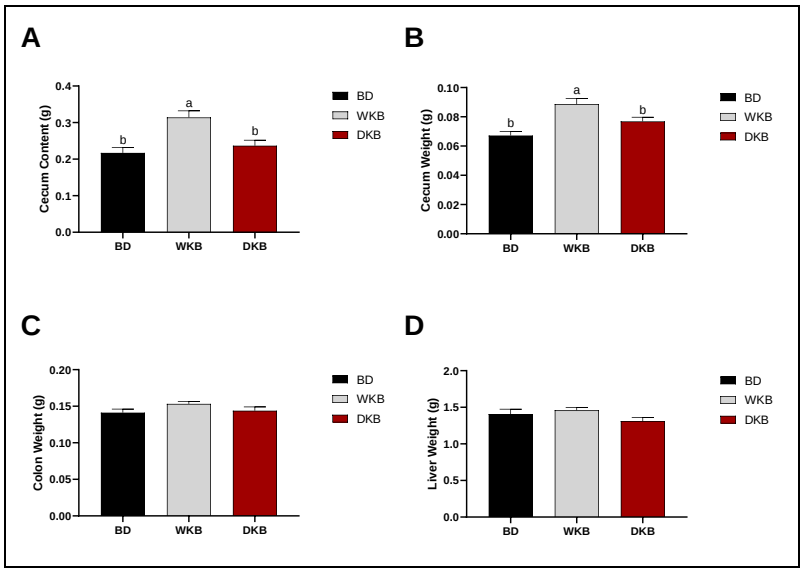

Figure 5-10: Cecum content, cecum, colon, and liver weights in BD, WKB, and DKB fed mice.
A: Cecum content (g) in BD, WKB, and DKB. **B:** Cecum weight (g). **C:** Colon weight (g). **D:** Liver weight (g). Data was analyzed by one-way ANOVA, followed by Tukey's post-hoc test (n=11 per group). Bars not sharing a lower-case letter are significantly different ($p<0.05$). BD=Basal Diet, WKB=White kidney bean supplemented basal diet, DKB= Dark red kidney supplemented basal diet.

5.3.4 Proximal Colon Histomorphometry

Crypt length was assessed in proximal colon cross sections stained with H&E and is presented in μm. There were no significant differences in crypt length between dietary groups ($p>0.05$) (**Table 5-2**). Mucus content score was measured in proximal colon cross sections stained with AB/NFR and is presented as mucus/μm^2. There were no significant differences in mucus content between dietary groups ($p>0.05$) (**Table 5-4**).

Table 5-4: Proximal Colon Crypt Length and Mucus Content

	BD	WKB	DKB
Crypt Length (μm)	162.57 ± 7.036	172.00 ± 4.636	162.76 ± 6.082
Mucus Content (mucus/ μm^2)	0.2937 ± 0.0076	0.2905 ± 0.0101	0.3012 ± 0.0091

Crypt length was measured using ImageJ software in proximal colon cross sections stained with H&E, and mucus content score in proximal colon cross sections stained with Nuclear Fast Red/Alcian Blue. Data was analyzed by one-way ANOVA followed by Tukey's post-hoc test (n=10-11/group). BD=Basal Diet, WKB=White kidney bean supplemented basal diet, DKB= Dark red kidney supplemented basal diet.

5.3.5 Microbial Community Structure

Microbial community α-diversity was assessed using the Choa-1 index, a measure of species richness at a read depth of 20,000 basepairs. Choa-1 index was similar across all dietary group ($p>0.05$) (**Figure 5-11A**). As an additional measure of α-diversity, the Observed species index was analyzed, a measure of the amount of unique species observed in a sample, however there were no differences between dietary groups ($p>0.05$) (**Figure 5-11B**). A third α-diversity metric was assessed, PD whole tree method, a measure of phylogenetic diversity which was not significantly different between groups ($p>0.05$) (**Figure 5-11C**), therefore all diets had similar α-diversity (Chao-1 index, observed species, and PD whole tree). Principle Coordinate Analysis (PCoA), a β-diversity visualization of the microbiota community composition, exhibited clustering based on diet, with both bean diets, WKB and DKB, being significantly different than BD group ($p<0.05$). WKB and DKB groups clustered together and showed no significant differences from one another ($p>0.05$) (**Figure 5-11D**).

The most abundant phyla in fecal samples from all dietary groups were *Firmicutes* and *Bacteroidetes*, making up approximately 90% of the observed phyla (**Table 5-5**). The phylum *Proteobacteria* was significantly increased in both WKB and DKB groups when compared to BD group ($p<0.05$). All other phyla were not significantly different between dietary groups ($p>0.05$) (**Figure 5-12A**) (**Table 5-5**). At the genus level, both WKB and DKB groups had a significant reduction in *Bacteroides* when compared to BD group ($p<0.05$), and *Bacteroides* in DKB group was significantly reduced compared to WKB group ($p<0.05$). Both WKB and DKB groups had significant increases in *Prevotella* and *Sutterella* compared to BD group ($p<0.05$). In DKB group, the family *S24-7* was significantly increased compared to both BD and WKB groups ($p<0.05$), and *Parabacteroides* was also significantly increased in the WKB and DKB group compared to BD, with abundance in the WKB group being significantly higher than the DKB group ($p<0.05$) (**Figure 5-12B**) (**Table 5-5**).

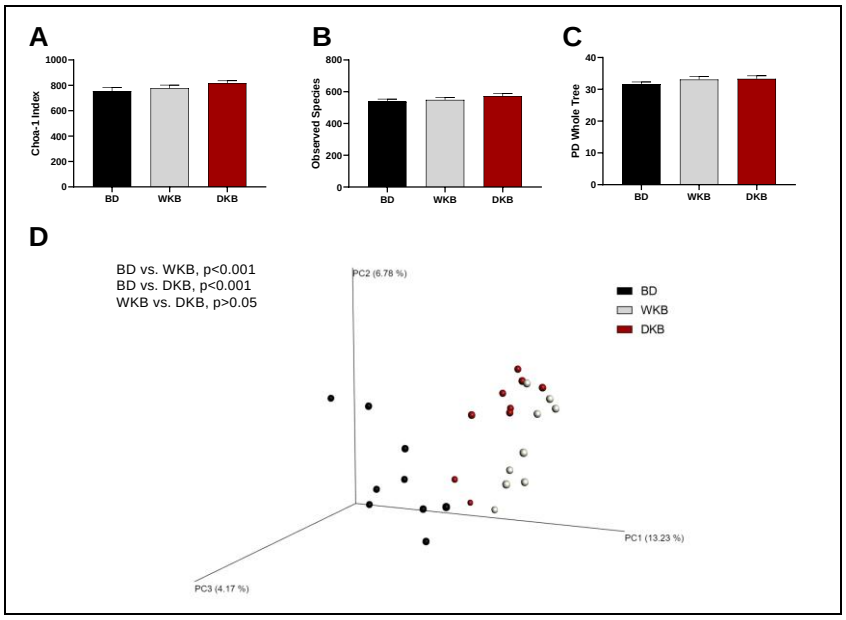

Figure 5-11: Week 7 Fecal Microbiota in BD, WKB, and DKB fed mice

Fecal microbiota community α-diversity (**A**-**C**), as measured by Choa-1 index (**A**), Observed species index (B), and PD-Whole tree method (**C**). **D**) β-diversity as visualized by principal component analysis (PCoA) of unweighted UniFrac distance matrices showing bacterial communities cluster within dietary groups and the percent of dataset variability explained by each principal component is shown in brackets in the axis titles. Each dot represents one mouse and each dietary group is denoted by a different coloured symbol (BD: black circles; WKB: white circles; DKB: red circles). Differences between groups were measured by PERMANOVA and P-values are shown. BD, basal diet; WKB, white kidney bean diet; DKB, dark kidney bean diet.

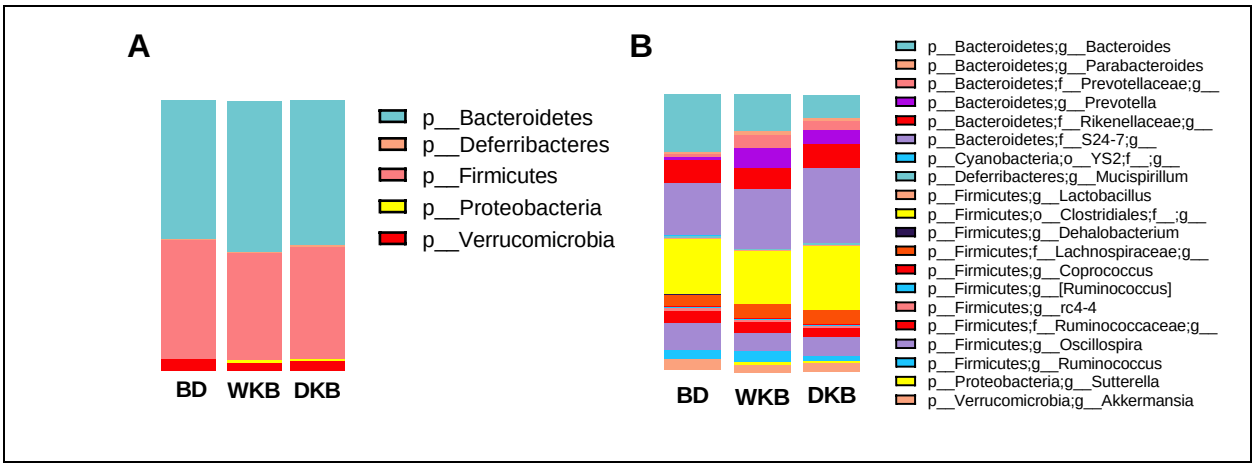

Figure 5-12: Taxonomic structure of fecal microbiota at phylum level (A) and genus (B) level.
Microbiota taxa composition at the phylum (A) and genus (B) including taxa representing >0.5% total composition in at least one sample. BD, basal diet; WKB, white kidney bean diet; DKB, dark kidney bean diet.

Table 5-5: Relative abundance of fecal microbiota in BD, WKB, and DKB fed mice

Taxonomy	% Relative Abundance		
	BD	WKB	DKB
Bacteroidetes	50.92 ± 2.761	55.84 ± 1.694	53.51 ± 2.629
g__Bacteroides	20.69 ± 2.364	13.31 ± 1.217*	9.01 ± 0.583*§
g__Parabacteroides	0.91 ± 0.199	1.54 ± 0.130*	1.03 ± 0.133§
f__Prevotellaceae;g__	0.90 ± 0.213	4.55 ± 0.506*	3.32 ± 0.583*
g__Prevotella	1.09 ± 0.191	7.27 ± 0.765*	4.73 ± 0.732*
f__Rikenellaceae;g__	8.26 ± 1.106	7.17 ± 0.855	8.55 ± 1.014
f__S24-7;g__	19.06 ± 1.835	22.00 ± 1.401	26.88 ± 2.216*
Deferribacteres	0.64 ± 0.126	0.30 ± 0.073	0.49 ± 0.106
g__Mucispirillum	0.64 ± 0.126	0.30 ± 0.073	0.49 ± 0.106
Firmicutes	43.49 ± 2.177	39.48 ± 1.774	41.52 ± 2.134
g__Lactobacillus	0.62 ± 0.339	0.29 ± 0.066	0.43 ± 0.166
o__Clostridiales;f__;g__	19.55 ± 2.173	18.70 ± 1.150	21.75 ± 2.137
g__Dehalobacterium	0.45 ± 0.067	0.40 ± 0.056	0.38 ± 0.049
f__Lachnospiraceae;g__	3.83 ± 0.727	4.18 ± 0.938	5.02 ± 0.731
g__Coprococcus	0.36 ± 0.120	0.58 ± 0.135	0.60 ± 0.144
g__[Ruminococcus]	0.55 ± 0.128	0.31 ± 0.050	0.32 ± 0.049
g__rc4-4	0.85 ± 0.170	0.67 ± 0.094	0.64 ± 0.120
f__Ruminococcaceae;g__	4.36 ± 0.661	3.96 ± 0.360	3.54 ± 0.410
g__Oscillospira	9.20 ± 0.986	5.98 ± 0.493*	6.59 ± 0.531
g__Ruminococcus	3.31 ± 0.964	3.95 ± 0.614	1.86 ± 0.345
Proteobacteria	0.30 ± 0.084	1.14 ± 0.128*	0.87 ± 0.161*
g__Sutterella	0.20 ± 0.074	1.05 ± 0.127*	0.76 ± 0.173*
Verrucomicrobia	4.10 ± 1.185	2.88 ± 0.851	3.34 ± 0.940
g__Akkermansia	4.10 ± 1.185	2.88 ± 0.851	3.34 ± 0.940

Relative abundance (mean ± SEM) of fecal bacterial taxa (n=11/group) at the phylum level (highlighted in grey rows), and corresponding order (o); family (f), and/or genus (g) with abundance great than 0.5% in at least one group. Values marked with an asterisk (*) differ from BD and values marked with a (§) symbol denotes differences between WKB and DKB assessed by the Kruskal-Wallis test with p-values adjusted for FDR<0.05 using the Benjamini-Hochberg method.

5.3.6 Microbial Activity

SCFAs (acetic, butyric, propionic, and valeric acid), a product of carbohydrate fermentation, and BCFAs (iso-butyric and iso-valeric acid), a product of protein fermentation, were measured in week 7 fecal samples and in cecum content collected at the study end-point (week 9) and are presented in μmol/g DW. Cecum acetic acid was significantly increased in DKB compared to BD ($p<0.05$), however concentrations did not differ between WKB and BD or DKB ($p>0.05$) (**Figure 5-13A**). Cecum propionic and valeric acid were similar across all groups ($p>0.05$) (**Figure 5-13B & E**), whereas both bean diets, WKB and DKB, significantly increased cecum butyric acid ($p<0.05$) (**Figure 5-13D**). Total SCFAs were measured by taking the sum of acetic, butyric, propionic, and valeric acid, which resulted in DKB having the highest levels of total cecum SCFAs, being significantly higher than the BD ($p<0.05$). WKB total cecal SCFAs was also increased however it was not significantly different than the BD or DKB ($p>0.05$) (**Figure 5-13F**). Cecum total BCFAs, iso-butyric, and iso-valeric acid, were comparable across all dietary groups ($p>0.05$) (**Figure 5-13G-I**). Cecum content pH was measured and was not significantly different between groups ($p>0.05$) (**Figure 5-13C**).

