ABSTRACT

MHAWISH, REHAM. The Role of Small Phenolic Acids and Their Metabolites in Modulating Metabolic Pathways in Obesity and Inflammation. (Under the direction of Dr. Slavko Komarnytsky).

Obesity is a global health issue affecting 1.5 billion overweight and 500 million obese individuals, nearly doubling in the last 30 years. It increases the risk of metabolic diseases like T2DM, cardiovascular disease, and nonalcoholic fatty liver disease, possibly due to chronic inflammation in adipose tissue. Factors contributing to obesity include genetic, metabolic, and environmental influences, with the Western diet being a significant risk factor. Conversely, the Mediterranean diet, rich in plant foods and polyphenols, is associated with a lower risk of metabolic syndrome. Phenolic acids, a major class of polyphenols, have numerous beneficial effects, including antioxidant, anti-inflammatory, and anti-obesity properties.

The goal of this dissertation is to determine the effects of phenolic acids and their metabolites on obesity and associated metabolic disorders. The project aims to address the gaps regarding the bioactivity of phenolic acids in regulating metabolic pathways and their potential mechanism promoting metabolic health in both animal and human models. The central hypothesis is that phenolic acid-rich dietary interventions can alleviate obesity, reduce fat mass, and improve metabolic health by modulating gene expression in white adipose tissue and maintaining postprandial glucose and insulin responses in animal models, and enhance overall health in obese/overweight individuals by enhancing satiety and improving antioxidant status.

The first chapter of the project provides an overview of recent research on phenolic acids and their derivatives, emphasizing their role in regulating metabolic pathways. These compounds are commonly found in blood, urine, and feces, reflecting their absorption, metabolism, and excretion. We discussed the crucial role of these compounds on metabolic health, with a focus on

glucose homeostasis, insulin sensitivity, and carbohydrate metabolism through weak interactions with multiple molecular pathways rather than a single high-affinity receptor.

The second chapter focuses on a specific types of phenolic compounds, which are bitter phenolic compounds in herbs and spices—originally plant defenses—that can enhance metabolic health by regulating glucose transport and stimulating metabolic hormones. These effects offer potential for food-based strategies to boost metabolic resilience and well-being.

In the next two chapters we investigated the effects of mixing different hydroxylated and methylated phenolic acids on obesity and low-grade inflammation in vivo and vitro with intention to understand more about the mechanism of phenolic acids and how their metabolites affect metabolism. Our in vivo research involved treating obese mice with a high-fat diet supplemented with these phenolic acids over an eight-week period, finding that methylated phenolic acids were effective in reducing body weight by decreasing fat mass. This reduction was linked to an improvement in the thermogenic and metabolic capacity of adipose tissue, suggesting a potential mechanism for their treatment effect on obesity-induced inflammation status.

In the last chapter, to overcome the main problem associated with polyphenols, which is their chemical instability, and to enhance bioavailability, we have designed a randomized controlled clinical trial to evaluate the impact of protein-polyphenol bars on metabolic health. The study involved healthy individuals aged 18-64 randomly assigned to one of four groups: conventional whey protein bars enriched with blackcurrant polyphenols, plant-derived pea protein bars, pea protein bars enriched with blackcurrant polyphenols, and pea protein bars with complexed blackcurrant polyphenols for eight weeks. Several metabolic factors were studied to determine whether the pea-blackcurrant polyphenol complex is an effective snack for curbing

hunger, preventing overeating, and reducing metabolic risk factors associated with suboptimal lifestyles.

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The Role of Small Phenolic Acids and Their Metabolites in Modulating Metabolic Pathways in Obesity and Inflammation

by
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DEDICATION

To myself, for working hard and persevering through every obstacle. To my esteemed family, whose love, support, and guidance have been my sanctuary and foundation. To my dear husband, my partner, and a constant source of strength. To my kids, who bring joy and laughter even during my most challenging times.

BIOGRAPHY

Reham Mhawish was born and raised in Jordan. She holds a bachelor's degree in nutrition and food technology from Jordan University of Science and Technology (JUST), where she graduated at the top of her class. Her outstanding academic performance earned her a scholarship from JUST for both her master's and Ph.D. studies. She completed her master's degree in nutrition science at JUST and began her Ph.D. in nutrition science with a minor in physiology at North Carolina State University (NCSU) in 2022, under the guidance of Dr. Komarnytsky at the NC State Plants for Human Health Institute (PHHI) in Kannapolis, NC.

Reham has always been deeply interested in human health, particularly obesity, and how different types of nutrition affect our well-being. This passion led her to study nutrition and work as a research assistant (RA) and teaching assistant (TA) at JUST. Her dedication and hard work earned her full funding from JUST to complete her Ph.D.

Throughout her academic journey, Reham has developed extensive knowledge and research skills in nutrition. She has published research papers in reputable journals. In addition to her academic achievements, Reham is passionate about conducting impactful research and enhancing her teaching abilities. Reham enjoys photography, cooking, and learning new things outside of her professional life. She has a keen interest in exploring different cuisines and capturing the beauty of everyday moments through her lens.

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I also express my profound gratitude to my parents, especially my father, who believed in me from the beginning of my bachelor's degree and always knew I would become a successful Ph.D. student. To my brother and sisters for their unwavering support, continuous encouragement, and prayers throughout my years of study and the process of researching and writing this thesis.

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Chapter 1: Small Phenolic Metabolites at The Nexus of Nutrient Transport and Energy Metabolism

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1.0. Abstract

Over time, human metabolism evolved to accommodate the challenges and benefits of plant foods that contain high amounts of carbohydrates and polyphenols. The latter are typically metabolized into small phenolic metabolites, including phenolic acids and their endogenous and microbial derivatives, that influence interconnected metabolic pathways involved in nutrient transport, energy metabolism, and neurotransmitter balance. Unlike other natural products, their biological effects arise from weak interactions with multiple molecular pathways rather than a single high-affinity receptor, making them versatile regulators of metabolic health. These compounds also modulate glucose transporters and carbohydrate metabolism, playing a crucial role in postprandial glucose and insulin responses. This review addresses the critical role of phenolic metabolites in metabolic health, with a focus on glucose homeostasis, insulin sensitivity, and carbohydrate metabolism. Incorporating polyphenols and phenolic acids into dietary strategies offers significant potential for improving insulin sensitivity, reducing metabolic disorder risks, and promoting whole-body glucose homeostasis. Furthermore, understanding how phenolic metabolites interact with metabolic pathways is essential for developing future effective nutritional strategies to support metabolic health.

1.1. Introduction

The variability in diets among early hominids underscores the importance of ecological flexibility rather than exclusive reliance on a specific type of food [1]. While the increased consumption of meat may have contributed to shorter digestive tracts—freeing up metabolic energy to support brain development—and enabled complex cognitive abilities by providing essential nutrients such as iron, zinc, and vitamin B12, it was the continuous inclusion of diverse plant-based foods that enabled survival and persistence despite ever-changing ecological and social pressures [2]. Australopithecus from the 3.5-million-year-old Sterkfontein site in South Africa ate a varied plant-based diet that was broadly vegan [3]. Proteins from green leaves, though relatively low compared to animal sources, can still contribute essential amino acids when consumed in sufficient quantities, and polyphenols are an inevitable component of plant-based diets [4]. Over time, the ability of humans and other species to metabolize or tolerate polyphenols likely co-evolved with their reliance on plant-based foods, reflecting a balance between challenges and benefits in plant consumption [5].

Plant small phenolic metabolites, including phenolic acids, phenolic acid esters, as well as endogenous and microbial catabolites of flavonoids, chalcones, stilbenes, lignans, and tannins (loosely defined as polyphenols), represent a promising nexus in the regulation of interconnected metabolic pathways [6]. These small molecules exert pleiotropic effects across many biochemical networks [7], including nutrient transport [8], energy metabolism [9], immune regulation [10], and neurotransmitter synthesis [11], making them ideal candidates for redefining healthy metabolic states.

Unlike many other natural products from plants, small phenolic metabolites do not have a common receptor or a primary molecular target in the human body, with the possible exception of

bitter taste chemoreceptors (TAS2Rs) [12]. Instead, their biological effects arise from interactions with multiple molecular pathways and cellular processes. These metabolites are well-known for neutralizing free radicals and reducing oxidative stress [13], a function that is not mediated by a specific receptor but occurs through direct chemical interactions with reactive oxygen species. Additionally, phenolic metabolites modulate cell signaling pathways involved in energy metabolism (e.g., AMP-activated protein kinase), immune function (e.g., nuclear factor kappa B), and beta-estrogen receptor signaling [14]. However, these effects are achieved through a combination of weak interactions and broader regulatory mechanisms rather than a single, high-affinity target.

Small phenolic metabolites also share striking similarities with certain endogenous biochemical pathways, including those involving serotonin, dopamine, and catecholamines [15], as well as direct conjugation to amino acids in mitochondria as a part of the phenolic detoxification and nitrogen deportation systems [16]. These similarities arise from their structural features, such as hydroxyl groups attached to aromatic rings, which enable them to interact with similar enzymes, transporters, and metabolic systems. This dual role in mimicking endogenous pathways and engaging in cellular detoxification underscores the unique and versatile nature of small phenolic metabolites in human physiology. In this review, we therefore aim to highlight the complex roles of small phenolic metabolites, focusing on their ability to influence metabolic pathways and enhance resilience, as well as act as modulators of nutrient transport, energy metabolism, mitochondrial function, and neurotransmitter balance.

1.2. Baseline Phenolic Acid Metabolites in Humans

1.2.1. Blood and urine

Baseline phenolic acid metabolites in humans are derived from the endogenous metabolic pathways and are heavily influenced by dietary polyphenols, serving as important markers of metabolic health and healthy gut microbiome [17]. These metabolites, including four major series of benzoic, phenylacetic, phenylpropanoic, and cinnamic acids, are commonly found in blood, urine, and feces in different quantities, reflecting their absorption, metabolism, and excretion [18].

In blood, phenolic acids typically exist in conjugated forms such as glucuronides, sulfates, or methylated derivatives, as they undergo extensive phase II metabolism in the liver [19]. Blood phenolic acid concentrations are typically lower compared to urine due to rapid metabolism and excretion [20].

In urine, phenolic acids are highly abundant, as they represent a primary route of excretion for systemically metabolized and gut-derived compounds, with phenylacetic acid, 4-hydroxyphenylacetic acid, and hippuric acids often being the predominant waste metabolites [21]. As such, these metabolites show low to no biological activity when compared with other series of phenolic acids [22]. Higher levels of urinary phenolic acids are often observed in individuals with a plant-rich diet, while lower levels may indicate metabolic dysfunction or poor gut microbial diversity.

Blood profiles are typically dominated by the benzoic and phenylacetic acids, their respective 4-hydroxy metabolites, as well as their glycine and glutamine conjugates. Most benzoic acids are rapidly metabolized into hippuric acid and its derivatives prior to their accumulation in urine, while the bulk of phenylacetic acids are converted into 4-hydroxyphenylacetic acid and are also targeted for renal excretion. Their methylated derivatives, including vanillic acid, homovanillic acid, ferulic

acid, isoferulic acid, and dihydroferulic acid. These are found in baseline circulation in small quantities due to their increased hydrophobicity and affinity for other human tissues. Nearly all phenylpropanoic acids in human circulation are derived from microbial transformation of other phenolic acid metabolites in the gut, the primary site of their abundance (Figure 1.1) including Benzoic acids (blue) included benzoic acid (BA), 2-hydroxybenzoic acid (2HBA), 3-hydroxybenzoic acid (3HBA), 4-hydroxybenzoic acid (4HBA), 3,4-dihydroxybenzoic acid (3,4DHBA, protocatechuic acid), 3-methoxy-4-hydroxybenzoic acid (3M4HBA, vanillic acid), and 3,4,5-trihydroxybenzoic acid (345THBA, gallic acid). Phenylacetic acids (green) included phenylacetic acid (PAA), 3-hydroxyphenylacetic acid (3HPAA), 4-hydroxyphenylacetic acid (4HPAA), 3,4-dihydroxyphenylacetic acid (3,4DHPAA, DOPAC), and 3-methoxy-4-hydroxyphenylacetic acid (3M4HPAA, homovanillic acid). Phenylpropanoic acids (orange) included phenylpropanoic acid (PPA), 3-hydroxyphenylpropanoic acid (3HPPA), 4-hydroxyphenylpropanoic acid (4HPPA, desaminotyrosine), 3,4-dihydroxyphenylpropanoic acid (3,4DHPPA, dihydrocaffeic acid), and 3-methoxy-4-hydroxyphenylpropanoic acid (3M4HPPA, dihydroferulic acid). Cinnamic acids (pink) included cinnamic acid (CA), 3-hydroxycinnamic acid (3HCA, m-coumaric acid), 4-hydroxycinnamic acid (4HCA, p-coumaric acid), 3,4-dihydroxycinnamic acid (3,4DHCA, caffeic acid), 3-methoxy-4-hydroxycinnamic acid (3M4HCA, ferulic acid), and 4-methoxy-3-hydroxycinnamic acid (4M3HCA, isoferulic acid). Amino acid conjugates (red) included those bound to glycine (hippuric acid) and glutamine (phenylacetylglutamine).

1.2.2. Feces and Microbial Biotransformation

Polyphenols is a major group of plant secondary metabolites found throughout plant tissues, collectively forming thousands of distinct chemical structures characterized by hydroxylated aromatic rings [26]. The estimates for daily total polyphenol intake vary across the country and date of the study, but it is reasonable to assume the upper limit of intake of 1370 mg/day total polyphenols among coffee and tea consumers, and a 540 mg/day intake in people who do not consume these drinks regularly, as described in a recent 77,441 participant study [27]. Fruits (apples, oranges), vegetables (onions, spinach, lettuce), cocoa products, and wine were the major other sources, with additional contributions from potatoes, cereals, legumes, or berries depending on the geographical region. The mean overall intakes were summarized as 910 mg total polyphenols, including 360 mg of total flavonoids and 410 mg of total phenolic acids [28].

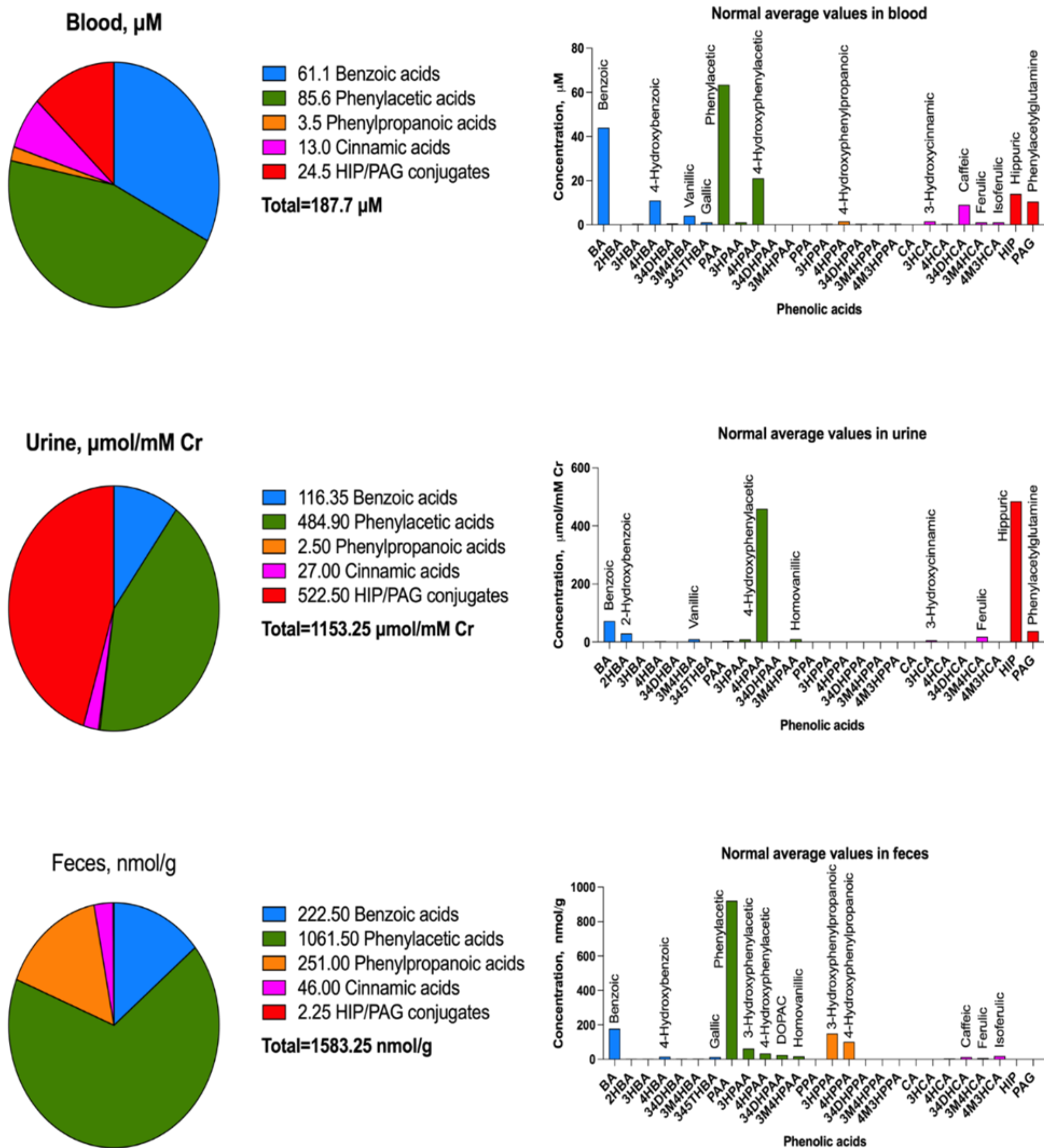

Figure 1.1. Major phenolic acids found in human blood (µM), urine (µmol/mM creatinine), and feces (nmol/g wet weight). Normal levels of each phenolic acid were retrieved and averaged across multiple clinical studies summarized in the Human Metabolome database [23].

Stomach absorption of intact polyphenols is minimal, but passive absorption of aglycones can occur in the upper gastrointestinal tract after enzymatic hydrolysis by lactase-phloridzin hydrolase and β-glucosidases in the small intestine brush border [29]. Once absorbed, polyphenols undergo phase I (oxidation, reduction) and phase II (conjugation) metabolism to convert less-polar molecules into water-soluble metabolites for renal excretion. In humans, phase II conjugation (glucuronidation in the endoplasmatic reticulum, sulfation in the cytoplasm, methylation in both the cytoplasm and ER, and conjugation with amino acids in mitochondria) predominates over phase I metabolism [30]. The degree of phase II conjugation depends on the physiochemical properties of phenolics, with diverse fragmentation patterns creating distinct metabolic signatures of benzoic and cinnamic acid metabolites [6]. In turn, phenylacetic and phenylpropanoic acids have no significant dietary sources but are derived from colonic microbial metabolism of dietary polyphenols [18]. Microbial transformation of caffeoylquinic acids produces large quantities of dihydrocinnamic acids that also enter circulation [31]. Beyond detoxification, phase II metabolism of dietary phenolic metabolites plays a critical role in regulating physiological levels of amino acid precursors (glycine, glutamine), supporting mitochondrial energy metabolism, and nitrogen waste deportation [32].

A study on healthy volunteers consuming coffee containing 310 mg of chlorogenic acid identified 56 phenolic metabolites in plasma [33]. Caffeic acid metabolites were primarily sulfated, ferulic acids were equally glucuronidated and sulfated, and coumaric acids were present in small amounts, while dihydro metabolites, likely formed by gut microbiota, appeared mainly in free forms (Figure 2, top panel). The study highlighted a shift from caffeic acid derivatives to ferulic and isoferulic metabolites in plasma, mediated by catechol-O-methyltransferase (COMT), and likely omitted the presence of downstream benzoic acid metabolites such as vanillic and 3-

hydroxybenzoic acids, possibly resulting from partial endogenous β-oxidation as shown previously [34].

1.3. Phenolic Acids Released from Dietary Polyphenols

Polyphenols is a major group of plant secondary metabolites found throughout plant tissues, collectively forming thousands of distinct chemical structures characterized by hydroxylated aromatic rings [26]. The estimates for daily total polyphenol intake vary across the country and date of the study, but it is reasonable to assume the upper limit of intake of 1370 mg/day total polyphenols among coffee and tea consumers, and a 540 mg/day intake in people who do not consume these drinks regularly, as described in a recent 77,441 participants' study [27]. Fruits (apples, oranges), vegetables (onions, spinach, lettuce), cocoa products, and wine were the major other sources, with additional contributions from potatoes, cereals, legumes or berries depending on the geographical region. The mean overall intakes were summarized as 910 mg total polyphenols, including 360 mg of total flavonoids and 410 mg of total phenolic acids [28].

Stomach absorption of intact polyphenols is minimal, but passive absorption of aglycones can occur in the upper gastrointestinal tract after enzymatic hydrolysis by lactase-phloridzin hydrolase and β-glucosidases in the small intestine brush border [29]. Once absorbed, polyphenols undergo phase I (oxidation, reduction) and phase II (conjugation) metabolism to convert less-polar molecules into water-soluble metabolites for renal excretion. In humans, phase II conjugation (glucuronidation in the endoplasmatic reticulum, sulfation in cytoplasm, methylation in both cytoplasm and ER, and conjugation with amino acids in mitochondria) predominates over phase I metabolism [30]. The degree of phase II conjugation depends on the physiochemical properties of phenolics, with diverse fragmentation patterns creating distinct metabolic signatures of benzoic

and cinnamic acid metabolites [6]. In turn, phenylacetic and phenylpropanoic acids have no significant dietary sources but are derived from colonic microbial metabolism of dietary polyphenols [18]. Microbial transformation of caffeoylquinic acids produces large quantities of dihydrocinnamic acids that also enter circulation [31]. Beyond detoxification, phase II metabolism of dietary phenolic metabolites plays a critical role in regulating physiological levels of amino acid precursors (glycine, glutamine), supporting mitochondrial energy metabolism and nitrogen waste deportation [32].

A study on healthy volunteers consuming coffee containing 310 mg chlorogenic acid identified 56 phenolic metabolites in plasma [33]. Caffeic acid metabolites were primarily sulfated, ferulic acids were equally glucuronidated and sulfated, and coumaric acids were present in small amounts, while dihydro metabolites, likely formed by gut microbiota, appeared mainly in free forms (Figure 1.2, top panel). The study highlighted a shift from caffeic acid derivatives to ferulic and isoferulic metabolites in plasma, mediated by catechol-O-methyltransferase (COMT), and likely omitted the presence of downstream benzoic acid metabolites such as vanillic and 3-hydroxybenzoic acids, possibly resulting from partial endogenous β-oxidation as shown previously [34]. A study on eight healthy male volunteers consuming 500 mg of 13C-labeled cyanidin-3-glucoside revealed extensive phase II metabolism and microbial biotransformation. Free, methylated, and glucuronidated forms peaked at 760 nM within 1–2 hours post-bolus [35]. Vanillic acid was the dominant plasma and urine metabolite, surpassing protocatechuic acid, with both preferring sulfation over glucuronidation. Hydroxycinnamic metabolites such as caffeic acid and ferulic acid were found in smaller amounts, with ferulic acid consistently dominating, indicating significant methylation of phenolic substrates. Ring fission reactions of anthocyanins

appear to favor the production of benzoic acid metabolites, although this requires further investigation (Figure 1.2, bottom panel).

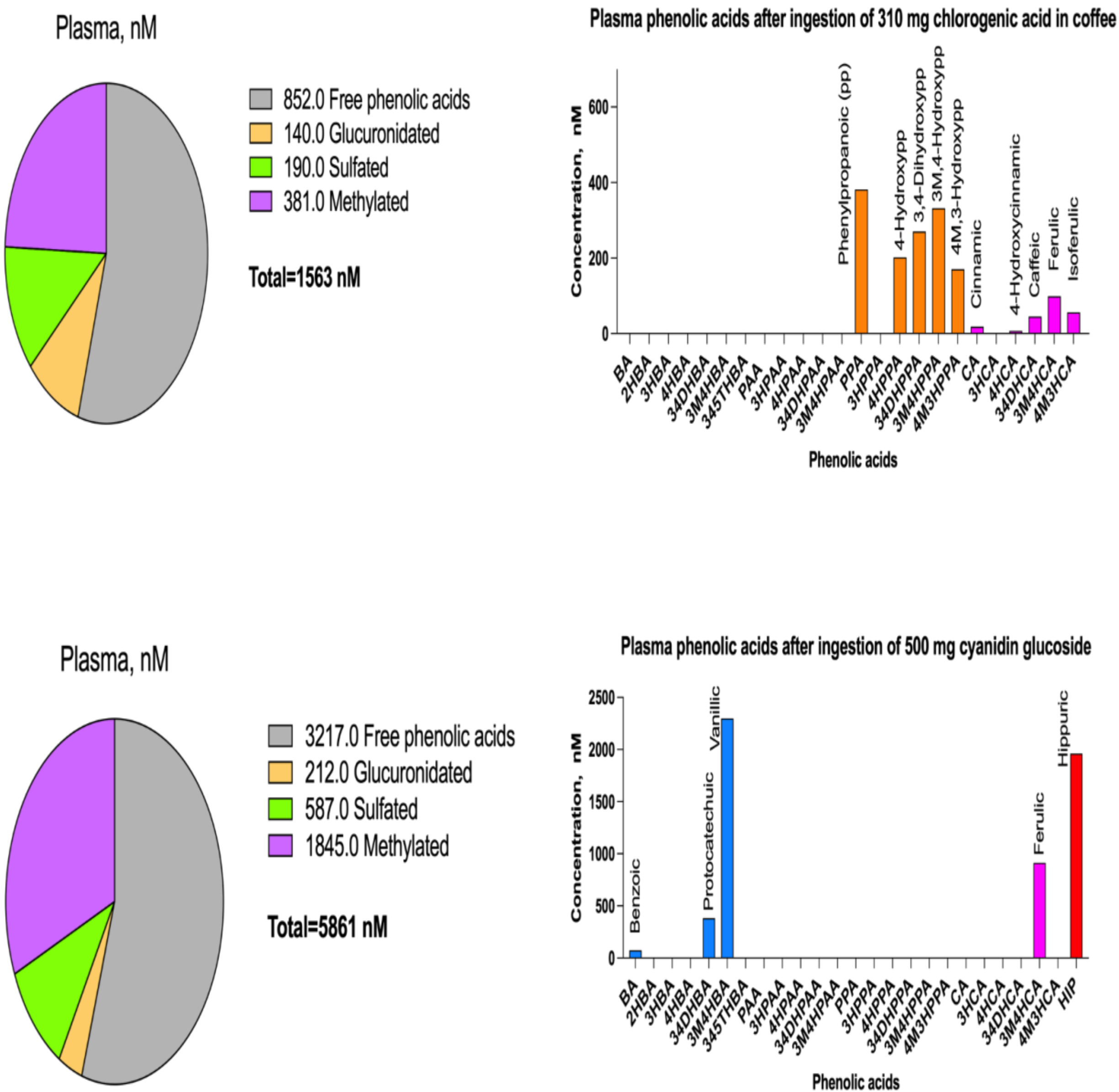

Figure 1.2. Major phenolic acids found in human blood (nM) after ingestion of 310 mg of chlorogenic acid in coffee (top) and 500 mg cyanidin glucoside (bottom), after [33] and [35]. The nomenclature of phenolic acid metabolites is listed in Figure 1.1.

