THE EFFECTS OF MANGO ON GUT MICROBIAL POPULATION AND ITS IMPACT ON BODY COMPOSITION AND GLUCOSE HOMEOSTASIS IN MICE FED HIGH FAT DIET

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Abstract

The gut microbial population is significantly compromised in high fat feeding. These changes are associated with obesity and type 2 diabetes (T2D). Fermentation of non-digestible carbohydrates and fiber from plant food sources are suggested to prevent gut dysbiosis due to high fat feeding. Various parts of mango have been studied for their anti-obesogenic, immunomodulatory and gastroprotective abilities. This study investigated the effects of 12-week freeze-dried mango pulp supplementation on the gut microbiota and its impact on body composition, glucose homeostasis and inflammatory markers in C57BL/6 mice fed a high fat (HF) diet. Male C57BL/6 mice were randomly assigned to 4 dietary treatment groups: Control (AIN-93M, 10% kcal from fat), HF (60% kcal from fat), and HF+1% or 10% mango. Cecal sample analyses by 16S rDNA sequencing show that HF feeding resulted in a significant loss of bacteria, most notably Bifidobacteria and Akkermansia while mango supplementation prevented the loss of these bacteria in a dose-dependent manner similar to control. Mango supplementation did not reduce body weight or fasting blood glucose. Plasma lipids were elevated with HF feeding compared to control, with both mango doses lowering plasma triglyceride. The HF+10% mango significantly lowered plasma non-esterified fatty acids but increased plasma total cholesterol. In comparison to the HF group, a dose-dependent increase in microbial fermentation was observed with mango supplementation, as evident in increased fecal and cecal acetic and butyric acid but not propionic acid. Furthermore, mango supplementation modulated gut inflammation, as observed with an increase in ileal and colonic IL-10 gene expression compared to the HF group. These results demonstrate that mango supplementation in high fat feeding modulated some of the adverse effects that accompanies high fat diet-induced obesity.

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CHAPTER I

INTRODUCTION

The incidence of obesity is reaching pandemic proportions globally and in the United States [1, 2]. Apart from genetic predisposition, obesity results from a long-term positive imbalance between energy intake and expenditure which may be regulated by multiple pathways. Importantly, the evolutionary change in our food behavior has introduced a diet rich in fat and refined sugars which are associated with increased incidence of various chronic diseases including obesity [3]. High fat diet-induced obesity has been reported to be associated with insulin resistance and type 2 diabetes (T2D) characterized by a state of inflammation and increased susceptibility to infection due to the malfunction of the immune system [4]. Furthermore, obesity induced by a high fat diet results in dyslipidemia which is a significant risk factor for cardiovascular diseases [5]. Therefore, these debilitating impacts of obesity make it an epidemic which is an unprecedented challenge to public health and civilized societies [6].

The human intestine serves as an environment for various bacteria and some archaea species living in a commensal relationship with the host [7, 8]. The gut microbial population has been reported to have a role in the maintenance of host's immune response and also in digestion, absorption and metabolism of nutrients [7, 9].

Through the hydrolysis and fermentation of dietary polysaccharides that the host cannot otherwise digest, the gut microbiota may increase host energy harvest up to ~150 kcal [10]. The undigested portion of food moves into the colon in humans or cecum in mice where it is fermented predominantly into short chain fatty acids (SCFA) [11]. SCFA resulting from microbial fermentation is vital for the maintenance of the colonic epithelial cells' integrity and may also be absorbed in the colon to impact liver and adipocyte metabolism [12, 13]. Moreover, SCFA have been shown to play a role in pancreatic insulin secretion by stimulating incretin production in the small intestine via the receptor GPR43 [14]. Importantly, the release of incretin is known to be responsible for 50-70% of insulin's postprandial response to glucose [15]. Thus, these reports show that the gut microbiota directly and indirectly influences the host metabolism.

Accumulating evidence has established that changes in the composition of the gut microbiota due to high-fat feeding play a significant role in obesity, insulin sensitivity, and obesity-associated inflammation characterized by an imbalance in pro-inflammatory and anti-inflammatory immune response [16, 17]. Cani and colleagues showed that high fat feeding in mice resulted in the dysbiosis of cecal microbiota compared to mice on a control diet, accompanied with increased body weight, fat accumulation, glucose intolerance and low grade inflammation [18]. Therefore, preserving the balance of the gut microbiota by feeding the commensal bacteria may help reduce the incidence of obesity and its detrimental outcomes [19].

There have been reports suggesting that the gut microbial population can be effectively preserved, manipulated or regulated through good quality dietary patterns [20]. Dietary food components believed to play a role in the preservation of the gut

microbiota include polyphenols, fiber and non-digestible carbohydrates [21]. These food components are known as promoting factors for growth and survival of the beneficial gut bacteria [21]. These reports suggest that foods high in fiber and polyphenols may help maintain the balance of the intestinal microbiota and thus, reducing the incidence of chronic diseases like obesity and T2D. This is important since gut microbial dysbiosis by high fat diets encourages colonization of the gut by pathogens, impairs the intestinal epithelial barrier, thus promoting the passage of bacteria and their products such as lipopolysaccharide into the blood, which are initiating factors of obesity and its comorbidities [22].   One of such foods rich in fiber and polyphenols is mango [23].

Mango (*Mangifera indica*), a popular tropical fruit, has been reported to possess a variety of therapeutic effects that are often dependent on the part of the plant used. Studies have indicated that polyphenolic extracts from various mango parts have antioxidant, antidiabetic, immunomodulatory and gastroprotective activities [24]. Moreover, recent studies involving high-fat feeding indicate that supplementation with freeze-dried mango pulp modulates blood glucose and reduces adiposity without a negative impact on skeletal health unlike the widely-prescribed glucose-lowering medication, rosiglitazone [25, 26]. Depending on the variety, mango pulp has a total fiber content ranging between 1.3 – 3.8 g/100g, with about 50% soluble fiber [27] which may be substantially available to the gut bacteria for fermentation. Observations from these previous studies strongly suggest that mango is a functional food that may be beneficial in supplying nutrients to maintain a healthy living of the gut bacteria and the host.

Since the gut bacteria requires fiber, non-digestible carbohydrates and polyphenols as growth substrates which help maintain a balance in the gut microflora, our

current knowledge is lacking in the impact of mango on maintaining the balance of the gut microbiota in the event of a high-fat diet challenge which results in dysbiosis. Therefore, the **purpose** of this study is to determine the effects of freeze-dried mango pulp on the gut microbiota and its impact on body composition, blood glucose, inflammatory markers and gut integrity in mice fed a high fat diet. The **hypothesis** of this study is that mango supplementation will preserve the balance of the gut microbiota while maintaining blood glucose and body fat composition in C57BL/6 mice fed high fat diet. Apart from contributing to the scientific literature available on mango, this study will provide novel evidence on the possible effects of mango fruit in preventing gut dysbiosis with possible corresponding impacts on glucose homeostasis and body composition. The **specific aims** of the study are to investigate:

1. The effects of dietary mango supplementation on cecal microbial population at the genus level.

    Working hypothesis: Mango supplementation will preserve the balance of the cecal microbiota by preventing the loss of beneficial bacteria in the gut which is usually associated with high-fat feeding.

2. The impact of mango supplementation on body composition, blood glucose and plasma lipids in C57BL/6 mice fed a high-fat diet

    Working hypothesis: Mango supplementation will modulate body composition and blood glucose while preventing dyslipidemia in C57BL/6 mice fed a high-fat diet.

3.   The impact of mango on cecal and fecal content of short chain fatty acids, and

GPR43 (SCFA receptor) expression in the ileum and colon mucosa of C57BL/6

mice fed a high-fat diet.

Working hypothesis: Mango supplementation will significantly increase short

chain fatty acid production, with a subsequent increased expression of their

receptor, GPR43 in C57BL/6 mice fed a high-fat diet. This will indicate a gut

protective effect of mango via SCFA-induced stimulation of GPR43 which is

known to mediate gut inflammatory activities and incretin secretion.

4.   The effects of mango on anti-inflammatory [i.e. interleukin (IL)-10] and pro-

inflammatory (IL-1β, IL-6) cytokine expression in the ileum, colon and plasma of

C57BL/6 mice fed a high-fat diet.

Working hypothesis: Mango supplementation will significantly attenuate the

overexpression of pro-inflammatory cytokines which is prevalent in high-fat diet-

induced obesity, with a subsequent increase in anti-inflammatory cytokines. This

will indicate that mango supplementation mediates a balance between pro- and

anti-inflammatory markers which is necessary for gut and body homeostasis.

CHAPTER II

LITERATURE REVIEW

The review includes an overview of the growing trend of obesity and type-2 diabetes, (T2D), composition of the gut microbiota, effect of diet on gut microbiota, the role of gut microbiota in nutrition and health, the role of gut microbiota in obesity and T2D, and the nutritional and health value of mango.

The Growing Trend of Obesity and T2D

Obesity is increasingly becoming a major public health problem globally. Available data suggest that obesity in adults is likely to keep accelerating in the near future due to increasing obesity in children and adolescents [28]. A study conducted among preschool children in urban areas of China indicated that the prevalence of obesity increased from 1.5% to 12.6% within a 7-year period [29].

In Europe, obesity is prevalent among women and especially in Southern and Eastern European countries [30]. In Great Britain specifically, it has been reported that the prevalence of obesity among adults more than doubled between 1980 and 2002 [31]. Somewhat surprisingly, this increasing trend of obesity has also been noted in

low-income countries, where most of the humanitarian assistance is driven towards combating starvation [32]. The United States is not immune from this pandemic. The prevalence of obesity (BMI ≥ 30) has continued to increase and the prevalence of overweight (BMI: 25 – 29.9) has also continued to increase in children and adolescents [33, 34]. Using the data from the National Health and Nutrition Examination Survey (NHANES 2003-2004), Ogden and colleagues [34] reported that 17.1% of US children and adolescents were overweight and 32.2% of adults were obese. Furthermore, 31% of those aged 60 years or older were reported to be obese in the year 2003 – 2004. Also, there is significant evidence that the United States has one of the highest obesity prevalence rates among western countries in the world [35]. Therefore, there is evidence that obesity is a growing problem globally and especially in the United States where it has been shown to be prevalent among all the age groups of the population.

The direct and indirect implications of obesity for a society are detrimental. For example, obesity plays a key role in development of other clinical disorders such as T2D, dyslipidemia, hypertension and cardiovascular disorders, with a prominent one being T2D [36, 37]. Various studies have reported the association of obesity with T2D and the increasing prevalence of T2D among the global population and especially in the United States. Wild *et al*., [38] estimated the global prevalence of T2D to be 2.8% in the year 2000 and are predicted to rise to 4.4% in 2030 among all age-groups [38]. Specifically, the total number of people with T2D is expected to rise from 171 million in 2000 to about 366 million in 2030 [38]. Moreover, the prevalence of T2D is higher in men than in women and with the proportion of people aged >65 years are the most affected [38].

Given the increasing prevalence of obesity, it is probable that these figures represent an underestimate of future T2D prevalence.

In the United States, T2D occurrences have been reported to have increased significantly over the years. Data from the National Health and Nutrition Examination Survey (NHANES, 1999-2002) showed that T2D cases increased from 5.1% between 1988-1994 to 6.5% from 1999- 2002 [39]. Moreover, a follow-up study on the data from NHANES reported that the prevalence of diagnosed T2D increased from 6.5% (1999-2002) to 7.8 % from 2003 to 2006, with significant increase noted in women, non-Hispanic whites and expectedly, in obese individuals [40].

The increasing prevalence of obesity and T2D comes with detrimental implications. Thus, there is an escalating demand for researchers and health professionals to find possible answers to curb the growing trend of these chronic and unhealthy states.

The Gut Microbiota

The human body habituates a unique population of microorganisms called the microbiota [41]. The microorganisms found in the human digestive tract, called the gut microbiota, are the subject of intense research. Microbial population densities may reach their maximum values in the colon with 10^{11} bacteria per gram of stool [42, 43]. These microorganisms colonize the gut right after birth, forming a complex community of organisms over the years that act as a barrier against the colonization of the gut by non-established or pathogenic species [43]. At the moment, a thorough description of all intestinal bacteria does not exist for two main reasons:

- Microscopic observations can only enumerate about 30% of the microorganisms via culture-based characterization [44-46].

- The gut bacterial species are highly diverse. Moreover, the use of molecular tools has indicated that the majority of the dominant bacterial species observed in the fecal microbiota of an individual is specific to that individual and is influenced by environment and diet [47-49].

Most of the dominant taxonomic groups of the gut microbiota have been known for a long time and may be determined through culture techniques while others have been demonstrated only recently via molecular approaches such as metagenomics and single gene approach based on ribosomal RNA [43]. Culturable genera of the dominant fecal microbiota of adults are: *Bacteriodes, Eubacterium, Ruminococcus, Clostridium* and *Bifidobacterium* [46, 50].

The non-culturable microorganisms include members of the phylum Firmicutes which comprises the *Eubacterium rectale – Clostridium coccoides,* representing up to 31% of total bacteria in the gut [51-53]. The phylum Firmicutes also comprises of the *Clostridium leptum* group with the species *Faecalibacterium prausnitzii, Ruminococcus albus* and *Ruminococcus flavefaciens* which represent about 16-22% of the dominant microbial group [53, 54]. Bacteroidetes are often present and share dominance with the above groups [43]. The phylum Actinobacteria is less consistently detected as dominant, but represents a few percentages of total bacteria. The phylum Actinobacteria comprises of bifidobacteria (0.7-10%) and bacteria of the *Collinsella-Atopobium* group (0.3-3.7% on average) [43]. Enterobacteria are more seldom observed in the top two logs of population of fecal microbiota (0.4-1%), similar to lactobacilli and streptococci (2%)

[54]. Other species that are found occasionally are related to *Clostridium ramosum, Eubacterium cylindroides, Phascolarctobacterium, Verrucomicrobium, Sporomusa, Selenomonas or Veillonella* [43].

The ability to isolate and grow microorganisms *in vitro* remains a key step in building our gut microbiota knowledge base, especially considering that phylogeny does not provide information on the in situ activity of microbes. Thus, phylogenetic reassessment of the intestinal microbiota has been essentially restricted to the dominant fraction and the available knowledge of the subdominant bacteria, i.e., below 10^8 per gram of stool, may be incomplete [43]. Our present knowledge indicates that the gut microbiota is constituted by seven phyla, namely Bacteroidetes, Firmicutes, Fusobacteria, Verrucomicrobia, Proteobacteria, Actinobacteria and Cyanobacteria [55]. Among these, the most abundant species are found in the Firmicutes and Bacteroidetes as they constitute about 90% of the human gut microbiota [55].

Methods for Assessing the Gut Microbiota

Sampling for gut microbiota analysis is often based on mucosal or stool samples [55]. However, ideal samples for gut microflora studies in humans may be mucosal samples since studies have shown that the biodiversity of fecal microbiota markedly differs from mucosal samples which may be more reflective of human disease [56, 57] . Due to the practical challenges posed by mucosal sampling in humans, stool samples are however often used as a proxy for gut microbiota studies due to the ease in collection compared to biopsy samples [55].

