ROLE OF THE AMPK-C/EBPα/PPARγ AND AMPK-UCP1/SIRT1 AXES IN THE ANTI-OBESOGENIC ACTIVITIES OF GALLOTANNIN DERIVATIVES FROM MANGO (*MANGIFERA INDICA* L.)

A Dissertation

by

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ABSTRACT

Obesity is an escalating global epidemic associated with increased risk of developing type 2 diabetes, cardiovascular diseases and cancer. Dietary modifications, particularly increasing consumption of polyphenol-rich foods, are considered some of the most effective strategies in the prevention of obesity-related chronic diseases. Mango (*Mangifera indica* L.) contains high content of phenolic compounds (e.g., gallic acid (GA), gallotannin (GT), and galloyl glycosides), showing anti-inflammatory and anti-obesogenic potential in chronic diseases. *Lactobacillus plantarum* (*L.plantarum)* possesses enzymatic activities to degrade GT into GA and PG, allowing for absorption and excretion. Thus, the interaction between gut microbiota and GT derivatives may affect the subsequent biological activities exerted by the microbial metabolites.

Anti-obesogenic potential of GT derivatives from mango in modulating lipid metabolism was investigated in 3T3-L1 adipocytes. GT derivatives suppressed adipogenesis and increased thermogenesis in adipocytes in part through the interactions with the AMPK-C/EBPα/PPARγ and AMPK-UCP1/Sirt1 axes. In gnotobiotic mice fed a high-fat diet (HFD), GT alone decreased lipid accumulation in white adipose tissue and increased thermogenesis in brown adipose tissue. Intestinal colonization with *L.plantarum* enhanced these effects and additionally lowered levels of inflammation and insulin resistance. GT and *L.plantarum* reduced HFD-induced inflammation and insulin resistance and promoted thermogenesis in adipose tissue potentially through the activity of GT-metabolizing bacterial enzymes yielding absorbable bioactive GT metabolites,

which implies the potential role of prebiotic-probiotic interactions in the prevention of diet-induced metabolic disorders.

These findings were expected to translate into a human clinical trial, which examined the influence of 6 weeks of daily mango supplementation on inflammation and metabolic functions. Mango supplementation improved the plasma levels of pro-inflammatory cytokines and metabolic hormones in obese participants partly due to increased systemic exposure to polyphenolic metabolites.

In summary, health benefits of mango-derived polyphenols in obesity and insulin resistance are mainly attributed to the production of microbial metabolites of GT, which is in part through the interactions with the AMPK-C/EBPα/PPARγ and AMPK-UCP1/Sirt1 axes in adipose tissue. Improving the abundance of probiotics in gut microbiota may improve the bioavailability of mango-derived polyphenols, resulting in enhanced efficacy of the microbial metabolites in the prevention of lipid accumulation and metabolic dysfunction in obesity.

DEDICATION

To my grandfather, Pinde Fang, who raised me and taught me nothing in this world can take the place of persistence.

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This work was supervised by a dissertation committee consisting of Professor Susanne Talcott, advisor and committee chair, and Professors Stephen Talcott and Yuxiang Sun of the Department of Nutrition and Food Science, and Professor Robert Alaniz of the College of Medicine.

All work for the dissertation was completed by the student independently.

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CHAPTER I

INTRODUCTION

Obesity is an escalating global epidemic associated with increased risk of developing type 2 diabetes (T2D), hypertension, and cardiovascular diseases. Excessive fat accumulation can cause dysfunction of adipokines involved in energy homeostasis and metabolic functions, contributing to chronic low-grade inflammation and insulin resistance in multiple tissues and organs. Dietary modifications, particularly increasing consumption of polyphenol-rich foods, are considered some of the most effective strategies in the prevention of obesity-related chronic diseases.

Emerging evidence demonstrates the two-way relationship between dietary polyphenols and the composition of the intestinal microbiota where an increased intake of polyphenols may shape the composition of the intestinal microbiota by increasing species with the ability to metabolize polyphenols. Pharmacokinetics of dietary polyphenols is influenced by gut microbiota, where inter-individual microbial biodiversity may translate into great variability of associated pharmacodynamics endpoints. Mango (*Mangifera indica* L.) contains high content of phenolic compounds (e.g., gallic acid (GA), gallotannin (GT), and galloyl glycosides), showing anti-inflammatory and anti-obesogenic potential in chronic diseases. Particularly, *Lactobacillus plantarum* (*L.plantarum)* possesses enzymatic activities to degrade GT into GA and PG, allowing for absorption and excretion. Thus, the interaction between gut microbiota and GT derivatives may affect the subsequent biological activities

exerted by the microbial metabolites. For this reason, this project targets further mechanistic and translational investigation.

Based on previous evidence and our preliminary data, we hypothesized that 1) GT derivatives suppress adipogenesis and increase thermogenesis in 3T3-L1 adipocytes in part through the interactions with the AMPK-C/EBPα/PPARγ and AMPK-UCP1/Sirt1 axes, 2) the consumption of GT in addition to a high-fat diet (HFD) in mice modulates obesity-associated biomarkers, 3) The presence of intestinal *L.plantarum* in germ-free (GF) mice modulates biological activities of GT, 4) the consumption of mango-derived GT modulates obesity-associated biomarkers in a human clinical study.

These hypotheses were tested in the following Specific Aims:

Specific Aim 1. Determine the actions and molecular mechanisms of mango-derived GT metabolites in 3T3-L1 adipocytes. 3T3-L1 preadipocytes were differentiated into adipocytes and treated with mango polyphenols (MG), or PG for 6 days. The anti-adipogenic activity was examined using Oil-Red-O staining and analyzing the expressions of molecules involved in adipogenesis. In addition, mature adipocytes were treated with MG and PG for 24 hours to examine the expressions of thermogenic molecules. Knockdown of AMP-activated protein kinase (AMPK) α1 with small interfering RNA (siRNA) was applied to confirm the hypothesis that MG and PG suppress adipogenesis at least in part through the activation of the AMPK pathway.

Specific Aim 2. Investigate the extent that health benefits of mango-derived GT metabolites are associated with intestinal *L. plantarum* in HFD-induced obese gnotobiotic mice. GF C57BL/6J mice were divided into three groups, GF control, GF

gavaged with GT, and mice colonized with *L.plantarum* and gavaged with GT. After 4 weeks of HFD feeding, mice were sacrificed and biomarkers for inflammation, insulin resistance, lipogenesis and thermogenesis were assessed in plasma, epididymal white adipose tissue, and interscapular brown adipose tissue.

Specific Aim 3. Investigate the influence of daily mango supplementation for 6 weeks on inflammation and metabolic functions in lean and obese individuals. Healthy lean (BMI 18-25 kg/m^2) and obese (BMI>30 kg/m^2) participants were recruited and received daily 400 g of mango pulp for 6 weeks. Inflammatory cytokines, metabolic hormones and lipid profiles were examined in plasma before and after 6 weeks of mango consumption. The modulation of obesity-associated disease biomarkers by mango consumption was correlated to systemic exposure of polyphenolic metabolites.

Specific Aim 4. Determine the actions and molecular mechanisms of microbial metabolites of GT in RAW 264.7 macrophages. RAW 264.7 macrophages were treated with lipopolysaccharides (LPS) in the presence of GA, PG or a vehicle control. Levels of LPS-induced pro-inflammatory cytokines and oxidative stress were examined. Their anti-inflammatory activity and molecular mechanisms were further investigated, with a focus on the AMPK and nuclear factor kappa b (NF-κb) activities, which may provide a linkage between the AMPK activation and regulation of inflammatory response in macrophages.

We expect that the chronic exposure of mango-derived polyphenols exerts health benefits in both lean and obese individuals. These health benefits in obesity, inflammation, and insulin resistance are mainly attributed to the production of the

bioactive microbial metabolites after mango consumption. Improving the abundance of probiotics (e.g., *L.plantarum*) in human gut microbiota may help improve the bioavailability of mango-derived polyphenols, resulting in enhanced bioefficacy of the microbial metabolites in the prevention of lipid accumulation and metabolic dysfunction in obesity and its-related chronic diseases. Findings from this research project are expected to link the biological activities of dietary polyphenols to gut microbial composition and provide novel insights into dietary recommendations that include probiotics into our diet to increase bioavailability and bioefficacy of dietary polyphenols.

CHAPTER II

LITERATURE REVIEW*

2.1 Obesity, Inflammation and Chronic Disease

Obesity is an escalating global epidemic, nearly 30% of the adult population is considered either obese or overweight (1). Obesity, represented by higher body mass index (BMI) >30 kg/m^2, is associated with increased risk of developing a large number of chronic metabolic diseases, including type 2 diabetes (T2D), hypertension, cardiovascular diseases and cancer (2). Chronic disease clusters are the leading causes of death in the United States, representing approximately 60% of all death, based on the data released in 2010 by the Centers for Disease Control and Prevention (CDC). About half of U.S. adults have at least one chronic disease (3, 4). Chronic disease-associated morbidity, mortality, and health-care costs pose major challenges to our society **(Figure 1)**. Therefore, preventive measures are expected to significantly reduce health care expenditures associated with obesity-related chronic conditions in the United States (5). Excessive accumulation of fat in obesity can cause dysfunction of adipokines involved in energy homeostasis and metabolic functions, contributing to chronic low-grade inflammation and metabolic abnormalities in multiple tissues and organs through the

* Part of this chapter is reprinted with permission from Fang, C., Xu, H., Guo, S, Mertens-Talcott, S. U., & Sun, Y (2018). Ghrelin Signaling in Immunometabolism and Inflamm-Aging. In *Neural Regulation of Metabolism* (pp. 165-182). Springer, Singapore.Copyright [2019] by Springer Nature; Barnes, R. C., Kim, H., Fang, C., Bennett, W., Nemec, M., Sirven, M. A., ... & Talcott, S. T. (2019). Body Mass Index as a Determinant of Systemic Exposure to Gallotannin Metabolites during 6-Week Consumption of Mango (Mangifera indica L.) and Modulation of Intestinal Microbiota in Lean and Obese Individuals. *Molecular nutrition & food research*, *63*(2), 1800512. Copyright [2019] by John Wiley and Sons.

pro-inflammatory cytokine secretion - immune cell infiltration - tissue dysfunction cascade (6, 7).

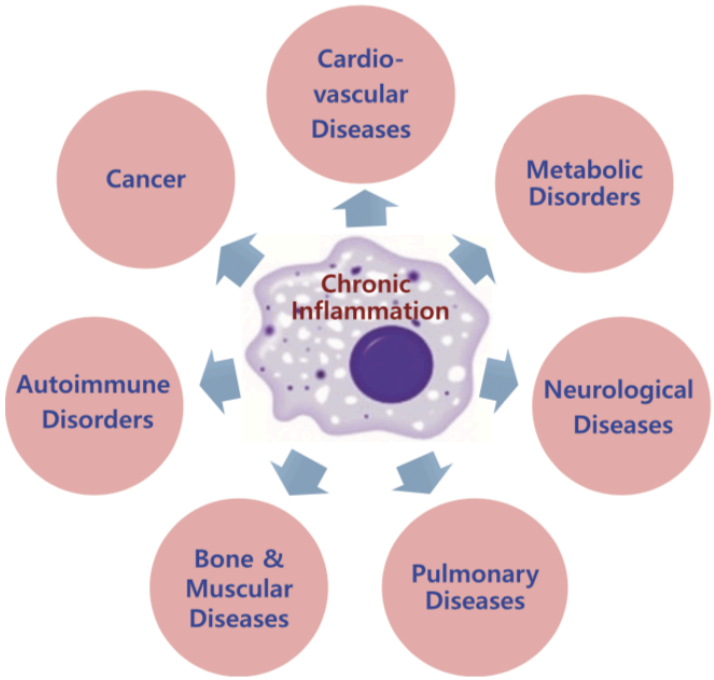

Figure 1 Obesity-associated chronic inflammation and chronic diseases. Reprinted with permission from *Neural Regulation of Metabolism* (pp. 165-182). Springer, Singapore.Copyright [2019] by Springer Nature.

2.1.1 Types of Adipose Tissue and Their Functions

Obesity is characterized by the expansion of adipose tissue, with increases in both number and size of adipocytes. Adipocyte differentiation from fibroblasts to adipocytes, also called adipogenesis, determines the number of adipocytes formed in the

development of obesity (8). Elevated adipogenesis is linked to increased fat deposition in adipose tissue (9). The differentiation of preadipocytes into adipocytes is a chronological event composed of distinct stages where transcriptional factors and adipocyte gene expressions are highly regulated by exogenous effectors. A number of key transcription factors are required in the early stage of adipocyte differentiation, including CCAAT-enhancer-binding proteins (C/EBP) α, C/EBPβ, C/EBPδ and peroxisome proliferator-activated receptor (PPAR) γ (10). After the early stage of differentiation, adipogenic genes encoding for glucose transporter 4 (GLUT4), lipoprotein lipase (LPL), fatty acid synthase (FAS), fatty acid binding protein 4 (FABP4) and adiponectin are necessary to induce the formation of mature adipocytes (11).

Adipose tissue is classified as either white adipose tissue (WAT) or brown adipose tissue (BAT). The size of lipid droplets in adipocytes is associated with the types of adipose tissue, with WAT being larger than BAT. Thermogenesis associated with adipocyte browning is the process through which WAT takes on the characteristics of BAT upon physiological and nutritional stimuli, such as cold exposure, pharmacological treatment, and phytochemical consumption. This process is characterized by enhanced expressions of thermogenic markers such as uncoupling protein 1 (UCP1) and sirtuin1 (Sirt1) (12, 13). Recently, a new type of brown-like adipocytes was discovered to have an increased number of mitochondria and reduced lipid droplet size within WAT. This novel type of cells is called beige adipocytes. Promoting thermogenesis is considered a potential therapeutic strategy in the prevention

of obesity and its co-morbidities (14). Due to limited amount and activity of BAT in adults, the brown remodeling of WAT is being investigated as a potential anti-obesogenic strategy **(Figure 2)**.

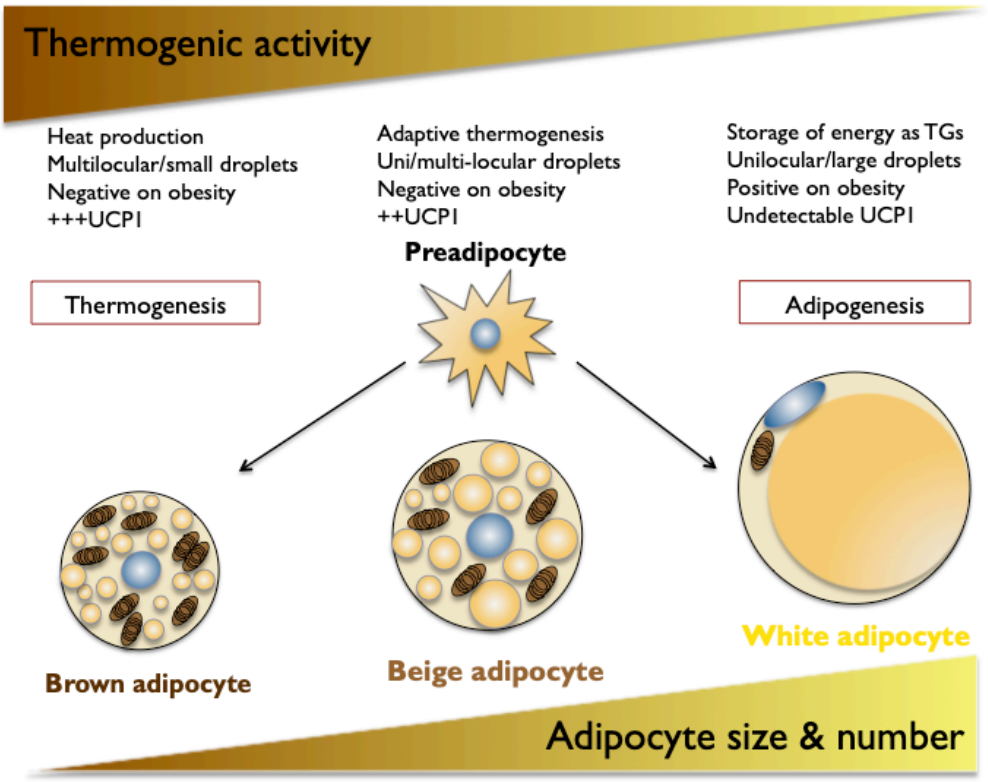

Figure 2 Regulation of adipogenesis and thermogenesis in adipocytes.

Understanding adipose tissue functions and molecular regulation is vital in the investigation of the therapeutic potential of bioactive compounds in the prevention of obesity and its related chronic diseases. Adipose tissue is known for its key role in energy storage by accumulating fat in the form of triacylglycerols (TAG) within lipid

droplets during energy excess, and mobilizing fat during energy deprivation. WAT also acts as an endocrine organ secreting adipokines, such as interleukin (IL)-6, IL-1β, and tumor necrosis factor-α (TNF-α), as well as hormones such as leptin and adiponectin; these peptides modulate inflammatory states and metabolic functions of tissues in the entire body (15, 16). In contrast, BAT contains large amounts of mitochondria that use lipids to generate heat (non-shivering thermogenesis) to maintain normal body temperature and protect against cold stress under cold exposure. Upon cold stimulation, the sympathetic nervous system (SNS) is activated. Nerve terminals release norepinephrine (NE) into BAT; NE binds to β3-adrenergic receptor (β3-AR). The activation of β3-AR leads to lipolysis, which converts TAG into free fatty acid (FFA). As a result, UCP1 recruits FFA into mitochondria to enhance respiration, subsequently promoting substrate oxidation and heat production (17, 18).

2.1.2 Role of Adipose Tissue in Obesity and Inflammation

Over the last two decades, adipose tissue has been considered a highly metabolically active endocrine organ that modulates energy and glucose homeostasis. WAT contains various cell types such as adipocytes, preadipocytes, dendritic cells, T cells, and adipose tissue macrophages, all of which secrete adipokines into the systemic circulation (19). Adipokines, a wide range of protein signals and factors, function as circulating hormones to communicate with other organs including brain, liver, skeletal muscle, as well as itself, adipose tissue, thus affecting metabolic functions of these

tissues and organs. Dysregulation of adipokines has been implicated in obesity, T2D, and cardiovascular disease (20).

Obesity is the consequence of sustained positive energy balance due to excessive energy intake and low energy expenditure (e.g., physical activity). Obesity is characterized by the expansion of WAT, the most effective lipid storage organ in the body. When higher nutrient flux into adipose tissue, adipocytes primarily respond to the higher demand for energy storage by increasing their size, called adipocyte hypertrophy. Adipocyte hypertrophy is typically associated with decreased insulin sensitivity, increased hypoxia and stress within adipose tissue along with increased production of pro-inflammatory cytokines (e.g., TNF-α, IL-1β, monocyte chemoattractant protein-1 (MCP-1) and plasminogen activator inhibitor-1 (PAI-1)). Increased autophagy is frequently reported in visceral adipose tissue as a major mechanism that adipocytes protect themselves from chronic condition-induced death. Cellular components released upon adipocyte apoptosis may further increase immune cell recruitment into adipose tissue. As a result, chemoattractant molecules bind to its receptors on monocytes subsequently recruiting immune cells (e.g., adipocytes, T cells, pre-adipocytes, dendritic cells and macrophages) into adipose tissue. Consequently, adipose tissue inflammation develops and causes impaired insulin signaling or the activation of pro-inflammatory pathways in target tissues through multiple mechanisms, including increased lipolysis and higher FFA release from adipose tissue, reduced glucose uptake, altered adipokines secretion and increased secretion of pro-inflammatory cytokines. This may ultimately

cause systemic insulin resistance and inflammation in other insulin-sensitive tissues and organs, such as brain, liver, muscle and heart (21) **(Figure 3).**

Collectively, diet-induced obesity is associated with increased adiposity and pro-inflammatory cytokine secretion in adipose tissue, which may have detrimental impacts on systemic insulin resistance, further exacerbating insulin resistance in metabolic tissues, particularly in insulin-targeting organs of the liver, muscle, and adipose tissues. Given the pivotal role of inflammation in insulin resistance and T2D, control of inflammatory response would have profound effects on insulin resistance and is a therapeutic strategy for chronic diseases.

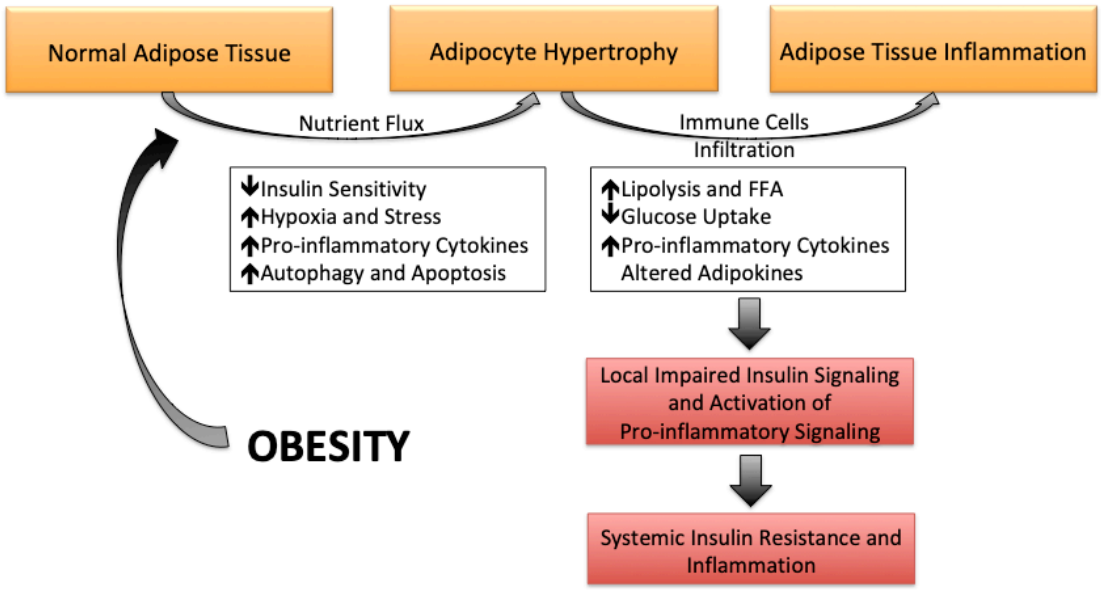

Figure 3 Mechanisms of obesity-induced insulin resistance and inflammation in adipose tissue.

2.2 Dietary Polyphenols in Obesity and Chronic Diseases

Our diet is an essential factor modulating risk factors for obesity, insulin resistance and cardiovascular diseases. Preventive measures and dietary modifications, particularly increasing consumption of fruits and vegetables, are considered some of the most effective strategies in the prevention of obesity-related chronic diseases (22). Fruits and vegetables contain a wide variety of nutrients, fibers, vitamins, minerals and phytochemicals that can potentially modulate disease-preventive mechanisms. A minimum of 400 g of fruits and vegetables daily is recommended for the prevention of chronic diseases. United States Department of Agriculture (USDA) recommends the use of MyPlate, a healthy eating style built on a mixture of food groups, including fruits, vegetables, grains, proteins and dairy products (23). However, a typical Western diet is characterized by high intake of saturated fat, refined sugar, salt, alcohol and processed foods while low intake of fiber and nutrient-dense foods, contributing to the increased risk/occurrence of chronic diseases (24). According to World Organization of Health (WHO), approximately 1.6 millions of deaths worldwide are attributed to insufficient intake of fruits and vegetables (25).

2.2.1 Chemical Structure and Classes of Polyphenols

Polyphenols are naturally occurring phytochemicals found in fruits, vegetables, cereals, chocolate, coffee and tea. They are secondary metabolites of plants that protect plants against pathogens. In food, polyphenols contribute to bitterness, astringency, color, oxidative stability, and importantly, antioxidant and anti-inflammatory activities

(26). Based on the number of phenol rings contained and the way the phenols are bound together, polyphenols are divided into four main classes, including phenolic acids, flavonoids, stilbenes and lignans. Phenolic acids are further divided into hydroxybenzoic acids and hydroxycinnamic acids. Phenolic acids are found in a variety of plant-based foods, accounting for nearly one third of the polyphenolic compounds. They are particularly abundant in the seed/kernel, bark, leaves and peel of fruits and vegetables. Phenolic acids are present both as free aglycones and in conjugated form as esters with a carbohydrate, commonly quinic acid or glucose (26). Gallic acid (GA), also known as 3,4,5-trihydroxybenzoic acid, is one of the most common types of phenolic acid found in both free and as part of hydrolysable tannins. GA has emerged as an antioxidant and anti-inflammatory agent over the last few decades (27).

Tannins (tannic acid, TA) are water-soluble phenolic compounds having high molecular weights ranging from 500 to over 3000 Da, widely distributed in plants such as berries, banana, apple, grape, wine and tea. Tannins can form cross-linkages with proteins and other macromolecules to form precipitation due to the presence of several galloly, hydroxyl and other functional groups. As a result, tannin-rich foods have traditionally been considered to be of low nutritional value for monogastric animals and poultry. The anti-nutritional property of tannins has been reported to decrease feed intake and efficiency, as well as growth rate in farm ruminants. Recently, more studies have shown that when applied appropriately, tannins possess various biological activities, including anti-microbial, anti-parasitic, anti-inflammatory, anti-virus properties that are beneficial in enhancing defense systems against parasites and

pathogens (28). Tannins are classified into hydrolysable and non-hydrolysable (condensed) tannins. Hydrolysable tannins contain esters of GA (gallotannins, GT) or ellagic acid (ellagitannins), and a core polyhydric alcohol (e.g., glucose and hydroxyl group). Once hydrolysis by acids or enzymes (e.g., tannin acyl hydrolase), GT break down into GA and glucose (29) **(Figure 4)**.

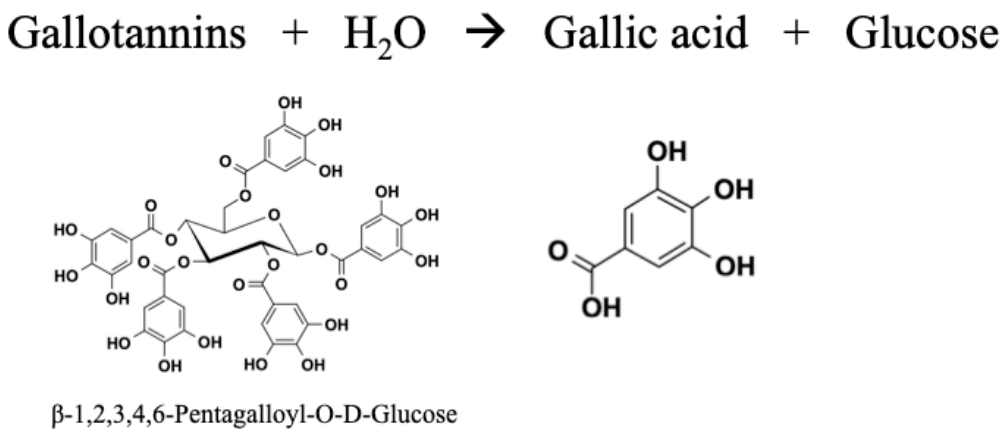

Figure 4 Chemical structure and metabolism of hydrolysable tannins.

2.2.2 Bioavailability and Metabolism of Polyphenols

Bioavailability refers to the rate and extent to which a drug or other substance enters systemic circulation and reaches its site of action (30). Therefore, the biological activities of dietary polyphenols depend largely on their bioavailability. Depending on structural complexity and polymerization, bioavailability of dietary polyphenols may vary between each individual polyphenol and generally considered low in humans.

Upon digestion, only a small percentage (5-10%) of less complex dietary polyphenols is believed to be absorbed into small intestine while 90-95% of dietary polyphenols is expected to reach the colon where hydrolysis, decarboxylation and other reactions are executed by intestinal microbiota, degrading to simple absorbable phenolic compounds. After absorption, polyphenols can be effluxed back into the intestinal lumen through bile acid or undergo structural modifications by Phase I (oxidation, reduction, and hydrolysis) and II (the introduction of a hydrophilic endogenous species, such as methyl, glucuronide or sulfate groups, to the molecule) biotransformation in the enterocytes and then the hepatocytes, resulting in a series of conjugated metabolites passaging into the systemic circulation for further distribution to organs and tissues before ultimately excreted in the urine and feces (31) **(Figure 5)**.

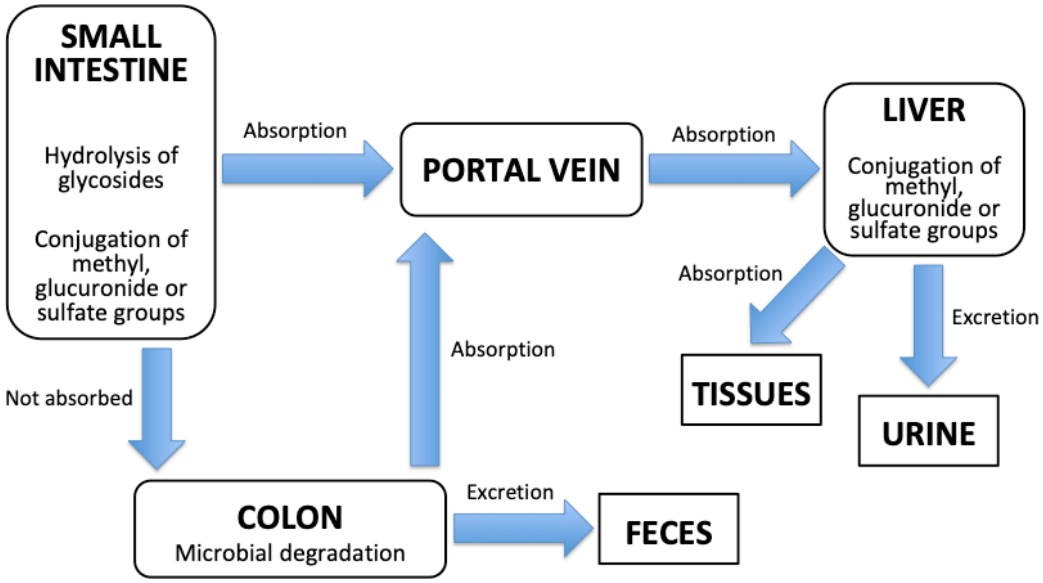

Figure 5 Metabolic fate of dietary polyphenols.