Flavanol breakdown in the form of epicatechin resulted in phase II metabolites, predominantly undergoing methylation and sulfation, with γ-valerolactones as the dominant group of compounds [36]. The breakdown pathway from γ-valerolactones to hippuric acid may involve phenylhydracrylic intermediates. Additionally, there appears to be a trend toward forming phenylacetic acids, hydracrylic acids, and flavandiols from flavonols [37], flavanones [38], and isoflavones [39], respectively, highlighting gaps in understanding the differences in metabolism of small phenolic metabolites from diverse parent compounds.

An additional source of variation in phenolic metabolites in circulation is their affinity for binding human serum albumin (HSA), which increases with the number of free aromatic hydroxyl groups [40]. HSA, the most abundant protein in human plasma, contains two binding sites for small organic molecules, including most phenolic acids [41]. Binding to HSA regulates the free, active concentration of a metabolite in the blood, and methylation of aromatic hydroxyls typically decreases this binding, potentially increasing the free fraction of methylated metabolites (methylated sink), enhancing their potency and tissue distribution. This is demonstrated by the detection of ferulic acid, but not caffeic acid, in blood cells after ingesting a polyphenol-rich extract, despite both being present in serum [42]. Deficiency in catechol-o-methyltransferase activity was preliminary linked to disruption of glucose homeostasis in an animal model [43].

1.4. Overlap with amino acid metabolism

Phenolic acid metabolism in humans shares several key pathways and intermediates with amino acid metabolism, highlighting their interconnected roles in health and physiology. Both phenolic acids and amino acids undergo biotransformation by gut microbiota, producing bioactive metabolites that influence systemic functions, such as neurotransmission and energy metabolism [44]. Enzymatic processes, including transamination and decarboxylation, are common to both

metabolic pathways. These pathways also intersect in cellular antioxidant systems, where phenolic acids and amino acid-derived metabolites, such as glutathione, contribute to redox homeostasis [45]. Furthermore, phenolic acids can modulate the metabolism of aromatic amino acids, such as tryptophan, influencing pathways linked to serotonin production and immune regulation [46].

Phenylalanine is metabolized into phenylacetic acid, a key metabolite derived from its catabolic pathway. This occurs primarily through deamination to produce phenylpyruvic acid, which is further metabolized to phenylacetic acid [47]. This pathway is part of phenylalanine catabolism in humans and is particularly active in conditions like phenylketonuria, where a deficiency in the enzyme phenylalanine hydroxylase disrupts the conversion of phenylalanine to tyrosine, leading to the accumulation of phenylacetic acid and its derivatives [48].

Tyrosine is metabolized into p-hydroxyphenylacetic acid as a primary phenolic acid metabolite. This occurs through deamination of tyrosine to form p-hydroxyphenylpyruvic acid, which is further converted into p-hydroxyphenylacetic acid [49]. Additionally, tyrosine can also be metabolized into homovanillic acid (HVA) via the dopamine pathway, depending on its involvement in neurotransmitter metabolism [50]. These metabolites play significant roles in both physiological processes and diagnostic assessments of metabolic or neurological disorders.

Tryptophan is metabolized into indole-3-acetic acid (IAA), a primary phenolic acid derivative. This occurs via the microbial metabolism of tryptophan in the gut, where it is converted to indole by bacterial enzymes and subsequently oxidized to indole-3-acetic acid [51]. Indole-3-acetic acid and related metabolites are involved in various physiological and signaling processes, including gut–microbiota interactions. Additionally, tryptophan can be metabolized into kynurenic acid and other metabolites through the kynurenine pathway [52].

The interconnected metabolic pathways of phenolic acids and aromatic amino acids provide a fascinating framework for understanding the health-promoting effects of polyphenols. The nitrogen deportation system, essential for nitrogen balance, is closely linked to these metabolic interactions. Compounds like hippuric acid and phenylacetylglutamine (PAG) serve as important mediators of nitrogen excretion. Phenolic metabolites can influence the nitrogen deportation system by modulating the microbial enzymes involved in aromatic amino acid catabolism, thereby altering the production of these substrates (Figure 1.3).

1.5. Energy transport and metabolism

1.5. Competitive interactions with organic anion transporters

Endogenous esterases, found in pancreatic secretions, brush border layers of the small intestine, and colonic microbiota, efficiently release phenolic metabolites from their parent compounds to facilitate absorption [53]. Most phase II metabolism, including glucuronidation, sulfation, and methylation, occurs in the enterocyte and liver, while processes like demethylation and dehydrogenation are restricted to the liver, establishing it as a key regulator of methylated phenolic metabolite levels [54]. Metabolites conjugated with a larger glucuronic acid moiety are more likely to enter enterohepatic circulation compared to sulfated or methylated derivatives, explaining their lower abundance in circulation. Absorption of phenolic metabolites from the gastrointestinal tract increases with hydrophobicity, while more polar structures are often methylated to improve uptake via monocarboxylate transporter MCT1 (SLC16A1) and transferred to the blood through MCT4 (SLC16A3) [55].

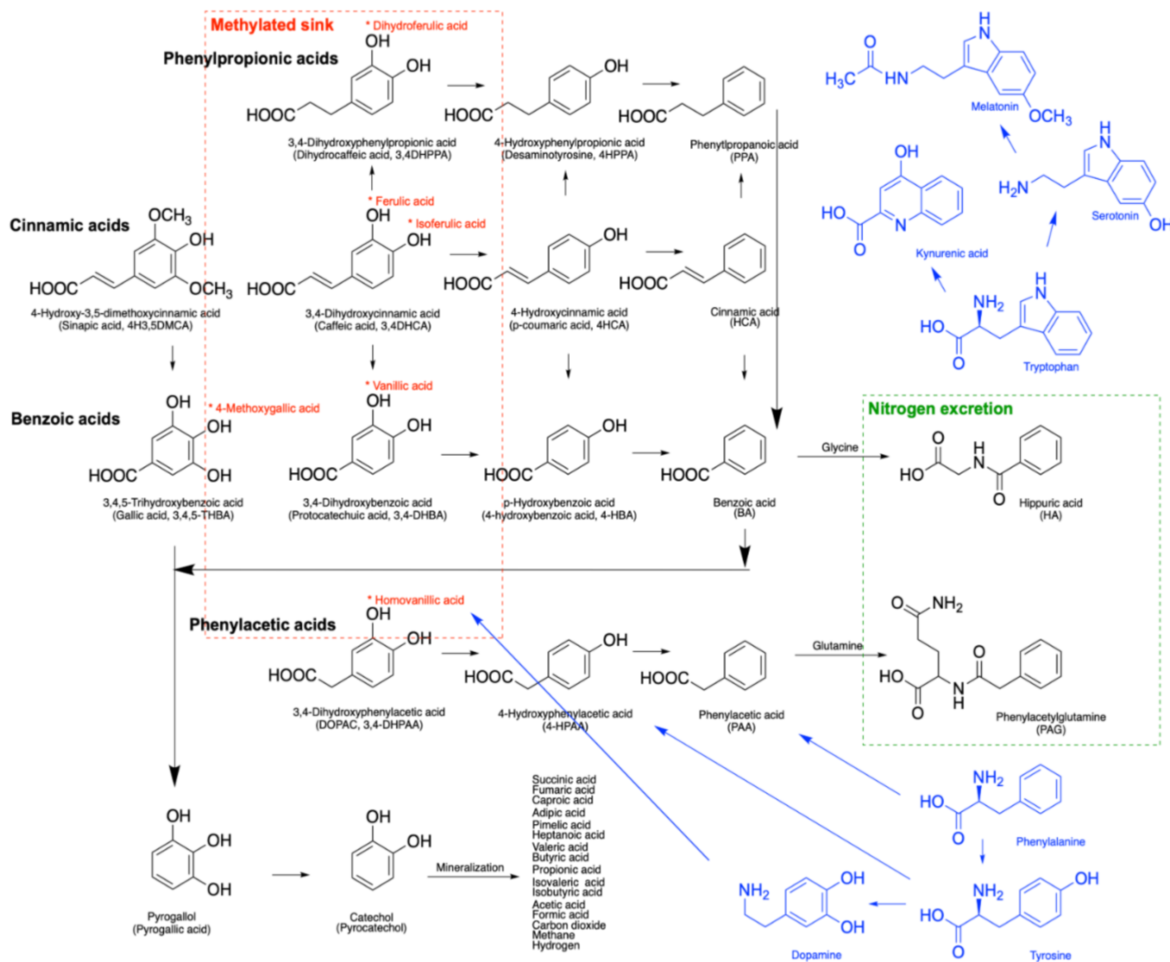

Figure 1.3. Major phenolic acid metabolites generated by host metabolism, as well as degradation and microbial fermentation of dietary polyphenols towards conjugation with amino acids (glycine and glutamine) and mineralization into small organic acids on the way to carbon dioxide, methane, or hydrogen production. The nomenclature of phenolic acid metabolites is listed in Figure 1. Red asterisk (*) denotes methylation sites and the respective names of the methylated phenolic metabolites. The red dashed area identifies these metabolites as a possible methylated reservoir of these compounds in human tissues. The green dashed area denotes two major metabolites responsible for nitrogen excretion. Blue color denotes aromatic amino acids and their metabolites.

Urinary excretion of phenolic metabolites is regulated by another set of organic anion transporters (OATs) in the proximal kidney tubules. Different transporters, such as OAT1 (SLC22A6), OAT2 (SLC22A7), OAT3 (SLC22A8), and OAT4 (SLC22A11), exhibit variable affinities for free, glucuronidated, and sulfated metabolites [56]. Phenolic acids can also enter the endoplasmic reticulum (ER) through ATP-binding cassette (ABC) transporters like ABCG2, which play a role in regulating the availability of these bioactive compounds for further processing and secretion [57]. In mitochondria, phenolic acids enhance antioxidant defenses by supporting glutathione metabolism. They can interact with SLC7A11, the cystine/glutamate antiporter, to regulate the availability of cysteine for glutathione synthesis, indirectly impacting mitochondrial redox homeostasis [58]. Finally, SLC25A1, a citrate carrier in the mitochondrial inner membrane, may indirectly facilitate the trafficking of phenolic metabolites that interact with citrate metabolism [59]. These transport systems play a pivotal role in determining the levels of phenolic metabolites in cells and determining their ultimate routes of excretion.

1.5.2. Glucose absorption in the gut

Phenolic acids interact with glucose transporters in the gut and other tissues, influencing glucose absorption and cellular uptake. Two primary transporter families are involved are sodium–glucose co-transporters (SGLTs) and facilitative glucose transporters (GLUTs). Phenolic acids can modulate these transporters either directly by binding to them or indirectly by altering their expression and activity [60].

SGLT1, found in the small intestine, facilitates active glucose transport into enterocytes. Phenolic acids can inhibit SGLT1 activity, thereby reducing glucose absorption and dampening postprandial blood glucose spikes [8]. Phenolic acids have been shown to reduce glucose transport via SGLT1 and GLUT2-mediated glucose absorption, particularly when GLUT2 translocates to

the brush border membrane of enterocytes in response to high glucose concentrations [61]. Phenolic acids interact with glucose transporters through various physicochemical mechanisms, including direct binding, modulation of transporter expression, and alteration of membrane dynamics. Studies suggest that certain phenolic acids can inhibit SGLT1-mediated glucose uptake by competing for binding sites or altering transporter conformation [62]. An extensive discussion on these effects and their application to glucose consumption was published recently [8]. Further research is needed to elucidate the structural basis of these interactions, the specificity of different phenolic acid derivatives for glucose transporters, and their dose-dependent effects in physiological conditions.

In peripheral tissues, phenolic acids influence GLUT4, a key transporter responsible for insulin-stimulated glucose uptake in muscle and adipose tissue. Phenolic acid metabolites, such as ferulic acid and its derivatives, have been found to modulate GLUT4 translocation to the plasma membrane, improving glucose utilization and insulin sensitivity [63]. This mechanism is particularly relevant for managing insulin resistance and type 2 diabetes mellitus. Moreover, phenolic acids can indirectly affect glucose transporters by either altering gut microbiota metabolism or directly modulating the activity of the bitter taste receptors [5]. In animal models, gastrointestinal cells co-express the bitter receptor mTAS2R108, the glucagon-like peptide-1 (GLP-1), and the GLP-1R receptor, and their expression levels are upregulated in response to bitter substances [12]. Model bitter substances also increase intracellular [Ca2+] in neuroendocrine STC-1 cells, a recognized model for glucose absorption and gastrointestinal hormone secretion [64], highlighting their potential for modulating glucose absorption and hormone secretion. These interactions contribute to the beneficial effects of phenolic acids on glycemic control, insulin sensitivity, and metabolic health.

1.5.3. Energy metabolism and insulin resistance

Beyond direct effects on glucose transport, phenolic acids also modulate signaling pathways that regulate transporter activity. For instance, phenolic acids can activate AMP-activated protein kinase (AMPK), a critical regulator of GLUT4 expression and glucose metabolism [65]. Activation of AMPK by phenolic acids has been linked to reduced hepatic glucose production and enhanced glucose uptake in peripheral tissues [66]. Similar effects can also modulate insulin secretion, glucose release from the liver, insulin receptor activation, and glucose uptake by insulin-sensitive tissues [67]. Additionally, they influence hepatic glucose output and other aspects of metabolism, demonstrating their potential in managing diabetes and obesity [68].

p-Coumaric acid, a key phenolic acid in plant foods, exhibits antioxidant, anti-inflammatory, and anticancer properties. It lowers blood glucose, total cholesterol, and triglycerides in diabetic models while protecting pancreatic β-cells from oxidative stress and improving hepatic glucose metabolism by enhancing hexokinase and glucose-6-phosphate dehydrogenase activity [69]. Additionally, its antioxidant effects reduce liver oxidative stress, alleviate diabetic nephropathy, and, when present in highland barley grain alongside procyanidin B1, enhance glucose uptake and insulin sensitivity to help combat insulin resistance [70].

Caffeic acid, found in various fruits, vegetables, and beverages, lowers plasma glucose and enhances insulin sensitivity [71]. It regulates blood glucose by inhibiting α-glucosidase and α-amylase, reducing postprandial hyperglycemia [72]. In rodent models, caffeic acid decreases body weight, visceral fat mass, and plasma lipid levels while promoting fatty acid oxidation and suppressing lipogenesis via AMP-activated protein kinase [73]. Ferulic acid, abundant in cereal grains and fruits, reduces insulin resistance by modulating inflammatory pathways such as JNK, ERK, and NFκB [74]. In diabetic rat models, ferulic acid improves insulin sensitivity, enhances

hepatic glycogenesis, and inhibits gluconeogenesis [75], with additional benefits observed when combined with dietary fibers like arabinoxylan for improved glucose tolerance and intestinal health [76]. However, most of these findings stem from animal studies, highlighting the need for caution in translating these results to human contexts. Given the compelling preclinical evidence, it is critical to advance ferulic acid and other phenolic acids into clinical trials to fully realize their therapeutic potential in metabolic health management.

1.6. Conclusions

Phenolic acid metabolites in humans are shaped by both endogenous metabolic processes and dietary polyphenol intake, with distinct profiles observed in blood, urine, and feces. These metabolites act as valuable biomarkers of metabolic health, reflecting the interplay between diet and metabolism. They directly influence nutrient transport and energy metabolism, further emphasizing their role in maintaining postprandial glucose and insulin responses. While studies have identified key metabolites in blood, urine, and feces, significant gaps remain regarding their transport, tissue-specific distribution, and long-term metabolic effects. Future research should address these discrepancies, explore interindividual variability in phenolic acid metabolism, and establish standardized methodologies for assessing their role as biomarkers of metabolic health. This will allow for developing novel dietary strategies for improving insulin sensitivity and reducing the risk of metabolic disorders by modulating carbohydrate metabolism in the gut, central to the whole-body glucose homeostasis.

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Chapter 2: Spices And Herbs as Natural Compact Delivery Systems of Phenolic Phytochemicals with Metabolic Benefits to Humans

2.0. Abstract

Bitter phenolic compounds in plants, originally evolved as chemical defenses, are preserved within the dry matrices of spices and culinary herbs, making them bioactive additions to the human diet that engage bitter taste receptors (TAS2Rs) throughout the gastrointestinal tract. A subset of these receptors may have evolved to anticipate carbohydrate intake from bitter plant foods by regulating intestinal glucose transporters and stimulating the release of metabolic hormones such as glucagon-like peptide-1(GLP-1) and cholecystokinin (CCK). These mechanisms may explain why diverse classes of bitter phytochemicals, despite their varied structures, consistently influence glucose metabolism and appetite regulation. The interplay between bitter receptor activation and incretin signaling represents a largely untapped opportunity for developing novel, food-based interventions to improve metabolic and immune health outcomes. Leveraging the natural bitterness of herbs and spices in the extraoral regions of the gastrointestinal tract offers a simple, sustainable, and culturally adaptable strategy to enhance metabolic resilience and quality of life.

Keywords: spices, culinary herbs, bitters, bitter receptors, extraoral, gastrointestinal modulation, glucose metabolism, postprandial glucose, blood sugar spikes, hypoglycemic

2.1. Introduction

Bitter compounds in plants have evolved as chemical defenses to protect against herbivores, pathogens, and environmental stress. These molecules, including alkaloids, terpenes (isoprene scaffold), polyphenols, glucosides, saponins and tannins, are concentrated in specific plant tissues such as seeds, roots, and some aerial parts [1]. In herbs and spices, these substances are packed into natural, compact reservoirs that preserve their stability, making them resistant to degradation over time [2]. The dry nature of spices and herbs ensures that these bitter compounds remain chemically stable and can withstand the rigors of storage and transportation. When consumed, these plant-based matrices are easily incorporated into the human diet, offering potential health benefits due to their bioactive properties [3].

Humans, like other vertebrates, recognize bitter taste through bitter taste receptors (TAS2Rs). These receptors appeared for the first time in cartilaginous and bony fishes around 460-430 million years ago, and rapidly expanded during the subsequent transition from aquatic to terrestrial life [4,5]. The emergence of bitter plants and insects on land, around 480-500 million years ago [6], is often considered as a key driver behind the subsequent expansion of the TAS2R receptor family. In contrast, animals generally lack bitter-tasting tissues, with bile being one of the primary exceptions [7]. Thus, developing a broad and sensitive bitter detection system provided a survival advantage, helping animals avoid toxins and navigate a new, chemically rich environment.

Calling TAS2Rs "bitter receptors", however, was a significant disservice to their true biological chemosensory role and vast extraoral functionality [8]. The expansion and diversification of bitter taste receptors in the gut, airways, and immune cells led to their evolutionary repurposing for additional metabolic and immune functions, such as digestion, hormone release, inflammation, and microbial sensing [9]. Today, there is a growing support for

the notion that bitter substances routinely engage chemosensory receptors all over the human body. Spices and herbs are uniquely positioned to leverage this system, offering not just flavor but also potential modulatory effects through their concentrated bitter-tasting constituents. However, a significant knowledge gap remains in understanding how these compounds interact at the molecular level across different tissues, and how they can be effectively harnessed for health-promoting purposes. In this narrative review, we aim to address the gap by focusing on the effects of bitter-tasting substances on carbohydrate metabolism, and specifically, their influence on glucose uptake and appearance in the blood, using both human clinical data and cell culture models.

2.2. Methods

This narrative review was developed through a summary of our work on the subject combined with an extensive search of scientific literature using databases including PubMed, Google Scholar, Web of Science, and Scopus, accessed via institutional subscriptions at NC State University, covering publications up to March 2025. Only peer-reviewed studies published in English were considered. The review concentrated on research published in the last 10 years. Keywords used in the search strategy included: “Bitter Taste Receptors” OR “TAS2Rs” OR “Bitter Compounds” OR “Phytochemicals” AND “Glucose Regulation” OR “GLP-1” OR “CCK” OR “Postprandial Glucose” OR “Glucose Absorption” OR “Glucose Metabolism” AND “Spices” OR “Herbs” OR “Bitters” OR “Culinary Bitterness” OR “Dietary Interventions” OR “Metabolic Health”.

2.3. Spices and herbs in traditional bitter preparations

The use of spices and herbs predates modern humans as evident from dental calculus of Neandertals from El Sidron cave in Northern Spain dated to 50,600-47,300 BCE that contained bitter-tasting dihydroazulene and chamazulene found in yarrow (Achillea millefolium L.) and chamomile (Matricaria chamomilla L.) [10]. The same individuals were also heterozygous tasters by maintaining divergent alleles of the bitter taste receptor TAS2R38 [11]. This, together with a substantial expansion of α-amylase AMY1 copy variants in the genomes of early hominids, points to a prominent dietary transition towards increased consumption of plants and storage carbohydrates (tubers, roots, nuts and grains) outside of the rainforest areas [12]. As most wild plant foods are naturally more or less bitter, the increase of carbohydrate loads in human diets also increased the consumption of plant bitter phytochemicals. On the other hand, additional exposure of foods to fermentation or heating (Maillard reactions) also generates bitter-tasting substances [13]. Partial debittering of plant foods was achieved in the form of cooking [14,15] or nixtamalization with hot limestones [16], but it was not until the development of modern crop cultivars and industrial debittering that human diets were essentially depleted of bitter tastes [17]. Consistent with this knowledge, modern higher quality diets are intrinsically more bitter, and promoting greater acceptance of bitter flavors could contribute to improved dietary patterns in the general population [18].

Inherent bitterness of spices and herbs found its way into many formulations dating back to the Classical Antiquity [19]. Bitter-tasting mithridate and theriaca (tiryaq, treacle) remedies in the form of honey electuary typically contained gentian, St John's wort, parsley, anise, ginger, and cinnamon in a complex mixture of up to 70 ingredients as recorded by Celsus and Galen, and targeted the gastrointestinal tract as an antidote to ingested poisons [20,21]. The importance of

consuming bitter herbs was also recognized in many traditional texts, for example as a choice of maror ("with bitter herbs they shall eat it"). This tradition continued in the form of bitter aperitifs or digestifs targeting gastrointestinal health such as Chartreuse, Benedictine (France), Amaro, Fernet, Campari (Italy), Kräuterlikör, Jägermeister, Underberg (Germany), Appenzeller Alpenbitter (Switzerland), Becherovka (Czech), Angostura and Peychaud's (Americas), among others. While their bitter profiles vary, they are typically dominated by gentian, wormwood, angelica, hyssop, anise, caraway, yarrow, bitter orange, and cinchona [22]. Modern research supports the traditional use of these preparations, showing that bitter mixtures can stimulate digestive secretions, modulate gut motility, and impact satiety as discussed below.

2.4. Diversity of bitter phytochemicals in spices and herbs

Spices are defined by the FDA as any "aromatic vegetable substances… whose significant function in food is seasoning", while the term "herbs" is traditionally restricted only to dry aerial parts (leaves and flowers). In modern times, consumption of spices varies from 0.5 g/day in Europe to 1.8 g/day in Africa and 2.6-4.4 g/day in Asia and Latin America for an average adult [23]. While spices and herbs are traditionally used for their modulation of taste, flavor, color, texture, or food preservation, their bitter compounds contribute not only to the sensory complexity of culinary ingredients but also to their functional roles as chemical defenses in plants [24]. It is therefore plausible that many bitter phytochemicals evolved to interact with conserved chemosensory pathways in the mammalian gastrointestinal system, suggesting a co-evolutionary chemical dialogue between plants and animal physiology, particularly in the upper gastrointestinal tract, much like the well-established bitter signaling crosstalk between the microbiome and the colon [25].

Bitterness is a common sensory property of many spices and herbs. Table 2.1 presents a brief and incomplete summary of the distribution of bitter compounds across botanicals, their phytochemical classifications, and known activations of human bitter taste receptors. The dataset highlights a broad distribution of bitter phytochemicals across different plant tissues and chemical classes. Alkaloids are the most common bitter compounds, appearing in barks (quinine), fruits (berberine, piperine), seeds (xylopine, theobromine), and leaves (skimmianine), and activating a wide range of the bitter taste receptors (TAS2Rs). Sesquiterpene lactones, notably in flowers (chamomile), leaves (wormwood), and roots (dandelion, chicory), show strong activation of TAS2R46, suggesting this receptor plays a central role in detecting plant-derived lactones. Some receptors such as TAS2R14 and TAS2R46 are frequently activated by multiple compound classes, indicating broad ligand specificity. In contrast, receptors like TAS2R2 and TAS2R16 are selectively activated by specific compounds (e.g., curcumin and sinigrin, respectively), suggesting narrow functional roles. Certain phytochemicals, like amarogentin and humulone, are effective at very low concentrations, reflecting high receptor sensitivity.

Table 2.1. Bitter principles in common spices and herbs, as well as the corresponding bitter receptor activation profiles summarized after [71,87].

Plant tissues	**Spices or herbs**	**Bitter principles**	**Phytochemical groups**	**TAS2R activation (Effective concentration, µM)**
Bark	Cinchona	Quinine	Alkaloids	TAS2R1, 4, 7, 10, 14, 39, 40, 41, 43, 44, 46 (10-1000 µM)
	Cinnamon	Coumarin	Coumarins	TAS2R10,14 (300 µM)

Table 2.1 (continued).

	Quassia	Quassin	Triterpene lactones	TAS2R4, 10, 14, 30, 46, 47 (300 µM)
Flowers	Chamomile	Nobilin	Sesquiterpene lactones	TAS2R46 (0.1 µM)
	Clove	Gallic acid	Gallotannins	TAS2R4, 14 (0.2-220 µM)
	Hops	Humulone	Alpha acids	TAS2R1, 14, 40, 47 (0.01-30 µM)
	Saffron	Picrocrocin	Monoterpene glycosides	Unknown (22 µM)
Fruits	Barberry	Berberine	Alkaloids	TAS2R38, 46 (10 µM)
	Bitter orange (chenpi)	Naringin	Flavanone glycosides	Unknown (10-220 µM)
	Pepper, black	Piperine	Alkaloids	TAS2R14 (10 µM)
Leaves	Basil, oregano	Rosmarinic acid	Caffeic acid esters	Unknown (103 µM)
	Parsley	Apigenin	Flavone glycosides	TAS2R14, 39, 43 (1-30 µM)
	Rosemary, sage	Carnosic acid	Diterpenes	Unknown
	Rue (ruta)	Skimmianine	Alkaloids	TAS2R14

Table 2.1 (continued).