Though early techniques involved culture-based approaches, these could only provide limited information about the composition of the gut microbiota since most of these bacteria

species are obligate anaerobes and are thus difficult to culture [55, 58]. However, recent advancements with the development of microbial culture chips and gel microdroplets allow for the culture of previously uncultured microbes [55]. As further highlighted in Table 1, an advantage of the culture technique is its cost effectiveness although it is labor intensive [55].

Recently, culture-independent techniques have been developed to study the microbial composition of a sample. These methods target the 16S ribosomal RNA gene for bacterial identification since the 16S rRNA gene is the most conserved site and provides the highest variability for phylogenetic identification of different bacteria than the 5S and 23S rRNA genes [59, 60]. Initial analysis involves extraction of genomic DNA from mechanically disrupted stool samples. The double-stranded DNA is separated by high temperature, which is followed by annealing of the conserved regions on the 16S rRNA genes and subsequently followed by the creation of a new strand of dsDNA by DNA polymerase. Multiple repetitions result in the amplification of the 16S rRNA gene [55]. The 16S rRNA amplicons generated via PCR can then be subjected to various culture-independent techniques including denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), fluorescence *in situ* hybridization (FISH), DNA microarray and sequencing techniques [55]. The description of these techniques, advantages and disadvantages are highlighted in Table 1.

The most recent technique developed to study the microbiome is known as metagenomic sequencing (also called metagenomics) [55]. Unlike the 16S rRNA gene profiling which only allows for understanding the composition of the gut microbiota and making comparisons between a healthy state and a diseased state, metagenomics allows for a more detailed understanding the biological and clinical significance, or the functional potential of the bacterial community present [55, 58]. This has the potential to take investigators into the next step of identifying which organisms are present and what they do. However, the computational analysis of data and bioinformatics that comes with it can be very daunting (Table 1) [55].

Table 1: Assessment techniques for gut microbiota characterization. (Fraher *et al*., [55])

Technique	Description	Advantages	Disadvantages
Culture	Bacteria isolation on selective media	Cost-effective, semi-quantitative	Labor intensive, limited culturable organisms
qPCR	Amplification and quantification of 16S rRNA. Reaction mixture contains a compound that fluoresces when it binds to dsDNA	Plylogenetic identification, quantitative, fast	PCR bias, unable to identify unknown species
DGGE/TGGE	Gel separation of 16S rRNA amplicons using denaturant/temperature	Fast, semi-quantitative, bands can be excised for further analysis	No phylogenetic identification, PCR bias
T-RFLP	Fluorescently labelled primers are amplified and then restriction enzymes are used to digest the 16S rRNA amplicon. Digested fragments separated by gel electrophoresis	Fast, semi-quantitative, cheap	No phylogenetic identification, PCR bias, low resolution
FISH	Fluorescently labelled oligonucleotide probes hybridize complementary target 16S rRNA sequences. Following hybridization, fluorescence can be enumerated using flow cytometry	Phylogenenetic identification, semi-quantitative, no PCR bias	Dependent on probe sequences, cannot identify unknown species
DNA microarrays	Fluorescently labelled oligonucleotide probes hybridize with complementary nucleotide sequences. Fluorescence detected with a laser.	Phylogenetic identification, semi-quantitative, fast	Cross hybridization, PCR bias, species present in low levels can be difficult to detect
Cloned 16S rRNA gene sequencing	Cloning of full-length 16S rRNA amplicon, Sanger sequencing and capillary electrophoresis	Phylogenetic identification, quantitative	PCR bias, laborious, expensive, cloning bias
Direct sequencing of 16S rRNA amplicons	Massive parallel sequencing of partial 16S rRNA amplicons	Phylogenetic identification, quantitative, fast, identification of unknown bacteria	PCR bias, expensive, laborious
Microbiome shotgun sequencing	Massive parallel sequencing of the whole genome (e.g 454 pyrosequencing, or Illumina)	Phylogenetic identification, quantitative	Expensive, intense computational data analysis

Abbreviations: qPCR: quantitative polymerase chain reaction, DGGE: denaturing gradient gel electrophoresis; TGGE: temperature gradient gel electrophoresis, T-RFLP: terminal restriction fragment length polymorphism, FISH: fluorescence *in situ* hybridization

Effect of Diet on the Gut Microbiota

The impact of diet on the composition of the gut microbiota commences early in life. Colonization of the gut begins at birth and following an initial irregular community structure during the first year of life, the human gut microbiota becomes more stable and adult-like, coinciding with the introduction of solid foods into the diet [61, 62]. Pediatric studies have consistently shown that there is higher proportion of Bifidobacteria in breast-fed infants as compared to formula-fed infants [63, 64]. Human milk oligosaccharides are considered functional growth factors for the beneficial gut bacteria, as inhibitory receptors binding to different pathogens, and promote the development of the early immune system [65]. Generally, dietary components that impact the gut microbiota include non-digestible food components and polyphenols.

Non-digestible Food Components

Diet directly influences microbial composition and metabolic activity by making substrates available in the form of undigestible dietary residues that are resistant to digestive enzymes and the digestion process [66]. As the non-digestible food components reach the human colon, they interact with microbiota and epithelial cells, serving as food to the resident colonic bacteria and stimulating their fermentative capacity [66, 67]. All plant food constituents that are resistant to digestion and absorption in the human small intestine, with complete or partial fermentation in the large intestine are part of the indigestible fraction serving as dietary substrates to the microbiota [68]. This indigestible fraction comprises of non-digestible carbohydrates such as non-starch polysaccharides, resistant starch and oligosaccharides, and also other non-carbohydrate compounds such

as lignin, resistant protein, polyphenols and carotenoids which possess antioxidant properties [68].

The non-digestible carbohydrates are all together broken down to intermediate products such as lactate, succinate, pyruvate, and short chain fatty acids (SCFA) such as acetate, propionate and butyrate by the gut bacteria [69]. The SCFA may serve as food to the gut microbiota and they may also be absorbed for the body's use. For example, butyrate has been described to promote the proliferation of beneficial bacteria species in the gut [67], while acetate and propionate has been revealed to impact liver and adipose metabolism [12, 13] .

The diet undoubtedly has an influence on the gut environment. Along with a reduction in gut transit time, increase in fiber intake has been reported to also increase total bacterial numbers and concentrations of bacterial fermented products [70]. Simultaneously, an increase in bacterial colonic fermentation results in a decrease in the pH in the proximal colon which results from high concentrations of short-chain fatty acids [71]. Previous studies have shown the importance of this colonic decrease in pH made possible by high fiber intake. Duncan and colleagues [72] reported that a one-unit decrease in pH (6.5 to 5.5) had a significant effect on selective species of the colonic microbial community, with a tendency to suppress *Bacteroides spp.* and promoting butyrate-producing gram-positive bacteria, such as *Roseburia spp* and *Eubacterium rectale.*

Furthermore, a decreased total carbohydrate intake in weight loss diets is often expected to be accompanied by some reduction in dietary fiber and resistant starch. The

provision of such diets to obese subjects has been shown to result in decreased

concentrations of microbially-produced SCFA in fecal samples, together with a

significant decrease in the proportion and total numbers of Bifidobacteria and butyrate-

producing Lachnospiraceae related to Roseburia [73]. Therefore, it is expected that non-

digestible food fractions and diet-driven changes in pH and gut transit influenced by

fiber, will be reflective on the composition of the gut and fecal microbiota.

Polyphenols

Polyphenols are chemical compounds containing more than one hydroxyl group

attached to a benzene ring [74]. These compounds are produced by plants and are

generally classified as flavonoid and non-flavonoid compounds [74]. Polyphenols are

considered beneficial to human health due to their free-radical scavenging ability, thus

preventing membrane lipid oxidation [75]. Apart from the antioxidant ability of

polyphenols, they may also be anti-inflammatory due to their ability to modulate the

expression of NF-kB related genes involved in inflammation [74].

Unlike non-digestible carbohydrates, polyphenols do not lead to production of

SCFA [76]. In foods and beverages, a substantial part of total phenolic compounds can be

accounted for by the polyphenols bound to the food indigestible fraction [77]. While a

small proportion of some dietary polyphenols can be absorbed through the small

intestine, the majority are either not absorbed and become fermentable substrates for

bacterial microflora in the colon along with the non-digestible food fraction [78]. The gut

microbiota promotes the biotransformation of these polyphenols in the colon, making

them available for colonic absorption [79].

Our understanding of the impact of polyphenols on the gut microbiota is evolving. It has been reported that dietary polyphenols may play a significant role in the modification of the gut microbial community [80, 81]. *In vitro* evidence has shown that flavonoids such as naringenin, diosmetin, ponciretin, and hesperetin inhibited the growth of pathogenic bacteria such as *Helicobacter pylori* [82] while catechin prevented the growth of *Clostridium histolyticum* [83]. Furthermore, a diet rich in tannins or polyphenols significantly slowed down the growth of *Clostridium spp* with a simultaneous stimulation of *Bacteroides* and *Lactobacillus* [84, 85]. Therefore, apart from their antioxidant and free-radical scavenging capacity, polyphenols may play a role in modifying the gut microbiota.

The Role of Gut Microbiota in Nutrition and Health

As previously noted, the food non-digestible fraction serves as a substrate for the survival of the microbiota. In turn, the microbiota metabolizes the non-digestible fraction to produce a variety of products, including SCFA. Absorption of microbially-produced SCFA provides additional energy to the host from dietary components that have remained undigested in the small intestine [9]. Therefore, the gut microbiota contributes to the energy harvest from the diet, and this contribution might be essential under conditions of food scarcity [86].

The caloric value per mole of non-digestible carbohydrate is considerably lower than that of a fully digestible carbohydrate and is also dependent on the extent of microbial fermentation and SCFA absorption [87]. For example, glucose and fructose

with a caloric value of 3.9 kcal/g produces approximately 38 mol ATP/mol while inulin and oligofructose has a caloric value ranging between 0 and 2.5 kcal/g, and may produce up to 17 mol ATP/mol [87]. This implies that directly replacing digestible carbohydrate by non-digestible carbohydrate in the diet should reduce the net delivery of calories to the host, assuming equal intake [9]. Evidence indicates also that the action of the intestinal microbiota on non-digestible carbohydrates might contribute to satiety [88].

Furthermore, the gut microbiota is essential in the metabolism of polyphenols. They are capable of performing a range of biotransformations on polyphenols that pass into the colon, thus affecting polyphenol absorption and bioavailability [89]. The genera *Clostridium* and *Eubacterium* have been identified as involved in the metabolism of many phenolic compounds such as isoflavone (daidzein), flavonol (quercetin and kaempferol), flavonone (naringenin and isoxanthumol), and flavan-3-ol (catechin and epicatechin) which enhances their absorption [79]. In addition, colonic fermentation of polyphenols yield a broad spectrum of absorbable biotransformation products, which include valeric acid, valerolactone, phenylpropionic acid, phenylacetic acid, phenylbutyric acid, phloroglucinol, urolithin A and urolithin B [76]. The biotransformation and promotion of polyphenol absorption by the gut microbiota may thus be vital in assessing the wide health benefits of these plant chemicals.

Some species of *Bacteroides, Clostridium and Eubacterium* are known to differ from the beneficial bacteria and thus considered detrimental because they possess enzymatic activities related to the generation of carcinogens [90]. In contrast, certain species belonging to the genera *Lactobacillus* and *Bifidobacterium* are considered

beneficial microorganisms and are commonly used as probiotics in the manufacture of functional food products [76].

The gut microbiota is considered to play an important role in the prevention of sporadic colorectal cancer through the production of butyrate and the transformation of certain dietary polyphenols [91]. However, cancer-promoting compounds can also be produced by microbial activity, thus, the balance of procarcinogenic and anticarcinogenic microbial actions is vital and has been reported to be highly dependent on diet and xenobiotic intake [92]. A 2012 study conducted by Wang and colleagues [93] reported a change in gut bacteria, especially butyrate producers, in patients with colorectal cancer and healthy control groups.

Other health properties have been attributed to the beneficial bacteria in the gut. These include inhibition of a wide range of pathogens, improvement of lactose digestion, reduction of serum cholesterol, stimulation of anti-inflammatory cytokine production, reinforcement of intestinal epithelial cell tight junctions and increased mucus secretion [94-96].

Metabolism and Benefits of Short-Chain Fatty Acids

The production of SCFAs largely depends on the consumption of non-digestible carbohydrates from the diet such as resistant starch and fiber [97]. The main SCFAs resulting from colonic fermentation are acetate, propionate and butyrate [97]. The production of these SCFAs rely on the microbiota composition and environmental conditions such as substrate availability, pH and hydrogen partial pressure [97]. Acetate

is produced from H_2 and CO_2 by acetogenic bacteria such as *Blautia hydrogenotrophica,* a strict anaerobe belonging to the Firmicutes family [98]. Acetate may also be produced from formate through the Wood-Ljungdahl pathway by *B. hydrogenotrophica* [98].

Propionate is majorly produced by Bacteroidetes and few Firmicutes such as *Veillonella* spp. and *Dialister* spp. through the succinate pathway [97]. Moreover, propionate may also be produced via the acrylate pathway, which uses lactate as a substrate or via the propanediol pathway by some Firmicutes and Proteobacteria, using deoxyhexose sugars like rhamnose as substrates [97].

Butyrate is largely produced by some Firmicutes using the enzyme, butyryl-CoA:acetate CoA-transferase and to lesser extent, butyrate kinase and phosphotransbutyrylase [99]. The species belonging to the Firmicutes that utilizes acetate the butyryl-CoA:acetate CoA-transferase enzyme include *Anaerostipes spp, Roseburia spp., E. hallii, F. prausnitzii, and E. rectale* [97]. Furthermore, *E. hallii* and *Anaerostipes spp* are capable of producing butyrate from both acetate and lactate [100].

The SCFAs have been established to possess a wide range of beneficial effects both within and outside the gut. Within the gut, butyrate is preferred as a source of energy by intestinal epithelial cells while propionate is majorly metabolized in the liver [97]. Also, microbial production of SCFAs may play a vital role in glucose homeostasis via incretin secretion. Strong evidence from animal models show that SCFAs may interact with their receptors, free fatty acid receptors 2 and 3 (FFAR2 and FFAR3) also known as GPR43 and GPR41 respectively, to induce the secretion of incretins from the enteroendocrine L and K cells of the intestine [14, 101]. Furthermore, intracellular

propionate and butyrate have been reported for their anti-inflammatory ability, capable of down-regulation of interleukin-6 (IL-6) and IL-12 in colonic macrophages [102-104]. Moreover, butyrate and propionate have been shown to induce the differentiation of T-regulatory cells expressing the transcription factor, forkhead box P3 (FOXP3) [105, 106]. This process may be vital in the control of gut inflammation, since T-reg cells are important to tolerize the gut by tempering the pro-inflammatory status which may be created by the presence of commensal bacteria or pathogens [107] .

