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Cardiometabolic Health Biomarker Modification with Dietary Polyphenols: Effects of Olive Oil and Walnuts on established CVD biomarkers, inflammation, and gut microbiome.

By

TATIANA DIACOVA
DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

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Abstract

Cardiovascular disease is a major health concern in the United States. Dietary patterns like the Mediterranean diet have demonstrated beneficial effects on cardiovascular health. The health benefits of any dietary pattern likely stem from the combined effects of its individual dietary components that can exert their beneficial effects through affecting host biology directly, as well as through modulation of the gut microbiome composition and functionality. Two common dietary components, olive oil and walnuts, have been studied in relation to cardiometabolic and gut health in several randomized, controlled trials.

The first chapter in this dissertation summarizes the current literature published on the role polyphenol and lipid-rich foods play in the gut-heart-brain axis. The review focuses on individual components in walnuts and olive oil that could potentially modulate gut microbiome composition and or functionality and consequently affect the gut, heart and brain health. With increased knowledge of how individual dietary components affect the host directly and indirectly through the gut microbiome changes, recommendations of foods to include in dietary patterns can be made with more confidence.

Diet is one factor modulating gut microbiota composition and functionality. However, determining causal links between diet-gut microbiome interactions and human health is complicated by inconsistencies in the evidence, arising partially from differences in research methods and reporting. Widespread adoption of standardized best practices would help advance the field but requires those practices be identified, consolidated and discussed. The second chapter is an umbrella review that aims to identify recommended best practices, define existing gaps, and collate considerations for the design of studies investigating diet-gut microbiome interactions in relation to human health outcomes. Recommendations, considerations and gaps

relating to best practices within the categories of study design, participant selection, dietary intervention/assessment, biological sample collection, and statistical analysis were extracted and consolidated to identify agreement and knowledge gaps. Eight narrative reviews were included. Several general points of agreement were identified but a recurring theme was that best practices will depend on the research aims, outcomes and feasibility. Multiple gaps were also identified.

The third chapter investigates the effects of daily walnut consumption on established biomarkers of cardiovascular health, as well as gut inflammation and gut microbiome in adults between the age of 45 and 75 years with borderline elevated LDL-C levels. In this randomized crossover trial, thirty participants were randomized to consume 56 g of walnuts daily or follow a no-nut diet for 3 weeks. One-week run-in and a 3-week washout periods were also included in the study design. Blood, fecal, and urine samples were collected at the beginning and end of each intervention period. Blood lipids, circulating inflammatory markers, fecal gut inflammatory markers, walnut- and microbial-derived metabolites, as well as gut microbial relative abundancies were investigated. Walnuts decreased total cholesterol, LDL, lipocalin-2 and calprotectin. Fecal lithocholic bile acid and several short-chain fatty acids were also lower after walnut consumption compared to the no-nut diet. Percent relative abundance of *Roseburia* increased, while relative abundance of fecal *Bifidobacteria* and *Alistipes* decreased after walnuts. In addition, three urolithin metabotypes were identified and plasma/urine levels of various urolithin metabolites were correlated with blood lipids and fecal bile acid levels.

The fourth chapter investigates the effects of consumption of two types of olive oil, low-polyphenol (LPOO) and high-polyphenol (HPOO), on biomarkers of cardiometabolic health in acute and short-term consumption settings. This chapter focuses on the effects of circulating blood cytokines and cytokines released from whole blood and peripheral blood

mononuclear cells (PBMCs) either unstimulated or stimulated with bacterial lipopolysaccharide (LPS). Twenty-six generally healthy adults (BMI >18.5 kg/m^2) participated in this randomized crossover trial. Participants were randomized to consume 40 ml of LPOO or HPOO. Following the assessment of acute consumption (2 hrs and 4 hrs postprandial), participants consumed their respective treatment daily for 2 weeks to determine effects of short-term consumption. There were no notable differences between treatments in levels of systemic cytokines and cytokines released from stimulated or unstimulated whole blood. We did not observe any significant between-group differences in systemic cytokines and cytokines secreted from unstimulated whole blood with two weeks of HPOO and LPOO consumption. However, in LPS stimulated PBMC samples, IL-1ß secretion was lower after short-term HPOO consumption compared to LPOO. After acute consumption, IL-6 and TNF-a spontaneously secreted from PBMCs were increased 4hrs after HPOO but not LPOO consumption. In contrast, TNF-a secreted from LPS stimulated PBMCs was lower 4hrs after HPOO intake compared to LPOO.

The fifth chapter is the conclusion. This chapter briefly summarizes the major findings of the literature review, umbrella review and the walnut and olive oil studies. Furthermore, it provides overall interpretation of these findings, as well as several future directions.

Chapter 1

Role of microbial metabolites from bioactive and lipid-rich foods in the gut-heart-brain axis: focus on walnuts and olive oil. A narrative review.

1. Gut microbiota and host health

The human gut microbiota can be defined as a dynamic community of organisms including prokaryotes, eukaryotes, archaea, viruses and fungi [1], with bacteria from phyla *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia* being dominant [2]. The intestinal microbiota contributes to metabolic and physiological function through the Digestion and degradation of food components [3], harvesting of inaccessible nutrients [4], and can produce additional metabolic products originally derived from the host or other microbes in the community [5]. These metabolites are the main components of the crosstalk between gut microbes and the host [5] and can have a profound impact on host health to include immune system maturation, maintenance of gut barrier function [6-8], and regulation of brain function and behavior [9]. In addition to brain health, the gut microbiome has the potential to affect cardiometabolic health, with dysbiosis in the gut microbiome contributing to type 2 diabetes, hypertension, obesity [5], and overall mortality risk [10]. In fact, a recent long-term prospective study conducted in Finland (n = 7211) suggested a positive association between the Enterobacteriaceae family and host overall mortality risk [10].

While this evidence is compelling, it is also important to acknowledge that the concept of gut dysbiosis has not been well-defined yet [11]. It is generally believed that gut microbiota dysbiosis is characterized by compositional and functional alterations in the microbial community in diseased individuals compared to healthy subjects [12]. These alterations are commonly manifested as a decrease in beneficial microorganisms and an increase in potentially harmful ones, along with a loss of overall microbial diversity [13]. The challenge with these manifestations is the lack of understanding and agreement within the scientific community on

which microbes should be categorized as “beneficial” and which ones should be deemed “harmful”, making defining a “healthy” gut microbiome virtually impossible [14].

1.1 Gut microbiota and brain health

Bidirectional communication between the gut and brain involves the neuroendocrine, and neuroimmune, and central nervous system (CNS) [5], and includes CNS communication through the sympathetic, parasympathetic and enteric divisions of the autonomic nervous system [5]. This, in turn, affects gut mobility, permeability, innate immune cell activation and microbiota composition [5].

The communication from the gut to the brain happens mostly through gut microbial metabolites (short-chain fatty acids (SCFAs), neurotransmitters, and their precursors) that can affect the levels of related metabolites in the brain via the blood circulation, therefore regulating brain function and cognition [15-17]. For instance, evidence shows that *Lactobacillus* spp. can produce the neurotransmitters gamma-aminobutyric acid (GABA) and acetylcholine [15]. *Bifidobacteria* can also synthesize GABA, while *Escherichia* spp., *Bacillus* spp., and *Saccharomyces* spp. produce catecholamines and *Bacillus* and *Serratia* spp. produce dopamine [15]. Evidence demonstrates that several of the aforementioned metabolites have the potential to act on brain cells directly, while also being able to communicate with the brain via the afferent vagal nerve fibers [15]. Tryptophan is known to be converted to serotonin (5-HT) in the brain, which can then induce cognitive change [15]. In addition, 5-HT appears to be produced by enterochromaffin cells (EC) of the intestinal epithelium, with circulating and intestinal levels dependent on the gut microbiota composition [15]. It has been well-established that 5-HT is important for learning and memory, as well as for normal cognitive function and may be involved in treatment and/or pathogenesis of cognitive disorders [19].

Evidence shows that gut microbes can affect 5-HT levels by either directly affecting its biosynthesis pathways or by producing metabolites that can impact them. For instance, several spore-forming bacteria, including *Clostridium ramosum*, demonstrated the ability to promote 5-HT biosynthesis by inducing host colonic tryptophan hydroxylase-1 (Tph1) expression [20]. In addition, several lactic acid bacteria, including *Bifidobacterium dentium* [21], *Bifidobacterium longum* [22], *Lactobacillus rhamnosus* [23-24] and *Lactobacillus acidophilus* [22] can increase intestinal serotonin-selective reuptake transporter (SERT) concentrations. Furthermore, EC cells have SCFA receptors that can stimulate 5-HT release, while activation of G-protein-coupled bile acid receptor 1 (GPBAR1 aka TGR5) by microbial-derived secondary bile acids (BAs) promotes 5-HT release [25].

Additional evidence points to the impact of the gut microbiome on mood and behavior. In a rodent model, lactic and propionic acids produced by the gut microbes affect behavior and impact memory [5]. Moreover, immune activation mouse models demonstrate that maternal microbiome composition plays an integral role in the development of autism-like behavior in offspring [26]. There is also increasing evidence for the role of microbiome composition and metabolite production in host mood disorders [27]. It has recently been found that an increase in relative abundance of a few bacterial genera like *Akkermansia*, *Desulfovibrio*, *Lactococcus*, *Oscillopsia*, and *Sutterella* was linked with social behavior and autism disorders [28-29], while an increase in *Prevotella*, *Klebsiella*, *Streptococcus* and *Clostridium* XI were implicated in individuals with the major depressive disorder (MDD) [30]. This is in agreement with the hypothesis that microbial derived metabolites may be involved in depression [31-32]. Moreover, microbiome metabolites associated with depressive disorders may also assist with metabolic alterations that often lead to impaired cardiometabolic health [33]. Variations in lipids, including

triglycerides (TGs), low-density lipoprotein cholesterol (LDL-C), very low-density lipoprotein cholesterol (VLDL-C), high-density lipoprotein cholesterol (HDL-C), phosphatidylcholine, lysophosphatidylcholine, lysophosphatidylethanolamines, phosphatidylethanolamines, and sphyngomyelins have been associated with MDD in various studies [34-36]. Disturbances in the levels of these lipids may be involved in the development of mitochondrial dysfunction, with HDL-C inversely correlating with mitochondrial DNA damage [37]. A recent metabolic profiling of 5283 individuals with depression and 10145 controls demonstrated a shift towards decreased levels of HDL-C and increased levels of VLDL-C and TGs in depressed people [33]. The exact molecular mechanisms underlying these shifts have not been elucidated yet, but the gut microbiome has been strongly implicated in modulating blood lipid levels and regulating mitochondrial function [38].

1.2 Gut microbiota and cardiovascular health

Gut microbial community composition and metabolic capacity can strongly impact cardiovascular health of the host. For instance, a recent systematic analysis of host genome, gut microbiome, body mass index (BMI), and blood lipids in 893 human subjects from the Dutch LifeLines-DEEP cohort demonstrated that family Clostridiaceae/Lachnospiracease were positively associated with LDL-C levels, while family Pasteurellaceae, genus *Coprococcus* and *Collinsella*, and species *Stercoris* were strongly negatively associated with TG levels. A novel and strong association of the family Christensenellaceae and the phylum *Tenericutes* with lower TG levels and higher HDL-C levels was also identified [39]. Furthermore, this study reported that gut microbiota appeared to explain 4-6% of the variance in HDL-C and TG levels, but no significant associations were found with either LDL-C or TC levels [39]. Another study by Kurilshikov et al. explored the relationship between the gut microbiome and plasma metabolites,

metabolic risk of cardiovascular disease (CVD) and cardiometabolic phenotypes in 1275 individuals from a population cohort (LLD) and a cohort of overweight/obese individuals with cardiovascular and metabolic complications (300-OB) [40]. After correction for age, sex, BMI, and smoking microbial associations were identified for 210 metabolic traits in the LLD population cohort and for 35 metabolic traits in the 300-OB cohort [40]. Two microbial factors were found to be identified with cardiometabolic phenotypes: higher abundance of bacterial L-methionine biosynthesis was significantly associated with the presence of atherosclerotic plaque and maximum stenosis and *Ruminococcus* sp_5_1_39BFAA was positively associated with hepatic fat content [40]. Finally, assessment of stool metagenomics and metabolomics of 1,429 Framingham Heart Study participants showed that species from *Oscillibacter* genus, which possesses conserved cholesterol-metabolizing capabilities, were associated with decreased fecal and plasma cholesterol levels [41]. Authors concluded that host cholesterol metabolism may be a broad property of *Oscillibacter* spp. with potential benefits for lipid homeostasis and cardiovascular health [41].

Several associations between the gut microbiota and adult stroke have also been recently suggested. Adult stroke is the second main cause of mortality and the third cause of disability worldwide [42-43]. Evidence shows that patients with stroke have different gut microbiota compared to healthy controls, as well as decreased α-diversity [5]. In addition, patients with cardiometabolic disease (CMD) have an enriched abundance of gut microbiome species of Enterobacteriaceae, including *Escherichia coli*, *Enterobacter aerogenes* and *Klebsiella* spp. vs. healthy controls [44]. Similarly, the gut microbiome of patients with ischemic heart failure (IHF) has an increased abundance of *Acinetobacter*, *Ruminococcus*, and *Veillonella* spp. and a decreased abundance of *Oscillibacter* spp., *Alistipes* and *Faecalibacterium* [45]. It has also been

observed that intestinal barrier function in heart failure patients is usually impaired leading to migration of bacterial products from the lumen into the circulation, which can induce a pro-inflammatory state (discussed in more detail below) [46-47].

Both the gut microbiota and host metabolites derived from dietary sources interact in the gut and produce a wide range of new metabolites which consequently modulate the gut microbiome composition and the host metabolome [48]. These changes are also represented in the blood and may be responsible for cardiovascular disorders. Depending on the nature of microbiota-derived metabolites such as SCFAs, BAs, branched-chain amino acids (BCAA), tryptophan, indole derivatives, and trimethylamine (TMA), different kinds of cardiovascular disorders may arise [2]. For instance, TMA, which is converted by the hepatic flavin monooxygenase 3 (FMO3) into trimethylamine N-oxide (TMAO), is a compound known to be positively associated with death, myocardial infarction (MI), and stroke over a three-year period according to several recent studies that combined data from over 4000 individuals who underwent coronary angiography [49-50]. The first study linking the microbiome and CVD identified TMAO as a causing agent of CVD [49-51]. Evidence also shows that TMAO levels are increased in people with Parkinson's, Alzheimer's and ischemic stroke [52] linking gut microbiome with heart and brain health yet again.

1.3 Gut microbiota and immunity

Gut microbiota seem to be affecting host immunity through three potential pathways, with one of them being immune activation [53]. Several recent mouse studies provide evidence to support this. For instance, it has been demonstrated that microbiota transfer from Caspase $1^{-/-}$, or inflammatory dysregulation prone, mice into LDLR $^{-/-}$ (LDL receptor deficient) mice increases atherosclerotic plaque formation compared to mice receiving microbiota from $LDLR^{-/-}$ mice

[54]. Kim et al. suggested that a potential underlying mechanism could be that gut microbiota elevated systemic pro-inflammatory cytokine levels, including interleukin-1ß (IL-1ß), interleukin-2 (IL-2) and interferon-γ (IFN- γ). In addition, transplants with *Bacteroides vulgatus* and *Bacteroides dorei* have demonstrated the ability to attenuate atherosclerotic lesions and decrease plasma tumor necrosis factor-α (TNF-α) levels [55].

Another potential pathway through which gut microbiota impacts host immunity is modulation of gut permeability and intestinal barrier function [53]. Intestinal cells, tight junctions between the cells, mucus and mucosal immunity are all mechanisms via which the host ensures that gut microbes are residing in the gut without triggering host immune response [53]. Minimal bacterial translocation from the gut into the bloodstream is a common event and can prime innate immunity and promote immune system maturation [15]. Perturbations in gut microbiota, however, can contribute to compromised gut barrier integrity resulting in more than minimal translocation of bacteria, where lipopolysaccharide (LPS), a component of gram-negative bacteria, can activate toll-like receptor 4 (TLR4) triggering a systemic immune response [15]. Several recent studies reported that patients with CVD may have higher circulating levels of LPS compared to healthy individuals [56-57]. Another gut microbial cell wall component that may stimulate the immune system is peptidoglycan, which trigger the nucleotide-binding oligomerization domain containing protein-1 (NOD1) and enhance neutrophil function [15].

Aside from being able to take advantage of the compromised gut integrity, gut microbes can also help strengthen the intestinal barrier. A recent $ApoE^{-/-}$ mouse study demonstrated that administration of live *A. muciniphila* may reduce gut permeability, circulating endotoxin and aortic atherosclerosis [58].

Another way through which gut microbiota may affect host immunity is production of several beneficial metabolites. One category of these metabolites are SCFAs, including butyrate, acetate and propionate. Lachnospiraceae and Ruminococcaceae families are the main known butyrate producers [59-60], while *Bifidobacterium* species can produce acetate and lactate [61] and *Akkermansia muciniphila* has the metabolic capacity to produce acetate and propionate [61]. SCFAs can bind G protein-coupled receptors 41, 43/109A [62-64] which may lead to a decrease in blood pressure and has shown to regulate abundance of regulatory T cells (Treg) in mice [65-67]. In addition, mucus production in the gut can be upregulated by SCFAs [15]. Other microbial metabolites that have shown the potential to affect host immunity are TMA and tryptophan-derived metabolites including tryptamine, indole and indole-derived acids among others [62]. Tryptophan metabolites have various physiological functions, including anti-inflammatory and anti-oxidative systemic effects [68]. In fact, indole production can enhance expression of cell adhesion molecules in the gut by stimulating the Pregnane X receptors (PXR) [15].

The immune system, in turn, contributes to gut microbial homeostasis by selectively promoting growth of specific bacterial species [15]. This is done through production of antimicrobial molecules (REGIIIa, a-/b-defensins, IgA and T cells), reactive oxygen species (ROS), and alterations in nucleotide-binding oligomerization domain containing protein-2 (NOD2), NOD-like receptor family pyrin domain containing-6 (NLRP6), and TLR immune pathways [15].