Week 7 fecal acetic acid was significantly increased in DKB compared to BD ($p<0.05$) but remained similar to WKB ($p>0.05$), and WKB was not significantly different than BD ($p>0.05$) (**Figure 5-14A**). Fecal propionic acid was significantly increased in DKB when compared to the WKB ($p<0.05$), but there were no differences between DKB and BD or between WKB and the BD ($p>0.05$) (**Figure 5-14B**). Fecal butyric acid was comparable between the BD and WKB and between WKB and DKB ($p>0.05$), but DKB was significantly increased compared to the BD ($p>0.05$) (**Figure 5-14D**). There were no significant differences in fecal valeric acid between groups ($p>0.05$) (**Figure 5-14E**). Total fecal SCFAs were significantly

increased in DKB compared to only the BD ($p<0.05$), with WKB being similar to DKB and BD ($p>0.05$) (**Figure 5-14F**). Fecal pH was significantly reduced in both bean diets compared to BD ($p<0,05$), and there were no significant differences in fecal pH between the bean diets ($p>0.05$) (**Figure 5-14C**). Fecal BCFAs, iso-butyric and iso-valeric acid, as well as total fecal BCFAs were similar across all groups ($p>0.05$) (**Figure 5-14G-I**).

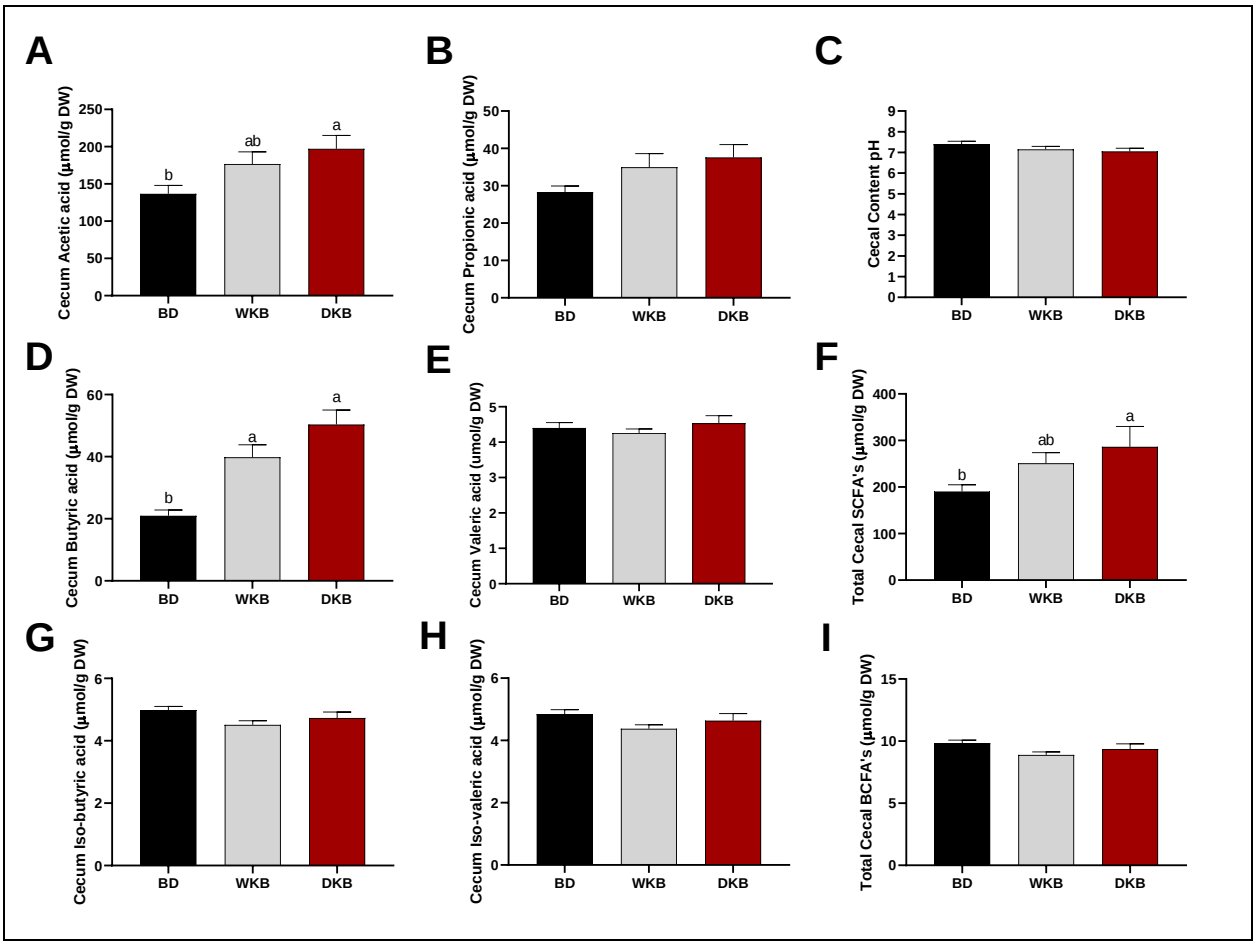

Figure 5-13: Week 7 Cecal pH, SCFAs, and BCFAs in BD, WKB, and DKB fed mice.
A: Cecum acetic acid (µmol/g DW). **B:** Cecum propionic acid (µmol/g DW). **C:** Cecal content pH. **D:** Cecum butyric acid (µmol/g DW). **E:** Cecum valeric acid (µmol/g DW). **F:** Total cecal SCFAs (µmol/g DW). **G:** Cecum iso-butyric acid (µmol/g DW). **H:** Cecum iso-valeric acid (µmol/g DW). **I:** Total cecal BCFAs **(**µmol/g DW). Data was analyzed by one-way ANOVA followed by Tukey's post-hoc test (n=10-11/group). Bars not sharing a lower-case letter are significantly different ($p<0.05$). BD=Basal Diet, WKB=White kidney bean supplemented basal diet, DKB= Dark red kidney supplemented basal diet.

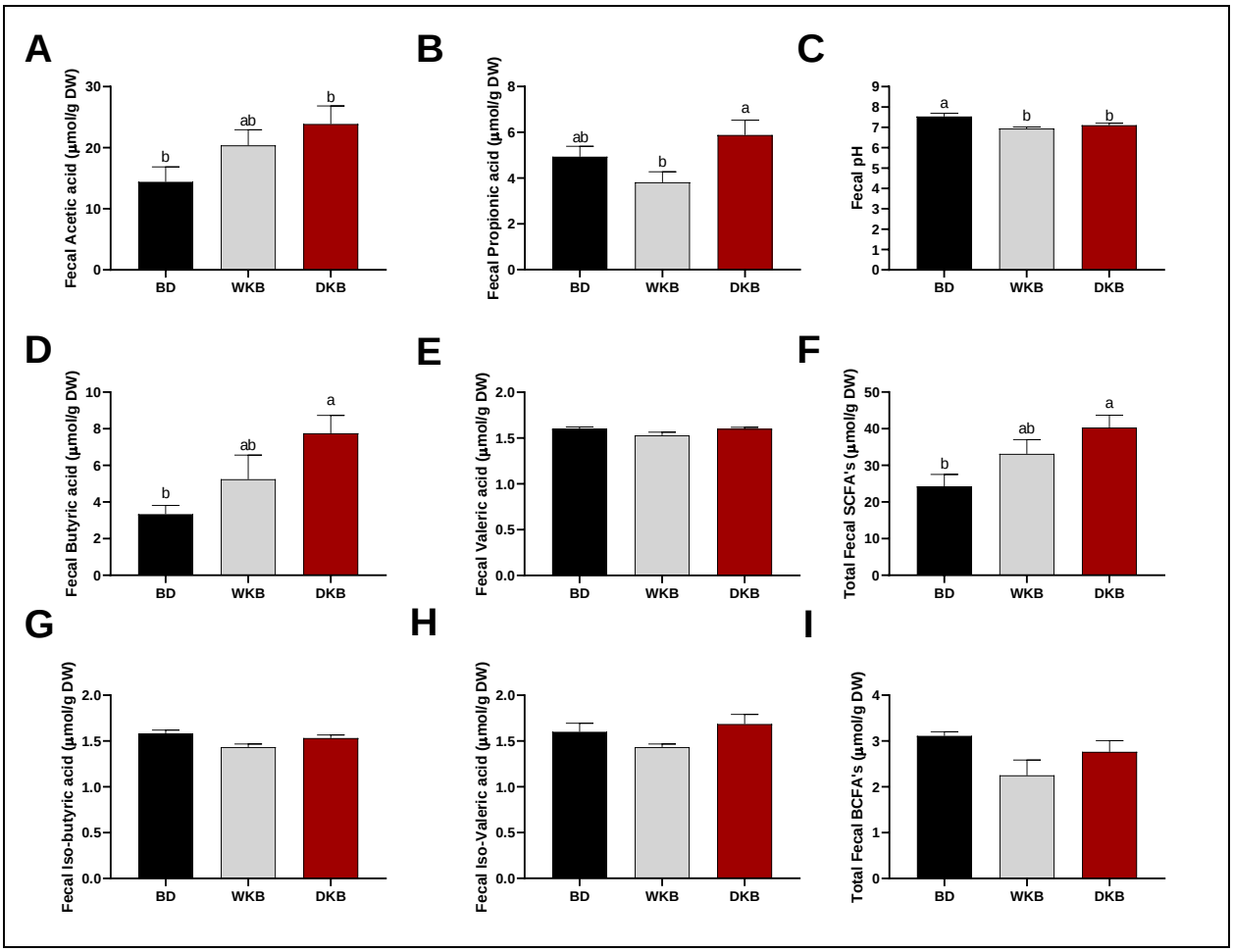

Figure 5-14: Week 7 Fecal pH, SCFAs, and BCFAs in BD, WKB, and DKB fed mice.
A: Fecal acetic acid (µmol/g DW). **B:** Fecal propionic acid (µmol/g DW). **C:** Fecal content pH. **D:** Fecal butyric acid (µmol/g DW). **E:** Fecal valeric acid (µmol/g DW). **F:** Total fecal SCFAs (µmol/g DW). **G:** Fecal iso-butyric acid (µmol/g DW). **H:** Fecal iso-valeric acid (µmol/g DW). **I:** Total fecal BCFAs **(**µmol/g DW). Data was analyzed by one-way ANOVA followed by Tukey's post-hoc test (n=10-11/group). Bars not sharing a lower-case letter are significantly different ($p<0.05$). BD=Basal Diet, WKB=White kidney bean supplemented basal diet, DKB= Dark red kidney supplemented basal diet.

5.3.7 Colon mRNA Gene Expression

Colon mRNA expression was analyzed in proximal colon tissues excised at the study end-point and is expressed as relative expression normalized to the expression of the reference genes (RPLP0 & EEF2). GPR41, GPR43, and GPR109a, are G protein-coupled receptors (GPCRs) of SCFAs in the colonic epithelium. GPR41, GPR 43, and GPR109a mRNA expression were similar across all dietary groups ($p>0.05$). Occludin and ZO-1, are TJ

transmembrane proteins and scaffolding/plaque proteins that hold colonic epithelial cells together[263]. Occludin mRNA expression was similar in diets ($p>0.05$), whereas ZO-1 was significantly increased in DKB group only, being significantly elevated compared to BD and WKB groups ($p<0.05$). TNF-α, IL-6, and IL-1β are pro-inflammatory cytokines capable of inducing immune system activation[264–266], were not significantly different between dietary groups ($p>0.05$). IL-10, an anti-inflammatory cytokine[267], was significantly increased in DKB compared to BD group ($p<0.05$), WKB group also showed an increase however it was not significantly different than BD or DKB groups ($p>0.05$). TLR2 and TLR4, are signaling proteins which recognize pathogen-associated molecular patterns (PAMPs) and are involved in the production of cytokines[268], remained comparable across all dietary groups ($p>0.05$) (**Figure 5-15**).

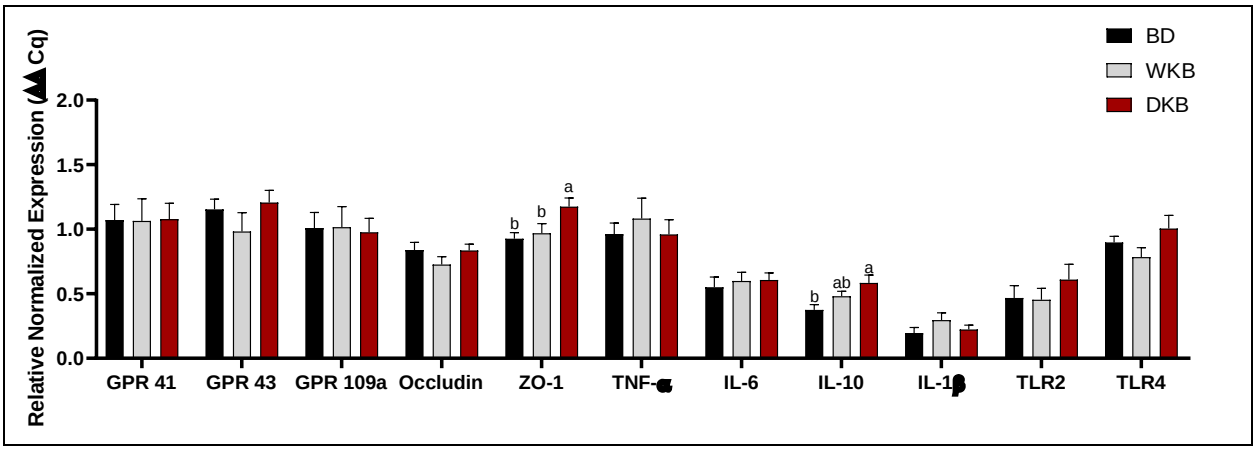

Figure 5-15: Proximal colon mRNA expression in BD, WKB, and DKB fed mice.
Relative normalized mRNA expression was calculated using the ΔΔCq method and is presented relative to zero. Data was normalized to the expression of the reference genes RPLP0 and EEF2. Data were analyzed by one-way ANOVA followed by Tukey's post-hoc test (n=9-11 per group). Bars not sharing a lower-case letter are significantly different ($p<0.05$). BD=Basal Diet, WKB=White kidney bean supplemented basal diet, DKB= Dark red kidney supplemented basal diet.