Extracellular SCFAs are also capable of vital interactions with a class of cell-surface receptors known as G protein-coupled receptors [108]. While GPR109A recognizes only butyrate, GPR43 interacts with all three major SCFAs [109, 110]. The anti-inflammatory and gut-protective abilities of these SCFAs associated with high fiber intake have been proposed to be via their interactions with these receptors [97, 111]. For example, the interaction of butyrate with GPR109A has been reported to promote the differentiation of Treg cells and IL-10 producing T cells, inhibit the activation of nuclear factor kappa B (NF-KB) [110, 112]. This is also similar to the effect of acetate and propionate interaction with GPR43 [113].   These beneficial effects of the SCFAs are not only important for host cells but also contribute to the gut microbiota homeostasis [97].

The Gut Microbiota in Obesity

Obesity is considered a worldwide epidemic, a major health problem in both developed and developing countries. It has many complications, as it is a significant risk factor for other diseases such as T2D, cardiovascular diseases and cancer [114, 115].

Apart from the widely known causes of obesity, it has recently been associated with a modification in microbiota, including a higher *Firmicutes/Bacteroidetes* ratio and a decrease in *Methanobrevibacter smithii* [86, 116].

In addition, other studies have further associated imbalances in gut microbial population with obesity [117, 118]. Ridaura and colleagues [117] notably reported that transplanting the fecal microbiota of obese humans into germ-free mice increased fat-mass and obesity-related metabolic phenotypes in these mice compared to when corresponding fecal microbiota from lean monozygotic twins was transplanted into another group of germ-free mice. Another study associated the mucin-degrading bacterium *Akkermansia muciniphila* with obesity and T2D [118]. The study showed that the abundance of *A. muciniphila* decreased in obese and T2D mice and that treatment with prebiotic normalized *A. muciniphila* levels, which in turn correlated with an improved metabolic profile [118].

Furthermore, high-fat diets have been reported to result in a high degree of gut microbial dysbiosis which is often characterized by reduction in Bifidobacterium spp, Bacteroides-related bacteria, Lactobacillus spp and Roseburia spp, with Firmicutes generally becoming more abundant [22, 119, 120].

Although alterations in the gut microbiome in obesity has been widely established, the mechanisms by which they might contribute to obesity is currently being investigated. A study from germ-free and conventionalized mice showed that conventionalization resulted in a significant increase in small intestine villi capillary density, leading to enhanced uptake of monosaccharide, with the promotion of hepatic

and adipose fat accumulation via sterol regulatory element binding protein-1 (SREBP-1) –regulated lipogenesis [121]. Furthermore, the gut microbiota promotes triglycerides storage in the adipocytes via the suppression of intestinal fasting-induced adipocyte factor (FIAF), which is known to inhibit adipose tissue lipoprotein lipase [121]. Moreover, the gut microbiota has been implicated in the promotion of liver steatosis, by reducing the bioavailability of dietary choline which is vital in VLDL synthesis and secretion in the liver [122]. In addition, the microbiome of mice fed a western diet (high fat, high sugar) was enriched in pathways involved in fermentation of simple sugars and glycans, coupled with a significant increase in phosphotransferase enzymes vital in the import of simple sugars [123]. These evidences strongly show that the gut microbiota may contribute to obesity via increase in dietary energy harvest, and distorted fatty acid metabolism.

Interestingly, the gut microbiota has been proposed as a significant link between obesity and its associated comorbidities [124]. Obesity is associated with elevated levels of bacterial lipopolysaccharide (LPS), which is the major component of the outer membrane of gram-negative bacteria [125, 126]. The transit of LPS into the circulatory system, known as metabolic endotoxemia, reflects passage of bacterial fragments across the intestinal epithelial layer into the systemic circulation, partly due to increased intestinal permeability [127]. Endotoxemia is associated with the loss of gut *Bifidobacterium* spp., which is known to increase/maintain mucosal barrier function against pathogenic bacteria and bacterial antigens [128, 129]. Furthermore, increased intestinal permeability in obesity may be due to an impaired epithelial tight junction [130]. Feeding a high-fat diet in mice significantly reduced the expression of occludin

and tight junction protein 1 (ZO-1) which are epithelial tight junction proteins, resulting into increased entry of LPS into the blood [130]. LPS is capable of triggering an innate immune response by binding to the CD14/TLR-4 complex [131]. The result of this is an inflammatory state, characterized by elevated NF-kB mediated production of proinflammatory cytokines, especially TNF-α and IL-6, thereby promoting tissue insulin resistance [131, 132]. Moreover, it has also been reported that endotoxemia is a significant risk factor for development of early atherosclerosis, linking LPS and cardiovascular disease [133]. The results of these studies reveal that the gut microbiota might play a significant role in the pathogenesis of obesity and its detrimental outcomes.

Nutrition and Health Value of Mango

Mango, also known as *Mangifera indica*, is a tropical fruit which varies in shape and size. It contains a thick yellow pulp, single seed and thick yellowish-red skin when ripe [24]. The seed is solitary, ovoid and enclosed in a hard, compressed fibrous endocarp [24].

Although there are variations in the nutritional composition of mango based on the variety, it is generally a rich source of fiber and polyphenols which, as discussed previously, are essential substrates in maintaining the balance of the gut microbiota, and thus, preventing immune dysregulation [23, 70, 77]. A study which investigated the total phenolic content and *in vitro* antioxidant capacity of five varieties of mango showed that Kent, Keitt, Haden and Tommy Atkins had similar total phenolic contents and antioxidant capacity [134]. However, this study observed that in comparison to other

varieties, the *Ataulfo* mango had significantly higher total phenolics and *in vitro* antioxidant capacity as measured by DPPH radical scavenging activities [134]. Furthermore, a recent study which reported the composition of two mango varieties noted that the total dietary fiber content of Ataulfo and Tommy Atkins mangoes were 225mg/g and 387mg/g (dry weight), respectively [23]. Furthermore, mango has a total fiber content comprising of about 50% soluble fiber [135, 136].

The medicinal value of mango seems to be dependent on the parts of mango tree and its cultivar. Although mango is often obtained from the diet in the form of its pulp, other parts of the mango fruit (skin) and the tree (bark and leaves) have been predominantly studied [26]. Mango is regarded as a valuable dietary source of phytochemicals which provide health benefit for the nervous system [135]. Also, mango fruit is a good source of phenolic compounds such as mangiferin and flavonoids, which may contribute to its antioxidant and anti-inflammatory properties [137]. Other phenolic compounds which has been reported in mango fruit are gallic acid, chlorogenic acid, vanillic acid and protocatechuic acid, with increasing antioxidant contributions during ripening [138].

Various evidences exist of the beneficial effects derived from different parts of mango. *In vitro* studies revealed that extracts from mango fruit peel inhibit adipogenesis in a 3T3-L1 pre-adipocyte cell line, with inhibition potency dependent on the variety of mango [139]. Similarly, treatment of 3T3-L1 adipocytes with mango seed kernel extract was reported to inhibit adipogenesis by down-regulating the adipogenic transcription factors peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT/enhancer binding protein alpha (C/EBPα) [140]. Also, evidence showed that

mango may possess chemopreventive property as reported in the study of Noratto *et al* [141] , which revealed that mango pulp extract of the Ataulfo variety inhibited growth of colon SW-100 cancer cells by 72% and induced the expression of pro-apoptotic biomarkers.

Furthermore, various studies using animal models have established the potential health benefits of mango. Sharma and others [142] studied the hypoglycemic potential of mango leaves in diabetic rats and reported a significant hypoglycemic effect of the leaf extract at a dose of 250 mg/kg suggesting an anti-diabetic effect of mango leaves. In addition, ethanolic extract of mango fruit has been reported to improve age-related and scopolamine-induced cognitive deficit in mice [143]. Using a high-fat animal model, Lucas and colleagues [26] reported that freeze-dried mango pulp modulates blood glucose in a similar pattern to the popular glucose-lowering medication, rosiglitazone (Avandia), used in the treatment of T2D and which has been associated with increased bone fractures and rapid bone loss. Interestingly in this study, it was reported that mango preserved bone parameters and thus, skeletal health was not affected in contrast to rosiglitazone [26]. In addition, 1% and 10% mango pulp supplementation in high fat diet-fed mice resulted in a significant reduction in percent body fat to levels similar to control animals while the 1% dose lowered blood glucose levels independent of body weight gain [25].

Few studies have investigated the benefits of mango supplementation in humans [144, 145]. A 12-week freeze-dried mango supplementation in obese adults resulted in the reduction of fasting blood glucose in these subjects [144]. Furthermore, normolipidemic volunteers who consumed whole mango or fresh cut mango for 30 days

had a reduction in fasting plasma triglyceride levels with an increase in plasma antioxidant capacity [145]. These studies suggest that mango may be an affordable alternative in managing high fat diet-induced fat accumulation and T2D. Other reported health benefits of mango has been reviewed in detail by Shah *et al*. (12). Due to the reported health benefits of mango, the purpose of this study is to determine the effects of freeze-dried mango pulp on the gut microbiota and its impact on body composition, blood glucose, inflammatory markers and gut integrity in mice fed a high fat diet.

CHAPTER III

METHODS

Animals and Treatment Groups

Sixty 6-week old male C57BL/6 mice were purchased from Charles River Laboratory (Portage, MI). Following a 1-week acclimatization period, mice were randomly assigned to one of four treatment groups (Table 2) for 90 days. Mice were housed in groups of 3-4 mice per cage. To minimize corophagic activity, wire bottom cages were used for housing.

Table 2: Treatment groups

Group	Dietary Treatment (n= 15 mice/group)
1	Control diet (10% kcal from fat)
2	High fat diet (HF; 60% kcal from fat)
3	HF+1% freeze-dried mango diet (w/w)
4	HF+10% freeze-dried mango (w/w)

For the mango diet, the variety and doses were based on our earlier study [25]. Ripe mango of the Tommy Atkins variety were purchased from a local grocery store and peeled. The pulp were freeze-dried, ground, and added at a dose of 1 and 10 % (w/w).

Mango composition was determined at NP Analytical laboratory (St. Louis, MO) (Table 3). All diets were prepared at Harlan-Teklad Laboratories (Madison, WI) and have the same macronutrient, calcium, and phosphorus content (Table 4).

Table 3: Mango composition

Analyte	Amount (%)
Moisture	2.076
Protein	4.735
Fat	1.345
Fiber	4.225
Ash	2.235
Calcium	0.069
Phosphorus	0.0845
Carbohydrate	89.6

Mice were given access to food and deionized water *ad libitum*. Food intake was monitored thrice a week and body weights recorded on a weekly basis. All procedures strictly adhered to the guidelines set forth by the Oklahoma State University Animal Care and Use Committee.

Table 4: Diet composition (g/kg)[1]

Ingredients	Control	High Fat (60% Fat)	HF + 1% Mango	HF + 10% Mango
g / kg diet				
Mango	-	-	10	100
Casein	140.00	180.00	179.46	174.56
L-Cystine	1.80	1.80	1.8	1.80
Corn Starch	465.692	116.00	107.1145	27.327
Maltodextrin	155.00	155.00	155.0	155.00
Sucrose	100.00	106.826	106.826	106.826
Lard	-	310.00	310.0	310.00
Soybean Oil	40.00	40.00	39.871	38.569
Cellulose	50.00	50.00	49.5775	45.775
Mineral Mix[2]	35.00	13.4	13.4	13.40
Calcium Carbonate	-	6.979	6.978	6.97
Calcium Phosphate, dibasic	-	7.487	7.465	7.265
Vitamin Mix (TD 94047)[3]	10.00	10.00	10.0	10.0
Choline Bitartrate	2.50	2.50	2.5	2.5
Tert-butylhydroquinone (TBHQ) Antioxidant	0.008	0.008	0.008	0.008

[1]Prepared at Harlan-Teklad Laboratories (Madison, WI)
[2]Complete mineral mix (TD94049, Harlan-Teklad Laboratories) was used for the control diet and a calcium and phosphorus deficient mineral mix (TD 98057, Harlan-Teklad Laboratories) was used for the mango and HF diets.
[3] Harlan-Teklad Laboratories (Madison, WI)

Necropsy and Tissue Processing

At the end of the 90 days treatment, mice were fasted for three hours starting at 5 am, with mice having access only to water. After fasting, the body composition of each mouse was assessed using a whole body PixiMus scan (GE Lunar, Madison, WI). Afterwards, mice were bled from their carotid artery and blood was collected in microcentrifuge tubes (Axygen Inc, Union City, CA) coated with EDTA (Amresco,

Solon, OH) as an anticoagulant. Plasma samples were obtained by centrifugation of whole blood samples at 4ºC for 10 minutes at 1500 x g . An aliquot of each plasma sample was transferred into microcentrifuge tubes and stored at -80ºC until analyses.

The liver, white adipose tissue, pancreas, spleen, and thymus were snap-frozen for later analyses. The ileum and jejenum were flushed with ice-cold saline (0.9% NaCl) and Peyer's patches were removed from the ileum. The Peyer's patches and ileum were then snap-frozen and stored at -80ºC for future analyses. Furthermore, the colon was flushed with ice-cold saline, and an incision was made to open it up. The colon was placed on a glass board with the lamina propria (LP) facing up. The colon lamina propria was removed by gently scraping with the edge of a glass slide. The colon LP samples were collected in microcentrifuge tubes and stored at -80⁰C for gene expression analyses. In addition, the cecum was harvested and its contents were flushed into pre-weighed and pre-cooled 15 mL centrifuge tubes (VWR Intl. LLC Randor, PA) with ice-cold saline, after which the cecal tissue was weighed and snap-frozen. Flushed cecal contents were centrifuged, (4ºC, 5 minutes, 1800 x g) and the supernatant discarded. Samples were kept frozen at -80ºC until analyses of cecal microbiota.

Measurements and Assays

Gut Microbiota

For the determination of possible changes in the gut microbiota affected by mango supplementation, frozen cecal samples were shipped overnight on dry ice to Second Genome Inc (San Francisco, CA). From the cecal samples, Second Genome

performed DNA isolation with the MoBio PowerMag Microbiome kit (Carlsbad, CA) followed by concentration normalization. To ensure all samples met minimum concentration and DNA mass, samples were quantified using the Qubit Quant-iT dsDNA Broad-Range Kit (Invitrogen, Life Technologies, Grand Island, NY). This was followed by DNA amplification so as to enrich the samples for bacterial 16S V4 rDNA region. This was done using fusion primers designed against surrounding conserved regions which are tailed with sequences incorporating Illumina adapters and indexing barcodes (San Diego, CA). Samples were PCR amplified with two differently barcoded V4 fusion primers, and their amplification products quantified by qPCR. Samples that met post-PCR quantification minimum were used for pooling and sequencing. A pool containing 16S V4 enriched, amplified and barcoded samples were loaded into the cartridge on a MiSeq instrument (Illumina, San Diego, CA) for cluster formation. This was followed by sequencing for 2x250 cycles using custom primers designed for pair-end sequencing.