2.2.3 Biological Activities of Polyphenols

Dietary polyphenols serve as potent antioxidants and anti-inflammatory agents, which enables them to protect human cells again damages due to obesity-related chronic inflammation and oxidative stress leading to oxidation of lipids, nucleic acids and protein in the pathophysiological development of chronic diseases such as insulin resistance, cardiovascular disease and cancers (32). Major proposed mechanisms through which natural polyphenolic antioxidants protect against oxidative stress include Hydrogen Atom Transfer (HAT), Single Electron Transfer (SET) and Transition Metals Chelation (TMC) (33). Dietary polyphenols exert anti-inflammatory activity notably through inhibiting the synthesis of pro-inflammatory cytokines (e.g., TNF-α and IL-1β), immune cell infiltration (e.g., macrophages and T cells), the production of several enzymes (e.g., cyclooxygenase (COX), lipoxygenase (LOX) and nitric oxide synthase (NOS)), and mediating molecular signaling associated with the inflammatory response (e,g., nuclear factor kappa b (NF-κb) and mitogen activated protein kinase (MAPK)) (34).

A deep understanding of *in vitro* and *in vivo* findings will greatly enhance the successful translation of novel drugs and dietary interventions to clinical settings. In regards to obesity, it has been well established that some polyphenols, such as catechins, epigallocatechin gallates, resveratrol and curcumin have shown pronounced effects on modulating obesity-related inflammation, dyslipidemia, glucose intolerance and insulin resistance (35). Hormonal and nutritional regulation of lipid metabolism by dietary polyphenols has gained increasing attention over the last decade due to their potential as

a dietary approach in the prevention of chronic diseases. Adipogenesis inhibition and thermogenesis promotion are promising targets in the prevention of obesity and obesity-related chronic diseases (36). Dietary polyphenols have shown to reduce adipogenesis (8, 37-39), as well as increase thermogenesis by converting white adipocytes into beige adipocytes (40-43). For example, in 3T3-L1 adipocytes, green tea catechins treatment dose-dependently reduced lipid accumulation by modulating transcriptional activity of C/EBPα, sterol regulatory element-binding protein 1c (SREBP-1c), PPARγ, Forkhead box protein O1 (FOXO1) and acetyl CoA carboxylase (ACC) (44-47). Administration of resveratrol and curcumin induces thermogenesis by increasing the expressions of thermogenic markers, such as PPARγ coactivator 1α (PGC-1α) and UCP1, both *in vitro* (48) and *in vivo* (49, 50). In high-fat diet-induced obese mice, green tea catechins supplementation decreased levels of body fat mass, cholesterol, glucose, insulin resistance and inflammation (51, 52).

Human clinical trials have also shown promising cardio-protective, neuro-protective effects, as well as anti-cancer and anti-aging activities of dietary polyphenols (26). Epidemiological studies have repeatedly reported an inverse correlation between the consumption of polyphenol-rich foods and the risk/occurrence of chronic diseases (53). It's worth noting that dietary supplementation of polyphenol-rich foods exert inconsistent effects in obese subjects with metabolic disorders, varying from reducing body weight and BMI in 2 months (54), to no effects after 3 months of supplementation (55). Discrepancy in human clinical trials might be attributable to the subject recruitment criteria, sample size, supplementation form and duration of the study. Major

controversies have been made around the bioefficacy of dietary polyphenols due to their low bioavailability in humans. Therefore, these clinical investigations fail to yield clear guidelines for intake recommendations.

2.3 Nutritional and Phytochemical Contents of Mango

Mango (*Mangifera indica* L.) is a tropical fruit believed to be originated from Southeast Asia and nowadays consumed worldwide for its flavor, exotic taste and high nutritional values. Among hundreds of mango cultivars, Tommy Atkins is the most common cultivar in the U.S. food market, followed by Ataulfo, Francis, Haden, Keitt, Kent and Palmer. Mango fruit has been reported to contain high content of carbohydrates, fiber, vitamin A, vitamin C, essential amino acids and phytochemicals, notably GT and mangiferin in the bark, leaves, peel and seed, while little information is available on the mango flesh/pulp, which is primarily consumed as fresh produce, fruit juice and dried fruits (56).

2.3.1 Mango Polyphenolic Composition and Metabolism

Mango is a rich source of phenolic compounds, including GA, GT, galloyl glycosides, and flavonoids, showing high antioxidant potential (57, 58); this promotes the application of mango-derived polyphenols in the prevention of obesity-related chronic diseases. In mango pulp, 11 GT and their isomers have been identified, from mono galloyl-glycosides to hepta galloyl-glycosides (59). Simple absorbable compounds such as GA and mono galloyl-glycosides are likely to either be absorbed or

undergo Phase I metabolism in the small intestine. GT are not absorbable in their polymerized form but can be hydrolyzed in the gut into absorbable GA and galloyl-glucose by tannase (tannin acyl hydrolase, EC 3.1.1.20), which hydrolyzes galloyl-ester bonds to produce free GA and smaller galloyl-glucose. Additionally, gallic acid decarboxylase (EC 4.1.1.59), which metabolizes GA into PG and occurs less frequently than tannase, may contribute to inter-individual variability in GT metabolism. Upon absorption, the main biotransformational pathways include methylation and sulfation by endogenous phase II enzymes, such as catechol-O-methyl transferase (COMT), uridine diphosphoglucuronosyl transferase (UGT) and sulfotransferase (SULT). These metabolize GA and PG mainly to 4-O-methylgallic acid and 4-O-methylgallic acid-3-O-sulfate, and pyrogallol-O-sulfate and methylpyrogallol-O-sulfate, respectively (60).

2.3.2 Inter-individual Variability of Polyphenolic Metabolism in Obesity

Inter-individual variability of polyphenolic metabolism is mainly attributed to genetic factors relevant to xenobiotic metabolism, particularly pharmacokinetics (known as digestion, absorption, metabolism, distribution and excretion) of polyphenols. However, other factors, such as differences in the composition of the intestinal microbiota between lean and obese individuals, contribute to inter-individual variability. Previous reports indicate that obese individuals may experience a lower systemic exposure to polyphenols than lean individuals. In addition to inter-individual differences in polyphenol pharmacokinetics, repeated intake, and duration of intake may also result in enhanced systemic exposure. For instance, recurring consumption of grape seed

extract by rats for 10 days yielded a 395% increase in GA in plasma, while 800 mg of green tea polyphenols daily in humans for 4 weeks led to a 60% increase (60).

A few possible explanations may provide insights into the pharmacokinetic differences of polyphenols between lean and obese individuals. First, obese individuals provide larger blood volumes to distribute polyphenols, resulting in lower concentration of polyphenols compared to lean individuals. After systemic exposure was normalized to plasma volume, higher BMI was associated with lower systemic exposure of catechin, epicatechin, and quercetin after 10 days of repeated dose of 2125 mg total polyphenols sourced from resveratrol, grape seed extract, and grape juice (61).

In addition, pharmacokinetic changes of polyphenols in obesity are associated with polyphenolic transporters, as well as enzymes involved in Phase I and II biotransformation in the liver. Transport of phenolic acids across brush border of small intestine is deemed to be regulated by transporters including sodium-dependent glucose transporter 1 (SGLT1), glucose transporter 2 (GLUT2) (62), and monocarboxylate transporter 1 (MCT1) (63). After absorption, polyphenols can be effluxed back into the intestinal lumen via multidrug resistance-associated protein (MRP1/2) (63) or can be conjugated through methylation regulated by COMT (64), glucuronidation by UGT (65), or sulfation by SULT (66), ultimately excreted in the urine and feces.

Last but not least, lean and obese individuals differ in gut microbial composition. Intestinal microbiota affects the hydrolysis of unabsorbable, high molecular weight polyphenols, which may translate into variations of biological activities in lean and obese individuals. In regards to mango-derived polyphenols, some

intestinal microbiota produce tannase and decarboxylase to degrade GT to GA and PG. In support of this hypothesis, tannase treatment on tea polyphenols *in vitro* enhanced the efficacy of bio-transformed tea polyphenols in reducing obesity (67).

Taking into account these physiological factors in obesity, repeated exposure of polyphenols may not convey the same health benefits in humans as in animals and cells. Therefore, there is a need to evaluate BMI-associated differences in pharmacokinetics and their impacts on polyphenol-based health effects.

2.4 Biological Activities of Mango-derived Polyphenols in Obesity

Natural phytochemicals sourced from mango fruits and their byproducts show potent anti-inflammatory, anti-obesogenic and anti-diabetic activities that are relevant to the prevention of obesity, T2D and cardiovascular disease. However, the underlying mechanisms by which polyphenols exert health-promoting effects in humans are complex and remain to be elucidated.

Much of the work on their molecular mechanisms has been conducted using multiple *in vitro* and *in vivo* models sharing characteristics of human obesity-related chronic diseases. Reviewing the literature we found 5 *in vitro* and 5 *in vivo* investigations. A majority of cell culture work with polyphenolic compounds was conducted using 3T3-L1 mouse preadipocytes with a focus on preadipocyte differentiation, the major process affecting lipid accumulation in adipocytes. Taing *et al.* (2012) found that mango peel extracts inhibited adipogenesis in 3T3-L1 adipocytes through a molecular mechanism similar to resveratrol, most likely associated with the

suppression of mitotic clonal expansion (68), while mango flesh extracts showed no inhibitory effects. However, this study only assessed the effects of mango extracts on the process that preadipocytes differentiating into adipocytes without considering their effects in mature adipocytes. Growing evidence indicates the involvement of dietary polyphenols in inducing adipocyte browning and the formation of beige adipocytes (14). Zhang *et al.* (2013) demonstrated that Benzophenone C-glucosides, iriflophenone 3-C-β-glucoside and foliamangiferoside A, the predominant polyphenolic metabolites after the consumption of mango leaf extract, were associated with lipid and glucose homeostasis in 3T3-L1 adipocytes. They further proposed the AMP-activated protein kinase (AMPK)-dependent mechanism in the down-regulation of lipid accumulation and the phosphatidylinositol-3-kinase (PI3K)/AKT-dependent mechanism in the up-regulation of glucose uptake (69). Several investigations have been undertaken using single purified compounds such as TA (Liu *et al.* 2004) and GA (Pandey *et al.* 2014) showing similar effects in inhibiting adipogenesis as the whole mango extract (70, 71). More attention should be paid that the bioavailability of dietary polyphenols is reported to be consistently low in humans. As a result, physiological concentrations of GA and PG identified in plasma have been low. The concentration range of polyphenolic compounds in these *in vitro* studies where the highest concentrations were not likely to be reached after the consumption of foods or dietary supplements makes it difficult to translate into *in vivo* effects.

The use of animal models in obesity and diabetes research is critical in the discovery, validation and optimization of novel therapies for their clinical translation in

humans. In the context of effects of dietary polyphenols on obesity-related chronic diseases, diet-induced obese animal model (e.g., mouse and rat) and transgenic (knock-out or knock-in) animal model are frequently used to investigate the anti-obesogenic and anti-diabetic potential and actions of mechanisms of dietary polyphenols. In animal studies, mangos have primarily been supplemented in the form of freeze-dried mango pulp and mango juice. Lucas *et al.* (2011) found that freeze-dried mango pulp (1 and 10% of diet) exert similar effects in reducing high-fat diet (HFD)-induced adiposity and glucose intolerance compared with the lipid-lowering drug fenofibrate, and the glucose-lowering drug rosiglitazone, with 1% mango exerting the most prominent effect. However, Ojo *et al.* (2016) reported that 10% mango greatly prevented the HFD-associated decrease in the microbial population of *Bifidobacteria* and *Akkermansia* along with greater short-chain fatty acids (SCFAs) (i.e., acetic and butyric acid) and IL-10 production while 1% showed no effect on these biomarkers (72). It remains to be investigated that the optimal dose of the freeze-dried mango pulp and the mechanisms underlying their health-promoting effects in obesity and insulin resistance (73). Natal *et al.* (2016) found that *Ubá* mango juice enriched with phenolic compounds from mango peel extract reversed HFD-induced weight gain, adiposity and inflammation (decreased TNF-α and increased IL-10) in obese Wistar rats. This study suggests that the effects are due, at least in part, to the inhibition of lipid accumulation (i.e., LPL, FAS and PPARγ) in and hypertrophy of epididymal adipose tissue (74). Finally, Zhang *et al.* (2013) proposed mechanisms that mango leaf extract modulated glucose and lipid homeostasis through the PI3K/AKT and AMPK signaling pathway (69). Growing evidence indicates

that GA is one of the bioactive compounds of mango-derived polyphenols (71, 75). GA regulated lipid metabolism through activating the AMPK/Sirt1/PGC1α axis in interscapular brown adipose tissue, therefore inducing thermogenesis and energy expenditure (75).

Eleven human clinical trials have been conducted to investigate the health effects of mango supplementation in healthy individuals, overweight and obese individuals, as well as individuals with T2D. In the three clinical trials with healthy lean adults, mango supplementation has shown to improve microcirculation and endothelial function (76), glycemic control by lowering postprandial glucose level (77), lipid profiles (e.g., triglycerides (TG) and very low-density lipoprotein (VLDL)) and antioxidant activity (78). Three clinical trials with mango supplementation have been reported in healthy overweight and obese individuals. Evans *et al.* found that daily supplementation of 10 g freeze-dried mango pulp for 12 weeks reduced fasting blood glucose in obese subjects and additionally lowered hip circumference in the male subjects, while no effect on obesity-related chronic inflammation was observed in the study (79, 80). Overweight subjects with hyperglycemia received a daily dose of 150 mg mangiferin, one of the natural phytochemicals sourced from mango fruits and their byproducts, for 12 weeks exhibited reduced levels of TG and FFA, as well as increased high-density lipoprotein cholesterol (HDL) (81). Finally, discrepancy in effects of mango supplementation has been reported in five clinical trials with subjects diagnosed with T2D. Contractor *et al.* (1999) reported that mango exerted no effect on postprandial blood glucose in 3 hours after consumption (82). However, other three

studies demonstrated that mango supplementation improved plasma glucose response (83, 84) and insulin level (85) after acute mango consumption. Unexpected results have been reported by Fatema *et al.* (2003) that mango induced higher glucose response as evidenced by the higher area under the curve (AUC) $_{0\text{-}3\ h}$ levels compared that of bread, a control dietary component used in this study (86). Therefore, the safety of incorporating mango fruit into a healthy diet should be taken into considerations in the therapeutic management of T2D conditions. These findings indicate that mango-derived polyphenols exert potential anti-inflammatory, anti-obesogenic and anti-diabetic effects that are relevant to the prevention of obesity-related chronic diseases **(Figure 6)**. However, the underlying mechanisms by which polyphenols exert health-promoting effects in humans are complex and remain to be elucidated.

2.5 Role of the AMPK Signaling in Obesity and Chronic Diseases

AMPK is a key cellular and whole-body energy homeostasis regulator due to its ability in stimulating ATP-generating pathways, as well as suppressing ATP-consuming pathways. AMPK is composed of three subunits: catalytic unit α (1, 2) and regulatory units β (1, 2) and γ (1, 2, 3). In adipose tissue, α1 is the predominant subunit accounting for the major part of the AMPK activity. AMPK is activated under conditions where there is decreased ATP level. Previously, two AMPK kinases have been identified: liver kinase B1 (LKB1) and $Ca2^{+}$/calmodulin-dependent protein kinase kinase β (CaMKKβ), and both can catalyze the activation of AMPK from its dephosphorylated form to the phosphorylated form. Once activated, AMPK modulates key proteins involved in the

regulation of glucose and lipid metabolism, and adipose tissue inflammation (87). Therefore, AMPK activation is considered a potential therapeutic target in the prevention of obesity-related chronic diseases.

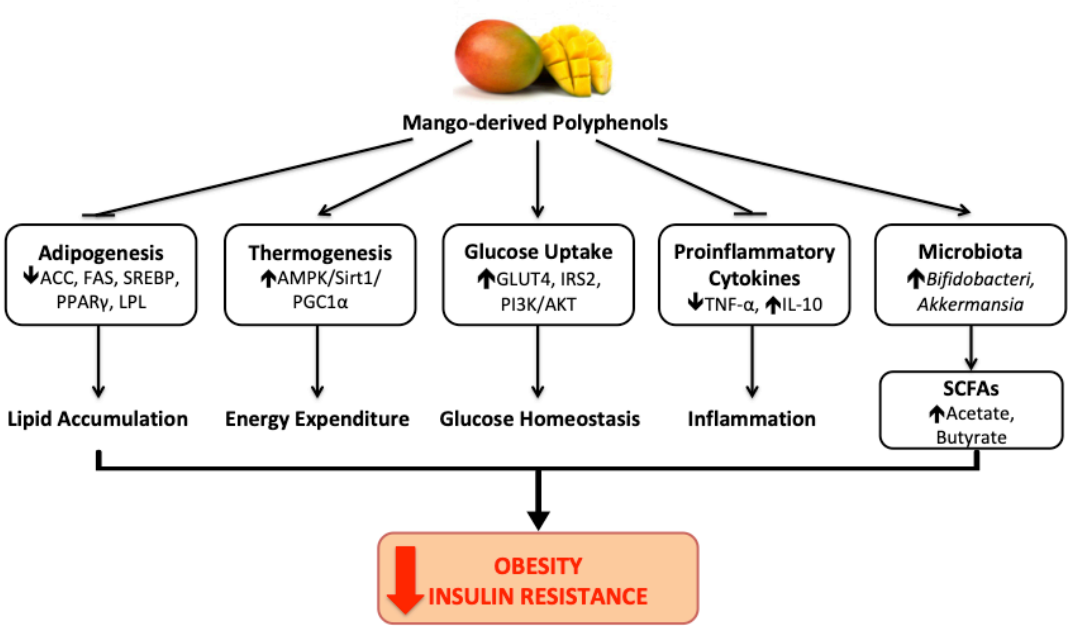

Figure 6 Proposed mechanisms of action of mango-derived polyphenols in obesity and insulin resistance.

AMPK is recognized as a major regulator in a series of lipid metabolism, including adipogenesis, lipogenesis, lipolysis and fatty acid oxidation. AMPK inhibits lipid accumulation by reducing the expressions of adipogenic markers such as PPARγ and C/EBPα. During lipogenesis, AMPK phosphorylates and inactivates ACC and FAS in adipocytes, reducing FA synthesis. It has also been suggested that AMPK activity

inhibits FA oxidation due to reduced FA transport (5) mediated by FA transporters, including CD36/FATP; however, this remains to be examined in adipocytes. Lipolysis, the breakdown of fats to release FFA, is stimulated by β-adrenergic receptor (β-AR)-mediated stimulation of cAMP production, resulting in protein kinase A (PKA) activation. PKA phosphorylates hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL), increasing lipolysis. AMPK activation has been reported to influence lipolysis by the phosphorylation and activation of HSL and ATGL. ATP consumption during re-esterification of FA after lipolysis may also activate AMPK (88).

Growing evidence suggests that AMPK activity is associated with inflammatory response in various types of cells constituting adipose tissue. AMPK activation inhibited production of pro-inflammatory cytokines such as IL-6 and IL-8 in adipocytes, and TNF-α, IL-1β and IL-6 in macrophages (89, 90). AMPKα1-deficient mice exhibited increased secretion of pro-inflammatory cytokines (i.e., TNF-α, IL-1β and IL-6) and macrophage infiltration of the pro-inflammatory M1 phenotype in WAT. Furthermore, stable knockdown of AMPKα1 in 3T3-L1 adipocytes increased the production of MCP-1, TNF-α and IL-1β in response to an inflammatory stimuli, FFA treatment (91). Findings from a co-culture system of macrophages and adipocytes further demonstrated the effects of AMPK in the regulation of inflammation and insulin resistance. AMPK inhibit the NF-κb signaling via a Sirt1-dependent manner (92). Additionally, effect of AMPK activation in BAT has gained increasing attention due to the fat-oxidizing and thermogenic activities possessed by BAT, leading to greater energy expenditure upon

cold exposure (93). Together, these findings reveal that AMPK activation can serve as a novel therapeutic strategy linking obesity-related nutrient metabolism to inflammation.

Previously, some dietary polyphenols (e.g., resveratrol, curcumin, genistein and berberine) exert anti-inflammatory activity in macrophages through the regulation of the AMPK-NF-κb axis (94). The microbial metabolites of GT, including GA and PG have shown health-promoting effects in reducing the risk of obesity, inflammation and cancers through the activation of the AMPK pathway. The administration of GA in obese mice reduced high-fat diet-induced weight gain and improved glucose homeostasis via targeting the activation of the AMPK/Sirt1/PGC1α signaling pathway in the liver, muscle, and interscapular BAT (75). Additional *in vitro* and *in vivo* mechanistic studies have also shown similar findings that GA exerts health benefits via the activation of the AMPK pathway (95). In a DSS-induced colitis rat model, mango beverage intake exert beneficial effects in mitigating inflammation in colitis, at least in part, through the production of PG that modulates the histone deacetylases 1 (HDAC1)/AMPK/ microtubule-associated protein light chain 3 (LC3) axis and induced autophagy, contributing to improved intestinal health (96). PG was also shown to decrease breast cancer cell proliferation possibly through the up-regulation of the AMPK pathway and the down-regulation of the AKT/mTOR pathway. An *in silico* docking modeling further indicates that PG can bind to the allosteric site of AMPK, thus inducing the activation of AMPK (97).

More comprehensive knowledge of the association between mango-derived polyphenols and the AMPK pathway in obesity and its related chronic diseases will

further elucidate the underlying mechanisms of these biological functions **(See Figure 7)**.

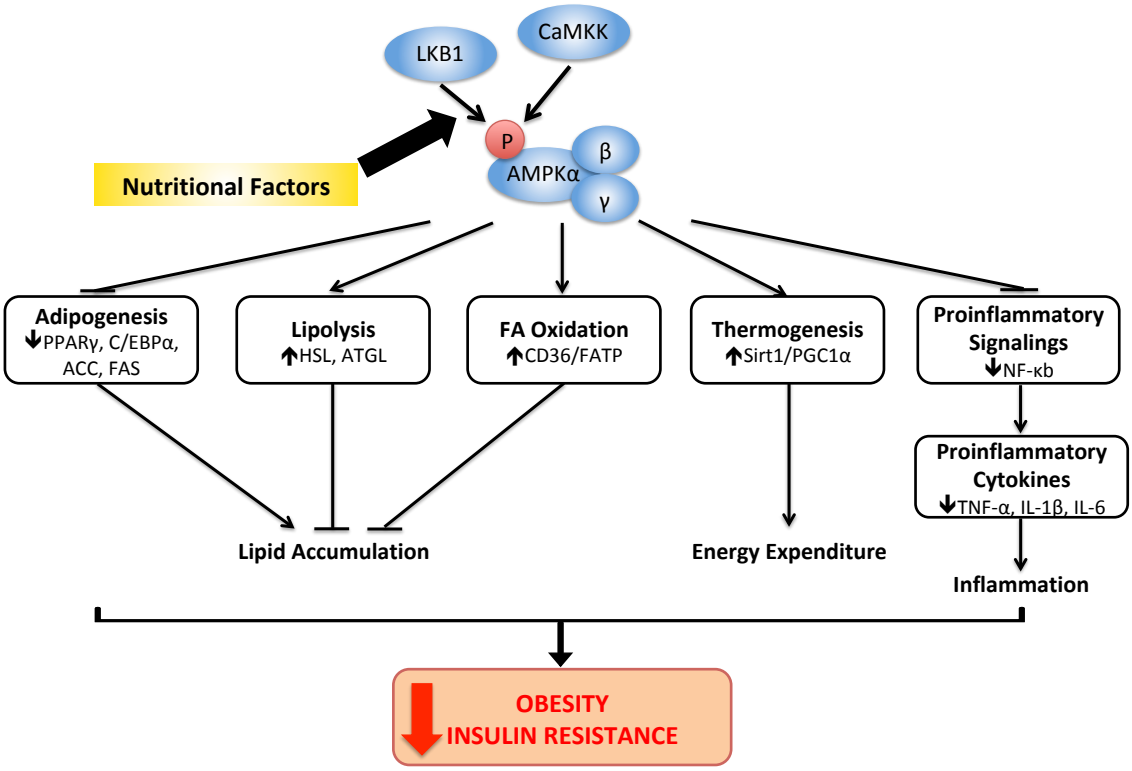

Figure 7 Proposed mechanisms of AMPK signaling in obesity and insulin resistance.

2.6 Potential Role of Gut Microbiota in Obesity

Gut microbiota are abundantly distributed in gastrointestinal tract (mouth, stomach, jejunum, terminal ileum and large intestine), with the highest number residing in the large intestine. Gut microbiota is associated with several hallmarks of obesity-

related chronic disease (e.g., insulin resistance, T2D, cardiovascular disease, and non-alcoholic steatohepatitis) due to their key role in nutrient absorption and low-grade inflammation (98, 99).

2.6.1 Effects of Obesity on Gut Microbial Composition

Gut microbiota affect energy harvest, storage and expenditure after food intake. In healthy individuals, approximately 85% of carbohydrates, 66-95% of protein, and all fats can be absorbed before entering the large intestine. Indigestible nutrients (e.g., resistant starch, unabsorbed sugars and mucins) account for approximately 10-30% of total energy ingested from food. Gut microbiota can, therefore, ferment these indigestible nutrients and generate SCFAs, such as acetate, propionate, and butyrate; and gases such as CO_2, CH_4, and H_2, all of which coexist in different proportions depending on the capability of fermentation (100). The production of SCFAs by gut microbiota is associated with obesity, demonstrated by germ-free (GF) mice after transplantation of gut microbiota from obese mice exhibited lower production of acetate and butyrate than lean mice (101).

In obesity, perturbation of gut microbiota can result in changes in intestinal permeability, a major contributor to low-grade inflammation characterized in obesity. Intestinal mucosa integrity and permeability can be maintained via intracellular tight junctions (102). Colonization of probiotics in GF mice, as well as mice with conventional microbiota, results in up-regulation of key tight-junction proteins and improvement of intestinal barrier function (103, 104). Endotoxin (e.g., LPS) is a cell

wall component of Gram-negative bacterial species. The presence of LPS in the circulation may be attributed to the compromised intestinal mucosa integrity and increased permeability (105).

Stably maintained gut microbial composition and activity contribute to the maintenance of energy homeostasis and inflammatory response. The four main phyla of gut microbiota consist of *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria*. Composition of gut microbiota can be influenced by a wide range of factors, including host genetics, disease, medicine and diet. In general, lean and obese individuals differ in gut microbial composition. Observational studies have shown that obese individuals have a lower bacterial diversity and abundance of *Bacteroidetes*, but a higher abundance of *Firmircutes* (106). Thus, obese individuals are characterized by a high ratio of *Firmicutes/Bacteroidetes* (107). With polyphenolic (e.g., resveratrol) treatment, HFD-induced obese mice had significant decreases of *Firmicutes/Bacteroidetes* ratio and increases of *Lactobacillus* (108). However, inconsistent results have been increasingly reported possibly due to the large inter-individual variation of gut microbial composition (109-112). These findings provide novel insight for future investigation into the relationship between body composition/BMI and gut microbial composition.

2.6.2 Dietary Polyphenols Re-shape the Gut Microbial Composition

Emerging evidence demonstrates the two-way relationship between dietary polyphenols and the composition of the intestinal microbiota where an increased intake

of polyphenols may shape the composition of the intestinal microbiota by increasing species with the ability to metabolize polyphenols (96, 113-116). Many so-called probiotic species such as *Streptococcus gallolyticus, Lonepinella koalarum, Bacillus licheniformis*, and several *Lactobacilli* species fall into this category (117). Recently, we have reported that daily consumption of GT-rich mango pulp for 6 weeks increases levels of tannase-producing bacteria (*Lactoccoccus lactis*) in healthy human subjects, and this increase was correlated with increased tannase enzyme activity in fecal samples. The production of a SCFA, namely butyrate showed a trend towards increased levels after 6 weeks of mango consumption (60). This evidence suggests that the health-promoting effects of GT and *L.plantarum* may be at least in part based on prebiotic-probiotic interactions between GT and *L.plantarum*. Potentially, non-absorbable GTs mediate their anti-inflammatory and anti-obesogenic activities indirectly through increasing the abundance of *L.plantarum*. In support of this hypothesis, the supplementation (22 weeks) of green tea polyphenols combined with *L.plantarum* reduced body fat content and cholesterol accumulation, and additionally promoted the growth of *Lactobacillus* species in the intestine and attenuated inflammation in HFD-induced obese mice (118). In addition, GT-induced increased abundance of *L.plantarum* may result in increased SCFA production. In antibiotic-associated diarrhea patients, a significant increase was noted for the production of butyrate in fecal samples of patients receiving an *L.plantarum*-fermented fruit drink compared to patients receiving a placebo fruit drink (119). Butyrate as a microbiota-induced fermentation product has shown

anti-inflammatory and anti-obesogenic potential possibly due to its ability in enhancing intestinal barrier integrity and function (120).