2. Gut-heart-brain axis

As discussed above, diet is known to modulate gut microbiota composition and functionality. Changes in gut microbiota then have effects on the gut, cardiovascular and brain health, which also seem to be tightly interconnected. For instance, accumulating evidence

suggests that the gut microbiota may play an important role in controlling atherosclerosis progression to support the link between the gut and CVD [69-70]. Bacteria with the capacity to produce butyrate, TMAO, LPS and phenylacetyl glutamine (PAGln) have been found to affect the gut-heart axis in atherosclerosis [71]. Several studies have shown that a reduction in butyrate in the gut promotes local inflammation and foam cell formation, induces dysbiosis and contributes to impairments in the gut barrier function [69-70]. These then exacerbate the release of bacterial toxins (e.g., LPS, TMAO, etc.) and fuel local and systemic inflammation, CVD, and cognitive dysfunction [69-70].

In parallel, a crosstalk between the gut microbiota and the brain has also been established. This crosstalk involves multiple pathways and signaling molecules, including immune, metabolic, and neural systems [72]. A few communication channels have been established thus far. These include the vagus nerve, the enteric nervous system, the immune system, products of microbial metabolism (e.g., SCFAs), microbial structural components (e.g. LPS), and microbial membrane vesicles [72]. Since there are many communication channels, there are also many opportunities for their disruption. For instance, decreased SCFA levels may affect gut and brain barrier function, gut microbiota-induced impairments in gut integrity may lead to the leakage of bacterial-derived products into the circulation which, in turn, can reach and potentially alter brain barriers [72]. As discussed previously, aside from affecting the brain, these disruptions in communication between microbiota and the brain may also affect host cardiometabolic health. In fact, alterations in gut-brain-microbiome interactions have been identified in rodent models of not only digestive, psychiatric, neurological but also several CVD disorders [71].

3. Effect of diet on gut-heart-brain axis

Available evidence indicates that obesity is positively associated with cognitive impairment [73], which may lead to an increased risk of neurodegenerative disease [74] and accelerated brain aging [75]. It has also been suggested that diet and dietary quality may be associated with cognitive decline independent from weight gain or obesity [76-77]. A population-based cross-sectional study by Geda et al. reported that high caloric intake increased the odds of mild cognitive impairment as much as two-fold in adults without dementia aged 70-92 years old [78]. Another study by Jacka et al. showed that higher intake of unhealthy typical Western diet (WD) foods over four years were associated with smaller hippocampal volumes in adults 60-64 years old [79]. WD, being rich in saturated fats and simple sugars, is also commonly associated with cognitive impairment and an increased risk of dementia [80]. Most studies exploring the effects of the WD on gut microbiome and cognitive function together are sparse, however, and have mostly been conducted in rats. Significant changes in the gut microbiome seem to be consistent across studies but the results do not always show cognitive changes, except for high sucrose diets [80]. Rodents that are fed WD for prolonged periods of time generally also develop various cardiometabolic impairments, which makes it difficult to directly link the diet to gut microbiota and cognition [80].

On the contrary, the Mediterranen diet (MedDiet), being high in fruits, vegetables, and fish can help reduce the risk of age-related cognitive impairment and improve overall cognitive performance as shown in several recent studies [81-86]. It is also associated with an improved gut microbiome composition/functionality. This diet is high in fiber that promotes the production of SCFAs like butyrate, propionate, and acetate. These can regulate anti-inflammatory responses by inhibiting histone deacetylases (HDACs), suppress nuclear factor kappa B (NF-kB) pathway in peripheral blood mononuclear cells (PBMCs) and neutrophils, which results in decreased

TNF- α production [5]. SCFAs actions on HDACs can also have a similar effect in macrophages and dendritic cells inducing production of Treg cells [5]. SCFAs can play a role in maintaining blood brain barrier (BBB), provide support to glial cells and modulate neurotrophic factors [80] providing benefits for brain health. For instance, beta-glucan, a type of fiber mostly found in oats and barley, has shown to affect cognition in several rodent models [80]. In Spain, Galie et al. examined whether following a MedDiet modified gut microbiota composition and fecal metabolomics profiles, as well as cardiometabolic risk factors, compared with a non-MedDiet supplemented with 50 g nuts daily. They reported for the first time that the 50 participants with metabolic syndrome following the MedDiet, compared with a non-MedDiet diet supplemented with nuts, significantly changed specific microbial genera and fecal metabolites [87]. However, it was concluded that further intervention studies were needed to understand the effects of different healthy dietary patterns on gut microbiota composition and functionality [87].

MedDiet is also rich in healthy omega-3 polyunsaturated fatty acids (PUFAs). PUFAs have shown the potential for immunoregulation, maintenance of gut epithelial integrity, and anti-inflammatory actions [80]. Studies using PUFAs as the dietary intervention consistently demonstrate a positive impact on the gut microbiome and cognition at the same time [80]. There is now plenty of evidence to support this. For instance, Devkota et al. demonstrated that saturated and unsaturated fats differentially affect gut microbiota through modulation of BA secretion and bacterial metabolism of primary BAs to secondary BAs in a mouse model [88]. Reddy et al. rat experiments also showed that the amount and type of dietary fat affected the excretion of microbially derived pro-inflammatory secondary BAs [89]. Moreover, they demonstrated that excretion of these secondary BAs was decreased when rats were fed a high omega-3 PUFA diet compared to omega-6 rich diet [90].

Phenolic compounds are another group of components in the MedDiet that can interact with gut microbes resulting in production of metabolites that provide protection against neurotoxic injury, suppress inflammation, and promote cognitive function [80]. Polyphenols are molecules with at least one aromatic ring with one or more hydroxyl groups attached [91-92]. They are secondary plant metabolites serving multiple vital functions including helping plants to survive under environmental stresses, protect them from microbial infections, provide defense against herbivorous organisms and lure pollinators [93]. Polyphenols include many various compounds that significantly differ in the complexity of their chemical structure/composition, molecular weight, etc. [91-92]. Polyphenols can be classified into flavonoids, phenolic acids, stilbenes, curcuminoids, lignans, ellagitannins (ET) and ellagic acid (EA) and coumarins [91-92]. The most common polyphenolic compounds are flavonoids [91-92]. The main flavonoid subclasses include flavonols, flavan-3-ols, flavanones, flavones, isoflavones, anthocyanins, dihydrochalcones and proanthocyanidins [91-92]. Among the non-polyphenols, phenolic acids can be divided into hydroxycinnamic acids and hydroxybenzoic acids [91-92].

Over 8000 polyphenolic structures have been identified in plants to date [94]. Evidence shows that their health-promoting benefits are performed through anti-inflammatory, antioxidant, and signal transduction pathway activities [94]. For these reasons, polyphenols have been widely investigated for the prevention and treatment of several pathological conditions, including cancer, neurodegenerative disorders, metabolic and cardiovascular diseases, and stroke [95-98]. Bioavailability of polyphenolic compounds is important to consider when exploring their health effects *in vivo*. Native polyphenol bioavailability is generally poor, however, only 1-10% of total polyphenol intake is detectable in blood and urine samples [96]. Bioavailability is especially

poor for flavones, including quercetin and rutin (0.3-1.5%), but higher for flavanols (e.g., tea catechins), flavanones (e.g., naringenin), soy isoflavones (e.g., genistein) and red wine anthocyanidins (3-30%) [96]. Bioavailability of native polyphenols is usually maintained in the nM or low μM range [96]. Consumption of 10-100 mg of a simple polyphenol results in plasma concentrations rarely exceeding 1 μM [99]. Polyphenols are usually present in foods as glycosides, which contributes to the poor absorption in their native forms [100]. Some glycosides do get hydrolyzed in the intestine, however [96]. In addition to poor absorption, polyphenols can be extensively metabolized by the intestine and liver. This means that while they can be found in plasma and tissues in their native intact forms, most are present in glucuronidated, sulfated and methylated forms [100-102]. As a result, polyphenol metabolism and their low stability under physiological conditions contributes to low aglycone levels in plasma even in individuals who consume a polyphenol-rich diet [96]. Notably, interindividual variations in polyphenol metabolism have been previously reported [99].

Colonic microorganisms play a crucial role in determining bioavailability and activity of polyphenols by transforming them into readily absorbable molecules of biologically active metabolites [95, 103-104]. For instance, gut microbiota is potentially modulating the activity of polyphenols that may be active against stroke and cognitive decline. Flavan-3-ols in particular are extensively metabolized by microbial enzymes [105]. Phenyl- γ -valerolactones and phenylvaleric acids, the main microbial metabolites of flavan-3-ols, may be responsible for neuroprotective actions of its parent compound [105]. Interestingly, there was a significant inverse association between olive oil, fruit and vegetable (all rich in polyphenols) intake with both systolic and diastolic blood pressure in the Greek arm of the EPIC study [106].

Daidzein, from soy products, gets metabolized to equol, which has more antioxidant activity and affinity to estrogen receptors than the parent compound [107]. The neuroprotective flavone glycoside baicalin can only be absorbed after hydrolysis by gut microbiota β-glucuronidase to the aglycone form baicalein [108]. The neuroprotective anthocyanin cyanidin-3-O-glucoside shows a poor bioavailability, while its microbiota degradation products are more easily absorbed [109]. EA and ET are degraded by gut microbes to form urolithins (Uros), molecules characterized by higher bioavailability and better anti-inflammatory and antioxidant properties than their parent compounds [102]. The relationship between gut microbiota and polyphenols, however, is bidirectional where gut microbiota can modulate polyphenol metabolism and polyphenolic compounds in turn influence the composition of the microbial population [83, 110]. Polyphenols can interfere with quorum sensing and limit the formation of pathogen biofilms [111]. There might also be a competition for the nutrients that favors the bacteria capable of extracting sugar molecules attached to the polyphenols as demonstrated in a recent review focusing on grape pomace and its potential as a cardiometabolic health-promoting ingredient [111].

4. Walnuts and their effect on gut-heart-brain axis

The edible part of walnut (kernel or meat) is globally recognized for its nutritional, health, and sensory characteristics [97]. Walnuts can be a part of the heart healthy MedDiet [113] and have been broadly studied due to their ability to modulate gut microbiota and exert beneficial effects on heart and brain health [114-120]. It is important to keep in mind, however, that the specific cultivar (genetics), harvest season, geographical origin, environmental conditions, soil composition, maturity level, methods of cultivation, processing and storage highly influence the chemical/nutritional composition of walnuts [112]. Moreover, in most cases,

consumers don't have the option of choosing the variety of walnut to purchase, and often mixtures of several varieties are sold [112].

Regardless of the potential differences in composition, the predominant components in walnuts are fat (58.3% - 65.2%), followed by protein (15.1 - 17.4%) and carbohydrates (13.9 - 19.4%) [112]. In general, all cultivars also show high levels of magnesium, potassium and calcium and, in contrast, very low levels of sodium, zinc, copper, manganese and iron [112]. Other notable compounds present are melatonin (1.90 ng/g), serotonin (34.3 ng/g), vitamins (e.g., folate, gamma tocopherol, etc.), and polyphenols (58.2 mg/g) [112]. Several of the mentioned components have the potential to interact with gut microbiota. This is likely due to the chemical structure of their cell wall that limits digestion of walnut components in the upper gut. Consequently, many of them end up being metabolized by microbial communities in the distal gut, while also affecting their composition and functional capacity.

A systematic review by Fitzgerald et al. and a meta-analysis by Creedon et al. assessed the effects of various nuts on fecal microbiota for over 600 adults in the US, Germany, Italy and Spain [121-122]. Fitzgerald et al. reviewed a total of eight studies, three of which had walnuts as dietary intervention. The amount of walnuts provided in these studies were 33, 42 and 43 grams daily with the intervention duration being three days, three weeks and eight weeks, respectively. Two of the studies reported a significant change in ß-diversity after walnut consumption compared to the control group [114, 123]. Furthermore, Holscher et al. study demonstrated an increase in relative abundance of *Faecalibacterium*, *Clostridium*, *Roseburia* and *Dialister* and a decrease in relative abundance of *Ruminococcus*, *Dorea*, *Oscillospira* and *Bifidobacterium* after walnut consumption compared to the control group [123]. Evidence from Bamberger et al. study did not support these findings as they observed an increase in two unclassified OTUs from the

Ruminococcaceae family and one OTU from the *Bifidobacterium* genus, while also reporting a decrease in relative abundance of *Anaerostipes* and *Blautia* from the Lachnospiraceae family [114]. The study by Garcia-Mantrana et al. mostly focused on identifying associations between shifts in relative abundance of gut microbes and the specific urolithin metabotypes of participants. They concluded that the shifts in microbial community were significant only for the Urolithin-B metabotype, which exhibited an increase in the Coriobacteriaceae family, as well as in the genera *Coprococcus*, *Collinsella*, *Bifidobacterium* and *Blautia* [124].

Creedon et al. meta-analysis also included eight studies, three of which were walnut interventions [122]. Two of these were the Bamberger and the Holscher et al. studies also reviewed by Fitzgerald et al. The third study was by Tindall et al. and was unique to this meta-analysis. This was a RCT comparing the effect of three isocaloric diets on gut microbiome composition: a diet containing whole walnuts (WD, 57-99 g daily), a fatty-acid matched diet devoid of walnuts (WFMD), and a diet replacing ALA with oleic acid without walnuts (ORAD)[119]. This study reported no distinct clustering of samples by diet using UniFrac distances, indicating no effect of walnuts compared to control, which is in disagreement with the findings from Holscher et al. and Bamberger et al. studies. They observed an increase in relative abundance of *Gordonibacter* following the WD compared to WFMD and ORAD diets. Both the WFMD and WD diets also led to a significant increase in relative abundance of *Roseburia*, *Eubacteria eligens* and *Butyricicoccus* relative to the standard western diet [119].

As evident from the discussed walnut intervention studies, walnuts have the potential to modulate gut microbiota composition. The results, however, are generally inconsistent across studies, which may be explained by variations in trial design, overall methodology, and inter-individual variability in gut microbiota [121].

4.1 Fatty acids

Lipids comprise anywhere between 30 and 70% of walnut composition [125]. Approximately 90% of total fatty acids in walnuts are unsaturated [125]. PUFAs represent 72.6 - 78.0% of total fatty acids, monounsaturated (MUFA) 13.3 - 17.8%, whereas saturated acids do not usually exceed 10% of total fatty acids [125]. Linoleic (LA) is the most abundant fatty acid, constituting from 59.0 to 60.6% of total fatty acids [125]. MUFAs are represented almost exclusively by oleic acid (12.3 - 16.9%) [125]. Foods of plant origin rarely have a high content of omega-3 fatty acids, however, compared to other nuts that contain mostly MUFAs, walnuts are unique due to their high levels of omega-3 alpha-linolenic acid (ALA) [125].

As mentioned above, varieties within the same species, cultivation methods, climatic characteristics profoundly affect chemical composition of nuts in general [126]. Geometrical properties of nut shells and kernels (length, width, thickness, density, surface area, volume, specific gravity) affect the quality of nuts as well [126]. Nut structure can then influence lipid digestibility [126]. Physicochemical properties of the cell wall, as well as their composition can influence nutrient digestibility [126]. Phospholipids and proteins can also limit access to hydrolytic enzymes [126]. For instance, Creedon et al. demonstrated a greater lipid bioaccessibility for ground almonds compared to whole almonds after mastication [127]. Additionally, studies on almonds show that chewing breaks down a large fraction of the first outer layer of cells, while the majority of parenchyma cells where most lipids and proteins are encapsulated remain intact [128-129]. Mandalari et al. showed in ileostomy patients that the lipids from walnuts trapped in the intact cells with fractured outer layers tend to leak out only after a prolonged incubation in the upper intestinal tract [130]. Recently, McArthur et al. subjected masticated samples of almonds, pistachios and walnuts obtained from healthy adults to

a static model of gastric and intestinal digestion [131]. While there was no significant difference in the total lipid release between the three nuts after intestinal digestion, walnuts produced a significantly larger particle size after chewing vs. almonds and after digestion vs. almonds as well as vs. pistachios [131].

The relatively large particle size may mean that some of the walnut lipids potentially travel all the way to the colon and interact with gut microbiota. A recent study by Devillard et al. surveyed 30 strains of human gram-positive intestinal bacteria for the ability to metabolize LA [132]. They found that different *Roseburia* spp. were among the most active in metabolizing it [132]. From previous experiments, it is also known that *Roseburia* spp. can metabolize fatty acids, including conjugation of LA [132]. Evidence also shows that *Roseburia* spp. have been linked to improved insulin sensitivity in adults with metabolic syndrome after a fecal microbiota transplant, independent of weight loss [133]. Interestingly, a recent RCT by Holscher et al. demonstrated that daily consumption of 42 g of walnuts for three weeks increased relative abundance of several bacterial genera, including *Roseburia*, in healthy adults [123].

Another study by Huyan et al. aimed at exploring the differences in microbial lipid metabolites among sunflower seeds, soybeans, and walnuts [134]. The matrices were subjected to *in vitro* digestion and colonic fermentation. The results showed that a higher lipid content in a food matrix and a reduced lipid digestibility resulted in higher levels of LA metabolites, such as conjugated linoleic acids (CLAs) and hydroxy FAs [134]. However, their data also suggested that other components of the food matrix, like fiber and phenolics, could also play an important role in formation of LA metabolites by modulating microbial community or activity. Specifically, in relation to the three food matrices studied in this experiment the lipid profiles

were produced when the same amount of each matrix was digested and fermented by the gut microbiota and walnuts produced the higher levels of CLAs [134].

Several reviews examined the effects of CLA on human health concluding that various CLA isomers exert potent physiological functions such as anticarcinogenic, anti-obese, antidiabetic and antihypertensive properties [135-137]. This means CLA may be effective to prevent lifestyle diseases or metabolic syndrome [135]. CLA's ability to bind to peroxisome proliferator activated receptor-α (PPARα), a nuclear receptor and the key regulator of fatty acid metabolism and inflammatory responses, is at least partially responsible for its beneficial health effects [138]. Evidence shows that CLA is incorporated and metabolized into brain tissue where it can induce biosynthesis of endogenous PPARα ligands including palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) [138]. These can also bind to other receptors like TRPV1, which extends CLAs anti-neuroinflammatory activities [138].