In order to determine the Operational Taxonomic Unit (OTU) present in the samples, sequenced pair-end reads were merged and dereplicated with USEARCH as described by Edgar [146]. Unique sequences were then clustered at 97% similarity by UPARSE and a representative consensus sequenced per *de novo* OTU was determined. Representative OTU sequences were then assigned taxonomic classification via Mothur's Bayesian classifier trained against the Greengenes reference database of 16S rRNA gene sequences with 80% classification confidence.

Glucose Tolerance Test (GTT)

GTT was performed on the 87th day of dietary treatment. Mice were fasted for 6 hours prior to GTT. Each mouse was injected intraperitoneally with a 20% glucose solution at a dose of 2 g/kg body weight. Blood glucose was measured from tail blood samples at 0, 5, 15, 30, 60, and 120 minutes post glucose challenge, using an AlphaTrak glucometer (Abbott Laboratories, Illinois, USA).

Determination of Plasma Lipids

Frozen plasma samples were allowed to thaw on ice and the concentrations of total cholesterol, triglycerides, high density lipoproteins (HDL), and non-esterified fatty acids (NEFA) was determined using the automated analyzer, BioLis 24i (Carolina Chemistry, Winston-Salem, NC) following manufacturer's instructions. For cholesterol determination, cholesterol esters are hydrolyzed to free cholesterol and fatty acids by cholesterol esterase. The free cholesterol generated is subsequently oxidized by cholesterol oxidase to cholesterol-4-en-3-one and hydrogen peroxide which upon the action of peroxidase, forms a quinone dye which can be read at 505 nm giving a proportional value to the total cholesterol present in the sample. The principle of triglyceride determination involves lipase hydrolysis of triglycerides in the sample to glycerol and free fatty acids. This is followed by 3 coupled enzymatic steps which uses glycerol kinase, glycerophosphate oxidase and peroxidase, resulting in the formation of a colored complex which forms a red quinoneimine which can be read at 520 nm with the value directly proportional to the triglyceride concentration in the sample. HDL determination involves an initial disruption of the HDL lipoprotein to release cholesterol

followed by enzymatic reactions used for total cholesterol. NEFA measurement follows

the principle of formation of acyl-CoA when NEFA is exposed to acyl-CoA synthetase in

the presence of ATP and CoA. Acyl-Coa is oxidized by acyl-CoA oxidase to produce

hydrogen peroxide which allows for the condensation of 3-methyl-N-ethyl-N-(b-

hydroxyethyl)-aniline with 4-aminoantipyrine in the presence of an added peroxidase to

form a purple-colored product that can be measured at 550 nm, correlating to the amount

of NEFA present in the sample.

Determination of Plasma Incretins, Cytokines and Adipokines

Concentrations of plasma cytokines and adipokines were determined using the

Bio-Plex suspension array system (Bio-Rad Laboratories Inc, Hercules, CA) according to

manufacturer's instructions. This system uses fluorescently dyed nanobeads with unique

individual spectral address to allow multiple detection of different molecules in a single

well of a 96-well plate [147]. For the determination of plasma cytokines, a mouse 23-Plex

assay (Bio-Rad Cat No: #M60-009RDPD), consisting of fifteen cytokines (IL-1α, IL-1β,

IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-17A, interferon

gamma [IFN-γ], TNF-α), six chemokines (eotaxin, monocyte chemoattractant protein

[MCP-1], macrophage inflammatory protein alpha [MIP1α], MIP-1β, regulated on

activation, normal T expressed and secreted [RANTES], keratinocyte chemoattranctant

[KC]) and two growth factors (granulocyte colony stimulating factor [G-CSF],

granulocyte macrophage colony stimulating factor [GM-CSF]) was used. Moreover, for

the determination of plasma adipokines, a mouse 8-plex assay kit (#171-F7001M),

consisting of ghrelin, gastric inhibitory peptide (GIP), glucagon-like peptide 1 (GLP-1),

glucagon, insulin, leptin, plasminogen activator inhibitor 1 (PAI-1) and resistin was used. Both assays were run according to instructions included with the Bio-Rad kits.

Briefly, plasma samples were reconstituted in sample diluent (1:4 v/v). Thereafter in a 96-well plate, 50 μL of diluted samples and standards (in duplicates) were mixed with 50 μL diluted 1x capture antibody-coated magnetic beads, covered with aluminum foil, and shaken with an orbital shaker at 850 rpm at room temperature for 30 minutes. After three washes with 100 μL of wash buffer, 25 μL of detection antibodies was added, the plate was covered with aluminum foil, and shaken again at 850 rpm for 30 minutes. Following three washes with 100 μL of wash buffer, 50 μL of streptavidin-phycoerythrin (SA-PE) was added to detect each captured analyte, covered with aluminum foil and shaken at 850 rpm for 10 minutes. Beads were resuspended in 125 μL of assay buffer and analytes were thereafter quantified using a Bio-Plex MAGPIX Multiplex Reader (Bio-Rad Laboratories Inc, Hercules, CA). Concentrations of analytes were determined using the Bio-Plex Manager (6.1) software.

SCFA analysis

The gut microbiota metabolize non-digestible food fraction into a variety of product, most notably short chain fatty acids [69, 78]. Thus, cecal and fecal SCFA content from mouse samples was determined according to a previously published method with modifications [148]. Three to four cecal samples were pooled in order to obtain a representative sample. Pooled cecal or fecal samples (1 g) were suspended in 900 μL or 4.5 mL of ice-cold Millipore H_2O (for cecal and fecal samples, respectively). An internal standard (10 mM of 2-ethylbutyric acid in 12% formic acid) was spiked into the sample

suspension resulting in a final concentration of 1 mM internal standard, and homogenized for 1 minutes or 3 minutes (for cecal and fecal samples, respectively). The pH of the resulting cecal and fecal homogenates was adjusted to 2 -3 using 5 M HCl. Samples were incubated at room temperature for 10 min followed by centrifugation (2200 x g, 20 min, 15°C). The resulting supernatants were filtered using 0.45 μm PTFE syringe filters (VWR, Cat No: 28145497) into a GC glass vials (Agilent Technologies, Santa Clara, CA). Gas chromatographic analyses were done at Robert M. Kerr Food and Agricultural Products Center (Oklahoma State University, Stillwater, OK) .

GC analysis was carried out using an Agilent 6890N GC system with a flame ionizable detector (FID) and an N10149 automatic liquid sampler (Agilent Technologies, Santa Clara, CA). The column used was a fused-silica capillary column with a free fatty acid phase (DB-FFAP, Product #: 125-3237, Agilent Technologies, Santa Clara, CA), with hydrogen supplied as the carrier gas at a flow rate of 14.4 mL/min. The initial oven temperature was held for 0.5 minutes at 100°C. This was raised to 180°C for 1 minute at a rate of 8°C/min, then to 225°C for 10 minutes at 45°C/min. Sample (1 μL) was injected into a splitless injection port with an initial temperature of 200°C while the temperature of the FID was 240°C. Calibration was done using standard solutions containing 400 mM of acetic acid, propionic acid and butyric acid, 200 mM for valeric and isovaleric acid, 100 mM for isobutyric acid, 50 mM for caproic acid and 15 mM for heptanoic acid which were all purchased from Sigma-Aldrich (Milwaukee, WI).

Gene Expression Analysis by Quantitative Polymerase Chain Reaction (qPCR)

SCFAs (propionate, acetate and butyrate) bind to G-protein coupled receptors to enhance secretion of gut hormones [108]. Prominent among these receptors is GRP43 which is equally sensitive to each SCFA and is expressed in the ileum and colon [108]. GPR43 has been reported to mediate gut inflammation and stimulate incretin secretion [14, 111]. Therefore, relative gene expression of GPR43, the pro-inflammatory cytokines IL-1β and IL-6, and the anti-inflammatory cytokine IL-10, in each treatment group was determined from the colon mucosa and the ileum using qPCR. This assay was carried out following a previously described protocol [25]. Total RNA was extracted from the frozen colon mucosa and ileum using TRIzol reagent (Sigma-Aldrich). The concentration of the extracted RNA was verified using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE) and agarose gel electrophoresis was used to verify the quality of the 18S and 28S rRNA. To generate cDNA, total RNA (2 μg) was treated with DNase I (Roche, IN) and reverse-transcribed using the SuperScript II synthesis system (Invitrogen, Grand Island, NY). Real-time quantitative PCR analysis was performed using SYBR Green chemistry on an ABI 7900HT sequence-detection system instrument and 2.4 SDS software (Applied Biosystems, CA). The complete list of the oligonucleotide primers used can be found in Table 5. The relative mRNA transcript levels were calculated according to the 2^{-ddCt} method [149]. The results were presented relative to the control group.

Table 5: Primer sequences for gene expression analysis

Gene	Forward Primer	Reverse Primer
mCyclo	5`-tgg aga gca cca aga cag aca-3`	5`-tgc cgg agt cga caa tga t-3`
mFfar2	5`-ctt ccc ggt gca gta caa gt-3`	5`-gct ctt ggg tga agt tct cg-3`
mIL-10	5`-ggt tgc caa gcc tta tcg ga-3`	5`-acc tgc tcc act gcc ttg ct-3`
mIL-1b	5`-caa cca aca agt gat att ctc cat g-3`	5`-gat cca cac tct cca gct gca-3`
mIL-6	5`-gag gat acc act ccc aac aga cc-3`	5`-aag tgc atc atc gtt gtt cat aca-3`

Western Blot Analysis

The interaction of short-chain fatty acids with GPR43 stimulates the release of incretins from the enteroendocrine L-cells of the small intestines [14]. Glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are anti-hyperglycemic incretins, which increase insulin secretion, increase glucose sensitivity and enhance proliferation of β-cells [150]. To determine the possible effect of these incretins on pancreatic metabolism, relative protein expression of the incretin receptors, GLP-1R and GIPR was determined from the pancreas using western blot following the approach of Huang et al [151].

To extract total protein, 50 mg of pancreas samples were lysed in 300 µl of RIPA buffer containing phosphate and protease inhibitor (Sigma Aldrich, St. Louis MO). The lysate was sonicated three times, (Misonix Inc, Farmingdale, NY) centrifuged at 8000 x *g*, for 10 mins at 4ºC, and the total protein concentration was determined using Pierce BCA Protein Assay Kit (Pierce Biotechnology Inc, Rockford, IL). Protein samples (20

µg) were separated by SDS-PAGE using 10 % acrylamide-bis gels (acrylamide:bis, 29:1). Thereafter, the samples were transferred to polyvinylidene fluoride (PVDF) membranes (ThermoScientific, Rockford, IL, Product # 88518). This was followed by incubation of the PVDF membranes with 5% powdered milk (Nestle, Colon, OH) at room temperature for 1 hour with gentle shaking. The membranes were thereafter probed with the following rabbit polyclonal antibodies: GLP-1R (1:200 dilution, Santa Cruz Biotech, Dallas, TX, Cat # sc-66911), GIPR (1:200 dilution, Santa Cruz Biotech, Dallas, TX, Cat # sc-98795) and β-Actin (1:1000 dilution, Cell Signaling Technology, Danvas, MA, Cat # 4967). The PVDF membranes were incubated with the antibodies overnight at 4ºC with gentle shaking. The following day, the PVDF membranes were washed twice in PBS for 5 minutes and incubated with 5% powdered milk (Nestle, Solon, OH) at room temperature for $1^1/_2$ hours in order to block non-specific sites on the membrane. Thereafter, an anti-rabbit IgG, HRP-linked antibody (1:1000 dilution, Cell Signaling Technology, Danvas, MA) was incubated with the membrane for 1 hour on an orbital shaker. The membrane was then washed in PBS for $1^1/_2$ hours with the PBS changed every 15 minutes. Immunodetection of expression bands was carried out with Ponceau S Stain (Sigma-Aldrich, St Louis, MO) and viewed with FluorChem R Imaging System (ProteinSimple, San Jose, CA). The resulting bands were thereafter quantified using UNScanIT software, version (Silk Scientific Inc, Orem, UT).

Liver Lipids Determination

Total lipids, triglycerides and cholesterol were determined following a protocol described by Carr *et al.*,[152] with few modifications. Approximately 100 mg of mashed frozen liver samples was weighed on a filter paper, folded and sealed with a paper clip,

transferred to a 50 mL centrifuge tube, and extracted with a 2:1 (v/v) mixture of chloroform: methanol at room temperature for 3 days to allow for efficient lipid extraction. After the third day, 3.5 mL of 0.05% sulfuric acid was added to each tube, vortexed and allowed to stand for few minutes. The top layer was aspirated off and the remaining volume was estimated. A duplicate aliquot (500 uL) was transferred into new test tubes for the triglyceride and total cholesterol determination. The triglyceride and total cholesterol content of the liver was determined from the previously taken aliquots using the BioLis 24i chemical analyzer (Carolina Chemistry, Wiston-Salem, NC) following manufacturer's instructions.

For determination of total lipids, aluminum weighing pans were oven-dried (100^0C for 1 hour) and cooled in the desiccator for 30 minutes. The remaining chloroform: methanol solution in the 50 mL centrifuge tubes was poured into the pre-weighed aluminum pans and the solvent was evaporated off under the fume hood. Thereafter, the aluminum pans were again oven-dried (100^0C for 1 hour) and cooled in the desiccator for 30 minutes. The weights of the aluminum pans were taken and the total liver lipid was calculated.

Statistical Analyses

For the gut microbiota data, univariate differential abundance of OTUs and the genus level was tested using a negative binomial Generalized Linear Model (GLM) for the overdispersion and Poisson process intrinsic to the data, which was implemented in the DESeq2 package [153] and described for microbiome applications by McMurdie and Holmes [154]. DESeq2 was run under default settings and p-values were corrected for

false discovery rates with the Benjamini-Hochberg procedure [155]. Only OTUs with the adjusted p-value below 0.05 and more than one log2 fold change were reported.

Apart from the gut microbial data, statistical analyses for other data involved computation of least square means and standard deviation of the means for each of the treatment groups using SAS version 9.1 (SAS Institute, Cary, NC). Data was checked for normal distribution using residual plots. Analysis of variance and least square means was calculated using the general linear model procedure and the means were compared using Fisher's least significant difference for comparing groups. P value < 0.05 was considered significant for all analysis.

CHAPTER IV

RESULTS

This study investigated the effects of mango supplementation in modulating the gut microbiota, and body composition, glucose and lipid metabolism in C57BL/6 mice fed a high fat diet. Furthermore, mango's effect on microbial short chain fatty acid production, and modulation of gut inflammation was determined.