5.3.8 Serum Biomarkers of Metabolic Health

Week 7 fasting serum biomarkers were assessed via multiplex and are presented in pg/mL. GIP, an incretin[47], was significantly reduced in WKB compared to BD ($p<0.05$), and was

further reduced in the DKB compared to both BD ($p<0.05$) and WKB ($p<0.05$) (**Figure 5-16A**). GLP-1, also an incretin[49], was similar across all groups ($p>0.05$) (**Figure 5-16B**). Ghrelin, a hormone involved in appetite regulation by inducing hunger[55,56], was significantly decreased in DKB when compared to the BD ($p<0.05$), while there were no significant differences between BD and WKB ($p>0.05$) (**Figure 5-16C**). PAI-1 (plasminogen activator inhibitor), is involved in adipose tissue insulin signaling[269], was significantly decreased in DKB compared to BD ($p<0.05$) but not significantly different than WKB ($p>0.05$). WKB PAI-1 was also not significantly different than BD ($p>0.05$) (**Figure 5-16D**). Leptin is a hormone secreted by adipose tissue and is involved in appetite regulation by suppressing hunger[270], was not significantly different between any dietary group ($p>0.05$) (**Figure 5-16E**). Resistin, is produced by adipose tissue and colonic epithelial cells amongst others[271], is involved in insulin resistance[272], was significantly decreased in DKB when compared to both BD and WKB ($p<0.05$) (**Figure 5-16F**). Insulin, is involved in blood glucose down-regulation[273], was not significantly different between dietary groups ($p>0.05$) (**Figure 5-16G**). Glucagon, is produced by α-cells in the pancreas and is involved in gluconeogenesis[50], was not significantly different between dietary groups ($p>0.05$) (**Figure 5-16H**). Adiponectin, is involved anti-inflammatory pathways[274], was significantly increased in DKB compared to BD, but remained comparable to WKB ($p>0.05$). WKB adiponectin levels were not significantly different than BD ($p>0.05$) (**Figure 5-16I**).

Week 3 fasting blood glucose was elevated in WKB compared to DKB ($p<0.05$), but neither bean diet differ from BD ($p>0.05$) (**Figure 5-16J**). Week 7 fasting blood glucose displayed a similar pattern, however there were no significant differences between groups ($p>0.05$) (**Figure 5-16K**). Week 7 fasting blood glucose and week 7 fasting insulin were used to calculate HOMA-IR, which was comparable across all groups ($p>0.05$) (**Figure 5-16L**).

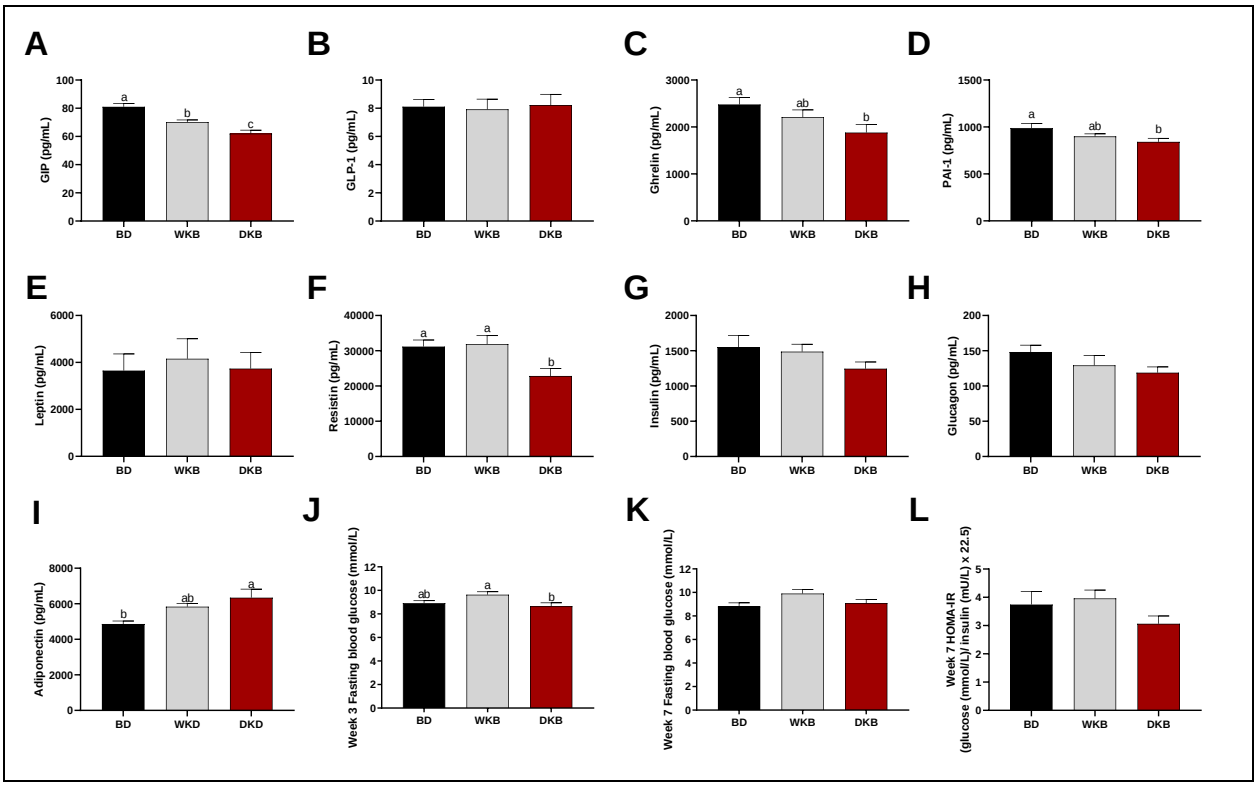

Figure 5-16: Week 7 Fasting Serum Biomarkers in BD, WKB, and DKB fed mice.
A: GIP (pg/mL). **B:** GLP-1 (pg/mL). **C:** Ghrelin (pg/mL). **D:** PAI-1 (pg/mL). **E:** Leptin (pg/mL). **F:** Resistin (pg/mL). **G:** Insulin (pg/mL). **H:** Glucagon (pg/mL). **I:** Adiponectin (pg/mL) **J:** Week 3 fasting blood glucose (mmol/L). **K:** Week 7 fasting blood glucose (mmol/L). **L:** Week 7 HOMA-IR (glucose (mmol/L)/Insulin (mU/L) x 22.5). Data were analyzed by one-way ANOVA followed by Tukey's post-hoc test (n=9-11 per group). Bars not sharing a lower-case letter are significantly different ($p<0.05$). BD=Basal Diet, WKB=White kidney bean supplemented basal diet, DKB= Dark red kidney supplemented basal diet.

5.4 Discussion

The objective of this study was to determine if long-term kidney bean supplementation at physiologically relevant doses would improve intestinal and metabolic health, and if seed coat colour would impact measured outcomes. Previous studies have demonstrated that bean supplementation can promote intestinal health in male C57Bl/6 mice by *i)* improving microbiota community structure by increasing the abundance of carbohydrate fermenters, *S24-7* and *Prevotella, ii)* increasing epithelial crypt length, mucus content and mRNA expression of mucins[182–184]. These studies utilized shorter study lengths with smaller sample sizes, therefore in this study, white and dark-red kidney beans were supplemented into a low-fat basal diet and fed to male C57Bl/6 mice for a period of 9 weeks. To assess components of intestinal health, the fecal microbiota composition and activity, as well as mucosal and epithelial barrier integrity were measured.

With regards to altering the microbiota composition through dietary means, previous studies on bean consumption have demonstrated that changes in the microbiota composition and activity can occur in as little as 3 weeks[170,183]. The changes in microbiota composition that arose from navy and black bean supplementation into a mouse model were mainly decreases in the genus *Oscillospira* and increases in the genera *Rumminococcus, Prevotella*, and *S24-7* family[170]. Similar to navy and black bean supplementation, in this current study kidney bean supplementation into a basal diet led to increased abundance of *Prevotella*, irrespective of bean seed coat colour. This genus has been demonstrated to ferment different fibres to produce high levels of SCFAs, and has been associated with improved glucose metabolism[275,276]. Interestingly, *Proteobacteria* is a phylum that has been associated with obesity and certain diseases[277], was increased by both bean diets, notably driven by increases in the genus *Sutterella*. Species of the

Sutterella genus are increased in the duodenum with decreasing abundance along the intestinal tract of humans, and have been increased in healthy controls compared to subjects with inflammatory diseases such as Multiple Sclerosis and IBD[278–281], therefore the increase in *Proteobacteria* is not expected to be associated with any negative effects in this study and may be associated with improved inflammatory tone. Also, as hypothesized there were differences in microbiota composition based on seed coat colour, as only WKB diet had significantly decreased abundance of *Oscillospira* compared to BD group. The genus *Oscillospira*, a member of the *Firmicutes* phylum, has been associated with slower transit time, harder stools, and increased colonic methane production[282], and reductions in transit time may clarify the increased cecum content weight seen in WKB. Another plausible reason for increased cecum weight, is despite having equivalent amounts of dietary fibre, the WK bean powder may have had more resistant starch reach the colon because of the increased presence of α-amylase inhibitors in white beans inhibiting starch digestion in the small intestine[283].

DKB groups also had significantly decreased abundance of the genus *Bacteroides* compared to both WKB and BD groups, which may indicate differences in lipid and protein metabolism, as *Bacteroides* are positively associated with animal protein and fat consumption[70]. Further differences between bean diets were seen as DKB significantly increased the abundance of the family *S24-7* compared to BD. The *S24-7* family was reportedly increased in diabetic mice fed a HFD supplemented with GOS compared to diabetic and non-diabetic mice fed a high-fat carbohydrate free diet[284], and has been associated with increased fermentation[187]. As expected, microbial fermentation was increased in DKB as seen by increases in cecal acetic acid, acetic acid is the SCFA produced in the largest molar quantity, leading to a significant increase in total cecal SCFAs in the DKB group compared to BD. Further increases in fecal acetic acid, butyric

acid, propionic, and total SCFAs were seen in DKB, suggesting that improvements in microbiota community structure in the DKB diet, namely rises in carbohydrate fermenters, increased microbial activity.

Moreover, proximal colon gene expression of TJ barrier protein ZO-1 was significantly upregulated in DKB, suggesting an enhancement in colonic barrier integrity. Further increases were seen in IL-10 expression only in DKB group, suggestive of improved inflammation as IL-10 is anti-inflammatory[267]. Previous studies on mice fed kidney beans prior to an inflammatory challenge found that bean supplementation led to increase colonic gene expression of IL-10, MUC1 and MUC3, suggestive of improved epithelial barrier integrity[184]. Collectively, these data validate my hypothesis that bean supplementation can modify the fecal microbiota composition and activity of healthy mice, which may impact the intestinal epithelial barrier and the development of intestinal barrier related pathologies. These data further support my hypothesis that darker coloured beans, which contain higher levels of polyphenolic compounds, would provide enhanced beneficial effects on intestinal health.

To determine if bean consumption affected metabolic health, BW, fat pad weight and body composition, and serum biomarkers of metabolic health were assessed. Previous studies have demonstrated that bean or bean extract consumption could induce weight loss and improve body composition[232,233,235], however there were no significant differences in BW, body composition, and fat pad weight between dietary groups. This suggests that 15% bean supplementation is not a sufficient dose to affect body composition measures, but it may affect other aspects of metabolic health such as fasting serum hormones and adipokines. For instance, ghrelin was significantly decreased in DKB group, which is suggestive of improved metabolic health as ghrelin is a hormone involved in hunger, glucose metabolism, food intake and satiety,

and is known to decrease after food consumption and rebounds faster after a meal in subjects with obesity[56,285]. PAI-1 was significantly decreased in DKB group demonstrating improvements in adipose tissue function as PAI-1 is a hormone involved in adipose tissue insulin signaling and produced in response to macrophages infiltrating adipose tissue; it's reduction has been associated with weight loss, and is elevated in subjects with obesity, type 2 diabetes, or metabolic syndrome[269]. Further enhanced effects were seen in DKB group, as serum GIP and resistin were significantly decreased. Reductions in resistin are associated with reductions in adipocyte insulin-stimulated glucose uptake, as resistin is produced by adipose tissue and colonic epithelial cells, is involved in insulin resistance[271,272]. Reductions in GIP are associated with reductions in obesity and insulin resistance in mice fed a HFD, as GIP is involved in fat storage and the regulation of glucose-induced insulin secretion[47,48,286]. Lastly, adiponectin is released by adipose tissue and is involved in glucose homeostasis and in anti-inflammatory pathways[274], was significantly increased in DKB. There were no significant differences in insulin, glucagon or HOMA-IR suggesting that adipose tissue function and signaling rather than β-cell function were mainly affected. As with measures of intestinal health, these results demonstrate that DK beans had additional metabolic health benefits over WK beans, as only DK bean supplementation led to improvements in adipose tissue function, signaling, and inflammatory tone.

Although the health effects demonstrated in this study cannot be attributed to a specific bioactive component of beans, the DK beans used in this study contained approximately twice the amount of free polyphenolic acids, and as hypothesized the DK beans enhanced intestinal and metabolic health over WK beans.

Chapter 6: The effects of cooked kidney bean supplementation into a high-fat diet on mouse intestinal and metabolic health

6.1 Introduction

Obesity is characterized by an increase in chronic systemic inflammation driven by dysfunctional adipose tissue, leading to unregulated circulation of pro-inflammatory mediators combined with a decrease in the production of anti-inflammatory mediators[131,132]. The intestinal microenvironment also contributes to chronic low-grade inflammation associated with obesity, in part through a dysbiotic microbial community, reduced intestinal barrier integrity, and an increase in inflammatory bacterial-derived lipopolysaccharide (LPS) crossing the intestinal barrier and entering the host (endotoxemia) [65,163]. LPS is known to initiate inflammation in adipose tissue, leading to adipose tissue dysfunction and increased production of pro-inflammatory adipokines[65,163].

Certain animal models are able to recapitulate the hallmarks of the obese phenotype by long-term feeding of HFD's. For instance, C57Bl/6 mice fed a HFD will become obese as demonstrated by increased BMI and fat pad weight, and they will also have increases in dysfunctional adipose tissue, insulin resistance and rises in colonic and systemic inflammation if persistently fed a HFD[157,163,287]. Dietary modulation of the microbial dysbiosis may play an important role in preventing/attenuating inflammation associated with obesity thereby reducing adipose tissue dysfunction and improving intestinal barrier integrity and function.

Beans contain many nutritional components that been demonstrated to improve many aspects of the obese phenotype such as non-digestible carbohydrates, including insoluble and soluble fibres, GOS and resistant starch[23]. Dietary fibres can improve intestinal health and the

gut microbiota composition by increasing the number of beneficial microbes [25,26], and are associated with many metabolic health benefits[27–32]. In addition, polyphenolic compounds have been shown to improve aspects of intestinal[33–35] and metabolic health[36]. The intestinal microbiota produces metabolites that help regulate host homeostasis, and can affect the immune and hormonal systems[38,61].