2.6.3 Gut Microbial Composition Affects Pharmacokinetics of Dietary Polyphenols

In addition to enhancing probiotic activities, the production of bioactive GT metabolites via microbial degradation can be associated with decreased inflammation and risk of developing obesity-related metabolic disorders (121). Pharmacokinetics of dietary polyphenols is influenced by gut microbiota, where inter-individual microbial biodiversity may translate into great variability of associated pharmacodynamics endpoints (31). In humans, only a small percentage of dietary polyphenols is believed to be absorbed into small intestine while 90-95% are expected to reach the colon where hydrolysis, decarboxylation and other reactions are executed by intestinal microbial bacteria. As a result, the biological activity of polyphenols depends at least for a major part on microbial metabolism (31, 122, 123). *L.plantarum* is a lactic acid bacterium widely used in probiotic products (124). Promising effects of *L.plantarum* in the prevention and alleviation of gut dysbiosis prompt its application in patients with irritable bowel syndrome (125). In addition, *L.plantarum* is known to encode for both tannase- and gallic acid decarboxylase-producing activities that catalyze the hydrolysis of galloyl ester bonds in hydrolysable GT, yielding glucose and GA. GA will then be decarboxylated to PG (126, 127). Small, absorbable, bioactive compounds GA and PG are easily distributed into tissues where they can act as anti-inflammatory and anti-obesogenic agents (31, 128, 129). This may enhance the health-promoting effects

derived from GT-rich food in reducing obesity and its related chronic diseases. Findings from these studies provide initial evidence of the beneficial role of probiotics in context with a polyphenol-rich diet.

2.7 Innovation of the Current Research

In this research project, the proposed human clinical trial is the first to correlate the systemic exposure to polyphenolic metabolites of GT to their biological activities. Inter-individual variability in both lean and obese individuals, specifically the lower exposure to polyphenolic metabolites, may explain the limited response of functional biomarkers in obese individuals.

In addition, very few studies have compared the effects of the fruit extract of polyphenols with their microbial metabolites in the regulation of lipid metabolism and inflammation in obesity. In our study, we used not only the mango extract, but also PG to treat 3T3-L1 adipocytes to determine the health benefits and underlying molecular mechanisms in obesity. Furthermore, effects of the central microbial metabolites of GT, including GA and PG, on LPS-induced inflammation and oxidative stress were further investigated in RAW 264.7 macrophages. This may contribute to increasing the physiological and nutritional significance of microbial metabolites of GT as anti-inflammatory and anti-obesogenic agents.

The application of the GF mouse model in our study allows us to examine the role of gut microbiota in pharmacokinetics of GT derivatives and the effects of the microbial metabolites on diet-induced obesity. The anti-obesogenic activity of

polyphenols has mostly been investigated in animals with conventional microbiota. Emerging evidence has shown that there is a two-way relationship between polyphenols and the composition of gut microbiota. The role of each individual species of microbiota in obesity and metabolic disorders has yet to be investigated. With the mono-colonization of GF mice with *L.plantarum*, we were able to determine the polyphenol-probiotic interactions. These interactions may lead to future studies analyzing the relation between probiotics, bioavailability, and bioefficacy of fruit-derived polyphenol derivatives.

Collectively, the proposed research has the potential to illustrate the health benefits of mango-derived polyphenols in obesity, and unveil the importance of gut microbiota in pharmacokinetics and pharmacodynamics of dietary polyphenols **(See Figure 8)**.

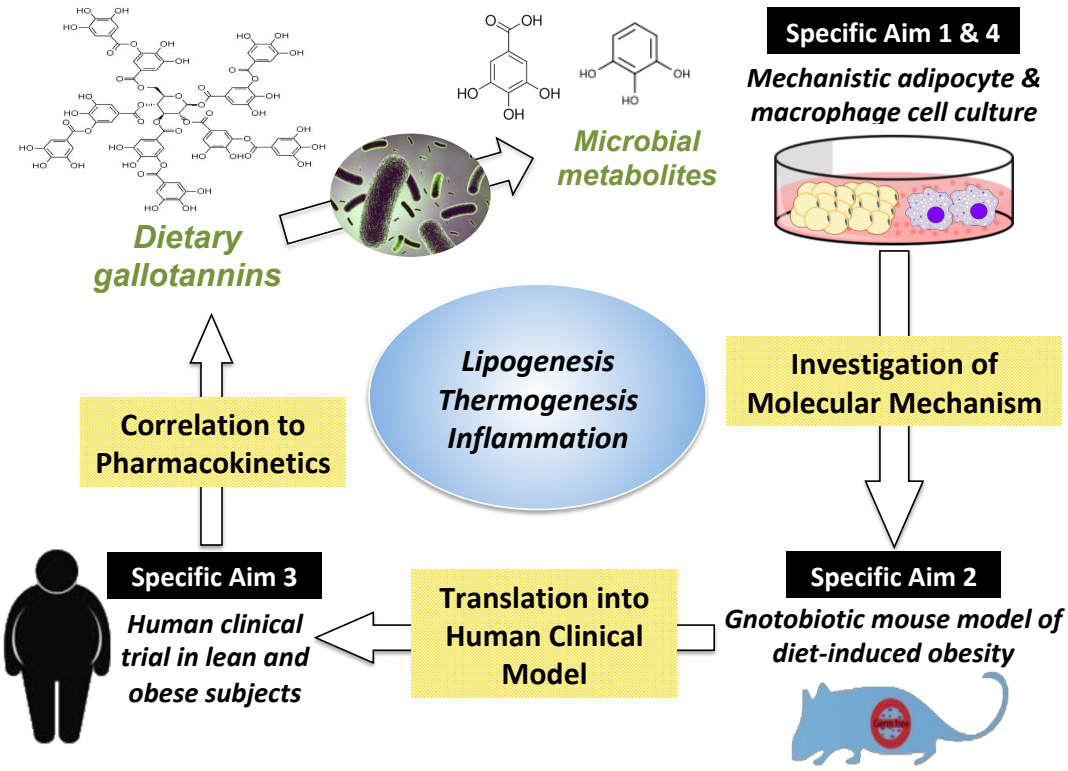

Figure 8 Overview of specific aims.

CHAPTER III

ACTIONS AND MOLECULAR MECHANISMS OF MANGO-DERIVED GALLOTANNIN METABOLITES IN 3T3-L1 ADIPOCYTES[†]

The major function of adipocyte is to store energy in the form of triacylglycerol (TAG) during energy excess, as well as mobilize TAG during energy deprivation. Long-term regulation of body weight and energy homeostasis is accompanied by the changes in both adipocyte number and size (10). In the last few years, adipocyte differentiation and thermogenesis have gained widespread interest to study the nutritional and hormonal regulation of adipocytes, therefore leading into implications for the prevention and treatment of obesity-related chronic disease. *In vitro* models (e.g., 3T3-L1 and 3T3-F422A) are widely used and considered a reliable proof-of-principle approach to study the actions and molecular mechanisms of bioactive compounds in obesity.

The 3T3-L1 cell line, derived from Swiss 3T3 mouse embryos (130), is a well-characterized and frequently used model in the study of lipid metabolism. At confluence, 3T3-L1 preadipocytes can be differentiated into adipocytes after exposure to an adipogenic cocktail containing insulin, which acts through the insulin-like growth factor 1 (IGF-1) receptor; dexamethasone, a synthetic glucocorticoid agonist that elevates intracellular cAMP levels; and methylisobutylxanthine, a cAMP-phosphodiesterase inhibitor to stimulate the cAMP-dependent protein kinase pathway.

[†] Reprinted with permission from Fang, C., Kim, H., Noratto, G., Sun, Y., Talcott, S. T., & Mertens-Talcott, S. U. (2018). Gallotannin derivatives from mango (Mangifera indica L.) suppress adipogenesis and increase thermogenesis in 3T3-L1 adipocytes in part through the AMPK pathway. *Journal of Functional Foods*, *46*, 101-109. Copyright [2019] by Elsevier.

Approximately 24 hours after the addition of adipogenic cocktail, differentiating preadipocytes undergo a post-confluent mitosis, allowing transcriptional factors to modulate adipocyte phenotype and subsequent growth arrest. Afterwards, cells are subject to at least one round of DNA replication and cell division, and are committed to becoming adipocytes. Round, mature adipocytes can be formed within 5-7 days (10, 131, 132). A sequence of transcriptional factors are involved throughout the cell differentiation at different stages, including lipoprotein lipase (LPL) (133), CCAAT/enhancer binding proteins (C/EBP) α (15), C/EBP β, C/EBP δ (132), and PPARγ (134).

Previously, anti-obesogenic effects of mango-derived polyphenols have been rarely examined *in vitro*. Very few studies have compared the effects of the whole fruit extract with the microbial metabolites in the regulation of lipid metabolism and identify the signaling signatures. Therefore in this chapter, the actions and molecular mechanisms of mango-derived gallotannin metabolites in 3T3-L1 adipocytes are investigated and discussed.

3.1 Introduction

Obesity is a rapidly growing epidemic in the United States that currently affects nearly 35% of the adult population and is predicted to affect 75% by 2020 (135, 136). The risk for several medical complications is increased in obese individuals, including diabetes, hypertension, cardiovascular disease and various cancers. Due to the increased morbidity and mortality associated with obesity, preventive measures are expected to

significantly reduce health care expenditures associated with obesity-related chronic conditions in the United States (137, 138).

Obesity is characterized by the expansion of adipose tissue, with increases in both number and size of adipocytes. Adipocyte differentiation from fibroblasts to adipocytes, also called adipogenesis, determines the number of adipocytes formed in the development of obesity (8). Elevated adipogenesis is linked to increased fat deposition in adipose tissue (9). The two types of adipose tissue formed during adipogenesis are white adipose tissue (WAT) and brown adipose tissue (BAT), though the formation of BAT is limited and considered not physiologically relevant in adults (139).

The size of lipid droplets in adipocytes is associated with the types of adipose tissue, with WAT being larger than BAT. WAT is known for its key role in energy storage (by accumulating fat within large single lipid droplets), along with energy metabolism and endocrine functions. BAT, primarily observed in infants and small mammals, consists of many mitochondria that use multiple, surrounding lipids as fuel to generate heat for survival in cold environments. Adipocytes with an increased number of mitochondria and reduced lipid droplet size can co-exist within WAT. These new, brown-like adipocytes are called beige adipocytes (140). The process through which white adipocytes transdifferentiate into beige adipocytes upon thermogenic stimulation is called adipocyte browning (14). Numerous i*n vitro* studies have been performed in 3T3-L1 mouse preadipocytes to investigate the underlying mechanisms of hormonal and nutritional regulation of adipogenesis and thermogenesis. Recently, dietary interventions for obesity, particularly polyphenols in fruits and vegetables, have been

shown to reduce adipogenesis (8, 37-39), as well as increase thermogenesis by converting white adipocytes into beige adipocytes (40-43). Adipogenesis inhibition and thermogenesis promotion are promising targets in the prevention of obesity and obesity-related chronic diseases (36).

Mango (*Mangifera Indica L.*) is a rich source of polyphenols, including gallotannins and gallic acid (141). Gallotannins are high molecular weight polyphenols that cannot be directly absorbed during digestion and require hydrolysis by gut microbiota. Some microbiotic species (e.g., *Lactobacillus plantarum*) produce tannin acyl hydrolase (tannase), which converts gallotannins into gallic acid, as well as gallate decarboxylase, further decarboxylates gallic acid to pyrogallol (PG) (142). Recently, preclinical studies have indicated that mango polyphenols (MG) and PG possess anti-inflammatory and anti-carcinogenic activities in different types of cancer (143-145). However, the role of these gallotannin derivatives has not been clarified in adipogenesis and thermogenesis.

AMP-activated protein kinase (AMPK) is a key cellular and whole-body energy homeostasis regulator that plays a central role in lipid metabolism (146), particularly adipogenesis (147) and thermogenesis (148). The activation of the AMPK pathway is associated with health benefits in peripheral metabolic tissues, such as in the liver and skeletal muscle. In these tissues, AMPK inhibits the synthesis of fatty acids and cholesterol while stimulating fatty acid oxidation and glucose transport (149, 150). As a result, AMPK activation is considered a therapeutic target in the prevention of obesity-related chronic diseases. PG has been shown to activate the AMPK pathway in breast

cancer cells. This activity was further confirmed by *in silico* modeling, implying direct binding of PG to the allosteric binding site of AMPK (97). However, the signaling signatures of MG and PG in adipocytes have not been clarified.

To address these gaps in knowledge, this study analyzed the role of gallotannin derivatives of mango (MG and PG) in regulating lipid metabolism of 3T3-L1 adipocytes. It was hypothesized that MG and PG possess anti-adipogenic and thermogenic activities, at least in part, through the modulation of the AMPK pathway.

3.2 Materials and Methods

3.2.1 MG Extraction and Analysis

Keitt mangos were provided by the National Mango Board (Orlando, FL), and PG was purchased from Sigma-Aldrich (St. Louis, MO). Gallotannin compounds were extracted from mango pulp using a solvent mixture containing 1:1:1 methanol, acetone, and ethanol. The mixture was stirred and filtered. Afterwards, the solvents were evaporated under reduced pressure at 45°C and re-dissolved in water acidified with 0.01% HCl. Following centrifugation and filtration, the phytochemical composition in mango pulp was characterized with a high-performance liquid chromatography-mass spectrometry (HPLC-MS) using Thermo Finnigan Surveyor HPLC-PDA coupled to a Thermo Finnigan LCQ Deca XP Max Msn ion trap mass spectrometer equipped with an ESI ion source (Thermo Fisher, San Jose, CA, USA) as previously described (141). Mango extracts were quantified for total phenolics using the Folin-Ciocalteu assay. The concentration of MG was expressed in mg/L gallic acid equivalents (GAE). Extracts

were prepared under standard operating procedures and monitored qualitatively and quantitatively throughout the study duration.

3.2.2 Cell Culture and Differentiation

3T3-L1 mouse preadipocytes were purchased from American Type Culture Collection (ATCC, Rockville, MD) and cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S, Life Technologies, Gaithersburg, MD) at 37°C in a humidified 5% CO_2 atmosphere. Two days post-confluence (Day 0), preadipocytes were differentiated into adipocytes as previously described by adding 10μg/ml insulin, 1μM dexamethasone, and 0.5mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich, St. Louis, MO). On Day 2, the medium was replaced with DMEM supplemented with 10% FBS, 1% P/S, and 10μg/ml insulin for another two days. On Day 4, the cells were incubated in DMEM containing 10% FBS and 1% P/S until Day 6 when the preadipocytes had reached complete differentiation into adipocytes (151).

3.2.3 Cell Viability of 3T3-L1 Preadipocytes and Adipocytes

Two days post-confluent 3T3-L1 preadipocytes were differentiated into adipocytes in the presence of MG (2.5-20 mg GAE/L) or PG (2.5-20 mg/L), or a vehicle control for 48 hours. Cell viability was determined by 10% Resazurin assay (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's protocol. Fluorescence intensity was measured at 560 nm excitation and 590 nm emission using a microplate

reader (BMG Labtech Inc., Durham, NC). Likewise, the cell viability was also examined in mature adipocytes after 6 days of differentiation. Results of cell viability were quantified as percentage of treated controls after background adjustment (152).

3.2.4 Effects of MG and PG on Aadipogenesis of 3T3-L1 Preadipocytes

Preadipocytes were treated with a vehicle control, MG, or PG from Day 0 to Day 6 to determine the effects of MG and PG on adipogenesis. Cell culture medium was replaced every two days.

3.2.4.1 Oil-Red-O Staining and Quantification

Briefly, on Day 6, cells were fixed with 10% neutral buffered formalin for 30 minutes at room temperature and washed twice with distilled water. Cells were incubated with 60% isopropanol for 5 minutes and stained with Oil-Red-O working solution for 30 minutes. Then, cells were washed 2-5 times with distilled water as needed to remove stain excess. Pictures were taken with a Zeiss Axioplan 2 microscope (Carl Zeiss, Thornwood, NY) fitted with an Axiocamhigh resolution digital camera and Axiovision 4.1 software. Finally, cells were incubated with 100% isopropanol for 10 minutes to extract Oil-Red-O stain. The absorbance was measured at 492 nm using a microplate reader (151).

3.2.4.2 Gene Expression

Total RNA was isolated from 3T3-L1 adipocytes on Day 6 using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The concentration of the extracted RNA was determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Briefly, 1000ng of RNA was used to synthesize cDNA using a Reverse Transcription Kit (Bio-Rad, Hercules, CA). The gene expression levels of AMPKα1, peroxisome proliferator-activated receptor γ (PPARγ), CCAAT/enhancer binding protein α (C/EBPα), fatty acid synthase (FAS), carnitine palmitoyltransferase I (CPT1) and liver kinase B1 (LKB1) were analyzed by qPCR (CFX384 Touch Real-Time PCR Detection System, Bio-Rad, Hercules, CA). Each reaction was performed in triplicate, and data were normalized to β-actin as an endogenous control (151).

3.2.4.3 Protein Expression

3T3-L1 adipocytes were harvested on Day 6, and cellular proteins were extracted. Approximately 50ug of total protein, quantified by Bradford assay (Invitrogen, Carlsbad, CA), was loaded and run on a 4-12% sodium dodecyl-polyacrylamide gel and transferred to a PVDF membrane using the iBlot Dry Blotting system (Invitrogen, Carlsbad, CA). The membrane was blocked in 5% non-fat milk solution for 1 hour and probed with primary antibodies against phospho-AMPKα1 (pAMPKα1), total-AMPKα1 (t-AMPKα1), LKB1, C/EBPα, PPARγ, fatty acid-binding protein 4 (FABP4) and perillipin (Cell Signaling Technology, Danvers, MA).

3.2.5 Effects of MG and PG on Thermogenesis of 3T3-L1 Adipocytes

Differentiated adipocytes were treated with MG or PG for 24 hours (Day 6 to Day 7) to examine thermogenic effects. The gene and protein expression levels of AMPKα1, LKB1, uncoupling protein 1 (UCP1) and sirtuin 1 (Sirt1) were analyzed after the treatments as described above. The effect of MG and PG on the size of lipid droplets contained in adipocytes was examined and photographed with Oil-Red-O staining.

3.2.6 Reactive Oxygen Species (ROS) Assay

After 6 days of differentiation, mature adipocytes were pre-treated with MG (2.5-10 mg GAE/L), PG (5-20 mg/L), or a vehicle control for 1 hour. The adipocytes were then incubated with 10ng/mL tumor necrosis factor α (TNF-α, Sigma-Aldrich, St. Louis, MO) along with the previous treatments for another 23 hours. After 24 hours, cells were washed once in PBS (pH 7.0) and stained with 10μM 2',7'-dichlorofluorescein diacetate (DCFH-DA, Sigma-Aldrich, St. Louis, MO) in no-phenol red DMEM (Life Technologies, Gaithersburg, MD) for 30 minutes. Fluorescence intensity was measured at 485 nm excitation and 520 nm emission using a microplate reader. The change of ROS production was determined as percentage of vehicle control after background subtraction (151).

3.2.7 Small Interfering RNA (siRNA) Transfection

At 70-80% confluence, 3T3-L1 preadipocytes were transfected with 100nM siRNA targeting endogenous AMPKα1 (Santa Cruz Biotechnology, Santa Cruz, CA) or

with a scrambled, non-silencing control siRNA using 2 μg/mL lipofectamine (Invitrogen, Carlsbad, CA) in Opti-MEM media (Life Technologies, Gaithersburg, MD) without antibiotics. After 24 hours of transfection, the medium was replaced with DMEM (10% FBS, 1% P/S), and treated cells were differentiated with a vehicle control, MG (10 mg GAE/L), or PG (20 mg/L) for 6 days (153). Cell lysates were then collected to analyze mRNA and proteins involved in adipogenesis as previously described.

3.2.8 Statistical Analyses

Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, Lo Jolla, CA). Results were presented as means ± SEM. Comparison of means between different groups was analyzed by *t*-test for two groups or Dunnett's test for multiple groups. A p value less than or equal to 0.05 indicates statistical significance.

3.3 Results and Discussion

3.3.1 Polyphenolic Composition of Mango Extracts

Polyphenolic profile of gallotannins in Keitt mango extracts determined by HPLC-MS included ester-monogalloly glucoside, gallic acid, ether-monogalloyl glucoside, *p*-hydroxybenzoic acid glycoside, penta-galloyl glucoside, hexa-galloyl glucoside, nona-galloyl glucoside, and gallotannins of different degrees of polymerization **(Figure 9).** The extract composition did not change qualitatively or quantitatively throughout the study duration.

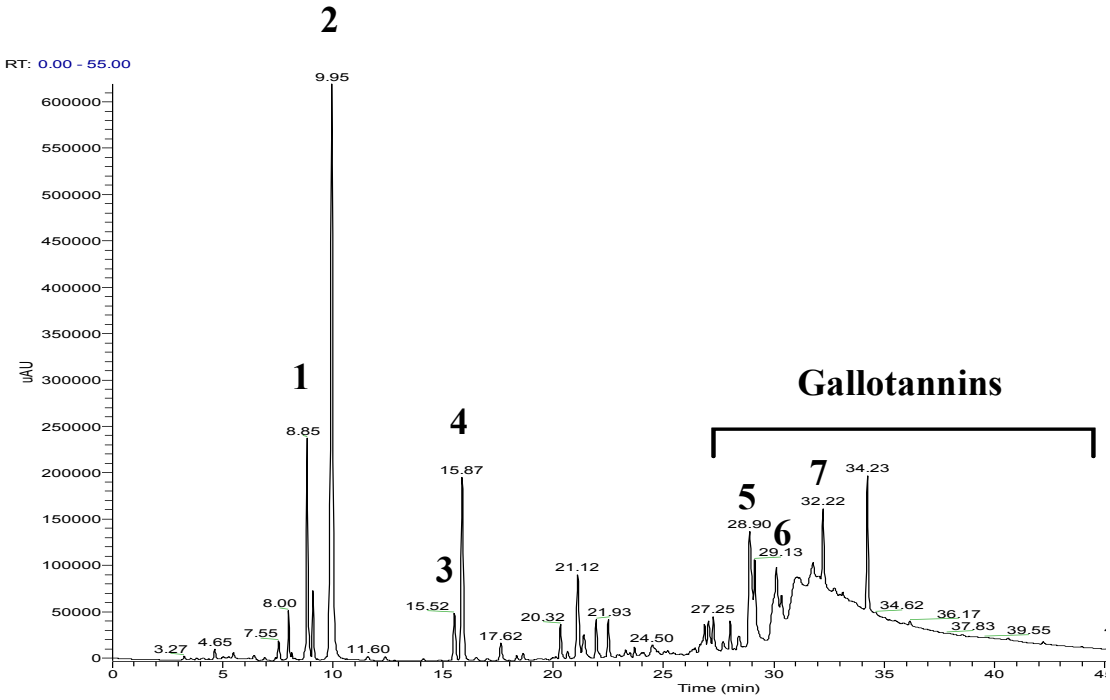

Figure 9 HPLC chromatogram of compounds present in mango polyphenols (Keitt) at 280 nm. Reprinted with permission from *Journal of Functional Foods*, *46*, 101-109. Copyright [2019] by Elsevier.

3.3.2 Effects of MG and PG on Cell Viability

The experimental design of this study is shown in **Figure 10A**. Results showed no reduction of cell viability in preadipocytes and mature adipocytes **(Figure 10B, C)** by PG within the tested dose range (2.5-20 mg/L). However, MG at 20 mg GAE/L reduced cell viability by nearly 10% during preadipocyte differentiation **(Figure 10B)**. In mature adipocytes, neither MG nor PG inhibited cell viability **(Figure 10B, C)**. Therefore, MG at 2.5, 5, 10 mg GAE/L and PG at 5, 10, 20 mg/L were selected for further experiments.

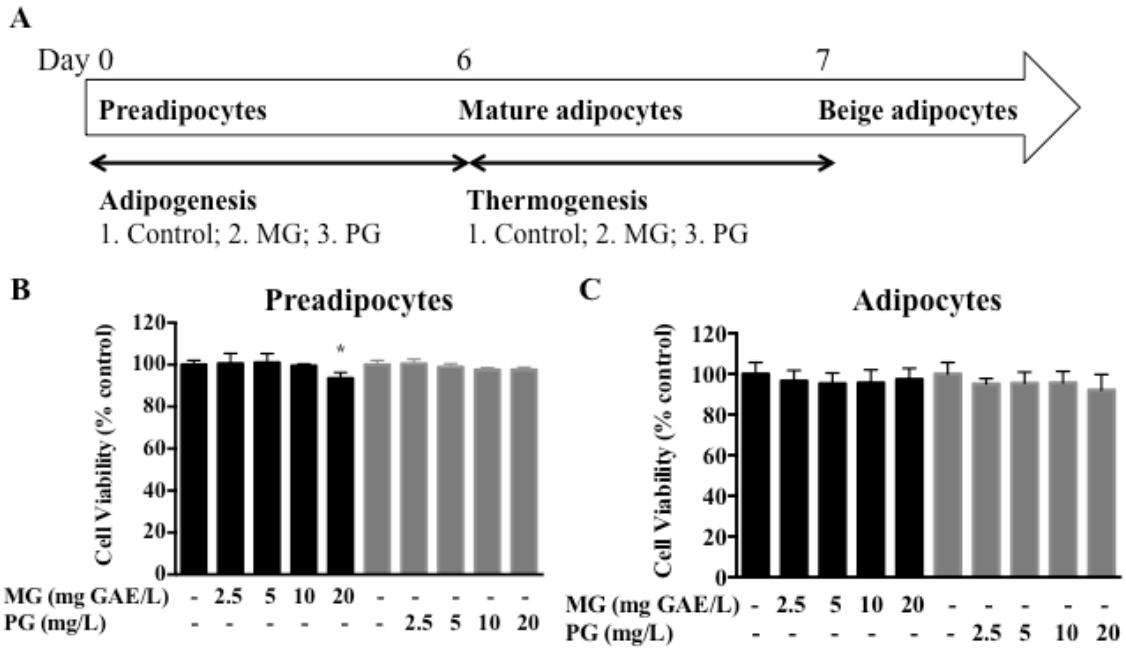

Figure 10 Experimental design and cell viability in 3T3-L1 preadipocytes and adipocytes. Reprinted with permission from *Journal of Functional Foods*, *46*, 101-109. Copyright [2019] by Elsevier.

3.3.3 MG and PG Inhibited Lipid Accumulation and Prevented ROS Production

MG and PG reduced lipid accumulation during adipogenesis in a dose-dependent manner, as shown by Oil-Red-O staining **(Figure 11A)**. Furthermore, lipid accumulation was reduced by 38.5% and 24.8% by MG (10 mg GAE/L) and PG (20 mg/L), respectively **(Figure 11B)**. Increased oxidative stress, measured by ROS production, is a pathological consequence of excessive lipid accumulation (154). Results showed that MG and PG prevented ROS production, induced by TNF-α, by up to 50% compared to cells treated with the vehicle control **(Figure 11C)**.

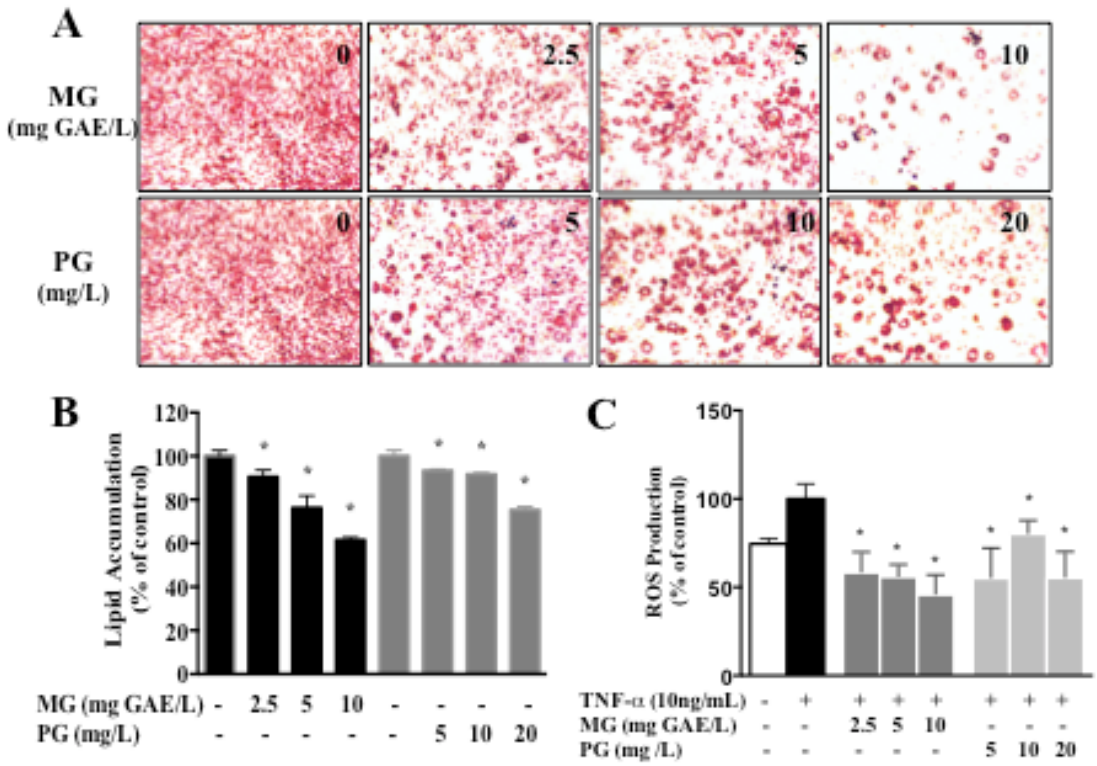

Figure 11 MG and PG inhibited lipid accumulation and prevented generation of ROS in 3T3-L1 adipocytes. Reprinted with permission from *Journal of Functional Foods*, *46*, 101-109. Copyright [2019] by Elsevier.