Effects of Mango Supplementation on the Gut Microbiota in High Fat Diet-fed Mice

We investigated the effect of 90-day mango supplementation in preventing the loss of beneficial gut bacteria due to high fat feeding. First, inter-sample relationships using a phylogenetic tree showed that phylum Firmicutes are the most abundant in the samples while the archaea phylum - Euryarchaeota was the least abundant (Figure 2a). Looking at genus-level changes in the cecal gut bacteria due to dietary feeding, the high fat diet resulted in a significant loss of the genera Akkermansia, Bifidobacterium, Sutterella, Ruminococcus, Collinsella, Coprobacillus, Staphylococcus and Oscillospora in comparison to the control group (Figure 2b). The bacterial genera that were significantly elevated in the high fat diet group are at the moment unclassified at the

genus level but majority belonged to the phylum Firmicutes (Figure 2b). The genus

Dorea, Sutterella and Ruminococcus were more abundant in the control group compared

to the 1% mango diet group (Figure 2c) while the genus Lactococcus was more abundant

in the 1% mango group compared to control. Similar results were observed with the 10%

mango group as there was a significant increase in abundance of the lactic acid producing

genera, Lactococcus and Lactobacillus in the 10% mango groups relative to the control

(Figure 2d). In comparison to the high fat diet-fed group, the 1% mango group did not

modulate any significant changes in any of the presently classified bacteria genera

(Figure 2e). However, the greatest bacterial modulation was seen with the 10% mango

group, as the genus Prevotella, Akkermansia, Bifidobacterium, Aldercreutzia,

Ruminococcus were more abundant compared to the high fat diet-fed group, indicating a

dose-dependent modulation of the gut bacteria by mango supplementation (Figure 2f).

Impact of mango supplementation on body and tissue weights, food intake, body composition, glucose homeostasis and lipid metabolism

Body weights were similar prior to initiation of the dietary treatments (Table 6).

However, after 90 days of treatment, there were significant differences in body weight

with the 10% mango supplemented group having the highest body weight. The mango

supplemented groups also had a significantly higher caloric intake levels compared to the

other groups (Table 6). Similarly, the HF+10% mango group have a significantly higher

liver weight compared to the control while the high fat and HF+1% mango groups had an

intermediate effect. Cecal tissue weight was highest in the 10% mango supplemented

group. Spleen, pancreas and thymus weights were unaffected by mango supplementation.

In addition to isolating and weighing the abdominal fat, whole body composition was also assessed by a densitometer (PixiMus) at the end of dietary treatment. Our results showed that mango supplementation had no effect in preventing abdominal fat accumulation due to high fat diet intake (Table 6). Mice fed the 10% mango have the highest abdominal fat weight, total fat mass, and % body fat. Additionally, lean mass was also high for this group.

To determine the effects of mango supplementation on glucose homeostasis, glucose tolerance test was conducted. Furthermore, incretin (GLP-1 and GIP) and insulin levels were assessed in the plasma. Subsequently, since incretins are known to impact pancreatic metabolism and insulin release via the enteroinsular axis [156], protein expression of the incretin receptors (GLP-1R and GIPR) was assessed in the pancreas.

In a similar pattern to the results on body composition, mango supplementation at both 1% and 10% doses were unable to modulate glucose homeostasis as shown by the area under the curve values obtained after the glucose tolerance test, which were not statistically different from the HF group (Table 7). However, there was a dose-dependent increase in plasma insulin levels with statistical significance only seen with the 10% mango (Table 7). Incretin assessment in the plasma showed that the control group had a significantly lower GLP-1 level compared to other groups (Table 7). However, the 10% mango supplementation induced the greatest GLP-1 secretion (Table 7). GIP levels in the plasma remained unaffected by dietary supplementation (Table 7). In the pancreas, both mango doses had no significant effect on the expression of GLP-1R protein (Figure 3a). However, mango supplementation significantly increased the expression of GIPR in the

pancreas compared to the high fat group with the 1% mango dose having the greatest impact (Figure 3b).

The effects of dietary treatment on lipid metabolism were assessed by measuring plasma and liver lipids as well as plasma adipokines (Table 8). Mango supplementation had a mixed effect on plasma lipid levels. Mango supplementation was not able to reduce plasma cholesterol levels due to high-fat feeding. Surprisingly, the 10% mango group showed significantly higher plasma cholesterol levels compared to other groups. HDL levels was significantly higher in HF, HF+1% and HF+10% mango groups compared to the control, with non-HDL levels showing a similar pattern to the total cholesterol levels. Interestingly, triglyceride levels were significantly lower in both mango doses with the 10% mango dose showing the highest reduction similar to the control. Furthermore, the 10% mango dose modulated a significant reduction in plasma non-esterified fatty acids (NEFA) levels compared to all other groups. The modulation of plasma lipids by the 10% mango supplemented group could not be explained by changes in liver lipid metabolism as the HF and mango supplemented groups had significantly higher total lipid and triglyceride levels compared to the control. Finally, both mango doses showed a tendency to reduce the pro-inflammatory plasma adipokines (leptin and resistin), however, this did not reach statistical significance.

Impact of mango supplementation on short chain fatty acid production and GPR43 expression in mice fed a high-fat diet.

Following 90 days of treatment, microbial SCFA production due to dietary treatment was assessed in the cecal and fecal contents via gas chromatography. In

addition, mRNA expression of an SCFA receptor, GPR43 was assessed in the ileum and colon mucosa.

SCFA analysis (Table 9) showed the impact of mango supplementation in modulating SCFA production as measured in cecal and fecal samples. High fat feeding resulted in a decrease of all SCFA measured in cecal samples while mango supplementation mediated an increase in SCFA production with the higher dose (10% mango) being the most effective at raising SCFA levels. Specifically, 1% mango supplementation significantly increased the levels of n-butyric acid and n-valeric acid compared to the high fat group in the fecal samples. The 10% mango-supplemented group however, had a significant increase in all the measured fecal SCFA (except propionate) in comparison to the high fat group. Similar results were seen with the cecal SCFA content as 10% mango supplementation led to a significant increase in all the SCFA measured compared to the high fat group, with the exception of propionic acid and valeric acid. However, mango supplementation had no significant impact on the expression of the SCFA receptor, GPR43 in the ileum and colon (Figure 4a and 4b).

Impact of mango supplementation on gut and plasma inflammatory markers in mice fed a high-fat diet.

In order to determine the possible immunomodulatory effect of mango supplementation in mice fed a high fat diet, we assessed the gene expression of proinflammatory markers (IL-6 and IL-1β) and an anti-inflammatory marker (IL-10) in the ileum and colon lamina propria. Furthermore, we assessed levels of various inflammatory markers in the plasma using the Bioplex Multiplex assay.

In this study, dietary treatment had no effect on the gene expression of IL-1b in the ileum (Figure 5A). The high fat diet caused an increase in IL-6 expression in the ileum compared to the control (Figure 5A). Interestingly, mango supplementation significantly decreased IL-6 expression in the ileum in a dose-dependent manner (Figure 5A). Mice fed the 1% mango diet had the highest expression of the anti-inflammatory marker, IL-10 in the ileum (Figure 5a). Similarly, results from the colon showed that both mango doses significantly increased the expression of IL-10 (Figure 5b). The reduction noticed in the expression of IL-1b and IL-6 in the colon with mango supplementation did not reach statistical significance. In the plasma, the immunomodulatory effect of mango supplementation noticed in the ileum and colon was not evident as dietary supplementation with mango had no significant impact on plasma cytokine levels except IL-10 in mice fed a high fat diet (Table 10).
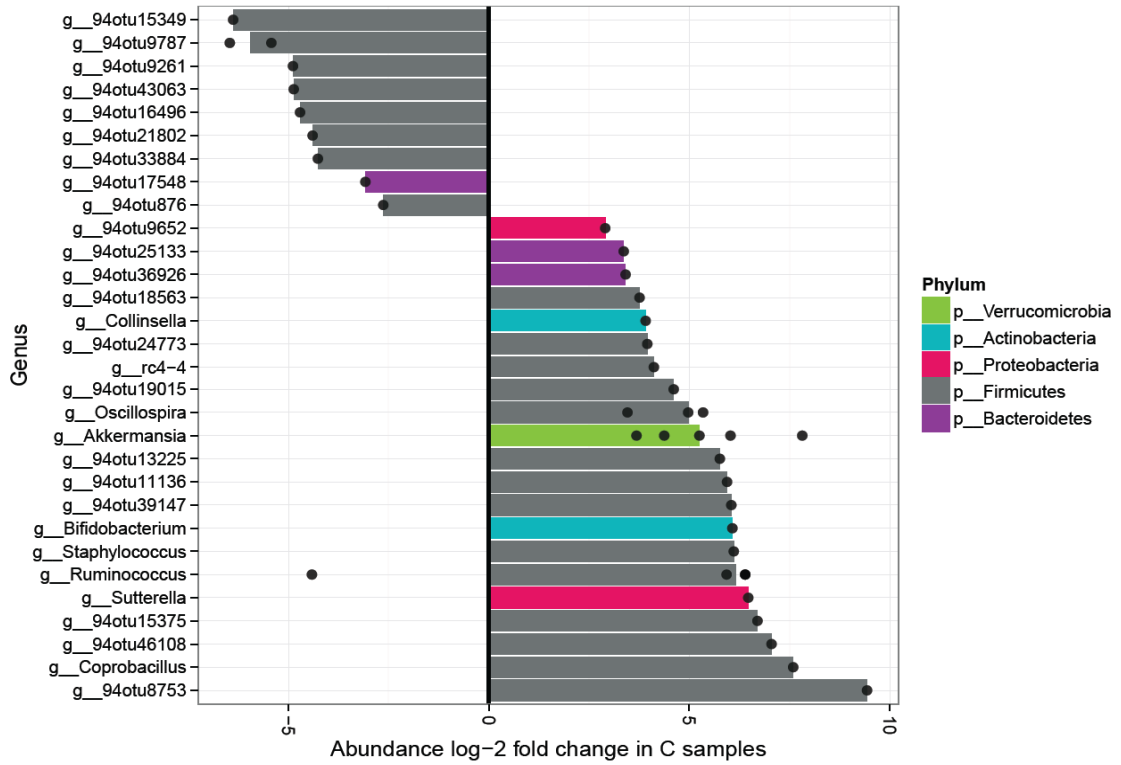
Figure 1: Effects of dietary mango supplementation on cecal gut microbial population
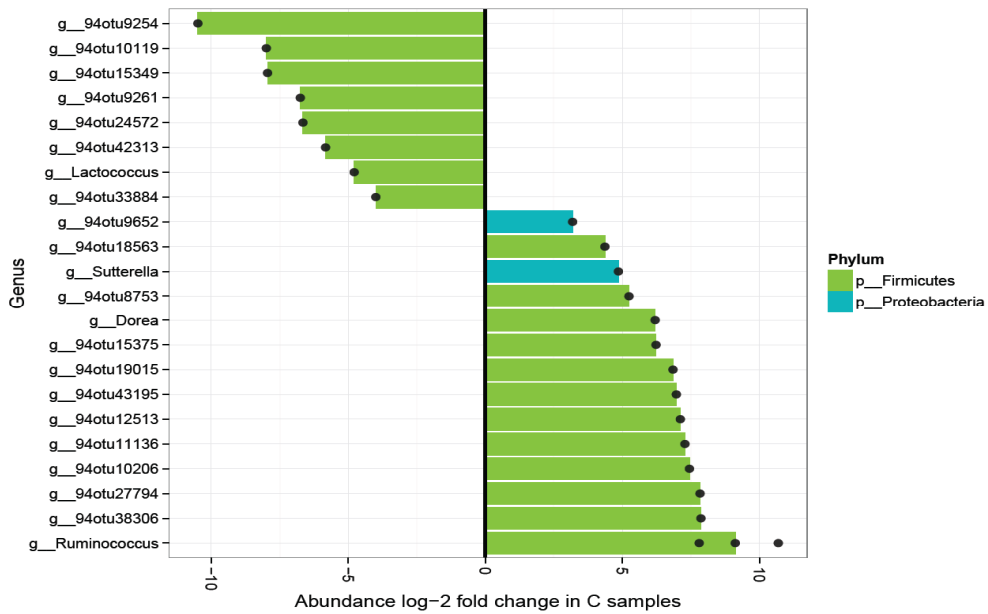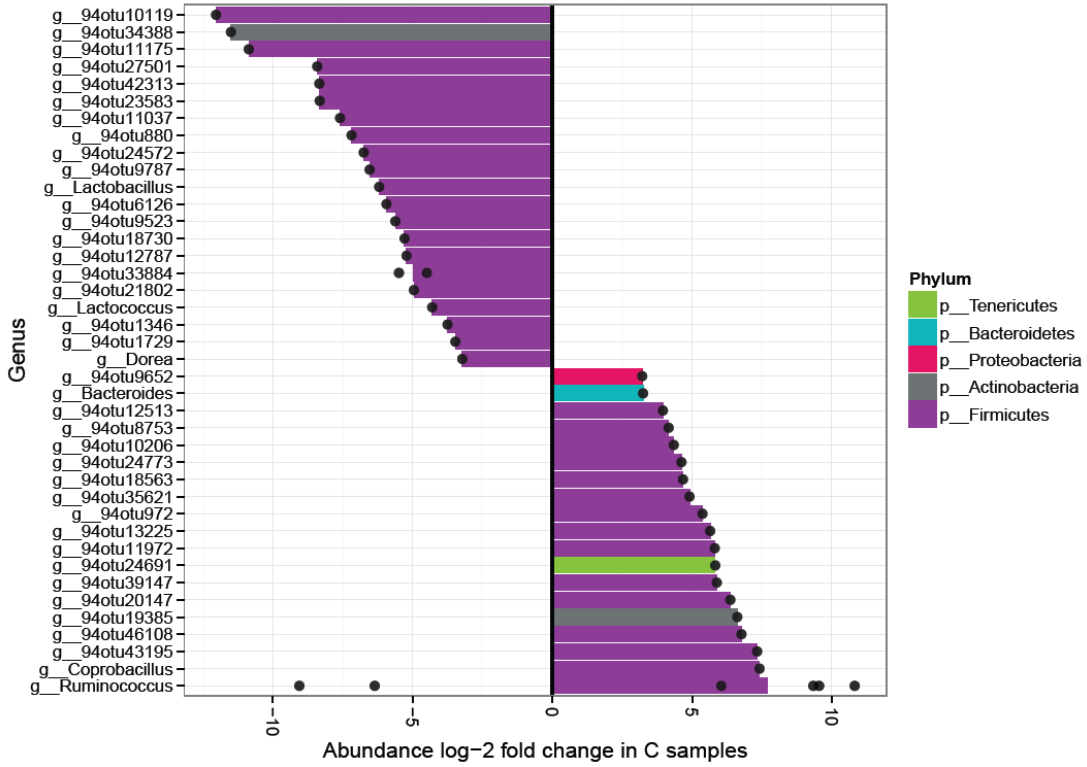
(a) Family-Level Phylogenetic tree
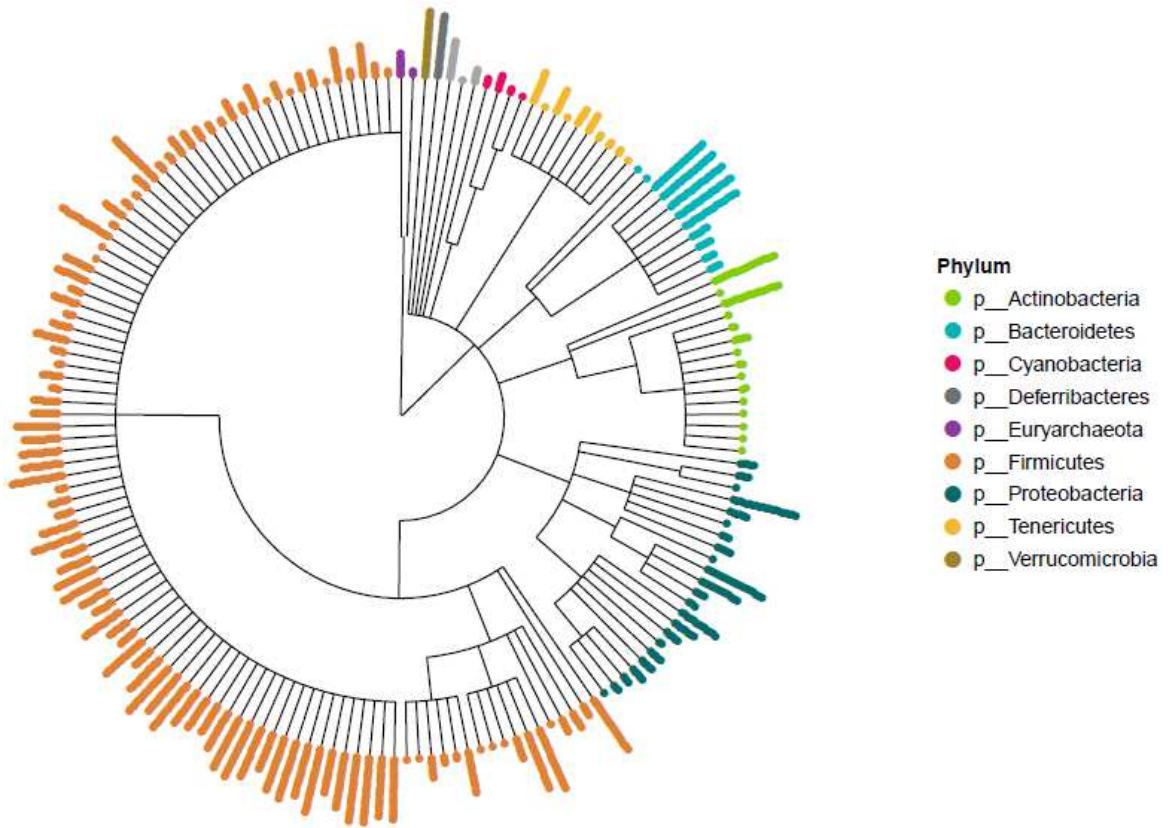
(b)   Feature selection - control (C) vs high fat (F) diet
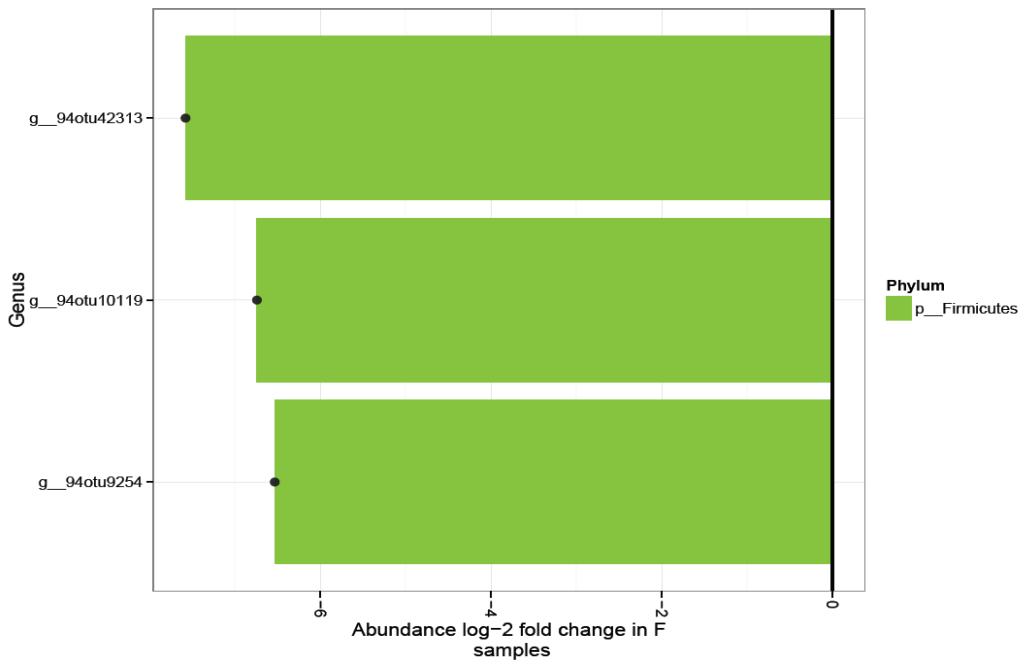