Therefore, the objectives of this study were to determine if *i)* kidney bean supplementation into a high-fat diet would attenuate diet-induced obesity and its associated intestinal and metabolic health impairments, and *ii)* intestinal and metabolic health outcomes were differently affected by kidney bean seed coat color.

6.2 Methods

6.2.1 Animals

5-week old male C57Bl/6 mice were purchased from Charles River, (Kingston, USA), and were acclimatized for 1 week with free access to water and BD. Mice were individually caged under controlled light cycles (12 light cycle/12 dark cycle), temperature (23°C), and humidity (~40%). Mice had free access to food and water and were kept on cob bedding. All experimental conditions were reviewed and approved by the uOttawa Animal Care Committee (ACC).

6.2.2 Experimental procedures

Thirty-six 6-week old mice were assigned to the three experimental diet groups (HF, WKH, DKH) (n=12 mice/group) such that there were no differences in mean BW between groups. BW was measured twice weekly using a digital scale, diet intake was measured twice weekly by using the following equation: DI=diet in (g) – diet out (g)/2 days and presented as daily DI (g/mouse/day), and energy intake (EI, kcal/mouse/day) was measured by multiplying DI (g/mouse/day) by the energy density of the diets (5.1 kcal/g).

Whole-body composition (lean and fat mass) was measured at weeks 2 and 6 using nuclear magnetic resonance technology (EchoMRI) as described above (Chapter 5.2.2).

At weeks 3 and 7, mice were fasted for 6 hours by removing food from the cages at 8:00 am (at 10 min intervals to account for time required for fasting blood collection). Fasted blood and serum were collected, and fasting blood glucose was measured as previously described (Chapter 5.2.2).

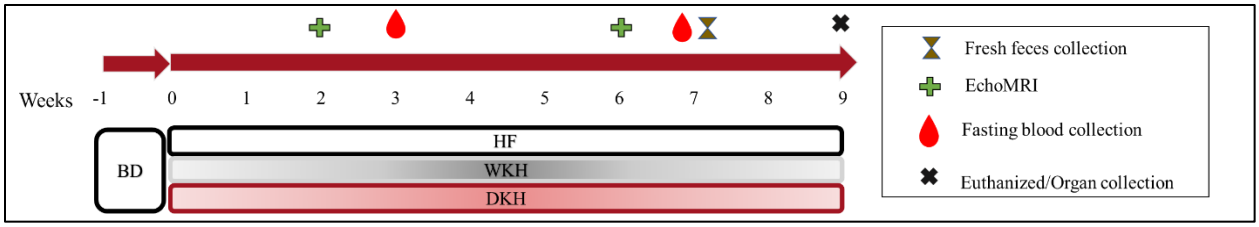

Figure 6-1. Experimental Procedural Timeline
Mice were acclimatized on BD for 1 week, after being randomly assigned to either HF, WKH, DKH, and kept on their diets for 9 weeks. Body composition was assessed at weeks 2 and 7. Fasted blood was taken at weeks 3 and 7. Fresh feces were collected at week 7, and tissues were collected upon study termination at week 9.

6.2.3. BMI & Organ weights

Upon study termination, final BW (g) was measured and body length (cm) was recorded by taking the lengths from the tip of the nose to the base of the tail with the mouse flat on its stomach. Body mass index (g/cm^2) was calculated using the equation[252]: BMI= Body Weight (g) / Body Length (cm)^2. After excision, the cecum and cecum content, colon, liver, perinephric adipose tissue (PAT), epididymal adipose tissue (EAT), and brown adipose tissue (BAT) were weighed and expressed in milligrams. Colon length was also measured by ruler and expressed in cm.

6.2.4 Histology

Upon termination, 1cm sections of proximal colon were placed in histology cassettes and immediately fixed in 10% buffered formalin phosphate (Fisher chemical, Cat. #SF100-4) for 24 hours and then switched to 70% ethanol and stored at 4°C before being paraffin-embedded and sectioning by the University of Ottawa Histology Core (Roger-Guindon Hall, Ottawa). Proximal colon cross sections (4 μm) were stained with hematoxylin and eosin stain (H&E) to assess colon crypt length and stained with alcian blue/nuclear fast red to assess mucus content as previously described (Chapter 5.2.4).

6.2.5 Microbial Community Structure

Week 7 fecal DNA was extracted as previously described (Chapter 5.2.5.1). The 16S V3-V4 region were amplified in fecal DNA samples following Illumina guidelines as described above (Chapter 5.2.5).

6.2.6 Microbial Activity

Fecal (collected at week 7), and cecal samples (collected at week 9) were analyzed by GC for SCFAs and BCFAs as previously described (Chapter 5.2.6). Fecal (week 7) and cecal (endpoint) pH were measured as previously described (Chapter 5.2.6). Data is presented as μmol/g DW.

6.2.7 Proximal Colon Gene Expression Analysis

Proximal colon mRNA gene expression was assessed by qPCR as previously described (Chapter 5.2.7). RPLPO and EEF2 were used as reference genes. Data were analyzed using CFX Maestro Software, and relative gene expression was calculated using the $\Delta\Delta C_q$ method, expression levels of each gene were normalized to that of the reference genes (EEF2 and RPLP0). Data is expressed as normalized expression relative to zero.

6.2.8 Serum Biomarkers of Metabolic Health

Week 7 fasting serum adiponectin was analyzed using Bio-Plex Pro™ Diabetes Adiponectin Assay (Bio-Rad™, Cat. #171-F7002M) as previously described (Chapter 5.2.8). Week 7 fasting serum was also analyzed for ghrelin, GIP, GLP-1, glucagon, insulin, leptin, PAI-

1, resistin using Bio-Plex Pro™ Diabetes Panel 8-Plex (BIO-RAD, Cat. #171-F7001M, USA) as previously described (Chapter 5.2.8). HOMA-IR was calculated using the formula: (glucose (mmol/L)/ insulin (mU/L) x 22.5) [262].

6.2.9 Statistical analysis

Data were analyzed using GraphPad Prism (version 8.1.2), and data were analyzed by either one-way ANOVA with Tukey's post-hoc analysis, or two-way ANOVA. Data with p-value less than 0.05 was considered significant. Mice of C57Bl/6 genetic background are known to contain under responders to diet-induced obesity (e.g. remaining lean on high-fat diets)[287,288]. Therefore, outliers were detected using the final BW data with a box/whisker plot, with the lower 10% as a cut off (diet-induced obesity resistant). Once outliers (n=2) were detected they were removed from all data analysis. For some figures shown below, the mean values from the healthy control group (BD) determined in Chapter 5 were included as a lean phenotype point of reference (dashed blue line), but were not included in the statistical analyses.

6.3 Results

6.3.1 Mice body weight, diet intake and energy intake

BW was measured twice weekly over the 9-week feeding trial, and there were no significant differences in BW between groups at any time point ($p>0.05$), and there were no significant differences in final BW between groups ($p>0.05$) (**Figure 6-2A & B**). DI and EI was measured twice weekly over the feeding trial, and there were no significant differences in DI and EI throughout the trial ($p>0.05$), suggesting the diets had similar palatability (**Figure 6-2C & D**).

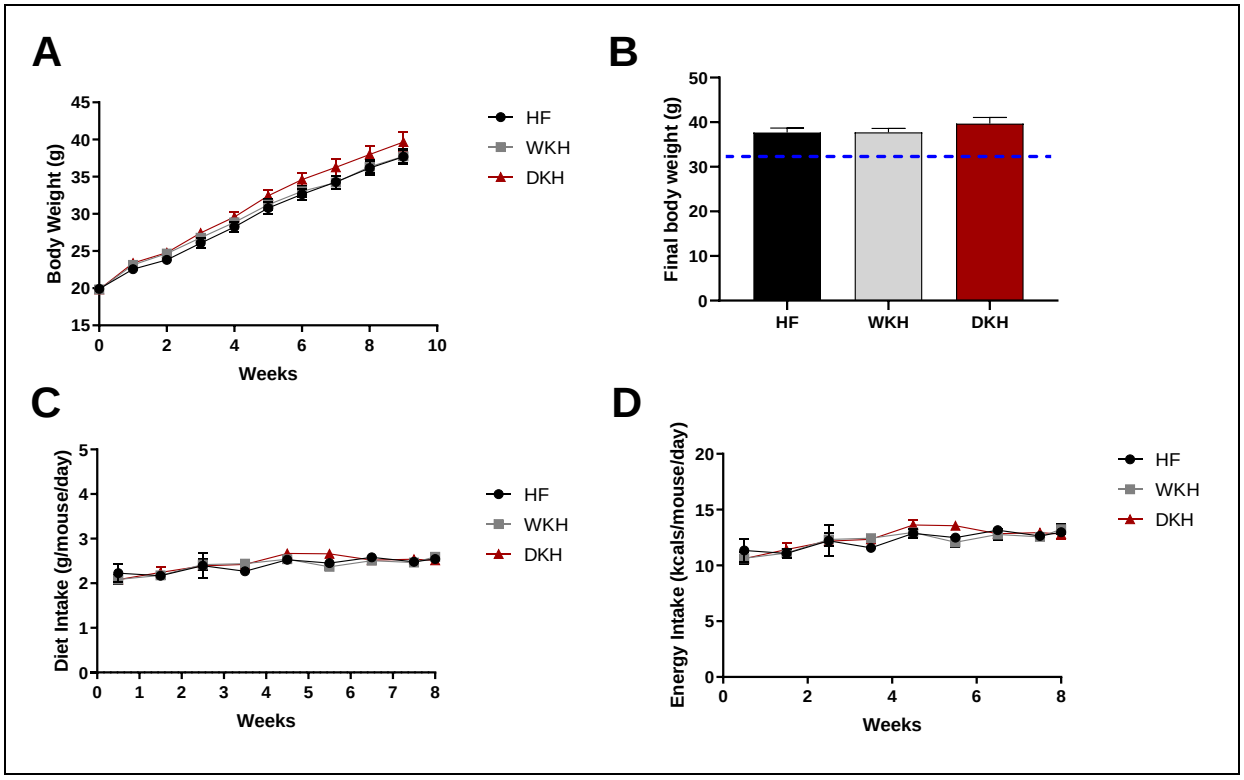

Figure 6-2: The effects of bean supplementation into a HF diet on mouse body weight and diet intake

A: Body weight (g) over time (weeks) in HF, WKH, and DKH fed mice. **B**: Final body weight (g). **C:** Diet intake (g/day). **D:** Energy intake (kcal/day). Diet intake and body weight were analyzed by two-way ANOVA followed by Tukey post hoc test (n=10-12 per group). Final body weight was analyzed by one-way ANOVA followed by Tukey post hoc test (n=10-12 per group.) Data points or bars not sharing a lower-case letter are significantly different ($p<0.05$). The blue dashed line represents the BD reference values of the same measure. HF=High-fat basal diet, WKH= white kidney bean supplemented high-fat diet, DKH= Dark red kidney bean supplemented high-fat diet.

6.3.2 BMI and body composition

BMI was calculated using the formula[252]: BMI= Body Weight (g) / Body Length (cm)^2. There were no significant differences in BMI between the dietary groups ($p>0.05$) (**Figure 6-3**), confirming the final body weight data (**Figure 6-2B**). Body composition was assessed by EchoMRI at weeks 2 and 6, and lean mass and fat mass are expressed in grams and as a percentage of body weight (% BW). There were no significant differences between groups in lean mass, (% BW), at both weeks 2 and 6 ($p>0.05$) however when presented in grams, both bean diets had significantly higher lean mass than HF at weeks 2 and 6 ($p<0.05$) (**Figure 6-4A & C**). Fat mass, (g) or (% BW), was comparable across groups at both weeks 2 and 6 ($p>0.05$) (**Figure 6-3B & D**). Both lean mass (g) and fat mass (g) and (%BW) increased over time, while lean mass (%BW) decreased over time.

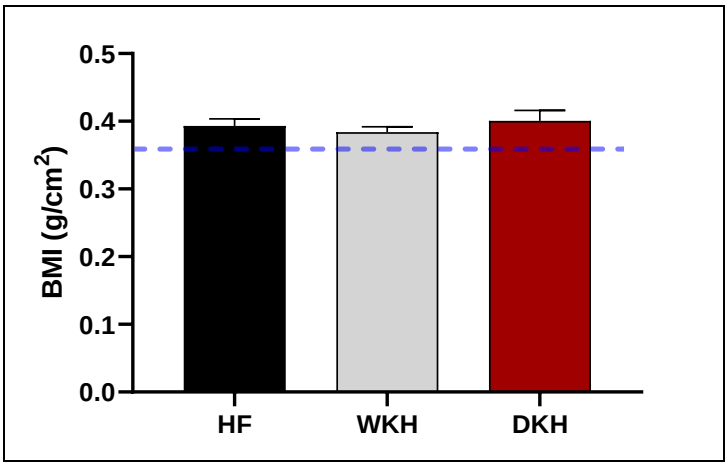

Figure 6-3: BMI in HF, WKH, and DKH fed mice.
BMI was calculated with formula: BMI= Body Weight (g) / Body Length (cm)^2. Data was analyzed by one-way ANOVA followed by Tukey's post-hoc test, (n=10-12/group). Bars not sharing a lower-case letter a significantly different ($p<0.05$). The blue dashed line represents the BD reference value of the same measure. HF=High-fat basal diet, WKH= white kidney bean supplemented high-fat diet, DKH= Dark red kidney bean supplemented high-fat diet.