Another way that dietary fats may affect host health is through modulation of the BA pool. Generally, a high consumption of fat stimulates hepatic synthesis of BAs, consequently increasing the amounts of BAs that escape ileal re-absorption and enter the colon where they are biotransformed by the gut microbes [139]. Aside from the amount of fat, the type of fat consumed also plays a role. For instance, mice fed a high-lard diet showed significantly higher weight gain compared to mice fed fish oil [140]. Their gut microbial profiles were also different with the lard-fed mice demonstrating an increase in *Bilophila*, *Bacteroides*, and *Turicibacter* that promoted increased TLR4 activation in white adipose tissue (WAT) [140]. Fish-fed mice, in turn, showed an increase in *Akkermansia* and *Lactobacilllus* genera [140]. Interestingly, when lard-fed mice were inoculated with the gut microbiota of the fish-fed mice they showed a reduction in the pro-inflammatory effects of the lard-rich diet [140]. Consumption of milk fat

diets promoted colitis in IL10$^{-/-}$ mice compared to safflower-based diets [88]. This diet-induced colitis was due to the presence of taurocholic bile acid, a primary conjugated BA, also present in humans. However, this mouse model is genetically predisposed to GI inflammation and more experiments are needed to assess whether the same result would be seen in a healthy host [141].

While the exact mechanism of the link between the type of dietary fat and chronic inflammation is not well understood, one potential mechanism could be the emulsifying properties of BA reaching the colon. GI inflammation, which may be promoted by diets rich in saturated fats, has been linked with malabsorption of BA in the small intestine, increasing the concentration of primary bile acids in the colon. Interestingly, ingestion of physiological concentrations of common synthetic dietary emulsifiers resulted in compromised gut mucosal lining integrity leading to gut microbiota moving closer to the intestinal epithelium, inflammation and metabolic syndrome in IL10$^{-/-}$ mice [142]. Ingestion of emulsifiers derived from soybean polar lipids resulted in the same effect in conventional mice as well [143].

The best-studied bile acid receptor is farnesoid X receptor (FXR), which is known to modulate the BA biosynthesis genes and affect glucose and lipid metabolism [144-145]. Studies have shown that chenodeoxycholic primary bile acid is the most potent FXR ligand. Activation of FXR may lead to induction of FMO3 enzyme in the liver resulting in conversion of TMA to TMAO [146]. Multiple mouse model experiments demonstrated that changes in FXR function may be associated with atherosclerosis [147-149]. Another nuclear hormone receptor that can also affect bile acid biosynthesis is PXR that is mostly activated by the secondary BAs (e.g. lithocholic and deoxycholic). PXR activation may contribute to increased lipid accumulation and CD36 expression in macrophages, while also elevating plasma LDL-C and VLDL-C levels [150], which are known to be atherogenic. G-protein coupled membrane receptor, TGR5, which

can be activated by the lithocholic bile acid can also be involved in atherosclerosis. Activation of TGR5 may inhibit pro-inflammatory cytokine production by inducing cAMP signaling and NF-kB inhibition [151].

Another group of microbial metabolites that may play a role in how dietary fats affect host health are SCFAs. The major SCFAs are butyrate, acetate, and propionate. Gut microbial composition shifts associated with atherosclerosis demonstrated that there is a decrease in *Roseburia* and *Eubacterium* [152-153]. Butyrate has shown the potential to inhibit NF-kB and modulate vascular cell adhesion molecules and consequently inhibiting leukocyte adhesion to endothelial cells [154]. Interestingly, a higher SCFA concentration (20 mmol/L) has shown to be pro-inflammatory in neutrophils by producing IL-8, IL-1ß and IL-6, while a lower concentration (0.02-2 mmol/L) did not induce cytokine secretion [155]. Exact mechanisms by which gut microbial SCFA induce their beneficial cardiovascular effects has not been elucidated yet and it has been mostly attributed to their ability to reduce systemic inflammation [156]. Production of L-lactate by *Lactobacillus paracasei* promoted increased lipid storage in enterocytes through its conversion to malonyl-CoA also resulting in inhibition of carnitine palmitoyltransferase 1 (CPT-1) and fatty acid oxidation [157]. *E. coli* production of acetate, however, led to an increase in FA oxidation in mice via upregulation of the AMPK/PGC-1α/PPARα pathway [157]. This means that gut microbes can impact enterocyte function and FA absorption in a diet-dependent manner [141].

4.2 Protein

Walnut kernels contain 14.92 g protein per 100 g [158]. The content of glutelin, albumin, globulin and prolamin are approximately 72.06%, 7.54%, 15.67% and 4.73% of the total extractable protein, respectively, as reported by Mao et al [159]. Walnut kernels are also

abundant in essential amino acids, like histidine (2.34 g/100 g protein), isoleucine (4.74 g/100 g protein), leucine (6.87 g/100 g protein), lysine (2.18 g/100 g protein), methionine (0.73 g/100 g protein), phenylalanine (4.41 g/100 g protein), threonine (3.08 g/100 g protein), tryptophan (1.04 g/100 g protein), and valine (5.78 g/100 g protein) [158]. Among non-essential amino acids, major amino acids are arginine (2.28 g/100 g), aspartic acid (1.83 g/100 g), glutamic acid (2.82 g/100 g), and serine (0.93 g/100 g) [158].

In general, approximately 3-18 g of dietary protein enters the human distal gut on a daily basis [160] and the source of protein seems to matter in regard to effects on health. High consumption of red meat, for example, which is rich in protein as well as heme iron may lead to elevated risk of colorectal cancer [161]. In general, dietary proteins are hydrolyzed into peptides and amino acids by the host and bacterial proteases and peptidases [162-163]. The resulting peptides can be utilized by both the host and the bacterial communities. Peptide and amino acid-fermenting bacteria include *Bacteroides*, *Prevotella*, *Clostridium*, *Veillonella*, *Megasphaera*, *Acidaminococcus* and *Slenomas* [160]. Most gut bacteria utilize amino acids and ammonia as their preferred nitrogen source [160]. The levels of protein and its metabolic products are relatively high in the proximal colon and reduced in distal colon [160]. Evidence shows that amino acids are not absorbed by the colonic mucosa but are rather metabolized by the resident microbial communities [164]. Microbial metabolism of amino acids results in production of ammonia and amines including cadaverine, agmatine, putrescine, tyramine, histamine [165]. These are usually produced as a result of arginine, ornithine, lysine, tyrosine, histidine and methionine metabolism [166], several of which are present in walnuts. Microbial synthesis of polyamines is considered as a therapeutic target but evidence from human trials is still limited [160]. Some minor SCFAs, iso-butyrate and iso-valerate, are formed by bacterial fermentation of

BCAA [160]. Other metabolites also include phenols (e.g., p-cresol) and indoles, which may have some health benefits as well [160]. High levels of protein in the colon could also lead to production of detrimental microbial metabolites, however. For instance, Magee et al. demonstrated high levels of sulphide and decreased levels of butyrate after a high protein diet in humans [167]. Evidence shows that this may result in increased incidence of colorectal cancer [160].

Although walnut kernels are the most consumed part of walnuts, most studies exploring the beneficial health effects of walnut protein are done using walnut meal. Walnut meal is the main by-product of oil extraction and contains more than 40% protein [168]. A large proportion is used to prepare edible protein powder because of its high essential amino acid content, while the remaining walnut meal is used as animal feed [168]. Walnut peptides have been reported to have immunomodulatory effects, tyrosinase inhibitory activity, antioxidant activity, and lipid metabolism regulatory activity [169-171]. Furthermore, several walnut peptides derived from defatted walnut meal hydrolysates showed protection against neuroinflammation [171-173]. In addition, results from a recent Zhi et al. mouse study showed that administration of walnut peptides can alleviate the clinical symptoms and pathological features of colitis and regulate gut microbiota dysbiosis and production of inflammatory factors [168].

4.3 Fiber

Walnut fiber is composed of typically neutral polysaccharides with the primary constituents of arabinose (40.16%), galactose (26.67%), and glucose (10.25%), along with rhamnose (7.58%), xylose (7.57%), galacturonic acid (5.05%), glucuronic acid (1.6%), mannose (0.89%) and fucose (0.23%) [174]. A recent study by Wang et al. explored the effect of walnut meal dietary fiber (WMDF) on metabolic syndrome in mice fed a high

fructose diet [174]. Results demonstrated an increase in relative abundance of *Bifidobacterium*, which is in alignment with evidence that *Bifidobacterium* can degrade a variety of arabinose and galactose polysaccharides [175]. It was also shown that levels of acetic acid significantly increased after WMDF consumption, which met the expectations of Bifidobacterium being a known producer of acetate. Interestingly, butyric acid was also significantly increased in WMDF- treated mice compared to control group. Butyrate producers can use acetic acid to synthesize butyrate via the butyryl CoA:acetate CoA transferase pathway [176-177]. *Faecalibaculum* and *Roseburia* belong to butyrate-producing bacteria, which can employ this route for butyrate formation and are generally considered to be beneficial bacteria associated with health promotion and were significantly increased in WMDF-treated mice as well [174].

In a recent short-term study of walnuts, a 3-day consumption in healthy individuals was found to modify the gut microbiome, while also increasing SCFA levels [178]. Importantly, these effects were dependent upon the composition of the individual microbiome [179]. Microbiota analysis showed significant increases in two bacterial species, namely, *Coprococcus* and *Anaerostipes*, each established producers of butyrate [180]. In addition, *Phascolarbacterium*, a known producer of acetate and propionate, was also increased by walnut consumption [181].

As mentioned above, SCFAs are key mediators of the microbiota-gut-brain crosstalk and are involved in the modulation of microglia, production of neurotrophic factors and maintenance of the BBB integrity, as well as in neurogenesis [182]. SCFAs can cross the BBB to control microglia structure and maturity [183]. Acetate, specifically, is metabolized by microglia to acetyl-CoA, fueling metabolic pathways, such as the tricarboxylic acid cycle (TCA) and the production of citrate and adenosine triphosphate (ATP), ultimately supporting the maturation of microglia [184]. The role of SCFA on glucose metabolism, fatty acid metabolism, and their

effects on the immune system are also well-known [185]. Moreover, evidence shows that SCFAs possess anticancer, anti-diabetic, and hepatoprotective effects, while also exhibiting protective effects against brain-related diseases, kidney diseases, cardiovascular damage, and IBD [185].

4.4 (Poly)phenolic compounds

Walnuts have the third highest (1156 mg GAE/100 mg) total phenolic content among the major nine tree nuts [186-187]. The phenolic acid content of walnuts is 36 mg/100 g, which is the highest among nuts [188]. The total phenol content of 11 walnut varieties studied by Pycia et al., ranged from 0.82 to 2.09 g GAE/100 g. Different parts of the walnut tree have been reported to contain phenolic compounds, including kernel (145–15,540 mg/100 g estimated average), shell (1804–2300 mg/100 g estimated average), husk (3428.11 mg/100 g), leaves (2136 mg/100 g), bark (3483 mg/100 g), and pellicle (5205 mg/100 g) [189].

Evidence shows that the majority of polyphenolic compounds in walnut kernel are presented in free form (51.1%–68.1%), followed by bound (21.0%–38.0%) and esterified forms (9.7%–18.7%) [190]. The content of phenolic acids (hydroxybenzoic acid and hydroxycinnamic acids) exceeds 80% of all phenolic compounds, followed by flavanols (6.4–12.5%), flavonols (1.3–4.8%), and flavanes (0.2–1%) [190]. In a recent Wu et al. study, a total of 37 phenolic compounds in free, esterified, and bound forms were identified in walnut kernels [190]. The major component, EA, was quantified in free (112.71 μg/g), esterified (66.38 μg/g) and bound form (46.38 μg/g), respectively [190]. Other phenolic acids were in abundance in free form including gallic acid (11.30 μg/g), ferulic acid (5.42 μg/g), sinapic acid (2.60 μg/g) and caffeic acid (1.19 μg/g) [190]. Moreover, 22 flavan-3-ols were identified in walnut kernel with catechin being the major flavonoid identified [190]. Epigallocatechin gallate, (−)-epicatechin gallate,

catechin gallate, rutin, quercetin-3-o-glucopyranoside, dihydroquercetin, dihydrokaempferol, naringin and juglone were also identified [190].

ETs and EA are polyphenols present in different fruits, including pomegranates, strawberries, black raspberries, raspberries, walnuts, and almonds [191]. *In vivo*, ETs are hydrolyzed to EA which in turn are metabolized by intestinal microbiota to different types of urolithins (Uros) [192]. The Uro families include UroM-5, UroD, UroM-6, UroE, UroC, UroM-7, Iso-UroA, UroB, and UroA [193-194]. Recently, new "R" Uros were discovered and classified as Uro M7R, Uro CR, and Uro AR [195]. Evidence demonstrates that several specific gut metabotypes are associated with the release of specific Uros, including UroA, iso-UroA, and UroB [196]. Individuals classified as metabolically healthy release higher concentrations of active UroA, therefore belonging to metabotype A [124, 197]. Interestingly, those classified as metabolically unhealthy produce minimal UroA and release the less active forms of urolithin including iso-UroA and UroB, thus belonging to metabotype 0 and metabotype B, respectively [124, 197]. Based on the correlations between metabotypes and metabolites, it has been suggested that the gut microbial community may play an important role in determining the production of active UroA [195]. Of these microbes, *Gordonibacter urolithinfaciens* and *Gordonibacter pamelaeae* are involved in converting EA to UroA [198-199]. *Akkermansia muciniphila* levels are reported to be associated with UroA levels but can vary in UroA activity depending on interindividual differences [200-201]. Based on this evidence, UroA production and activity may be associated with the gut microbial community and metabotype classification, more research is needed to provide stronger associations, however [195].

All Uros possess a common 6H-dibenzo [b,d]-pyran-6-one nucleus and differ by hydroxyl groups [202]. Once absorbed, Uros reach the liver and undergo phase II

biotransformation, to yield various conjugated forms [203]. For UroA, conjugated forms include UroA-glucuronide and UroA-aglycones [203]. UroA conjugates are found at the highest concentrations in human plasma ranging from 0.024 to 35 μM [202]. Gasperotti et al. recently performed a pharmacokinetics experiment in which a single bolus of 23 polyphenol microbial metabolites (total 2.7 μmol) was administered intravenously to rats to reliably reproduce a physiological post absorption situation [204]. Tissues and urine were collected shortly thereafter (15 s to 15 min). Results showed that both UroA and UroB were negligibly excreted in the urine as glucuronyl derivatives [205]. Rapid uptake of UroA into the kidneys was observed, whereas UroB appeared in the kidneys only after 2 min, when it was also present in the blood [204]. The liver slowly absorbed UroA up to 5 min, but no UroB could be detected in the liver at 15 min [204]. This shows that the liver could neither convert UroA to UroB nor take the latter up from the blood [204]. Interestingly, the organ that displayed the highest uptake capacity was the heart, where both UroA and B were found at 15s, at approximately 300 and 2000 pmol/g, respectively [204].

Evidence of high uptake of Uros by the cardiovascular tissues is also supported by in vivo trials. For instance, cardioprotective effects of UroA were investigated in a mouse model of myocardial reperfusion injury [206]. The results showed reduced ROS production, which was likely due to UroA ability to regulate the PI3K/Akt pathway and enhance antioxidant activities in cardiomyocytes [206]. In another study Cui et al. examined whether UroA had the potential to attenuate atherosclerotic lesions in rats fed a high-cholesterol diet and subjected to a balloon injury of the aorta [207]. UroA reversed inflammatory lipid levels, decreased levels of angiotensin II, and decreased foam cell development through activation of the Nrf2 pathway and inhibition of p-ERK [207]. Additionally, in macrophage foam cells, UroA suppressed mIR-33-a

by altering ERK/SREBP1/AMPKα signaling pathways to regulate cholesterol efflux to reduce intracellular cholesterol load [208].

Han et al. investigated UroA's effect on impairing oxidized low-density lipoprotein (oxLDL)-induced endothelial dysfunction and inflammation in human artery endothelial cells treated with oxLDL [209]. Even low concentrations of UroA were shown to increase nitric oxide (NO) production due to improvement of endothelial nitric oxide synthase (eNOS), decrease monocyte adhesion factors such as intercellular adhesion molecule-1 (ICAM-1), and decrease ERK-mediated inflammation along with IL-6 and TNF-α [209].

Additionally, it has been shown that EA can provide protection against ischemic injury in both cellular and in vivo models of brain ischemia by regulating Bcl-2/Bax expression [210]. It also showed the potential to mitigate oxygen glucose deprivation-induced damage in N2a neuroblastoma cells and primary neurons and to reduce ischemic brain injury in mice by inhibiting endoplasmic reticulum stress [211]. UroA has exerted anti-inflammatory properties in macrophages, inhibiting neuroinflammation in aged transgenic R1.40 mouse hippocampus, human neuroblastoma cells, and BV2 mouse microglial cells [212]. UroA decreased total NO concentration and pro-inflammatory cytokines IL-6 and TNF-α, as well as increasing cell viability by inhibiting apoptosis [212].

4.5 Phytosterols

Phytosterols are a type of lipid molecule that structurally and functionally is similar to cholesterol. Cholesterol is a primary component of animal cell membranes, phytosterols are common components of plants, including nut kernels [213]. Initially, it was believed that there are only 3 phytosterols in walnuts: β-sitosterol, campesterol, and sigmasterol [213]. A recent report, however, stated that there are actually 13 phytosterols [214]. These include campesterol,

stigmasterol, $\Delta^{5,23}$-stigmastadieenol, clerosterol, β-sitosterol, stigmastanol, Δ5-avenasterol, $\Delta^{5,24(25)}$-stigmastadiene, cycloeucalenol, cycloartenol, 28-methylobtusifoliol, 24-methylenecycloartenol, and citrostadienol [214]. Average phytosterol levels in walnuts range from 1130-1679 ug/g for English walnuts and 1236-1770 ug/g for Black walnut kernels. β-sitosterol seems to be the predominant form (63-89% of total sterols in walnuts) [213]. Genetic factors, geographic origin, seasonal variations, environmental factors may affect amounts of phytosterols in walnuts [213].