(c)   Feature selection- control (C) vs high fat+1% mango (1P) diet
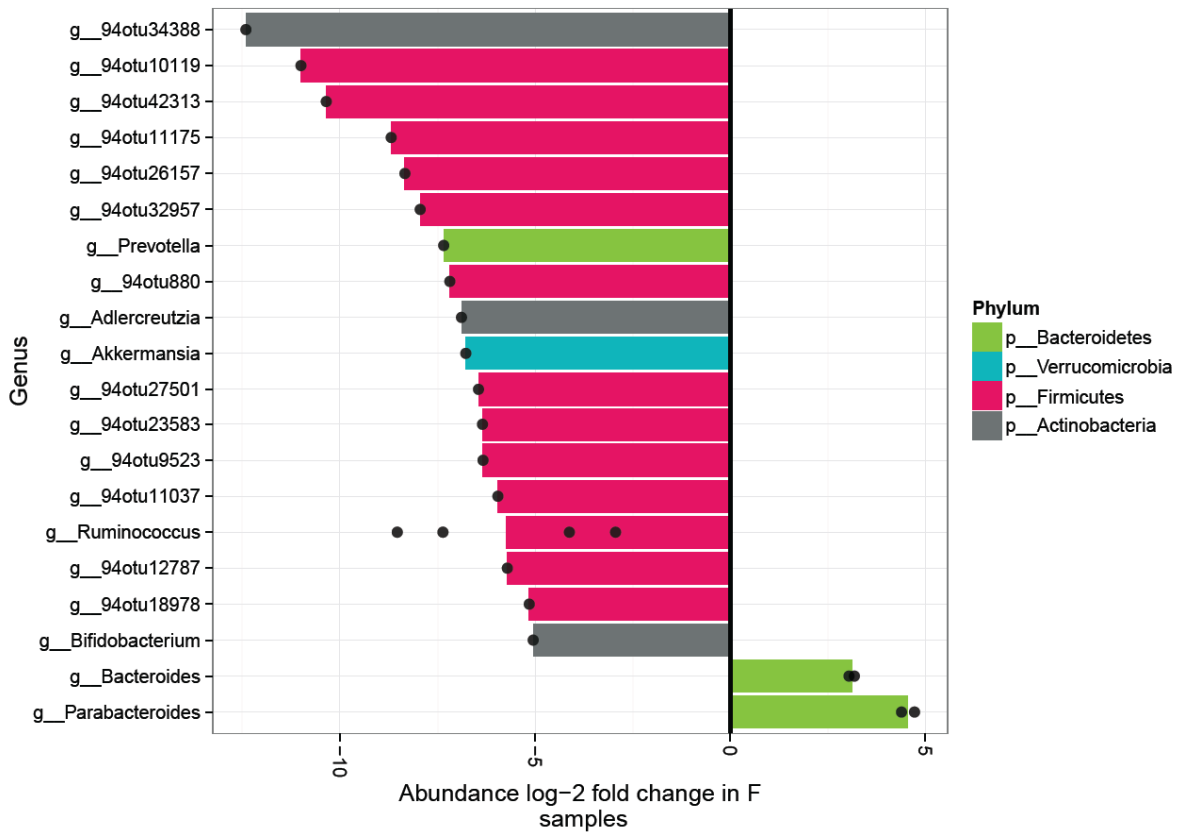
(d)   Feature selection - control (C) vs high fat+10% mango (10P) diet

(e)   Feature selection - high fat (F) vs high fat+1% mango (1P) diet

(f)   Feature selection - high fat (F) vs high fat+10% mango (10P) diet

DNA isolated from cecal samples were subjected to 16S rDNA sequencing. n = 4 mice/group. C=Control, F=High Fat, 1P=High fat+1% Mango, 10P = High fat+ 10% Mango. (a) Phylogenetic tree at the Family level. The height of each bar indicates the number of samples containing that particular family. (b-f) Genus level changes due to dietary treatment as presented by log 2 fold changes versus genus. Bars represent median value of each Genus and points are OTUs belonging to that Genus. (b,c,d) Significantly elevated genera in C are on the positive axis (right) while elevated genera in F, 1P and 10P are on the negative axis (left). (e, f) Significantly elevated genera in F are on the positive axis (right) while elevated genera in 1P and 10P are on the negative axis (left). Features were considered significant if their FDR-corrected P-value ≤ 0.05, and the absolute value of their Log-2 Fold Change was greater than or equal to 1. OTU = Operational Taxonomic Unit, FDR = False Discovery Rate

Table 6: Effects of freeze-dried mango supplementation on food intake, body and tissue weights, and body composition of mice fed a high fat diet for 90 days.

	Control	High fat (HF)	HF + 1% Mango	HF + 10% Mango	P-value
Food Intake (Kcal/day)	10.63 ± 0.78^c	12.43 ± 1.13^b	12.99 ± 1.29^a	12.58 ± 1.42^{ab}	<.0001
Body weights					
Initial	20.74 ± 1.28	20.81 ± 1.09	20.94 ± 1.18	20.82 ± 1.09	0.9929
Final	30.89 ± 1.97^c	38.79 ± 3.75^b	40.43 ± 3.83^b	43.33 ± 3.85^a	<.0001
Tissue weights					
Liver (g)	1.34 ± 0.17^b	1.49 ± 0.27^{ab}	1.48 ± 0.29^{ab}	1.68 ± 0.43^a	0.0385
Cecal tissue (mg)	71.33 ± 7.43^{ab}	70.67 ± 11.62^{ab}	64.37 ± 10.94^b	76.00 ± 13.52^a	0.0436
Abdominal fat (g)	1.10 ± 0.32^b	2.60 ± 0.32^a	2.60 ± 0.36^a	2.78 ± 0.72^a	<.0001
Spleen (mg)	88.00 ± 27.83	103.33 ± 26.64	94.38 ± 26.83	105.33 ± 22.95	0.2424
Pancreas (mg)	160.67 ± 39.00	175.33 ± 32.92	153.13 ± 44.08	156.00 ± 36.41	0.3976
Thymus (mg)	56.00 ± 14.04^b	68.00 ± 18.21^a	68.13 ± 17.21^a	73.33 ± 14.48^a	0.0328
Body composition					
Lean mass (g)	22.13 ± 1.24^b	23.98 ± 2.87^a	24.19 ± 2.29^a	24.85 ± 2.33^a	0.0121
Fat mass (g)	10.04 ± 1.79^c	18.31 ± 1.71^b	18.69 ± 2.55^b	21.17 ± 2.18^a	<.0001
% body fat	31.02 ± 3.73^c	43.37 ± 2.28^b	43.49 ± 3.55^b	45.99 ± 2.89^a	<.0001

n=15 mice/group. Mean ± S.D. Within a row, values with unlike superscript letters are significantly different (P ≤ 0.05) from each other.

Table 7:   Effects of freeze-dried mango supplementation on fasting blood glucose, glucose area under the curve (AUC), plasma insulin and incretin levels of mice fed a high fat diet for 90 days.

Parameter	Control	High fat (HF)	HF + 1% Mango	HF + 10% Mango	P-value
Fasting blood glucose (mg/dL)	161.69 ± 31.61^c	193.08 ± 32.62^b	233.31 ± 37.07^a	208.46 ± 30.94^{ab}	<.0001
Glucose AUC (mg x min/dL)	38643.35 ± 7193.69^b	57989.23 ± 7623.42^a	60871.46 ± 8476.39^a	61961.15 ± 5584.92^a	<.0001
Insulin (ng/mL)	0.64 ± 0.15^b	0.88 ± 0.22^b	0.91 ± 0.31^b	1.45 ± 0.48^a	0.0002
GLP-1 (pg/mL)	16.34 ± 5.05^c	23.69 ± 8.22^{ab}	18.67 ± 5.75^{bc}	28.02 ± 8.05^a	0.01
GIP (pg/mL)	122.06 ± 16.99	171.00 ± 53.04	165.49 ± 56.88	160.81 ± 58.14	0.2048

Glucose AUC was calculated following glucose tolerance tests in mice at the end of the study. n=13 mice/group. Plasma insulin, glucagon-like peptide 1 (GLP-1) and gastric inhibitory peptide (GIP) were measured at the end of the study as part of an 8-plex assay on a bioplex multiplex reader as previously described under methods. n=8 mice/group. Mean ± S.D. Within a row, values with unlike superscript letters are significantly different ($P \leq 0.05$) from each other.

Figure 2: Effects of freeze-dried mango supplementation on the relative protein expression of the incretin receptors (a) GLP-1R and (b) GIPR in the pancreas of mice fed a high fat diet.
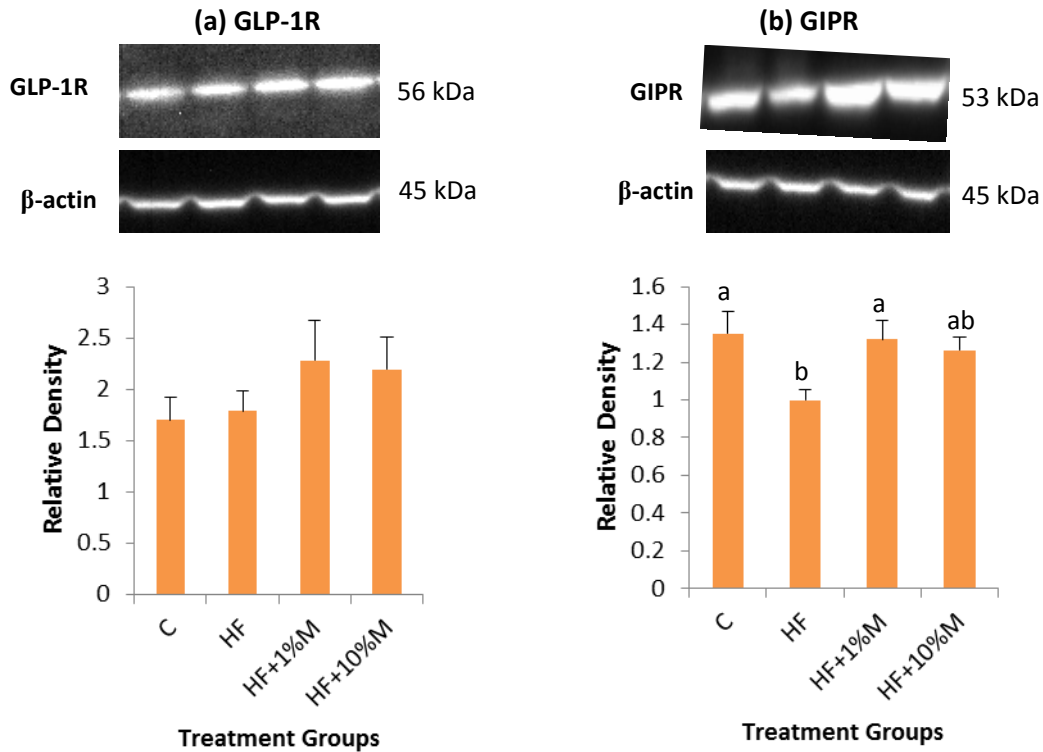
n = 6 mice/group. Data = Mean ± SEM. Bars with unlike superscript letters are significantly different from each other. GLPR-1- glucagon-like peptide 1 receptor, GIPR – gastric inhibitory peptide receptor

Table 8: Effects of freeze-dried mango supplementation on plasma and liver lipids of mice fed a high fat diet for 90 days.