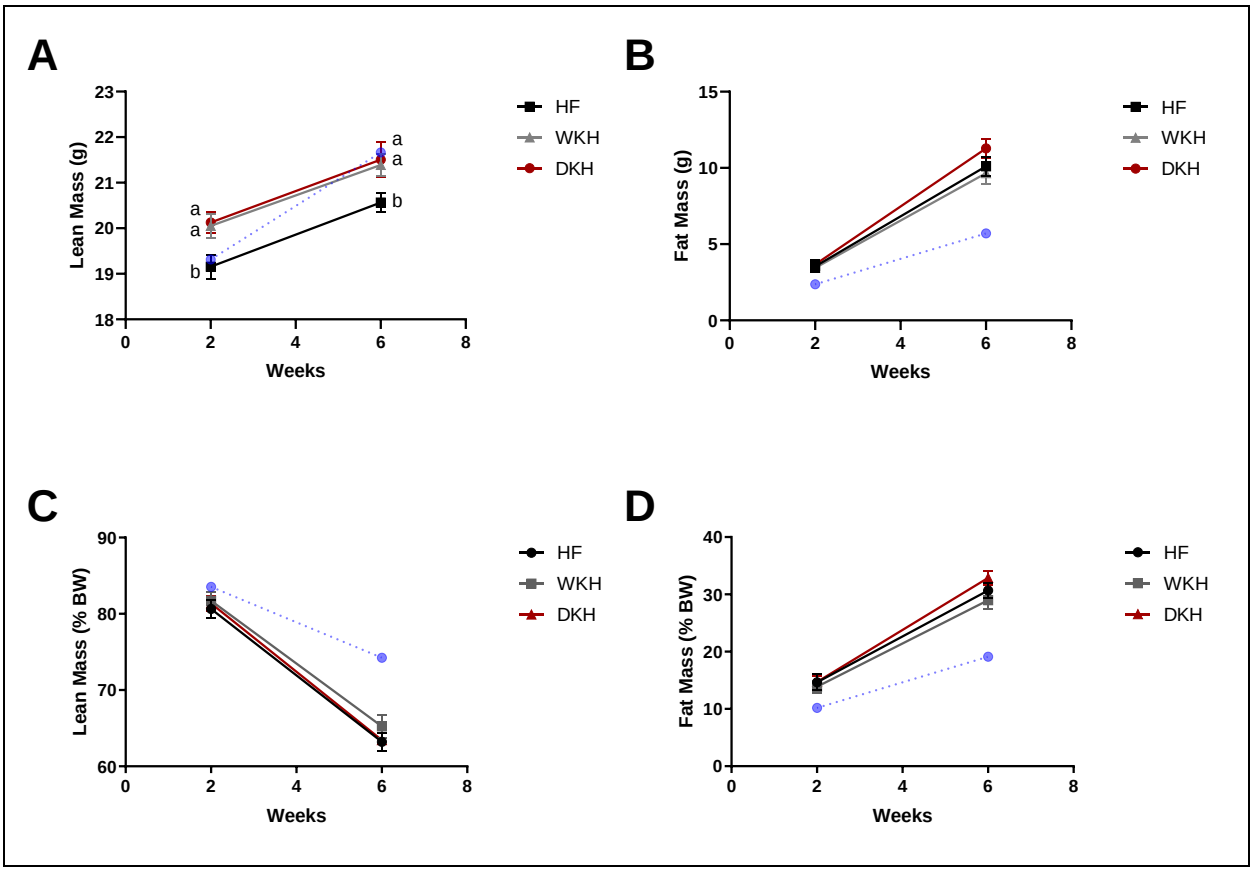

Figure 6-4: Body Composition, lean mass and fat mass, assessed by EchoMRI in HF, WKH, and DKH fed mice.
A: Lean mass (g) in mice at weeks 2 and 6 in HF, WKH, and DKH fed mice. **B:** Fat mass (g) in mice at weeks 2 and 6. **C:** Lean mass (% BW) in mice at weeks 2 and 6. **D:** Fat mass (%BW) in mice at weeks 2 and 6. Data was analyzed by two-way mixed model ANOVA followed by Tukey's post-hoc test, (n=10-12/group). Data points or bars not sharing a lower-case letter are significantly different ($p<0.05$). The blue dashed line represents the BD reference values of the same measure. HF=High-fat basal diet, WKH= white kidney bean supplemented high-fat diet, DKH= Dark red kidney bean supplemented high-fat diet.

6.3.3 Organ and fat pad weights

After the 9-week feeding trial, mice were euthanized, and organs (cecum, colon, and liver) and fat pads (EAT, PAT, and BAT) were excised, weighed and are presented as the mean in g and % BW ± SEM. Mice in all dietary groups had similar EAT, BAT and PAT weights when presented in both grams or as a percentage of body weight ($p>0.05$) (**Table 6-1**). Upon study termination, cecum and cecum content, colon and liver were weighed, and are expressed in (g). Mice consuming bean diets, WKH and DKH, had significantly elevated cecum and cecum

content weight compared to HF group ($p<0.05$). There were no significant differences between WKH and DKH for both cecum content and cecum weight ($p>0.05$) (**Figure 6-5A & B**). Colon weights were similar between all dietary groups ($p>0.05$) (**Figure 6-5C**). There were no significant differences in liver weight between dietary groups ($p>0.05$) (**Figure 6-5D**).

Table 6-1: Mean fat pad weight in HF, WKH, and DKH fed mice.

	HF	WKH	DKH
Epididymal (g ± SEM)	2.087 ± 0.1343	2.035 ± 0.1106	2.340 ± 0.1333
Epididymal (% BW ± SEM)	5.499 ± 0.2363	5.363 ± 0.2127	5.858 ± 0.1729
Perinephric (g ± SEM)	1.047 ± 0.04808	0.9653 ± 0.05011	1.157 ± 0.07507
Perinephric (% BW ± SEM)	2.782 ± 0.08080	2.545 ± 0.08770	2.884 ± 0.09970
Brown (g ± SEM)	0.2050 ± 0.008142	0.2243 ± 0.01779	0.2463 ± 0.01774
Brown (% BW ± SEM)	0.5435 ± 0.01468	0.5893 ± 0.03510	0.6137 ± 0.03002

Data was analyzed by one-way ANOVA followed by Tukey's post-hoc test (n=10-/group). Data not sharing a lower-case letter are significantly different. ($p<0.05$). HF=High-fat basal diet, WKH= white kidney bean supplemented high-fat diet, DKH= Dark red kidney bean supplemented high-fat diet.

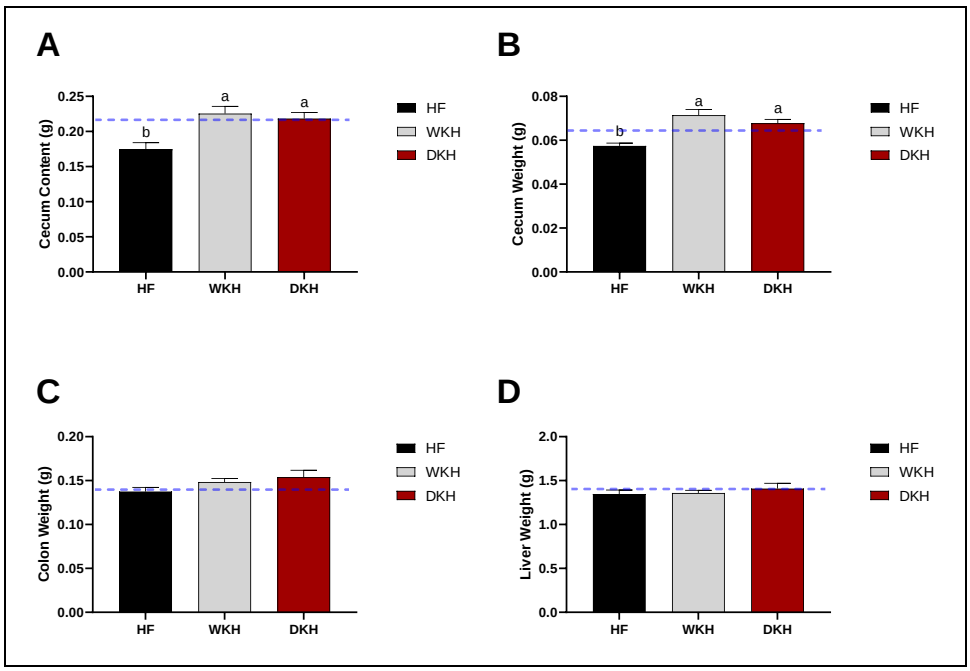

Figure 6-5: Cecum content, cecum, colon, and liver weights in HF, WKH, and DKH fed mice.
A: Cecum content (g) in HF, WKH, and DKH. **B:** Cecum weight (g). **C:** Colon weight (g). **D:** Liver weight (g). Data was analyzed by on-way ANOVA, followed by Tukey's post-hoc test (n=10-12 per group). Bars not sharing a lower-case letter are significantly different ($p<0.05$). The blue dashed line represents the BD reference values of the same measure. HF=High-fat basal diet, WKH= white kidney bean supplemented high-fat diet, DKH= Dark red kidney bean supplemented high-fat diet.

6.3.4 Proximal Colon Histomorphometry

Crypt length was assessed in proximal colon cross sections stained with H&E and is presented in μm. Crypt length from largest to smallest was DKH, WKH, followed by HF, however there were no significant differences in crypt length between dietary groups ($p>0.05$). Mucus content score was measured in proximal colon cross sections stained with AB/NFR and is presented as mucus/μm^2. Mucus content was highest in DKH, followed by HF then WKH, but there were no significant differences between groups ($p>0.05$) (**Table 6-2**).

Table 6-2: Proximal Colon Crypt Length and Mucus Content

	HF	WKH	DKH
Crypt Length (μm)	164.40 ± 7.066	165.14 ± 6.125	171.68 ± 6.766
Mucus Content (mucus/ μm^2)	0.3098 ± 0.0087	0.2952 ± 0.0161	0.3224 ± 0.0037

Crypt length was measured using ImageJ software in proximal colon cross sections stained with H&E, and mucus content score in proximal colon cross sections stained with Nuclear Fast Red/Alcian Blue. Data was analyzed by one-way ANOVA followed by Tukey's post-hoc test (n=10-11/group). HF=High-fat basal diet, WKH= white kidney bean supplemented high-fat diet, DKH= Dark red kidney bean supplemented high-fat diet.

6.3.5 Microbial Community Structure

Microbial community α-diversity was assessed using the Choa-1 index, Observed species, and PD whole tree, at a read depth of 20,000 basepairs. Choa-1 index was significantly increased in DKH compared to only the HF group ($p<0.05$), with WKH and DKH groups having no significant differences in Choa-1 index ($p>0.05$) (**Figure 6-6A**). DKH group had significantly higher level of observed species when compared to HF group ($p<0.05$), but not compared to WKH group ($p>0.05$) (**Figure 6-6B**). PD whole tree was significantly increased in both WKH and DKH diets compared to HF($p<0.05$), with no difference between bean groups ($p>0.05$) (**Figure 6-6C**). PCoA, a β-diversity visualization of the microbiota community composition, showed clustering based on diet, with both bean diets being significantly different from HF

group ($p<0.05$), and both bean diets being significantly different from each other ($p<0.05$) (**Figure 6-6D**).

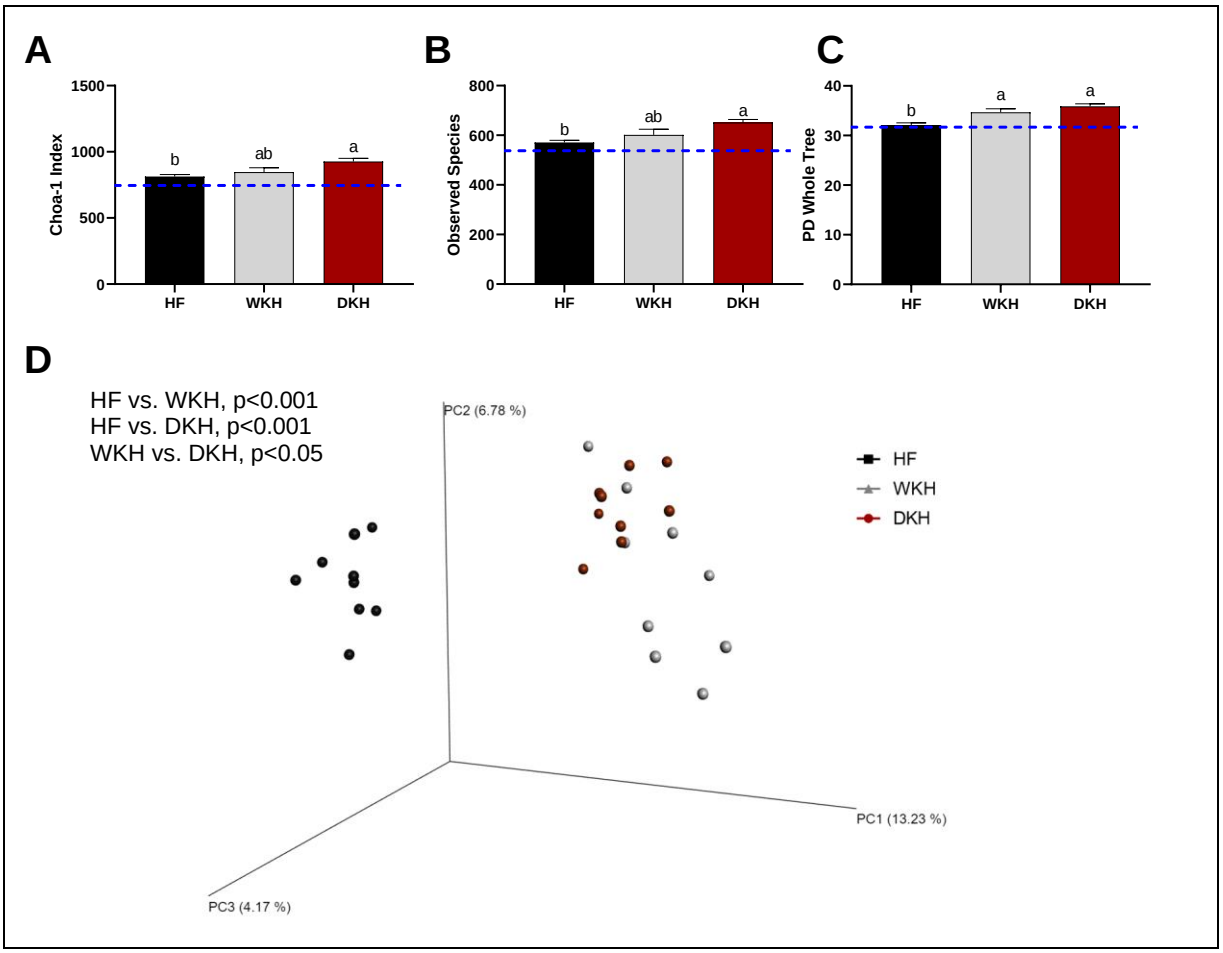

Figure 6-6: Week 7 Fecal Microbiota in HF, WKH, and DKH fed mice
Fecal microbiota community α-diversity (A-C), as measured by Choa-1 index (**A**), Observed species index (**B**), and PD-Whole tree method (**C**); Significant differences were measured by One-way ANOVA followed by Tukey's post-hoc test; bars not sharing a lower-case letter are significantly different ($p<0.05$). **D**) β-diversity as visualized by principal component analysis (PCoA) of unweighted UniFrac distance matrices showing bacterial communities cluster within dietary groups and the percent of dataset variability explained by each principal component is shown in brackets in the axis titles. Each dot represents one mouse and each dietary group is denoted by a different coloured symbol (HF: black circles; WKH: white circles; DKH: red circles). Differences between groups were measured by PERMANOVA and P-values are shown. HF=High-fat basal diet, WKH= white kidney bean supplemented high-fat diet, DKH= Dark red kidney bean supplemented high-fat diet.