	Wormwood	Absinthin	Sesquiterpene lactones	TAS2R10, 14, 46, 47 (0.1-100 μM)
Roots or rhizomes	Angelica	Furanocoumarins	Furanocoumarins	TAS2R10, 14, 49
	Chicory	Lactucopicrin	Sesquiterpene lactones	TAS2R43, 46
	Dandelion	Taraxacin	Sesquiterpene lactones	TAS2R46 (0.1-100 μM)
	Gentian	Amarogentin	Secoiridoid glycosides	TAS2R1, 4, 39, 43, 46, 47, 50 (3-300 μM)
	Turmeric	Curcumin	Curcuminoid	TAS2R2
Seeds	Cacao	Theobromine	Alkaloids	TAS2R14 (1000 μM)
	Celery	Butylphthalide	Phthalide lactones	Unknown
	Fenugreek	Diosgenin	Saponins	Unknown
	Grains of Selim (diarr)	Xylopine	Alkaloids	Unknown
	Hyssop	Marrubiin	Diterpene lactone	TAS2R46 (0.3 μM)
	Mustard	Sinigrin	Glucosinolates	TAS2R16, 38 (100 μM)

2.5. Bitter receptors from a functional perspective

Classical TAS2Rs in the oral cavity enable a general aversion to the unpleasant bitter taste as an early signal to avoid ingestion of poisonous plants, insects, scavenged animal carcasses, and other spoiled foods [26]. While in primates the number of functional TAS2R genes varies from 18

to 26, humans maintain 25 active TAS2R genes and 8 non-functional pseudogenes, all clustered on three chromosomes [27]. This clustering is evolutionary preserved in mammals, as mouse mTAS2R genes exist in three similar clusters, although some subgroups of the TAS2R genes show a clear tendency for both expansion and contraction [28,29]. This process may have increased or decreased the functional redundancy of bitter taste perception, as well as allowed for additional new functionality of the broad-specificity human TAS2R10, TAS2R14, TAS2R43, and TAS2R46 genes that also exist as the expanded mTAS2R gene subgroups in mice (Figure 2.1). Expansion of TAS2R gene clusters had a clear evolutionary advantage on land as their number peaked at 74 loci in coelacanth, 50-136 loci in anuran frogs, and 36-50 loci in lizards [30]. Yet there was no advantage of TAS2R functionality in the marine environment, as both birds (pinguins) and mammals (cetacean) that returned to the ocean experienced a near complete loss of TAS2Rs [31,32]. The second peculiar feature of the bitter TAS2R receptors is their spatial distribution. Beyond the classical localization throughout the oral cavity and the increased abundance in its posterior part where TAS2Rs recognize bitter gustatory stimuli and lingering bitter aftertastes [33,34], these receptors can be also found in other tissues exposed to the external environment such as respiratory, urinary, and extraoral gastrointestinal systems, where they operate independently of conscious taste. Here they are often associated with the ciliated epithelial cells, and contribute to innate immune defenses, production of type 2 immune cytokines IL-4 and IL-13, and prevention of pathogen invasion [35,36]. Additionally, bitter ligands induce relaxation of smooth muscles in airways [37], vascular system [38], and the gut where the gastric emptying is also delayed [39]. Additionally, many of the blood cells express functional TAS2Rs, including leukocytes [40] and monocytes [41] that seem to respond to bitter ligands with the chemotactic transmigration. Since blood cells, as well as brain and heart tissues, are not directly exposed to the

external environment, there is also a high chance that endogenous TAS2R ligands exist, as it was shown for bile acids (TAS2R1, TAS2R4, TAS2R14, TAS2R39, TAS2R46) [42] and bitter peptides [43].

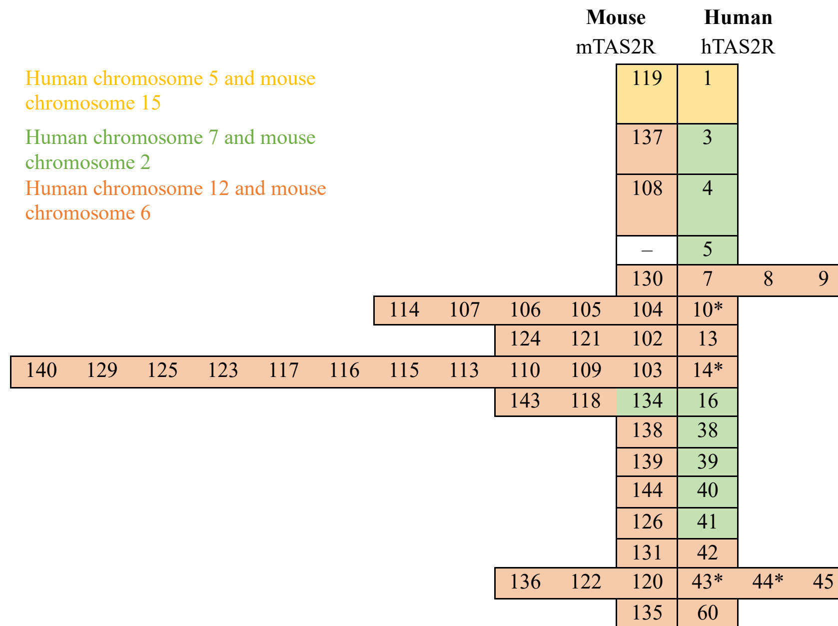

Figure 2.1. Evolutionary relationships between the ortholog human (hTAS2R) and mouse (mTAS2R) bitter taste receptors, summarized after [28,29]. Clustering is based on multiple sequence alignment of the individual bitter receptors, and their chromosomal localization is color-coded. Human TAS2R2, 12 (26), 15, 18, 62, 63, and 64 are not listed due to nonfunctional pseudogene status. Human TAS2R44 (31), 47 (30), 48 (19, 23), 49 (20), and 50 (51) gene names are synonymous. Human TAS2R5 does not seem to have an ortholog in mice. Asterisk (*) denotes human TAS2R with broad specificity.

2.5.1. Gastrointestinal bitter receptors and neuroendocrine regulation

The extraoral distribution of the human TAS2R receptors follows several clear trends. While all 25 TAS2R receptors are expressed in the oral cavity, the colon tissues do not express a cluster of related receptors TAS2R7, TAS2R8, and TAS2R9 (mouse ortholog mTAS2R130), as

well as two receptors with broad specificity, TAS2R16 and TAS2R41 (mouse ubiquitous orthologs mTAS2R143 and mTAS2R126). This abundance of bitter receptors may be driven by higher amounts of microbiota and the microbial bitter ligands at these sites. Finally, two TAS2R genes from the chromosome 7, TAS2R4 and TAS2R38, are ubiquitously expressed throughout the gut, and this pattern is evolutionally conserved (mouse orthologs mTAS2R108 and mTAS2R138). The ubiquitous gut expression of related TAS2R48 and TAS2R49 is also observed in humans, but not rodents that express mTAS2R143 and mTAS2R126 instead (Figure 2.2).

Location	**Chr.**																
Oral cavity	5	1															
	7	3	4	5	16	38	39	40	41								
	12	7	8	9	10 *	13	14 *	42	43 *	44 *	45	46 *	47	48	49	50	60
Stomach	5																
	7		4	5													
	12				10 *		14 *			44 *				48	49		
Duodenum	5																
	7		4	5		38											
	12				?		14 *		?	?	?			?	49		
Jejunum	5																
	7	3	4	5		38	39	40									
	12	7	8		10 *	13	14 *		43 *	44 *	?	46 *	47	48	49		
Ileum	5																
	7	3	4	5		38											
	12				10 *		14 *		43 *	44 *				48	49		
Colon	5	1															
	7	3	4	5		38	39	40									
	12				10 *	13	14 *	42	43 *	44 *	45	46 *	47	48	49	50	60

Figure 2.2. Expression profile of the TAS2R bitter receptors in the different regions of the human gastrointestinal tract, summarized after [88]. Human TAS2R44 (31), 47 (30), 48 (19, 23), 49 (20), and 50 (51) gene names are synonymous. Asterisk (*) denotes human TAS2R with broad specificity. Question marks (?) indicate no current data availability.

The early connection between TAS2R receptors, bitter chemosensing, and metabolic regulation was established when a large number of the TAS2R promoters were reported to contain the binding sites for the sterol regulatory element-binding protein 2 (SREBP-2, encoded by the SREBF2 gene), so that low-cholesterol diet increased the sensitivity of the intestinal TAS2R signaling system [44], stimulated CCK secretion, delayed gastric emptying, and decreased food intake [45]. These findings were also extended in enteroendocrine cell models STC-1 and NCI-H716 to PYY [46] and GLP-1 in the Amish Family Diabetes Study [47]. The effects on TAS2R gene expression and correlation with GLP-1 increases in response to different classes of bitter plant phytochemicals were confirmed in a preclinical model [48]. The colocalization of TAS2R5 and GLP-1 was also confirmed in human duodenal and ileac tissues [49].

2.5.2. Bitter receptor activation and carbohydrate metabolism in humans

The multitude of data suggests that beyond the two primary functions of the gastrointestinal bitter TAS2R chemoreceptors (recognition of bitter toxins in the upper gut and the bitter signaling crosstalk with microbiome in the distal portions of the tract), they also contribute to the luminal content sensing in the small intestine [50]. More specifically, since plant-based foods are the only sources of both bitter tasting phytochemicals and carbohydrates in human diets, our earlier studies hypothesized that a particular subset of the gastrointestinal TAS2Rs gained the function to prime or modulate the body carbohydrate metabolism in anticipation of carbohydrate loads associated with bitter plant foods [51]. This can be achieved with a direct inhibition of glucose uptake in the jejunum where TAS2R receptors, the sodium-glucose cotransporter 1 (SGLT1), and the fructose transporter SLC2A5 (GLUT5) colocalize [52], or with a possible indirect effect on the low-affinity basolateral monosaccharide transporter SLC2A2 (GLUT2) that enables sugar transfer from

enterocytes into the bloodstream. At the same time, TAS2R receptors also colocalize in the gastrointestinal enteroendocrine cells that express and secrete GLP-1 [53], with direct effects on insulin secretion and improved postprandial glucose responses. This hypothesis provides a possible explanation why diverse, unrelated classes of nontoxic bitter phytochemicals rapidly modulate carbohydrate metabolism while not sharing a common chemical structure or pharmacophore [48]. In addition to GLP-1 (22%), the incretin-mediated insulin response is also dependent on the glucose-dependent insulinotropic polypeptide (GIP, 44%) and glucose itself (33%) [54], suggesting a vast underexplored area of metabolic regulation that could be harnessed for novel dietary interventions.

Cinchona bark (Cinchona officinalis L.) is a bitter spice that yields alkaloid quinine found in a variety of modern drinks including tonic water, gin cocktails, wine blends (Dubonnet, Malaga Quina, Barolo Chinato), and soft drinks (Irn-Bru, Paso de los Toros, Faxe Kondi). Quinine content in foods is limited to 83 mg/l in the US and 100 mg/l in Europe [55]. The hypoglycemic effect of quinine is known in association with the treatment of malaria [56] and consuming gin & tonic cocktails [57]. Intragastric administration of quinine at 275 and 600 mg to 15 healthy subjects decreased the glycemic response (AUC120) to a nutrient drink by -9% to -14% without slowing gastric emptying [58]. Similarly, both intragastric and intraduodenal administration of 600 mg quinine to 14 healthy subjects prior to a nutrient drink decreased peak postprandial blood glucose by -11% to -14% [59]. These effects were slightly more pronounced in female subjects (n=13/sex), and were also associated with plasma GLP-1, CCK, C-peptide, and insulin levels [60].

Gentian root (Gentiana lutea L.) is a bitter herb that contains secoiridoid glycosides amarogentin and gentiopicrin and is widely used in bitter preparations (Suze, Salers, Aveze, Amaro, Angostura, among others) and soft drinks (Moxie). The aqueous extract of the root was

coated with ethylcellulose [61] to provide 100 mg of secoiridoids to 20 healthy subjects and resulted in 30% decrease in energy intake, as well as a trend for a higher GLP-1 response [62]. A 1:1:1 mixture of gentian root, cinchona bark, and chicory root in 600 mg capsules was tested in 31 overweight subjects consuming a 40% hypocaloric diet for 90 days, and showed a prolonged satiety accompanied by -5.9% decrease in fasting blood glucose and -11.4% decrease in body weight [63].

Hops flower (Humulus lupulus L.) is a bitter herb rich in alpha acids (humulone, α-lupulic acid) commonly used as a bittering agent in beer. The 100 mg and 250 mg capsules containing 51.5% alpha acids were given to 30 healthy fasted subjects and resulted in 10% reduction in the self-reported hunger scores [64]. In another study, capsules containing 8-48 mg of isohumulones were given to 94 prediabetic subjects daily for 4 months and resulted in -4.6% reduction in fasting blood glucose and -0.3% reduction in HbA1c [65]. Similar findings were observed for the model bitter substance, denatonium benzoate, following its intragastric infusion in healthy female subjects [66].

2.6. Polyphenols, small phenolic acid metabolites, and bitter receptors

The multitude of studies also point to the fact that bitter polyphenols in herbs and spices can also improve glucose tolerance by stimulating gastrointestinal hormone secretion, although many of the studies focused primarily on coffee chlorogenic acids [67], tea catechins [68], and blackcurrant anthocyanins [69] without a direct connotation to their interactions with the gastrointestinal bitter receptors. The realization that many polyphenols and their metabolites taste bitter to a certain degree was largely obscured by the fact that this bitterness is highly variable and depends on their glycosylation status, changes in hydroxylation and methylation profiles, as well

as the degree of polymerization. At some point, condensation and/or polymerization reactions in polyphenols shift the perception of bitterness towards astringency, which does not depend on direct interactions with bitter receptors but instead relies on formation of stable complexes with proteins that convey a drying or puckering sensation.

This perception has changed in the recent years as the information about interactions of different phenolic compounds with the individual bitter taste TAS2R receptors started to accumulate in cell culture [70] and preclinical models [48], appeared in databases dedicated to bitter ligands and the associated bitter taste receptors, such as BitterDB [71], and was utilized in the ML-based prediction tools for identifying putative ligand-TAS2R interactions, such as BitterX [72]. The current prediction algorithms routinely achieve 76-82% accuracy, which allows for the effective modeling of large bitter compound libraries [73].

6.1. Anthocyanins

A significant number of herbs and spices are rich in anthocyanins, including blackcurrants (Ribes nigrum L.), roselle (Hibiscus sabdariffa L.), kokum (Garcinia indica Chois.), and dark varieties of basil (Ocimum basilicum L.) and perilla (Perilla frutescens (L.) Britton). The parental structures of anthocyanin glucosides showed a high potency score for putative activation of up to 11 human TAS2Rs. These scores diminished when the parental structures were broken down to small phenolic acids and their metabolites, together with a clear change in the predicted TAS2R activation profiles. The final phenolic breakdown metabolites immediately prior their mineralization [74] were predicted to virtually not be recognized by the human TAS2Rs (Table 2. 2).

Table 2.2. Putative interactions (% binding probability) of a model anthocyanin, its aglycone, and small phenolic metabolites with TAS2R receptors calculated using the BitterX ML-based model [72]. The thresholded weighted potency score (PS) was calculated as sum of probabilities above 50% threshold (activation strength) divided by a total number of receptors and multiplied by the number of activated receptors (activation breadth).

Broad								*		*							*	*		*		PS
hT2R	**1**	**3**	**4**	**5**	**7**	**8**	**9**	**10**	**13**	**14**	**16**	**38**	**39**	**40**	**41**	**42**	**43**	**44**	**45**	**46**	**47**	
C-GA				75	73					72	65	57	76		61		67	60		62	57	319
C-R				78	75					65	61	52	69		60		60	57		54	60	304
C-G				75	67					71	65	51	74		60		66	59		61	54	309
C	62			62						73			78		58		63					95
CA	67							53		71	66		59									63
FA	68		54					60		72	70		58		54							122
DHC	67							53		69	67		53		53							87
DHF	67							57		69	69		55		56							90
COA	59		69					59		69												41
PCA	57									55												9
VA	59									57	52						53					35
PAA	68		78					76		66	64		55		58							130
HVA	64							52		71	66		54		52							86
HBA	73		53					59		77	69				56							93
BA	72		77					74		68	66				54							99
PHG										60			51									9
PG													51									2
CAT	60									59			60		57							38

Asterisk (*) denotes human TAS2R with broad specificity. The parent compounds were cyanidin-3-rutinoside (C-RG), cyanidin-3-(6-acetylglucoside) (C-GA), cyanidin-3-glucoside (C-G), as well as the cyanidin aglycone (C) and its small phenolic metabolites caffeic acid (CA), ferulic acid (FA), dihydrocaffeic acid (DHC), dihydroferulic acid (DHF), p-coumaric acid (COA), protocatechuic acid (PCA), vanillic acid (VA), phenylacetic acid (PAA), homovanillic acid (HVA), 4-hydroxybenzoic acid (HBA), phloroglucinol (PHG), pyrogallol (PG), and catechol (CAT).

2.6.2. Proanthocyanidins

Proanthocyanidins is another group of polyphenols abundant in herbs and spices, particular in cinnamon (Cinnamomum cassia (L.) J.Presl) and cocoa powder (Theobroma cacao L.). However, proanthocyanidins are found in these powders in an average degree of polymerization that ranges from 4 to 10, with monomers and dimers present only at the level of 5-10% of the

mixture. The thresholded weighted potency scores of these compounds suggested that they do not interact with bitter TAS2R receptors at the level of trimers and above. Also similar to anthocyanins, small phenolic metabolites generated from proanthocyanidin breakdown showed diminished capacity to activate TAS2Rs (Table 2.3). This observation may explain why large doses on cinnamon are necessary to observe its effects on postprandial glycemia levels in humans, and why these observations remain inconsistent among the different studies [75].

Table 2.3. Putative interactions (% binding probability) and the potency score (PS) of a model proanthocyanidin, its monomeric units, and small phenolic metabolites with TAS2R receptors calculated using the BitterX ML-based model [72].

Broad								*		*							*	*		*		PS
hT2R	**1**	**3**	**4**	**5**	**7**	**8**	**9**	**10**	**13**	**14**	**16**	**38**	**39**	**40**	**41**	**42**	**43**	**44**	**45**	**46**	**47**	
E1																						0
D1												51										2
C1																						0
B1				74	77					72		56	74	71	58		63	56		53	57	313
A1				73	75					69		51	67	63	51		62	61		56	58	302
CT	64			59						72	54		72		63		59					124
ECT	64			58						71	52		73		62							91
VAL	55		63					63		75			62		59		57			51		155
HAA	60									68			52		54							37
EGC	59			57						68			72		55		55					88
GCG				64	67					71			77		51		65			55		126
GA	93									98	96		87				94					94

Asterisk (*) denotes human TAS2R with broad specificity. Large proanthocyanidin pentamers (E1), tetramers (D1) and trimers (C1) were predicted not to interact with TAS2Rs. The parent proanthocyanidin dimers were B1 and A1, as well as their metabolites catechin (CT), epicatechin (ECT), 5-(3'-hydroxyphenyl)-γ-valerolactone (VAL), 3-(3'-hydroxyphenyl)-hydracrylic acid (HAA), and gallo derivatives epigallocatechin (EGC), epigallocatechin gallate (GCG), and gallic acid (GA). Asterisk (*) denotes human TAS2R with broad specificity.

2.6.3. Other phenolic compounds

Spices and herbs are also particularly abundant with flavonols (quercetin, kaempferol, myricetin) and flavones (apigenin, luteolin), among others. These flavonoids interact with bitter TAS2RS similar to anthocyanins in that the respective di- and mono- glucosides are perceived as more bitter, and their predicted bitterness decreases as these structures are metabolized (Table 2.4).

Table 2.4. Putative interactions (% binding probability) and the potency score (PS) of model flavonoids with TAS2R receptors calculated using the BitterX ML-based model.

Broad								*		*							*	*		*		**PS**
hT2R	**1**	**3**	**4**	**5**	**7**	**8**	**9**	**10**	**13**	**14**	**16**	**38**	**39**	**40**	**41**	**42**	**43**	**44**	**45**	**46**	**47**	
QR				78	72					62	52		68		57		53				53	158
QG				76	64					68	58		72		57		62	53		55		203
Q	57			66						73			76		55		62					93
API				73	71					69	58	52	70		58		62	57		58	57	301
AG	54			68	57					75	60		78		59		64			57		206
A	62			57						77			81		61		56					95

Asterisk (*) denotes human TAS2R with broad specificity. Parent structures were quercetin rutinoside (QR), quercetin glucoside (QG), and quercetin aglycone (Q), as well as apiin (apigenin diglycoside, API), apigenin-7-O-glucoside (AG), and apigenin aglycone (A).

Lower postprandial glycemia was confirmed in clinical studies after consumption of fenugreek (Trigonella foenum-graecum L.) [76], amla (Phyllanthus emblica L.) [77], basil (Ocimum tenuiflorum L.) [78], and turmeric (Curcuma longa Linn.) [79], among others. The molecular mechanisms behind these effects were also evaluated in combination studies with healthy volunteers that consumed 150 mg of coffee chlorogenic and 540 mg of green tea catechols, resulting in acute beneficial effects on postprandial glucose, insulin, and incretin responses to a high-fat and high-carbohydrate cookie meals [68]. This is also true for other glycosylated secondary metabolites, such as steviol glycosides [80], secoiridoid glycosides [81], glucosinolates [82], and sesquiterpene lactones [83]. These findings indicate that consuming herbs and spices that

contain polyphenols capable of activating bitter TAS2Rs may stimulate the release of incretin hormones from specialized cells in the gastrointestinal tract, trigger insulin secretion, and ultimately reduce postprandial blood glucose levels within a few hours after a meal.

2.7. Rediscovering bitter in modern diets

Consistent with the human studies described above, both ancestral and modern higher quality diets are expected to be intrinsically more bitter, as they tend to include a greater diversity of plant-based foods rich in secondary metabolites. Encouraging the consumption of bitter foods or adding the desired bitterness to foods in the form of spices and herbs, may help recondition taste preferences, especially in populations habituated to hyper-palatable, highly processed foods [84]. Over time, this could contribute to greater dietary variety, improved nutrient density, and enhanced metabolic resilience [85]. Therefore, promoting acceptance of bitterness may be a powerful strategy to shift eating behaviors toward healthier, more sustainable diets.

This statement also extends to modern cultivars of spices, herbs, and grains that were selectively bred for milder flavors, often at the expense of their original bitter and astringent phytochemical profiles [17]. As a result, many of these cultivars may have reduced concentrations of bioactive compounds that contribute to metabolic health. Reintroducing or preserving the bitter traits of traditional varieties could enhance both the functional and nutritional value of these dietary staples. This is substantiated by observing the rates of glucose uptake in the intestinal cells after exposure to digests from the AFRI CORE oat worldwide diversity panel with different levels of bitter-tasting secondary metabolites (Figure 2.3).

These observations imply that the reduction of bitter phytochemicals during crop domestication may have inadvertently diminished natural glucose-regulating mechanisms and

adaptive hormonal responses that optimize nutrient handling. An alternative approach to achieve similar dietary effects is incorporating select spices and herbs into foods and beverages as a practical way to reintroduce beneficial bitterness into modern diets. Unlike purified metabolites, spices and herbs deliver these phytochemicals in complex fiber-rich or oil-based matrices [86] that facilitate delayed release in the gastrointestinal tract, unless left to cook for a long time. Reintroducing these traditional flavors also aligns with a broader movement toward functional, health-promoting diets.

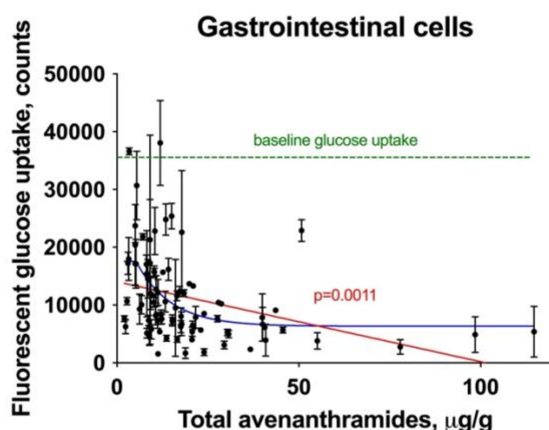

Figure 2.3. Fluorescent 2-NBDG glucose uptake in the STC-1 intestinal cell model after exposure to aqueous oat digests from the 109 AFTI CORE phenotypic oats panel. Cells were incubated with treatments for 2 hours, presented with 2-NBDG for 30 min, and fluorescence was quantified at excitation/emission of 465/540 nm.

2.8. Conclusions

The widespread distribution of TAS2R bitter receptors throughout the gastrointestinal tract highlights their multifunctional roles beyond taste, including site-specific effects on nutrient absorption and microbiome interactions. Activation of gastrointestinal TAS2Rs by plant-derived bitter compounds stimulates the release of key hormones such as GLP-1 and CCK, promoting better glucose regulation, insulin secretion, and appetite control. Thus, rediscovering and

reintegrating bitter flavors from common spices and herbs into modern diets offers a promising strategy to improve metabolic health and dietary quality. Broadening dietary exposure to bitter phytochemicals could represent a simple yet powerful step toward more resilient and health-promoting food systems.

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Chapter 3: Direct Supplementation with Small Phenolic Acids Improves Adiposity and Voluntary Physical Activity in Obese Mice

3.0. Abstract

Small phenolic metabolites can modulate the interconnected metabolic pathways, influencing nutrient transport, energy metabolism, and wellbeing. We previously observed that berries enriched with methylated phenolic metabolites counteracted mitochondrial dysfunction and reduced adiposity in preclinical studies. Here, we aimed to investigate the effects of methylated phenolic metabolites by supplementing diets with phenolic acids (PA) and specifically methylated (MPA) or non-methylated (NMPA) phenolic acids, to assess their ability to mitigate metabolic damage from high-fat diets. C57BL/6J mice were fed either low-fat (LFD) or high-fat (HFD) control diets for 6 weeks, followed by supplementation with phenolic acids for an additional 6 weeks. The HFD mice supplemented with phenolic acids showed significant reductions in body weight (-13.5% to -16.2%, $p<0.05$) and fat mass (-37.2% to -40.6%, $p<0.05$), with MPA intervention leading to the most pronounced effects. Animals on MPA supplementation also exhibited increased heat production (5.5%-8.6%, $p<0.05$) and more active behavior (23.0% to 55.2%, $p<0.01$). In vitro, protocatechuic and caffeic acids were better at suppressing LPS-induced inflammation in macrophages, while methylated derivatives improved thermogenic and metabolic capacity of the adipose tissues. These findings highlight the potential of methylated phenolic metabolites to improve metabolic health, warranting further investigation in clinical settings.

3.1. Introduction

Small phenolic metabolites, including phenolic acids and flavonoid-derived catabolites, play a crucial role in regulating interconnected metabolic pathways [1]. Rather than acting through a single receptor, they influence multiple biochemical networks, including nutrient transport, energy metabolism, immune regulation, and neurotransmitter synthesis [2]. Their biological effects stem from broad regulatory interactions, such as modulating oxidative stress, AMP-activated protein kinase (AMPK) signaling, and nuclear factor kappa B (NF-κB) activity, and beta-estrogen receptor signaling among many others [3]. Some of them have a bitter taste and can activate TAS2R receptors in the gut, reducing glucose absorption into the bloodstream [4]. Additionally, they participate in mitochondrial detoxification and nitrogen deportation processes, further integrating into core metabolic functions [5]. Structurally, they also resemble endogenous catecholamines, allowing them to possibly interact with similar enzymes and transporters [6].