Phytosterols are found in abundance in MedDiet and are known to promote beneficial changes in bacterial communities, while they are not present in a WD [215]. In total, 20-80% of the cholesterol consumed daily (average recommended intake of 300 mg cholesterol per day) is absorbed, while the absorption of phytosterols is only 2–3% (average intake of phytosterols is less than 500 mg per day) [216-217], which means that non-absorbed sterols can be further processed by the gut microbiome. Evidence shows that phytosterols have the potential to modulate cholesterol metabolism and potentially reduce the risk of hyperlipidemia, but the exact mechanism is not yet known [218]. Lv et al. recently conducted a study where they aimed at elucidating this mechanism in high-fat diet (HFD) mice [218]. The results showed that the phytosterol treatment reduced the accumulation of TC, TG, and LDL-C in the serum of HFD mice, while increasing the serum levels of HDL-C. Moreover, the results of 16S rRNA sequencing analysis demonstrated that phytosterols modulated the gut microbiota by reducing the relative abundance of *Lactobacillus* and other bile salt hydrolase- (BSH-) producing gut microbiota [218]. Nonetheless, according to estimations, the dietary intake of phytosterol (150–400 mg phytosterols/day) does not reach the necessary established levels (1500–3100 mg phytosterols/day) in order to exert its hypocholesterolemic effect [213]. Consequently, the above-

mentioned levels can only be achieved in the daily diet through phytosterol-enriched foods, such as dairy products [218]. Cuevas-Tena et al. investigated the impact of plant sterol enrichment dose on the gut microbiota of lean and obese subjects using an in vitro fermentation model [219]. In this study, the phytosterol-enriched supplement, but also β-sitosterol alone, was able to increase the proportion of the genera belonging to the *Firmicutes* phylum. This increase suggested a potential modification of SCFAs and of the microbial profile of both lean and obese populations [219]. In another study by Xiao et al. glucocorticoid-induced osteoporosis mice were treated with high and low concentrations of phytosterols for four weeks [220]. Results demonstrated that the phytosterol treatment increased the relative abundance of *Ruminococcus* and decreased the relative abundance of *Bilophila*, which were significantly associated with glucocorticoid-induced osteoporosis indications [220].

In summary, although the exact associations of phytosterols and the intestinal microbiome are still under study, there is enough evidence showing that these compounds are potential regulators of cholesterol modifiers of the gut microbiota composition [220]. At the same time, even though the body of evidence regarding the impact of phytosterols on gut microbiota alterations and on diet-induced health or disease conditions is growing, there are a limited number of well-designed and controlled human studies [221-222].

4.6 Vitamins

100 g of walnuts contain 1.59 μg of Vitamin A, 13.05 mg of biotin, 0.88 mg of Vitamin C, 57.95 mg of total folates, and 4.12 α-tocopherol equivalent (Vitamin E) [158].

Some vitamins, when provided in large doses or when delivered to the large intestine directly, have been shown to beneficially modulate the gut microbiome by increasing the abundance of several commensals (vitamins A, B_2, D, E, and β-carotene), increasing or

maintaining microbial diversity (vitamins A, B_2, B_3, C, K) and richness (vitamin D), increasing SCFA production (vitamin C), or increasing the abundance of SCFA producers (vitamins B_2, E) [223]. Others, such as vitamins A and D, modulate the gut immune response or barrier function, thus, indirectly influencing intestinal health or the microbiome [223]. But the data on the impact of vitamins on microbial populations and health status control are quite controversial [223]. On one hand, the administration of vitamins D, A, and K favored the prevalence of *Lactobacillus* [223]. On the other, the intake of the same vitamins led to the increase of opportunistic pathogens or the depletion of synergistic bacteria belonging to several bacterial categories, such as *Proteobacteria*, *Deferribacteres*, *Enterobacteriacae*, *Clostridiaceae*, *Ruminococcus*, and *Odoribacter*, or *Verrucomicrobia*, *Bifidobacterium*, and symbiotic *Bacteroidetes*, respectively [224]. In another study, inclusion of vitamin A in obesogenic diet patterns in three-week-old male C57BL/6J mice precluded changes in microbiota α-diversity and enriched the abundance of *Lachnospiraceae* [225].

Vitamin E is considered a group of fat-soluble compounds and includes two main sub-categories: (a) α-, β-, γ-, and δ-tocopherols and (b) α-, β-, γ-, and δ-tocotrienols [226]. Among these, α-tocopherol is one of the most important fat-soluble antioxidants of cellular membranes as it is the most biologically active form retrieved from human tissues [227]. In a recent study, where five-week-old C57BL/6 male mice followed a high- and low-vitamin E diet, the phyla *Bacteroidetes* and *Verrucomicrobia* (*Akkermansia muciniphila* species) were associated with lower body weight [228]. More specifically, a dose-dependent relationship was highlighted between α-tocopherol and different gut microbial compositions, as the authors observed an increase in *Proteobacteria* and a decrease in *Verrucomicrobias* phylum [228]. Another study revealed that α-tocopherol supplementation was associated with changes in gut microbiota

composition [194]. Particularly, it was shown that α-tocopherol can reduce levels of *Bacteroides* and *Lactobacillaceae*, as well as the *Firmicutes/Bacteroidetes* ratio in humans [229]. In addition, δ-tocotrienol, and its hydrogenated metabolite present in human feces, δTE-13′-carboxychromanol, has shown to exert cancer-preventive effects in mice [230]. Although these compounds showed no significant effect on bacterial richness, they exhibited a modulating role in gut microbiota composition, by inducing an increase in health-promoting *Lactococcus* and *Bacteroides* [230]. Focusing on δTE-13′-carboxychromanol, this tocotrienol metabolite counterbalanced the reduction in *Roseburia* in IBD patients and uniquely facilitated the elevation of *Eubacterium coprostanoloi* gene levels [230].

4.7 Minerals

In general, walnut kernels have high levels of magnesium, potassium and calcium and, in contrast, very low levels of sodium, zinc, copper, manganese and iron [125]. Studies investigating the effect of minerals on gut microbiota are scarce, likely due to the lack of knowledge about the amounts of minerals reaching the distal gut and the microbial capacity for metabolizing them. Most studies on this topic focus on how gut microbiota affect absorption of minerals. Although this is not in direct alignment with the focus of the present review, a brief overview of this evidence for calcium and magnesium, minerals present in high amounts in walnuts, is provided below.

Studies show that gut microbiota can increase calcium absorption and modulate the production of gut serotonin, which is believed to interact with bone cells and to regulate bone metabolism [231]. Whisner et al. investigated the effect of soluble maize fiber on calcium absorption and retention in pubertal children [232]. A moderate daily intake of soluble maize fiber resulted in increased calcium absorption during a 3-week period [232]. When children

consumed a soluble maize fiber, lower abundance of *Firmicutes* and higher abundance of *Bacteroidetes* were detected in the gut microbiota. Moreover, calcium absorption was positively correlated with the genera *Bacteroides*, *Butyricicoccus*, *Oscillibacter*, and *Dialister* [232].

Animal studies provide convincing evidence for an association between dietary magnesium availability and microbial composition in the gut [233-235]. Jorgensen et al. investigated the impact of dietary magnesium deficiency on the composition of the gut microbiota in C57BL/6 mice [233]. A magnesium-deficient diet for six weeks altered the gut microbiota and was associated with altered anxiety-like behavior [233]. Garcia-Legorreta et al. studied the effect of low and high magnesium diets on the modulation of the intestinal microbiota of male Wistar rats [236]. Interestingly, after two weeks, the control and low magnesium groups both had higher bacterial diversity than high magnesium group [236]. As the authors concluded for mice with no magnesium deficiency, supplementation above recommended magnesium intake levels can result in the development of intestinal dysbiosis [236].

5. Olive oil and its effect on gut-heart-brain axis

OO is commonly consumed around the world due to its pleasant flavor, odor and health benefits [237]. The concentrations of various compounds in OOs are affected by many factors, including the cultivar [238], environmental factors [239], olive fruit maturity [240], the technical processing [241], as well as storage of the fruit [242], or storage of OOs [243]. Approximately 98-99% of OO are fatty acids; however, OO is also rich in several unsaponifiable compounds, which comprise about 1-2% of its composition [244]. These contain over 200 chemical compounds including polyphenols, tocopherols, hydrocarbons, β-carotene, esters, aldehydes,

ketones, alcohol, sterols, etc. [244]. Extra virgin olive oil (EVOO) generally has higher nutritional value vs. refined oils (OO, pomace oil) due to the much higher content of phenolic compounds and squalene, which provide protection from excessive fatty acid hydrolysis and oxidation [245].

EVOO and its phenolic compounds are promising for the prevention of certain neurodegenerative diseases, such as Alzheimer's disease [246-247]. For instance, several observational human studies demonstrated that the EVOO-rich MedDiet may help reduce the risk of Alzheimer's [248-249]. This aligns with data from rodent models that either the intake of EVOO or its specific phenolic components led to reduced Aβ accumulation, resulting in improved memory and cognition [250-254]. Much of this effect as well as the demonstrated benefits for cardiometabolic health and mucosal immunity may be due to the EVOO ability to modulate gut microbiota composition and/or functionality. EVOO consumption is generally associated with an increase in genus *Bacteroides*, which is known to be negatively associated with blood TG levels in human and animal hosts [255-256]. In addition, presence of members of the *Lachnospiraceae* and *Lactobacillus*, known to increase with EVOO consumption [257-261], positively correlate with blood HDL-C and negatively correlate with TC [256, 262-264].

EVOO may affect the gut microbiota by reducing the abundance of pathogenic bacteria, while also increasing abundance of bacterial species capable of producing SCFAs, which in turn exert anti-inflammatory effects and can modulate the expression of a variety of genes [265-267]. For instance, EVOO consumption has been related to the increase in *Clostridium* XIVa genus, which is responsible for butyrate production [268]. Additionally, EVOO consumption may stimulate immunoglobulin A (IgA) production, dampen down inflammatory cytokine production

[268] and protect against intestinal oxidative injury through the potent antioxidant activity of EVOO phenolic compounds [269].

Based on large scale epidemiological studies like PREDIMED, it has been concluded that consumption of at least four tablespoons or approximately 59 ml of EVOO daily may be associated with a 30% decrease in cardiovascular event risk and improved cognitive function vs. a low-fat diet [270-272]. The European Food Safety Authority recommends replacing saturated fats with EVOO and consuming at least 20g of EVOO daily, which can assist with reaching optimal blood lipid/lipoprotein levels and provide protection from LDL oxidation [273-274]. In regard to beneficial effects on the gut microbiota, two intervention studies demonstrated that daily consumption of 40-50 g of EVOO is necessary to observe an effect [257].

5.1 Lipids

All types of OO have a high concentration of MUFAs that can be as high as 85g/100g due to the presence of oleic acid (55–85 g/100g) specifically [275]. There are also high amounts of PUFAs mainly LA and ALA. LA, an omega-6 fatty acid, accounts for approximately 2.5 - 21 g/100g, while ALA, an omega-3 fatty acid, comprises up to 1g/100g [275] . In terms SFAs, palmitic acid constitutes nearly 7.0-20g/100g, while stearic acid constitutes 0.5-5g/100g. The remaining fatty acids including behenic acid, arachidic acid, and myristic acid occur in relatively small quantities [275-276].

Evidence shows that each type of fatty acid exerts different impacts on gut microbiota, but isolated mechanisms are still unknown. For instance, oleic acid has exerted beneficial effects in several rodent models [277]. A study comparing the effects of diets enriched with peanut oil high in oleic acid, EVOO, and chow supplemented with fructose-infused drinking water in Swiss Webster rats, found that both the peanut oil and EVOO significantly increased the relative

abundance of *Bifidobacterium* and reduced *Blautia* levels [277]. Peanut oil and EVOO also had favorable effects on body weight, cholesterol levels, and insulin sensitivity [277]. Moreover, when researchers exposed the mice to HFD or a HFD supplemented with either an oleic acid-derived compound (1500 mg/kg/day) or a combination of omega-3 fatty acids (EPA and DHA, 3000 mg/kg/day), they found that mice supplemented with the oleic acid compound had greater gut microbial diversity as well as lower body weight compared with HFD-fed mice [278]. Another similar study showed that diets high in oleic acid-rich OO fed to C57BL6/J mice were associated with significantly greater abundance of members of the *Bacteroidaceae* in comparison to mice fed diets high in palm oil, flaxseed or fish oil, and high levels of sucrose [279]. A recent mouse experiment by Howe et al. demonstrated that pre-exposure to either palmitic or oleic acid attenuated cytokine secretion from microglia stimulated with TLR2 agonist lipoteichoic acid and improved sensitivity to NO production [280].

5.2 Phytosterols

Approximately 30-60% of the unsaponifiable fraction of OO are in the form of phytosterols [281]. There are generally 100 different sterol forms in plant species, but the most common ones are -sitosterol, campesterol, and stigmasterol [281]. The concentration of phytosterols in OO generally exceeds 100 mg/100 g, with β-sitosterol being the predominant type [281]. The amount of β-sitosterol in an OO depends on the cultivation season [281], while the amount of campesterol and stigmasterol fluctuate with the maturity of the fruit [244]. The total sterol amount usually depends on the genetic factors and the oil extraction technique [244].

Ma et al. recently conducted a study where they explored the effects of β-sitosterol on the allergic activity in ovalbumin allergic mouse model [282]. Results showed that β-sitosterol supplementation for seven weeks attenuated allergic symptoms and intestinal inflammation. β-

sitosterol also enhanced physical and biochemical intestinal barrier by upregulating expression of claudin-1, occludin, and zonulin [282]. Increased IL-10 levels and reduced Th2 cell associated factor levels in the gut were also observed [282]. Another study by Wu et al. investigated whether β-sitosterol can inhibit TMAO production in ApoE$^{-/-}$ mice by modulating the gut microbiota composition [283]. Results indicated that β-sitosterol supplementation led to inhibition of bacterial genera containing cholintrimethylamine lyase gene. Reduced levels of TMA, FMO3, and TMAO were also observed [283]. Moreover, high-dose phytosterol esters intragastrically administered to rats daily for 12 weeks restored HFD-induced dysbiosis, modulating levels of *Firmicutes*, *Proteobacteria*, *Actinobacteria*, and *Verrucomicrobia* [284].

5.3 Phenolic compounds

Phenolic compounds are responsible for the bitterness and pungency of OO [244]. They are also responsible for neutralizing free radicals, which contributes to the stability of the OO [244]. Amount of phenolics may range anywhere between 50 and 1000 mg/kg with the typical concentration being around 100-300 mg/kg [244, 285]. EVOO in particular, contains high levels of various phenolic compounds consisting of phenolic acids (gallic acid and ferulic acid), secoiridoids (oleuropein), phenolic alcohols (hydroxytyrosol and tyrosol), and flavonoids (luteolin and rutin) [286-287]. The three phenolic compounds in highest concentration, however, are the glycoside oleuropein (OLE), hydroxytyrosol (3,4-dihydroxyphenyl ethanol) (HTyr) and tyrosol (Tyr) [285]. Several factors including cultivar, fruit ripening level, climatic conditions, growing region, processing technology, and irrigation determine the olives phenolic composition [288].

After ingestion of OO, a micelle is normally formed [289]. First modification takes place in the mouth with the help of the hydrolytic action of saliva [289]. This means that OO

polyphenols reach the stomach already partially hydrolyzed and then travel to the small intestine, where most are absorbed [289]. The ones that do not get absorbed keep traveling down to the distal parts of the GI tract where they interact with the resident microbial communities. OO phenols could reach the colon by different ways: non-absorbed food fraction, metabolites resulting from the enterohepatic recirculation or as a consequence of the basolateral-apical transport of intestinal cells [290].

OO polyphenols have shown to be protective against the oxidation of human intestinal cells of colorectal adenocarcinoma and regulating the expression of IL-8 [255]. They can also provide barrier protection and prevent pathogen invasion via effects on mucosal immunity [255]. In an RCT including 10 patients with hypercholesterolemia, ingestion of a 500 mg/kg polyphenol rich EVOO increased intestinal IgA, which has an important role in the homeostasis of the microbiota community [268]. In another study the effects of EVOO phenolics for 30 days in mice with DSS–induced colitis were investigated. Results showed a significant reduction in MCP-1, TNF-α, cyclooxygenase-2, and NO synthase expression levels [291].

HTyr and Tyr content in OO may vary between 3.0 and 25.6 mg/kg [292]. HTyr is considered amongst the most potent natural antioxidants [292]. In general, it is the best absorbed phenolic compound in the intestinal tract with an efficiency ranging from 40% to 95% [293]. The amount of HTyr and Tyr that arrive in the small intestine following incubation and passage through the acidic conditions of the stomach may be considerably higher than the amount initially present in ingested OO [294]. Evidence shows that the highest HTyr and Tyr plasma concentrations are reached around 0.5 - 1hr after oral consumption and are no longer detectable after 4 hrs [295]. HTyr uptake in the intestine is accomplished via bidirectional passive diffusion and is largely influenced by the matrix in which it was delivered [296]. For example, an oily

matrix will allow for easier absorption, while aqueous solutions or yogurt-like texture will make absorption more difficult [297-298]. Phenolic bioavailability in OO (and other foods in general) can also be influenced by the host age, hormonal status, or gender, as evident from available literature [299-300]. Once absorbed, HTyr is distributed through capillaries mainly to the muscles, testicles, liver, kidneys, and brain, with renal uptake being 10 times higher than other organs [292, 301]. In the blood, HTyr can bind to HDL-C to carry out its antioxidant and cardioprotective capacity [302]. It can also downregulate PPAR-α and γ- expression, which may result in reduction in adipocyte size [303]. In addition, an increase in AMPK and lipase was reported in adipocytes exposed to Htyr [304].

Similar to other phenolics, HTyr and Tyr are mainly subjected to Phase I and II metabolism but they are also capable of reaching the colon and interacting with the gut microbes [292]. For instance, evidence shows that the non-digested Tyr is biotransformed into its catabolites phenylacetic and phenylpropionic acid derivatives [292]. It has also been demonstrated that HTyr and Tyr can be degraded by *Lactobacilli* and *Bifidobacteria* in vitro and both of these organisms are known for the ability to produce GABA. Moreover, daily intake of 50 mg/kg of HTyr increased concentration of *Lactobacillus* (especially *L. johnsonii*), while also hindering the genera *Proteobacteris*, *Deferribacteres*, *Rikenella* and restoring the gut microbiota depressed by a fat-rich diet as well increasing the integrity of the intestinal barrier [305].