At the phylum level, HF had significantly higher abundance of *Firmicutes* compared to WKH and DKH ($p<0.05$) (**Figure 6-7**) (**Table 6-3**). Along with a decrease in *Firmicutes* in DKH

and WKH, *Bacteroidetes* were significantly increased in DKH and WKH groups when compared to HF group ($p<0.05$). The abundance of the phylum *Verrucomicrobia* and its associated genus member, *Akkermansia,* was highest in WKH group, however significance was reached only when compared to DKH group ($p<0.05$). While the phylum *Proteobacteria* and genus *Sutterella* were significantly increased in both WKH and DKH groups compared to HF group ($p<0.05$). Furthermore, there were significant increases in the genus *Prevotella*, *S24-7*, and *Lactobacillus*, and significant decreases in the genus *Oscillospira* in both WKH and DKH groups compared to the HF group ($p<0.05$). Lastly, there were significant decreases in the family *Ruminococcacae* in WKH group compared to DKH and HF groups ($p<0.05$) (**Table 6-3**).

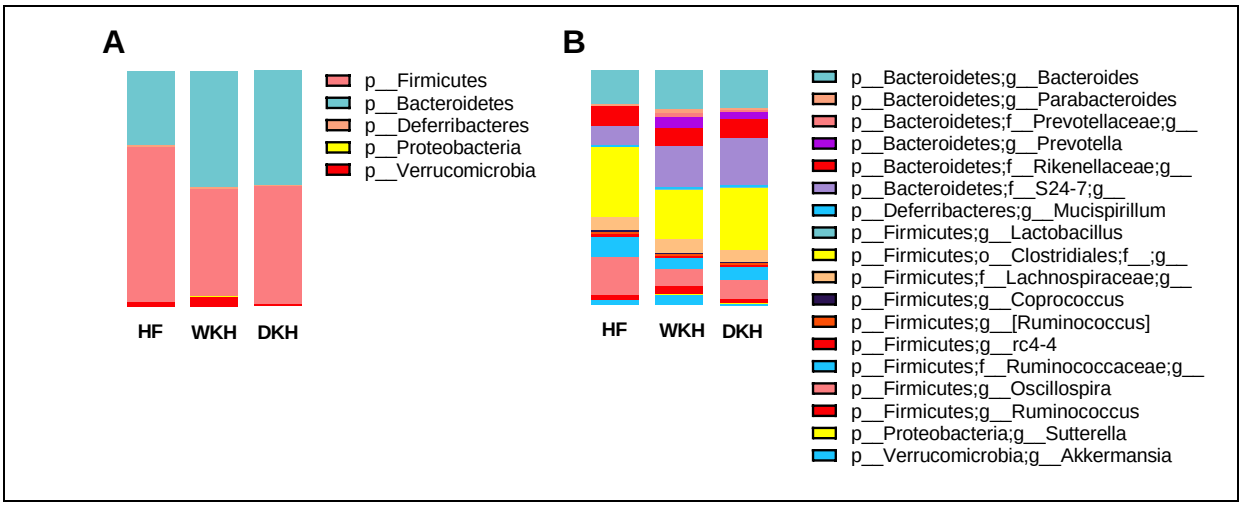

Figure 6-7: Taxonomic structure of fecal microbiota.
Microbiota taxa composition at the phylum (A) and genus (B) including taxa representing >0.5% total composition in at least one sample. HF=High-fat basal diet, WKH= white kidney bean supplemented high-fat diet, DKH= Dark red kidney bean supplemented high-fat diet.

Table 6-3: Relative abundance of fecal microbiota in HF, WKH, and DKH fed mice

Taxonomy	% Relative Abundance		
	HF	WKH	DKH
Bacteroidetes	31.33 ± 3.707	48.82 ± 4.00*	48.24 ± 3.313*
g__Bacteroides	14.22 ± 1.637	16.03 ± 1.902	16.03 ± 1.821
g__Parabacteroides	0.71 ± 0.238	1.68 ± 0.543	0.74 ± 0.111
f__Prevotellaceae;g__	0.00 ± 0.001	1.91 ± 0.385*	0.88 ± 0.199*
g__Prevotella	0.14 ± 0.039	4.33 ± 0.739*	2.82 ± 0.424*
f__Rikenellaceae;g__	8.40 ± 1.887	7.82 ± 2.325	7.94 ± 1.386
f__S24-7;g__	7.85 ± 0.701	17.05 ± 2.260*	19.83 ± 1.141*
Deferribacteres	0.92 ± 0.172	0.84 ± 0.267	0.83 ± 0.210
g__Mucispirillum	0.92 ± 0.172	0.84 ± 0.267	0.83 ± 0.210
Firmicutes	65.14 ± 3.608	45.07 ± 4.229*	49.35 ± 3.301*
g__Lactobacillus	0.11 ± 0.032	0.48 ± 0.159*	0.36 ± 0.076*
o__Clostridiales;f__;g__	28.84 ± 2.738	20.61 ± 2.097	25.97 ± 2.317
f__Lachnospiraceae;g__	5.81 ± 0.616	5.63 ± 1.041	5.03 ± 0.664
g__Coprococcus	0.60 ± 0.193	0.74 ± 0.135	0.61 ± 0.104
g__[Ruminococcus]	0.82 ± 0.115	0.63 ± 0.077	0.61 ± 0.070
g__rc4-4	1.52 ± 0.403	1.09 ± 0.151	1.05 ± 0.156
f__Ruminococcaceae;g__	8.36 ± 1.020	4.51 ± 0.590*	5.35 ± 0.613
g__Oscillospira	15.98 ± 1.627	7.25 ± 0.977*	7.84 ± 0.780*
g__Ruminococcus	2.13 ± 0.489	3.28 ± 0.635	1.65 ± 0.254
Proteobacteria	0.09 ± 0.027	0.71 ± 0.116*	0.47 ± 0.104*
g__Sutterella	0.04 ± 0.023	0.64 ± 0.106*	0.41 ± 0.097*
Verrucomicrobia	2.09 ± 0.848	4.05 ± 0.927	0.75 ± 0.277§
g__Akkermansia	2.09 ± 0.848	4.05 ± 0.927	0.75 ± 0.277§

Relative abundance (mean) of fecal bacterial taxa (n=10-12/group) at the phylum level (highlighted in grey rows), and corresponding order (o); family (f), and/or genus (g) with abundance great than 0.5% in at least one group. Values marked with an asterisk (*) differ from HF and values marked with a (§) symbol denotes differences between WKH and DKH assessed by the Kruskal-Wallis test with p-values adjusted for FDR<0.05 using the Benjamini-Hochberg method. HF=High-fat basal diet, WKH= white kidney bean supplemented high-fat diet, DKH= Dark red kidney bean supplemented high-fat diet.

6.3.6 Microbial Activity

SCFAs (acetic, butyric, propionic, and valeric acid), a product of carbohydrate fermentation, and BCFAs (iso-butyric and iso-valeric acid), a product of protein fermentation, were measured in week 7 fecal samples and in cecum content collected at the study end-point (week 9) and are presented in μmol/g DW. Cecum acetic acid, propionic and valeric acid were similar across all groups ($p>0.05$) (**Figure 6-8A, B & E**). Cecum butyric acid was increased in

both bean diets compared to HF; however, this was not significantly different (p>0.05) (**Figure 6-8D**). Cecum total SCFAs did not differ significantly between groups (p>0.05) (**Figure 6-8F**). Cecum BCFAs, iso-butyric and iso-valeric acid, were reduced in DKH but there were no significant differences between groups (p>0.05). Cecum content pH was significantly decreased in DKH compared to WKH group (p<0,05), however neither bean group differed from HF (p>0.05) (**Figure 6-8C**).

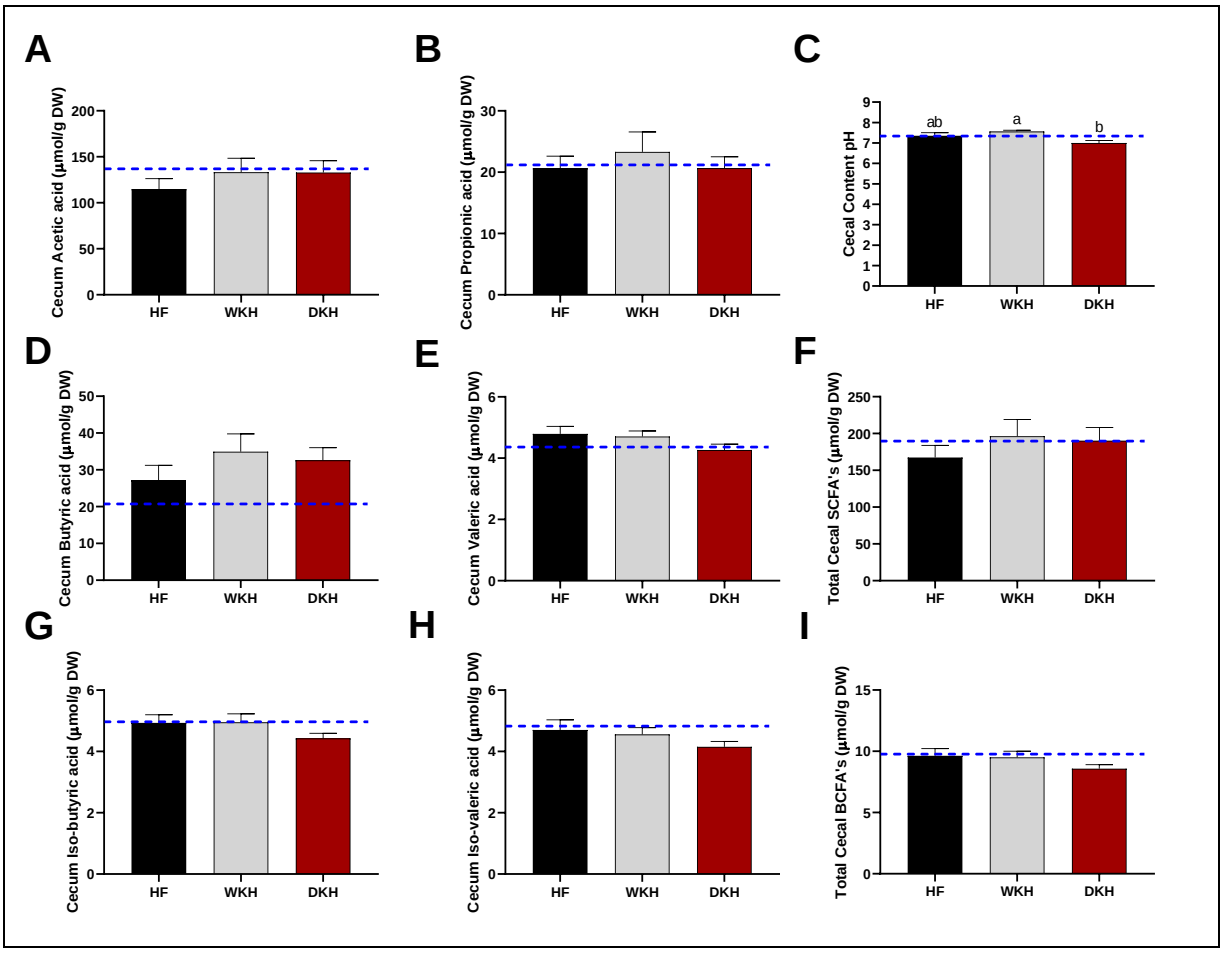

Figure 6-8: Cecal pH, SCFAs, and BCFAs in HF, WKH, and DKH fed mice.
A: Cecum acetic acid (µmol/g DW). **B:** Cecum propionic acid (µmol/g DW). **C:** Cecal content pH. **D:** Cecum butyric acid (µmol/g DW). **E:** Cecum valeric acid (µmol/g DW). **F:** Total cecal SCFAs (µmol/g DW). **G:** Cecum iso-butyric acid (µmol/g DW). **H:** Cecum iso-valeric acid (µmol/g DW). **I:** Total cecal BCFAs **(**µmol/g DW). Data was analyzed by one-way ANOVA followed by Tukey's post-hoc test (n=10-11/group). Bars not sharing a lower-case letter are significantly different (p<0.05). The blue dashed line represents the BD reference values of the same measure. HF=High-fat basal diet, WKH= white kidney bean supplemented high-fat diet, DKH= Dark red kidney bean supplemented high-fat diet.