Dietary phenolic acids are most abundant in coffee, tea, red wine, berries, bread (flour), some vegetables and spices [7]. It is generally estimated that about 40% to 60% of phenolic acids are ingested in the form of simple esters or glucosides, while the remaining amounts are generated from the ingested polyphenols and flavonoids [8]. A counterintuitive trend of increasing phenolic consumption from southern to northern Europe is primarily driven by higher intake of coffee and whole grains, resulting in a mean adjusted phenolic acid intake of 512 mg/day, ranging from 213 to 1,265 mg/day across different European countries [8]. These findings align with other estimates of total polyphenol intake, which range from 540 to 1,370 mg/day, with higher levels observed in regular coffee and tea consumers [9]. The most recent systematic review reaffirmed these proportions, reporting a mean total polyphenol intake of 910 mg per day, including 360 mg of flavonoids and 410 mg of phenolic acids [10]. Cinnamic acids dominate the dietary phenolic acid

profile in the descending order of hydroxycinnamic acids (84-95%), hydroxybenzoic acids (5-14%), hydroxyphenylacetic acids (0.1-0.8%), and hydroxyphenylpropanoic acids (<0.1%) [8].

Similar to other polyphenols, phenolic acids differ in their hydroxylation and methylation patterns that directly affect their bioactivity, bioavailability, tissue distribution, and excretion [11]. Gut epithelial cells, smooth muscle cells, liver, and blood vessels are likely exposed to higher concentrations of polar phenolic acids, while dihydro- and methylated phenolic metabolites have greater potential to reach distant sites, where they may exert their biological effects [12]. Increased hydrophobicity of the phenolic ring metabolites reduced plasma residence time and enhanced tissue affinity, suggesting these molecules are more likely to reach cellular and mitochondrial targets by crossing multiple membranes [13]. This impact of methylation and hydrophobicity on the structure-activity relationships of phenolic metabolites remains largely unexplored, leaving a critical gap in understanding their varying abilities to modulate health and well-being outcomes in different tissues.

We first noticed that berries enriched with methylated phenolic metabolites enhanced mitochondrial respiration in adipose tissue, helping to counteract mitochondrial dysfunction linked to metabolic stress and reducing adiposity in cell models [14]. This study aims to build on these findings, demonstrating that supplementation with phenolic acids alone can accomplish similar metabolic outcomes, and that higher methylated metabolites are more effective at reducing metabolic damage from high-fat diets. We therefore recreated the total phenolic acid profile (PA) based on the nutritional data of 36,037 subjects aged 35-74 years from the European EPIC study [8], and tested this hypothesis by reformulating this profile to include only methylated (MPA) or only non-methylated (NMPA) phenolic acids. Our main objective was to selectively increase

phenolic acids, and specifically their methylated derivatives, in the diet to evaluate a novel strategy for mitigating metabolic stress and improving overall metabolic health.

3.2. Materials and Methods

3.2.1. Reagents and supplies

Phenolic acids were purchased from Sigma (St. Louis, MO, USA), mixed in the appropriate proportions as described in Table 1, and incorporated into the respective low-fat diet (LFD, D12450J, 10 kcal % fat, 3.85 kcal/g) or high-fat diet (HFD, D12492, 60 kcal % fat, 5.24 kcal/g) at 0.28% total added phenolic acids by Research Diets (New Brunswick, NJ, USA).

Table 3.1. Study diets with added phenolic acids (%).

			LFD or HFD diets, mg/kg food			
Phenolic acid	**Abbreviation**	**Methylated**	**Controls**	**PA**	**MPA**	**NMPA**
Protocatechuic acid	3,4-DHBA	No	–	13.2	–	34.0
Gallic acid	3,4,5-THBA	No	–	155.2	–	186.4
3,4-Dihydroxy-phenylacetic acid	3,4-DHPAA	No	–	1.0	–	21.8
Caffeic acid	3,4-DHCA	No	–	2,074	–	2,540
Vanillic acid	3M-4HBA	Yes	–	20.8	34.0	–
Syringic acid	3,5DM-4HBA	Yes	–	31.2	186.4	–
Homovanillic acid	3M-4HPAA	Yes	–	20.8	21.8	–
Ferulic acid	3M-4HCA	Yes	–	466.4	2,540	–
Total added NMPA		Yes	0.00%	0.21%	–	0.28%
Total added MPA		No	0.00%	0.07%	0.28%	–
Total added PA		All	0.00%	0.28%	0.28%	0.28%

3.2.2. Animals and diets

Male 4-week-old C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and housed four animals per cage under controlled temperature (24 ± 2 °C) and light conditions (12 h light–dark cycle, lights on at 7:00 a.m.). Immediately upon arrival, animals were allowed to adapt to the new conditions for 7 days, and the animals were handled

daily to reduce the stress of physical manipulation. Mice were then randomized based on the initial body weights into ad libitum access to LFD (n = 32) or HFD (n = 48) for 6 weeks to induce development of obesity and metabolic disfunction.

At this point, obese animals were evaluated based on their body weights and sorted into normally obese mice (HFD, n=32), mice that were resistant to obesity (NR, n=8), and the super-responder animals that gained extreme amounts of body weight in the first weeks of the study (SR, n=8) as described previously [15]. NR and SR animals were removed from the study into a separate arm that was evaluated independently (Figure 3.1).

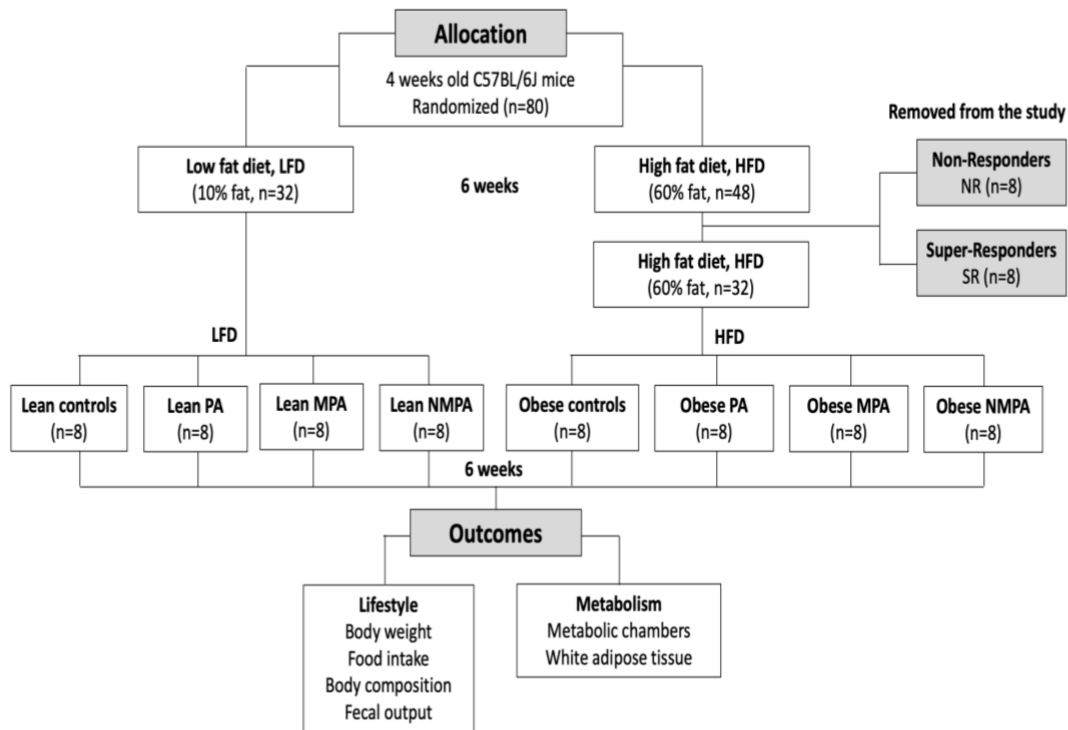

Figure 3.1. Flow chart of the study. All lean animals consumed low-fat diet (LFD), and all obese animals consumed high-fat diet (HFD) for the duration of the study. The intervention diets were supplemented with 0.28% (w:w) of 8 phenolic acids as an equal mixture of non-methylated and methylated compounds (PA), the matching amount of 4 methylated phenolic acids only (MPA),

and the matching amount of 4 non-methylated phenolic acids only (NMPA) as summarized in Table 3.1.

Both lean LFD mice (n=32) and obese HFD mice (n=32) were then randomized into four dietary treatment groups each (n=8), including the respective controls (LFD or HFD), or animals supplemented with a mixture of 4 non-methylated phenolic acids (NMPA), a mixture of 4 methylated phenolic acids (MPA), or an equal mixture (1:1) of both non-methylated and methylated phenolic acids (PA) at the same total concentration of 0.28% (w:w) in the respective diet (Table 1). All animal experiments were performed according to procedures approved by the NC Research Campus Institutional Animal Care and Use Committee in the David H. Murdock Research Institute (Kannapolis, NC, USA), an AAALAC-accredited animal care facility (protocol No. 19-009, approved on 6 May 2019).

3.2.3. Body weights, food intake, and body composition

Animal weights and food intakes (accounting for spillage) were recorded weekly for the duration of the study. Body composition analysis was performed on unanesthetized mice using EchoMRI (Echo Medical Systems, Houston, TX, USA) during the last week of the study.

3.2.4. Energy expenditure

An open-circuit LabMaster Metabolism Research Platform (TSE Systems, Bad Homburg, Germany) was used to assess indirect calorimetry and activity at the Animal Metabolism Phenotyping Core, UNC Nutrition Obesity Research Center. Rates of oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were recorded in accordance with a reference cage every 30 min for 48 h. VCO_2/VO_2 was defined as the respiratory exchange ratio (RER), and energy expenditure (EE) was estimated using the equation = $[3.815 + (1.232 \times RER)] \times VO_2$. The non-protein respiratory quotient table [16] was used to compute lipid and carbohydrate oxidation rates.

The ActiMot system (TSE) was applied to measure movement activity by measuring infrared beam breaks in horizontal (XT+YT directions, running) and vertical (ZT direction, rearing) planes, as well as the distance traveled within the cage (DistD). All measurements were performed in individual animals (n=4) and averaged in 30 min intervals.

3.2.5. Fecal parameters

Fecal pellets were collected over one day the end of the study. Fresh trays were gently removed, and pellets were collected in Eppendorf tubes using clean forceps. Pellets in contact with other surfaces were excluded. After collection, pellets were counted, weighed individually, and immediately frozen at −80 °C. The 24 h fecal outputs were obtained from the animals housed in the metabolic chambers (TSE Systems).

3.2.6. Macrophage RAW 264.7 Cell Culture

The mouse macrophage cell line RAW 264.7 (ATCC code TIB-71) were maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies), supplemented with 10% fetal bovine serum (Life Technologies), 100 IU/ml of penicillin, and 100 µg/ml of streptomycin (Fisher Scientific, Pittsburg, PA) at a density not exceeding 5 × 105 cells/ml. Cells were routinely passaged every 3-4 days in Nunc cell culture dishes (Nalge Nunc International, Rochester, NY) maintained at 37°C and 5% CO2 in a humidified Thermo Forma Series II incubator (Fisher Scientific). Cells were seeded in 24-well plates in triplicate at the concentration of 5x104 cells/well in 1 ml culture medium and allowed to adhere for 24 h. The cells were then treated with 10 µM individual phenolic acids or their mixture as indicated and elicited with 1 µg/ml LPS for additional 6 h.

3.2.7. Adipocyte 3T3-L1 cell culture

The mouse embryonic cell line 3T3-L1 (ATCC CL-173) that undergoes a preadipose to adipose conversion and expresses major pathways of glucose and lipid metabolism was obtained

from ATCC (Manassas, VA). Cells were routinely passaged every 3-4 days and maintained in high glucose DMEM containing 10% fetal bovine serum FBS (Life Technologies, Carlsbad, CA) and 1% penicillin-streptomycin (Fisher Scientific, Pittsburg, PA) at 37°C and 5% CO2. Cells were subcultured into 6-well dishes at a density of 105 cells/well (day 0), changed to fresh DMEM/FBS medium once confluent (day 2), and induced to adipose conversion by subsequently changing to D4 medium (DMEM/FBS, 1 µg/ml insulin, 500 µM 3-isobutyl-1-methylxanthine, and 0.25 µM dexamethasone) on day 4 and to D6 medium (DMEM/FBS, 1 µg/ml insulin) on days 6, 8, and 10. Cells were treated with 1000x stocks of individual phenolic acids or their mixtures in DMSO every time the cell culture media was changed on days 4, 6, 8, and 10, and compared with vehicle (DMSO at a final concentration of 0.1%) in triplicate.

3.2.8. Lipid quantification

Cells were stained with Oil red O on day 12 to visualize differentiated adipocytes and quantify lipid accumulation following an established protocol with some modifications (Mehra et al., 2007). After medium removal, cells were fixed in 10% neutral buffered formalin at room temperature for 10 min. Cells were then washed twice with ice-cold PBS and stained with 1% Oil red O in isopropanol, diluted 3:2 in PBS, for 1 h at room temperature. Following two additional PBS washes, fresh PBS was added to cover the cell surface and prevent dehydration. Cells were photographed at 40x magnification using EVOS FL Cell Imaging System (Thermo Fisher Scientific, Waltham, MA). Lipid-bound Oil red O stain was eluted by 30 min incubation with isopropanol and quantified by absorbance read at 520 nm on a Synergy H1 spectrophotometer (BioTek, Sunnyvale, CA).

3.2.9. RNA extraction, purification, and cDNA synthesis

The total RNA was isolated from Macrophage RAW 264.7 and Adipocyte 3T3-L1 cell using TRIzol reagent (Life Technologies, Carlsbad, CA) following the manufacturer's instructions. RNA was quantified using the Biotek SynergyH1/Take 3 plate (Agilent, Santa Clara, CA). The cDNAs were synthesized on ABI GeneAMP 9700 using the high-capacity cDNA Reverse Transcription kit and 2 μg of RNA (Life Technologies).

3.2.10. Quantitative PCR analysis

The resulting cDNA was amplified by real-time quantitative PCR using SYBR green PCR master mix (Life Technologies). To avoid interference due to genomic DNA contamination, only intron-overlapping primers were selected using Primer Express software, version 2.0 (Applied Biosystems, Foster City, CA).

The following primers were used to capture the core cluster of the short-lived inflammatory gene expression networks and adipocyte-related genes, including TNF-α, forward primer: 5'-GTT CTA TGG CCC AGA CCC TCA CA-3', reverse primer: 5'-TAC CAG GGT TTG AGC TCA GC-3'; IL-1β, forward primer: 5´-CAA CCA ACA AGT GAT ATT CTC CAT G-3´, reverse primer: 5´-GAT CCA CAC TCT CCA GCT GCA-3´; IL-6, forward primer: 5′-TAG TCC TTC CTA CCC CAA TTT CC-3′, reverse primer: 5′-TTG GTC CTT AGC CAC TCC TTC-3′; IL-17, forward primer: 5'-ATC TGG TCC TAC ACG AAG CC-3', reverse primer: 5'-GTC CCG GAC TTC AAG ACC C-3'; Adiponectin, forward primer: 5′-TGT TCC TCT TAA TCC TGC CCA-3′, reverse primer: 5′-CCA ACC TGC ACA AGT TCC CTT-3′; Leptin, forward primer: 5′-GAG ACC CCT GTG TCG GTT C-3′, reverse primer: 5′-CTG CGT GTG TGA AAT GTC ATT-3′; Uncoupling protein-1 (Ucp1), forward primer: 5′-AGG CTT CGA GTA CCA TTA GGT-3′, reverse primer: 5′-CTG AGT GAG GCA AAG CTG ATT T-3′; and β-actin as a housekeeping

gene, forward primer: 5′-AAC CGT GAA AAG ATG ACC CAG AT-3′, reverse primer: 5′-CAC AGC CTG GAT GGC TAC GT-3′.

Gene expression profiles in the liver were analyzed for inflammation-related TNF-α, IL-1β, IL-6 genes as well as a nuclear farnesoid X receptor FXR, forward primer 5′-CCA CCG GCT GTC AGG ATT T-3′, reverse primer 5′-GCA TAC CTT TAG CTG GCT TCA CA-3′; sterol regulatory element-binding protein 1 SREB1, forward primer 5′- GAT GTG CGA ACT GGA CAC AC-3′, reverse primer 5′- CAT AGG GGG CGT CAA ACA G-3′; proglucagon (GCG) precursor of GLP-1, forward primer: 5′-TGA AGA CCA TTT ACT TTG TGG CT-3′, reverse primer: 5′-CCA AGT GAC TGG CAC GAG AT-3′; and the associated receptor GLP-1R, forward primer: 5′-CAG GGC TTG ATG GTG GCT ATC-3′, reverse primer: 5′-CGC TCC CAG CAT TTC CG-3′.

Quantitative PCR (qPCR) amplifications were performed on an ABI 7500 Fast real-time PCR (Life Technologies) using 1 cycle at 50 °C for 2 min and 1 cycle at 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The dissociation curve was completed with 1 cycle of 1 min at 95 °C, 30 s at 55 °C, and 30 s at 95 °C. mRNA expression was analyzed using the ΔΔCT method and normalized with respect to the expression of the β-actin housekeeping gene. Amplification of specific transcripts was confirmed by obtaining melting curve profiles.

3.2.11. Statistical Analysis

Data were analyzed by one-way ANOVA followed by Dunnett's multiple-range tests using Prism 8.0 (GraphPad Software, San Diego, CA, USA). Temporal measures were analyzed by two-factor repeated-measures ANOVA, with time and treatment as independent variables.

All data were presented as means ± SEM. Significant differences were accepted when the p-value was <0.05.

3.3. Results

3.3.1. Phenolic acid composition of intervention diets

The total added phenolic acid profile (PA) was formulated based on the nutritional data of 36,037 subjects aged 35-74 years from the European EPIC study [8] and included 31.2 mg/kg food of protocatechuic acid, 20.8 mg/kg food of vanillic acid, 155.2 mg/kg food of gallic acid, 31.2 mg/kg food of syringic acid, 1.0 mg/kg food of 3,4-dihydroxyphenyl acetic acid, 20.8 mg/kg food of homovanillic acid, 2,074 mg/kg food of caffeic acid, and 466.4 mg/kg food of ferulic acid for a total of 2,800 mg/kg food (Table 3.1). The respective methylated (MPA) and non-methylated (NMPA) diets were formulated by including only methylated or unmethylated phenolic acids from each matched pair (Figure 3.2). The resulting PA diet matched the previously reported ratios [8], and the MPA and NMPA diets were developed to investigate the effects of consuming similar amounts of phenolic acids with only methylated (MPA) or only non-methylated (NMPA) moeities. Phenylpropanoic acids have not been included in formulations due to negligible amounts present in foods.

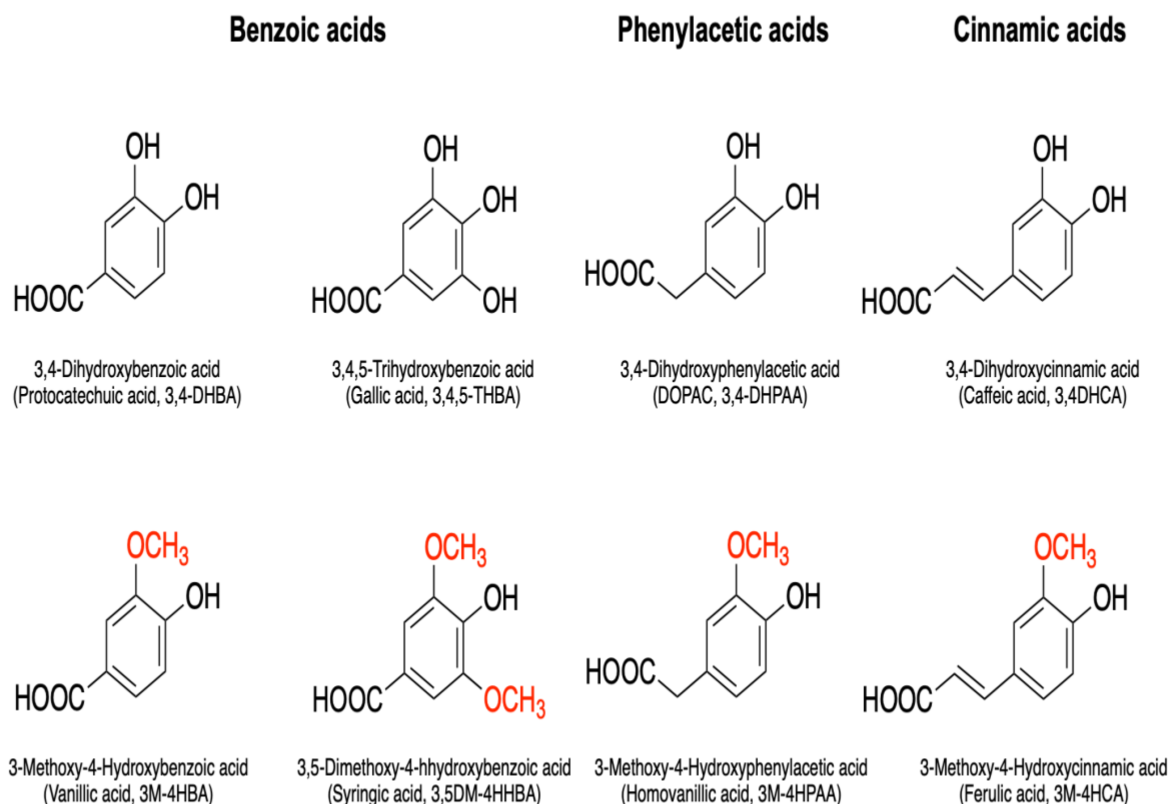

Figure 3.2. Eight phenolic acids reported as most abundant in diet were selected as matched non-methylated (top) and methylated (bottom) pairs, and incorporated in animal food as various mixtures (Table 3.1) at 0.28% total phenolic acids (w:w). Methylation sites are highlighted in red.

3.3.2. Small phenolic acids improve obesity in DIO model

C57BL/6J, a well-recognized polygenic developmental model of diet-induced obesity [17], was fed either LFD or HFD control diets for the first 6 weeks of this study. This allowed for detection of mice that showed a reduced response to the high fat diet (non-responders), as well as animals that gained excessive weight rapidly (super-responders) [15]. These two sets of animals fell within the top and bottom 15% of the HFD group, and were removed from the study to allow

for selection of 32 “obese control” mice matched to the 32 “lean control” animals on the LFD diet (Figure 3.1).

The selected animals were randomized into the respective LFD and HFD control, as well as animals receiving the interventions consisting of mixed phenolic acids (PA), methylated phenolic acids (MPA), and non-methylated phenolic acids (NMPA) incorporated into the respective diet at the same level of 0.28% (w:w) for additional 6 weeks. Among the LFD animals, there were no changes in body weight (Figure 3a) or food intake (Figure 4A) between the control and treatment animals. The HFD group, however, showed a progressive decrease in body weight gain in animals consuming the PA (-13.3% at week 11 and -16.2% at the end of the study, and MPA treatments (-12.6% and -13.5%, respectively) ($p<0.05$ and $p<0.01$), but not the animals on the NMPA intervention (Figure 3.3B). This decrease did not correlate with the food intake that showed a trend to increase in the HFD-PA and HFD-MPA mice for the duration of the study but did not reach significance (Figure 3.4B).

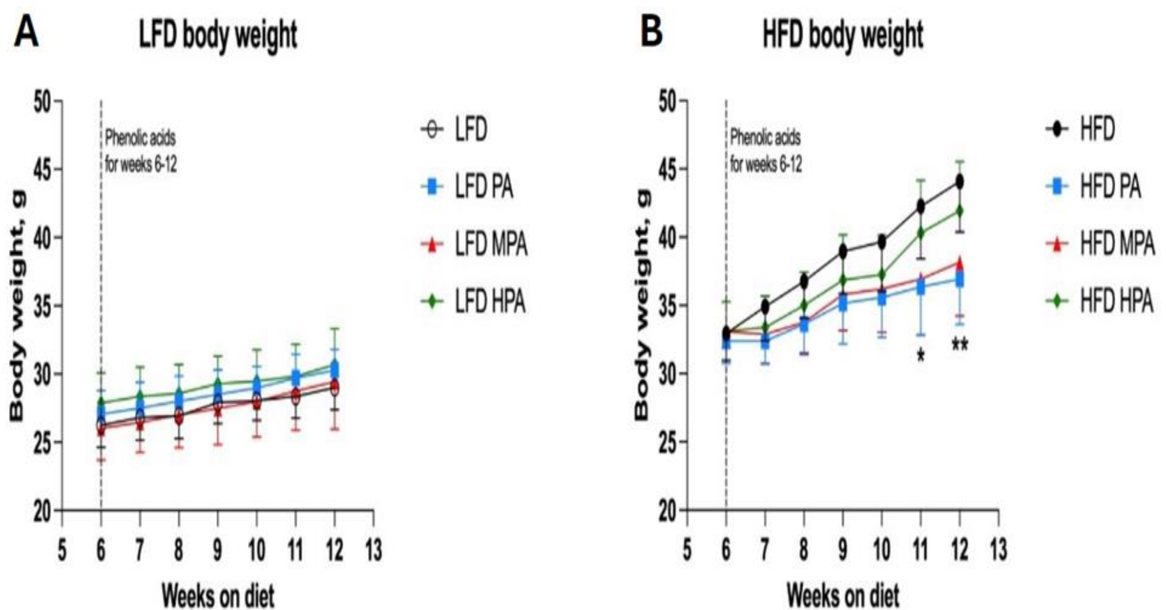

Figure 3.3. Body weight of (A) LFD lean controls or (B) HFD obese controls in comparison to the respective interventions that included 0.28% total phenolic acids (PA), four methylated phenolic acids (MPA), or the four matching non-methylated phenolic acids (NMPA) for 6 weeks of supplementation (week 6-12 of the study). Results are expressed as means ± SD (n = 8). Data were analyzed using a two-factor repeated-measures ANOVA; * $p < 0.05$, ** $p < 0.01$ versus the HFD controls.

This decrease did not correlate with the food intake that showed a trend to increase in the HFD-PA and HFD-MPA mice for the duration of the study but did not reach significance (Figure 3.4B).

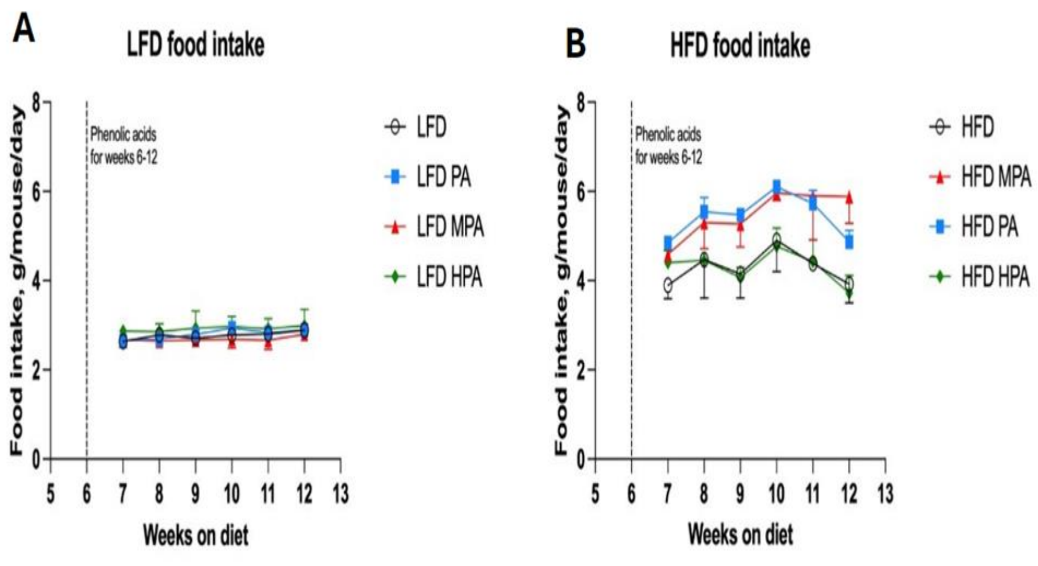

Figure 3.4. Weekly changes in food intake of (a) LFD lean controls or (b) HFD obese controls in comparison to the respective interventions that included 0.28% total phenolic acids (PA), four methylated phenolic acids (MPA), or the four matching non-methylated phenolic acids (NMPA)during the 6 weeks of supplementation. Results are expressed as means ± SEM (n = 8). Data were analyzed using a two-factor repeat-ed-measures ANOVA.