3.3.4 MG and PG Modulated the Expressions of Molecules Involved in Adipogenesis

The differentiation of preadipocytes into adipocytes is a chronological event composed of distinct stages where transcriptional factors and adipocyte gene expressions are highly regulated by exogenous effectors (10). In this study, the effects of MG and PG on the gene expressions of key transcription factors (C/EBPα and PPARγ), and adipogenic genes (FAS, FABP4 and perilipin) were examined after 6 days of differentiation. MG (10 mg GAE/L) decreased the expressions of C/EBPα, PPARγ and FAS by 59.2%, 49.2% and 43.1%, respectively; PG (20 mg/L) decreased by 23.6%,

38.4% and 40.9%, respectively **(Figure 12A-C)**. Gene expressions of CPT1 and AMPKα1 were increased 1.6 and 3.3-fold by MG (10 mg GAE/L), respectively; and 2 and 1.5-fold by PG (20 mg/L), respectively **(Figure 12D, F)**. Furthermore, both MG and PG down-regulated protein expressions of C/EBPα, PPARγ, FABP4 and perilipin **(Figure 12G)**. PG (20 mg/L) up-regulated the protein expressions of p-AMPKα1, t-AMPKα1 and LKB1, while MG showed no significant effects **(Figure 12G)**.

3.3.5 MG and PG Modulated the Expressions of Molecules Involved in Thermogenesis

Thermogenesis associated with adipocyte browning is the process through which WAT takes on the characteristics of BAT upon stimulus. This process is characterized by enhanced expressions of thermogenic markers such as UCP1 and Sirt1 (12). PG (20 mg/L) increased gene expressions of UCP1, Sirt1, LKB1 and AMPKα1 1.4, 2.2, 1.3 and 12.0-fold, respectively **(Figure 13A-D)**. MG (10 mg GAE/L) only increased gene expression of Sirt1 1.5-fold **(Figure 13B)**. These results were further confirmed by up-regulated protein expressions of UCP1, Sirt1, p-AMPKα1 and AMPKα1, in addition to a lack of effect on LKB1 **(Figure 13E, F)** in PG-treated (20 mg/L) adipocytes. In MG-treated (10 mg GAE/L) adipocytes, only up-regulation of Sirt1 was observed **(Figure 13E)**. Furthermore, PG (20 mg/L) reduced the size of lipid droplets contained in adipocytes compared to the vehicle control **(Figure 13G)**.

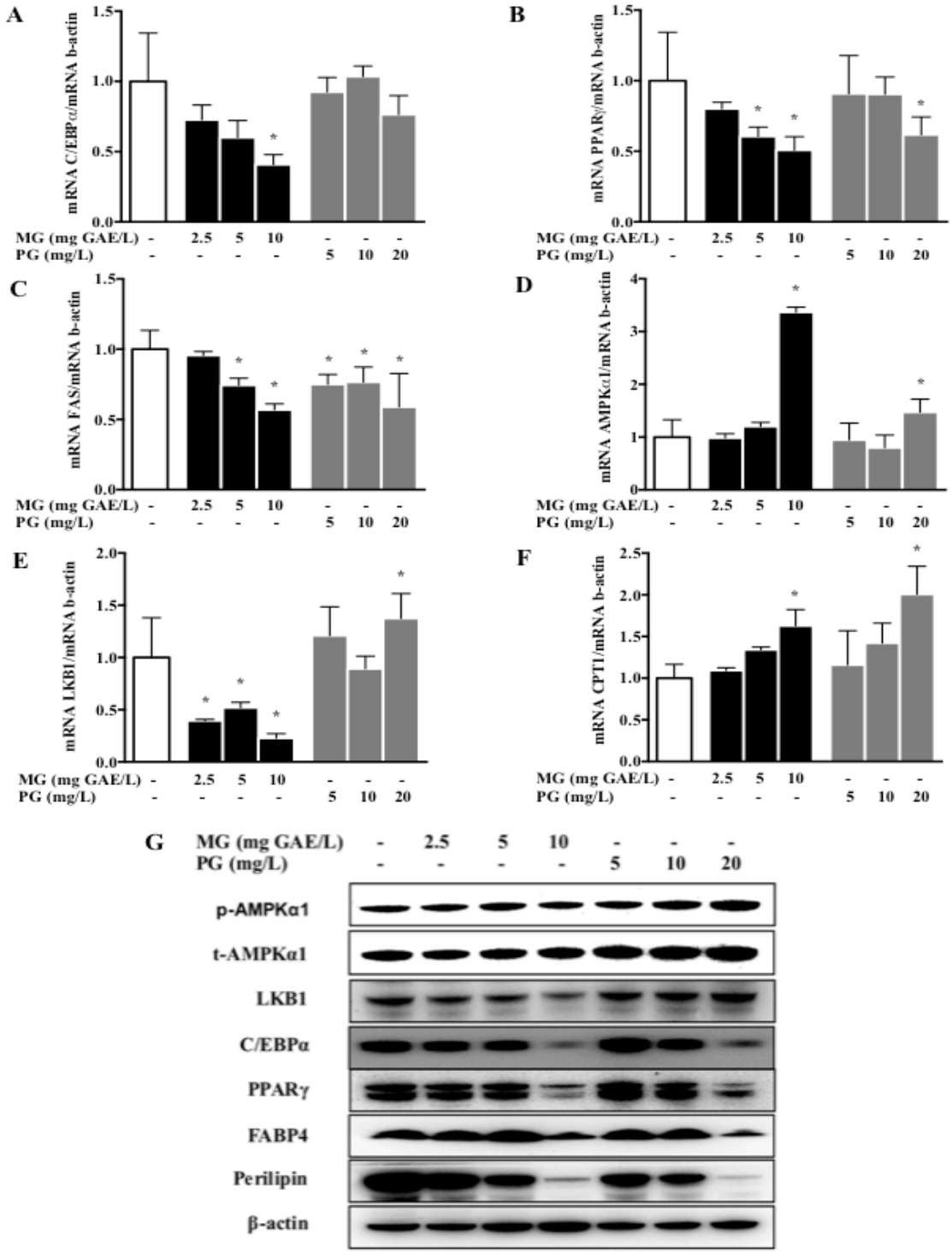

Figure 12 MG and PG modulated the expressions of molecules involved in adipogenesis in 3T3-L1 adipocytes. Reprinted with permission from *Journal of Functional Foods*, *46*, 101-109. Copyright [2019] by Elsevier.

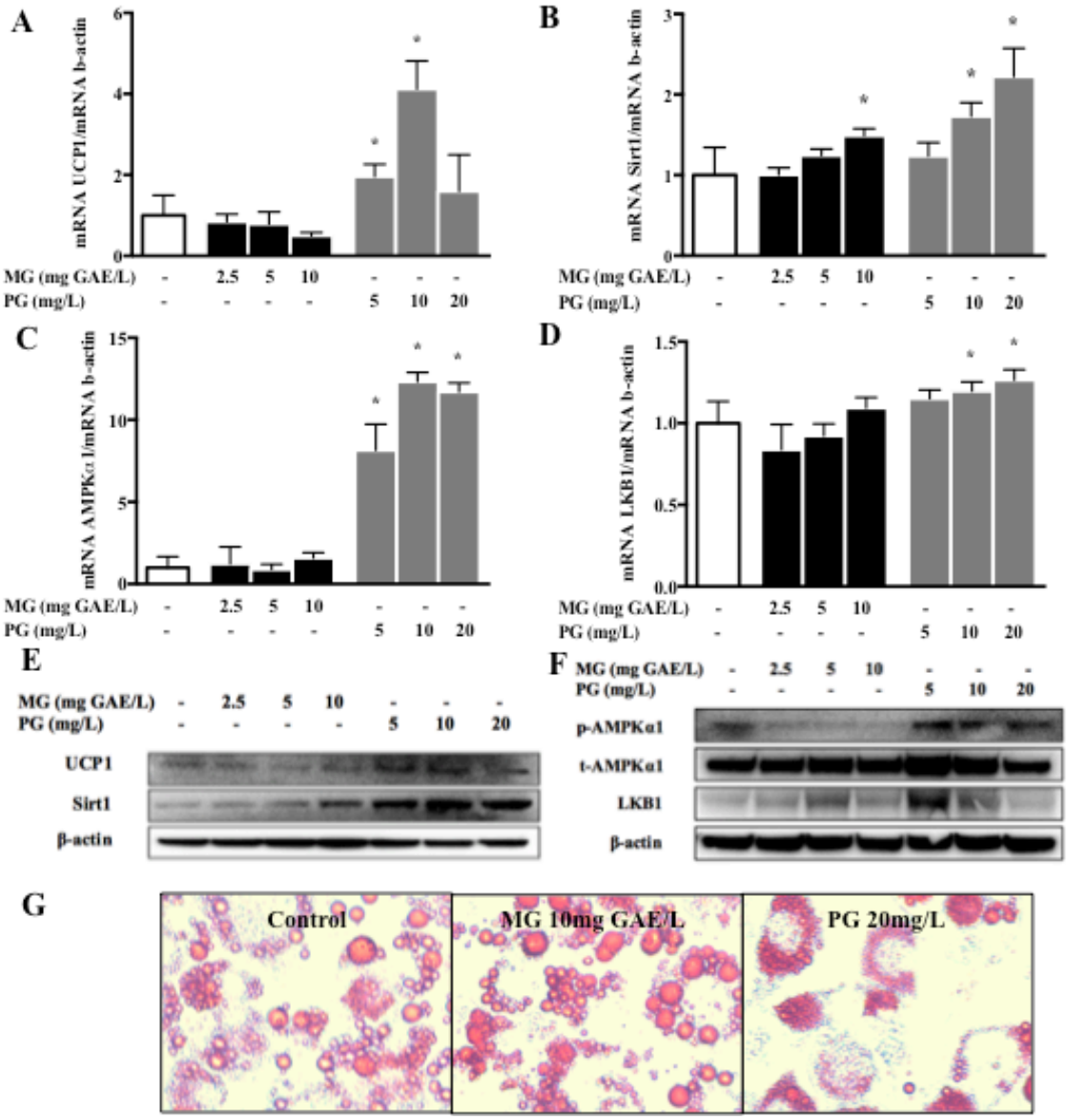

Figure 13 MG and PG modulated the expressions of molecules involved in thermogenesis and increased multi-locularity of lipid droplets contained in 3T3-L1 adipocytes. Reprinted with permission from *Journal of Functional Foods*, *46*, 101-109. Copyright [2019] by Elsevier.

3.3.6 Effects of MG and PG on the AMPK Pathway

To test our hypothesis that PG exerts anti-adipogenic effects, partly through modulation of the AMPK pathway, 3T3-L1 preadipocytes were transfected with AMPKα1 siRNA or a scrambled siRNA control for 24 hours. AMPKα1 knockdown decreased the AMPKα1 mRNA expression by nearly 60% **(Figure 14A).** AMPKα1 siRNA partially abolished the effects of MG (10 mg/L GAE) and PG (20 mg/L) on adipogenesis. Specifically, the protein levels of AMPKα1 were significantly reduced following the gene knockdown; reduced protein levels of C/EBPα and PPARγ were slightly increased **(Figure 14B) (see Figure 15).**

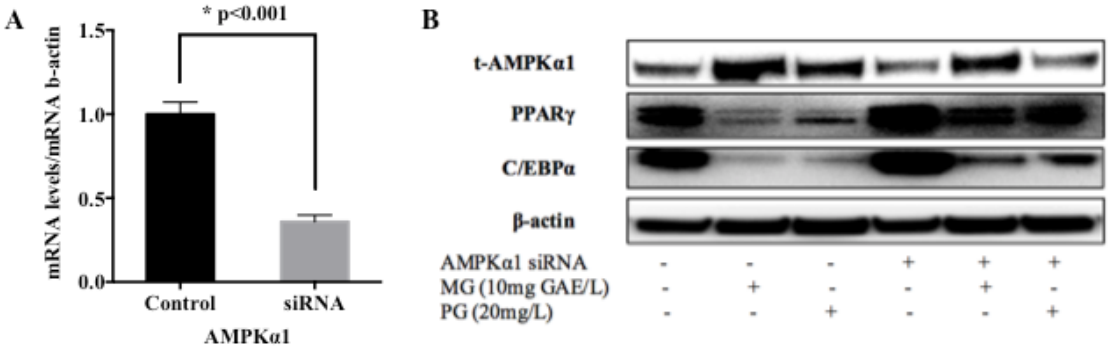

Figure 14 AMPKα1 siRNA partially abolished the effects of MG and PG on adipogenesis in 3T3-L1 adipocytes. Reprinted with permission from *Journal of Functional Foods*, *46*, 101-109. Copyright [2019] by Elsevier.

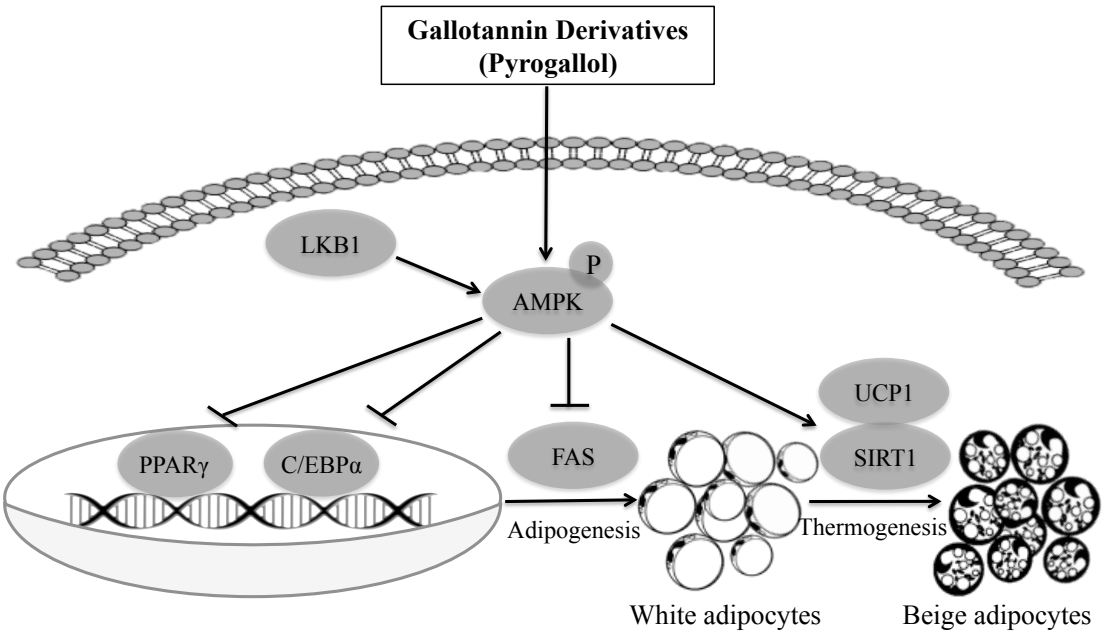

Figure 15 Schematic overview of a proposed mechanism for PG in adipogenesis and thermogenesis of 3T3-L1 adipocytes. Reprinted with permission from *Journal of Functional Foods*, *46*, 101-109. Copyright [2019] by Elsevier.

3.4 Discussion

Overall, PG and MG demonstrate significant anti-obesogenic potential in adipocytes through the inhibition of adipogenesis while PG additionally promotes thermogenesis. PG enhanced the expression of LKB1, an upstream regulator of the AMPK pathway that directly phosphorylates and activates AMPK (146), and modulated the expression of downstream molecules (C/EBPα, PPARγ, FAS, FABP4, perilipin, UCP1 and Sirt1). Therefore, the following two signaling cascades are proposed for the regulation of lipid metabolism by PG-induced AMPK activation: adipogenic signaling cascade LKB1-AMPKα1-C/EBPα/PPARγ/FAS/FABP4 and thermogenic signaling

cascade LKB1-AMPKα1-UCP1/Sirt1. Additionally, both MG and PG exerted antioxidant property by reducing oxidative stress in adipocytes.

PG is a bioactive gallotannin derivative with physiological relevance to human health. After mango consumption, both gallotannins and gallic acid can be degraded by tannase-and/or decarboxylase-producing bacteria into PG (155). Overall, physiological concentrations of PG identified in plasma have been low. An intake of 2650 mg black tea extract combined with 50 mg of selected mass-labeled phenolic acids resulted in an increase of plasma concentration of gallic acid and PG to up to 1.2 μmol/L and 7.1 μmol/L, respectively (156). In a human clinical trial, approximately 50 mg of PG, mainly in its sulfated form, has been detected in urine samples following 400 g of mango intake for 10 days demonstrating the intestinal absorption of PG followed by urinary excretion upon consumption of gallotannins (157). The recovery rates for phenolic acids from biological matrices are low in general due to extensive protein-binding (158, 159) and therefore are likely to be underestimated (30). Thus, the concentration range in this study was selected within a proof-of-principle approach to evaluate overall mechanistic activities where the highest concentrations were not likely to be reached after the consumption of foods or dietary supplements. PG has shown promising health benefits in the prevention and treatment of multiple types of cancer, including lung and breast cancer, partly due to the anti-proliferative effect on human cancer cell lines (160, 161).

In this study, MG and PG treatments suppressed the expressions of C/EBPα and PPARγ, two transcriptional factors required in the early stage of adipocyte

differentiation. Preadipocytes lacking either C/EBPα or PPARγ have reduced potential for adipogenesis, resulting in less lipid accumulation in the late stage of cell differentiation (162). After the treatment with siRNA targeting AMPKα1, the decreased C/EBPα and PPARγ protein expressions by MG and PG treatments were significantly recovered. This suggests that the anti-adipogenic activity of MG and PG are partly associated with the modulation of the AMPK pathway. However, it's worth noting that the inhibitory effects of PG on C/EBPα and PPARγ were not completely reversed by AMPKα1 siRNA. This indicates that other signaling pathways might be involved in the anti-adipogenic activity of MG and PG, possibly the feedback loop of the phosphatidylinositol-3-kinase (PI3K)/AKT/ the mammalian target of rapamycin (mTOR) pathway as previously demonstrated (97, 163).

In this study, both MG and PG reduced the expressions of FAS and FABP4. After the early stage of differentiation, FAS and FABP4 are necessary to induce the formation of mature adipocytes (11). Previously, increased expressions of FAS and FABP4 have been implicated in the development of obesity, insulin resistance, and atherosclerosis (9, 164); their reduction by gallotannin derivatives is indicative of the therapeutic potentials of gallotannin derivatives in obesity-related chronic conditions.

The MG extract is primarily comprised of monogalloyl glucoside, gallic acid and gallotannins. It suppresses adipogenesis partly through the modulation of the AMPK pathway without any involvement of PG. As a result, we propose that other gallotannin derivatives in MG extract might possess anti-adipogenic activities similar to PG. Previous studies indicate that gallic acid plays a critical role in regulating body

weight and glucose homeostasis through the activation of the AMPK pathway in the liver, muscle and interscapular brown adipose tissue (75). Benzophenone C-glucosides, iriflophenone 3-C-β-glucoside and foliamangiferoside A that can be found to be absorbed after the consumption of mango leaf extract have been associated with the up-regulation of the AMPK activity in 3T3-L1 adipocytes, leading to reduced lipid accumulation in vitro (69). These findings suggest that the anti-obesogenic activities of MG extract may be attributed to MG-derived polyphenols or combined activities of several polyphenols.

Adipocyte browning is the process that converts white adipocytes to beige adipocytes through thermogenesis under physiological and nutritional stimuli, such as cold exposure, pharmacological treatment (12), and phytochemical consumption (13). Upon the activation of browning, WAT takes on characteristics of BAT, notably the induction of UCP1 expression and the existence of multi-locular lipid droplets (36). Therefore, promoting thermogenesis is considered a potential therapeutic strategy in the prevention of obesity and its co-morbidities. Due to the limited amount and activity of BAT in adults, the brown remodeling of white adipose tissue is being investigated as a potential anti-obesogenic strategy.

Growing evidence indicates that the administration of phytochemicals, such as resveratrol and curcumin, increases the expressions of thermogenic markers, such as PPARγ coactivator 1α (PGC-1α) and UCP1, both *in vitro* (48) and *in vivo* (41, 49). In this study, gallotannin derivatives demonstrated thermogenic activity in mature adipocytes, rather than affecting lipid accumulation in the process of adipocyte

differentiation (Data not shown). This study showed that PG enhanced the expression of UCP1 and Sirt1, whereas MG only increased Sirt1 expression at a high concentration (10 mg GAE/L). Additionally, the brown remodeling of WAT by PG might be due, at least in part, to the activation of the AMPK pathway as shown by the increased expression of LKB1 and AMPKα1. These results are in concordance with previous reports that polyphenols exert anti-obesogenic property partly by promoting the formation of beige adipocytes and thermogenesis (40, 48, 165).

3.5 Conclusions

Overall, this study demonstrates that gallotannin derivatives suppress lipid accumulation and promote thermogenesis in adipocytes, at least in part, through the activation of the AMPK signaling pathway. More comprehensive knowledge of the health benefits of mango-derived polyphenols in specific tissues of animals, such as WAT and BAT, will further elucidate the underlying mechanisms of these biological functions. Given that PG is a microbial metabolite of MG, a well-controlled, double-blinded clinical trial with oral administration of mango-derived polyphenols supplemented with or without probiotics is necessary to identify the biological activities of MG and PG in humans.

CHAPTER IV

HEALTH BENEFITS OF ORALLY ADMINISTERED GALLOTANNINS IN THE PRESENCE OR ABSENCE OF *LACTOBACILLUS PLANTARUM* IN GNOTOBIOTIC MICE FED A HIGH-FAT DIET[‡]

Dietary polyphenols are secondary metabolites of fruits and vegetables that have shown potent anti-oxidant and anti-inflammatory activities. Dietary polyphenols and their metabolites beneficially influence the composition and function of the gut microbiome. The intestinal microbiome plays a key role in modulating the bioavailability of polyphenols. This reciprocal interaction between gut microbiome and polyphenols exerts potential health effects in modulating intestinal health and the risk of developing chronic diseases. However, gut dysbiosis in chronic diseases such as inflammatory bowel disease, irritable bowel syndrome, obesity, and cardiovascular disease would greatly affect the host gut microbiome and reduce the biological activity of therapeutic agents such as dietary polyphenols. In this chapter, the polyphenol-microbiome interactions in chronic diseases are investigated using a gnotobiotic mouse model with high-fat diet feeding. This study will give a comprehensive understanding regarding the role of dietary polyphenols within a host microbiome environment. These findings have the potential in explaining lack of efficacy of polyphenol-rich treatments

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in experimental models with chronic diseases and contributing to improved health outcomes with polyphenol-rich treatments.

4.1 Introduction

Obesity leads to adipose tissue expansion with hypertrophic adipocytes that secrete adipokines and initiate immune cells infiltration, which may further exacerbate the propagation of immune cell recruitment and secretion of pro-inflammatory cytokines (166). This environment negatively affects inflammatory and insulin signaling pathways in metabolic tissues such as the liver, skeletal muscle and adipose tissue, and increases the risk of developing insulin resistance, type 2 diabetes, and cardiovascular disease (19, 21). The modulation of adipose tissue function, both white adipose tissue (WAT) and brown adipose tissue (BAT), by dietary intervention has been increasingly recognized as a potential anti-obesogenic therapy (13, 167, 168). The classical role attributed to WAT is energy storage in the form of lipid. The last few decades have seen major advances in the thermogenic potential of WAT, which may convert white adipocytes into beige adipocytes under certain stimuli, such as cold exposure, catecholamines, thyroid hormone, and plant-based polyphenolic compounds such as resveratrol, curcumin, genistein, and quercetin (13). In distinction from WAT, BAT dissipates energy by non-shivering thermogenesis that generates heat in response to cold exposure. This thermogenesis process is mediated by a series of molecular factors that can affect the brown adipocyte development and functions (12). Recently, BAT is thought to be a viable target of dietary polyphenols to stimulate energy expenditure and

energy dissipation and therefore protect from developing obesity and metabolic disorders (169).

Polyphenols are considered therapeutic agents in the prevention of chronic diseases due to their antioxidant and anti-inflammatory activities (170). Recently, the anti-obesogenic activity of polyphenols, such as gallotannins (GT) and their metabolites gallic acid (GA) and pyrogallol (PG), have been increasingly reported in *in vitro*, *in vivo*, and human clinical studies (68, 73, 74, 79, 121, 128, 129). In mouse 3T3-L1 adipocytes, mango polyphenolic extract containing primarily GT and GA, as well as a pure standard of PG suppressed adipocyte differentiation and PG additionally promoted trans-differentiation into beige adipocytes. These activities were at least in part mediated through the activation of the AMP-activated protein kinase (AMPK) pathway that modulates down-stream enzymes involved in lipid metabolism (128). The administration of GA in obese mice caused weight loss and decreased the levels of triglyceride, low-density lipoprotein (LDL), very low-density lipoprotein (VLDL), and leptin (129). The consumption of either a polyphenol-rich mango juice or freeze-dried mango pulp decreased high-fat diet (HFD)-induced adiposity and inflammation and modulated plasma glucose levels (73, 74). Previously, 6 weeks consumption of a daily dose of 400 g of mango pulp decreased plasma concentrations of pro-inflammatory cytokines such as interleukin 8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1), plasminogen activator inhibitor 1 (PAI-1), and hemoglobin A1c (HbA1c) in obese subjects. The health-promoting activities of daily mango consumption were positively correlated to the systemic exposure to GT derivatives from mango, including catechol

sulfate, 4-*O*-methylgallic acid, and methylpyrogallol-*O*-sulfate (121). These findings suggest that the consumption of GT-rich fruits, teas, spices and nuts might be beneficial in the prevention of obesity and metabolic disorders.

The pharmacokinetics of dietary polyphenols is influenced by gut microbiota, where inter-individual microbial biodiversity may translate into great variability of associated pharmacodynamics endpoints (31). In humans, only a small percentage of dietary polyphenols is believed to be absorbed into small intestine while 90-95% are expected to reach the colon where hydrolysis, decarboxylation and other reactions are executed by intestinal microbial bacteria. As a result, the biological activity of polyphenols depends at least for a major part on microbial metabolism (31, 122, 123). *L.plantarum* is a lactic acid bacterium widely used in probiotic products (124). Promising effects of *L.plantarum* in the prevention and alleviation of gut dysbiosis prompt its application in patients with irritable bowel syndrome (125). In addition, *L.plantarum* is known to encode for both tannase (tannin acyl hydrolase, EC 3.1.1.20)- and gallic acid decarboxylase (EC 4.1.1.59)-producing activities that catalyze the hydrolysis of galloyl ester bonds in hydrolysable GT, yielding glucose and GA. GA will then be decarboxylated to PG (126, 127). The application of *L.plantarum* in degrading large, unabsorbable compounds into small, absorbable compounds may enhance the health-promoting effects derived from GT-rich food in reducing obesity and its related chronic diseases.

The anti-obesogenic activity of polyphenols has mostly been investigated in animals with conventional microbiota. The role of each individual species of microbiota

in obesity and metabolic disorders still remain unclear. The use of a germ-free (GF) mouse model provides an invaluable tool to understand not only the interaction between *L.plantarum* and GT, but also the subsequent effects on diet-induced obesity exerted by the microbial metabolites (171). Therefore, the current study aimed to investigating the health benefits of orally administered GT in the presence or absence of *L.plantarum* in gnotobiotic mice fed a HFD.

4.2 Materials and Methods

4.2.1 Extract Preparation and Characterization

Tannic acid and GA were purchased from Sigma-Aldrich (St. Louis, MO). Sephadex LH-20 (Sigma-Aldrich, St. Louis, MO) was used to isolate GT and remove residual GA (172). A 250 mL column was filled to 25% capacity with Sephadex-LH-20 and the resin was rehydrated with 100% ethanol. 1% tannic acid in 0.1% formic acid was loaded onto the column, washed with 1 column volume of 100% ethanol, and eluted with acetone and 0.1% formic acid (80:20). Acetone was evaporated under reduced pressure at 45°C. The resulting concentration of the tannic acid isolate was 11,423 mg L^{-1} gallic acid equivalent (GAE). To confirm the purity of GT, the extract was analyzed using a Thermo-Finnigan Surveyor high-performance liquid chromatography-photodiode array detector (HPLC-PDA) in tandem with a LCQ Deca XP Max ion trap spectrometer with an ESI source as previously described (173). GTs and their derivatives were characterized at 280 nm.