Oleuropein (OLE) amounts in olives average at approximately 19.2 mg/kg [306]. OLE is susceptible to the stomach environment where it is further hydrolyzed to produce free HTyr and elenolic acid [289, 307]. OLE is a glycoside and can be absorbed via passive and paracellular diffusion. It can also be taken up into epithelial cells of the small intestine via sodium-glucose co-transporter-1 (SGLT1) on the apical side and then it can then reach the bloodstream via

glucose transporter 2 (GLUT2) by facilitated diffusion [296, 308]. Unlike HTyr and Tyr, which are well absorbed in the small intestine, OLE reaches the colon unchanged and is rapidly degraded by the colonic microflora to produce HTyr, which can then be absorbed [294].

In vivo, OLE is preferentially degraded by lactic acid bacteria, such as *Lactobacillus* and *Bifidobacterium* species [309]. These species may help maintain intestinal barrier by inducing mucin production, reducing pro-inflammatory cytokine secretion, and lowering the TC and LDL-C levels [296, 310-311]. It was also found that OLE may be able to ameliorate advanced-stage type 2 diabetes in mice by regulating gut microbiota [312]. Consistent with the previously reported effects of HTyr administration, OLE was shown to significantly reduce the relative abundance of *Bacteroidetes*, including the *Parabacteroides* genus, and increase that of *Verrucomicrobia*, including the *Akkermansia* genus, confirming their importance in improving diabetes symptoms and insulin resistance [312]. Moreover, OLE is involved in energy metabolism and adiposity. It has shown the potential to reduce expression of PPAR-γ, compromise adipocyte differentiation, and improve insulin sensitivity [313]. It may also be able to increase uncoupling protein 1 (UCP1) expression, which will likely result in a decrease of visceral fat and formation of beige adipose tissue [314]. In addition, several preclinical studies showed that OLE can serve as a ligand to PPAR-α that regulates CYTP450 and controls lipid homeostasis, the activation of which mediates the pleiotropic effects of various vital functions of the body, making OLE a cardioprotective and neuroprotective agent [315].

5.4 Tocopherols

There are eight different isomers of vitamin E (α, β, γ, and δ) and tocotrienols (α, β, γ, and δ) present in OO with α-tocopherol being the most abundant (90-95%) [316]. Tocotrienols

are present in trace amounts only (0.1 mg/100g) [316]. Cultivar, geographic origin, fruit ripening, soil and climatic conditions affect tocopherol/tocotrienol composition [316].

Like phenolic compounds, tocopherols may also influence gut microbiota composition, although studies are limited. In a study of dietary intake of 28 nutrients, assessed by food frequency questionnaires completed by 60 pregnant women in the Norwegian Microbiota Study cohort, dietary intake of the tocopherol, vitamin E, was associated with a decrease in pathogenic *Proteobacteria* [317].

5.5 Squalene

OO is the richest source of squalene after the cod liver oil [244] representing 90% of the OO hydrocarbon fraction. Squalene acts as a peroxyl radical scavenger and protects PUFAs from autoxidation related to high temperatures [244]. Cultivar, fruit ripening and storage conditions can affect squalene content [244]. The reported concentrations in OO range between 20 and 900 mg/100g [318-319]. A recent study on dietary supplementation of squalene on the growth performance of early-weaned piglets found that squalene significantly increased anti-inflammatory markers (e.g. IL-10), while also decreasing the pro-inflammatory ones including TNF-a and IL-6 cytokines [320-321]. It was also demonstrated that squalene supplementation increased jejunal villus height and expression of tight junction proteins [320]. An increase in abundance of beneficial bacteria such as *Gemmiger* and a decrease in abundance of generally harmful bacteria such as *Alloprevotella*, *Desulfovibrio*, and *Barnesiella* were also observed [320].

5.6 Pigments

Besides all the previously described compounds, OO may also contain several pigments that provide the olive fruit with its green or reddish color [244]. Carotenoids are found in more

mature olives, while chlorophyll derivatives are mostly present in green olives [244]. The various types of pigments within these two pigment groups are chlorophyll α and β, β-carotene, violaxanthin, lutein, and other xanthophylls [244]. Carotenoids can range from trace amounts to as much as 20 mg/100g with β-carotene and lutein being the main components [244]. The main chlorophyll type found in olives is pheophytin-α. Amounts of these pigments can vary based on chemical and enzymatic transformations that take place during OO production, cultivar, climate, and growing conditions [322-323].

The bioavailability and consequently absorption of carotenoids have some limitations due to factors such as the dietary sources, food composition, cooking temperature, season, the breakup of the food matrix, presence of lipids, dosage, and presence of other soluble compounds/carotenoids [324-325]. These factors can lead to the release of carotenoids from the food matrix, improving its bioavailability or transforming the carotenoids into isomers that are better absorbed by the organism [325]. In a recent study supplementation with β-carotene increased the abundance of *Bacteroidetes* and *Proteobacteria* and decreased the abundance of harmful bacteria such as *Dialister* and *Enterobacter*, which corroborates the positive effects of this carotenoid in intestinal health [325]. In addition, several studies showed that some unknown metabolites were produced during the carotenoids' pathway through the intestinal tract [326]. These metabolites may include apocarotenoids, that have a shorter chain length and oxygen modification, which increases their aqueous solubility and electrophilicity, and consequently, improves the target of some transcription factors such as NF-κB, giving these metabolites some biological effects [327-328]. Furthermore, it was reported that certain microbes produce carotenoids in the colon, demonstrating a prebiotic-like effect that results in bacterial shifts with health-associated properties [327].

Evidence shows that chlorophyll, another pigment in olives, may have an effect on gut microbiota as well. A recent study by Li et al. investigated the effects of early-life dietary supplementation with chlorophyll on male mice with HFD-induced obesity [329]. Results demonstrated that chlorophyll slowed down weight gain, improved glucose tolerance and reduced low-grade inflammation. In addition, it reduced the *Firmicutes* to *Bacteroidetes* ratio, while also affecting specific microbial species. For instance, *Blautia* relative abundance increased and *Lactococcus* and *Lactobacillus* relative abundance decreased [329]. Another study by Li et al. tested chlorophyll-rich spinach extract in early- and adult-life intestinal digestion and colonic fermentation in the murine model in vitro [330]. Chlorophylls favored pheophytinization and oxidative reactions, leading to the formation of pheophytin-α, pheophytin-β, 13^2-hydroxy pheophytin α, and 15^1-hydroxy-lactone pheophytin α. Notably, *Blautia* in the gut microbiota of 3-week-old mice (early life) and unclassified *Lachnospiraceae* in 8-week-old mice (adult life) were advantageous for transforming the pheophytins to pheophorbide-α, pheophorbide-β, 15^1-hydroxy-lactone pheophorbide-α, and 13^2-hydroxy pheophorbide-α, thereby demonstrating the loss of the phytol chain in the pheophytins [330]. Meanwhile, total SCFA, as well as acetic, propionic, and butyric acids, were increased by the process of microbial fermentation in the presence of pheophytins [330].

6. Conclusions

Scientific evidence strongly points to the fact that there is a tight interconnection between what we eat and the gut, heart, and brain health. This sometimes is referred to as the gut-heart-brain axis. Various dietary patterns like the MedDiet diet have the potential to exert beneficial effects on the axis via either direct or indirect pathways (e.g. through gut microbiota modulation). The benefits of any dietary pattern, including the MedDiet, likely result from

synergistic effects of the individual dietary components. The purpose of this review was to summarize evidence on how two common MedDiet components, walnuts and olive oil, affect the gut-heart-brain axis. The specific focus was the interaction between individual components in walnuts and OO and the gut microbiome, as well as the effect of that interaction on host health and gut-heart-brain axis in particular. Due to our current inability to draw causal relationships between dietary interventions and changes in gut microbiota composition and functionality, as well as the specific links between these changes and host health, it is extremely difficult to make any definitive conclusions. Our lack of understanding of what a "healthy" or "unhealthy" microbiome looks like further obstructs the big-picture view. Therefore, more well-designed human trials investigating the relationships between diet and gut-heart-brain axis are warranted. With increased knowledge of how individual dietary components affect the axis, recommendations of foods to include or exclude in dietary patterns can be made with more confidence.

7. References

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Chapter 2

Best Practices and Considerations for Conducting Research on Diet-Gut Microbiome Interactions and their Impact on Health in Adult Populations: An Umbrella Review

This project was developed in collaboration with the Institute for Advancement of Food and Nutrition Sciences Microbiome Expert Group. Authorship: Tatiana Diacova, Chris Cifelli, Cindy Davis, Hannah D. Holscher, Mary Kable, Johanna W. Lampe, Marie Latulippe, Kelly S. Swanson, J. Philip Karl

Abstract

Diet is one factor modulating gut microbiota composition and functionality. However, determining causal links between diet-gut microbiome interactions and human health is complicated by inconsistencies in the arising evidence, partially, from differences in research methods and reporting. Widespread adoption of standardized best practices would help advance the field but requires those practices be identified, consolidated and discussed. This umbrella review aimed to identify recommended best practices, define existing gaps, and collate considerations for the design of studies investigating diet-gut microbiome interactions in relation to human health outcomes. Reviews discussing these and published after 2013 were identified using a systematic search. Recommendations, considerations and gaps relating to best practices within the categories of study design, participant selection, dietary intervention/assessment, biological sample collection, and statistical analysis were extracted and consolidated to identify agreement and knowledge gaps. Eight narrative reviews were included. Several general points of agreement were identified but a recurring theme was that best practices will depend on the research aims, outcomes and feasibility. Multiple gaps were also identified. Some such as suboptimal diet assessment methods and lack of validated biomarkers are relevant across nutrition science. Others, including defining a “healthy” gut microbiome and absence of standardized sample and data collection/analysis protocols are relevant across gut microbiome research. Gaps relatively unique to diet-gut microbiome research include underrepresentation of microbiota-modulating dietary components in food databases, lack of knowledge regarding intervention durations required to elicit lasting changes in the gut microbiome, and the need to

further develop and refine statistical approaches for integrating diet and gut microbiome data. Ultimately, future research and discussions will help address these gaps and evolve best practices. In the interim, the best practices and considerations discussed herein, and the publications from which that information was extracted, can provide a guide for designing and conducting diet-gut microbiome studies.

1. Introduction

The human gut microbiome encompasses a dynamic community of organisms including prokaryotes, eukaryotes, archaea, and fungi residing in the gastrointestinal tract. Bacteria are the dominant and most commonly studied domain with *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, *Proteobacteria*, *Verrucomicrobia* and *Fusobacteria* phyla predominating (9). These gut microbes contribute to various host physiological functions throughout the body including maturation and modulation of the immune system (10), maintenance of gut barrier health (11), and regulation of brain function and behavior (12) among others. Accordingly, the gut microbiome is increasingly recognized as a critical mediator of human health and disease risk (13-18).

Diet is one important factor affecting gut microbiome composition, function and metabolic activity (9, 19-24). Evidence shows that diet may explain as much as 50% of interindividual variation in gut microbiota composition in mice and 20% in humans (25-26). Dietary components impact the gut microbiome in several ways. These include providing substrate for microbial metabolism, modulating gut barrier integrity and morphology, directly or indirectly altering the gastrointestinal environment, and influencing quorum sensing and biofilm formation (27). Gut microbes, in turn, modulate the digestion, bioavailability and absorption of various dietary components (28-31). The result of this bidirectional relationship is production of

metabolites that play an important role in host health and disease by altering function of biological systems throughout the body from the gut to the brain (32).

Despite evidence supporting a strong connection between diet, the gut microbiome and human health, conflicting results regarding specific diet-gut microbiome interactions and their influence on host health are widespread (33). Reasons for this inconsistency vary but may be caused in part by the diverse approaches used for studying diet-microbiome interactions and their impact on human health. The diversity is attributable in part to a rapidly developing field in which new technologies and techniques are constantly being developed. While allowing for new insights into diet-gut microbiome interactions, these advancements complicate comparisons across studies and make drawing definitive conclusions extremely difficult. The resulting uncertainty impedes development of evidence-based dietary recommendations aimed at nourishing the gut microbiome to improve human health.

Widespread adoption of standardized best practices for studying the role of diet-microbiome interactions in human health would facilitate the comparison and integration of studies ultimately needed to develop evidence-based recommendations. However, doing so first requires best practices and considerations for study design and conduct be identified, consolidated and debated. Narrative reviews focused on best practices for the design of gut microbiome (e.g., 34-50) and nutrition studies exist (51-54). However, fewer have specifically considered best practices for study design in research focused on diet-gut microbiome interactions and human health (1-8) and, among those reviews, foci and recommendations have in some cases differed. This umbrella review therefore aimed to identify current recommended best practices in diet-gut microbiome research, define existing gaps where best practices have not been identified, and collate considerations for the design of studies aiming to determine effects of

diet-gut microbiome interactions on human health-related outcomes. The overarching goal of this work is to improve study design and reporting to facilitate synthesis and comparison of results across studies and ultimately advance understanding of the causal effects of diet-microbiome interactions on human health and disease.

2. Methods

The protocol for this umbrella review was developed according to the Joanna Briggs Institute Manual for Evidence Synthesis for umbrella review and registered on OSF (https://doi.org/10.17605/OSF.IO/FUPZS) and PROSPERO (CRD42023437645). The central question of the review was: What are the established and/or suggested best practices and considerations for study design and research methods in human studies exploring the effects of nutrition interventions on gut microbiome-related health outcomes? The specific objectives were to identify perspectives, reviews, systematic reviews and meta-analyses exploring best practices and considerations in diet-microbiome research; consolidate, summarize and discuss best practices and study design considerations; and identify gaps where best practices have not been established.

Search strategy

Two separate literature searches using the search strings below were conducted using PubMed on 2/24/2023 and 3/3/2023, respectively. Both searches were then duplicated on 10/6/2023. All searches restricted the data range to manuscripts published in the past 10 years. Manual search of reference lists from identified articles and personal knowledge of the authors were used to identify additional relevant publications.

Search 1:

(microbiome[Title] or microbiota[Title] or microflora[Title]) AND (gut[Title] OR

intestinal[Title] OR gastrointestinal[Title] OR enteric[Title] OR diet*[Title] OR nutrition*[Title] OR prebiotic*[Title] OR probiotic*[Title] OR fiber*[Title]) AND ("best practices"[Title] OR challenge*[Title] OR "study design"[Title] OR recommendation*[Title] OR guide*[Title] OR consideration*[Title] OR guideline[Title])

Search 2:

(microbiome[Title] or microbiota[Title] or microflora[Title]) AND (gut[Title] OR intestinal[Title] OR gastrointestinal[Title] OR enteric[Title] OR diet[Title] OR nutrition[Title] OR “diet therapy’[mesh]) AND ("best practices"[Title] OR challenge*[Title] OR "study design"[Title] OR recommendation*[Title] OR guide*[Title] OR consideration*[Title])

Eligibility criteria

Only narrative reviews, systematic reviews, meta-analyses, consensus statements, perspectives, and commentaries published in the last 10 years (2013-2023) were included. Included papers had to provide recommendations for methods related to randomized controlled trials or non-randomized study designs relevant to any non-patient human population and discuss methods or study design considerations relating to both nutrition-related interventions and gut microbiome related outcomes. Gut microbiome related outcomes included composition, microbiome-derived compounds, food-derived compounds altered by the gut microbiome, gut microbiome function and gut microbiome mediated health effects.

Exclusion criteria included not discussing best practices, standardization, considerations or gaps relating to best practices and method standardization; focus on design of animal, in vitro or in silico studies or design of studies for strictly human infant or in-patient populations; focus on microbiome populations in locations other than the GI tract; and focus on interventions that are not nutrition related. Publications discussing development of a new method or dietary therapy

and publications not including methodological considerations relating to both nutrition and the gut microbiome were excluded.

Screening and data extraction

Abstracts were screened for relevancy using Abstrakr software (55) by two independent reviewers blinded to each other's responses. The full text was retrieved for all abstracts considered as maybe or definitely relevant by at least one reviewer. Full texts were then screened by the same two independent reviewers who were blinded to each other's responses and according to the eligibility criteria described above. Discrepancies were resolved through discussion.

Data were extracted from all manuscripts meeting the inclusion/exclusion criteria by two independent reviewers using the same data extraction template. The template was in the form of an Excel spreadsheet divided into tabs corresponding with the predetermined structural review elements and sub-elements of interest. The first element focused on study design and participant selection. Sub-elements included research question and aims, trial design, blinding, duration (run-in, intervention, washout), effect size, sample size calculations, statistical analysis plan , addressing interindividual variability, inclusion/exclusion criteria , important metadata to collect, and population diversity. The second element focused on dietary data. Sub-elements included intervention design and development (diets, foods, supplements, dose, placebo/control), assessing adherence/compliance (biomarkers, ensuring compliance), describing diet/intervention, diet standardization, methods (emerging methods, commonly used methods), database selection (from nutrition- to gut microbiome-centered), other considerations (timing with microbiota analysis, timing and location of meal/supplement consumption). The third element described sample analysis. Sub-elements within this element were biological sample collection (types of

sample, quantity, frequency, timing, collection/transport/storage/processing), metadata to collect, sequencing, relative vs. absolute measures. The last element pertained to statistical analysis. It include such sub-elements as data transformation and normalization, model (general characteristics, analyzing diet-microbiome interactions), type I error, reporting taxonomy, diversity measures, interpreting and reporting of results, data sharing.

Text related to the elements/sub-elements were extracted, placed under the appropriate element/sub-element and coded as a "best practice", "consideration", or "gap in knowledge". Extracted text was then compared between the two reviewers and consolidated if in agreement. Discrepancies were resolved through discussion.

Review quality

Methodological quality for narrative reviews, perspectives and opinions was assessed by two blinded reviewers using the Joanna Briggs Institute Critical Appraisal Checklist for Text and Opinion Papers (Supplemental Table 2.5). Disagreements were resolved through discussion.

Role of the expert group

Conceptualization and design of the umbrella review was informed by all co-authors who comprised a panel of scientists with expertise in diet-gut microbiome interactions formed by the Institute for the Advancement of Food and Nutrition Sciences (IAFNS). The group provided feedback on the review protocol prior to registration on OSF/PROSPERO and reviewed extracted results relating to best practices, considerations and gaps in knowledge. In several cases, expert group opinion differed from the extracted information. Those differences are noted in the text and tables.