Lipid	Control	High fat (HF)	HF + 1% Mango	HF + 10% Mango	P-value
Plasma lipids and adipokines					
Total Cholesterol (mg/dL)	110.14 ± 23.22[d]	163.00 ± 11.27[b]	155.33 ± 18.55[b]	178.93 ± 23.28[a]	<.0001
Triglycerides (mg/dL)	41.21 ± 9.09[b]	52.27 ± 20.81[a]	44.60 ± 13.32[ab]	35.93 ± 7.18[b]	0.0164
HDL (mg/dL)	61.79 ± 14.71[c]	81.27 ± 5.09[a]	80.80 ± 5.59[a]	81.73 ± 7.11[a]	<.0001
Non-HDL (mg/dL)	48.36 ± 10.98[c]	81.73 ± 7.47[b]	74.53 ± 14.55[b]	97.20 ± 18.79[a]	<.0001
NEFA (mEq/L)	0.85 ± 0.14[a]	0.81 ± 0.14[a]	0.80 ± 0.11[a]	0.69 ± 0.09[b]	0.0076
Leptin (ng/mL)	3.23± 1.52[b]	12.15 ± 3.79[a]	8.62 ± 3.31[a]	11.84 ± 4.71[a]	<.0001
PAI-1 (ng/mL)	0.19 ± 0.05[b]	0.35 ± 0.07[a]	0.35 ± 0.12[a]	0.32 ± 0.09[a]	0.0064
Resistin (ng/mL)	31.320 ± 9.15[b]	76.45 ± 28.36[a]	60.39 ± 20.43[a]	65.23 ± 31.52[a]	0.0058
Liver lipids (mg/g tissue)					
Total Lipids	107.81 ± 17.69[b]	149.89 ± 28.43[a]	143.53 ± 35.51[a]	168.97 ± 49.20[a]	0.0123
Total Cholesterol	3.72 ± 0.47	3.65 ± 0.74	4.00 ± 0.85	3.34 ± 0.55	0.2902
Triglycerides	23.59 ± 12.19[b]	60.79 ± 23.36[a]	58.34 ± 19.51[a]	66.23 ± 24.52[a]	0.0010

n=15 mice/group or 8 mice/group for plasma and liver lipids, respectively. Mean ± S.D. Within a row, values with unlike superscript letters are significantly different ($P \leq 0.05$) from each other. HDL- high density lipoprotein; NEFA- non-esterified fatty acids; PAI-1- plasminogen activator inhibitor
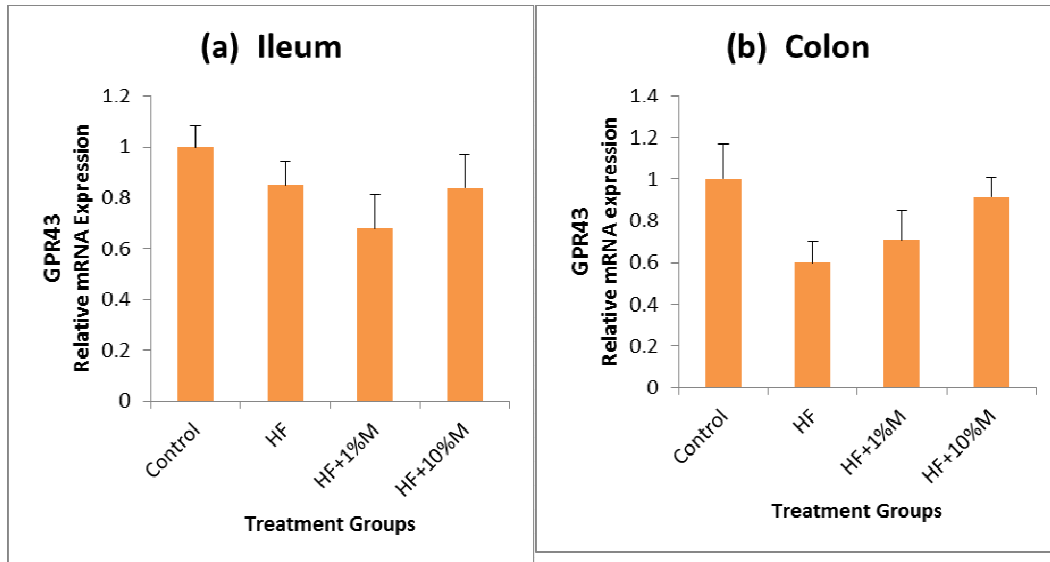
Table 9: Effects of mango supplementation on fecal and cecal short chain fatty acid (SCFA) content of mice fed a high fat diet for 90 days

	Baseline	Control	High fat (HF)	HF + 1% Mango	HF + 10% Mango	P-value
Fecal SCFA (mM)						
Acetic acid	12.19 ± 3.11^a	7.41 ± 0.88^c	3.92 ± 1.56^d	4.52 ± 0.95^d	9.38 ± 1.21^b	<.0001
Propionic acid	0.22 ± 0.05^{ab}	0.17 ± 0.05^b	0.20 ± 0.08^{ab}	0.23 ± 0.07^{ab}	0.28 ± 0.01^a	0.0120
Isobutyric acid	0.16 ± 0.03^a	0.03 ± 0.01^c	0.03 ± 0.01^c	0.03 ± 0.01^c	0.04 ± 0.01^b	<.0001
n-Butyric acid	0.04 ± 0.01^c	0.02 ± 0.01^d	0.05 ± 0.02^c	0.08 ± 0.02^b	0.11 ± 0.02^a	<.0001
Isovaleric acid	0.18 ± 0.02^a	0.06 ± 0.01^c	0.05 ± 0.01^c	0.05 ± 0.01^c	0.08 ± 0.02^b	<.0001
n-Valeric acid	0.03 ± 0.01^d	0.03 ± 0.01^c	0.06 ± 0.01^b	0.09 ± 0.01^a	0.10 ± 0.01^d	<.0001
Cecal SCFA (mM)						
Acetic acid	NA	8.41 ± 0.81^a	5.80 ± 0.92^b	5.50 ± 0.56^b	7.91 ± 1.05^a	0.0007
Propionic acid	NA	1.26 ± 0.17^a	0.82 ± 0.06^b	0.74 ± 0.05^b	0.84 ± 0.08^b	<.0001
Isobutyric acid	NA	0.10 ± 0.01^a	0.06 ± 0.01^b	0.07 ± 0.01^b	0.09 ± 0.01^a	<.0001
n-Butyric acid	NA	0.64 ± 0.11^b	0.46 ± 0.02^c	0.50 ± 0.09^{bc}	0.83 ± 0.11^a	0.0003
Isovaleric acid	NA	0.10 ± 0.02^a	0.07 ± 0.01^b	0.08 ± 0.01^b	0.10 ± 0.01^a	<.0001
n-Valeric acid	NA	0.26 ± 0.03^a	0.20 ± 0.02^b	0.21 ± 0.02^b	0.23 ± 0.02^{ab}	0.0170

n=15 mice/group or 11 mice/group for fecal and cecal SCFA, respectively. Fecal baseline samples were obtained from 20 mice at random (4 cages containing 4 mice/cage). Values aremean ± S.D. Within a row, values with unlike superscript letters are significantly different ($P \leq 0.05$) from each other.

Figure 3: Effects of freeze-dried mango supplementation on the relative mRNA expression of the short chain fatty acid receptor, GPR43 in the (a) ileum and (b) colon of mice fed a high fat diet.

n = 6 mice/group. mean ± SEM

Figure 4: Effects of freeze-dried mango supplementation on the relative mRNA expression of IL-1β, IL-6, and IL-10 in the (a) ileum and (b) colon of mice fed a high fat diet.
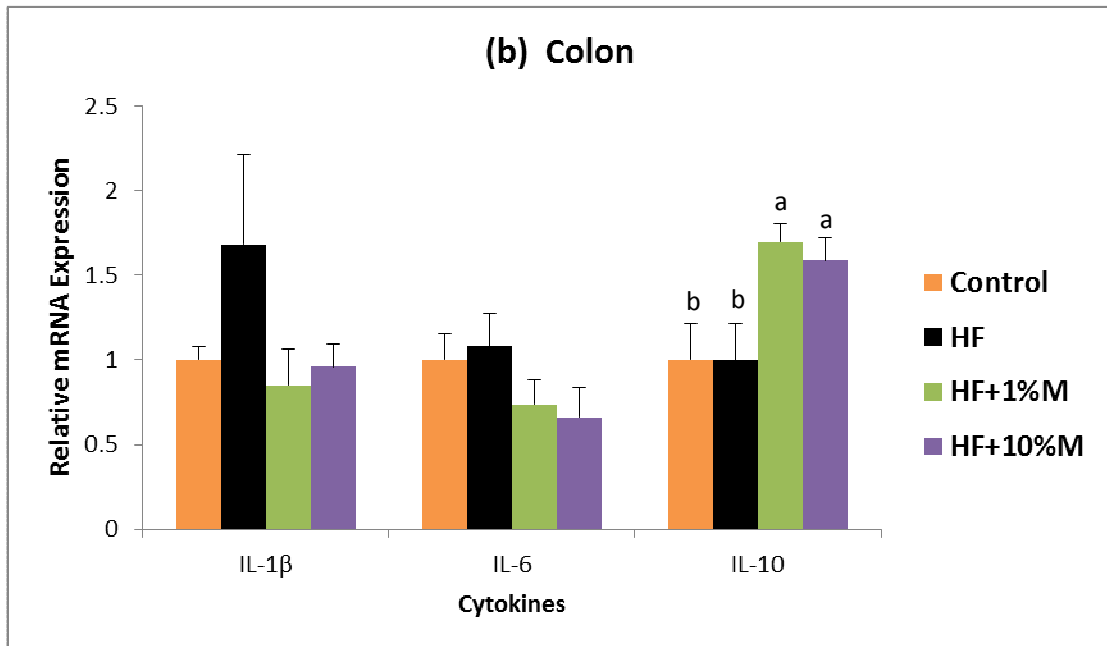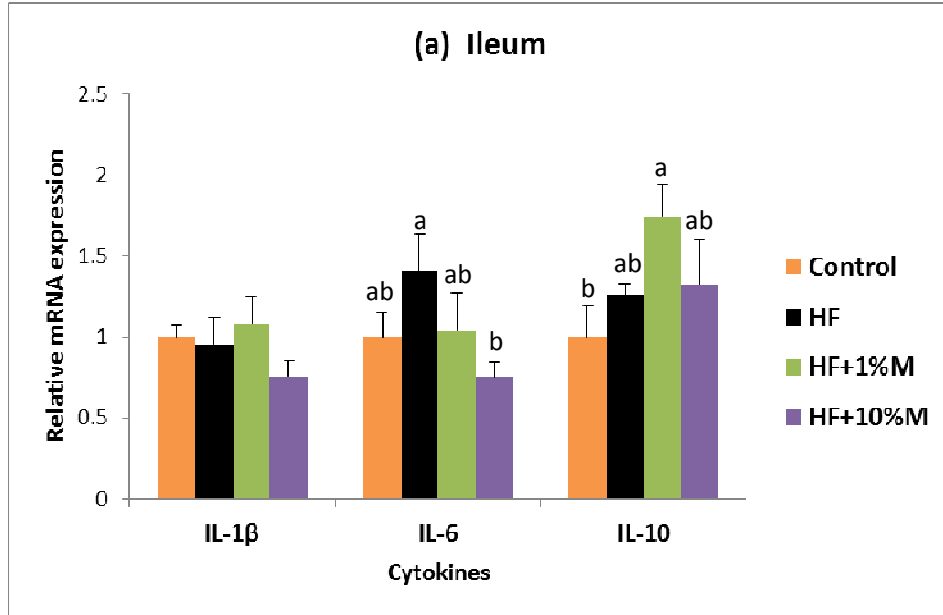
n = 6 mice/group. Mean ± SEM. Bars with unlike superscript letters are significantly different from each other.