4.2.2 Enzymatic Activities of L.plantarum WCFS1

L.plantarum WCFS1 was purchased from American Type Culture Collection (ATCC, Rockville, MD) and routinely grown in MRS broth (Difco, Detroit, MI) in an anaerobic environment at 37°C. For characterizing tannase and decarboxylase activities of *L.plantarum*, bacteria were grown on MRS broth until early exponential phase (optical density at 600 nm [OD600]=0.3). Cultures were added to a fresh modified medium (6 g $(NH_4)_2SO_4$, 0.4 g $MgSO_47H_2O$, 7 g KH_2PO_4, 0.02 g $FeSO_47H_2O$, 3 g Casamino acids (Sigma-Aldrich, St. Louis, MO) in 1 L of water, pH: 5.5) (174)) supplemented with 0.5 mM GT or 1.5 mM GA. Cultures were continued to grow to mid-exponential phase (OD600=0.6), centrifuged at 4^0C for 10 minutes at 3,000 rpm, and washed twice with phosphate-buffered saline (PBS) (pH 5.8). Afterwards, cultures were re-suspended in PBS supplemented with 0.5 mM GT or 1.5 mM GA. To investigate tannase activity of *L. plantarum,* aliquots of sample were removed at 0, 1, 6, 12, 24 hours after the addition of GT. As for the decarboxylase activity, aliquots of sample were removed at 0, 15, 30, 60, and 120 minutes after the addition of GA. Acidified methanol was added to samples. Samples were filtered and analyzed by high-performance liquid chromatography-mass spectrometry (HPLC-MS) (175).

4.2.3 Animal Study Design

GF C57BL/6J mice were maintained under GF conditions in a room with a 12-hour light-dark cycle and routinely monitored for GF status by standard microbiological methodologies (176). All procedures were performed inside a sterile, flexible-film

isolator, unless microorganisms were intentionally introduced. GF mice were randomly divided into three groups: non-colonized GF mice received a vehicle solution (GF-Con) or GT (GF-GT), and one group was colonized with *L.plantarum* and received GT (Lp-GT), for five weeks. After 1 week of acclimation with regular diet (D12450J, Research Diets, New Brunswick, NJ), mice were orally gavaged with 100 μL of either saline or *L.plantarum* (10^8 CFU/100 μL) for three consecutive days (Week 0-1: Day 1 to Day 3), followed by GT oral gavage (1.6 mg/mouse/every other day) for 5 weeks. GF condition and colonization of gnotobiotic mice were monitored weekly by fecal 16S ribosomal RNA (rRNA) analysis and confirmed the colonization with *L.plantarum* and the absence of cultivation with other strains (data not shown) (177). After adaptation of animals to intestinal colonization and administration of GT, a HFD containing 60% kcal fat (D12492-1.5V, Research Diets, New Brunswick, NJ) was given for the last 4 weeks of this study. Mice were sacrificed, and blood, tissues and feces were collected, weighed, and stored at -80°C until further analysis. The animal use protocol was approved by the Institutional Animal Care and Use Committee of Texas A&M University (IACUC# 2016-0087).

4.2.4 Dosage Information

A dosage of 1.6 mg/mouse/every other day of GT was selected in this study based on our previous findings that mice orally gavaged with 0.8 mg/day of mango polyphenols (primarily GT) experienced significantly decreased tumor volume and oxidative stress involving the activation of the AMPK pathway and suppression of the

mammalian target of rapamycin (mTOR) pathway. This dosage was considered safe and showed no toxicity or any other adverse effects to mice (97). Therefore, an equivalent dose was expected to be effective in this study.

4.2.5 Inflammatory Cytokines and Metabolic Hormones

Diet-induced obesity is associated with low-grade systemic inflammation and insulin resistance (178). In this study, the plasma levels of inflammatory cytokines, including tumor necrosis factor α (TNF-α) and MCP-1; and metabolic hormones, including insulin and leptin were determined by multiplex bead assay (Millipore, Billerica, MA). These experiments were performed on a Luminex L200 machine (Luminex, Austin, TX) and data were analyzed by Luminex xPONENT software version 3.1. Differences of TNF-α, MCP-1, and leptin were compared between Week 2 and Week 6. Fasting blood glucose levels were determined using a Cayman glucose colorimetric assay kit (Cayman Chemical Company, Ann Arbor, MI). Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) was calculated based on the following formula: fasting plasma glucose (mmol/L)×fasting plasma insulin (μU/mL)/99.95 (179).

4.2.6 Quantitative RT-PCR

Total RNA was isolated from adipose tissues using the mirVana™ miRNA Isolation Kit (Applied Biosciences, Foster City, CA) according to the manufacturer's protocol. The concentration of the extracted RNA was determined using the NanoDrop

ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Briefly, 1000 ng of RNA was used to synthesize cDNA using a Reverse Transcription Kit (Invitrogen, Grand Island, NY). SYBR Green PCR Master Mix (Applied biosystems, Foster City, CA) was used for the qPCR analysis on the CFX384 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Gene expression levels of carnitine palmitoyltransferase I (CPT1), perilipin 1, hormone-sensitive lipase (HSL), nuclear factor erythroid-2-like 1 (Nfe2l1), transmembrane protein 26 (Tmem26), T-box transcription factor 1 (Tbx1), peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α), cyclooxygenase 2 (Cox2), PR domain containing 16 (PRDM16), and cytochrome c oxidase 7a1 (Cox7a1) were analyzed by qPCR, and data were normalized to β-actin as an endogenous control (128).

4.2.7 Western Blotting

Adipose tissues were homogenized and lysed in T-PER tissue protein extraction reagent (Pierce, Rockford, IL) containing 1% Halt protease and phosphatase inhibitor cocktail (Thermo Scientific), and centrifuged at 12,000 g for 15 minutes at 4°C. The layer below the fat was collected and centrifuged again (180). Protein was then quantified by the Bradford assay (Invitrogen, Carlsbad, CA), loaded and run on a 4-12% sodium dodecyl-polyacrylamide gel and transferred to a PVDF membrane using the iBlot Dry Blotting system (Invitrogen, Carlsbad, CA). The membrane was blocked in 5% non-fat milk solution for 1 hour and probed with primary antibodies against phosphorylated AMPKα1 (p-AMPKα1), total-AMPKα1 (t-AMPKα1),

CCAAT/enhancer binding protein α (C/EBPα), peroxisome proliferator-activated receptor γ (PPARγ), fatty acid synthase (FAS), Sirtuin1 (SIRT1), uncoupling protein 1 (UCP1), and β-actin (Cell Signaling Technology, Danvers, MA) (128). The band intensity in Western blot was determined using ImageJ software (National Institutes of Health, Bethesda, MD, USA; http://rsb.info.nih.gov/ij/).

4.2.8 Histological Analyses

Adipose tissue was dehydrated, embedded in paraffin, and sectioned at 5 μm of thickness. Hematoxylin and eosin (H&E) staining was performed as previously described (50). Images of each section from each mouse were obtained with a Zeiss Axioplan 2 microscope (Carl Zeiss, Thornwood, NY) fitted with an Axiocamhigh resolution digital camera and Axiovision 4.1 software using the same settings.

4.2.9 Statistical Analyses

The data were analyzed using GraphPad Prism 6 (GraphPad Software, Lo Jolla, CA). Results are presented as means ± standard error of the mean (SEM). p values were calculated using one-way ANOVA if data were normally distributed or the Kruskal–Wallis test if data were not normally distributed. A p value less than or equal to 0.05 indicates statistical significance between groups and is marked with different letters above the data.

4.3 Results

4.3.1 Tannase and Decarboxylase Activities of L.plantarum

Activities of GT-metabolizing enzymes in *L.plantarum* cultures were assessed using HPLC-MS. GA, the product of microbial hydrolysis of GT by tannase produced by *L.plantarum*, was detected in *L.plantarum* cultures at 280 nm after 24 hours incubation with 0.5 mM GT **(Figure 16A, B)**. *L.plantarum* is known to produce gallate decarboxylase that catalyzes the decarboxylation of GA to produce PG (175). In this study, PG was detected in *L.plantarum* cultures incubated with 1.5 mM GA for 2 hours at 280 nm **(Figure 16C, D)**.

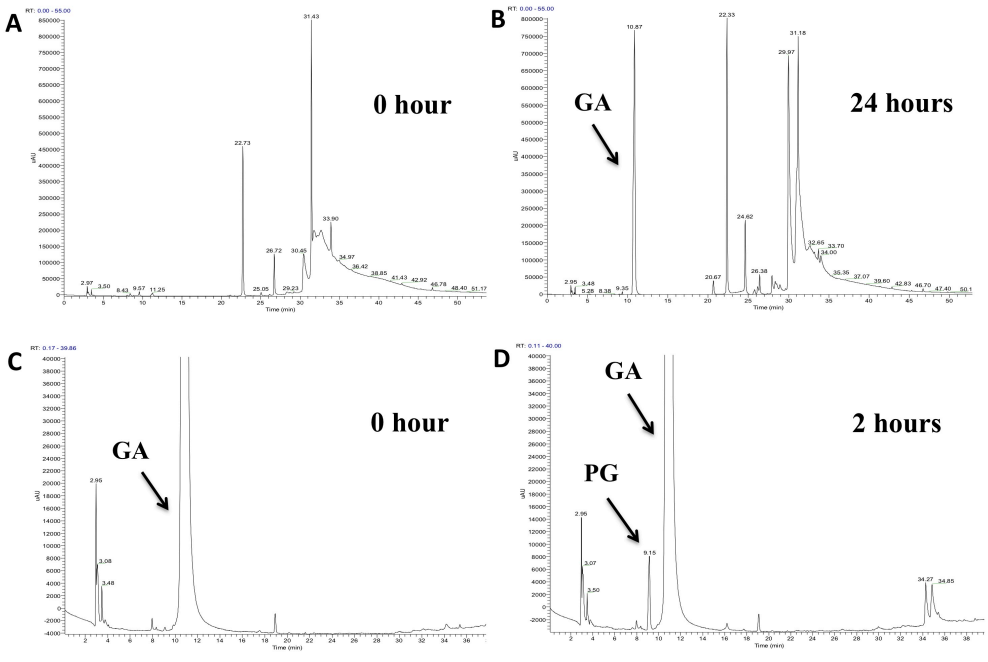

Figure 16 Polyphenolic production by *L.plantarum*. Reprinted with permission from *Molecular nutrition & food research*, 1800937. Copyright [2019] by John Wiley and Sons.

4.3.2 Characterization of GT Extract

Using liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS), GT with a degree of polymerization of 5 and greater were detected at 280 nm at a retention time of 25-55 minutes, and no GA or other small absorbable compounds were present in the GT extract **(Figure 17A)**.

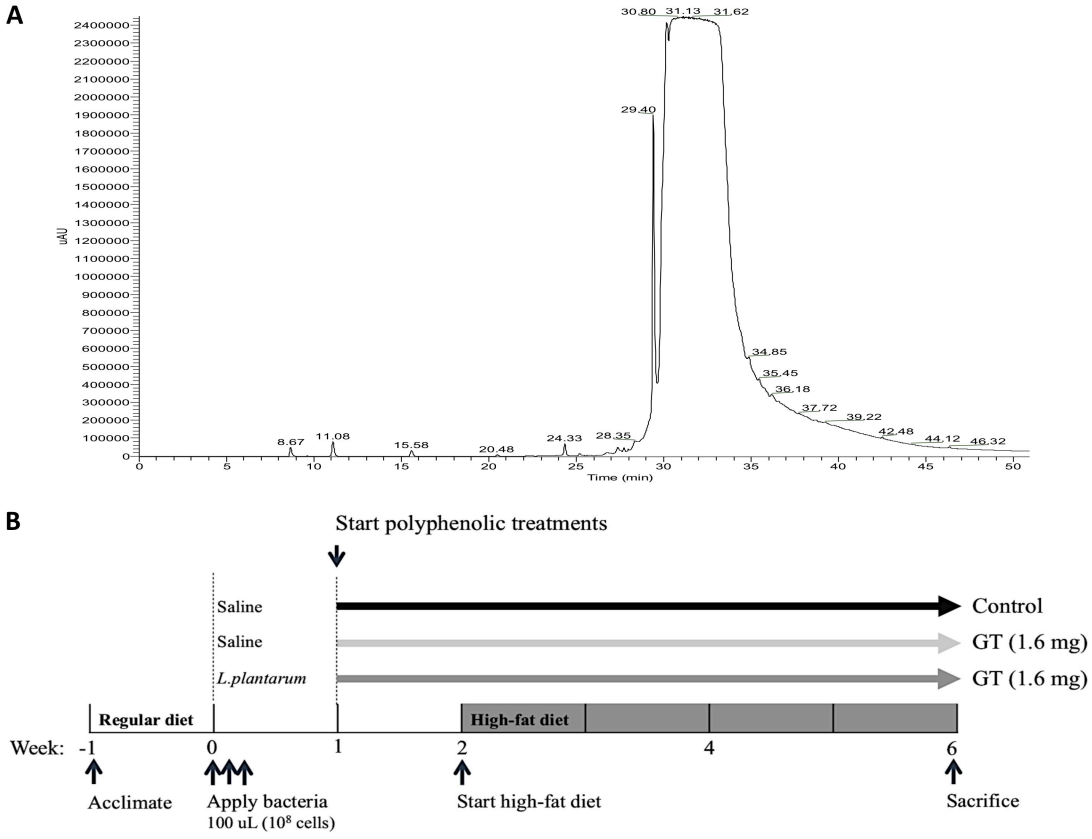

Figure 17 HPLC chromatogram of compounds present in gallotannin extract and experimental design of the gnotobiotic mouse study. Reprinted with permission from *Molecular nutrition & food research*, 1800937. Copyright [2019] by John Wiley and Sons.

4.3.3 L.plantarum Colonization did not Affect Average Body Weight and Adiposity but Improved Metabolic Functions

Overall, gnotobiotic mice in each study group (n=7, female=3, male=4, **Figure 17B**) showed no significant difference in average body weight **(Figure 18A-C)** and fat mass including epididymal WAT (eWAT) **(Figure 18D-F)**, and interscapular BAT (iBAT) **(Figure 18G-I)** after 4 weeks of HFD feeding. Gender-specific physiological differences between female and male mice may impact the results of this study; therefore male and female mice were evaluated separately as well as in data pools within each treatment group. Fasting plasma glucose levels were similar in three groups **(Figure 19A)**. Mice colonized with *L.plantarum* had significantly lower level of insulin ($p=0.0043$) and HOMA-IR ($p=0.0111$) compared to the GF group gavaged with GT **(Figure 19B, C)**. GF mice orally gavaged with GT experienced a trend towards decreased levels of TNF-α, MCP-1, and leptin. Intestinal colonization with *L.plantarum* significantly alleviated the HFD-induced increases of TNF-α, MCP-1, and leptin by 337.63% ($p=0.0183$), 330.53% ($p=0.0234$), and 59.94% ($p=0.0330$) between Weeks 2 and 6, respectively **(Figure 19D-F)**.

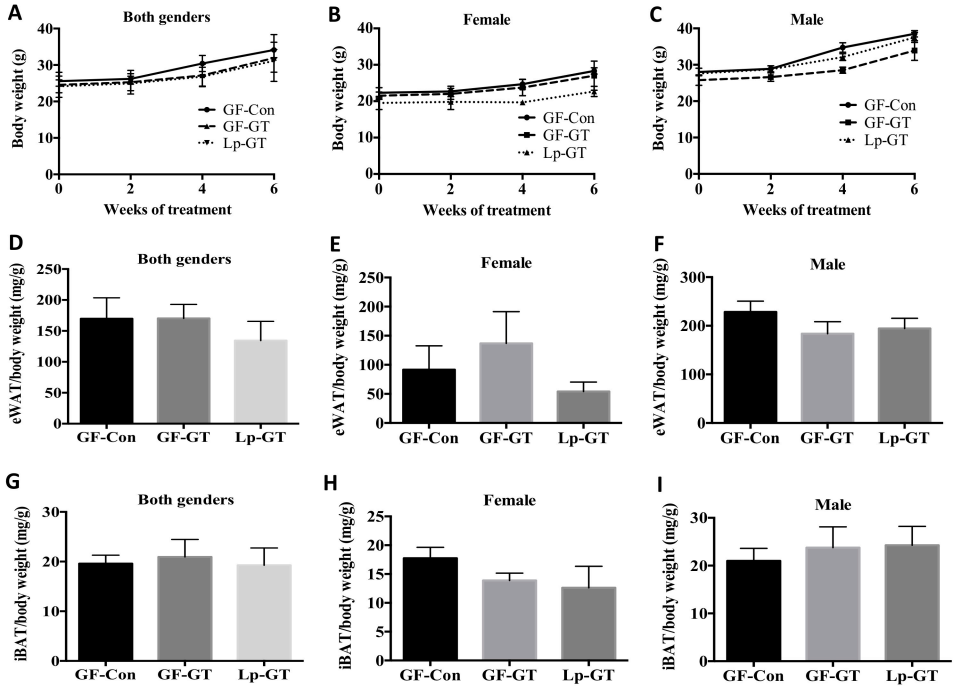

Figure 18 Body weight and adiposity of HFD-fed gnotobiotic mice. Reprinted with permission from *Molecular nutrition & food research*, 1800937. Copyright [2019] by John Wiley and Sons.

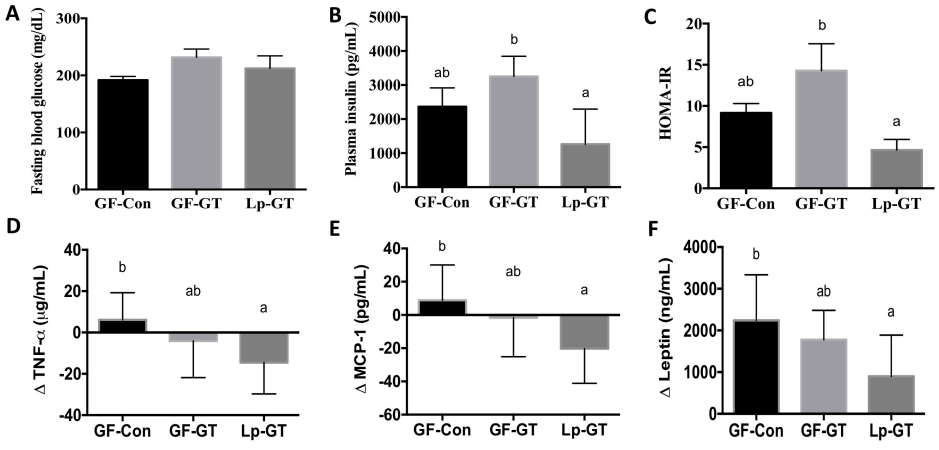

Figure 19 Plasma levels of metabolic hormones and inflammatory cytokines. Reprinted with permission from *Molecular nutrition & food research*, 1800937. Copyright [2019] by John Wiley and Sons.

4.3.4 GT and L.plantarum Colonization Modulated the Expressions of Molecules Involved in Lipid Metabolism and Reduced Lipid Size in eWAT

White adipose tissue has its classical role as a lipid storage organ, as well as other critical roles in endocrine function associated with a wide range of metabolic disorders (7, 181). In eWAT, both GF-GT and Lp-GT groups exhibited increased mRNA expressions of lipolytic (perilipin 1 and HSL) and thermogenic (Tmem26, Tbx1, PGC1α, Cox2) genes. In addition, Lp-GT group exhibited increased CPT1 mRNA expression compared to the GF groups **(Figure 20A, B)**. Lipid accumulation is highly regulated by key transcription factors (e.g., PPARγ and C/EBPα) and enzymes involved in fatty acid synthesis (e.g., FAS) (11, 128). GT treatment down-regulated the protein expressions of FAS and PPARγ. Additionally, C/EBPα showed a trend towards decreased levels **(Figure 20C, D)**. Morphologically, Lp-GT group was characterized by more of the multi-locular lipid droplets in the eWAT than the control group as shown by H&E staining **(Figure 20E)**, and this suggests reduced lipid size in L.plantarum-colonized mice. GT treatment alone did not affect the size of lipid droplets **(Figure 20E)**.

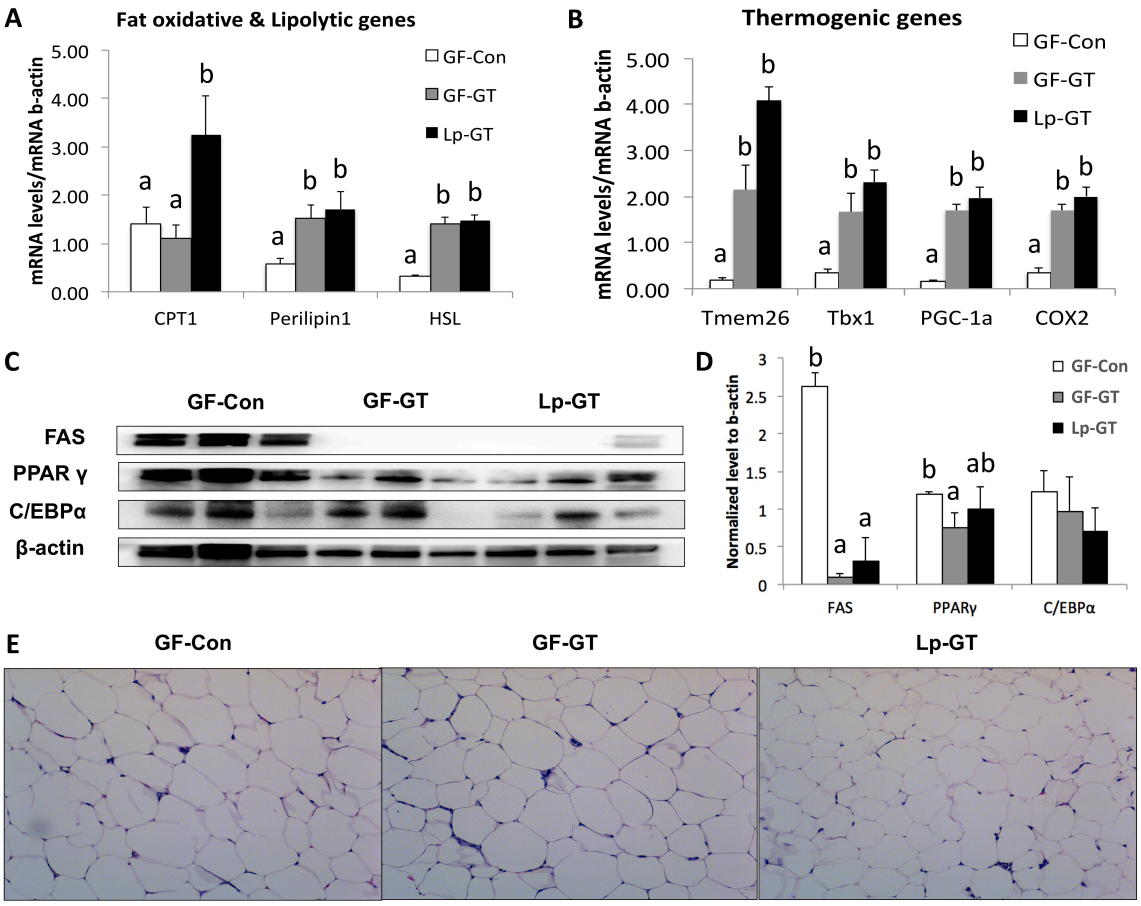

Figure 20 GT and *L.plantarum* colonization modulated the expressions of molecules involved in lipid metabolism and reduced lipid size in eWAT. Reprinted with permission from *Molecular nutrition & food research*, 1800937. Copyright [2019] by John Wiley and Sons.

4.3.5 GT and L.plantarum Colonization Modulated Lipid Metabolism and Enhanced Thermogenesis in iBAT

Brown adipose tissue is specialized in dissipating energy through thermogenesis and has been implied as relevant to the prevention and treatment of obesity (182). In iBAT, the mRNA expressions of thermogenic genes (Nfe2l1, Tmem26, Tbx1, PGC1α,

Cox2, PRDM16 and Cox7a1) were increased in both GF-GT and Lp-GT groups; Cox7a1 mRNA expression was further enhanced by *L.plantarum* colonization compared to the GT treatment alone, but not significantly so **(Figure 21A)**. The AMPK pathway plays a pivotal role in energy metabolism and is highly expressed in brown adipose tissue (183). Previously, GT derivatives (e.g., PG) from mango polyphenolic extract have been shown to induce the browning of white adipocytes into beige adipocytes, which might be associated with the activation of the AMPK pathway (128). Accumulating evidence suggests that some polyphenols (e.g., resveratrol and procyanidins) induce the formation of the brown-like adipocytes through the phosphorylation of AMPKα1 and enhancing the expressions of brown adipocyte markers such as UCP1, SIRT1, PGC1α, and PRDM16 (50, 184). The protein expressions of SIRT1 and UCP1 were up-regulated in the GT-treated groups while p-AMPKα1 were additionally up-regulated in the Lp-GT group, suggesting the activation of the AMPK pathway and enhanced thermogenesis in iBAT by *L.plantarum* colonization **(Figure 21B-E)**. The H&E staining further confirmed our hypothesis that GT in combination with *L.plantarum* induces thermogenesis and reduces lipid size in iBAT **(Figure 21F)**.

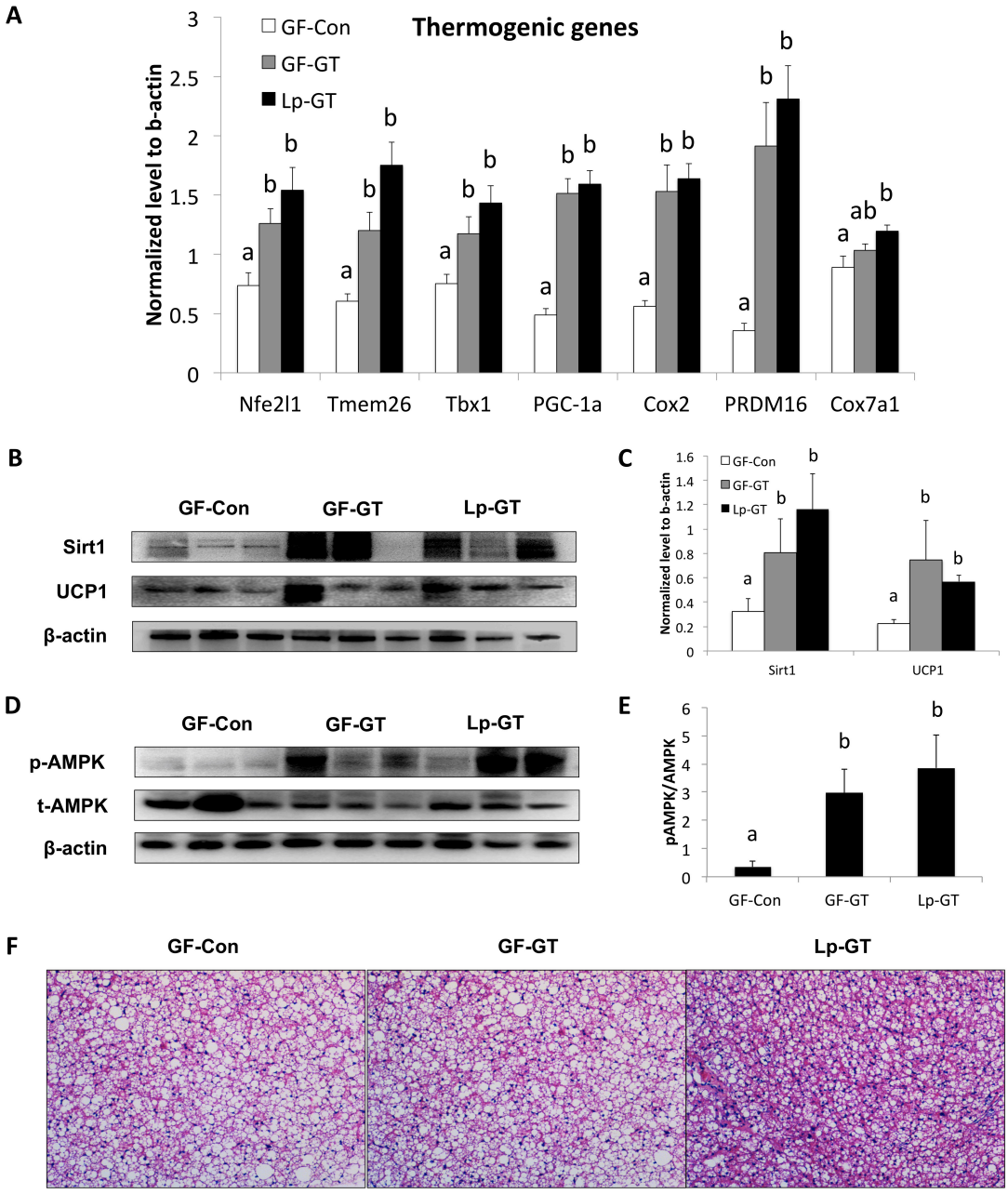

Figure 21 GT and *L.plantarum* colonization modulated lipid metabolism and enhanced thermogenesis in iBAT. Reprinted with permission from *Molecular nutrition & food research*, 1800937. Copyright [2019] by John Wiley and Sons.

4.4 Discussion

Plant-based bioactive compounds from fruits and vegetables have been found to inhibit lipogenesis while promoting brown and beige adipocyte development and thermogenesis and are therefore considered novel nutritional intervention strategies in the prevention of obesity and its related chronic diseases. Mechanisms underlying the anti-obesogenic activities of some polyphenols, including epigallolcatechin gallate (EGCG) (185, 186), resveratrol (187), quercetin (188, 189), and curcumin (190-192) have been investigated in *in vitro, in vivo,* and human clinical studies. While anti-inflammatory and anti-cancer activities of polyphenols such as GTs and their derivatives (GA and PG) have been examined in breast cancer (97) and colitis (143, 144) models, investigation of these polyphenols in obesity seems to be limited. Previously, we have shown that both, mango polyphenolic extract (high in GT and GA) and a purified compound PG inhibit adipogenesis and reduce lipid accumulation and PG additionally promotes thermogenesis in 3T3-L1 adipocytes (128). It has yet to be determined whether the beneficial effects are attributed to the parent compound GT or the production of microbial GT metabolites GA and PG by tannase and decarboxylase produced by gut microbiota (e.g. *L.plantarum*). Therefore, this study aimed to investigating whether the intestinal colonization with *L.plantarum* can improve the bioactivities of GT administered to GF mice.