3.3.3. Changes in body composition and fecal outputs

The decreased body weight gain occurred predominantly from the reduced gain of the adipose tissue as indicated by the body composition analysis (Figure 3.5). These findings were absent in the LFD mice (Figure 3.5A) reflecting stable body weights and food intake across all LFD groups. The fat mass was however reduced in all animals receiving the phenolic acids intervention, albeit to a different degree. HFD-PA mice showed -37.2% reduction in adipose tissue, similar to HFD-MPA mice (-40.6%, $p<0.05$), while HFD-NMPA animals showed -12.4% fat reduction that did not reach significance (Figure 3.5B).

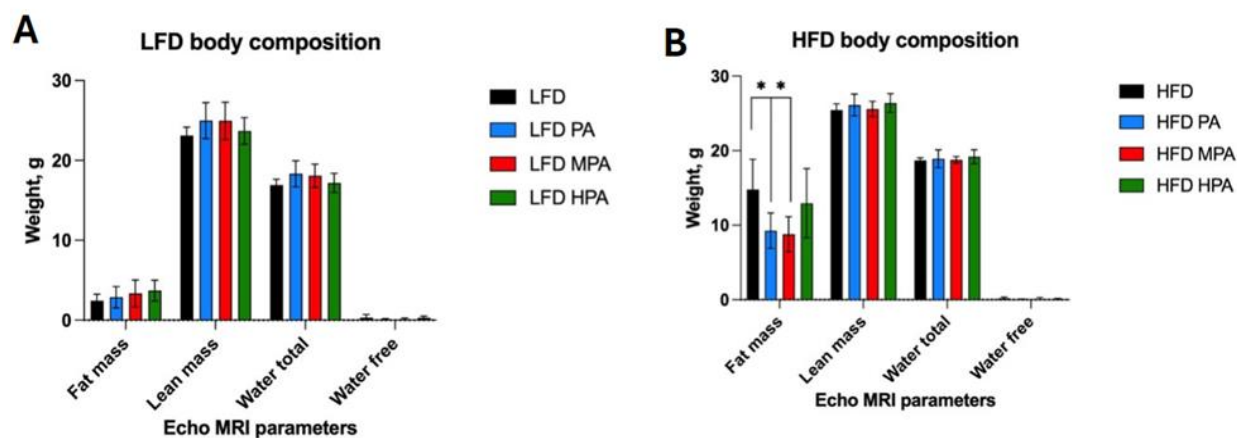

Figure 3.5. Body composition of (a) LFD lean controls or (b) HFD obese controls in comparison to the respective interventions that included 0.28% total phenolic acids (PA), four methylated phenolic acids (MPA), or the four matching non-methylated phenolic acids (NMPA) during the 6 weeks of supplementation. Results are expressed as means ± SEM (n = 8). Data were analyzed using one-way ANOVA followed by Dunnett's multiple comparisons; * $p < 0.05$ versus the HFD controls.

We next evaluated the corresponding fecal outputs in these animals. No changes were observed in the wet weights of the individual fecal pellets and 24-hour total fecal outputs in the LFD group. The individual fecal pellet weights showed much higher variability in animals consuming HFD diet and all phenolic acids interventions, moreover, the 24-hour fecal outputs were significantly reduced in all HFD-intervention groups, $p<0.05$ (Figure 6). Since food intakes remained the same, this could possibly indicate the reduced fat or water content of the feces in animals treated with the phenolic acid mixes.

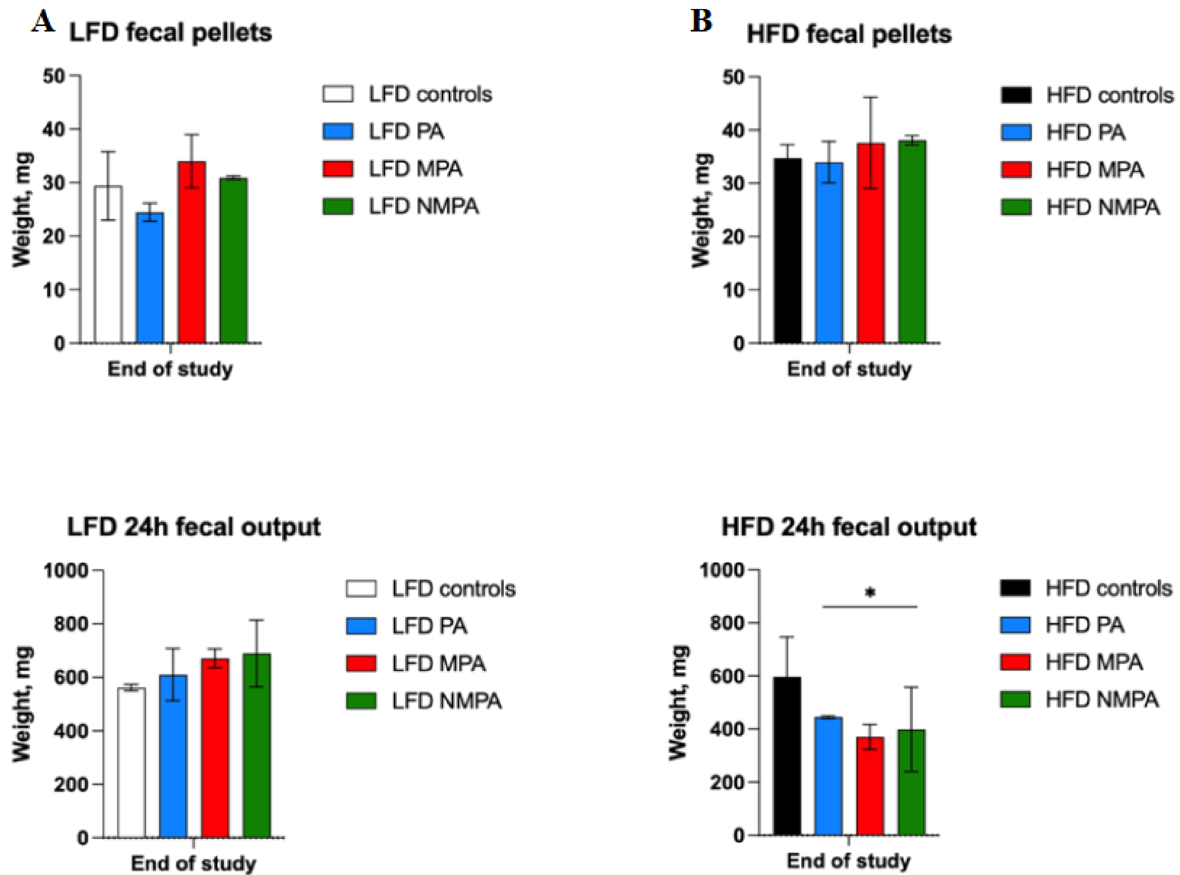

Figure 3.6. Changes in weights of individual fecal pellets after supplementation with phenolic acids, and the total fecal outputs over a 24 h period in (A) LFD lean controls or (B) HFD obese controls in comparison to the respective interventions that included 0.28% total phenolic acids (PA), four methylated phenolic acids (MPA), or the four matching non-methylated phenolic acids (NMPA) during the 6 weeks of supplementation. Results are expressed as means ± SEM (n = 8). Data were analyzed using one-way ANOVA followed by Dunnett's multiple comparisons; * $p < 0.05$ versus the HFD controls.

3.3.4. Metabolic response to phenolic acids in the diet

The open-circuit indirect calorimetry was used to estimate energy expenditure, including VO2, VCO2, heat production, respiratory quotient (RER), and voluntary movements in all animals. All metabolic parameters related to energy metabolism were normalized to lean body

mass to account for variations in animal body weight, following standard practice [18]. There were no significant changes in oxygen consumption or the respiratory quotient, indicating that all treated animals utilized energy fuels at the carbohydrate/fat ratio similar to those in the respective LFD and HFD controls without phenolic acid supplementation (Figure 7). The RER quotient was significantly higher in the LFD group (0.844) than in the HFD group (0.733), indicating the expected increase in carbohydrate metabolism in animals on a low-fat diet.

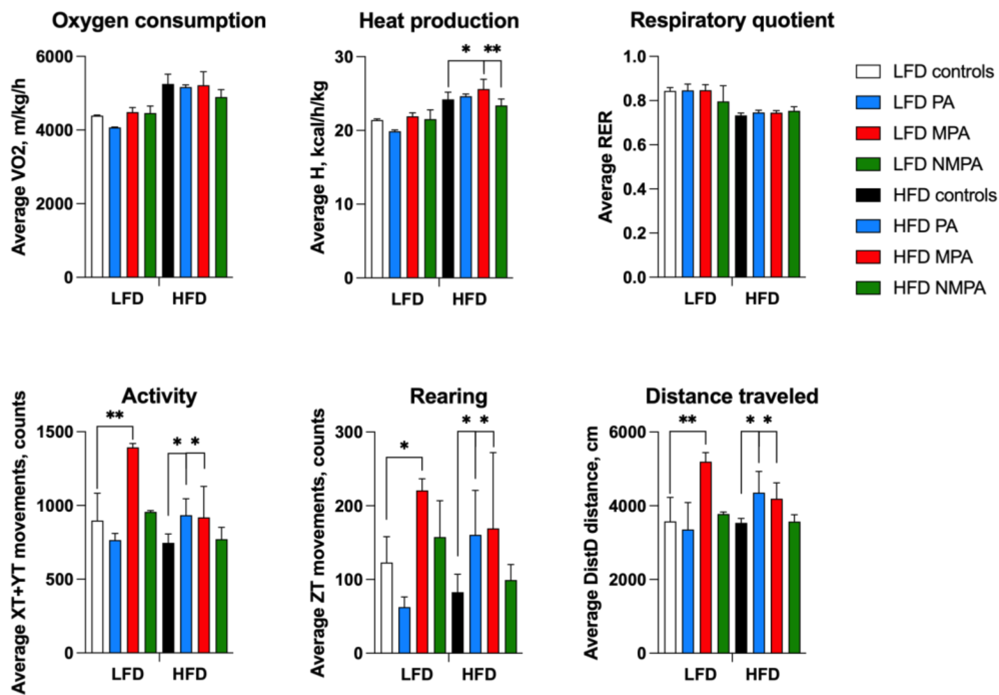

Figure 3.7. Changes in whole-body energy balance in (A) LFD lean controls or (B) HFD obese controls and in response to the respective interventions that included 0.28% total phenolic acids (PA), four methylated phenolic acids (MPA), or the four matching non-methylated phenolic acids (NMPA) during the 6 weeks of supplementation. Results are expressed as means ± SEM (n = 4). Data were analyzed using one-way ANOVA followed by Dunnett's multiple comparisons; * $p < 0.05$, ** $p < 0.01$ versus the LFD or HFD controls.

We also observed increased heat production in animals consuming methylated phenolic acid (MPA) mixture (+5.5%, $p<0.05$), and this difference was even more evident when compared to the animals on the non-methylated phenolic acid (NMPA) mixture (+8.6%, $p<0.01$) (Figure 3.7). These increases correlated with the more active behavior of animals consuming the MPA mixture, as compared to both LFD and HFD controls. This was evident from volunteer horizontal movements (X+Y), vertical rearing (Z), and the average distance traveled in the cage settings (Figure 3.7).

3.3.5. Phenolic acids in RAW macrophages

Gene expression of inflammatory genes was induced by LPS and cells were treated with individual phenolic acids or their mixtures in the physiologically relevant dose of 10 μM (Kahle et al., 2006). While there was a general trend to suppress pro-inflammatory effects by all phenolic acids, it was significant only for protocatechuic acid (TNF-α, IL-1β, and IL-6, $p<0.05$) and caffeic acid (IL-1β and IL-6, $p<0.05$) (Figure 3.8). The combinations of methylated and non-methylated phenolic acids, as well as the mixture of all phenolic acids, produced weaker overall effects. This suggests that the anti-inflammatory actions of phenolic acids in macrophages do not directly correlate with their metabolic effects in adipose tissue. However, these findings align with previous reports indicating that benzoic acids exhibit stronger anti-inflammatory properties than their cinnamic acid counterparts (Singh et al., 1984).

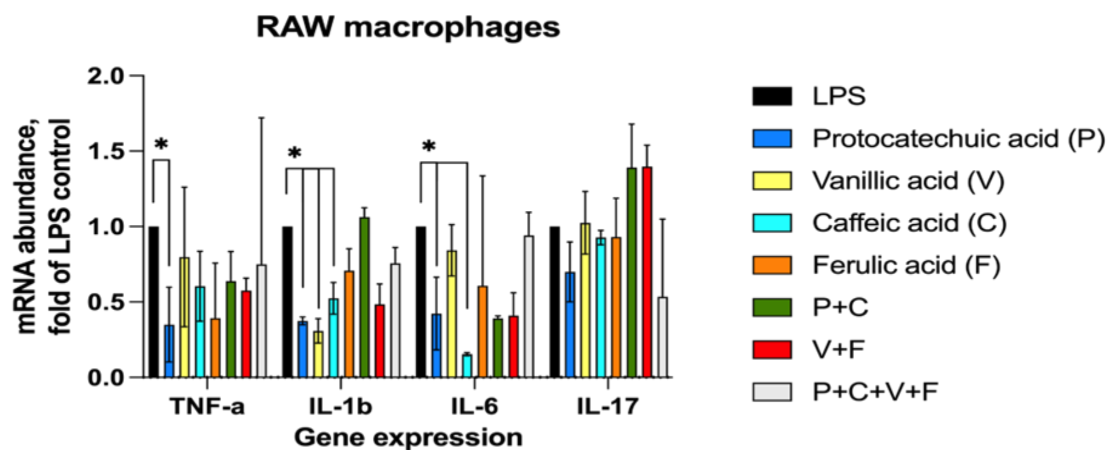

Figure 3.8. Gene expression profiles of pro-inflammatory genes TNF-α, IL-1β, IL-6, and IL-17 in RAW macrophages stimulated with LPS and treated with the physiological levels of small phenolic acids (10 μM). Individual phenolic acids, as well as mixtures of non-methylated (protocatechuic and caffeic acids), methylated (vanillic and ferulic acids), or all phenolic acids were applied to cells for 6 h prior to the analysis. Results are expressed as means ± SEM (n = 3). Data were analyzed using one-way ANOVA followed by Dunnett's multiple comparisons; * $p < 0.05$ versus the LPS controls.

3.3.6. Lipid accumulation in 3T3-L1 adipocytes

Treatment of 3T3-L1 adipocyte cell culture with two major benzoic acids protocatechuic acid (PCA) and its methylated counterpart vanillic acid (VA), two major cinnamic acids caffeic acid (CA) its methylated counterpart ferulic acid (FA), as well the combinations of unmethylated or methylated phenolic acids showed various levels of bioactivity, ranging from suppression of fat accumulation by unmethylated phenolic acids to increased lipid accumulation by methylated phanolic acids (Figure 3.9). All screenings were performed at the concentration of 10 μM, which

is above the upper range of physiologically relevant concentrations for these metabolites.

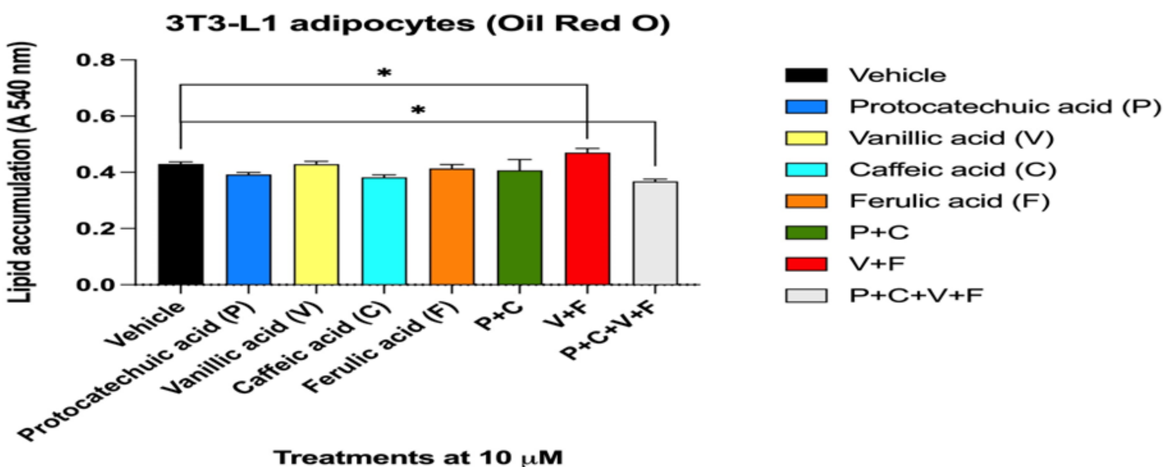

Figure 3.9. Spectrophotometric quantification of lipid accumulation in adipocytes exposed to 10 μM of phenolic acids or their mixtures as indicated. Results are expressed as means ± SEM (n = 3). Data were analyzed using one-way ANOVA followed by Dunnett's multiple comparisons; * $p < 0.05$ versus the vehicle controls.

3.3.7. Gene expression profiles in 3T3-L1 adipocytes

Treatment with phenolic acids significantly altered gene expression during the differentiation of adipocytes, with notable downregulation of pro-inflammatory markers such as TNF-α and IL-6. Genes associated with lipid metabolism and insulin sensitivity, specifically adiponectin, were upregulated, indicating improved adipocyte function. There was a marked increase in the expression of genes linked to uncoupling reactions in the mitochondria, such as Ucp1, suggesting metabolic upregulation (Figure 4.4).

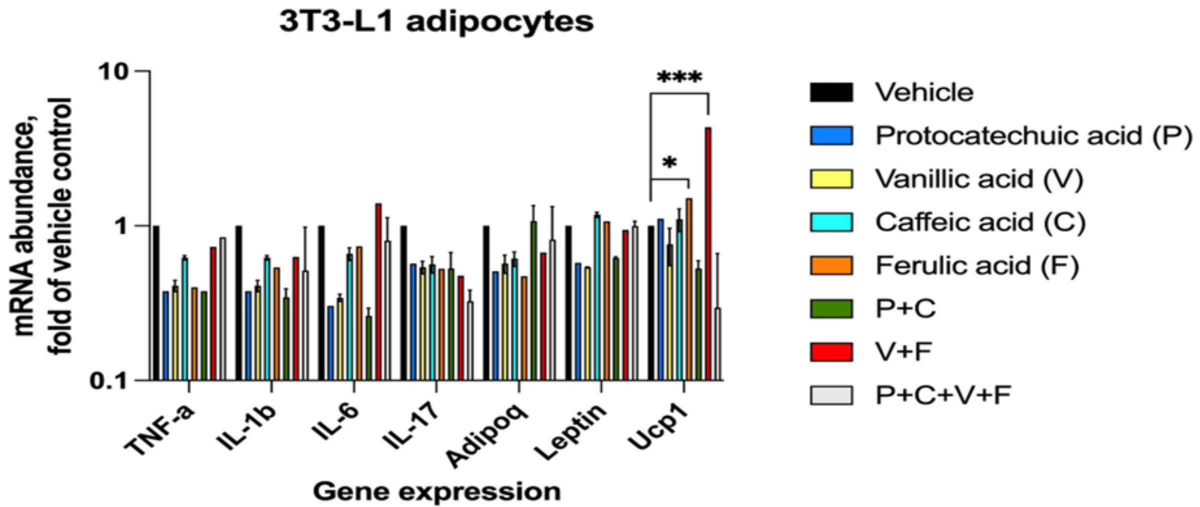

Figure 3.10. Gene expression profiles of pro-inflammatory genes TNF-α, IL-1β, IL-6, and IL-17 and adipogenic genes adiponectin, leptin, and uncoupling protein 1 (Ucp1) in the differentiatd 3T3-L1 adipocytes (day 12). Cells were treated with the physiological levels of small phenolic acids (10 μM) for the duration of the adipogenic induction (days 4-12). Results are expressed as means ± SEM (n = 3). Data were analyzed using one-way ANOVA followed by Dunnett's multiple comparisons; * p< 0.05 versus the vehicle controls.

3.4. Discussion

The low bioavailability of polyphenols has been a longstanding topic of research due to their resistance to digestion [19]. It is now understood that microbial catabolism and chemical degradation convert these complex molecules into smaller, more bioavailable metabolites, such as phenolic acids [20]. These compounds play a crucial role in gut biochemical interactions and host metabolism [21]. Additionally, metabolites of polyphenol-rich foods contribute to gastrointestinal mucoprotection, nourish intestinal epithelial cells, and help maintain gut barrier integrity [12].

Polyphenol digestion and the absorption of phenolic acids can be altered in obesity due to differences in gut microbiota composition and intestinal permeability [22]. Obese individuals often have a dysbiotic microbiome with reduced microbial diversity, which can impact the enzymatic activity and induce the pro-inflammatory profile [23]. Altered expression of transporters, such as monocarboxylate transporters (MCTs) and ATP-binding cassette (ABC) transporters, in obesity [24] may also affect the uptake and efflux of phenolic acids. Finally, reduced metabolic flexibility in obese states can impact how these metabolites are processed in the metabolically active tissues, potentially diminishing their beneficial effects [25].

The metabolism of dietary polyphenols is significantly influenced by catechol-O-methyltransferase (COMT), an enzyme that modifies catechol-like structures through methylation [26]. While COMT is best known for its role in catecholamine metabolism, it also affects the bioavailability and distribution of phenolic metabolites, creating the pools of methylated phenolic metabolites [11] with lower binding capacity to serum albumins [27] and higher affinity for body tissues [28]. Lower COMT activity generally correlates with higher blood pressure and abdominal obesity [29]. Direct supplementation with phenolic acids, and especially with their methylated derivatives, may help counteract obesity-related impairments by not relying on microbial and enzymatic conversions altered in obese states. This could enhance phenolic metabolite distribution and provide additional metabolic benefits, such as improved glucose regulation, reduced inflammation, and enhanced gut barrier function in obese individuals.

This study was therefore designed to explore direct supplementation with phenolic acids in lean and obese animal models, at the physiological range of phenolic acid intake recorded in human longitudinal studies. The selected supplementation dose for animals (0.28% or 2.8 g/kg of diet) corresponds to an average daily intake of 240 mg/kg/day. Adjusting for the mouse-to-human

equivalent dose factor of 12.3 [30], this translates to 19.5 mg/kg/day for humans, or approximately 1,170 mg per day for an average adult.

In the LFD cohort, neither body weight nor food intake differed between control and intervention animals, indicating no effect of phenolic acids on body weight under normal dietary conditions. However, HFD-PA and HFD-MPA mice showed significant reductions in body weight gain (-16.2% and -13.5%, respectively) without a corresponding decrease in food intake, suggesting metabolic effects independent of calorie consumption. These effects were twice as pronounced as the 3-6% decreases observed with previous dietary interventions involving whole berry supplementation standardized to 0.4 g/kg anthocyanins in the diet [14]. Body composition analysis revealed that the reduced weight gain was primarily due to lower fat mass accumulation, with HFD-PA and HFD-MPA groups showing most significant reductions. This suggests that specific phenolic acid formulations, particularly PA and MPA, may reduce fat accumulation and body weight gain in diet-induced obesity without suppressing food intake.

Assessing supplemented mice for metabolic parameters showed no significant changes in oxygen consumption or RER between treated and control animals, suggesting that phenolic acid supplementation did not alter the relative use of carbohydrate versus fat as an energy source. Heat production significantly increased in animals receiving methylated phenolic acids (MPA). These increases in thermogenesis were also accompanied by greater voluntary movements, especially in the MPA-treated animals. Therefore, the methylated phenolic acids may enhance energy expenditure and physical activity, potentially contributing to their effects on body weight regulation. The molecular mechanisms behind these effects are not clear. One possibility is that methylated phenolic acids have higher affinity for body tissues due to increased hydrophobicity

that promotes their diffusion into the peripheral tissues [28] and directly modulates the adipocyte metabolism in the cytoplasm or mitochondria [31].

Another possibility is suggested by a marked increase in voluntary physical activity observed in animals supplemented with phenolic acids, and particular the group of methylated compounds (MPA). This observation may be linked, directly or indirectly, to may be linked to interactions of small phenolic acids with the catecholamine and dopamine networks [1]. This can occur by potentially influencing catecholamine metabolism or mimicking their effects at neurotransmitter receptors [32]. This unusual increase in voluntary physical activity was also observed in our earlier animal studies and was associated with whole food interventions (berries) with enhanced methylation profiles [14]. It can be hypothesized that by increasing dopamine levels or acting on similar neural targets, MPA supplementation may improve mood, motivation, and reward-related behaviors, leading to increased physical activity, although this needs to be confirmed in the future studies.

Further mechanistic insights were obtained using RAW macrophage and 3T3-L1 adipocyte cell lines. Among individual compounds, protocatechuic and caffeic acids demonstrated the strongest anti-inflammatory effects in macrophages, while mixtures were generally less potent. This supports the notion that individual phenolic acids may exert distinct cellular actions, not always synergistic when combined. The concurrent increase in IL-17 expression in both adipose tissue and macrophages remains unclear and may point to a broader immunomodulatory role beyond classical inflammation. In differentiated adipocytes, phenolic acids improved expression of metabolic markers such as adiponectin and Ucp1, and reduced pro-inflammatory cytokine levels, further confirming their beneficial impact on adipocyte functionality.

3.5. Conclusions

This study demonstrates that direct supplementation with small phenolic acids, particularly methylated derivatives, can effectively reduce body weight gain and fat accumulation in diet-induced obesity without altering food intake. The observed increases in thermogenesis and voluntary physical activity, especially in animals supplemented with methylated phenolic metabolites, highlight the potential role of these compounds in enhancing energy expenditure. Further research is needed to clarify these molecular interactions and determine whether similar benefits can be replicated in human studies.

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Chapter 4: Changes In Gene Expression Profiles from Adipose Tissue in Response to Small Phenolic Acids

4.0. Abstract

Unmethylated and methylated phenolic acids, common dietary metabolites of polyphenols, were investigated for their immunometabolism effects in white and brown adipose tissue, macrophages, and adipocyte models. In white adipose tissue, phenolic acid supplementation enhanced the expression of Ucp1 and adiponectin in both lean and obese mice, while attenuating pro-inflammatory markers (TNF-α, IL-1β, IL-6) in obese animals. Notably, IL-17 was upregulated across treatments, suggesting immune remodeling. No significant gene expression changes were observed in intestinal or skeletal muscle tissues. These findings support the potential of small phenolic acids to beneficially modulate adipose tissue inflammation and metabolism, with differential effects based on structural features and tissue context.