Table 10: Effects of freeze-dried mango supplementation on plasma cytokine levels in mice fed a high fat diet for 90 days

Cytokine (pg/mL)	Control	HF	HF + 1% M	HF + 10% M	P-value
IL1a	30.07 ± 6.28	15.24 ± 3.20	14.71 ± 1.92	24.56 ± 7.49	0.1306
IL1b	871.38 ± 159.93	562.02 ± 85.55	508.73 ± 26.89	730.76 ± 201.16	0.2430
IL2	156.07 ± 27.08	74.01 ± 18.02	85.84 ± 7.71	107.13 ± 32.28	0.0862
IL3	58.40 ± 16.74	27.82 ± 4.73	28.60 ± 3.53	42.70 ± 11.05	0.1527
IL4	37.70 ± 6.70	20.80 ± 3.95	21.67 ± 1.51	28.80 ± 8.34	0.1597
IL5	51.70 ± 11.05	29.84 ± 8.14	31.05 ± 3.69	51.63 ± 13.44	0.2183
IL6	28.12 ± 5.33	18.36 ± 2.17	19.84 ± 1.65	19.31 ± 4.21	0.2296
IL10	192.50 ± 33.32[a]	110.33 ± 11.60[b]	115.60 ± 6.32[b]	127.39 ± 23.89[b]	**0.0418**
IL12p40	219.20 ± 16.46	203.34 ± 22.69	191.16 ± 16.26	206.44 ± 7.65	0.6996
IL12p70	605.30 ± 106.50	411.67 ± 47.28	404.84 ± 20.34	494.53 ± 100.94	0.2505
IL13	1077.80 ± 171. 45	685.66 ± 97.95	734.53 ± 42.74	932.57 ± 229.65	0.2611
IL17A	177.15 ± 24.25	151.67 ± 12.75	175.34 ± 19.26	189.56 ± 13.71	0.5234
Eotaxin	1381.87 ± 193.64	1049.82 ± 118.80	999.57 ± 103.16	1090.72 ± 296.27	0.4658
G-CSF	105.47 ± 19.44	114.82 ± 51.32	131.30 ± 34.42	87.79 ± 15.92	0.8274
IFN-γ	76.71 ± 15.82	47.33 ± 6.59	47.54 ± 4.69	60.56 ± 15.78	0.2725

KC	108.62 ± 9.73	95.97 ± 5.23	136.03 ± 26.37	121.32 ± 11.71	0.3157
MCP1	582.80 ± 80.27	366.72 ± 59.48	383.05 ± 22.07	464.75 ± 94.57	0.1352
MIP1α	37.28 ± 5.18	25.47 ± 2.41	24.14 ± 1.14	29.84 ± 4.89	0.0869
MIP1β	139.87 ± 28.81	77.17 ± 12.25	77.95 ± 5.99	98.92 ± 23.99	0.1140
RANTES	53.19 ± 8.73	37.72 ± 2.12	32.71 ± 4.34	33.68 ± 6.03	0.0731
TNF-α	1244.92 ± 225.09	768.25 ± 101.58	768.30 ± 61.70	983.72 ± 208.36	0.1532

Plasma cytokines were measured at the end of the study with a Bioplex multiplex reader as previously described under methods. n=8 mice/group. Data = Mean ± S.D. Values with unlike superscript letters are significantly different ($P \leq 0.05$) from each other. IL= Interleukin, G-CSF = Granulocyte-colony stimulating factor, IFN-γ = Interferon gamma, KC = Keratinocyte Chemoattractant, MCP1 = Monocyte chemoattractant protein, MIP = Macrophage inflammatory protein, RANTES = Regulated on activation, normal T expressed and secreted, TNF-α = Tumor necrosis factor alpha

CHAPTER V

DISCUSSION

The present study was undertaken to determine if freeze-dried mango supplementation can prevent the loss of beneficial gut microbiota associated with high fat diet. Moreover, we also investigated the effects of mango supplementation on glucose and lipid parameters. The findings of this study revealed that mango supplementation in high fat diet-fed mice prevented the loss of beneficial gut bacteria in a similar pattern to control without decreasing body weight or fat accumulation. It also showed a mango-mediated modulation of gut inflammation and incretin secretion. Furthermore, supplementation with mango was shown to reduce dyslipidemia associated with high fat feeding.

Several studies in humans and rodent models have suggested that specific gut bacterial changes may play a part in the development of obesity and type 2 diabetes. For example, *Bifidobacteria* has been reported to be lower in obese [157] and type 2 diabetic individuals [158], with an inverse correlation between increase in fat mass in diet-induced obese mice supplemented with inulin-type fructans [119, 159]. In addition, similar results have been attributed with the mucin degrader, *Akkermansia muciniphila*

in a mouse model of diet-induced obesity and type 2 diabetes, as a loss of this bacterium induced by a high fat diet was normalized upon prebiotic feeding, leading to a reversal of fat mass gain and insulin resistance [118].

In this present study, genus level changes in the gut bacteria showed that mango supplementation restored *Bifidobacteria* and *Akkermansia* levels similar to control with the higher dose (10% mango) having the most bacterio-protective effect. Although mango supplementation prevented the loss of these beneficial gut bacteria due to high fat feeding in a similar pattern to some reported prebiotics [119], this did not translate into a prevention of weight gain or improved glucose tolerance. It is noteworthy that while the higher mango dose (10% mango) was the most effective in preventing the loss of bacteria due to high fat diet, these mice surprisingly had a higher body weight than other groups. The slightly higher caloric intake in the HF+10% mango may have contributed to their higher body weight. Additionally, the increase in body weight of the HF+10% mango group may have been due to some species from the genus *Ruminococcus* which have been reported to possess strong energy-harvesting capabilities from starch and cellulolytic fibers in the colon [160] which may have been absorbed and made available to the host. Moreover, some studies have also reported a failure of prebiotic feeding in preventing body weight gain [88, 161, 162]. A recent study using a prebiotic fiber constituting 1:1 ratio of inulin and oligofructose reported a lack of reduction in fat mass and body weight despite a dose-dependent increase in *Bifidobacteria* [88]. Similar results were reported in other studies with oligofructose supplementation [161, 162]. Results from the present study and others [88, 161, 162] suggest that bacterial changes at least at the genus level may not be the only significant factor modulating weight gain and fat

mass increase in obesity and its comorbidities. Therefore, metagenomic approaches such as shotgun sequencing, which may give an idea of upregulated or downregulated genes within the gut microbial genome [163] may provide an alternative to understand the link between mango's effect on gut microbial changes and weight gain in obesity. However, this study provided evidence that mango supplementation prevented changes in the community structure of the gut microbiota due to high fat feeding.

Prebiotic feeding is known to modulate the gut microbiome and promote the production of short chain fatty acids (SCFA) which possess various physiological roles [97]. We show in this study, that mango supplementation did not only prevent the loss of bacteria due to high fat feeding, but also stimulated an increase in SCFA production as measured in both fecal and cecal samples, which suggests an increase in colonic fermentation with mango supplementation. In agreement with the results on bacterial modulation, the 10% mango dose had the most SCFA stimulatory effect as evident with a significant increase in acetic and butyric acid compared to the high fat group in both cecal and fecal samples.

Butyrate has been established as the preferred energy source for gut epithelial cells [97, 164]. Importantly, colonocytes lacking butyrate may undergo autophagy [165]. Moreover, an essential function of butyrate is the mediation of intestinal inflammation and promotion of mucosal tolerance [97, 166]. Butyrate improves gut tolerance to bacteria and antigens by promoting the differentiation of T-regulatory cells expressing the transcription factor, FOXp3$^+$ [106]. Also, butyrate promotes the extra-thymic differentiation of T-cells in the colon into IL-10 – producing T-cells [110]. In agreement with increased butyrate production due to mango supplementation in this study, enhanced

gene expression of the anti-inflammatory cytokine, IL-10 was observed in the mango supplemented group compared to the high fat group. IL-10 is vital in maintaining epithelial layer integrity and homeostasis [167]. A possible mechanism by which mango may mediate an increase in IL-10 expression may be via SCFA's interaction with their receptors located in the colon mucosa and also on immune cells [109]. The SCFA receptor, GPR43, equally recognizes acetate, propionate and butyrate [109]. In the present study, a 35% increase in GPR43 expression was found in the HF+10% mango group compared to the HF group, although this was not statistically significant.

On the other hand, acetate is readily absorbed into the portal blood and taken up by the liver in conjunction with endogenous acetate [168]. Importantly, both acetate and butyrate stimulate the secretion of mucin, a key component of a healthy intestinal barrier, thus preventing the passage bacteria or their components such as lipopolysaccharide into the blood [169, 170]. Therefore, it can be concluded that mango supplementation prevents the loss of beneficial gut bacteria, especially SCFA-producing bacteria, leading to a subsequent increase in SCFA production despite high fat feeding. Also, mango supplementation exerts gut anti-inflammatory effects via an increase in SCFA, especially butyric acid production. This effect of mango feeding may be important in the promotion of gut immune tolerance.

Moreover, the modulation of gut bacteria and SCFA production by mango supplementation may have further implications. An important link between obesity and cancer, especially colorectal cancer (CRC), may be obesity-induced changes in gut bacteria and decreased SCFA production [97]. Ou and colleagues [171] reported that low fiber and high fat intake is associated with increased colon cancer risk in African

Americans due to a reduction in gut bacteria, especially butyrate producers, and colonic production of butyrate. The opposite was found in rural native Africans feeding on a high fiber diet [171]. Several studies have shown the antitumorigenic effects of butyrate mainly by inhibiting histone deacetylaces (HDACs) and promoting apoptosis via other HDAC-independent mechanisms [102, 112, 172]. It has been shown that the anti-inflammatory effects of butyrate in the colon is mediated via HDAC inhibition [104]. Thus, it is speculated that the increase in butyrate production mediated by mango supplementation may be vital in maintaining a healthy colon even in the face of high fat feeding. However, further evidence via histological studies of the colon may be needed to affirm the ability of mango supplementation to maintain a healthy colon despite high fat feeding.

This study also investigated the effects of mango supplementation on plasma and hepatic lipids. Mango supplementation had a mixed effect on plasma lipids. Plasma total cholesterol was elevated in the high fat group, and was further elevated significantly with 10% mango supplementation. A previous study which placed heathy volunteers on lactulose supplementation reported an increase in serum total cholesterol thereby concluding that certain types of fiber may raise serum cholesterol levels and this may be due to increased acetate incorporation into cholesterol in the liver [173]. In support of this concept, when Wolever et al. [174] gave rectal infusions of acetate to healthy subjects, an increase in serum acetate and cholesterol was observed. These studies present strong evidences that increased acetate metabolism in the liver may increase plasma cholesterol since acetate is a precursor of cholesterol synthesis [175]. In contrast, propionate is known as a strong inhibitor of acetate incorporation into cholesterol in the liver [176]. In

this present study, 10% mango supplementation resulted in a significant increase in both fecal and cecal acetate levels compared to the high fat group with only no significant increase observed in propionate levels. Since microbial-produced acetate and propionate are rapidly absorbed and metabolized in the liver [97], the elevation of plasma total cholesterol with 10% mango supplementation may be a result of mango's inability to produce sufficient propionate levels capable of inhibiting the incorporation of acetate into cholesterol in the liver.

Although mango supplementation seems to have negative effects on plasma cholesterol, the present study showed a dose-dependent decrease in plasma triglyceride levels compared to the high fat group. A similar decrease in triglyceride levels has been reported in healthy human volunteers supplemented with whole and fresh-cut mango [145]. Our study however showed that in an obese state induced by a high-fat diet, mango supplementation prevented an elevation in plasma triglycerides similar to control. Interestingly, the reduction in plasma triglyceride levels was also accompanied by reduction in plasma non-esterified fatty acid (NEFA) with 10% mango supplementation. It is noteworthy that this reduction in circulating TG and NEFA by mango supplementation is independent of a reduction in fat accumulation as the mango supplemented group had similar white adipose tissue weights as the high fat group. Since there were no noticeable changes in liver lipids, these results suggest a reduction in the lipolytic activity in the white adipose tissue which is usually elevated in high fat diet-induced obesity state characterized by increased fat accumulation [5, 177]. A possible mechanism by which mango may mediate this effect may be linked to an increase in microbial SCFA production. Although we did not measure serum SCFA or its effect on

adipose tissue metabolism, the study of Ge and colleagues [178] showed that SCFAs, especially acetate and propionate, inhibited adipose tissue lipolysis via the activation of GPR43 and this effect was nullified in GPR43-deficient animals. Therefore, mango supplementation reduced TG and NEFA, possibly via SCFA-mediated inhibition of adipose tissue lipolysis. This may be important since dyslipidemia may cause lipotoxicity of peripheral tissues and are linked to obesity-related complications such as cardiovascular occurrences [5].

As part of understanding the effects of mango supplementation on glucose homeostasis, plasma concentrations of incretins (i.e., glucagon-like peptide-1, GLP-1 and glucose-dependent insulinotropic polypeptide, GIP) which increase insulin secretion and glucose sensitivity as well as enhance proliferation of β-cells, were assessed [150]. The present data showed that 10% mango supplementation stimulated an increase in plasma GLP-1 similar to the HF group without any effect on GIP. This was accompanied by a concomitant increase in plasma insulin only in the HF+10% mango group. This is in agreement with a previous study which showed an increase in plasma insulin in a GLP-1-dependent manner in oligofructose-supplemented mice fed a high-fat diet [159]. Mango may act as prebiotics similar to oligofructose and increase plasma GLP-1 by increasing the number of enteroendocrine L-cells in the jejenum and colon [179]. The enteroinsular axis is known to be involved in pancreatic insulin release via incretins, in a glucose-dependent manner [156]. Since our high fat and mango-supplemented groups had similar blood glucose, our data showed mango supplementation improved the efficiency of insulin secretion via the enteroinsular axis. However, this increase in plasma GLP-1 and insulin may not have been sufficient to physiologically modulate blood glucose as we did

not observe any differences in glucose tolerance among the mango-supplemented and the high fat diet groups.

In contrast to the data on plasma incretin l seen in this study, it was intriguing to note that pancreatic expression of GIP receptor was elevated by mango supplementation compared to the high fat group while GLP-1 receptor expression remained unchanged. Two factors may have contributed to this contrasting result. First, is the ability of the enteroinsular axis to compensate for GLP-1 inaction by upregulating the GIP-insulin axis [180]. Pederson and colleagues [180] showed that GLP-1R knockout mice modified the insulinotropic action of GLP-1 by activating the GIP component of the enteroinsular axis. However, a recent study concluded that the enteroinsular axis requires the concerted action of both incretins (GLP-1 and GIP) to maintain glucose homeostasis [156]. This may have been the reason that mango supplementation had no effect on glucose tolerance despite an increase in insulin levels. A second reason may have been our inability to determine the protein levels of these receptors specifically on B-islets as against the whole pancreas used in this study which is a limitation of our study. However, our data suggest that mango may be effective in improving insulin secretion by possible stimulation of the enteroinsular axis, although this concept needs to be further explored.

Based on the current evidence provided in this study, future studies may focus on the contribution of microbial production of SCFA due to mango supplementation on serum SCFA and the direct impact on liver and adipose metabolism. Furthermore, the implication of butyrate production due to mango supplementation in this study may be further investigated in a model of colorectal cancer, since previous *in vitro* evidence has shown that treating colon cancer cells with mango pulp extract strongly inhibited their

growth and induced the expression of apoptotic markers [141]. To our knowledge, this is the first study to show 10% mango's ability at increasing the abundance of the genus *Aldercreutzia* despite high fat feeding. *Aldercreutzia* is a recently characterized genus capable of producing equol, an antioxidant, from dietary isoflavonoids especially from soy [181, 182]. Leaning on the knowledge that mango fruit is a rich source of various polyphenols [135, 137, 138], it will be interesting to extensively study the possible impact of mango isoflavonoids on equol production and potential physiological benefits that may be derived from it. This may provide evidence of mango's ability in improving both gut and systemic antioxidant status.

In conclusion, this study suggest that despite the inability of mango supplementation in reducing body weight gain, fat accumulation and glucose intolerance induced by a high fat diet, it modulates gut bacteria differently from the high fat group in favor of the beneficial Bifidobacteria and Akkermansia, and enhanced short chain fatty acid production. The results also indicate that mango supplementation in mice fed a high fat diet mediates the reduction of plasma triglycerides and NEFA, improves insulin secretion possibly via the action of incretins and enhanced anti-inflammatory cytokine production in the gut. These results imply that mango supplementation in high fat feeding may be useful in the beneficial modulation of some adverse effects that accompanies high fat diet-induced obesity.

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