Data summary

Review results were organized and are presented according to the structural review elements defined above. Each element is divided into a section summarizing suggested best practices and another summarizing considerations and gaps extracted from the included reviews. Each structural element also includes a "points of agreement and gaps in knowledge" table and a "best practices" figure with extracted data summaries. "Points of agreement" for the purpose of this manuscript were defined as best practices for which a majority of papers commenting on a particular element were in agreeance on a best practice without another review stating something contradictory. Both explicit identification of a best practice and inclusion of statements that allowed a best practice to be inferred from the authors' discussion were used to determine agreeance.

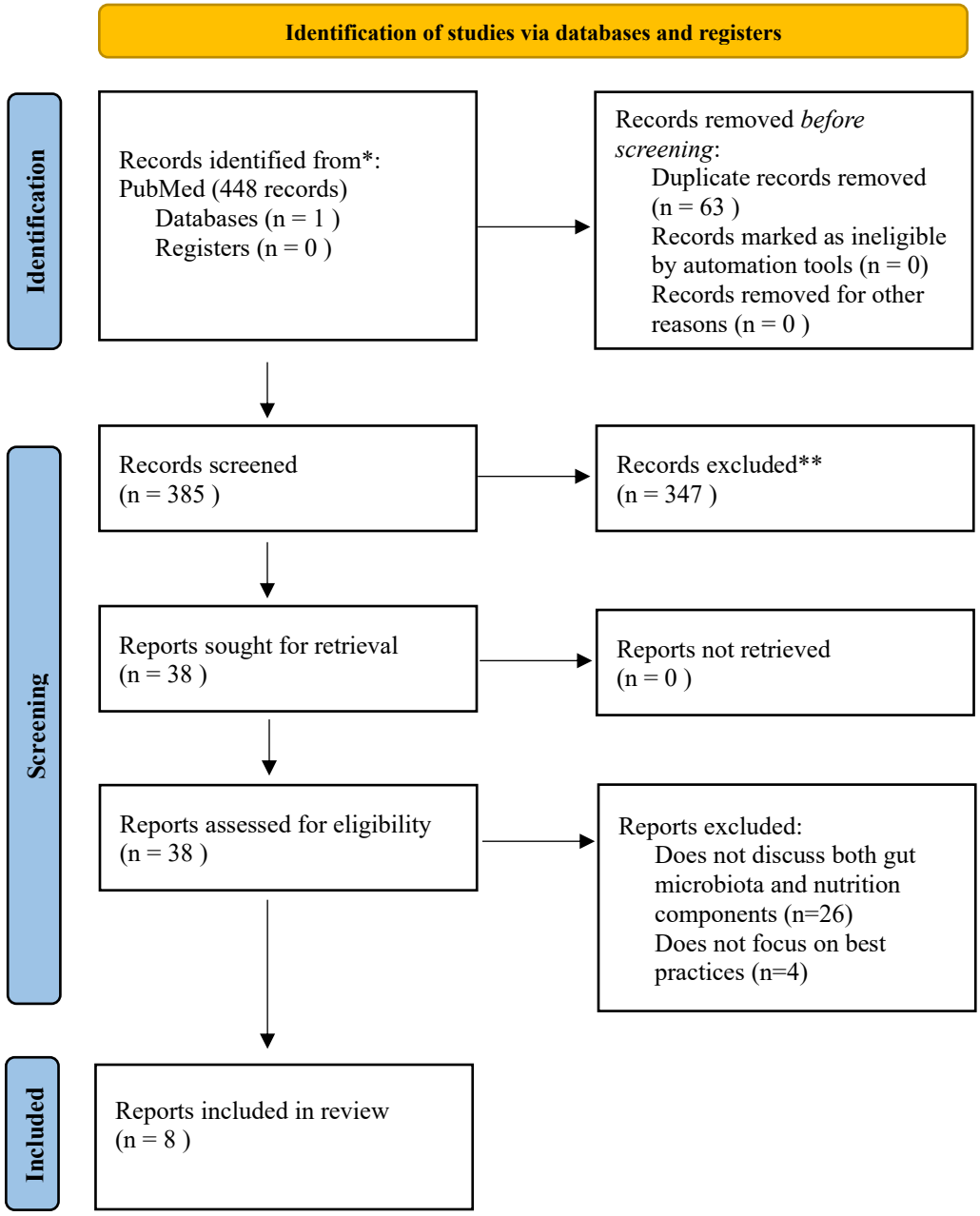
3. Results

The PubMed search identified 365 unique reports for screening. Of those, 38 abstracts were selected for full-text review, and eight publications were included in the present review (1-8). Countries represented in authorship lists included the United States, Australia, the United Kingdom, New Zealand, Ireland, Finland, Serbia, Switzerland, France, Belgium and Italy.

While all eight publications generally focused on best practices, considerations, gaps in knowledge for diet-gut microbiome studies, each presented a unique aspect. Choi et al. discussed approaches to applying computational models for diet-gut microbiome data integration. Hughes et al. focused on development of precision nutrition in the context of diet-gut microbiome interactions. Klurfeld et al. mainly focused on best practices and considerations for studies with fiber as the dietary intervention, while Mohr et al. discussed best practices for randomized controlled trials of of probiotics in physically active populations. Marques et al. presented guidelines for conducting gut microbiome studies in experimental and clinical hypertension

specifically. Swann et al. aside from discussing best practice guidelines for human gut microbiota intervention studies, also presented the EU legislative aspects in relation to foods and health claims. Finally, Johnson et al. and Shanahan et al. provided more of a general overview of current practices in diet-gut microbiome analysis, while also suggesting several best practices.

Figure 2.1 PRISMA diagram



3.1 Study design and participant selection

A summary of the results for the study design and participant selection element are provided in Table 2.1 and Figure 2.2.

3.1.1 Research questions and aims

Summary of best practice recommendations

Three publications (6-8) discussed best practices related to research questions and aims, and no points of agreement were identified. One publication stated that mechanisms, treatments, and outcomes of interest are all important to consider when developing research questions and aims (6). The other two publications emphasized that the question and aims should be hypothesis-driven (7) and clearly stated at the outset (8), respectively.

Considerations and gaps

One publication (8) discussed considerations for research questions and aims, describing several broad, common research questions often considered in diet-gut microbiome studies. These questions included the effects of the gut microbiome on metabolism and bioavailability of nutrients and non-nutrient foods, how that metabolism influences human health, the effects of diet, food or food components on gut microbiota composition and function, and the effects of gut microbiota changes on human health and biomarkers of health. The authors also noted that some health-related changes may be occurring in parallel but independently of shifts in the gut microbiome (8), which can complicate causal inference and therefore should be considered when developing research questions and aims.

3.1.2 Trial design and blinding

Summary of best practice recommendations

Seven publications (2-8) discussed best practices for trial design and four agreed that randomized controlled designs are optimal for most diet-gut microbiome studies (2, 4, 6, 8). Four publications discussed best practices for blinding, with three agreeing that double or triple blinding should be used (3, 6, 8). Two publications recommended crossover over parallel group designs (3,5), while another described parallel group designs as often being more suitable for certain interventions like probiotics (6). There was no agreement on any other best practices for trial design, with different single publications recommending that study designs be hypothesis-driven (7) and include both microbiota- and host-specific assessments (8).

Considerations and gaps

Four publications (4, 6, 7-8) discussed considerations for trial design and blinding: **Crossover designs** were discussed as having several advantages for diet-gut microbiome studies. Those advantages were described as providing the potential to test dose-response relationships and controlling confounding from habitual diet and baseline microbial communities that often demonstrate large inter-individual variability (7). Mohr et al. agreed that when responses are thought to strongly be driven by the underlying gut microbiome a crossover design is optimal (6). Klurfeld et al., however, pointed out that crossover studies usually are limited to relatively short-term interventions, increase volunteer burden, and prevent evaluating hard disease end points (4). **Parallel designs** were described as less burdensome than crossover trials on volunteers and potentially better suited for certain diet-gut microbiome studies where longer-term interventions, concerns of attrition, and lengthy washout periods preclude using a crossover design (6). **Adaptive trials** (56), which can be applied to both crossover and parallel studies, were identified as another trial design option to consider in diet gut-microbiome research (6). Mohr et al. explained that within an adaptive design, planning to be flexible is encouraged,

interim looks at data are allowed without compromising the integrity of the trial, and follow-up assessments are encouraged to obtain information on dissipation of effects and persistence of interventions (6).

An additional consideration discussed is that the longitudinal nature of both crossover and parallel studies allows for measuring the effect of temporal dietary variation and the effect of an intervention on microbial composition and stability within an individual (7). Design of those studies can often be improved when informed by observational and exploratory or pilot research that facilitate the generation of testable hypotheses (7) and provide information necessary to design and adequately power clinical trials. Swann et al. described **exploratory and pilot trials** as particularly useful in diet-gut microbiome research as optimal intervention doses, persistence of any effect, variability and effect sizes are often unknown but integral for study design (8).

A final consideration discussed by Klurfeld et al. is that demonstrating a diet-gut microbiome interaction causes a particular health effect in humans is extremely challenging (4). Doing so and identifying the underpinning mechanisms often requires a combination of well-designed human and animal studies.

3.1.3 Duration of run-in, washout, intervention

Summary of best practice recommendations

Four publications (3-6) discussed best practices for the duration of run-in, washout, and intervention periods in diet-gut microbiome research, but no points of agreement were identified. Only one publication (3) mentioned **run-in duration,** stating the run-in period should be at least 3-5 days based on the evidence that gut microbiome can change as soon as one day after the dietary intervention has reached the distal gut (57). Only two publications discussed **intervention durations** (5-6). One publication noted that intervention durations should be 2-3

weeks to allow the microbiota to stabilize (5), while another publication stated that intervention duration will depend on primary study outcomes and noted that interventions of 4-16 weeks are commonly used in probiotic studies (6). Though all four publications discussed **washout duration** for crossover studies, there was no agreement on the length of time required. Recommendations ranged from two (4, 6) to four (5) weeks and emphasized being sufficiently long to prevent carryover effects (3,6).

Considerations and gaps:

Three publications (3, 4, 6) discussed considerations for the duration of run-in, washout, and intervention. The summary of these considerations is provided below:

Mohr et al. stated that **run-in periods** may be beneficial depending on the research questions and study design, and to gauge intervention compliance and ability to complete outcome measures (6). **Intervention duration** will likewise vary based on the research question and primary outcomes. Notably, the duration required to demonstrate microbiota-mediated changes in host phenotype is likely on the order of weeks to months (3). Mohr et al. noted that for some interventions such as probiotics, a one-week **washout** may be adequate (6). For other types of interventions, the required washout duration may be ≥2 weeks (4). For parallel group studies, including follow-up assessments is critical for determining the extent to which any intervention-mediated changes in the gut microbiome persist long-term (6) and is useful for informing appropriate washout periods for future crossover studies.

3.1.4 Effect size and sample size calculations

Summary of best practice recommendations

Four publications (3, 5, 7-8) discussed best practices for sample size calculations, but no points of agreement were identified. Recommendations included hypothesis-driven calculations

(8), accounting for intrinsic human gut microbiome variation (5) and considering biochemical, chemical and physiological traits (8). Though one publication suggested that as many as 400-500 participants are needed for case-control and cross-sectional studies (3), no specific recommendations were given for experimental studies. One publication stated that sample size calculations should be based on the primary outcome, be hypothesis-driven, and ideally based on data from previous trials (8). Regarding the latter point, sample size calculations require determining expected effect sizes. Two publications discussing best practices for doing so (2, 5), and agreed that both the biological and clinical significance of the observed effects should be considered.

Considerations and gaps:

Two publications (2, 8) discussed considerations for conducting sample size calculations and determining expected effect sizes. Swann et al. stated that prediction of specific microbial organisms to be modulated by a dietary intervention beforehand is often difficult if not virtually impossible (8). Even if such predictions were possible, interpretation of specific microbial group compositional changes in terms of host health effects remains challenging (8). Both publications noted a challenge is that thresholds defining biologically meaningful effect sizes for changes in gut microbiome features or that differentiate individuals vary depending on outcomes of interest, are generally not standardized and often not known (2,8). For those reasons, effect sizes and sample size calculations for diet-gut microbiome studies commonly rely on host biochemical or clinical traits (which usually serve as primary study outcomes) thought to be influenced by diet-gut microbiome interactions rather than gut microbiome-related variables (8). Nevertheless, Swann et al. described several limited instances where sample size calculations may be based on a gut microbiome measure. For example, if measurement of bifidogenesis after ingestion of a test

fiber is sufficient to establish selective fermentation, sample size calculations may be performed based on effect sizes and standard deviation for fecal *Bifidobacteria* measured via quantitative approaches (8).

3.1.5 Statistical Analysis Plan

Summary of best practice recommendations

Two publications suggested best practices for statistical analysis plans (SAP) (7-8). Both publications stated the SAP should include a general description of how the primary outcomes will be analyzed and should be developed before submission to review boards for approval (7-8). One publication suggested that the SAP can be revised as a part of blind data review when indicated but that the SAP should not be changed after blinding is broken (8).

Considerations and gaps

No considerations or gaps in knowledge for SAP were extracted from the eight publications included in this review.

3.1.6 Interindividual variability

Summary of best practice recommendations

Three publications (3,5,7) discussed best practices for accounting for the marked interindividual variability observed across human gut microbiomes. Best practice recommendations for reducing the impact of that variability on results included increasing sample size (3, 7), collecting repeated samples (3, 7), and using baseline microbiome composition as a control (5). The main point of agreement among the three publications was that repeated measures can help reduce the impact of interindividual variability.

Considerations and gaps:

No considerations or gaps in knowledge for interindividual variability were extracted from the eight publications included in this review.

3.1.7 Inclusion and exclusion criteria (factors to consider)

Summary of best practice recommendations

Four publications discussed best practices for developing study inclusion and exclusion criteria (3, 5-6, 8). General points of agreement were that these criteria should consider a variety of factors such as baseline microbiome composition, habitual diet, demographics characteristics, health related factors, supplement and medication use, habitual diet, lifestyle/behavioral and environmental factors.

Inclusion criteria: four publications discussed best practices for inclusion criteria (3, 5-6, 8). Points of agreement were to consider baseline microbiome composition and habitual diet (3, 6, 8). Defining age range of interest (8), maintaining temporal, geographical, and demographic consistency in participant recruitment for case-control study designs (5) and including a diverse participant population (6) were also mentioned as best practices in separate publications.

Exclusion criteria: three publications discussed best practices for developing exclusion criteria (5-6, 8). Points of agreement were that pro-/prebiotic consumption in the habitual diet, antibiotic use, pre-existing health conditions and comorbidities should all be considered (5-6, 8). Only one publication mentioned demographics and anthropometrics (5). Three publications discussed -biotic use, specifically mentioning probiotics (5-6, 8), prebiotics (6,8), and fermented foods (6) though no points of agreement were identified in terms of an optimal washout period with recommendations including 3-4 (6) or 4-6 weeks (5). Two publications stated that antibiotic use should be considered when developing exclusion criteria (5-6) and one of the publications

recommended other medications be considered as well (5). There was no agreement in terms of the appropriate washout from medication use with recommendations ranging from 3-4 weeks (6) to 3-6 months (5). Other factors mentioned as important to consider for exclusion criteria were recent weight loss, special diets, plans to change exercise habits, and recent long-distance travel (8).

Considerations and gaps:

None of the publications discussed considerations for inclusion/exclusion criteria.

3.1.8 Metadata to collect

Summary of best practice recommendations

Six publications (3-8) discussed best practices for metadata to collect within the general categories of: demographics and anthropometrics, overall and gastrointestinal health-related factors, supplement and medication use, diet, lifestyle, geography, and environment. Overall, points of agreement were that the critical metadata to collect include sex/gender, age, ethnicity, health/disease state and history, medication use, habitual diet, and exercise habits. Additional recommendations extracted from one or more publications for metadata to collect can be found in Supplemental Table 2.1.

Considerations and gaps:

Only one publication (8) discussed considerations regarding metadata to collect in diet-gut microbiome studies. Swann et al. noted that while age is important to consider we still lack a good understanding regarding of when the gut microbiota becomes fully adult-like, and that the gut microbiota of individuals >65yr is distinct from younger individuals in both composition and function. As a result, the impact of dietary interventions on microbial-host interactions and their downstream effects on health may differ in certain age groups (8).