In this study, GT inhibited fat synthesis in eWAT and promoted thermogenesis in iBAT. In eWAT, GT-treated mice exhibited lower expressions of lipid synthesis enzymes (FAS, PPARγ and C/EBPα), and higher expressions of molecules involved in

lipolysis (perilipin1 and HSL) and thermogenesis (Tmem26, Tbx1, PGC-1α, and Cox2) compared to GF mice gavaged with a vehicle solution. Similarly, in iBAT the expressions of thermogenic markers (Nfe2l1, SIRT1, UCP1, Tmem26, Tbx1, PGC-1α, Cox2, and PRDM16) were significantly higher in GT-treated groups. However, feeding GF mice with GT alone did not affect fasting blood glucose, insulin, and HOMA-IR levels compared to vehicle-treated mice. In this study, GT was administered in the form of tannic acid that contains GA oligomers of 5 and greater. These GA oligomers are not absorbable and are subject to hydrolysis, decarboxylation, and other reactions by intestinal microbial bacteria that yield absorbable GT metabolites (126, 127). GT treatment alone without the addition of *L.plantarum* demonstrated beneficial effects on modulating inflammatory responses and adipose tissue functions. Previously, the polyphenols-lipid/protein binding activity was proposed as a possible mechanism of reduced obesity and inflammation after HFD feeding (193). In this study, the binding of dietary polyphenols GT to lipids and proteins in the intestine may interfere with the bioactivity of enzymes involved in signaling transduction, leading to impaired macronutrient digestion, metabolism, and absorption (193). These body weight- and fat-lowering effects might furthermore alleviate HFD-induced inflammation and adipose tissue dysfunction, which is in line with our findings.

In addition to the GT treatment, colonization with *L.plantarum* significantly improved biomarkers for inflammation and insulin resistance evidenced by lower plasma levels of insulin, TNF-α, MCP-1, and leptin in *L.plantarum*-colonized group compared to GF-GT group. No effect on average body weight, adiposity, and fasting

blood glucose level was observed for either treatment groups possibly due to the short duration of the study. The expressions of CPT1 in eWAT, as well as p-AMPKα1 and Cox7a1 in iBAT were further increased after the colonization with *L.plantarum*. *L.plantarum* encodes for GT-metabolizing enzymes that yield absorbable bioactive metabolites, namely GA and PG in the gnotobiotic mouse model. Both eWAT and iBAT of *L.plantarum*-colonized mice were characterized by smaller, multi-locular lipid droplets. These results support the hypothesis that *L.plantarum* has the potential in reducing HFD-associated inflammation, possibly through GT metabolism yielding absorbable GT metabolites **(Figure 22)**.

Emerging evidence demonstrates the two-way relationship between dietary polyphenols and the composition of the intestinal microbiota where an increased intake of polyphenols may shape the composition of the intestinal microbiota by increasing species with the ability to metabolize polyphenols (96, 113-116). Many so-called probiotic species such as *Streptococcus gallolyticus, Lonepinella koalarum, Bacillus licheniformis*, and several *Lactobacilli* species fall into this category (117). Recently, we have reported that daily consumption of GT-rich mango pulp for 6 weeks increases levels of tannase-producing bacteria (*Lactoccoccus lactis*) in healthy human subjects, and this increase was correlated with increased tannase enzyme activity in fecal samples. The production of a short-chain fatty acid, namely butyrate showed a trend towards increased levels after 6-week mango consumption (194). This evidence suggests that the health-promoting effects of GT and *L.plantarum* may be at least in part based on prebiotic-probiotic interactions between GT and *L.plantarum*. Potentially, non-

absorbable GTs mediate their anti-inflammatory and anti-obesogenic activities indirectly through increasing the abundance of *L.plantarum*.

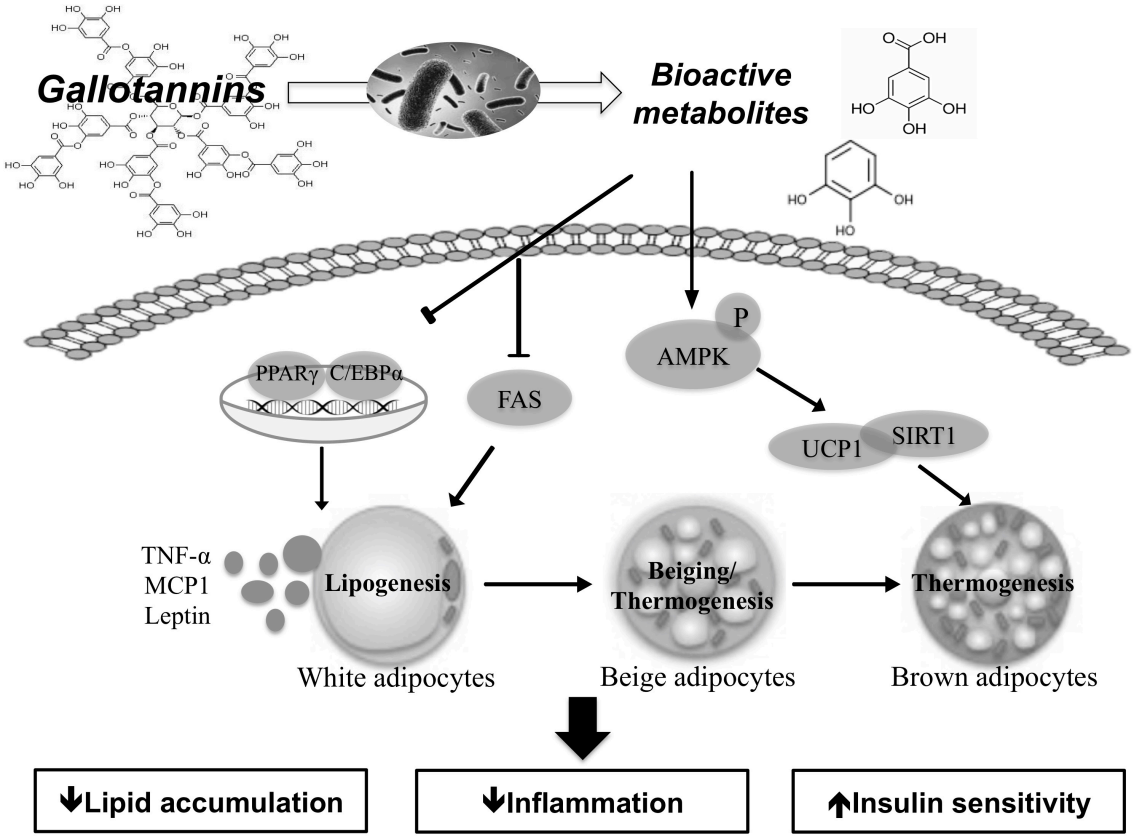

Figure 22 Schematic diagram of a proposed mechanism for GT with *L.plantarum* colonization in reducing obesity in gnotobiotic mice. Reprinted with permission from *Molecular nutrition & food research*, 1800937. Copyright [2019] by John Wiley and Sons.

In support of this hypothesis, the supplementation (22 weeks) of green tea polyphenols combined with *L.plantarum* reduced body fat content and cholesterol accumulation, and additionally promoted the growth of *Lactobacillus* species in the

intestine and attenuated inflammation in HFD-induced obese mice (118). In addition, GT-induced increased abundance of *L.plantarum* may result in increased short-chain fatty acid production. In antibiotic-associated diarrhea patients, a significant increase was noted for the production of butyrate in fecal samples of patients receiving an *L.plantarum*-fermented fruit drink compared to patients receiving a placebo fruit drink (119). Butyrate as a microbiota-induced fermentation product has shown anti-inflammatory and anti-obesogenic potential possibly due to its ability in enhancing intestinal barrier integrity and function (120).

In addition to enhancing probiotic activities, the production of bioactive GT metabolites via microbial degradation can be associated with decreased inflammation and risk of developing obesity-related metabolic disorders (121). *L.plantarum* possesses tannase- and decarboxylase-producing activities in degrading large, unabsorbable GT into small, absorbable, bioactive compounds GA and PG that are easily distributed into tissues where they can act as anti-inflammatory and anti-obesogenic agents (31, 128, 129). This may enhance the health-promoting effects derived from GT-rich food in reducing obesity and its related chronic diseases. Taken together, it remains to be investigated to what extent the beneficial effects of GT in combination with *L.plantarum* are attributed to the enhanced growth of GT-metabolizing bacteria or the increased systemic exposure to GT derivatives.

This pilot study has several limitations including a short study duration and low number of animals (n=7), which may lower the impact of this study. Overall, reports using gnotobiotic mouse models are still limited and several studies have reported that

GF mice exert a leaner phenotype due to the lack of gut microbiota compared to the mice with conventional microbiota (195, 196). However in a previous study, 5 weeks of HFD feeding significantly increased body weight gain and fat mass, and induced inflammation and glucose tolerance in GF mice compared to the low-fat diet feeding (197). This present study serves as a proof-of-principle indicating the role of the intestinal microbiota in the efficacy of dietary GT in diet-induced obesity. In addition, we demonstrated that GT increased the expressions of genes encoding beige fat activation and thermogenesis in WAT and BAT, respectively where future, long-term studies should be conducted to confirm this effect on actual energy expenditure, including body core temperature, oxygen consumption and CO_2 production, as well as physical activity in a larger number of animals.

Findings in this study provide initial evidence of the beneficial role of probiotics in context with a polyphenol-rich diet. Overall, it remains uncertain, to what extent the anti-obesogenic effects improving WAT and BAT functions are based on GT metabolites or the presence of the probiotic species *L.plantarum*. This study has the potential to link the biological activities of dietary polyphenols to gut microbial composition and provide novel insights into dietary recommendations that include probiotics into our diet to increase bioavailability and bioefficacy of dietary polyphenols. Future pharmacokinetic/pharmacodynamic analyses should characterize polyphenolic profiles in plasma and adipose tissue to understand the role of individual bioactive GT metabolites.

4.5 Conclusions

Overall, orally administered GT reduced HFD-induced inflammation and lipid accumulation in eWAT and promoted thermogenesis in iBAT. Colonization with *L.plantarum* further reduced adipose tissue expansion, inflammation, and insulin resistance. The potential role of prebiotic-probiotic interactions in the production of absorbable, bioactive microbial GT metabolites is suggested by this pilot gnotobiotic mouse study. Enhanced bioavailability and bioefficacy of GT derivatives might be responsible for their anti-inflammatory and thermogenic activities after the colonization with *L.plantarum*. Together, these findings have implications for a future human clinical trial with dietary GT and probiotics to investigate if health-promoting effects of GT are attributed to GT itself or the production of microbial GT metabolites. Human clinical studies are needed to lay the groundwork in the development of intake recommendations for prebiotic-probiotic combinations.

CHAPTER V

INFLUENCE OF DAILY MANGO SUPPLEMENTATION FOR 6 WEEKS ON INFLAMMATION AND METABOLIC FUNCTIONS IN LEAN AND OBESE INDIVIDUALS[§]

Preclinical findings indicate that mango-derived polyphenols exert potent anti-inflammatory and anti-lipogenic effects that are relevant to the prevention of obesity-related chronic diseases. However, findings from human clinical trials are inconsistent about the anti-obesogenic effects related to glucose and lipid metabolism, probably due to the various study designs and lengths, the forms of polyphenol supplementation used in the study, and subject inter-variability (e.g., BMI, microbial composition and intestinal enzymes) affecting pharmacokinetics and pharmacodynamics of dietary polyphenols. The underlying mechanisms by which dietary polyphenols exert health-promoting effects in humans are complex and remain to be elucidated. In this chapter, we put our research efforts into investigating the biological activities of mango-derived polyphenols in lean and obese individuals. Findings from this study can help explain the discrepancy in polyphenol-associated health benefits in human clinical trials.

[§] Reprinted with permission from Fang, C., Kim, H., Barnes, R. C., Talcott, S. T., & Mertens-Talcott, S. U. (2018). Obesity-Associated Diseases Biomarkers are Differently Modulated in Lean and Obese Individuals and Inversely Correlated to Plasma Polyphenolic Metabolites After 6 Weeks of Mango (Mangifera Indica L.) Consumption. *Molecular nutrition & food research*, 1800129. Copyright [2019] by John Wiley and Sons.

5.1 Introduction

Nearly 30% of the global adult population is considered to be overweight or obese (1). Obesity is associated with increased risk of developing a large number of medical conditions, including type 2 diabetes (T2D), hypertension, dyslipidemia and cardiovascular diseases (2). Excessive accumulation of fat in obesity has been proven to cause dysfunction of adipokines that are involved in energy homeostasis and metabolic functions, and can contribute to chronic low-grade inflammation and metabolic abnormalities in multiple tissues and organs (6). Dietary modifications, particularly increasing consumption of fruits and vegetables, are considered some of the most effective strategies in the prevention of obesity-related chronic diseases (22).

Mango (*Mangifera indica* L.) contains high content of phenolic compounds (e.g., gallic acid, gallotannin, galloyl glycosides, and flavonoids), showing high antioxidant potential (57, 58); this promotes the application of mango-derived polyphenols in the prevention of obesity-related chronic diseases. In preclinical studies, mango intake reduced adiposity and improved glucose tolerance, inflammatory cytokine expressions, and lipid profiles in rodents fed a high-fat diet (73, 74). An *in vitro* model using 3T3-L1 preadipocyte cells indicates that the inhibition of adipogenesis by mango extracts is most likely associated with the suppression of mitotic clonal expansion (68), a critical step in the initiation of adipocyte differentiation (128). In addition, human clinical trials have demonstrated that mango supplementation improves lipid profiles and antioxidant capacity in healthy individuals (78) and reduces blood glucose in obesity (198), as well as individuals with T2D (85). Natural phytochemicals sourced

from mango fruits and their byproducts, such as mangiferin (199), have been shown to reduce the triglycerides and free fatty acids levels, as well as increase high-density lipoprotein cholesterol (HDL) level in overweight individuals with hyperglycemia (81). These findings indicate that mango-derived polyphenols exert potent anti-inflammatory and anti-lipogenic effects that are relevant to the prevention of obesity-related chronic diseases. However, the underlying mechanisms by which polyphenols exert health-promoting effects in humans are complex and remain to be elucidated.

To further investigate the biological activities of dietary polyphenols in human health, it is necessary to understand that their bioavailability is influenced by multiple factors, such as dietary polyphenol exposure. Chronic, continuous exposure to dietary polyphenols contributes to a greater increase of plasma concentration in healthy individuals, as much as a 60% increase (200). However, this direct relationship between polyphenol administration and plasma production is not apparent in obese individuals (61). The mechanisms describing how obese individuals experience lower circulating levels of polyphenols, either parent compounds or metabolites, than lean individuals remain unclear. However, these findings indicate that different biological activities are associated with different systemic exposure to polyphenolic metabolites in lean and obese individuals.

Therefore, this study was designed to examine the effects of daily mango supplementation for 6 weeks on inflammation and metabolic functions in lean and obese individuals. Taking into account different baseline levels of plasma biomarkers in lean and obese individuals, we hypothesized that mango supplementation exerts health-

promoting benefits by differently modulating plasma levels of inflammatory cytokines and metabolic hormones in lean and obese individuals. Furthermore, the inconsistency in health benefits between lean and obese individuals might be correlated to the variance of the mango polyphenolic metabolite production.

5.2 Materials and Methods

5.2.1 Study Participants

Healthy lean (BMI 18-26.2 kg/m^2) and obese (BMI > 28.9 kg/m^2) individuals, aged 18-65 years old, were recruited in this study at the Vegetable and Fruit Improvement Center at Texas A&M University by university email list and flyers. Potential participants were screened for eligibility using an online survey prior to the initial screening. The exclusion criteria were: (1) obesity-related health conditions, such as high blood pressure, diabetes and heart disease; (2) insulin treatment; (3) pregnancy or lactation; (4) smoking more than 1 pack/week; (5) alcohol or substances abuse within the last 6 months, binge drinking (5 or more alcoholic drinks for males, or 4 or more alcoholic drinks for females); (6) allergy to mango fruit; (7) on blood thinner and aspirin. This intervention study was approved by the Institutional Review Board (2014-0802D) at Texas A&M University, and the protocol was registered at clinical.gov (NCT02227615). Eligible participants provided informed consent before the study initiation.

5.2.2 Study Design and Dietary Intervention

In this study, healthy lean and obese participants were assigned to receive daily 400 g of mango supplementation for 6 weeks. A daily dose of 400 g of mango pulp was selected based on our previous human clinical trial, where we observed that daily consumption of 400 g of mango pulp for 10 days resulted in significant increases in urinary excretion of mango-derived gallotannin metabolites (pyrogallol-O-sulfate and deoxypyrogallol-O-sulfate) (157). Based on these findings, we hypothesized that the observed significance in pharmacokinetics might translate into significant results for relevant pharmacodynamics endpoints investigated in this study. The variety Ataulfo was chosen due to the high content of gallotannins and gallotannin derivatives compared to other mango cultivars (201). After fully ripening, mangos were peeled and deseeded, then 400 g of pulp was vacuum-sealed and stored at -20ºC until use. The nutrient values of 400 g of mango pulp were based on USDA National Nutrient Database for Standard Reference Release 28. Participants were instructed to consume one bag of 400 g-mango pulp as part of their current, unmodified diet (breakfast) for 6 weeks on a daily basis. Three site visits were required by the study protocol: the initial screening, at Week 0, and at Week 6. Participants were fasted overnight. On study days, fasting blood samples were drawn. Afterwards, participants were instructed to consume 400 g-mango pulp, and post-prandial blood samples were collected at 0.5, 1, 1.5, 2, 3, 4, 6, and 8 hours by a registered study nurse. Participants were allowed to consume water and a standardized lunch (8 crackers and 326 g pasta Alfredo) after 4 hours of the first blood draw. This standardized lunch was based on an isocaloric diet that is low in

polyphenols. Blood samples were then centrifuged at 4,000 x g for 5 minutes, and the supernatant was collected and the amounts of metabolites from galloyl derivatives were analyzed by Liquid Chromatography–Mass Spectrometry (LC–MS) method for the pharmacokinetic evaluation (157, 202). The area under the curve (AUC) corresponding to each pharmacokinetic parameter was calculated. The remaining samples were stored at -80°C for further biochemical analysis.

5.2.3 Anthropometric Measurements and Blood Pressure

Following the initial screening interview, participants underwent anthropometric measurements at Week 0 and at Week 6 of the study, including height, body weight, BMI, pulse, body temperature, systolic blood pressure (SBP) and diastolic blood pressure (DBP). All measurements were conducted using standard methods after the participants fasted overnight. During each visit, participants were weighed on a flat surface with a Seca (Vogel & Halke, Hamburg, Germany) weighing scale and recorded to the nearest 0.1 kg. Height was measured without shoes using an Accustat Genentech Stadiometer (San Francisco, CA) and recorded to the nearest 0.1 cm. Body mass index (BMI) was calculated according to the formula: BMI = body weight / height^2 (kg/m^2). An INNOVO thermometer (INNOVO medical, Stafford, TX) was used to measure body temperature. Blood pressure was examined in millimeter mercury (mmHg) using Omron Automatic BP791IT (Omron Corporation, Kyoto, Japan).

5.2.4 Dietary Analysis

Participants were asked to maintain their dietary habits, except for the daily mango supplementation. Additionally, participants filled out a three-day food record twice: the three days prior to Week 0 and Week 6. These data were entered into iProfile 3.1 Nutrition Dietary Assessment software (Wiley, Hoboken, NJ) to analyze the intakes of total energy, carbohydrate, protein and fat. All data entry was performed by well-trained lab personnel.

5.2.5 Lipid Profiles

Plasma levels of total cholesterol and triglycerides were measured by Cayman quantification assay kits (Cayman, Ann Arbor, MI), and HDL and low-density lipoprotein cholesterol/very low-density lipoprotein cholesterol (LDL/VLDL) were measured by BioVision quantification kits (BioVision, Milpitas, CA) according to the manufacturer's protocol.

5.2.6 Inflammatory cytokines and metabolic hormones

Participants were fasted overnight. Plasma samples were collected on study days (Week 0 and Week 6) before mango consumption, and utilized to determine the levels of inflammatory cytokines, including tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), IL-6, IL-8, IL-10, interferon γ (IFNγ) and monocyte chemoattractant protein-1 (MCP-1); the cardiovascular biomarker C-reactive protein (CRP); and metabolic hormones, including ghrelin, resistin, peptide YY (PYY), plasminogen activator

inhibitor-1 (PAI-1), gastric inhibitory polypeptide (GIP), glucose, and insulin by multiplex bead assay (Millipore, Billerica, MA). Fasting (0 hour) and post-prandial (0.5, 1, 1.5, 2, 3, 4, 6, and 8 hours) blood samples after mango consumption were used to generate AUC for inflammatory cytokines (TNF-α, IL-10, IL-8 and MCP-1). These experiments were performed on a Luminex L200 machine (Luminex, Austin, TX) and data were analyzed by Luminex xPONENT software version 3.1. Glycated hemoglobin A1c (HbA1c) was measured in plasma using human HbA1c ELISA kit (Biotang, Inc, Lexington, CA). Plasma levels of glucose were determined by a colorimetric assay kit (Cayman). The values of Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) was calculated by the formula: fasting plasma insulin (μU/mL) x fasting plasma glucose (mmol/L)/22.5 (203).

5.2.7 Statistical Analysis

The data were analyzed by GraphPad Prism 6 (GraphPad Software, Lo Jolla, CA). Normality was checked by D'Agostino-Pearson normality test. If data were normally distributed, a paired *t* test was used to compare Week 6 to Week 0 measurements within lean or obese group. If data were not normally distributed, a Wilcoxon matched-pairs signed rank test was used. Baseline differences between lean and obese groups were assessed using a Student *t* test. The AUC for inflammatory cytokines was performed using the PkSolver Microsoft Excel Add-In (204). A Kruskal–Wallis test was used to compare the AUC of inflammatory cytokines in lean and obese groups. To determine the relationship between different variables, including the plasma

levels of mango polyphenolic metabolites versus plasma biomarkers and mango polyphenolic metabolites versus BMI, Spearman's correlation tests were performed. A correlation coefficient r (rho) of zero indicates no linear relationship, whereas -1 and +1 indicate perfect negative and positive relationships, respectively, between two variables. A p value less than or equal to 0.05 was considered statistically significant. All results were presented as means ± SD.

5.3 Results and Discussion

5.3.1 Anthropometric Measurements and Blood Pressure

Out of initial 23 participants, twelve lean (BMI 22.87 ± 2.22 kg/m^2, male= 9, female=3) and nine obese (BMI 34.60 ± 4.89 kg/m^2, male= 4, female=5) individuals completed this study, and two individuals dropped out due to scheduling difficulties. Eligible participants underwent anthropometric and blood pressure measurements on the first and last study days (Weeks 0 and 6, respectively). There was no statistically significant change in body weight, BMI, pulse and body temperature within lean or obese groups (**Table 1**). However, 6 weeks of mango supplementation lowered the SBP levels by more than 4 mmHg (119.83±13.16 vs. 115.42±12.33; p=0.05) in lean participants. Emerging evidence suggests that consumption of polyphenol-rich fruits protects against hypertension in both healthy and hypertensive individuals (205-208). Mango provides a high amount of tannins and other phenolic acids (57), which may exert anti-hypertensive effects after long-term supplementation. However, a previous study reported that continuous freeze-dried mango supplementation for 12 weeks

showed no improvement on both SBP and DBP in obese participants; any anti-hypertensive effects of mango may have been masked by the use of anti-hypertensive drugs in this study (198).

Table 1 Anthropometric characteristics and blood pressures of participants (Weeks 0 and 6). Reprinted with permission from *Molecular nutrition & food research*, 1800129. Copyright [2019] by John Wiley and Sons.

Variable	Group	Lean (n=12)			Obese (n=9)		
		Week 0	Week 6	p value	Week 0	Week 6	p value
Gender	Female	25.0%			55.6%		
	Male	75.0%			44.4%		
Age (years)	Mean	25.6			27.8		
	SD	(4.2)			(8.3)		
Height (cm)	Mean	172.6			168.3		
	SD	(6.0)			(12.1)		
Weight (kg)	Mean	68.2	68.6	0.70	98.5 (17.6)	98.1	0.31
	SD	(7.2)	(7.3)			(17.4)	
BMI (kg/m^2)	Mean	22.87	22.94	0.49	34.60	34.49	0.33
	SD	(2.22)	(2.39)		(4.89)	(4.58)	
Pulse (BPM)	Mean	71.33	66	0.08	77.78	75.67	0.39
	SD	(14.35)	(11.17)		(10.22)	(9.68)	
Body temperature (°C)	Mean	36.72	36.66	0.29	36.46	36.44	0.40
	SD	(0.32)	(0.39)		(0.32)	(0.27)	
Systolic blood pressure (mmHg)	Mean	119.83	115.42	0.05*	119.22	123	0.78
	SD	(13.16)	(12.33)		(16.93)	(13.36)	
Diastolic blood pressure (mmHg)	Mean	74.08	76.67	0.73	83.89	80.78	0.20
	SD	(12.75)	(7.80)		(11.05)	(6.06)	

*Asterisk indicates significant difference after 6 weeks of mango supplementation within each group. Paired *t* test if data were normally distributed or Wilcoxon matched-pairs signed rank test if data were not normally distributed, $p<0.05$.

Unlike the previous study, our study excluded participants with hypertension. Furthermore, no anti-hypertensive drugs were taken by participants in our study. Six weeks of mango supplementation significantly reduced SBP in lean participants, while no effect on blood pressure level of obese participants was observed.

5.3.2 Mango Supplementation and Dietary Intake

A serving of 400 g-mango pulp contains 240 kcal, 3.28 g protein, 1.52 g fat, 59.92 g carbohydrates (CHO), 6.40 g fibers, minerals and vitamins. Participants' intakes of total energy, CHO, fat and protein were evaluated before and after 6 weeks of mango supplementation using a three-day food record. Overall, at Week 0, obese participants had higher intakes of total energy, carbohydrate and fat compared to lean participants. In the lean group, total energy intake was significantly reduced from 1974.77±411.57 kcal to 1767.08±463.03 kcal ($p=0.02$) after 6 weeks. In comparison, obese participants had an increase in protein intake (86.42±40.60 g vs. 101.90±59.14 g; $p=0.05$), but no change in total energy intake after 6 weeks of mango supplementation **(Table 2)**.

It is widely accepted that obesity is a consequence of energy imbalance characterized by energy intake exceeding energy expenditure (209). In this study, no change in body weight was observed in either the lean or obese group (**Table 1**), while significant differences in macronutrient intakes were observed in three-day food record for both lean and obese groups. These findings need to be evaluated with care, due to the limited reliability of the three-day food record, as well as the misreporting occurred in self-reported dietary assessments (210).

Table 2 Dietary intake of macronutrients. Reprinted with permission from *Molecular nutrition & food research*, 1800129. Copyright [2019] by John Wiley and Sons.

Variable	Group	Lean (n=12)			Obese (n=9)		
		Week 0	Week 6	p value	Week 0	Week 6	p value
Energy (kcal)	Mean	1974.77	1767.08	0.02*	2313.49	2451.78	0.68
	SD	(411.57)	(463.03)		(918.99)	(785.88)	
Carbohydrates (g)	Mean	201.54	187.24	0.25	264.40	295.90	0.77
	SD	(46.18)	(44.83)		(96.08)	(47.46)	
Proteins (g)	Mean	88.69	80.58	0.19	86.42	101.90	0.05*
	SD	(21.66)	(26.30)		(40.60)	(59.14)	
Total fats (g)	Mean	82.43	85.12	0.28	99.38	92.57	0.32
	SD	(21.75)	(35.48)		(64.01)	(49.17)	

*Asterisk indicates significant difference after 6 weeks of mango supplementation within each group. Paired *t* test if data were normally distributed or Wilcoxon matched-pairs signed rank test if data were not normally distributed, $p<0.05$.

Table 3 Lipid profiles of participants (Weeks 0 and 6). Reprinted with permission from *Molecular nutrition & food research*, 1800129. Copyright [2019] by John Wiley and Sons.

Variable	Group	Lean (n=12)			Obese (n=9)		
		Week 0	Week 6	p value	Week 0	Week 6	p value
Biochemistry markers							
Total cholesterol (mM)	Mean	1.83	1.82	0.37	2.13	2.08	0.47
	SD	(0.35)	(0.42)		(0.39)	(0.50)	
Triglycerides (mg/dL)	Mean	77.03	89.60	0.08	125.87	118.66	0.67
	SD	(40.35)	(49.94)		(111.57)	(83.36)	
HDL (mg/dL)	Mean	37.53	32.46	0.36	34.88	32.49	0.59
	SD	(16.67)	(10.40)		(13.84)	(7.08)	
LDL/VLDL (mg/dL)	Mean	49.45	50.46	0.58	45.68	47.18	0.85
	SD	(7.04)	(10.43)		(8.78)	(7.60)	

HDL: high-density lipoprotein cholesterol; LDL: low-density lipoprotein cholesterol; VLDL: very low-density lipoprotein cholesterol.
*Asterisk indicates significant difference after 6 weeks of mango supplementation within each group. Paired *t* test if data were normally distributed or Wilcoxon matched-pairs signed rank test if data were not normally distributed, $p<0.05$.