Keywords: polyphenols; phenolic acids; methylated metabolites; metabolic stress; diet-induced obesity; thermogenesis; adipocyte function; energy expenditure

4.1. Introduction

Phenolic acids are a diverse group of small aromatic metabolites widely distributed in the human diet, where they occur as free compounds or as structural blocks of plant polymeric substances such as lignins, polysaccharides, and flavonoids (Kumar and Goel, 2019). Derived primarily from hydroxybenzoic and hydroxycinnamic acid backbones, these compounds have attracted growing interest due to their ability to influence systemic metabolic and immune functions (Sun and Shahrajabian, 2023). Once ingested, phenolic acids undergo extensive transformation by gut microbiota and host metabolic enzymes, generating a pool of low-molecular-weight metabolites that can be absorbed into circulation and interact with peripheral tissues (Kay et al., 2017), including adipose depots and immune cell populations (Vong et al., 2022). Increasing evidence suggests that these metabolites act as signaling molecules rather than simply functioning as antioxidants, modulating gene expression in metabolic tissues, enhancing mitochondrial performance, and shaping inflammatory responses (Mhawish and Komarnytsky, 2025).

White adipose tissue (WAT) plays a central role in energy storage and endocrine regulation but is also a site of chronic low-grade inflammation in obesity and metabolic syndrome (Kawai et al., 2021). The interplay between adipocytes and resident immune cells, including macrophages, underlies many of the pathophysiological changes associated with diet-induced obesity (Teixeira et al., 2024). Perturbations in this microenvironment are marked by a shift from anti-inflammatory to pro-inflammatory macrophage phenotypes (Thomas and Apovian, 2017), accompanied by altered expression of adipokines and mitochondrial uncoupling (Ucp) proteins (van Dierendonck et al., 2020). Targeting these regulatory circuits with dietary components such as phenolic acids offers a compelling strategy to restore metabolic balance (Skates et al., 2018).

Multiple preclinical studies have demonstrated that specific phenolic acids can modulate inflammatory signaling pathways in cells. For instance, protocatechuic and caffeic acids have been shown to inhibit lipopolysaccharide (LPS)-induced production of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 in macrophages, largely through the suppression of NF-κB and MAPK activation (Armutcu et al., 2015). These effects may be potentiated or attenuated by methylation, hydroxylation, and other modifications of the phenolic structure, which influence their bioavailability, cellular uptake, and interaction with intracellular signaling targets (Cosme et al., 2020). Notably, methylated phenolic acids are expected to exhibit higher membrane permeability and longer tissue retention times, enhancing their capacity to modulate intracellular responses (Ichiyanagi et al., 2006).

Despite the recognized immune-modulatory capacity of phenolic acids, their impact on adipose tissue function, including in the context of a low-grade inflammatory response, remains underexplored. Our previous observations have suggested that these compounds may influence both inflammatory gene expression and adipocyte-specific markers with potential implications for adipose browning and metabolic flexibility (Skates et al., 2018). We next performed an initial preclinical evaluation of the effects of direct supplementation with phenolic acids and their methylated derivatives on metabolic outcomes in lean and obese mice (Chapter 3). The study aimed to bypass microbial and enzymatic conversion of polyphenols by supplying physiologically relevant doses of phenolic acids directly in the diet. Results showed that in high-fat diet mice, phenolic acid supplementation significantly reduced body weight gain and fat mass accumulation without affecting food intake. These effects were more pronounced with methylated phenolic acids (MPA), which also increased thermogenesis and voluntary physical activity.

This complex activity profile underscores the need for more detailed investigation into the gene regulatory roles of dietary phenolic acids in adipose and immune cells. By elucidating how different classes and structural variants of phenolic acids influence gene expression in metabolically active tissues from lean and obese animals, we attempted to gain deeper insight into the potential of these compounds to mitigate metabolic dysfunction and inflammation through coordinated transcriptional effects.

4.2. Materials and Methods

4.2.1. Animal diets

Phenolic acids were purchased from Sigma (St. Louis, MO, USA), mixed in the appropriate proportions as described in Table 1, and incorporated into the respective low-fat diet (LFD, D12450J, 10 kcal % fat, 3.85 kcal/g) or high-fat diet (HFD, D12492, 60 kcal % fat, 5.24 kcal/g) at 0.28% total added phenolic acids by Research Diets (New Brunswick, NJ, USA). Both lean LFD mice (n=32) and obese HFD mice (n=32) were then randomized into four dietary treatment groups each (n=8), including the respective controls (LFD or HFD), or animals supplemented with a mixture of 4 non-methylated phenolic acids (NMPA), a mixture of 4 methylated phenolic acids (MPA), or an equal mixture (1:1) of both non-methylated and methylated phenolic acids (PA) for 6 weeks as described in Chapter 3.

4.2.2. RNA extraction, purification, and cDNA synthesis

The total RNA was isolated from the epidydimal white adipose tissue using TRIzol reagent (Life Technologies, Carlsbad, CA) following the manufacturer's instructions. RNA was quantified using the Biotek SynergyH1/Take 3 plate (Agilent, Santa Clara, CA). The cDNAs were synthesized on ABI GeneAMP 9700 using the high-capacity cDNA Reverse Transcription kit and 2 µg of RNA (Life Technologies).

4.2.3. Quantitative PCR analysis

The resulting cDNA was amplified by real-time quantitative PCR using SYBR green PCR master mix (Life Technologies). To avoid interference due to genomic DNA contamination, only intron-overlapping primers were selected using Primer Express software, version 2.0 (Applied Biosystems, Foster City, CA).

The following primers were used to capture the core cluster of the short-lived inflammatory gene expression networks and adipocyte-related genes, including TNF-α, forward primer: 5'-GTT CTA TGG CCC AGA CCC TCA CA-3', reverse primer: 5'-TAC CAG GGT TTG AGC TCA GC-3'; IL-1β, forward primer: 5´-CAA CCA ACA AGT GAT ATT CTC CAT G-3´, reverse primer: 5´-GAT CCA CAC TCT CCA GCT GCA-3´; IL-6, forward primer: 5′-TAG TCC TTC CTA CCC CAA TTT CC-3′, reverse primer: 5′-TTG GTC CTT AGC CAC TCC TTC-3′; IL-17, forward primer: 5'-ATC TGG TCC TAC ACG AAG CC-3', reverse primer: 5'-GTC CCG GAC TTC AAG ACC C-3'; Adiponectin, forward primer: 5′-TGT TCC TCT TAA TCC TGC CCA-3′, reverse primer: 5′-CCA ACC TGC ACA AGT TCC CTT-3′; Leptin, forward primer: 5′-GAG ACC CCT GTG TCG GTT C-3′, reverse primer: 5′-CTG CGT GTG TGA AAT GTC ATT-3′; Uncoupling protein-1 (Ucp1), forward primer: 5′-AGG CTT CGA GTA CCA TTA GGT-3′, reverse primer: 5′-CTG AGT GAG GCA AAG CTG ATT T-3′; and β-actin as a housekeeping gene, forward primer: 5′-AAC CGT GAA AAG ATG ACC CAG AT-3´, reverse primer: 5´-CAC AGC CTG GAT GGC TAC GT-3´.

Gene expression profiles in the liver were analyzed for inflammation-related TNF-α, IL-1β, IL-6 genes as well as a nuclear farnesoid X receptor FXR, forward primer 5′-CCA CCG GCT GTC AGG ATT T-3′, reverse primer 5′-GCA TAC CTT TAG CTG GCT TCA CA-3′; sterol regulatory element-binding protein 1 SREB1, forward primer 5′- GAT GTG CGA ACT GGA

CAC AC-3′, reverse primer 5′- CAT AGG GGG CGT CAA ACA G-3′; proglucagon (GCG) precursor of GLP-1, forward primer: 5′-TGA AGA CCA TTT ACT TTG TGG CT-3′, reverse primer: 5′-CCA AGT GAC TGG CAC GAG AT-3′; and the associated receptor GLP-1R, forward primer: 5′-CAG GGC TTG ATG GTG GCT ATC-3′, reverse primer: 5′-CGC TCC CAG CAT TTC CG-3′.

Gene expression profiles in the small intestine were analyzed for the bitter receptor TAS2R108, forward primer: 5′-GGT CAA CAG TCG CAG AAT TGC-3′, reverse primer: 5′-TGT CCT GGA GGG TAA GCA GC-3′; gustducin subunit alpha GNAT3, XXX; phospholipase C beta 2 PLCB2, XXX; a transient receptor potential melastatin-5 channel TRPM5, XXX; the proglucagon (GCG) precursor of GLP-1; the associated receptor GLP-1R; and a G protein-coupled receptor for bile acids TGR5, XXX.

Gene expression profiles in the skeletal muscle were analyzed for the myogenic differentiation 1 MyoD1, XXX; myogenic factor 5 Myf 5, XXX; a transcriptional activator myogenin Myog, XXX; a negative regulator myostatin Gdf8, XXX; a muscle atrophy regulator Trim63, XXX; insulin-like growth factor binding protein 5 Igfbp5, XXX; and transcriptional Pparg coactivator 1 alpha Pgc1a, XXX.

Quantitative PCR (qPCR) amplifications were performed on an ABI 7500 Fast real-time PCR (Life Technologies) using 1 cycle at 50 °C for 2 min and 1 cycle at 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The dissociation curve was completed with 1 cycle of 1 min at 95 °C, 30 s at 55 °C, and 30 s at 95 °C. mRNA expression was analyzed using the ΔΔCT method and normalized with respect to the expression of the β-actin housekeeping gene. Amplification of specific transcripts was confirmed by obtaining melting curve profiles.

4.2.4. Statistical Analysis

Data were analyzed by one-way ANOVA followed by Dunnett's multiple-range tests using Prism 8.0 (GraphPad Software, San Diego, CA, USA). Temporal measures were analyzed by two-factor repeated-measures ANOVA, with time and treatment as independent variables. All data were presented as means ± SEM. Significant differences were accepted when the p-value was <0.05.

4.3. Results

4.3.1. Differential gene expression in white adipose tissue

White adipose tissue is a complex environment of adipocytes, adipose-derived stem cells, stromal vascular endothelial cells and various immune cell populations. The changes in gene expression profiles of two groups of biomarkers responsible for characterizing the immune and adipose status of the tissues varied between the LFD and HFD animals. The 3 biomarkers of adipocyte status including adiponectin, leptin, and Ucp1 were upregulated by all phenolic acid treatments in LFD mice. The Ucp1 enhancing effect was most evident in PA and MPA interventions ($p<0.01$). Among the immune markers, no changes were observed in the expression levels of the early response inflammatory genes including TNF-α and IL-1β. Unexpectedly, all phenolic acid mixtures increased IL-6 and IL-17 expression in the white adipose tissue of LFD mice as well ($p<0.05$, Figure 4.1a).

In the animals consuming HFD diet, expression of 3 pro-inflammatory biomarkers TNF-α, IL-1β, and IL-6 was reduced by all phenolic acid mixtures ($p<0.05$, Figure 8b). However, the IL-17 mRNA was elevated as in the LFD interventions. No leptin-related effects were significant for all treatments. Upregulation of adiponectin and Ucp1 in response to phenolic acid

supplementation was observed like the LFD diet, with most significant effects recorded in MPA and PA mixes ($p<0.01$, Figure 4.1b).

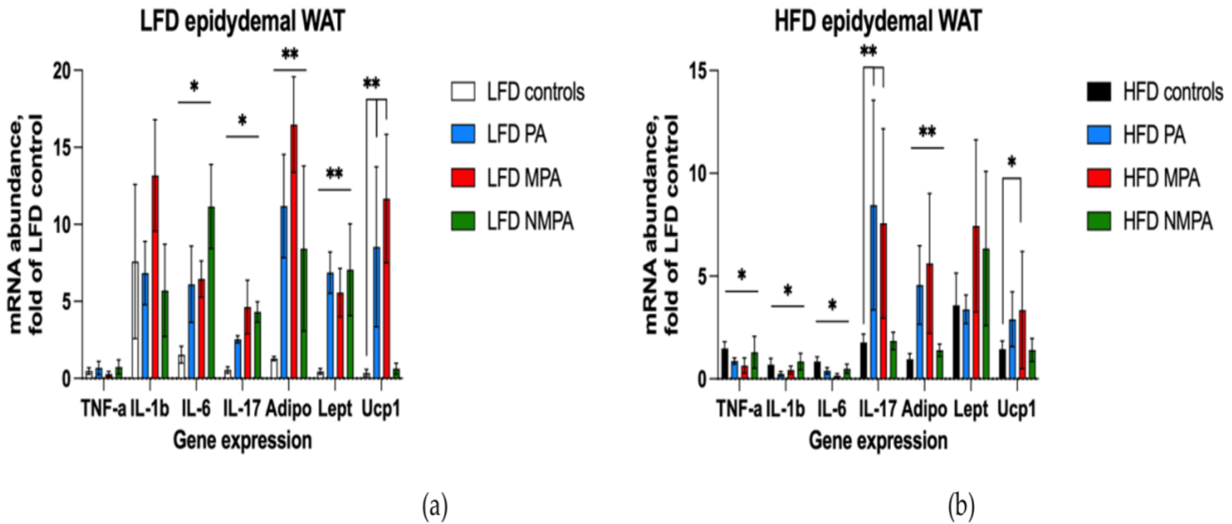

Figure 4.1. Gene expression profiles of white adipose tissue in (a) LFD lean controls or (b) HFD obese controls in comparison to the respective interventions that included 0.28% total phenolic acids (PA), four methylated phenolic acids (MPA), or the four matching non-methylated phenolic acids (NMPA) after 6 weeks of supplementation. Results are expressed as means ± SEM (n = 6). Data were analyzed using one-way ANOVA followed by Dunnett's multiple comparisons; * $p< 0.05$ versus the HFD controls.

4.3.2. Gene expression profiles in the interscapular adipose tissue of DIO mice

Treatment with phenolic acids modulated the expression of key genes involved in inflammation, adipogenesis, and mitochondrial function in brown adipose tissue. Inflammatory markers such as TNF-α and IL-1β were downregulated, indicating reduced adipose tissue inflammation in LFD, but not HFD animals. Leptin was below the detection level in LFD animals

and was upregulated by methylated phenolic acids in HFD mice. Ucp1 was significantly upregulated, suggesting enhanced thermogenic and metabolic activity associated with consumption of methylated phenolic acids (Figure 4.2).

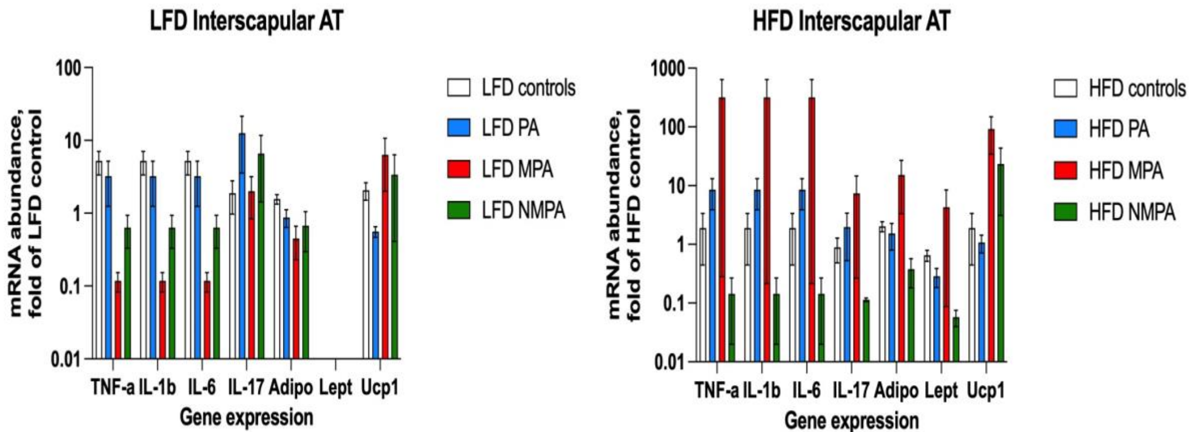

Figure 4.2. Gene expression profiles of pro-inflammatory genes TNF-α, IL-1β, IL-6, and IL-17 and adipogenic genes adiponectin, leptin, and uncoupling protein 1 (Ucp1) in the interscapular adipose tissue of LFD and HFD mice. Results are expressed as means ± SEM (n = 4). Data were analyzed using one-way ANOVA followed by Dunnett's multiple comparisons; * $p < 0.05$ versus the controls.

4.3.3. Gene expression profiles in the mesenteric adipose tissue of DIO mice

Supplementation with phenolic acids had little effects on the mesenteric adipose tissue of the LFD mice, except for Ucp1 that was upregulated. Phenolic acid treatment significantly downregulated inflammatory genes such as IL-1β and IL-6, but upregulated TNF-α, indicating a complex remodeling of chronic low-grade inflammation in the mesenteric fat. Expression of

adiponectin and Ucp1 increased, suggesting improved adipocyte function and insulin sensitivity (Figure 4.3).

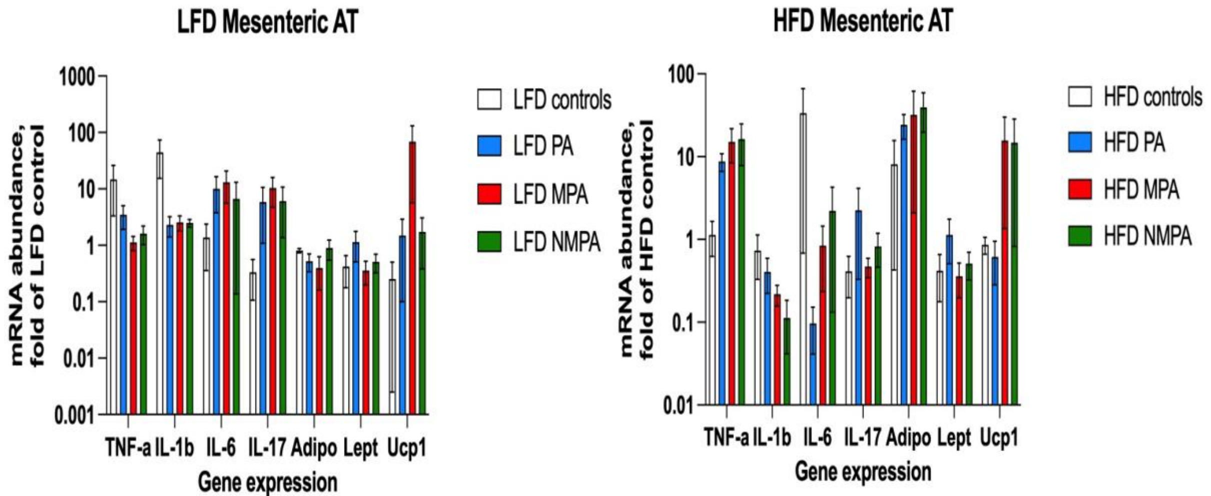

Figure 4.3. Gene expression profiles of pro-inflammatory genes TNF-α, IL-1β, IL-6, and IL-17 and adipogenic genes adiponectin, leptin, and uncoupling protein 1 (Ucp1) in the mesenteric adipose tissue of LFD and HFD mice. Results are expressed as means ± SEM (n = 4). Data were analyzed using one-way ANOVA followed by Dunnett's multiple comparisons; * $p < 0.05$ versus the controls.

4.3.4. Gene expression profiles in the small intestine of DIO mice

Treatment with phenolic acids was associated with no significant differences in the expression of several genes involved in bitter taste signaling in the small intestine of LFD and HFD mice (Figure 4.4).

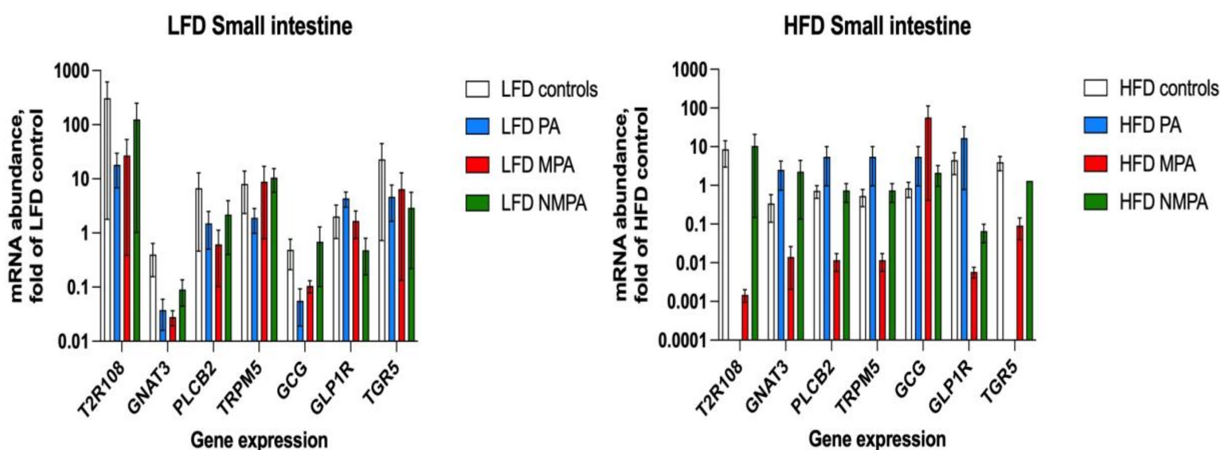

Figure 4.4. Gene expression profiles of the bitter receptor TAS2R108, gustducin subunit alpha (GNAT3), phospholipase C beta 2 (PLCB2), a transient receptor potential melastatin-5 channel (TRPM5), the proglucagon (GCG) precursor of GLP-1, the associated receptor GLP-1R, and a G protein-coupled receptor for bile acids (TGR5) in the small intestine of LFD and HFD mice. Results are expressed as means ± SEM (n = 4). Data were analyzed using one-way ANOVA followed by Dunnett's multiple comparisons; * $p < 0.05$ versus the controls.

4.3.5. Gene expression profiles in the muscle tissue of DIO mice

Treatment with phenolic acids was associated with no significant differences in the expression of several genes involved in regulation of skeletal muscle mass in the gastrocnemius muscle tissue of LFD and HFD mice (Figure 4.5).

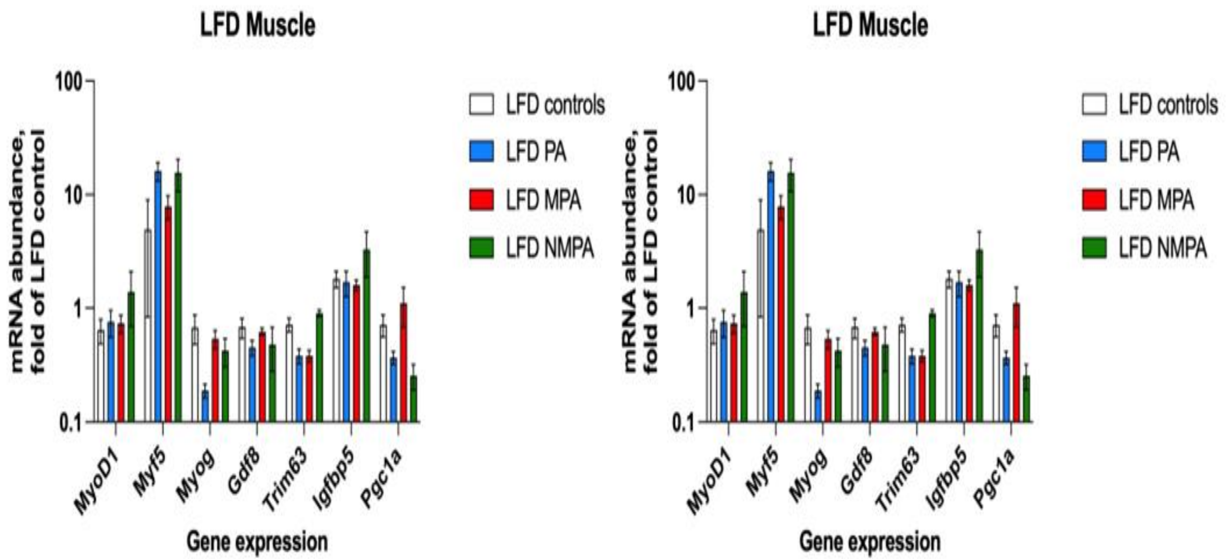

Figure 4.5. Gene expression profiles of the myogenic differentiation 1 (MyoD1), myogenic factor 5 (Myf 5), a transcriptional activator myogenin (Myog), a negative regulator myostatin (Gdf8), a muscle atrophy regulator (Trim63), the insulin-like growth factor binding protein 5 (Igfbp5), and the transcriptional PPARg coactivator 1 alpha (Pgc1a) in the skeletal muscle of LFD and HFD mice. Results are expressed as means ± SEM (n = 4). Data were analyzed using one-way ANOVA followed by Dunnett's multiple comparisons; * $p < 0.05$ versus the controls.

4.3.6. Gene expression profiles in the liver tissue of DIO mice

Treatment with phenolic acids was associated with no significant differences in the expression of several genes involved in regulation of liver function including gluconeogenesis in the liver tissue of LFD and HFD mice (Figure 4.6).

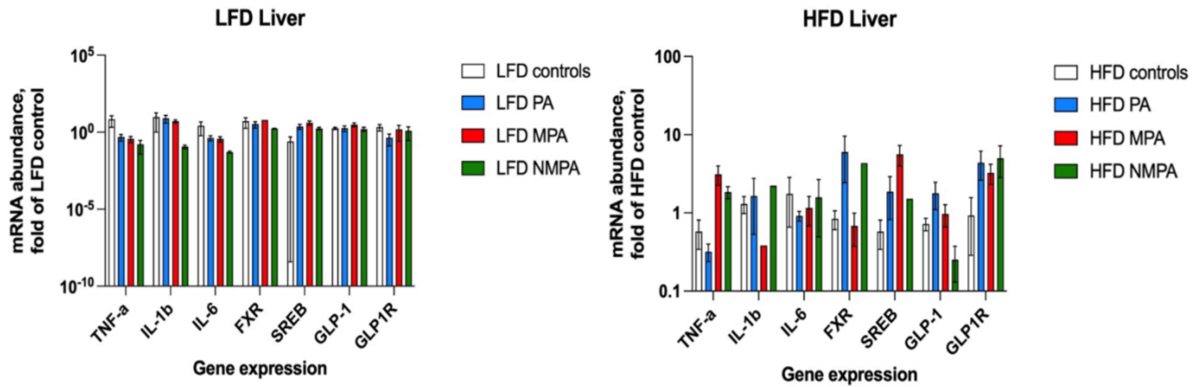

Figure 4.6. Gene expression profiles of pro-inflammatory genes TNF-α, IL-1β, IL-6, and other genes that control glucose metabolism in the liver of LFD and HFD mice. Results are expressed as means ± SEM (n = 4). Data were analyzed using one-way ANOVA followed by Dunnett's multiple comparisons; * $p < 0.05$ versus the controls.