Table 2.1. Points of agreement and gaps in knowledge: Study design & participant selection

Element		References	Points of agreement	Gaps
Research question and aims		6-8	None	
Trial design		2-8	Randomized-controlled trials	Best practices for integrating animal studies in human trials to establish causal relations and mechanisms
Blinding		3, 5-6, 8	Blinding should be used	
Duration	*run-in*	3	None	Need for a run-in period and, if required, the optimal duration
	intervention	5-6	None	- Duration of intervention required to demonstrate microbiota-mediated changes in host phenotype is often unknown - Duration of intervention needed to elicit long-term or permanent changes in gut microbiome is unknown
	washout	3-6	Washout period should be long enough to prevent carryover effect.	Duration of washout period required for any intervention effects on the gut microbiome to return to baseline is unclear and may vary by type of intervention.
Effect size		2, 5	Clinical and biological significance should both be considered	- Biologically meaningful effect sizes for changes in gut microbiome features often unknown. - Often not possible to select beforehand which bacteria are expected to be modulated by a dietary intervention. - No current consensus on definition of a normal or abnormal microbiota making it difficult to determine if an increase or decrease of specific microbiota groups is any indication of any specific health effect.
Sample size calculations		5, 7-8	None	The gut microbiome features (if any) on which sample size calculations should be based is often unknown
SAP		7-8	Should be written before data collection	
Interindividual variability		3, 5, 7	Repeated measurements help reduce impact of interindividual variability	Best approach for managing and reducing impact of inter-individual variability on results is undetermined

Inclusion criteria		3, 5-6, 8	Inclusion and exclusion criteria should consider a variety of factors such as baseline microbiome composition, habitual diet, demographics characteristics, health related factors, supplement and medication use, habitual diet, lifestyle/behavioral and environmental factors	Specific guidance on how baseline microbiome composition and habitual diet should be addressed is lacking
Exclusion criteria (factors to consider)	*demographics/anthros*	5	None	
	consumption of -biotics	5-6, 8	Consumption of pro/prebiotics in habitual diet should be considered	- No consensus on optimal washout period from pro/prebiotic consumption required prior to starting study - Whether a washout from intake of other -biotics (e.g., postbiotics, synbiotics) is needed is not always clear
	washout for -biotics consumption	5-6		
	medication use	5-6	Antibiotic use should be considered	- Effects of many medications on the microbiome are largely unknown - Requirements for washout or need to account for other specific medications besides antibiotics are not defined
	washout for medication use	5-6	None	
	other	5-6, 8	Comorbidities & pre-existing conditions should be considered	
Metadata to collect		3-8	Critical metadata to collect are sex/gender, age, ethnicity, health/disease state and history, medication use, habitual diet, exercise habits	The critical metadata to collect and include as covariates in analyses (or account for in participant selection and/or randomization) are undefined
Inclusion of diverse population		6	None	Importance of inclusion of diverse population not being discussed as best practice

Figure 2.2: Best practice recommendations for study design and participant selection

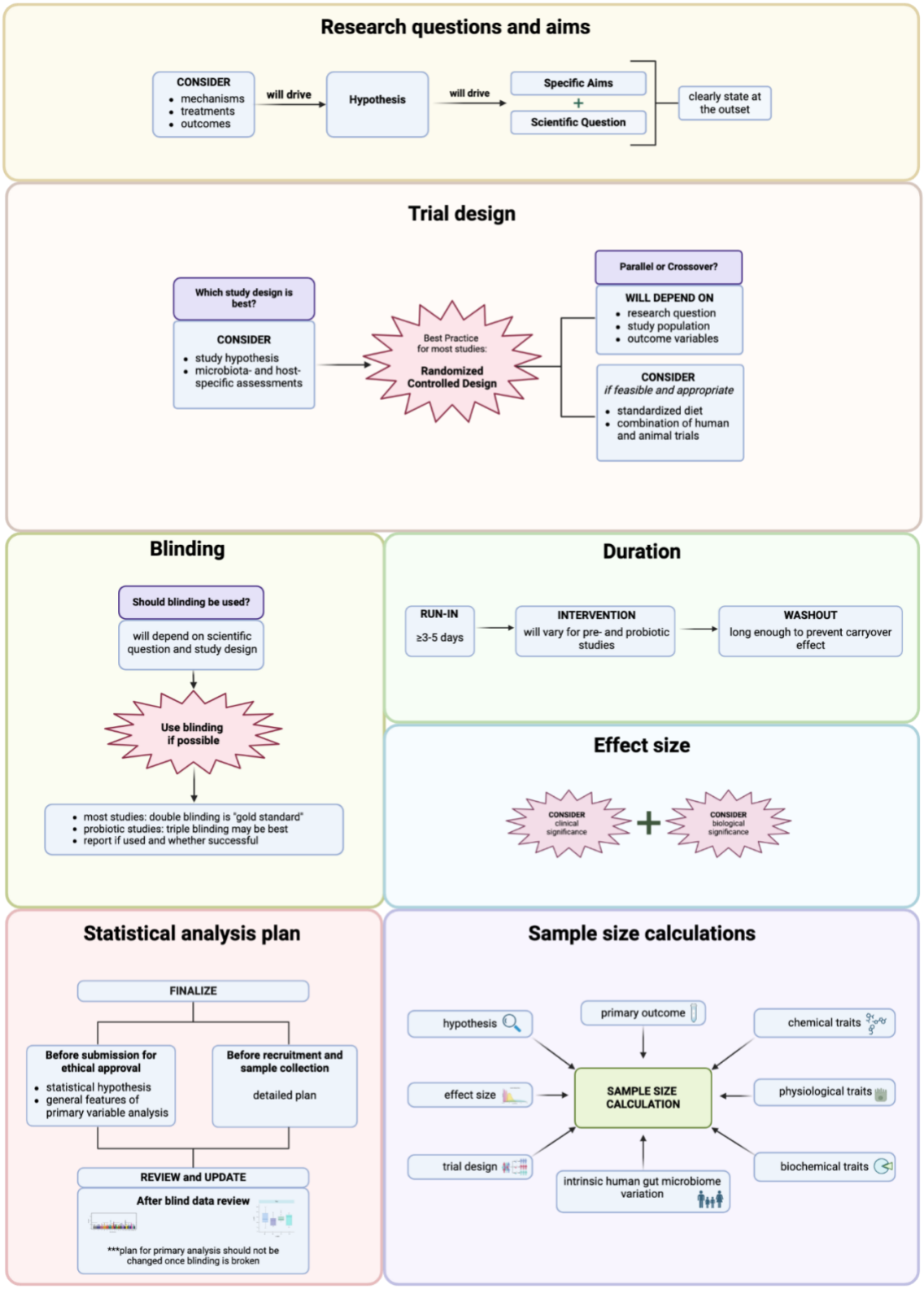
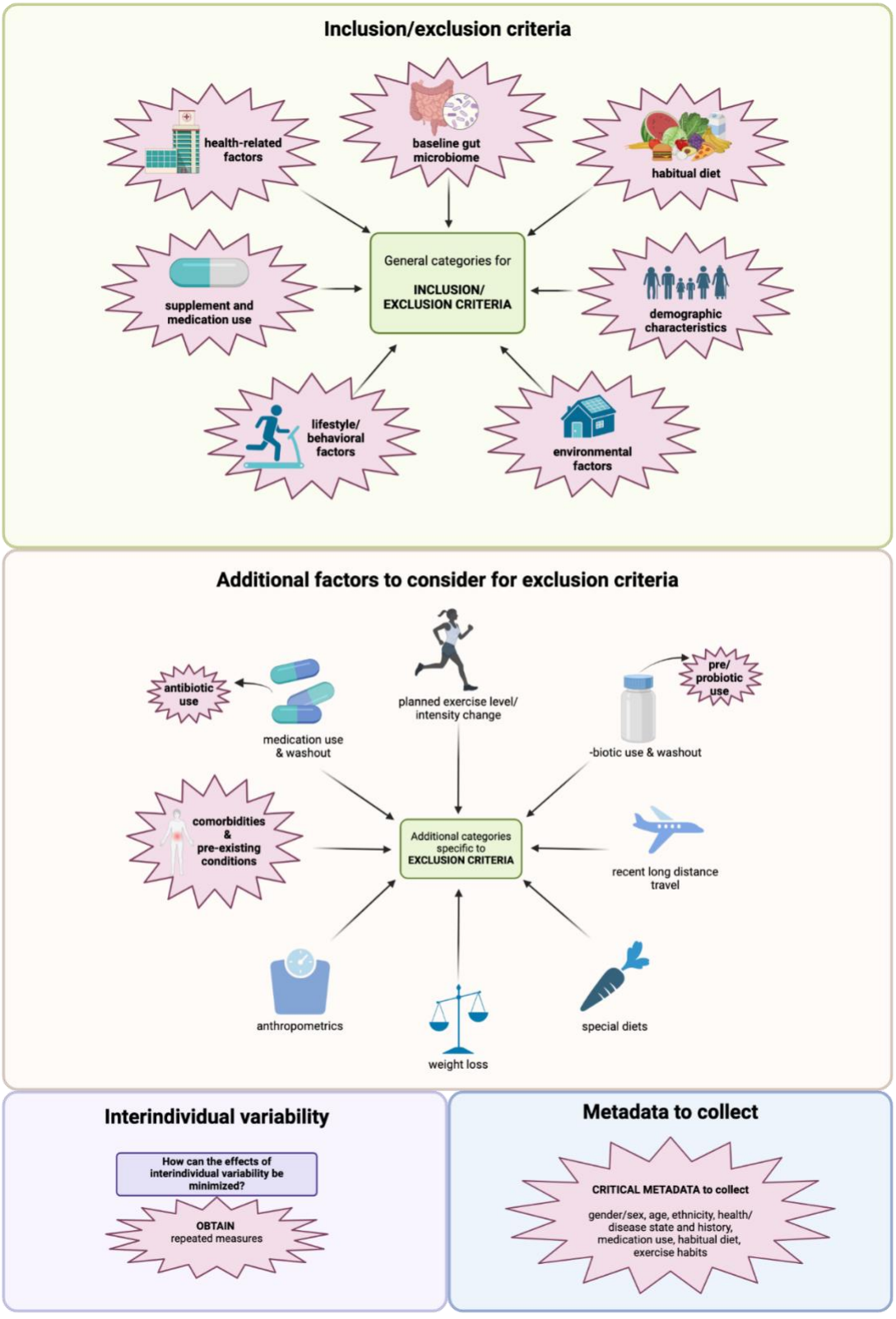


Figure 2.2: Best practice recommendations for study design and participant selection (continued)



3.2 Dietary data

A summary of the results for dietary data element are provided in Table 2.2 and Figure 2.3.

3.2.1. Intervention design and development

Summary of best practice recommendations

Six publications (2-6, 8) provided best practice recommendations for designing intervention diets, foods, supplements and doses, and developing appropriate controls. Points of agreement were that researchers should consider controlling intake of fiber, resistant starch, fructans and other oligosaccharides in intervention and control diets (4-5). An additional point of agreement was that the placebo or control diet should be indistinguishable from the intervention when possible and have minimal or no impact on the gut microbiome (2, 6, 8). Other best practice recommendations included providing all meals from a metabolic kitchen (4) and ensuring participants maintain weight stability when weight loss is not an outcome (5). For studies of isolated dietary fiber interventions, microcrystalline cellulose was recommended as an ideal placebo (8). Best practice recommendations for probiotic studies included basing the intervention and dose on in vitro, preclinical and/or human studies (6) and ensuring regulatory compliance (8).

Considerations and gaps

Three publications (2, 4, 6) discussed factors to consider when designing/developing dietary interventions. Within those publications multiple challenges inherent in designing interventions for diet-gut microbiome studies, particularly those using **whole food** interventions, were identified. Klurfeld et al. stated that, relatively unique to this field, both nutritive and non-nutritive compounds (e.g. polyphenols, emulsifiers, etc.) modulate the gut microbiota and therefore influence health outcomes (4). Additionally, factors like food structure, preparation

method, delivery mode and matrix can influence diet-gut microbiota interactions, but to what extent is often unknown (4). As a result, dietary ingredients and food preparation methods require careful consideration when designing interventions for diet-gut microbiome studies. Hughes et al. emphasized that those considerations extend to development of the placebo/control condition and make it particularly challenging to develop a strong control for studies with whole food(s) as a dietary intervention (2). Further, designing placebos/controls that allow for isolation of diet-microbiome interactions as a causal factor driving changes in host phenotype (versus microbiota-independent effect of the intervention) can be particularly challenging and requires careful consideration (4).

Inherent in designing any intervention is selecting the dose to be tested. Klurfeld et al. encouraged researchers to consider side effects when selecting dosing stages with certain microbiota-targeted interventions, such as **fermentable fibers** (4). An additional consideration is that minimum dose required to elicit effects on the gut microbiome may be undetermined and whether the provided dose should be based on absolute dose, energy intake or other factors (e.g., body weight) can be unclear (4). Mohr et al. described several unique challenges that exist for studies using **probiotic** interventions (6). They noted that important factors to consider related to probiotic dosing include effect sizes produced in previous studies, type of formulation, persistence within the host, colonization within the GI tract, manufacturing processes, and stability of the final probiotic product.

3.2.2 Describing diets and interventions

Summary of current recommendations

Six publications (1, 3-4, 6-8) discussed best practices for describing diets and interventions. Points of agreement included providing information on the overall diet and

detailed information regarding specific individual nutrients or foods being tested (1, 4, 7). One publication also recommended providing as much detail as possible when describing any type of dietary intervention (4). The recommended level of detail suggested in these publications for different interventions is discussed below.

For **whole foods and diets,** one publication recommended providing information on food preparation and cooking methods, food matrices, ripeness of foods, presence of additives, emulsifiers and artificial sweeteners, organic/conventional farming methods, microbial composition of non-fermented foods, and microbial load of dietary patterns (3). Other recommendations included using existing or data-driven dietary pattern indices (1) and quantifying "microbiota-accessible carbohydrates" (MACs) (7).

Related to MACs, two publications provided recommendations regarding details to describe in interventions using **isolated fibers, resistant starches** and **prebiotics** (4,8). Details included fiber source, carbohydrate composition, purity, degree of polymerization, particle size, average molecular weight and its distribution range, glycosidic bonds, solubility, viscosity. Confirming resistance to digestion was emphasized as well (4, 8). Similarly, the single publication providing recommendations for describing interventions using **isolated polyphenols** identified polyphenol source, degree of polymerization, chemical structure, and oligo-/polymer content as important characteristics to describe (8).

Regarding **probiotic** and **synbiotic** interventions, two publications agreed that phenotypic and physiological characteristics of the organisms used should be described (6,8). The same publications also recommended reporting safety, purity, potency, viability, and stability of these interventions. Reporting the method of inactivation for **parabiotic** interventions and

characterizing metabolites and inanimate microbes for studies testing **postbiotics** interventions were also recommended (6).

Considerations and gaps:

Two publications discussed factors to consider when describing dietary interventions (1, 4). Klurfeld et al. encouraged researchers to consider reporting more than just the amount and the chemical type of fiber, which may be insufficient and hamper reproducibility given the complexity and diversity of similar fiber types (4). Choi et al. discussed dietary pattern indices and their potential to be considered for describing diets in certain studies. The indices can capture multiple dimensions of diet, and often incorporate balance and variety (1). However, Choi et al also described limitations to that approach are existing indices rely on expert knowledge which fluctuates over time, while data-driven dietary patterns can be difficult to generalize beyond the study population and may not correspond with expert consensus and general nutritional knowledge (1).

3.2.3 Diet standardization

Summary of current recommendations

Four publications suggested best practices for diet standardization (3, 5-6, 8) but no points of agreement were identified. Recommendations included using provided diets when possible (6), attempting to stabilize participants diets (3, 8) and choosing the best approach based on study aims and outcomes (3). Standardizing the time and location of consumption of provided diets was recommended by one publication (3).

Considerations and gaps

Two publications included factors to consider when standardizing participant diets in diet-gut microbiome research (2-3). Several of those considerations pertained to the proposed

best practice of "stabilizing habitual diet". One reason why diet stabilization has been proposed as an alternative to providing complete standardized diets is that providing diets can be very expensive and burdensome for participants and research staff (2). Johnson et al. also commented that switching participants onto an identical, standardized diet could impact the gut microbiome, which could obscure intervention effects, that the optimal composition of any standardized diet is undefined and the extent to which diet standardization would reduce interindividual variability in the gut microbiome is unclear (3). Therefore, when considering individual nutrients or dietary components, it may be advantageous to allow participants to continue eating their habitual diets (2).

3.2.4 Assessing adherence/compliance

Summary of current recommendations

Six publications discussed best practices for assessing intervention adherence/compliance (3-8). Points of agreement were that measures of compliance should be incorporated into the study (3-8) and that biomarkers should be used to assess compliance metrics when feasible (5-8). Recommended methods of assessing compliance included collecting multiple food records and any uneaten provided foods and intervention products (3, 5). Two publications agreed that acceptable levels of compliance should be determined a priori, and non-compliant subjects excluded from analyses (3, 8).

Considerations and gaps

Six publications discussed considerations for assessing compliance/adherence (3-8). Marques et al. stated that as in any nutrition study, measuring adherence/compliance is important and assessment methods, to some extent, will vary based on the population, dietary intervention, and outcomes of interest (5). Shanahan et al. highlighted that very few established biomarkers for

diet-microbiome and microbiome-host interactions have been identified (7). Those that have been identified can be expensive to measure, and the validity, reproducibility, and sensitivity of some is suboptimal (7). Nonetheless, several biomarkers were described as potentially being particularly relevant to diet-gut microbiome studies:

Klurfeld et al. and Swann et al. both agreed that for interventions using **dietary or functional fibers,** fecal or blood SCFA concentration and fecal pH measurements can be potentially useful biomarkers (4, 8). Fecal SCFA concentrations, however, may not reflect in vivo exposure given that 95% or more are absorbed before elimination in the stool (4). Mohr et al. described breath hydrogen as also having the potential to serve as dietary fiber fermentation marker, while Marques et al. encouraged recovery of fecal fiber (5, 6). Of note, Klurfeld et al. explained that despite the availability of these methods, a gap is that there are no well-established biomarkers that reflect dietary fiber intake and researchers have to resort to relying on dietary histories that are often inaccurate, lack the necessary detail, and are highly subject to recall bias (4).

Biomarkers for several other foods and nutrients were discussed. Swann et al. described urinary alkylresorcinol concentrations as a short/medium-term biomarker of **whole grains** and **cereal brans** (particularly rye and wheat) intake (8). Urinary and fecal ferulic acid or serum dihydroferulic acid were listed as possible markers of **rye bran** and **whole grain wheat** intake (8). Urinary nitrogen is a well-validated biomarker of **protein** intake, as mentioned in Shanahan et al. publication (7). Circulating trimethylamine N-oxide (TMAO) can reflect dietary **choline** intake and may be particularly relevant to diet-gut microbiome interactions influencing cardiovascular health (7). Increasingly, various **polyphenols** are being studied in the context of diet-gut microbiome interactions. Shanahan et al. pointed out that for polyphenols with low

bioavailability that cannot be measured in blood directly, principle microbial metabolites can be used to monitor compliance (7).

Several emerging methods were discussed as also potentially being useful for measuring adherence/compliance in diet-gut microbiome studies. These include meta-barcoding to evaluate the plant component of diet (3, 7), metabolomics, metagenomics, and natural enrichment of stable isotopes (8).

3.2.5 Diet assessment methods

Summary of current recommendations

Five publications discussed best practices for diet assessment methods (1, 3, 6-8). Points of agreement were that multiple options are currently available and that the selection of the optimal method to use will depend on the study design and aims (6-8). Three publications also recommended selecting a diet assessment method based on the nature of study, participant population, feasibility, and strengths/limitations of the method in relation to the research question (6-8). An additional point of agreement was that the chosen method should allow for analysis of dietary patterns or quality (1, 3, 7). One publication recommended using technology and biochemical markers for diet assessment, if possible (3) while two publications recommended combining multiple 24-hr recalls (AMPM method via ASN24) or 3–4-day food records and FFQs (3, 6). Additional recommendations included providing clear instructions and training on how to include the level of detail necessary prior to completion of diet recording (3, 7), as well as performing comprehensive cross-checking of collected data (7).