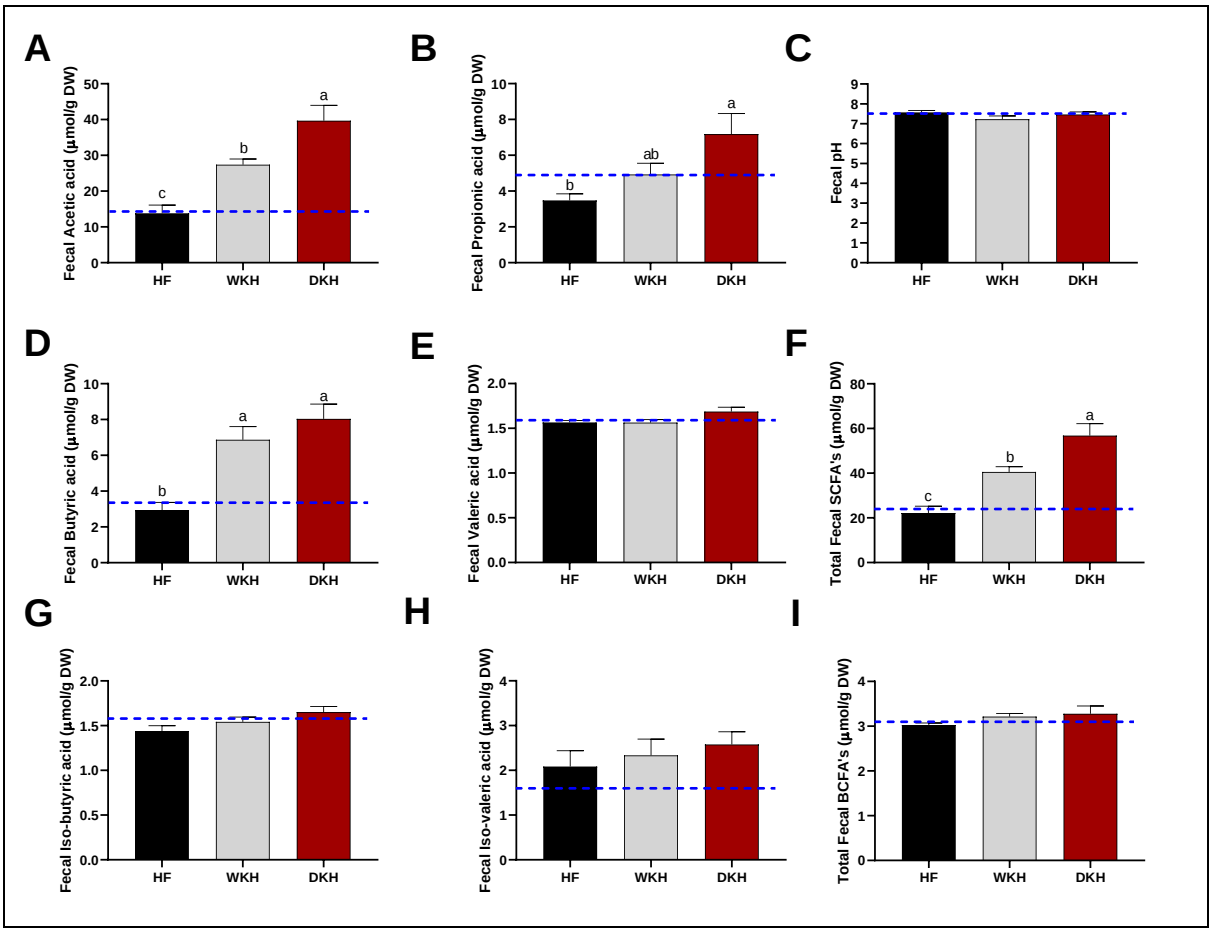

Figure 6-9: Week 7 Fecal pH, SCFAs, and BCFAs in HF, WKH, and DKH fed mice.
A: Fecal acetic acid (µmol/g DW). **B:** Fecal propionic acid (µmol/g DW). **C:** Fecal content pH. **D:** Fecal butyric acid (µmol/g DW). **E:** Fecal valeric acid (µmol/g DW). **F:** Total fecal SCFAs (µmol/g DW). **G:** Fecal iso-butyric acid (µmol/g DW). **H:** Fecal iso-valeric acid (µmol/g DW). **I:** Total fecal BCFAs **(**µmol/g DW). Data was analyzed by one-way ANOVA followed by Tukey's post-hoc test (n=10-11/group). Bars not sharing a lower-case letter are significantly different ($p<0.05$). The blue dashed line represents the BD reference values of the same measure. HF=High-fat basal diet, WKH= white kidney bean supplemented high-fat diet, DKH= Dark red kidney bean supplemented high-fat diet.

Week 7 fecal acetic acid was significantly increased in DKH compared to both WKH and HF groups ($p<0.05$) (**Figure 6-9A**). Fecal propionic acid was significantly increased in DKH when compared to HF group ($p<0.05$), but there were no differences between bean groups or between WKH and HF groups ($p>0.05$) (**Figure 6-9B**). Fecal butyric acid was significantly elevated in both bean diets ($p<0.05$), and there were no significant differences between WKH and DKH ($p>0.05$) (**Figure 6-9D**). There were no significant differences in fecal valeric acid between groups ($p>0.05$) (**Figure 6-9E**). Total fecal SCFAs were significantly increased in DKH

compared to WKH group and HF group ($p<0.05$) (**Figure 6-9F**). Fecal pH was not significantly different between dietary groups ($p>0.05$) (**Figure 6-9C**). Fecal total BCFAs, iso-butyric and iso-valeric acid were similar across all groups ($p>0.05$) (**Figure 6-9G-I**).

6.3.7 Colon mRNA Gene Expression

Colon mRNA expression was analyzed in proximal colon tissues excised at the study end-point, and is expressed as relative normalized expression, genes were normalized to the expression of the reference genes (RPLP0 & EEF2). GPR41 was significantly downregulated in DKH compared to HF group ($p<0.05$), but there were no significant differences between HF and WKH, or between WKH and DKH groups ($p>0.05$). GPR43 expression was significantly reduced in DKH compared to both WKH and HF ($p<0.05$), while GPR109a mRNA expression

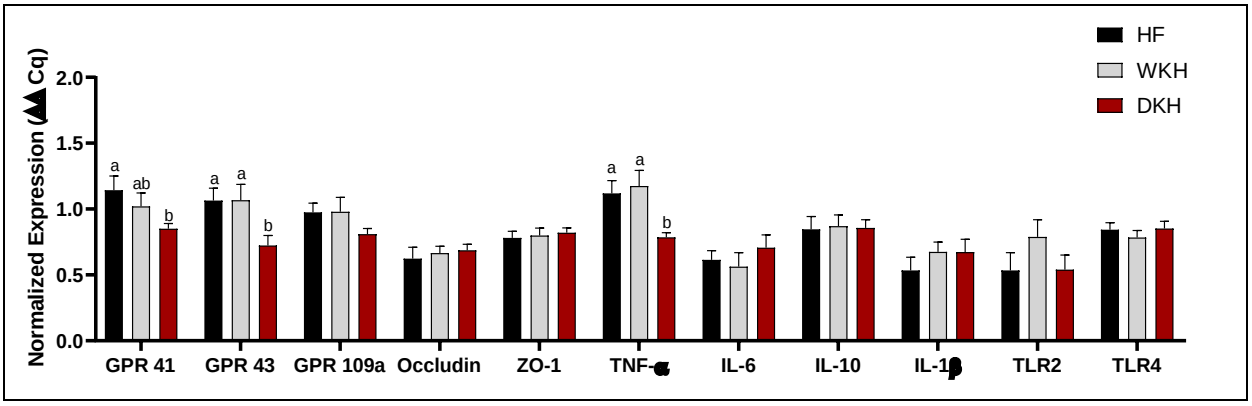

Figure 6-10: Proximal colon mRNA expression in BD, WKB, and DKB fed mice.
Relative normalized mRNA expression was calculated using the ΔΔCq method and is presented relative to zero. Data were normalized to the expression of the housekeeping genes RPLP0 and Eef2. Data were analyzed by one-way ANOVA followed by Tukey's post-hoc test (n=9-12 per group). Bars not sharing a lower-case letter are significantly different ($p<0.05$). HF=High-fat basal diet, WKH= white kidney bean supplemented high-fat diet, DKH= Dark red kidney bean supplemented high-fat diet.

was similar across all dietary groups ($p>0.05$). Occludin and ZO-1, were similar in all dietary groups ($p>0.05$). TNF-α was significantly downregulated in DKH compared to both HF and WKH groups ($p<0.05$). There were no significant differences between groups for the remaining

cytokines IL-6, IL-10, and IL-1β ($p>0.05$). TLR2 and TLR4, are signaling proteins, remained comparable across all dietary groups ($p>0.05$) (**Figure 6-10**).

6.3.8 Serum Biomarkers

Fasting serum collected at week 7 was analyzed for biomarkers of metabolic health, and are presented in pg/ml. There were no significant differences in serum GIP, or GLP-1 between any dietary groups ($p>0.05$) (**Figure 6-11A & B**). Serum ghrelin and resistin tended to decrease

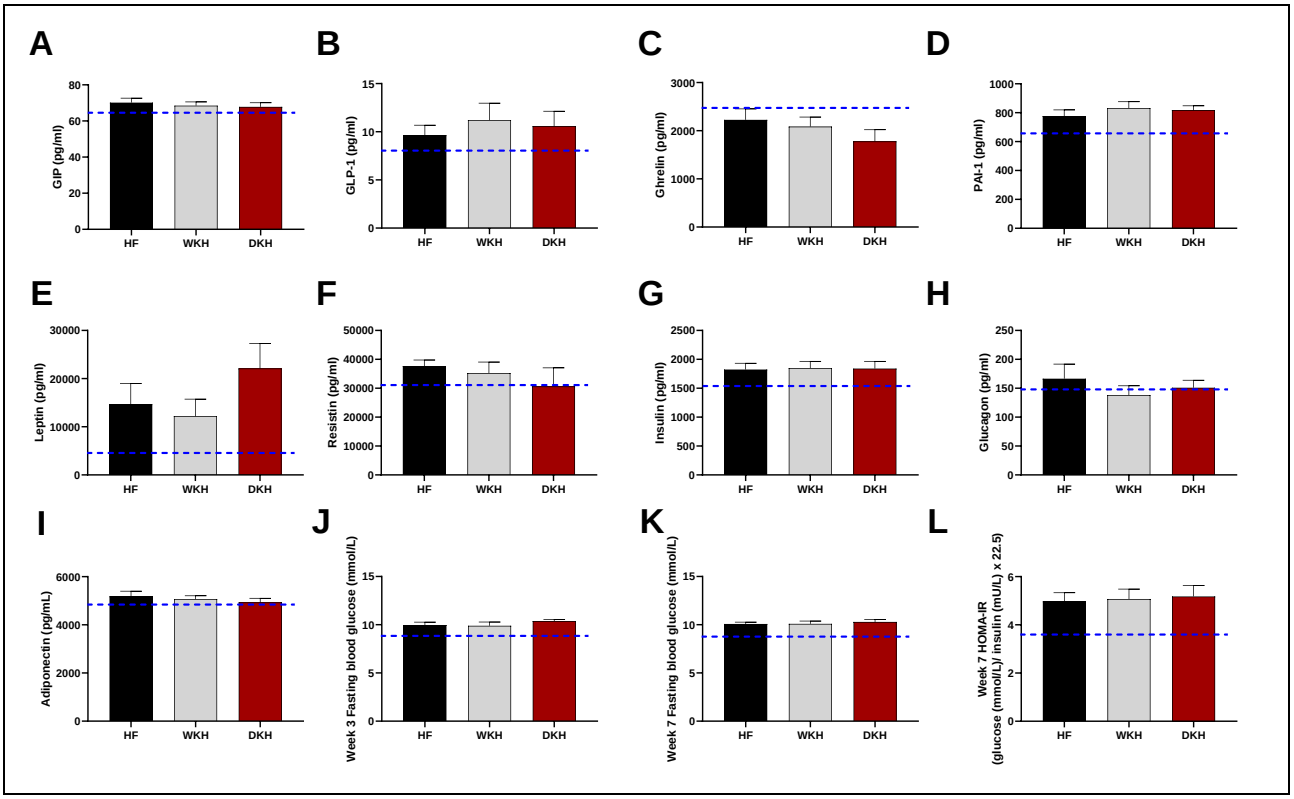

Figure 6-11: Week 7 Fasting Serum Biomarkers in HF, WKH, and DKH fed mice.
A: GIP (pg/mL). **B:** GLP-1 (pg/mL). **C:** Ghrelin (pg/mL). **D:** PAI-1 (pg/mL). **E:** Leptin (pg/mL). **F:** Resistin (pg/mL). **G:** Insulin (pg/mL). **H:** Glucagon (pg/mL). **I:** Adiponectin (pg/mL) **J:** Week 3 fasting blood glucose (mmol/L). **K:** Week 7 fasting blood glucose (mmol/L). **L:** Week 7 HOMA-IR (glucose (mmol/L)/Insulin (mU/L) x 22.5). Data were analyzed by one-way ANOVA followed by Tukey's post-hoc test (n=9-11 per group). Bars not sharing a lower-case letter are significantly different ($p<0.05$). HF=High-fat basal diet, WKH= white kidney bean supplemented high-fat diet, DKH= Dark red kidney bean supplemented high-fat diet.

in WKH followed by DKH however there were no significant differences between groups ($p>0.05$) (**Figure 6-10C & F**). Serum PAI-1, insulin, and glucagon were similar between all dietary groups ($p>0.05$) (**Figure 6-10D, G, H**). Serum leptin was increased in DKH group, however this was not significantly different as all diets had similar leptin concentrations ($p>0.05$) (**Figure 6-10E**). Serum adiponectin was not significantly different between groups ($p>0.05$) (**Figure 6-10I**). Fasting blood glucose at weeks 3 and 7 were not significantly different between groups, and as a result HOMA-IR was also not significantly different between groups ($p>0.05$) (**Figure 6-10J-L**).

6.4 Discussion

The objectives of this study were to determine if i) kidney bean supplementation into a high-fat diet would attenuate diet-induced obesity and its associated intestinal and metabolic health impairments, and ii) intestinal and metabolic health outcomes would be differently affected by kidney bean seed coat color. Overall, the findings in this study demonstrate that supplementation of HF diet with cooked kidney beans resulted in improvements in intestinal health, but did not impact measured biomarkers of metabolic health. These data demonstrate that supplementation of kidney beans into a high-fat diet are capable of attenuating microbial dysbiosis by increasing species richness and evenness and altering microbial activity. Furthermore, these findings show that some of the intestinal effects were dependent on seed coat colour since the DK bean group showed a greater microbial community diversity, increased fecal SCFA concentrations, and reduced colonic expression of pro-inflammatory mediator, TNF-α. This suggests that components unique to DK beans (e.g. polyphenolic compounds) provide additional benefits to improve obesity-associated intestinal health.

As described in the literature review, obesity is associated with a dysbiotic microbiota, characterized by a lower community diversity and altered community structure and function[63,64]. In this study, kidney bean supplementation resulted in enhanced fecal microbial community diversity compared to HF controls, with DK beans having the greatest effect. Furthermore, it has been demonstrated that disruptions to the microbiota caused by high-fat feeding can lead to an increase in species that can better harvest energy and decreases in species that are beneficial to the host[63,76,154,155]. This dysbiotic microbiota is manifested by increases in the *Firmicutes* phylum, and significant decreases in the *Bacteroidetes* phylum, which can lead to increased energy extraction as seen in subjects with obesity[63,65,76,154,163]. In this current study, kidney bean

diets attenuated these high-fat diet induced changes, as *Bacteroidetes* was significantly increased and *Firmicutes* was significantly decreased compared to HF group, however there were no difference between bean types suggesting additional phenolic compounds associated with darker seed coat colors did not play a role in this effect.