4.4. Discussion

This study provides a comprehensive evaluation of how structurally diverse small phenolic acids influence gene expression across multiple metabolic tissues, with a focus on adipose inflammation and metabolic function. In white adipose tissue, phenolic acid supplementation enhanced the expression of Ucp1 and adiponectin, markers of thermogenic and insulin-sensitive adipocytes, in both lean and obese animals. Interestingly, while pro-inflammatory cytokines such as TNF-α and IL-1β were unchanged in lean animals, IL-6 and IL-17 were unexpectedly upregulated by all treatments. In contrast, in obese animals, phenolic acids suppressed major inflammatory cytokines including TNF-α, IL-1β, and IL-6, suggesting an anti-inflammatory remodeling of white adipose tissue under conditions of chronic metabolic stress.

Tissue-specific effects were also observed in other adipose depots. In brown (interscapular) adipose tissue, methylated phenolic acids markedly increased Ucp1 expression, potentially

enhancing thermogenesis. In mesenteric fat, adiponectin and Ucp1 were also upregulated, but the inflammatory response was complex, with TNF-α unexpectedly elevated despite reductions in IL-1β and IL-6. No significant gene expression changes were observed in muscle or intestinal tissues, suggesting a possible tissue selectivity of phenolic acid action. Collectively, these findings suggest that methylated small phenolic acids modulate adipose inflammation and metabolism through distinct gene regulatory networks and may offer targeted nutritional strategies for improving metabolic health in obesity.

4.5. Conclusions

Dietary supplementation with phenolic acids modulated gene expression profiles in both white and brown adipose tissues, promoting adipocyte function and mitochondrial activity, especially through consistent upregulation of adiponectin and Ucp1. While these compounds suppressed key pro-inflammatory genes such as IL-1β and IL-6 in obese animals, they unexpectedly elevated IL-17 across multiple models, suggesting a nuanced and context-dependent immune response. The methylated phenolic acids were especially associated with changes in gene response networks that regulate thermogenic and metabolic capacity of the adipose tissues. Overall, these results highlight the tissue-specific and compound-specific metabolic benefits of phenolic acids, supporting their potential as modulators of adipose tissue function in obesity.

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Chapter 5: Impact Of Consuming Protein-Polyphenol Bars on Metabolic Health: Preliminary Results of a Randomized Controlled Trial

5.0. Abstract

Nutritional snacks in a bar form became a cultural trend recognized and accessible to everyone. Plant-based proteins are an increasingly popular substitute for those who strive to reduce or eliminate dairy ingredients from their diets for personal, allergy-related, or sustainability concerns. The nutritional value of protein bars can be further enhanced by berry polyphenols that are known to support metabolic health. The study involved 75 participants aged 18-64 with a body mass index of 18.5 and higher who were randomly assigned to one of four groups: [1] a pea protein bar with a matched nutritional and sensory profile (control), [2] a pea protein bar with complexed aggregate protein-blackcurrant polyphenol particles, [3] a pea protein bar with free black currant polyphenols, and [4] a conventional whey protein bar with free black currant polyphenols as a reference. There was one pre-study (screening) visit and two study visits 8 weeks apart (baseline and end of the study). Participants consumed their assigned bar during the study visits and then daily for 8 weeks. Data collection methods included health history assessments, food habit questionnaires, and measurements of satiety and gastrointestinal discomfort. Additionally, body weight and composition, blood biomarkers related to metabolic health, urine samples for polyphenolic metabolites, and fecal samples for phenolic metabolites and microbiome profiles were collected at each visit and analyzed.

5.1. Introduction

Recent scientific studies have shifted their focus from the fundamental role of food as a source of energy and nutrients to the subtler influence of biologically active dietary components on human health. Consumer interest in food's active role in health, life extension, and the prevention of non-transmissible chronic diseases has surged (Granato et al., 2010). This interest has led to the emergence of the term "functional food," which refers to foods that have a positive impact on health beyond basic nutrition (Foegeding et al., 2017).

Protein-polyphenol particles might be employed as a novel method for functional protein delivery in the formulation of high-protein bars since they not only boost the nutritional content of the protein bars but also prevent rheological and structural alterations (Xiong et al., 2020). Adding protein makes it possible to consume more protein while consuming fewer calories overall, aiding in weight reduction (Clifton, 2012). Additionally, these particles enhance the stability of fruit-derived phytochemicals, which are often unstable and susceptible to oxidation and heat degradation, compromising their absorption and health benefits (Jia et al., 2025).

Stability of fruit-derived phytochemicals is crucial for food industry utilization. Complexed protein-polyphenol particles are synthesized by sorbing health-protective polyphenols onto proteins at pH values near the proteins' isoelectric point. This forms non-covalent bonds that are reversible post-digestion, effectively excluding high-caloric sugars and oils from vegetable or fruit sources. The result is a concentrated form of polyphenols that provide health benefits, immune protection, and satiety (Foegeding et al., 2017).

These days, there are many different types of protein bars available in supermarkets, from whey protein bars (Szydłowska et al., 2020) to plant-based versions suitable for vegans and vegetarians (Alcorta et al., 2021). Despite the popularity of high-protein diets among active and

sedentary groups, there is little evidence on which kind of protein—vegetable or animal—should be consumed. To help both athletes and non-athletes make informed decisions about the type of protein they should consume, this paper examined and analyzed some major variables.

High-protein bars have been trending in recent years, leading to increased consumption of protein-rich foods. The protein bar market topped $200 million in 1997 and nearly $1.7 billion in 2010 (Rigik, 2011), and it is expected to grow further, targeting athletes and a diverse consumer base not only to increase protein intake and satisfy immediate hunger but also because they are perceived as a source of other nutrients or as a healthy mini-meal (Wyatt, 2011).

There is accumulating evidence suggesting that consuming foods made from phenol-rich plants may help prevent some diseases, such as obesity and insulin resistance, due to their antioxidant, antimutagenic, antithrombotic, and anti-inflammatory properties (Sun & Shahrajabian, 2023; Kuljarusnont et al., 2024).

There are many phenolic chemicals in plants, which collectively create thousands of distinct chemical structures distinguished by hydroxylated aromatic rings (Aragonès et al., 2017). These substances assist plants in several crucial ways. They are responsible for metabolic plasticity that allows plants to adapt to shifting biotic and abiotic environments and gives the plants color, flavor, and purported health advantages (Boudet, 2007). Phenolic compounds are widely present in several fruits and vegetables (Lima et al., 2014).

The blackcurrant (Ribes nigrum L., Grossulariceae), a small, perennial shrub native to central Europe and northern Asia, is cultivated throughout the world, including the United States (Gopalan et al., 2012).

In this study, we are particularly interested in blackcurrants because many phytochemical studies have indicated that blackcurrants are one of the fruits with high levels of polyphenols.

HPLC analysis revealed that they contain high amounts of delphinidin-3-O-glucoside, delphinidin-3-O-rutinoside, cyanidin-3-O-glucoside, and cyanidin-3-O-rutinoside, and they contain the highest amount of anthocyanins of all the commonly consumed berries, such as blueberries, blackberries, raspberries, and cranberries (Lee et al., 2015). Additionally, emerging evidence suggests that blackcurrants have potent anti-inflammatory and antioxidant abilities (Gopalan et al., 2012).

Most studies on complexed protein-polyphenol particles have focused on forming nano- or micro-scale particles to protect the bioactivity of polyphenols, rather than addressing the intake of nutritional bars from the complexed protein-polyphenol particles on human health, especially obesity (Da Silva et al., 2024; Xiao et al., 2023).

Pulses are under-consumed in the USA and Western world, and scalable, industrially friendly production strategies are needed to increase their use. Pilot-scale spray drying production of plant-based microparticles PP-BC made with pulse protein and blackcurrant extract was investigated as an effective strategy for creating new pulse ingredients. The microparticles were characterized for their physicochemical and morphological attributes and used to produce protein bars. The study found that complexed pulse protein-polyphenol particles had better sensory scores, making spray-dried protein-polyphenol particles a practical strategy for the emerging wellness-oriented market (Hoskin et al., 2025). Therefore, this clinical trial was developed to determine whether consuming these bars with a focus on complexed pea protein-blackcurrant polyphenol particles is more effective in improving the overall health of healthy individuals compared to other types of bars, specifically pea protein bars enriched with uncomplexed blackcurrant polyphenols and whey protein bars, due to the enhanced bioavailability of polyphenol delivered in the complexed particle format.

5.2. Objectives

Pulses are excellent sources of protein, fiber, antioxidant polyphenols, and micronutrients. Because they have low-production-cost crops with lower carbon footprints and water requirements. However, their consumption remains low in the USA and in most developed Western nations. As part of the USDA ARS Pulse Crop Health Initiative, we have developed stable aggregate ingredients comprised of high-protein pulse (pea protein) bound to health-protective fruit bioactive compounds (blackcurrant polyphenols). The ingredient was incorporated into a protein bar that can serve as a convenient, nutritious snack between meals, helping to curb hunger, prevent overeating, and attenuate metabolic risk factors associated with suboptimal lifestyles. Therefore, the aims of this study are to determine the feasibility and benefits of consuming the newly developed protein bars based on the following set of objectives:

Primary objective: The aim of this study was to investigate the effect of four different types of protein bars on postprandial glycemic response using a handheld glucometer at multiple time points (0, 30, 60, and 120 minutes), and to assess participants' sensory acceptance, gastrointestinal tolerability, and perceived quality of life following consumption. Sensory evaluation was conducted using the 9-point Hedonic Acceptance Scale, gastrointestinal symptoms were assessed using the Gastrointestinal Tolerability Questionnaire (GITQ), and overall wellbeing was measured using the Global Quality of Life (GQOL) Questionnaire.

Secondary objective: assess possible changes in biomarkers of metabolic risk factors, including fasting blood glucose, triglycerides, cholesterol, and blood pressure. Additional biochemical markers may include plasma insulin, leptin, adiponectin, glucagon-like peptide-1; a panel of inflammatory cytokines IL-1b, IL-6, IL-8, IL-10, IL-12p70, and TNF-a; as well as metabolic flexibility and oxidative stress in peripheral blood mononuclear cells.

Tertiary objective: quantify 24h phenolic secretion into urine and feces and assess the health status of the colonic microbiome using fecal DNA samples.

5.3. Materials and Method

5.3.1. Materials and Reagents

Protein bars that were used in this study were formed as mentioned in this article (Hoskin et al., 2025). The conventional whey protein bar with added black currant polyphenols, plant-derived pea protein bar, pea protein bar with added black currant polyphenols, and complexed pea protein-blackcurrant polyphenol particles standardized and matched for caloric density, nutritional profiles, and physical appearance (berry flavor and purple coloration). Each bar contained 17 g of carbohydrates, with specific formulations adjusted to include black currant polyphenols where applicable. The nutritional label is the same for all protein bars. The bars were manufactured by Nutreo SAS food manufacturing facility located in Guarne, Colombia. The facility is engaged in the manufacturing, processing, packing, or holding of food for human or animal consumption in the United States; List of ingredients and composition of the protein bars (Table 5.1).

5.3.2. Study Participants

Seventy-five adults (4 groups, n=19 each) recruited via email and flyers from the Charlotte metropolitan area. The inclusion criteria for this study were participants from both genders aged 18-64 years old who have a BMI of 18.5 or higher, generally healthy, not pregnant or nursing, non-tobacco users, and had no allergies to milk products. Exclusion criteria included having a body mass index lower than 18.5, being pregnant or nursing, using blood sugar-lowering medications such as metformin, semaglutide, or insulin, using dietary supplements with polyphenols, having a history of allergies or intolerance to protein bar ingredients, and noncompliance with the study

protocol. Subjects who met the recruitment criteria upon initial screening via the NCSU Qualtrics form were invited to the Komarnytsky lab at the Plants for Human Health Institute, NCRC, Kannapolis, NC, for further participation in the study.

Table 5.1. List of ingredients and composition of the protein bars

Group	**[1]**	**[2]**	**[3]**	**[4]**
Protein bar type	Pea	Pea	Pea	Whey
	No polyphenols	Complexed polyphenols	Added polyphenols	Added polyphenols (reference)
Puffed quinoa, g	5.71	5.71	5.71	5.88
Whey protein isolate, g	–	–	–	9.41
Pea protein isolate, g	9.14	–	9.14	–
Berry polyphenol powder, g	–	–	1.83	–
Pea protein polyphenol complex	–	11.43	–	–
Honey, g	11.43	11.09	11.42	11.76
Coconut oil, g	2.86	2.86	2.86	2.94
Sunflower seed, g	2.40	2.29	2.40	2.58
Chia seed, g	2.29	2.29	2.29	2.58
Sesame seed, g	1.14	1.14	1.14	1.18
Vanilla extract, g	0.91	0.91	0.91	0.94
Water, g	2.29	2.29	2.29	2.35
Berry flavor, g	0.05	–	–	0.05
Purple dye, g	0.33	–	–	0.33
Total weight, g	40.00	40.00	40.00	40.00
Calories per serving (1 bar)	160	160	160	160

5.3.3. Study Design

This is a randomized, double-blind, parallel intervention study with one pre-study and two study visits (baseline and end of study). Participants were assigned to one of four interventions in random order: [1] a pea protein bar with a matched nutritional and sensory profile (control), [2] a

pea protein bar with complexed aggregate protein-blackcurrant polyphenol particles, [3] a pea protein bar with free black currant polyphenols, and [4] a conventional whey protein bar with free black currant polyphenols as a reference. Participants consumed their assigned bar daily instead of a regular snack for 8 weeks (56 bars) (Figure 5.1).

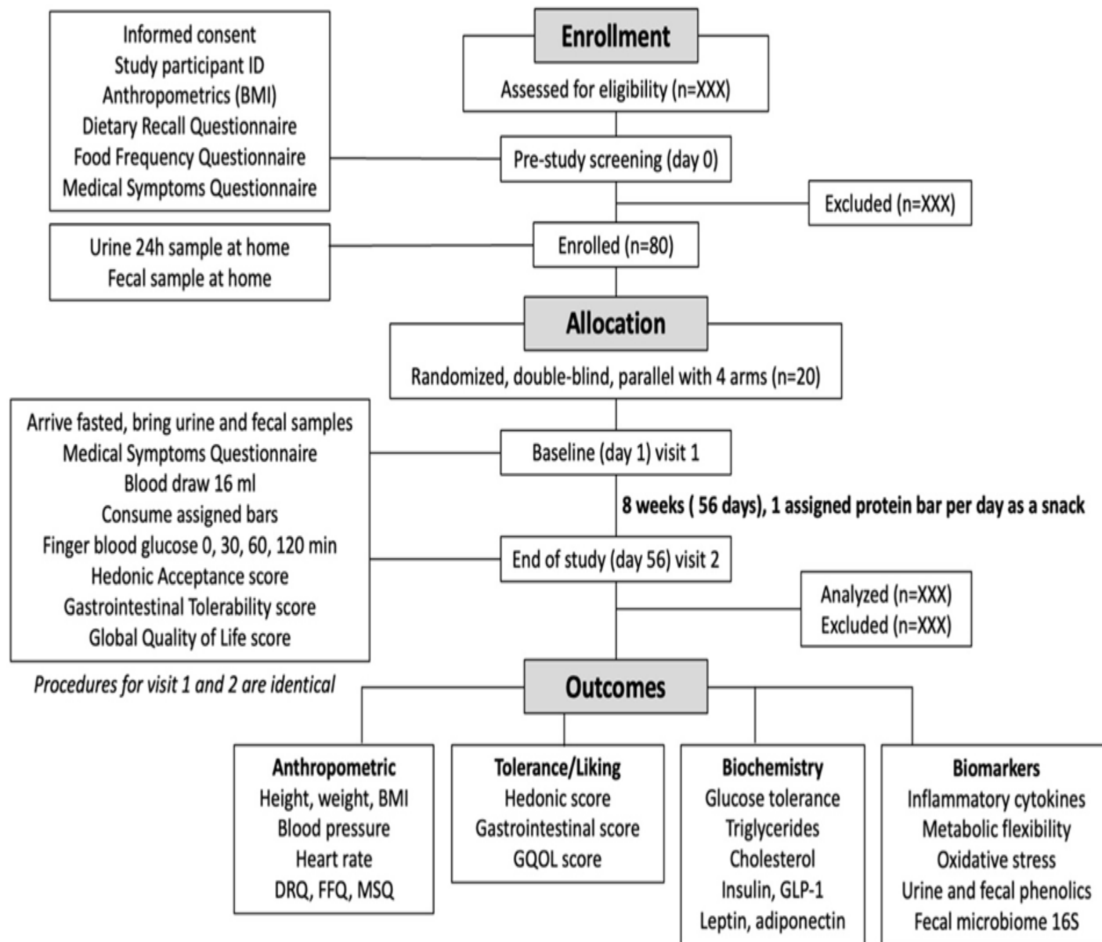

Figure 5.1. Study flowchart.

At the pre-study screening visit, participants arrived at the Komarnytsky lab in Kannapolis, NC, where they signed an informed consent form, received a study participant ID, and underwent anthropometric measurements using the InBody370 body composition analyzer and the Maguja RN-032A arm blood pressure monitor. During the pre-study screening visit, the participants were

taught how to complete the Dietary Recall Questionnaire (DRQ), Food Frequency Questionnaire (FFQ), and Medical Symptoms Questionnaire (MSQ) and asked to complete them during the pre-study visit. The completed questionnaires were reviewed with the research personnel.

Anthropometric measurements were taken in the private area of the lab. Height, body weight, and body fat percentage were recorded using a noninvasive InBody370 body composition analyzer through 8-point bioelectrical impedance. Blood pressure and heart rate were recorded using a noninvasive Maguja RN-032A arm blood pressure monitor. BMI was calculated by the research personnel.

Recruited participants were given 2 labeled 3,000 ml 24-hour urine collection containers and 2 plastic hats (specimen collectors for females only). The containers contain 10 g boric acid and 300 mg ascorbic acid for stabilization of the urine sample. Participants were instructed to collect all urine into this container for 24 hours and return the container to the research personnel during the next day's baseline visit. The second container was to be returned during the end of the study visit. The recruited participants were also issued 2 labeled Zymo Research DNA/RNA Shield Fecal Collection kits. Participants were instructed to collect the fecal sample into the kit at home and return the kit to the research personnel during the next day's baseline visit. The second kit was to be returned during the end-of-study visit.

On Baseline Visit 1 (Day 1), participants were instructed to arrive at 8:00 am after an overnight fast. A total of 14 ml of blood was collected by a butterfly needle inserted in a vein in the antecubital fossa or a forearm vein by a trained phlebotomist into two blood collection tubes: 6 ml into a BD Vacutainer Purple EDTA tube that was processed for isolating and storing the plasma sample and a 8 ml BD Vacutainer CPT mononuclear cell preparation tube for isolating and storing peripheral blood mononuclear cells (PBMCs).

Participants were instructed to measure fasting blood glucose using the handheld Care Touch glucometer (time 0 min), then consume 2 protein bars assigned to them, and perform 3 more blood glucose checks at times 30 min, 60 min, and 120 min after consuming the protein bar to determine how carbohydrates from the protein bar are processed. Each protein bar contained 17 g of carbohydrates. While waiting for blood glucose time points, the participants were asked to rate taste and palatability of the protein bar including after-taste, mouthfeel, and smell using the 1-9 point Hedonic Acceptance (9PH) scale (dislike extremely, dislike very much, dislike moderately, dislike slightly, neither dislike nor like, like slightly, like moderately, like very much, like extremely); Gastrointestinal Tolerability Questionnaire (GITQ) using 1-3 point scale (absent, mild, moderate, severe) and covering a range of GI symptoms like nausea, belching, feeling of fullness, satiety, vomiting, abdominal distension, flatulence, diarrhea, smooth stool, constipation, dry mouth and thirst; and a Global Quality of Life (GQOL) Questionnaire that focuses on the overall wellbeing using a 1-9 point scale (extremely bad, very bad, bad, somewhat bad, neither bad nor good, somewhat good, good, very good, extremely good).

Participants were given a bag labelled with 56 protein bars assigned to them and instructed to arrive at 8:00 am after an overnight fast after 8 weeks for the end of the study visit. Participants were asked to keep and return protein bar packages as proof of study compliance. All evaluations and procedures were to be performed again essentially as described for visit 1 at the end of Study Visit 2 (Day 56, i.e., 8 weeks).

5.3.4. Measurements of Blood Samples Biomarkers

Plasma aliquots were used to determine triglycerides, cholesterol, insulin, leptin, adiponectin, glucagon-like peptide-1, and a panel of inflammatory cytokines by the respective commercial kits, ELISA kits, and BD Accuri C6 flow cytometry. PBMC samples were used to determine metabolic flexibility and oxidative stress in peripheral blood mononuclear cells by Seahorse Extracellular Flux analyzer and BD Accuri C6 flow cytometry. All assays were performed in the Komarnytsky lab in the BSL-2 cell culture room using samples stored at -80°C.

5.3.5. Analysis of Phenolic Composition

Urine collection containers and stool collection kits were collected on Day 1 and at the end of the study. Urine samples were processed for storage at -80°C to extract and quantify phenolic metabolites. Two fecal samples were processed for storage: one for DNA extraction to assess the health status of the colonic microbiome and the other for extraction and quantification of phenolic acid metabolites. Phenolic composition analysis was performed on urine and fecal samples extracted with acidified methanol. Total phenolics were quantified using the Folin-Ciocalteu colorimetric method and Shimadzu Prominence LC-2030C HPLC profiling. All assays were conducted in the Komarnytsky lab in the BSL-2 cell culture room using samples stored at -80°C.

Fecal samples were also processed in the Komarnytsky lab to isolate microbial DNA. The DNA samples were analyzed externally for amplicon (16S/ITS) sequencing to detect changes in microbial abundance and profile ratios. All samples were coded with study participant ID numbers only.

5.3.6. Statistical Methods

Data were analyzed by one-way ANOVA using JMP. Tukey's range test is then performed for pairwise comparisons. Its significance was $P<0.05$.

5.4. Results and Discussion

5.4.1. Demographic and Anthropometric Characteristics

Of the 75 subjects initially enrolled, 69 completed their baseline study sessions and were included in the final analysis. Two participants withdrew after completing their baseline visit due to unstable blood glucose levels. An additional four participants withdrew prior to their baseline visit, citing scheduling conflicts and an inability to commit to the 3.5-hour session duration.

Participants were enrolled and randomly assigned to one of four intervention groups: Group P (Pea protein bar with purple dye, n = 17), Group P + BCP-C (complexed pea protein-blackcurrant polyphenol particles, n = 18), Group P + BCP (pea protein bar with added black currant polyphenols, n = 17), and Group W + BCP (whey protein bar with added black currant polyphenols as a reference control, n = 17),. Baseline demographic and anthropometric characteristics are presented in Table 5.2.

The mean age distribution across all groups ranged from 41.5 ± 10.7 to 51.8 ± 13.0 years. At baseline no significant differences were observed among groups in terms of height, weight, and BMI. Mean BMI values ranged from 25.1 ± 4.0 to 31.9 ± 12.2 kg/m^2. Additionally, no significant differences were observed in body composition (muscle mass, fat mass, and fat percentage) among all groups.

There were no significant differences in systolic and diastolic blood pressure values across all groups, averaging around 119.2 ± 11.0 mmHg for systolic and 75.6 ± 9.2 mmHg for diastolic

reads. Basal metabolic rate (BMR), heart rate, and oxygen saturation levels were also comparable at baseline, with no statistically significant between-group differences. These findings suggest balanced distribution of baseline characteristics across all intervention groups.

Table 5.2. Demographic and anthropometric data of the study participants (± SD).

Group code **Protein** **Polyphenols** **Subjects**	**B** **Pea** **Purple dye** **(n=17)**	**C** **Pea** **BCP complex** **(n=18)**	**D** **Pea** **BCP** **(n=17)**	**A** **Whey** **BCP** **(n=17)**
Age (years)	43.3 ± 11.3	41.5 ± 10.7	44.7 ± 13.9	51.8 ± 13.0
Gender (female/male, %)	10/7 (58.8%)	11/7 (61.1%)	13/4 (76.5%)	9/8 (52.9%)
Hight, cm	167.6 ± 11.2	169.8 ± 11.4	167.8 ± 9.2	173.1 ± 7.4
Weight, kg	74.5 ± 24.4	81.5 ± 21.3	73.5 ± 16.1	84.8 ± 17.2
Muscle mass, kg	28.7 ± 7.7	31.0 ± 7.9	28.2 ± 7.6	31.6 ± 5.8
Fat mass, kg	19.0 ± 8.7	26.0 ± 15.9	22.5 ± 7.0	28.2 ± 15.5
Fat mass, %	27.1 ± 10.4	30.3 ± 12.5	30.5 ± 7.0	31.9 ± 12.2
BMI, kg/m^2	25.1 ± 4.0	28.1 ± 6.3	25.9 ± 3.6	28.4 ± 6.6
BMR, cal	1482 ± 276	1568 ± 285	1473 ± 271	1592 ± 209
Systolic BP, mmHg	121.2 ± 11.3	113.8 ± 15.1	118.2 ± 12.2	119.2 ± 11.0
Diastolic BP, mmHg	82.2 ± 7.6	75.5 ± 9.9	76.5 ± 11.5	73.9 ± 6.6
Heart rate, bpm	72.2 ± 8.1	73.2 ± 14.0	69.5 ± 10.8	70.2 ± 12.5
Oxygen saturation, %	97.4 ± 3.2	97.5 ± 0.9	98.3 ± 1.6	95.3 ± 7.7

5.4.2. The 3-day Dietary Recall Questionnaire

Participants were asked to record their food and drinks and any supplements during three consecutive days, including one weekend day. Caloric intake assessed via a 3-day dietary recall indicated that there were no significant differences in daily caloric intake among the groups, with all participants reporting similar energy consumption. This suggests that subjects had similar dietary energy intakes at the start of the trial (Figure 5.1). We also calculated the grams of carbohydrates, protein, fiber, and total sugar for each day.

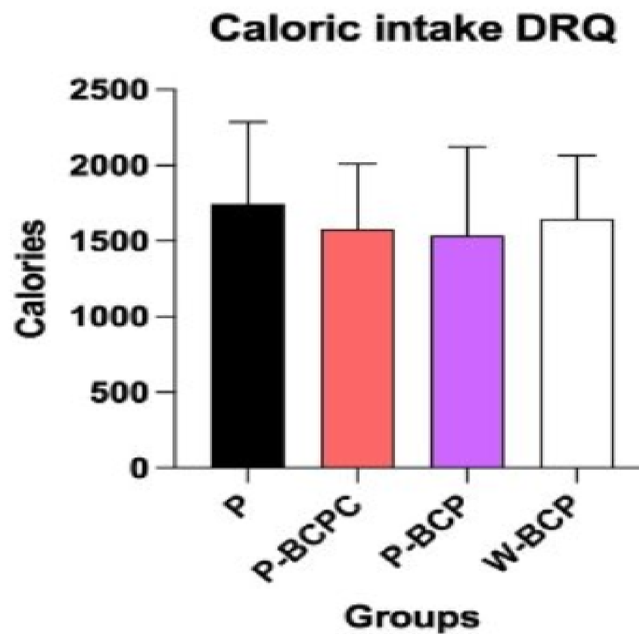

Figure 5.2. Subject-reported daily caloric intake at baseline using 3-day Dietary Recall Questionnaire.