5.3.3 Plasma Levels of Lipids, Inflammatory Cytokines and Metabolic Hormones

To identify the role of mango supplementation in dyslipidemia, lipid profiles (total cholesterol, triglycerides, HDL and LDL/VLDL) were analyzed before and after 6 weeks of mango supplementation. Baseline values of total cholesterol and triglycerides were slightly higher in the obese than the lean group (**Table 3**). Mango supplementation did not cause any significant changes in lipid levels for either study group. Expressions of biomarkers for inflammation and cardiovascular disease were compared before and after 6 weeks of mango supplementation. Significant differences were observed between the lean and obese groups at baseline in IL-10 (3.43±1.72 pg/mL vs. 1.83±0.92 pg/mL; p=0.02), CRP (2.96±4.32 mg/L vs. 17.73±19.37 mg/L; p=0.05), and resistin (20.59±5.01 ng/mL vs. 32.68±15.22 ng/mL; p=0.03) (**Table 4**). The plasma levels of inflammatory biomarkers and CRP were not significantly suppressed by mango supplementation **(Table 4)**. When plasma AUC of inflammatory cytokines (0-8 hours) were analyzed (**Figure 23**), IL-8 and MCP-1 were significantly decreased by 46% and 33%, respectively, in the obese group after 6 weeks, indicating improved acute inflammatory response in obesity after mango supplementation. Previous studies have shown the correlation of IL-8 and MCP-1 with the development of obesity-related diseases (211, 212). Lowered levels of IL-8 and MCP-1 in obese participants after mango supplementation may indicate lowered risk of developing obesity-related chronic diseases due to mango intake. Low levels of IL-10 are frequently observed in individuals with metabolic syndrome (213).

Table 4 Plasma levels of CRP and metabolic hormones of participants (Weeks 0 and 6). Reprinted with permission from *Molecular nutrition & food research*, 1800129. Copyright [2019] by John Wiley and Sons.

Variable	Group	Lean (n=12)			Obese (n=9)		
		Week 0	Week 6	p value	Week 0	Week 6	p value
Cardiovascular biomarker							
CRP (mg/L)	Mean SD	2.96 (4.32)	1.43 (1.16)	0.23	17.73 (19.37)	14.96 (15.82)	0.37
Hormone biomarkers							
Ghrelin (pg/mL)	Mean SD	8.94 (4.39)	9.70 (6.94)	0.77	7.43 (1.84)	7.86 (2.15)	0.46
Resistin (ng/mL)	Mean SD	20.59 (5.01)	19.69 (1.37)	0.35	32.68 (15.22)	33.75 (21.88)	0.63
PYY (pg/mL)	Mean SD	23.56 (15.58)	24.64 (12.38)	0.55	15.32 (10.46)	22.11 (9.05)	0.10
PAI-1 (ng/mL)	Mean SD	30.93 (18.12)	23.69 (17.58)	0.09	31.34 (8.09)	24.93 (12.20)	0.05*
GIP (pg/mL)	Mean SD	4.99 (4.11)	9.46 (6.37)	0.04*	10.82 (9.54)	9.87 (7.12)	0.42
Glucose (pg/mL)	Mean SD	73.16 (8.64)	72.06 (8.83)	0.26	77.57 (9.60)	80.51 (13.88)	0.73
Insulin (pg/mL)	Mean SD	318.93 (239.24)	303.33 (172.95)	0.58	485.94 (165.92)	506.29 (316.20)	0.50
HOMA-IR	Mean SD	1.76 (1.47)	1.63 (1.11)	0.56	2.71 (1.07)	3.13 (2.60)	0.58
HbA1c (mmol/mol)	Mean SD	12.28 (4.49)	11.65 (5.07)	0.62	17.53 (6.09)	14.35 (7.04)	0.06

*Asterisk indicates significant difference after 6 weeks of mango supplementation within each group. Paired *t* test if data were normally distributed or Wilcoxon matched-pairs signed rank test if data were not normally distributed, $p<0.05$.

In this study, as expected, plasma levels of IL-10 were higher in the lean compared to the obese group; mango supplementation induced a non-significant increase in IL-10 levels in both study groups (**Figure 23**). Elevated levels of CRP are considered to be independent predictors of cardiovascular diseases (214). In this study,

CRP levels were higher in the obese compared to the lean group; mango-induced decreases in this inflammation biomarker were non-significant after 6 weeks **(Table 4)**. Overall, no changes in AUC of inflammatory cytokines were observed in the lean group **(Figure 23)**. Taken together, these findings suggest that mango supplementation has the potential in modulating the inflammatory cytokine levels in obese individuals; this needs to be further confirmed in larger scale, longer-term clinical trials.

Obesity causes a number of metabolic changes, such as affecting the levels of metabolic hormones involved in insulin sensitivity, as well as appetite and glucose control (215). In this study, we observed a significant difference in HbA1c (12.28±4.49 mmol/mol vs. 17.53±6.09 mmol/mol; p=0.03) and a non-significant difference in insulin levels between lean and obese groups at baseline (318.93±239.24 pg/mL vs. 485.94±165.92 pg/mL; p=0.0893) **(Table 4**).

After 6 weeks of mango supplementation, GIP was increased by 47% (p=0.04) in lean participants, and HbA1c and PAI-1 were reduced by 18% (p=0.06) and 20% (p=0.05), respectively, in obese participants (**Table 4**). GIP is an incretin hormone released by enteroendocrine K-cells in response to nutrient stimulation (216). Conflicting results showed neither dietary modification nor weight loss had effects on fasting GIP levels (217). In this study, mango supplementation increased the fasting GIP levels in lean participants, but not obese participants. The role of GIP in metabolic functions in healthy lean individuals remains largely unknown.

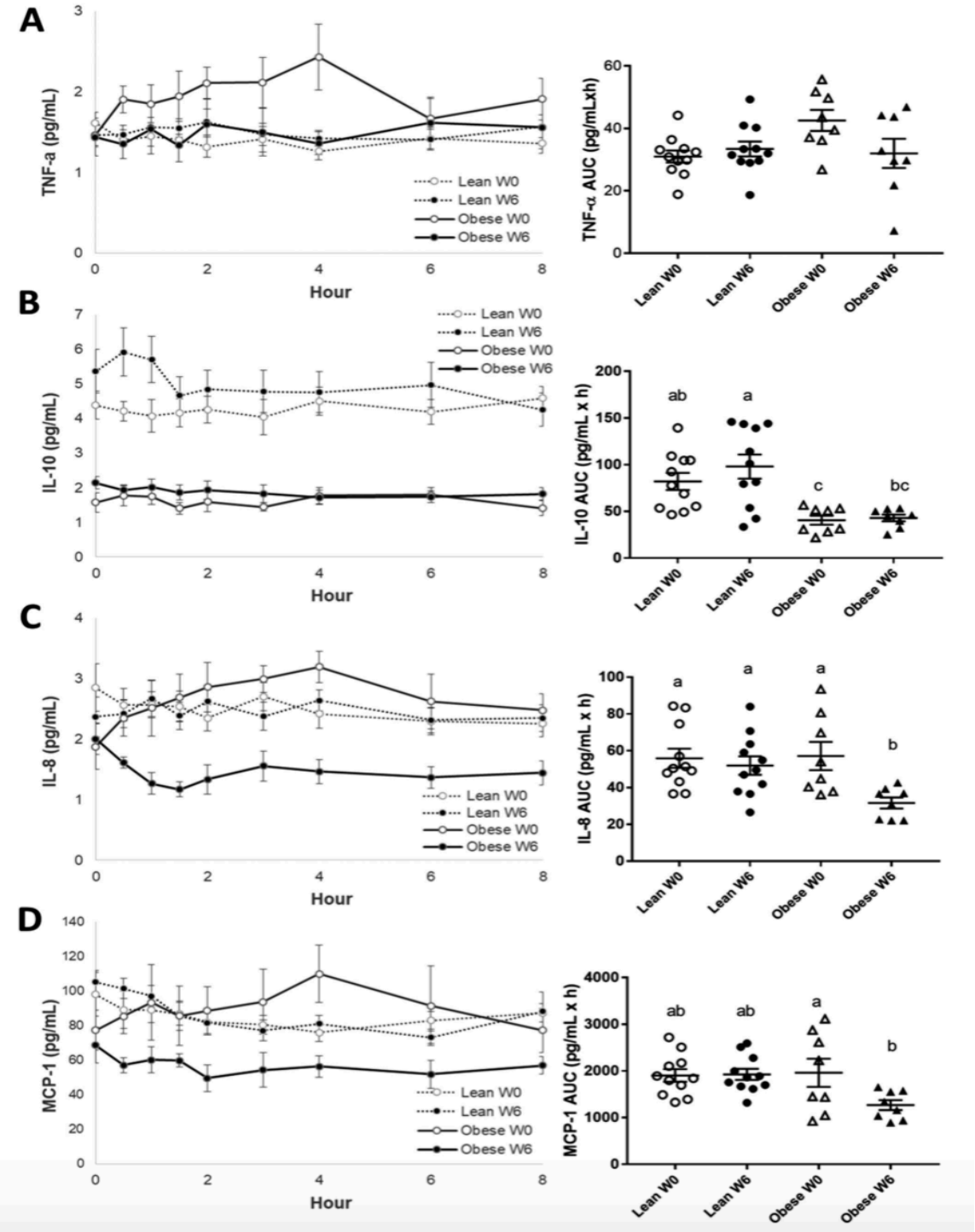

Figure 23 Plasma levels of inflammatory cytokines for eight hours after mango supplementation and area under the curve (AUC). Reprinted with permission from *Molecular nutrition & food research*, 1800129. Copyright [2019] by John Wiley and Sons.

PAI-1 is a metabolic hormone directly related to obesity (218). Circulating PAI-1 levels predict the development of insulin resistance, T2D, and atherosclerosis (219). In this study, mango reduced PAI-1 levels in the obese group after 6 weeks. PAI-1 was positively correlated with BMI in obese participants only ($r=0.5107$, $p=0.0450$) at baseline. Decreased PAI-1 levels might be associated with reduced fat accumulation and inflammation, thereby improving metabolic profiles in obese individuals (220). HbA1c is the gold standard in monitoring long-term (8-12 weeks) glycemic control (221). After 6 weeks of mango supplementation, HbA1c level were reduced by 18.3% ($p=0.06$) in the obese group, possibly indicating better long-term glucose homeostasis due to mango supplementation. In spite of the direct relationship between HbA1c and fasting blood glucose in this study ($r=0.4333$, $p=0.0041$) (data not shown), mango supplementation did not affect fasting plasma glucose levels in either group (**Table 4**). Previously, 12 weeks of freeze-dried mango supplementation lowered fasting blood glucose levels in obese individuals, but had no effects on HbA1c and insulin levels (198). Future clinical trials including participants with metabolic syndrome and diabetes may aid in the determination of the beneficial effects of mango supplementation on glucose homeostasis and insulin sensitivity.

5.3.4 Correlations of Plasma Concentrations of Polyphenolic Metabolites with BMI or Plasma Lipids, Inflammatory Cytokines and Metabolic Hormones

The amount of phenolic content in 400 g of mango pulp was quantified to be 259.4 mg of total galloyl derivatives, including 95.4 mg of non-tannin (3.64 mg gallic

acid and 91.7 mg monogallolyl glucose), and 164 mg gallotannins with different degree of polymerization. In LC-MS pharmacokinetic analysis, five galloly derivatives from mango were identified and quantified in plasma: 4-O-methylgallic acid, 4-O-methylgallic acid-3-O-sulfate, pyrogallol-O-sulfate, methylpyrogallol-O-sulfate and catechol-O-sulfate. It was worth noting that pyrogallol derivatives were not present in mango pulp, but detected in plasma as mango microbial metabolites. These five galloly derivatives were presented as correlation to functional biomarkers. Potential biological activity of mango-derived gallotannin metabolites was investigated using Spearman's correlation tests to examine the correlations of plasma polyphenolic metabolites with BMI, or plasma lipids, inflammatory cytokines and metabolic biomarkers in lean and obese participants (**Figure 24 and 25**). In lean participants, plasma concentrations of the sum of polyphenolic metabolite ($p=0.0607$, $r=-0.3969$), as well as catechol sulfate alone ($p=0.09$, $r=-0.3520$), were inversely correlated to BMI (**Figure 24A and B**). These findings suggest that increased BMI is associated with lower polyphenol absorption. Conversely, no such relationship was identified in obese participants. The level of total cholesterol was inversely associated with the production of catechol sulfate in lean ($p=0.0090$, $r=-0.5316$) but not obese participants (**Figure 24C**). IL-10 was correlated with catechol sulfate in both lean ($p=0.0149$, $r=0.5007$) and obese ($p=0.0540$, $r=0.4613$) groups, indicating the potential anti-inflammatory activity of catechol sulfate (**Figure 24D and 25A**). PYY was correlated to the sum of polyphenolic metabolites ($p=0.0324$, $r=0.5054$) in obese participants (**Figure 25B**). PYY is a satiety hormone that responds to nutrient intake. Some evidence showed low fasting PYY levels in obese individuals

compared to the healthy lean controls (222). PYY is involved in energy expenditure and lipid metabolism, thus contributing to long-term regulation of body weight (223). In obese participants, plasma levels of PAI-1 and HbA1c were inversely correlated with 4-O-methylgallic acid and methylpyrogallolsulfate, respectively (p=0.0223, r=-0.5687; p=0.0955, r=-0.4050) (**Figure 25C,D**).

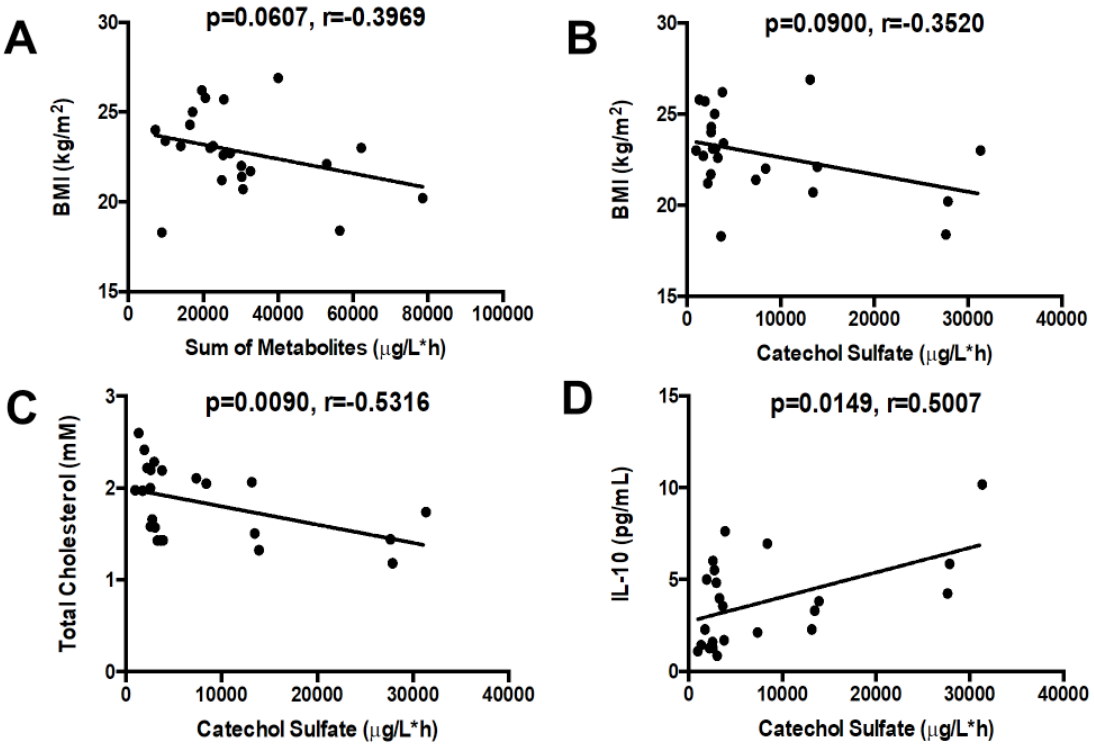

Figure 24 Correlation of pharmacokinetics of polyphenolic metabolites with BMI or plasma biomarkers in lean participants. Reprinted with permission from *Molecular nutrition & food research*, 1800129. Copyright [2019] by John Wiley and Sons.

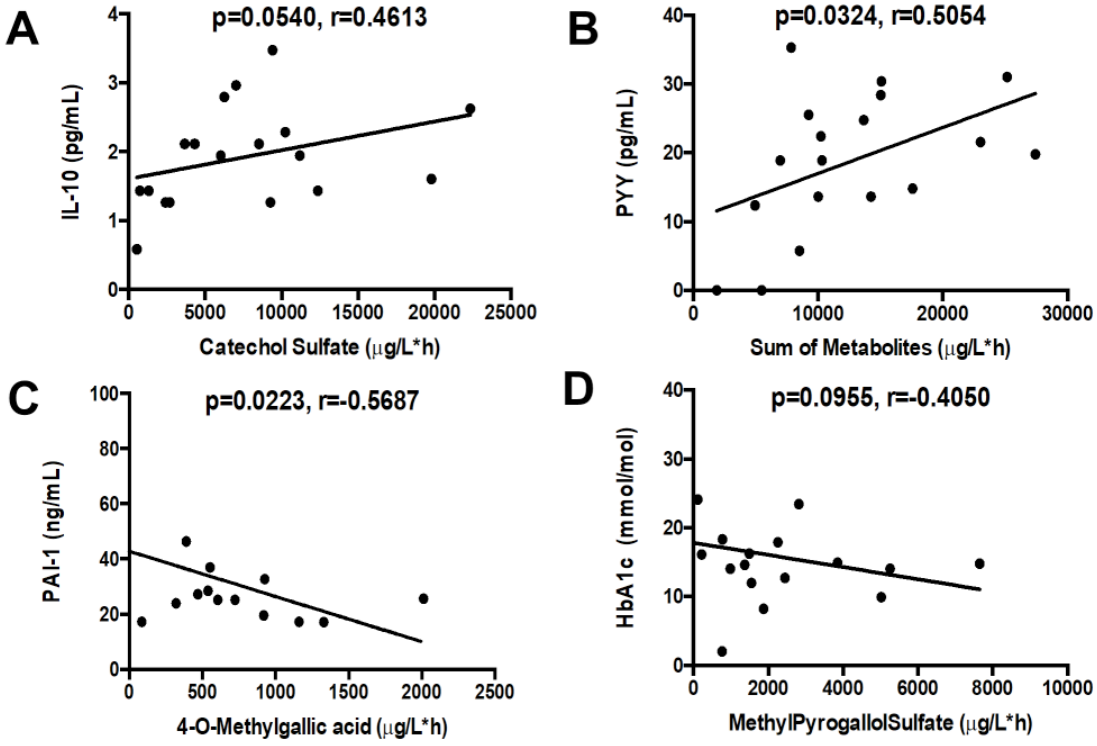

Figure 25 Correlation of pharmacokinetics of polyphenolic metabolites with plasma biomarkers in obese participants. Reprinted with permission from *Molecular nutrition & food research*, 1800129. Copyright [2019] by John Wiley and Sons.

Previously, four weeks repeated consumption of black tea polyphenol significantly increased urinary excretion of 4-O-methylgallic acid, but had no effect on the plasma levels of PAI-1 in healthy individuals (BMI 27.0 ± 0.6 kg/m^2) (224). Other studies indicate tannic acid inhibits PAI-1 expression by blocking the complex formation between PAI-1 and a protease (225). Gallic acid, the parent compound of 4-O-methylgallic acid and methylpyrogallolsulfate, has been shown to attenuate high-fat diet-induced diabetic nephropathy, partly by reducing the HbA1c levels in type 2 diabetic rats. A higher oral dose of gallic acid (50 mg/kg body weight) exhibited

stronger inhibitory effect on HbA1c levels compared to a lower dose (25 mg/kg body weight) (226). Higher concentration of mango polyphenolic metabolites might be associated with reduced appetite in obese individuals; however, this was not reflected in the three-day food record in this study.

In summary, daily mango supplementation for 6 weeks lowered blood pressure in lean participants, and benefits obese participants mainly by decreasing inflammatory cytokines (IL-8 and MCP-1), PAI-1 and HbA1c levels. Furthermore, the health-promoting effects of mango supplementation may be clearly present in a longer-term intake study. This study is the first to correlate the systemic exposure to polyphenolic metabolites from gallotannins to biological activities. The inter-individual variability in lean and obese individuals, specifically the lower exposure to polyphenolic metabolites may explain the limited response of functional biomarkers. In lean individuals, functional biomarkers are largely within normal ranges and not subject to diet-induced improvements (227, 228). Before performing the study in both lean and obese individuals, it has not been previously reported that the systemic exposure to mango polyphenols is lower in obese compared to lean individuals, which might have affected the significance of pharmacodynamics endpoints in obese individuals. This pilot clinical study should be followed by longer-term investigations with larger study cohorts including a control group consuming a placebo diet containing the same amount of vitamins and fibers as mango. The role of polyphenol bioavailability in its derived health benefits remains to be investigated. Results from this study enhance the

comprehension of potential health benefits of mango supplementation in lean and obese individuals and form a basis for future studies.

5.4 Conclusions

These findings support the central hypothesis that 6 weeks of daily mango supplementation exerts beneficial effects in lean and obese individuals, primarily by lowering blood pressure in lean individuals and decreasing inflammatory cytokines (IL-8 and MCP-1), PAI-1 and HbA1c levels in obese individuals, thus lowering the risk of developing obesity-related chronic diseases. The potential of mango-derived polyphenols to beneficially impact inflammation and metabolic functions is possibly correlated to the systemic exposure to mango-derived gallotannin metabolites, suggesting mango supplementation as a viable, preventive approach in metabolic disorders and other obesity-related chronic diseases.

CHAPTER VI

ACTIONS AND MOLECULAR MECHANISMS OF MICROBIAL METABOLITES OF GALLOTANNINS IN RAW 264.7 MACROPHAGES[**]

Adipose tissue macrophages are the major cell population in adipose tissue localized in the "crown-like structures" and "fat-associated lymphoid clusters". Macrophages infiltrate into adipose tissue and are responsible for the secretion of inflammatory cytokines and adipokines, which may exacerbate the progression of obesity-induced insulin resistance and other chronic conditions (229).

Murine macrophage cell line, RAW 264.7 cell line, originated from Abelson leukemia virus transformed cell line from BALB/c mice, is one of the most commonly used *in vitro* models in the study of inflammation and oxidative stress (230). Lipopolysaccharides (LPS) stimulation of RAW 264.7 macrophages induces transcription genes that encode for mediators of the inflammatory response, leading to the release of pro-inflammatory cytokines (e.g., TNF-α, IL-6, IL-1β), nitric oxide (NO) and reactive oxygen species (ROS) (231). This has been served as a well-established model to investigate the anti-inflammatory activity of dietary polyphenols and their underlying molecular mechanisms (232-235).

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Dietary polyphenols serve as potent antioxidants and anti-inflammatory agents, which enables them to protect human cells against damages due to chronic inflammation and oxidative stress in obesity leading to oxidation of lipids, nucleic acids and protein in the pathophysiological development of chronic diseases such as insulin resistance, cardiovascular disease and cancers (32). Previously, our studies have shown that the biological activities of gallotannin derivatives from mango in lowering the risk of developing obesity, insulin resistance and several types of cancer are mostly attributed to the production of the microbial metabolites, such as gallic acid (GA) and pyrogallol (PG) (96, 128, 161). However, the signaling signatures of these microbial metabolites in adipose tissue macrophages remain to be elucidated. Therefore in this chapter, the actions and molecular mechanisms of microbial metabolites of gallotannins in RAW 264.7 macrophages are investigated and discussed.

6.1 Introduction

Obesity is a leading and growing health concern in the United States. It was estimated that in 2016, nearly 18% of children and 39% of adults in the United States are obese, making obesity rates in the United States one of the highest in the world (236). Obesity-associated low-grade inflammation leads to many chronic diseases such as type 2 diabetes, cardiovascular diseases and cancers. Control of inflammatory response would have profound effects on insulin resistance, and is a therapeutic strategy for chronic diseases. Inflammatory response is a highly regulated process in which adipose tissue immune cells are involved in initiation, maintenance and resolution (237).

Adipose tissue macrophages (ATMs) are the major cell population in adipose tissue, localized in the "crown-like structures" and "fat-associated lymphoid clusters", constituting up to 40% in obese mice. ATMs infiltrate into adipose tissue and secrete monocyte chemotactic protein-1 (MCP-1), IL-6 and inducible nitric oxide synthase (iNOS), which exacerbates the progression of obesity-induced insulin resistance (238, 239).

AMP-activated protein kinase (AMPK) is an important metabolic sensor in the regulation of LPS-induced inflammation in macrophages. Macrophages AMPK activity is increased upon stimulation with IL-10 and decreased upon stimulation with lipopolysaccharides (LPS) (90). Activation of AMPK activity in macrophages is associated with increased sirtuin1 (Sirt1) and decreased LPS- or FFA-induced NF-κb activation (92); it helps maintain mitochondrial function and reduce inflammation and oxidative stress. This evidence indicates the anti-inflammatory effects of AMPK in macrophages. Activators of AMPK, including metformin, TZDs, glucagon-like peptide-1 agonists, resveratrol, and adiponectin are considered potential therapeutic agents in obesity and insulin resistance (240), which shed lights on the use of gallotannin derivatives from mango, for example, GA and PG as anti-obesogenic and anti-diabetic agents.

Polyphenols are secondary metabolites from fruits and vegetables that have demonstrated potent antioxidant and anti-inflammatory activities (170) through the mechanisms of hydrogen atom transfer, single electron transfer, and transition metals chelation (33). Previously, some dietary polyphenols (e.g., resveratrol, curcumin,

genistein and berberine) exert anti-inflammatory activity in macrophages through the regulation of the AMPK-NF-κb axis (94). The microbial metabolites of gallotannins, including GA and PG have shown health-promoting effects in reducing the risk of obesity, inflammation and cancers through the activation of the AMPK pathway. The administration of GA in obese mice reduced high-fat diet-induced weight gain and improved glucose homeostasis via targeting the activation of the AMPK/Sirt1/PGC1α signaling pathway in the liver, muscle, and interscapular brown adipose tissue (75). Additional *in vitro* and *in vivo* mechanistic studies have also shown the similar findings that GA exerts health benefits via the activation of the AMPK pathway (95). PG has demonstrated potent anti-obesogenic activities through mediating the AMPK/C/EBPα/PPARγ and AMPK/UCP1/Sirt1 axes in 3T3-L1 adipocytes, thus suppressing adipogenesis and promoting the brown remodeling of adipocytes (128). In a DSS-induced colitis rat model, mango beverage intake exert beneficial effects in mitigating inflammation in colitis, at least in part, through the production of PG that modulates the histone deacetylases 1 (HDAC1)/AMPK/ microtubule-associated protein light chain 3 (LC3) axis and induced autophagy, contributing to improved intestinal health (96). PG was also shown to decrease breast cancer cell proliferation possibly through the up-regulation of the AMPK pathway and the down-regulation of the AKT/mTOR pathway. An *in silico* docking modeling further indicates that PG can bind to the allosteric site of AMPK, thus inducing the activation of AMPK (97).

Given the pivotal role macrophages play in regulating inflammatory response, the investigation of dietary polyphenols and the potential relationship with the AMPK

pathway in macrophages has gained intense research attention. In this study, the actions and molecular mechanisms of the microbial metabolites of gallotannins, including GA and PG in RAW 264.7 macrophages were investigated. It was hypothesized that GA and PG reduce LPS-induced inflammation and oxidative stress in RAW 264.7 macrophages by mediating the AMPK signaling pathway. Overall, this study may contribute to increasing the physiological and nutritional significance of microbial metabolites of gallotannins as anti-obesogenic and anti-diabetic agents.

6.2 Materials and Methods

6.2.1 Cell Culture and Reagents

RAW 264.7 macrophages were purchased from American Type Culture Collection (ATCC, Rockville, MD), cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S, Life Technologies, Gaithersburg, MD), and were incubated at 37 °C in a humidified 5% CO_2 atmosphere. GA, PG and LPS were purchased from Sigma-Aldrich (St. Louis, MO). The concentration of GA and PG was expressed in mg/L (241).

6.2.2 Cell Viability of RAW 264.7 Macrophages

RAW 264.7 macrophages were seeded at a density of 1×10^4 cells/well in a 96-well, black-bottom plate and were treated with GA (1.5 -20 mg/L), PG (1.5 -20 mg/L), LPS (62.5-1000 ng/mL), or a vehicle control for 48 hours. Afterwards, cell viability was

determined by 10% Resazurin assay (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's protocol. Fluorescence intensity at 560 nm excitation and 590 nm emission was measured using a microplate reader (BMG Labtech Inc., Durham, NC). The cell viability is shown as the percentage of vehicle-treated control after background adjustment (241).

6.2.3 Effects of GA and PG on ROS Generation

RAW 264.7 macrophages were seeded at a density of 1×10^4 cells/well in a 96-well, black-bottom plate and were pre-treated with GA (1.25-5 mg/L), PG (1.25-5 mg/L), or a vehicle control for 1 hour. Cells were then incubated with 1 μg/mL LPS along with the previous treatments for another 23 hours. Afterwards, cells were washed with phosphate buffered saline (PBS) and stained with 10 μM 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Sigma-Aldrich, St. Louis, MO) in no-phenol red DMEM (Life Technologies, Gaithersburg, MD) for 30 minutes. Fluorescence intensity was measured at 485 nm excitation and 520 nm emission using a microplate reader. ROS generation is shown as the percentage of vehicle-treated control after background adjustment (242).