Further changes similarly impacted by WK and DK bean diets were increases in *Prevotella spp.* and *S24-7 g.*, which are fibre fermenters, and beneficial to the host[77,187], and *Sutterella* which may be involved in anti-inflammatory pathways[280,281]. Increases in the abundance of fecal SCFA-producing bacteria in the bean-fed mice may have contributed to the kidney bean-induced increases in fecal SCFA concentrations. Fecal SCFAs were significantly affected by bean consumption, with both bean diets significantly increasing fecal butyrate concentrations, while only the DK group significantly elevated acetic acid, propionic acid, and total SCFAs. Furthermore, DK beans significantly reduced expression of SCFA-signaling receptors, GPR41 and GPR43, but not GPR109a. Although signalling through these receptors can mediate numerous beneficial effects of SCFAs[102–104,107], there is evidence to suggest that these are upregulated during inflammation[289,290]. Therefore, down-regulation induced by the DK group may be indicative of an anti-inflammatory effect, which is inline with the observed reduction in colonic pro-inflammatory TNF-α in the DK group.

It is unknown from these results why DK beans promoted higher fecal SCFA production compared to WK beans. Perhaps the higher phenolic content in the DK beans increased the amount of fermentable polysaccharides reaching the distal colon as they have been previous shown to inhibit α-amylase activity and thus starch digestion[179,212,283], however this requires further investigation. Furthermore, the results of this study indicate that bean-induced increases in microbial carbohydrate fermentation differed between the cecum and distal colon sites (e.g.

feces). In the cecal content, there were no differences in SCFA concentrations between dietary groups, which may indicate interfering components of high fat diets in cecal fermentation and/or an altered cecal microbiota composition which did not favor carbohydrate fermentation. Further evaluation of the cecal microbiota community structure will help resolve this phenomenon.

Previous studies have shown that navy beans supplemented into a high-fat diet increases the abundance of *Akkermansia* and this genus is reduced in mice consuming a high-fat diet[182,291]. *Akkermansia muciniphila* have been associated with improved inflammation and improvements in insulin resistance[291]. In this current study however, kidney bean consumption did not significantly alter *Akkermansia* abundance compared to the HF group, however, the WK group had the highest abundance which was significantly greater than the DK group. Although speculative at this point, this may indicate that components associated with lighter colored beans (e.g. WK beans, navy beans) can promote *Akkermansia* abundance, or vice versa, components of dark colored beans have inhibitory effects on *Akkermansia*. However, this is unlikely to be caused by phenolic compounds, since studies have shown that phenolic-rich extracts enhance Akkermansia abundance in obese mice[292,293]. Other bean induced-changes in members of the *Firmicutes* were increases in *Lactobacillus* and decreases in *Oscillospira*. These changes are indicative of improved microbial community structure as members of *Lactobacillus* are probiotic and have been demonstrated to improve plasma leptin, insulin, and total cholesterol in high-fat diet induced obese mice[294].

SCFAs can also have metabolic effects, therefore serum gut hormones and adipokines were assessed. Surprisingly, there were no effects of bean consumption seen on serum adipokines, gut hormones, or blood glucose levels and insulin resistance (HOMA-IR). This could be due to large differences seen in metabolic adaptations to a high-fat diet in C57Bl/6

mice, wherein large differences exist in diabetes incidence and glucose utilization[295]. Future studies could use a larger subset of mice per group to account for the large variations seen in metabolic adaptions to a high-fat diet. Bean supplementation did not affect BW or BMI; remarkably bean consumptions increased lean mass in both bean diets while fat mass remained comparable, this may have been due to the increased fat allowing for greater protein digestibility, as has been demonstrated in previous studies on foxes[296,297]. It has also been demonstrated that cellulose can decrease protein digestibility, while consuming a high-fat diet can increase protein digestibility compared to high-starch diets[298]. Furthermore within the high-starch diets, the group consuming cooked starch had increased protein digestibility compared to the uncooked starch diet[298]. These results suggest that cooked bean powder combined with a high-fat diet may increase protein digestibility compared to a high-fat diet composed of added starch and cellulose.

Overall, these results support my hypothesis that kidney beans would improve intestinal in mice consuming a high-fat diet, as microbial dysbiosis was attenuated and intestinal inflammation improved through reductions in TNF-α colonic gene expression. Metabolic health was not impacted in this study as bean consumption did not alter any parameters of metabolic health. Lastly, as hypothesized, consumption of dark coloured beans led to additional benefits on intestinal health, as demonstrated by increased microbial species richness and diversity, as well as increased SCFAs and improved inflammatory tone.

Chapter 7: General Discussion & Future Directions

7.1 General Discussion

My thesis hypothesis was that kidney bean supplementation would improve intestinal and metabolic health, and that these effects would be increased by darker coloured beans. My hypothesis was supported as kidney bean supplementation improved measures of intestinal and metabolic health, and darker coloured beans provided enhanced effects on certain measures. Lastly, I hypothesized that the health effects seen by bean supplementation would differ based on background diet. This hypothesis was also supported as microbial community structure and activity, and metabolic and inflammatory markers were differentially affected based on background diet.

Beans did not affect overall body weight between groups in either study, suggesting that the diets were equally matched for macronutrient composition, and no weight gain or loss is expected to occur when consuming plant-based protein from whole kidney beans versus an isocaloric macronutrient matched animal-based protein diet. The HF group had elevated final BW compared to LF group, supporting the models for HFD-induced obesity[145,287]. Furthermore, the high-fat diets contained more calories per gram than the low-fat diets, which led to all mice consuming the high-fat diets having lower DI, but EI was similar between studies. This would reduce the overall bean consumption in the high-fat diets as beans were supplemented at the same level in both studies, therefore future studies should increase the bean supplementation into the high-fat diets to match the bean intake of the low-fat diet.

Intriguingly, lean mass was significantly increased by both bean diets in the high-fat study, but this effect was not seen in the low-fat study. Previous studies have indicated that increased fat content can increase the digestibility of protein[296,297], which may indicate that

higher fat content can increase the digestibility of plant-based proteins. Furthermore, cecum content and cecum weight were lower in the HF group, however bean supplemented HFDs attenuated this reduction. When beans were supplemented into a BD, only WK beans demonstrated enhancements in cecum content and cecum weight, further supporting my hypothesis that the effects of bean supplementation would be affected by background diet. Based on previous studies, in which beans were supplemented into a BD, results show that histological markers of improved barrier integrity were increased by beans[170,184]. However, in this study there were no differences in crypt length or mucus content between dietary groups regardless of background diet. This may be explained in part by the differences in length of study, but also because of the difference in background diet composition. In this current study pectin was added to the background diet while in the previous studies the background diet only contained cellulose as the source of fibre, suggesting the addition of soluble fibre to the background diets in the form of pectin may have played a role in increasing baseline mucosal barrier integrity.

Furthermore, microbiota composition was significantly impacted by the supplementation of kidney beans to both a BD or HFD. This was observed as significant differences in PCoA in both bean varieties were detected, signifying a shift in microbiota composition. In the low-fat study, both bean types were grouped together in PCoA plot, and were not significantly different from each other for PCoA and all α-diversity metrics (Choa-1, Observed species, PD Whole Tree), suggesting that both bean types similarly improved microbiota composition. Whereas, in the high-fat study there were significant differences between all three groups on the PCoA plot, as well as in α-diversity metrics, suggesting that high-fat content in the diet may interact with the polyphenols and fibre, as only in the high-fat study were there significant differences between bean diets on α- and β-diversity metrics.

Bean supplementation also affected the microbiota taxonomy, with both bean varieties increasing the relative abundance of *Prevotella spp.,* known producers of SCFAs[275], regardless of background diet. Other bean effects seen across both background diets were increases in *Proteobacteria* and *Sutterella spp.,* a genus that has been increased by fructo-oligosaccharide supplementation in dogs, which may indicate this genus plays a role in carbohydrate metabolism[299,300]. Differences exist between beans supplemented into a low- or high-fat diet on the microbiota composition, for example the high-fat study saw both bean diets increase the abundance of *Bacteroidetes*, this was not seen in the low-fat bean diets. Further differences were seen between studies; in the high-fat studies both bean diets reduced the abundance of *Firmicutes,* this was not seen in the low-fat bean groups as they had similar abundance of the two phyla compared to BD group. This suggests that the HF control group had enhanced microbial dysbiosis as demonstrated by a decrease in *Firmicutes* and an increase in *Bacteroidetes,* and that beans supplemented into a high-fat diet can attenuate this dysbiosis to resemble the microbiota of mice fed a low-fat diet. These results suggest that background diet is an important factor in the effect of bean supplementation on microbiota community structure, as both bean diets induced similar changes in the low-fat diet whereas in the high-fat diets, there were significantly more differences between bean types.

For SCFA measurements, bean supplementation led to enhanced SCFA production regardless of background diet, however in the high-fat study, only fecal SCFAs were enhanced which may indicate that certain components of high fat diets are interfering with cecal fermentation and/or an altered cecal microbiota composition which does not preferentially ferment carbohydrates. Additionally, DK beans enhanced microbial activity compared to WK beans irrespective of background diet.

SCFAs are known to be involved in the anti-inflammatory process and involved in appetite regulation, and can have metabolic effects[105,111,112,118]. In the low-fat fed mice, only DKB decreased serum GIP, ghrelin, PAI-, and resistin. DKB also significantly increased serum adiponectin, suggesting that DK beans can help improve serum biomarkers when consumed as part of a low-fat diet. In contrast, in the high-fat model, serum biomarkers of metabolic health were not impacted by bean consumption. As discussed in Chapter 6, this may be due to the fact that mice on the high-fat diets had decreased diet intake due to the high calorie density, this resulted in overall smaller levels of bean consumption in the high-fat diets versus the low-fat diets. Furthermore, C57Bl/6 mice demonstrate large variability in metabolic responses to a HFD wherein diabetes incidence and glucose utilization are not similarly impacted in all mice[295]

Lastly, colonic gene expression was differently impacted based on background diet, as mice in the low-fat study consuming DKB had significantly increased ZO-1 and IL-10 expression, demonstrating increased TJ and anti-inflammatory gene expression. However, in the high-fat studies, DKH decreased GPR 41 and GPR 43 colonic mRNA expression, while also decreasing TNF-α expression. This could be due to GPR 41 and GPR43 being involved in the inflammatory process, as their expression is increased during increased inflammatory processes[301].

This research is translational to humans, in that the level of kidney bean supplementation was representative of a physiologically relevant intake level that is attainable for most Canadians, as the highest level of intake for Canadians is reported to be around 300 g per day, or ~16% of the daily caloric intake (assuming 2500kcal/day diet)[7]. Furthermore, DIO mice are excellent models that manifest metabolic dysfunction, increased inflammation and intestinal permeability typically seen in the obese phenotype, allowing for pre-clinical evidence that bean

supplementation at a physiologically relevant dose may improve aspects of intestinal and metabolic health in both the healthy and obese.

7.2 Limitations and Future Directions

Current limitations to this study include the lack of direct measures of intestinal permeability, such as by fluorescent probe, or by measuring LPS in the blood through LBP assay. Future work will be conducted on analyzing serum LBP levels, and future studies will include the use of an Ussing chamber which will allow direct measurement of intestinal permeability, as the tissue will be mounted into the chamber and fluorescent probe will be used to assess how "leaky" the tissues are[302]. This will also allow for the measurement of permeability in different colon sections (proximal versus distal) to determine which parts of the intestinal tract may be affected and targeted by bean consumption. It should be noted that gene expression was only conducted in the proximal colon, since fermentation occurs more proximally, the results may be significantly different than in the distal colon, where protein fermentation occurs. Other limitations to gene expression is that gene expression may not be entirely representative of protein expression[303], and future work will be conducted on analyzing protein expression of the genes analyzed in this study. Additionally, only fecal samples were analyzed for microbiota composition, as these are the most translational to humans, future analysis will be conducted to analyze the cecum microbiota of the mice in these studies. Other limitations were the lack of microbial species identification, we could therefore not identify what a specific genus is doing, as certain species of a genus can be beneficial while others detrimental. Future work is being done to employ a pipeline to identify these species.

Furthermore, while the phenolic compounds found in kidney beans have been previously characterized[24], they were not directly assessed in this study, therefore health effects conferred

from kidney bean consumption in this study cannot be directly attributed to certain phenolic compounds. Future work is required to characterize the phenolic compound profile found in the kidney beans used in this study. It would also be beneficial to measure phenolic compounds in the intestinal tract/feces as polyphenols may interact with plant-fibres in such a way that they are not easily digested and may reach the colon[152]. Once in the colon they are transformed into secondary phenolic metabolites which may be more bioactive than there primary counterparts[189,190]. It would be beneficial to extract the phenolic compounds found in beans as well as their secondary metabolites and analyze them in isolation by incorporating them into an animal diet. The same could be done with the fibre found in beans; specific fibre types such as oligosaccharides and resistant starch could be isolated and analyzed in an animal model. By analyzing the components of the beans in isolation, it would be easier to identify the mechanisms by which kidney bean consumption improve intestinal and metabolic health.

Another hallmark of obesity, adipose tissue dysfunction and CLS, was not analyzed in these studies, therefore the impact of kidney beans on adipose tissue morphology and inflammatory tone were not investigated and will be pursued in the future. Since the high-fat diets contained 5.1 kcal/g, less diet was consumed for sufficient energy intake compared to the low-fat diets. The bean supplementation level was therefore lower in mice consuming a high-fat diet, as high-fat fed mice consumed significantly less grams of food than the mice on a low-fat diet. Future studies comparing bean supplementation into low or high-fat background diets should account for such and increase the bean supplementation dose in the high-fat model, consequently the total amount of beans consumed will be similar between background diets.

Chapter 8: Conclusion

Overall, the results from this thesis substantiate Health Canada's New Food Guide, in that increased consumption of plant-based proteins in the form of cooked kidney beans, in their whole food form, can have health benefits. These results demonstrate that consumption of cooked kidney beans, at supplementation levels achievable by Canadians, can improve intestinal and metabolic health, in part through modifying the microbiota community structure, as well as it's activity in the production of SCFAs, and by improving serum hormones. Consumption of kidney beans of different seed coat colours, within the same gene pool, can produce different health effects, such as enhanced microbial activity (SCFA production), and improved colonic barrier integrity and inflammatory tone. These improvements on intestinal and metabolic health also depended on the background diet with which they were consumed; when consumed as part of a healthy diet, beans induced beneficial effects on both intestinal and metabolic health parameters, while when consumed as part of a diet high in fat, beans only improved parameters of intestinal health, with DK beans having the greatest effect in both studies. These studies taken together suggest that darker coloured beans may provide additional health benefits compared to lighter coloured beans of the same variety (e.g. black vs. navy bean, cranberry vs. white, dark-red vs. white kidney bean). The health effects of beans on intestinal and metabolic health also depend on background diet, as differences were seen when mice consumed beans as part of a low- or high-fat diet.

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