5.4.3. Food Frequency Questionnaire and The Total Phenolic Content (TPC)

Several studies confirmed that FFQ is effective in estimating polyphenol intake (Yang et al., 2010; Li et al., 2024), therefore to estimate individual polyphenol intake and assess baseline dietary patterns, a semi-quantitative, food-based Food Frequency Questionnaire (FFQ) was considered in this study. The FFQ included 139 items with a focus on foods rich in polyphenols. Food items were categorized into several groups, including beverages (juices, milk-based drinks, meal replacements, and energy or high-protein beverages); grains and cereals (oatmeal, grits, other cooked cereals, rice, cracked wheat, millet, and pasta); fruits and vegetables; protein sources (meat, chicken, fish, eggs, and soups); snacks and sweets (pizza, crackers, nuts, and various sweets); fats (butter and oils from different sources); and other items such as coffee and tea. Participants

reported their consumption frequency for each item using eleven categories: never, one to six times per year, seven to eleven times per year, once a month, two to three times a month, once a week, one to two times a week, three to four times a week, five to six times a week, once a day, or twice a day or more. For seasonally available foods like fruits, participants also indicated the number of months per year they typically consumed each item, selecting from 3, 6, 9, or 12 months. To validate the FFQ, estimated polyphenol intake was compared with total polyphenol concentrations measured in 24-hour urine samples. The Folin–Ciocalteu reagent method was used for quantification, providing an objective biomarker for dietary polyphenol intake.

The left panel illustrates the average daily phenolic intake (mg/day) reported by participants across four groups: P, P-BCPC, P-BCP, and W-BCP. Estimated dietary phenolic intake was similar across groups at baseline, with no substantial differences observed. The right panel presents the total phenolic content (TPC) measured in urine (mg/L) for the same four groups. Baseline urinary TPC levels were below 60 mg/day and consistent across all groups, indicating minimal systemic exposure to phenolics before the intervention began and these findings support the validity of the FFQ in estimating polyphenol consumption, as reflected in biological markers (Figure 5.3).

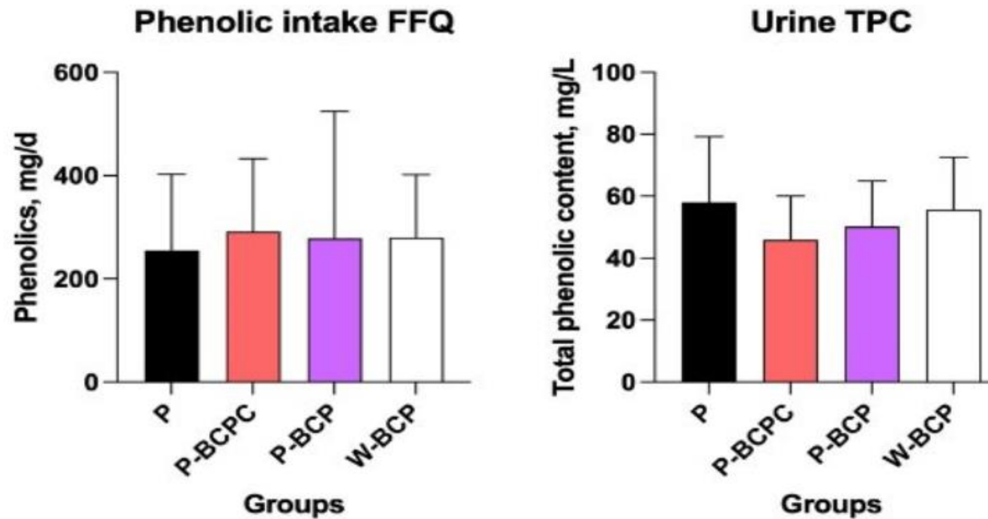

Figure 5.3. Subject-reported daily phenolic intake at baseline using a 6-months Food Frequency Questionnaire and urinary total phenolic content (TPC).

5.4.4. Food Acceptance Questionnaire

Participants evaluated four different bar formulations, P, P-BCPC, P-BCP, and W-BCP, using the Food Acceptance Questionnaire. The FAQ had 7 question items to assess the sensory characteristics of bars, including texture, flavor, taste, aftertaste, mouthfeel, smell, and appearance. Ratings were based on a 9-point hedonic scale, where 9 indicated "like extremely" and 1 indicated "dislike extremely," as appeared in Figure 5.4.

Across most sensory attributes, bars containing either pea protein alone or enriched with blackcurrant components received similar average scores compared to the W + BCP group. These results suggest that plant-based proteins, particularly pea protein used in these formulations, may gain the same overall consumer acceptability.

All the bars generally had a like-slightly rating on most domains such as flavor, taste, and mouthfeel, the attributes that are critical for consumer satisfaction and product success.

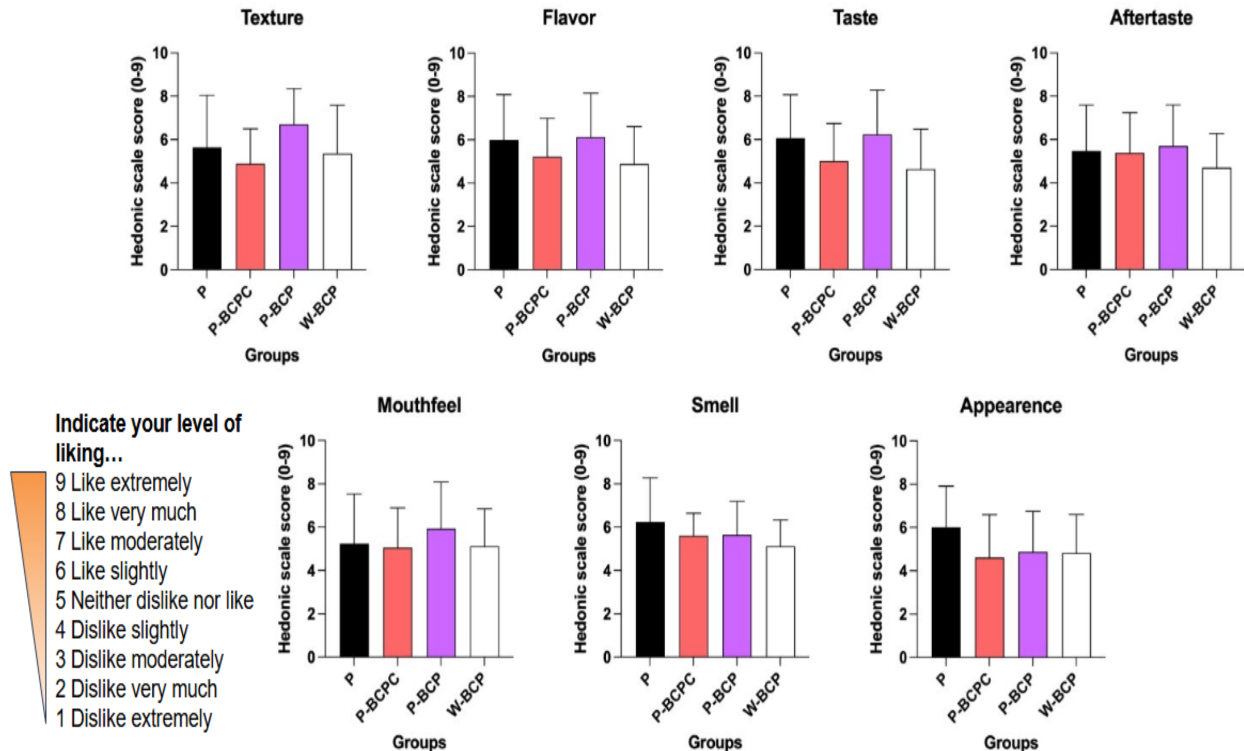

Figure 5.4. Subject-reported liking of the bars at baseline using Food Acceptance Questionnaire.

Similarly, previous research has shown that supplementing myofibrillar protein gels with polyphenol-rich plant additives from blackcurrant pomace significantly enhances sensory appeal, particularly improving color, flavor, and overall acceptability (Leicht et al., 2025). Additionally, previous research demonstrated that incorporating a moderate amount of pea protein into a tomato-based product improved both rheological properties such as viscosity and texture and sensory perception, particularly enhancing mouthfeel and overall acceptability (Bal et al., 2024).

5.4.5. Gastrointestinal Tolerance Questionnaire

Each participant was asked to evaluate the impacts of consuming bars on their gastrointestinal tolerance by evaluating different symptoms: nausea, belching, fullness, stomach

distension, vomiting, flatulence, diarrhea, smooth stool, constipation, dry mouth, and thirst. Each symptom was rated on a 4-point scale (0 = absent, 1 = mild, 2 = moderate, 3 = severe).

Across the groups, most symptoms were reported at low to moderate levels. Notably, statistically significant differences were observed in some symptoms, including fullness and stomach distention, particularly between the P group and the P-BCP groups. For other symptoms such as dry mouth, thirst, or vomiting, no significant differences were observed indicating that these were not major concerns across any of the interventions. (Figure 5.5).

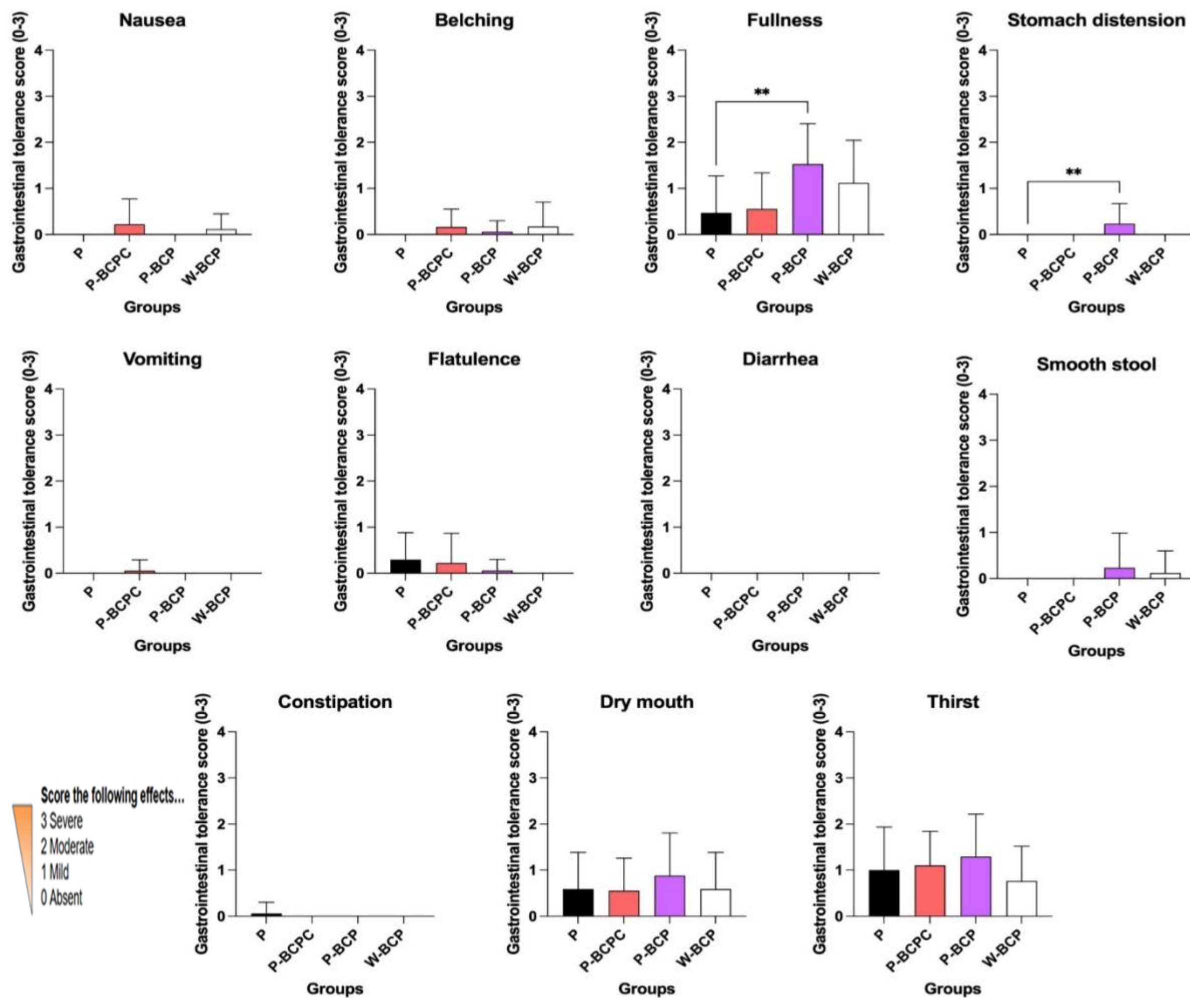

Figure 5.5. Self-reported gastrointestinal effects using Gastrointestinal Tolerance Questionnaire

In a previous randomized, double-blind study, consumption of 15 g and 30 g of NUTRALYS® pea protein significantly reduced subsequent energy intake and was associated with a greater feeling of fullness compared to a control condition, and this effect could be due to increase levels of cholecystokinin (CCK), a hormone linked to satiety, suggesting a physiological mechanism underlying the enhanced sensation of fullness (Re et al., 2016).

5.4.6. Global Health Questionnaire

Self-reported well-being was assessed using the PROMIS Global Health Questionnaire across several domains: social activities, physical activities, pain interference, and fatigue. The results were compared among all groups (Figure 5.6).

In most domains all groups reported similar levels, with average scores around 4, corresponding to a rating of "very good." No substantial differences were observed between groups in all domains. This aligns with previous research indicating that dietary interventions, particularly those rich in anti-inflammatory compounds like polyphenols, may reduce perceived pain or discomfort (Del Bo et al., 2019).

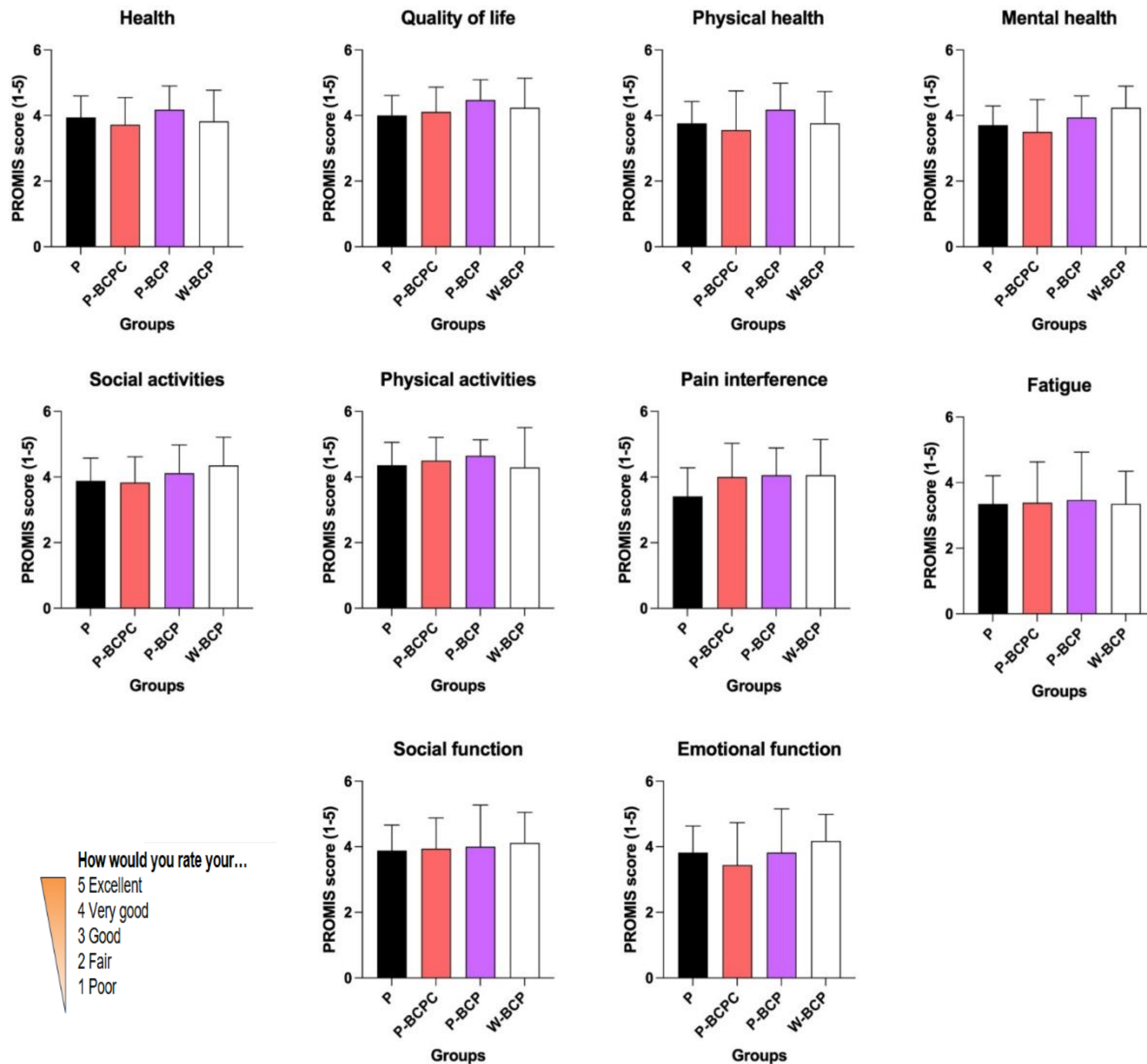

Figure 5.6. Self-reported wellbeing using PROMIS Global Health Questionnaire.

5.4.7. Postprandial Glucose Response

Postprandial glucose levels were measured over a 120-minute period following the consumption of two bars containing 34 grams of carbohydrates (Figure 5.7). At baseline, all groups exhibited comparable fasting glucose levels (approximately 80–90 mg/dL), indicating similar glycemic status prior to intervention.

Following ingestion, all groups experienced a rise in glucose levels at 30 minutes. Notably, the W-BCP group exhibited the highest peak, suggesting a stronger glycemic response and potentially less effective glucose regulation. In contrast, the P-BCP and P-BCPC groups demonstrated attenuated glucose excursions, with P-BCPC showing the lowest peak among all groups. This suggests that the interventions associated with these groups may have contributed to improved postprandial glucose control.

By 60 minutes, the P-BCPC group showed the most reduction in glucose levels, indicating a more rapid return toward baseline. This may reflect enhanced insulin secretion or improved insulin sensitivity in this group compared to others. The P group, while showing a moderated response, had a glucose profile more similar to W-BCP, suggesting that the presence of BCP or BCPC was necessary to achieve a more favorable glycemic outcome.

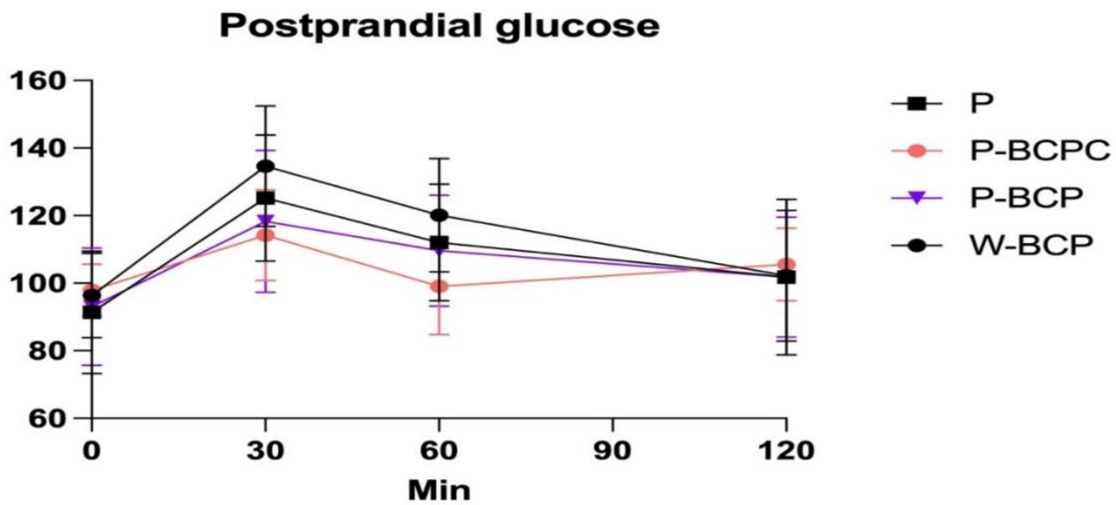

Figure 5.7. Postprandial glucose after 2 bars (34 g carbs)

This pattern aligns with previous research indicating that dietary components such as polyphenols and dietary fibers can modulate carbohydrate metabolism and reduce postprandial glycemic excursions. Polyphenols, in particular, have been shown to inhibit carbohydrate-

digesting enzymes (e.g., α-amylase and α-glucosidase), delay glucose absorption, and enhance insulin sensitivity (Hanhineva et al., 2010; Williamson, 2013). Additionally, protein and fiber-rich formulations may slow gastric emptying and reduce the glycemic index of foods, contributing to the observed effects. Additionally, pea protein supplements can significantly reduce food intake in both lean and obese individuals, with increased CCK and GLP-1 levels in obese individuals, suggesting enteric coating as an effective dietary strategy for obesity prevention and treatment (Geraedts et al., 2011). Also, pea protein reduced postprandial glycemic levels and stimulated insulin release in healthy adults (Thondre et al., 2021).

5.8. Conclusion

These findings support the potential role of functional food formulations based on plant proteins and polyphenols in managing postprandial glycemia, which is a critical factor in metabolic health and the prevention of insulin resistance and type 2 diabetes.

Future studies should explore these effects in larger populations and over extended durations, incorporating additional biomarkers such as insulin, incretins, and inflammatory markers to better understand the underlying mechanisms. To address this, we plan to continue this study over an 8-week period to investigate how different types of bars influence postprandial glucose responses and to elucidate the physiological pathways involved.

5.9. References

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Chapter 5: Conclusions And Future Directions

Plant-based foods such as tea, coffee, wine, cocoa, cereal grains, soy, fruits, and berries contain a diverse range of phytochemicals called polyphenols, which include flavonoids, phenolic acids, proanthocyanidins, and resveratrol. (Gharras, 2009). Most polyphenols are absorbed at a relatively low level in the small intestine (5–10%). The remaining (90–95%) enter the large intestinal lumen, where they are metabolized by the gut microbiota into bioactive metabolites with low molecular weight and possibly increased bioavailability (Vong et al., 2022). The regular consumption of polyphenol-rich foods and beverages and their delivered metabolites has been shown to reduce postprandial glucose responses and fasting hyperglycemia, enhance acute insulin production, improve insulin sensitivity, and decrease the incidence of cardiovascular diseases, colon cancer, liver disorders, obesity, and diabetes (Cory et al., 2018; Del Bo et al., 2019).

Nowadays, a growing interest in the botanical products generated derived from polyphenols is known for their antioxidant, anti-inflammatory, and antimicrobial (Albuquerque et al., 2020) properties as pharmaceutical agents and have diverse biotechnological applications in various industries, particularly in food preservation, food and packaging industries, cosmetics, and textiles (Rasouli et al., 2017).

Phenolic acids are the simplest class of PC, consisting of one phenolic ring and a carboxylic acid function. They are divided into two groups: hydroxybenzoic acids derived from benzoic acid and hydroxycinnamic acids from cinnamic acid, with important bioactivities (Kumar & Goel, 2019). We explained the role of small phenolics and their metabolites in regulating several metabolic pathways. Our review demonstrates that these compounds significantly influence nutrient transport, energy metabolism, and neurotransmitter balance. By interacting with multiple molecular pathways, phenolic acids and their metabolites exhibit versatile regulatory effects on

metabolic health. These results advance our understanding of the metabolic roles of phenolic acids and highlight their potential in dietary strategies for improving insulin sensitivity and reducing metabolic disorder risks (chapter one). We further explored the mechanisms by which phenolic compounds, particularly bitter ones found in spices and culinary herbs, can enhance metabolic status. Originally evolved as chemical defenses in plants, these compounds have been preserved in human diets and now play a role in modulating glucose metabolism and appetite regulation. Emerging evidence suggests that bitter phenolics may influence intestinal glucose transporters and stimulate the release of metabolic hormones(chapter two).

This evolutionary adaptation presents a promising avenue for developing food-based strategies to improve metabolic and immune health, ultimately enhancing metabolic resilience and overall quality of life. To address this, we developed an in vivo study to investigate the effects of hydroxylated and methylated phenolic acids on obesity, a serious health problem. The study involved treating obese mice with a high-fat diet mixed with these phenolic acids for eight weeks. Our major finding was that methylated phenolic acids were more effective in reducing body weight by decreasing fat mass. This effect was associated with an improvement in the expression of various genes in white adipose tissue, suggesting a potential mechanism for their anti-obesity effects. Understanding their actions and developing strategies to improve bioavailability, sustainable extraction, and stability are still crucial. The reason is because polyphenols derived from fruit and vegetables are essential for food industry utilization, as they are unstable and susceptible to oxidation and heat degradation, compromising their absorption and health benefits (Jia et al., 2025). To maintain the health benefits of polyphenols from fruits and vegetables, effective methods include encapsulation (Bartosz & Irene, 2016), nanoencapsulation (Xiao et al., 2023), use of antioxidants, pH adjustment, temperature control, and modified atmosphere

packaging (Pan et al., 2025). Studies on complexed aggregate protein–polyphenol particles excused on forming nano- or micro-scale particles to protect the bioactivity of polyphenols, rather than addressing the intake of nutritional bars from the polyphenol–protein complex on human health, especially obesity (Da Silva et al., 2024). Complexed aggregate protein–polyphenol particles could be used for functional protein delivery in high protein bars, boosting nutritional content and preventing alterations. Our clinical trial study showed the effectiveness of complexed pea protein bars- blackcurrant polyphenols particles in improving overall health (chapter 5).

However, some limitations should be considered in our research, including the animal study (Chapter 3&4), which is a method of treatment delivery. While encapsulated treatments are more effective in ensuring good delivery and bioavailability, mixing phenolic acids with the diet provides a unique opportunity to study their effects on taste receptors in the mouth. Although we did not specifically study this aspect, our study could provide valuable insights into the mechanisms of action. Additionally, clinical studies on humans are necessary to better address the effects of these compounds, as our current research is limited to animal models. While our research focused on studying the most common phenolic acids, there is still a limitation for studying a broader range of phenolic acids.

The impact of protein-polyphenol bars on metabolic health may have several limitations, such as consistent consumption and compliance with the study protocol, which can also affect results validity. Dietary and lifestyle variables can influence outcomes, and self-reported data can introduce bias.

Therefore, there are still some gaps that need to be better studied: (1) Further research to fully understand and utilize these compounds effectively in the food industry, ensuring their stability and bioactivity preservation throughout their useful life, without causing side effects. (2)

Studying differences between methylated and hydroxylated phenolic acids on various diseases, such as non-alcoholic fatty liver disease (NAFLD) and cardiovascular diseases. (3) Additionally, clinical studies on human models are essential to evaluate the efficacy of these compounds in diverse populations. Investigating the impact of dietary interventions rich in phenolic acids with different structures on metabolic health will provide valuable insights. (4) Studying the synergistic effects of phenolic acids with other bioactive compounds like flavonoids will further enhance their therapeutic potential and provide a deeper understanding of their combined impact on health. These will guide further research and improve our understanding of the role of phenolic acids and flavonoids in human health, ultimately contributing to the development of effective dietary strategies and therapeutic interventions.

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