6.2.4 Gene Expression

Cells were seeded at a density of 1.5×10^5 cells/well in a 12-well plate and were pre-treated with GA (2.5 and 5 mg/L), PG (2.5 and 5 mg/L), or a vehicle control for 1 hour. Cells were then incubated with 1 μg/mL LPS along with the previous treatments for another 4 hours. Total RNA was isolated using a using an RNeasy Mini Kit (Qiagen,

Valencia, CA) according to the manufacturer's protocol. The concentration of the extracted RNA was determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). cDNA was synthesized using a Reverse Transcription Kit (Bio-Rad, Hercules, CA). The mRNA expression levels of IL-1β, IL-6, TNF-α, MCP-1, CD163, AMPKα1, and β-actin were analyzed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) in a CFX384 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Each reaction was performed in triplicate, and data were normalized to β- actin as an endogenous control.

6.2.5 Protein Expression

Cells were seeded at a density of 3×10^5 cells/well in a 6-well plate and were pre-treated with GA (2.5 and 5 mg/L), PG (2.5 and 5 mg/L), or a vehicle control for 1 hour. Cells were then incubated with 1 μg/mL LPS along with the previous treatments for another 23 hours, and the supernatants were collected and centrifuged to remove cell debris. The levels of inflammatory cytokines, including IL-1β, IL-6, TNF-α, and MCP-1 were determined using a multiplex bead assay (Millipore, Billerica, MA). These experiments were performed on a Luminex L200 machine (Luminex, Austin, TX) and data were analyzed by Luminex xPONENT software version 3.1 (121). In a separate experiment, cells were pre-treated with GA (2.5 and 5 mg/L), PG (2.5 and 5 mg/L), or a vehicle control for 1 hour and stimulated with 1 μg/mL LPS along with the previous treatments for another 1 hour. Cell lysates were harvested and cellular protein was extracted using a Pierce RIPA buffer (Pierce, Rockford, IL) containing 1% Halt

protease and phosphatase inhibitor cocktail (Thermo Scientific). Western Blot was performed with primary antibodies against phosphorylated AMPKα1 (p-AMPKα1), total-AMPKα1 (AMPKα1), Sirtuin1 (Sirt1), phosphorylated NF-κb (p-NF-κb), NF-κb, and β-actin (Cell Signaling Technology, Danvers, MA). The band intensity was analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA; http://rsb.info.nih.gov/ij/) (128).

6.2.6 Statistical Analyses

Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, Lo Jolla, CA). Results are presented as means ± SEM. Comparison of means between different groups was analyzed by *t*-test for two groups or Dunnett's test for multiple groups. A p value less than or equal to 0.05 indicates statistical significance.

6.3 Results and Discussion

6.3.1 Effects of GA and PG on Cell Viability

Cell viability assay showed that GA at 20 mg/L and PG at 10 mg/L reduced the viability of RAW 264.7 macrophages by nearly 89% and 8%, respectively (**Figure 26**). Results did not show any significant difference in RAW 264.6 macrophages when treated with LPS within the tested concentration range of 62.5-1000 ng/mL (**Figure 26**). Therefore, further analysis was performed with 2.5 mg/L and 5 mg/L for both GA and PG and 1 μg/mL for LPS.

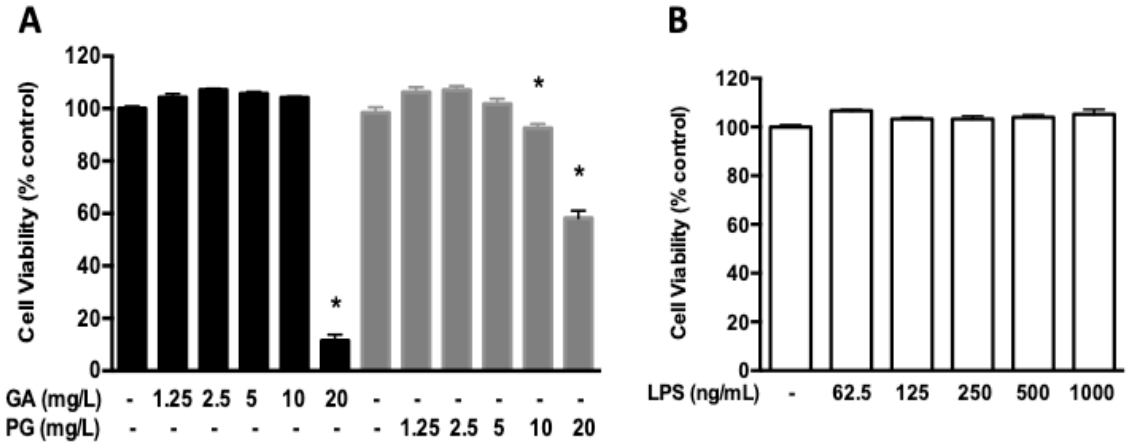

Figure 26 Cell viability in RAW 264.7 macrophages treated with GA, PG and LPS.

6.3.2 GA and PG Reduced LPS-induced ROS Generation and Inflammation

The production of ROS, known as the oxidative stress, is involved in the pathological processes such as obesity, insulin resistance and cardiovascular disease (243). Inflammation is considered a manifestation of increased oxidative stress. Elevated secretion of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6 from macrophages induces the production of ROS; this in turn damages the cellular structures and antioxidant properties and consequently leads to the development of obesity-related chronic diseases (244).

Previously, mango-derived polyphenols containing gallotannins and GA have shown to reduce inflammation in rats with DSS-induced colitis (144). Additionally, these polyphenols inhibited ROS generation and inflammation in both TNF-α-induced

non-cancer MCF-12A and MDA-MB231 breast cancer cells (245). The anti-inflammatory activities are associated with the down-regulation of the NF-κb signaling.

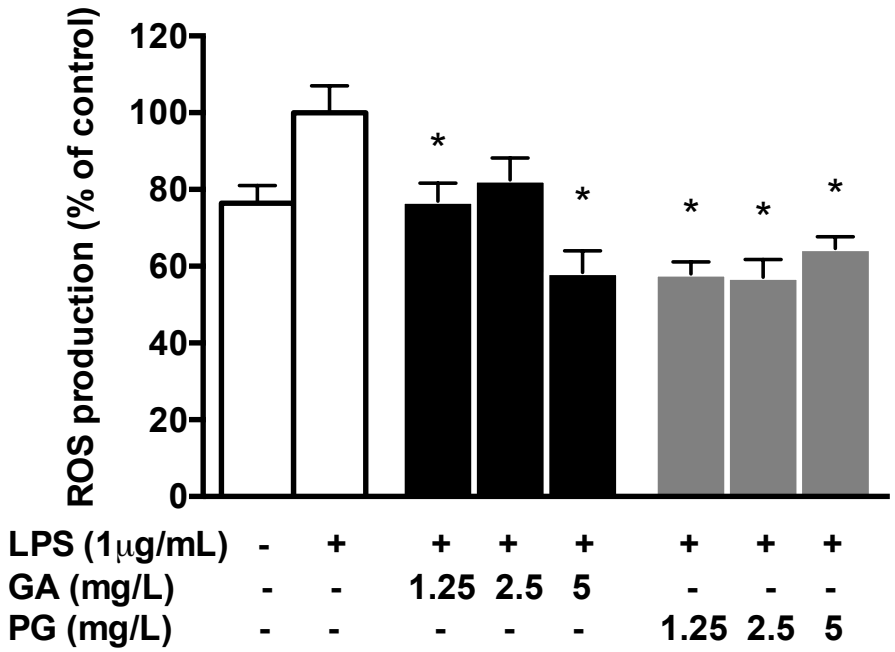

Figure 27 ROS generation in RAW 264.7 macrophages treated with GA and PG.

However, the actions and mechanisms of the bioactive compounds from mango-derived polyphenols, predominantly GA and PG in mediating inflammatory response remain largely unknown. In this study, results showed that GA and PG prevented LPS-induced ROS generation by up to 42% and 43%, respectively **(Figure 27).** LPS treatment alone dramatically increased the mRNA and protein expressions of pro-inflammatory cytokines including TNF-α, IL-1β, and MCP-1. Both GA and PG

treatments reduced the mRNA and protein expressions of TNF-α and IL-1β. The mRNA expression of CD163, an anti-inflammatory macrophage marker was significantly decreased by LPS whereas PG effectively reversed this inhibitory effect by LPS **(Figure 28)**. PG additionally decreased the protein expression of IL-6 and MCP-1 induced by LPS treatment **(Figure 29)**.

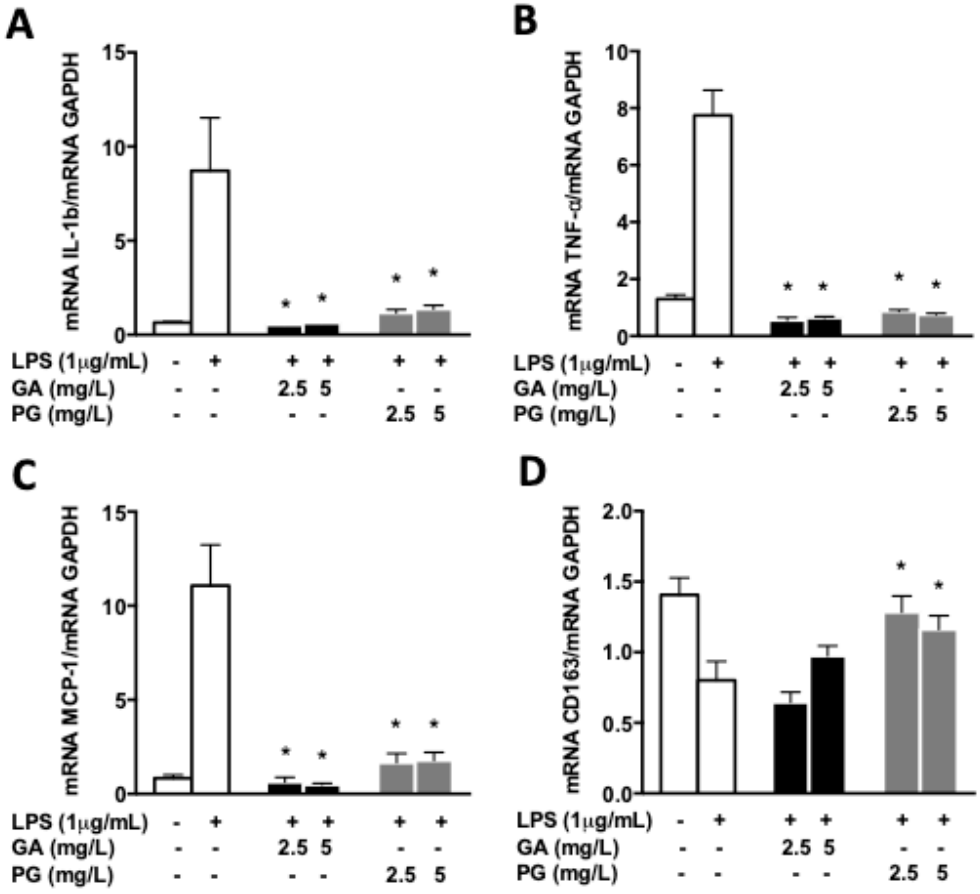

Figure 28 mRNA expressions of inflammatory cytokines in LPS-stimulated RAW 264.7 macrophages.

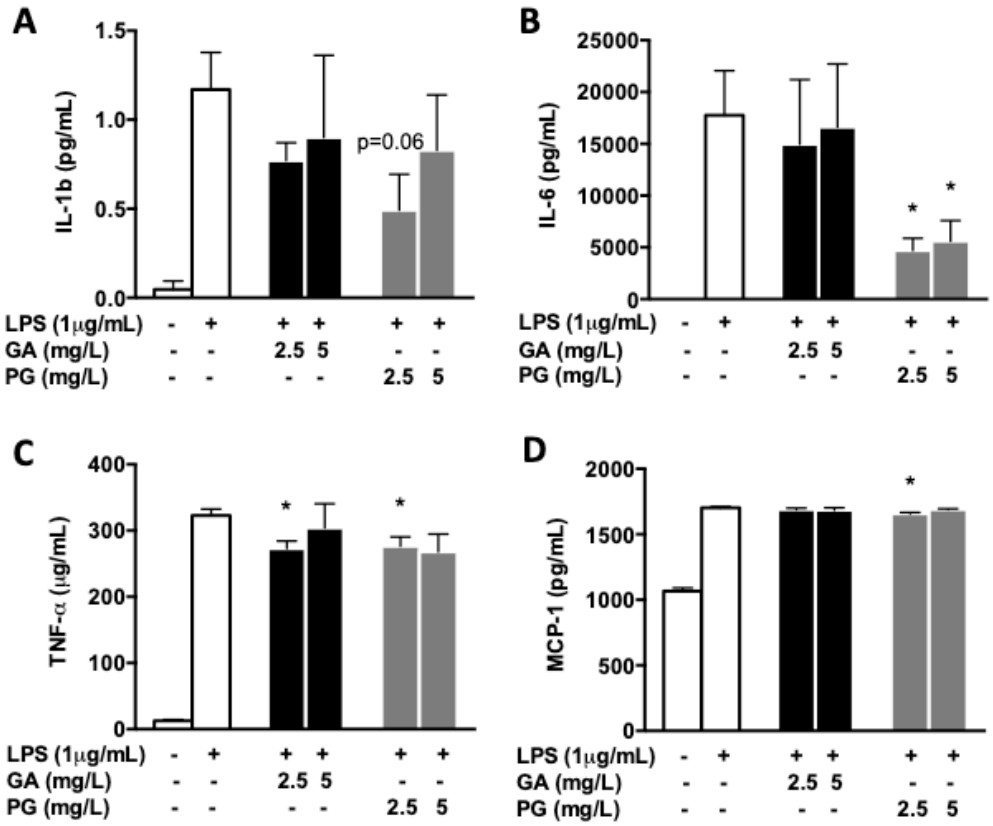

Figure 29 Protein expressions of inflammatory cytokines in LPS-stimulated RAW 264.7 macrophages.

6.3.3 GA and PG modulated the AMPK and NF-κb signaling pathway

Activation of AMPK activity in macrophages is associated with increased sirtuin1 (Sirt1) expression and decreased LPS- or FFA-induced NF-κb activation (92), which indicates the anti-inflammatory effects of AMPK pathway in macrophages. Previous *in vitro* and *in vivo* mechanistic studies have also shown that GA and PG exert health-promoting effects in reducing the risk of obesity, inflammation and cancers through the activation of the AMPK pathway. GA preferably mediated the

AMPK/Sirt1/PGC1α axis in the brown adipose tissue of diet-induced obese mice (75) while PG mediated the AMPK/C/EBPα/PPARγ and AMPK/UCP1/Sirt1 axes in 3T3-L1 adipocytes (128) and the histone deacetylases 1 (HDAC1)/AMPK/ microtubule-associated protein light chain 3 (LC3) axis in CCD-18Co colon cells. An *in silico* docking modeling further indicates that pyrogallol can bind to the allosteric site of AMPK, thus inducing the activation of AMPK (97). Together, this evidence suggests that GA and PG may possess potential anti-inflammatory and anti-obesogenic activities in macrophages through mediating molecules involved in the AMPK signaling pathway.

To investigate the mechanisms underlying the anti-inflammatory effects of GA and PG, we assessed the mRNA and protein expressions of molecules involved in the AMPK pathway that have shown to play key roles in mediating inflammatory response. GA and PG (5 mg/L) increased the gene expressions of AMPK 2.2-fold and 3.2-fold, respectively **(Figure 30A)**, along with up-regulated activity of AMPK and down-regulated NF-κb in LPS-stimulated RAW 264.7 macrophages **(Figure 30B-D)**. GA and PG reversed the inhibitory effect of LPS on the protein expression of Sirt1 **(Figure 30E)**. Collectively, these findings showed that both GA and PG reduced LPS-induced inflammation and oxidative stress, at least in part, via mediating the AMPK-Sirt1-NF-κb axis in RAW 264.7 macrophages. To test this hypothesis, future experiments are suggested to perform in RAW 264.7 macrophages pre-treated with or without the addition Compound C, an AMPK inhibitor, and stimulated with 1 μg/mL LPS along with the previous treatments. Molecules involved in the AMPK-Sirt1-NF-κb will be examined as described above.

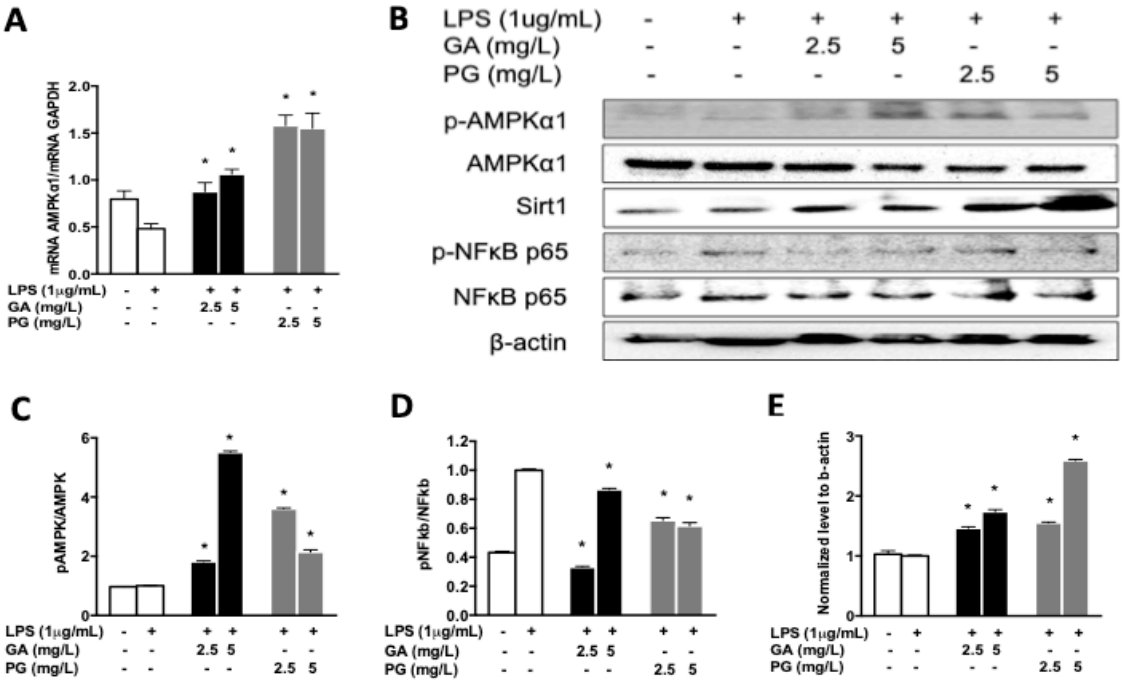

Figure 30 GA and PG modulated NF-κb and AMPK signaling pathway in LPS-stimulated RAW 264.7 macrophages.

6.4 Conclusions

Overall, this study demonstrates that the microbial metabolites of gallotannins from mango, including GA and PG, exert beneficial effects in reducing LPS-induced inflammation and oxidative stress, which are due, at least in part, to mediating the AMPK-Sirt1-NF-κb axis in RAW 264.7 macrophages. More comprehensive *in vitro* and *in vivo* studies are needed to fully elucidate the role and the molecular mechanisms of GA and PG in diet-induced obesity, inflammation and other related chronic conditions. It would be of great importance to compare the safety and bioefficacy of the two microbial metabolites (i.e., GA and PG) in the prevention and treatment of chronic

diseases, which may provide valuable knowledge in the development of innovative drugs and their clinical use.

CHAPTER VII

CONCLUSIONS AND CONSIDERATIONS FOR FUTURE RESEARCH

7.1 Summary and Conclusions

Obesity is an escalating global epidemic associated with increased risk of developing type 2 diabetes (T2D), hypertension and cardiovascular diseases. Excessive fat accumulation can cause dysfunction of adipokines involved in energy homeostasis and metabolic functions, contributing to chronic low-grade inflammation and insulin resistance in multiple tissues and organs. Dietary modifications, particularly increasing consumption of polyphenol-rich foods, are considered some of the most effective strategies in the prevention of obesity-related chronic diseases.

Emerging evidence demonstrates the two-way relationship between dietary polyphenols and the composition of the intestinal microbiota where an increased intake of polyphenols may shape the composition of the intestinal microbiota by increasing species with the ability to metabolize polyphenols. Pharmacokinetics of dietary polyphenols is influenced by gut microbiota, where inter-individual microbial biodiversity may translate into great variability of associated pharmacodynamics endpoints. Mango (*Mangifera indica* L.) contains high content of phenolic compounds (e.g., gallic acid (GA), gallotannins (GT), and galloyl glycosides), showing anti-inflammatory and anti-obesogenic potential in chronic diseases. Particularly, *Lactobacillus plantarum* (*L.plantarum)* possesses enzymatic activities to degrade GT into GA and pyrogallol (PG), allowing for absorption and excretion. Thus, the

interaction between gut microbiota and GT derivatives may affect the subsequent biological activities exerted by the microbial metabolites.

For this reason, this project targets further mechanistic and translational investigation via the following Specific Aims:

- Specific Aim 1. Determine the actions and molecular mechanisms of mango-derived GT metabolites in 3T3-L1 adipocytes.
- Specific Aim 2. Investigate the extent that health benefits of mango-derived GT metabolites are associated with intestinal *L. plantarum* in high-fat diet (HFD)-induced obese gnotobiotic mice.
- Specific Aim 3. Investigate the influence of daily mango supplementation for 6 weeks on inflammation and metabolic functions in lean and obese individuals.
- Specific Aim 4. Determine the actions and molecular mechanisms of microbial metabolites of GT in RAW 264.7 macrophages.

Based on findings from our *in vitro*, *in vivo* mechanistic studies and human clinical trial, we demonstrate that both mango polyphenols and PG possesses significant anti-obesogenic potential in 3T3-L1 adipocytes through the inhibition of adipogenesis while PG additionally promotes thermogenesis. The regulation of lipid metabolism by PG-induced AMPK activation is via the adipogenic signaling cascade LKB1-AMPKα1-C/EBPα/PPARγ/FAS/FABP4 and the thermogenic signaling cascade LKB1-AMPKα1-UCP1/Sirt1. Additionally, mango polyphenols and PG exerted antioxidant property by reducing oxidative stress in 3T3-L1 adipocytes. Mango polyphenols extract is primarily

comprised of monogalloyl glucoside, GA and GT. It suppresses adipogenesis partly through the modulation of the AMPK pathway without any involvement of PG. Therefore, the anti-obesogenic activities of mango polyphenols extract may be attributed to mango-derived polyphenols or combined activities of several polyphenols.

The central microbial metabolites of GT derivatives from mango (e.g., GA and PG) are considered the bioactive compounds in the anti-inflammatory, anti-obesogenic and anti-cancer activities. As a result, the application of *L.plantarum* in degrading large, unabsorbable compounds into small, absorbable compounds may enhance the health-promoting effects derived from GT-rich food in reducing obesity and its related chronic diseases. The use of a germ-free (GF) mouse model provides an invaluable tool to understand not only the interaction between *L.plantarum* and GT, but also the subsequent effects on diet-induced obesity exerted by the microbial metabolites. In this research project, the consumption of GT in addition to a HFD in GF mice modulates obesity-associated biomarkers by inhibiting fat synthesis in white adipose tissue and promoting thermogenesis in brown adipose tissue. Therefore, GT treatment alone without the addition of *L.plantarum* demonstrates beneficial effects on modulating inflammatory responses and adipose tissue functions. In addition to the GT treatment, colonization with *L.plantarum* significantly improved biomarkers for inflammation and insulin resistance. These findings provide initial evidence of the beneficial role of probiotics in context with a polyphenol-rich diet. This study has the potential to link the biological activities of dietary polyphenols to gut microbial composition and provide

novel insights into dietary recommendations that include probiotics into our diet to increase bioavailability and bioefficacy of dietary polyphenols.

Findings from *in vitro* and *in vivo* mechanistic studies were further investigated in a human clinical trial designed to study the effects of daily mango supplementation for 6 weeks on inflammation and metabolic functions in lean and obese individuals. In summary, daily mango supplementation for 6 weeks lowers blood pressure in lean participants and benefits obese participants mainly by decreasing inflammatory cytokines (IL-8 and MCP-1), PAI-1 and HbA1c levels. The potential of mango-derived polyphenols to beneficially impact inflammation and metabolic functions is possibly correlated to the systemic exposure to mango-derived GT metabolites, suggesting mango supplementation as a viable, preventive approach in metabolic disorders and other obesity-related chronic diseases. The inter-individual variability in lean and obese individuals, specifically the lower exposure to polyphenolic metabolites, may explain the limited response of functional biomarkers. In lean individuals, functional biomarkers are largely within normal ranges and not subject to diet-induced improvements.

Macrophages are the major cell population in adipose tissue responsible for the secretion of inflammatory cytokines, NO and ROS. Given that GT-rich diet significantly improves biomarkers for inflammation and insulin resistance, the anti-inflammatory potential and the molecular mechanisms of GT derivatives, including GA and PG, were further examined in RAW 264.7 macrophages. Both GA and PG exert beneficial effects in reducing LPS-induced inflammation and oxidative stress, which are due, at least in part, to mediating the AMPK-Sirt1-NF-κb axis in RAW 264.7 macrophages. It remains

to be investigated to what extent the anti-inflammatory activities of GA and PG are associated with the AMPK pathway.

In summary, chronic exposure of mango-derived polyphenols exerts health benefits in both lean and obese individuals. These health benefits in obesity, inflammation, and insulin resistance are mainly attributed to the production of microbial metabolites (e.g., GA and PG) after mango consumption, at least in part, through mediating the AMPK pathway. Improving the abundance of probiotics (e.g., *L.plantarum*) in human gut microbiota may help improve the bioavailability of mango-derived polyphenols, resulting in enhanced efficacy of the microbial metabolites in the prevention of lipid accumulation and metabolic dysfunction in obesity and its related chronic diseases.

7.2 Considerations for Future Research

Overall, this research project demonstrates the role of GT derivatives and their microbial metabolites in the prevention of obesity and its related chronic diseases with a focus on investigating the inflammatory response and metabolic functions of adipose tissues.

GA and PG are bioactive GT derivative with physiological relevance to human health. Bioavailability of dietary polyphenols is reported to be consistently low in humans. As a result, physiological concentrations of GA and PG identified in plasma have been low. The concentration range of polyphenolic compounds in this research project was selected within a proof-of-principle approach to evaluate overall

mechanistic activities where the highest concentrations were not likely to be reached after the consumption of foods or dietary supplements. Additionally, a wide range of concentration was selected to determine any cytotoxicity on the cell lines used in this research project. While physiological concentrations of a single metabolite (e.g., GA and PG) would not reach the selected concentration range, the combined exposure to different metabolites can be expected to be high after the consumption of dietary supplements for example. Currently, most published bioavailability studies use solvents to precipitate proteins before extraction of phenolics from biological matrices. The recovery rates for these methods are low; therefore, bioavailability of phenolic acids is likely under-estimated. Additionally, most bioavailability studies take place on one day and do not consider steady-state accumulation or the adaptation of the intestinal microbiota in producing metabolites. In this research project, we have shown that the systemic exposure and urinary excretion of GA and PG metabolites after the consumption of a single serving/day of mango for 6 weeks, and clearly demonstrates the adaptation and increased systemic exposure after prolonged consumption of mango when compared to control group.

Anti-inflammatory and anti-obesogenci activities of GA and PG are partly associated with the modulation of the AMPK pathway. It's worth noting that none of these studies have provided conclusive evidence. Inhibitory effects of PG on lipid accumulation in 3T3-L1 adipocytes were not completely reversed by AMPKα1 siRNA. This indicates that other signaling pathways might be involved in the anti-adipogenic activity of PG, possibly the feedback loop of the phosphatidylinositol-3-kinase

(PI3K)/AKT/ the mammalian target of rapamycin (mTOR) pathway as previously demonstrated. In addition, the involvement of the AMPK-Sirt1-NF-κb axis in reducing LPS-induced inflammation and oxidative stress in RAW 264.7 macrophages remains to be investigated in future *in vitro* and *in vivo* mechanistic studies. Transgenic and knockout mouse model with inhibition of AMPK signaling pathway would serve as a invaluable approach to elucidate the underlying mechanisms of the biological activities of GA and PG.

Additionally, it remains uncertain, to what extent the anti-obesogenic effects improving white adipose tissue and brown adipose tissue functions are based on GT metabolites or the presence of the probiotic species *L.plantarum*. This gnotobiotic study has the potential to link the biological activities of dietary polyphenols to gut microbial composition and provide novel insights into dietary recommendations that include probiotics into our diet to increase bioavailability and bioefficacy of dietary polyphenols. Future pharmacokinetic/pharmacodynamic analyses should characterize polyphenolic profiles in plasma and adipose tissue to understand the role of individual bioactive GT metabolites. Human clinical studies are needed to lay the groundwork in the development of intake recommendations for prebiotic-probiotic combinations. Given GA and PG are the central microbial metabolites of GT, a well-controlled, double-blinded clinical trial with oral administration of mango-derived polyphenols supplemented with or without probiotics is necessary to identify the biological activities of GA and PG in humans. Moreover, it would be of great importance to compare the safety and bioefficacy of GA and PG in the prevention and treatment of obesity and its

related chronic diseases, which may provide valuable knowledge in the development of innovative drugs and their clinical use.

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