Considerations and gaps

Four publications discussed considerations for diet assessment methods (1, 3, 7-8). Swann et al.

noted that in general best practice recommendations for diet assessment should follow those for any nutrition study (58) while being adapted to provide the level of detail needed to adequately describe diets and interventions within the context of diet-gut microbiota interactions (8).

Shanahan et al. highlighted the need for tools and methods that facilitate less onerous, yet accurate estimation of dietary intake, especially those that enable the measurement of nutrients in foods that are relevant to microbes and that estimate the availability of nutrients at the luminal substrate-microbe interface (7). Similarly, Choi et al. noted that connecting food servings to the quantity of food reaching the colon where it can ultimately impact the gut microbiome will require new approaches to dietary assessment and analysis (1). Also, towards those goals, Johnson et al. stated that several technology-based methods that incorporate image capture, wearable devices and apps are in development but still require validation (3). Devices for sampling throughout the GI tract have also been developed or are in development (3). Eventually these tools may enable researchers to follow a meal through the gut and collect data about that meal, as well as corresponding changes to the microbial community in situ (3). Unfortunately, while these approaches will have the potential to provide precise estimates of exposure to specific dietary components and interactions of those components with gut microbes, the technology is likely to be impractical for many studies, costly, and burdensome in the near-term (3).

3.2.6 Database selection

Summary of current recommendations

Two publications discussed dietary database selection (3, 8). No points of agreement were identified. One publication recommended that the diet database utilized be reported (8). Another publication discussed several characteristics of a database that would be suitable for a

diet-gut microbiome study, like inclusion of extensive information about foods and food components to enable the use of machine learning approaches to link dietary information and gut microbiome variables (3).

Considerations and gaps in knowledge

Three publications included factors to consider for database selection (1, 3, 7). Shanahan et al. stated that one of the challenges of comparing collected dietary data to food composition tables is that although many dietary constituents relevant to diet-microbe interactions have been measured in foods, not all are readily quantifiable in human diets or have not been measured comprehensively across the food supply (7). For instance, traditional macronutrient analysis alone may fail to uncover diet-microbiome relationships (7) and many non-nutritive food items containing high amounts of small molecules like polyphenols (e.g., spices and herbs) are underrepresented in dietary databases and software (1). Moreover, Choi et al. noted that because food composition tables are based on chemical analysis of foods, they do not account for variations in nutrient bioavailability and absorption (1), which ideally would be accounted for in diet-gut microbiome studies. Another factor that is not considered in the food composition tables is particle size of the ingested food, which combined with other components of eating context and environment (e.g. meal preparation, amount of chewing, etc.) can influence the amount of a food or nutrient reaching the gut microbiota (1).

Finally, Johnson et al. also emphasized that there is great variability in food databases used across studies (3). Even within the English-speaking regions of the Americas, UK, and Australia there is no single database that is being consistently utilized (3). Nutrient composition sources, analysis methods for specific nutrients, naming conventions used to identify foods, and food grouping structures vary between different databases (3). This makes comparison across

studies and cohorts extremely difficult (3). Therefore, Johnson et al. urged researchers to establish a shared food ontology that can harmonize dietary data collected in different global regions and by different tools (3).

3.2.7 Other considerations

Summary of current recommendations

Four publications discussed best practices for other elements relevant to dietary data collection and analysis (3, 5-7). Points of agreement included timing dietary data collection to coincide with microbiome measurements (3, 7), with one publication recommended collecting 2-3 days of food records prior to each microbiota measurement (3) and recording and reporting the timing and location of dietary intervention consumption (3, 5-6).

Considerations and gaps in knowledge

Two publications discussed several considerations related to dietary data collection and analysis (3, 7). Johnson et al. noted that a single stool sample may reflect different durations of diet history for different people due to differences in transit time and suggested that three days of recording intake should be sufficient to capture relevant short-term dietary factors affecting microbiota composition in a single stool sample (3). Similarly, Shanahan et al. encouraged collecting multiple dietary records to capture variability in daily dietary intake, regardless of the additional participant burden (7). Finally, Johnson et al. noted that timing of meals can also affect gut microbiota composition but is largely ignored in diet-gut microbiome studies (3).

Table 2.2. Points of agreement and gaps in knowledge: Dietary data

Element		References	Points of agreement	Gaps
Intervention design & development	*diets*	4-5	Control intake of fiber, resistant starch, fructans and other oligosaccharides	-To what extent factors like food structure, preparation method, delivery mode and matrix should be considered need to be determined. - Best strategies for designing intervention and placebo/control to isolate diet-microbiome interaction as causal factor driving changes in host phenotype (vs microbiota-independent effect of the intervention) are needed - 3D structures of dietary fibers are not uniform, have not been completely described and may impact diet-gut microbiome interactions. - Often unclear if dose should be based on absolute dose, energy intake or other factors. - Minimum dose required to elicit effects on gut microbiome may not be known - Ideal placebos for all interventions not defined.
	foods	8	None	
	supplements	6, 8	None	
	dose	6	None	
	placebo/control	2-3, 5-6, 8	Include placebo that, when possible, is indistinguishable from intervention and has minimal/no impact on the gut microbiome	
Describing diet/intervention		1, 3-4, 7-8	Overall diet should be considered. When describing intervention attention needs to be given to characteristics that would influence any interaction with the gut microbiome. These characteristics will vary based on the intervention.	Given the myriad dietary components that may impact the gut microbiome, the level of detail required to ensure reproducibility is unclear
Diet standardization		3, 5-6, 8	None	-Whether standardizing or stabilizing diet offers best approach to reducing/ controlling impact of inter-individual variability - Level of diet standardization/control required to definitively elucidate effects of diet-microbiome interactions on health outcomes.
Assessing adherence/compliance	*biomarkers*	4-8	Measure biomarkers when feasible	Few, if any, established biomarkers for diet-microbiome and microbiome-host interactions exist. Future research needed to refine existing and identify new biomarkers[7]
	ensuring compliance	3, 5-6, 8	Compliance should be measured with method used varying based on study design	Acceptable level of compliance to demonstrate efficacy is undefined

Diet assessment methods	*emerging methods*	3	None	- Commonly used existing methods may fail to account for/collect enough detail on dietary factors that may impact the gut microbiome, or may be sufficiently inaccurate to limit their applicability - Tools that facilitate less onerous, yet accurate estimation of dietary intake are needed. - There is a need for the further development of methods that enable measuring nutrients in foods that are relevant to microbes and methods that estimate the availability of nutrients at the luminal substrate-microbe interface - Connecting food servings to the quantity of food that reaches the distal colon where it can ultimately impact the gut microbiome will require new approaches to dietary assessment and analysis.
	commonly used methods	1, 3, 6-8	Multiple options available and choice depends on study design/aims. Should follow best practice recommendations reported elsewhere. Method should allow for analysis of dietary patterns/quality in addition to single nutrients if relevant for outcomes of interest	
Database selection		3, 8	None*	- No single existing databases appears to capture all information on dietary factors that may impact diet-microbiome interactions. - A shared food ontology that can harmonize dietary data collected in different global regions and by different tools is needed but does not yet exist[3]
Other considerations	*timing with microbiota analysis*	3, 7	Dietary data collection should be timed to coincide with microbiome measurement.	
	timing & location of meal/supplement consumption	3, 5-6	Record and report timing and location of consumption	Unclear how to account for variability in timing/ location of meal consumption in data analysis

***Expert group opinion:** while there is no agreement on the best database to use, it is essential to report which database was use

Figure 2.3: Best practice recommendations for dietary data

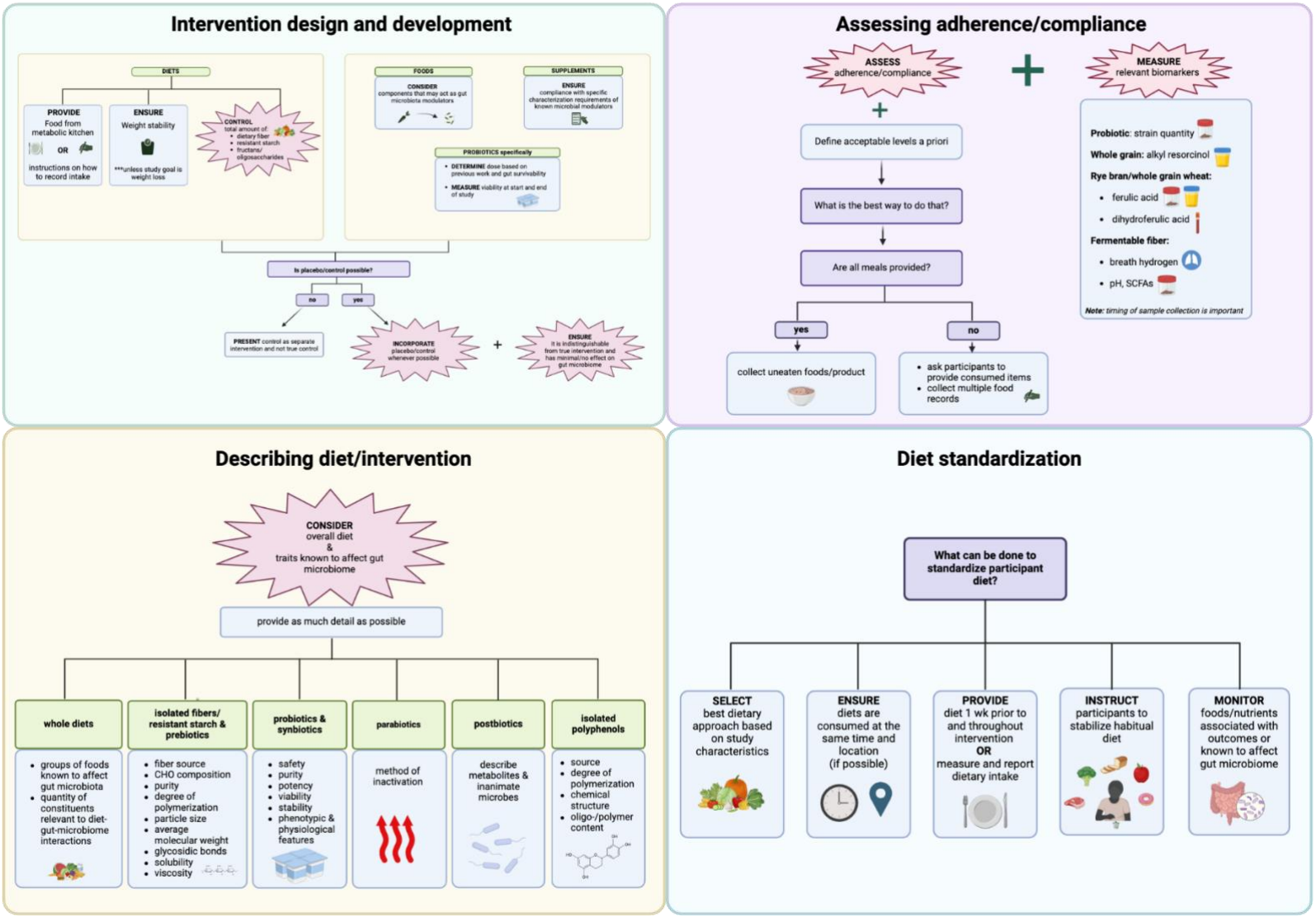
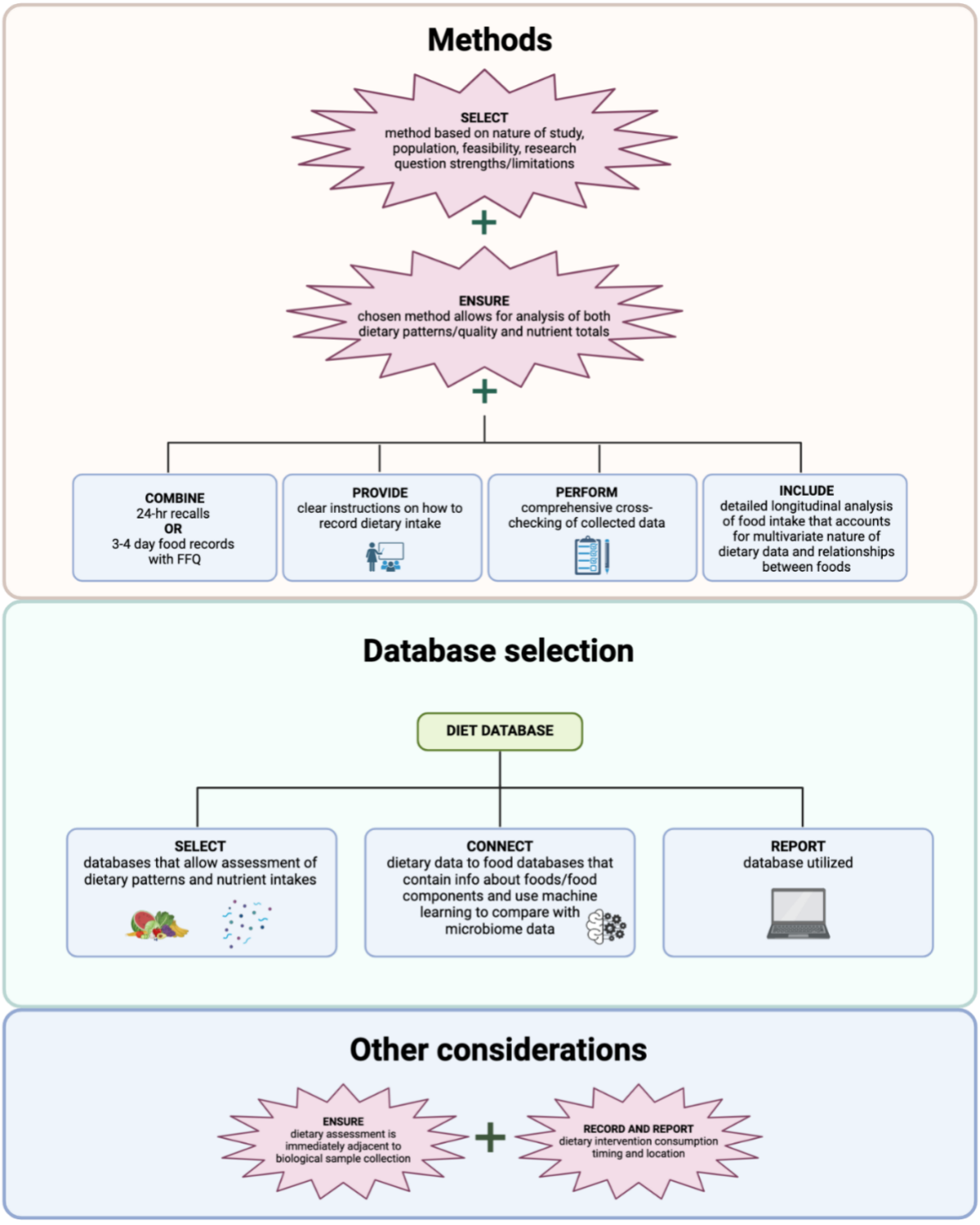


Figure 2.3: Best practice recommendations for dietary data (continued)



3.3 Biological sample analysis

A summary of the results for biological sample analysis element are provided in Table 2.3 and Figure 2.4.

3.3.1 Biological sample collection

Summary of best practice recommendations

Six publications discussed best practices for biological sample collection (3-8). The points of agreement included selecting sample types appropriate for the research question (7-8) and collecting sample quantities appropriate for the assay or measurement of interest (5) (Table S4). An additional point of agreement was to use standardized and consistent sample collection procedures throughout the study (3, 5, 7). With respect to fecal sample collection specifically, points of agreement included immediately freezing and storing samples at -80C after collection (3, 5, 8) and recording and reporting time of sample collection (3-5, 8).

Two publications (3, 5) suggested collecting multiple fecal samples per data collection period but did not agree on the ideal number of samples/days to collect, recommending 2-3 days (5) and 3-7 days (3), respectively. Three publications recommended collecting samples at several time points during a study (e.g. baseline, middle, endpoint, etc.) (6-8). Two publications stated sample collections should align with dietary and biological assessments (3, 7). Finally, two publications agreed that when collecting and transporting fecal samples freeze-thaw cycles should be minimized (5, 8) and cold chain should be maintained, or appropriate preservatives used (7-8).

Considerations and gaps in knowledge

Five publications discussed factors to consider for biological sample collection (3-5, 7-8). Most of the best practices identified dealt with **fecal samples**, which are commonly used in diet-

gut microbiome studies. Klurfeld et al. cautioned that feces can only provide spatially fixed data and does not accurately reflect longitudinal or horizontal environments of the colon (4). Additionally, the extent to which fecal samples capture the diet-microbiome interactions most relevant to host health outcomes is still largely unknown. Marques et al. noted that while fecal samples are generally considered as samples of the metacommunity, anatomically explicit samples, such as **mucosal biopsies**, are greatly enriched for a site-specific community and, for some outcomes, may be more relevant for detecting host-microbiota interactions (5). Further, regardless of the sample location and type, samples are only snapshots that do not distinguish between permanent and transient strains (4).

The **fecal metabolome** is thought to reflect the metabolic interplay between diet, host, and gut microbiota. However, Swann et al. noted that as fecal metabolites remain unabsorbed from the GI tract, they may be unseen by the host metabolic system (8). As such, **urine and blood samples** may be more reflective of diet-gut microbiome-host interactions (8). Swann et al. also highlighted a caveat present with collecting serum/plasma samples, which is that most metabolites produced by the gut microbiota from the ingested foods appear in blood 5-7 hours after ingestion and are cleared rapidly thereafter (8). This means that overnight fasted blood samples may not be informative (8). It is also essential to ensure that the collected sample is in alignment with the outcome of interest. For instance, Swann et al. suggested that for metabolomics analysis, 24-hr urine would more accurately reflect intake over the previous days compared to spot urine samples (8).

Several of the reviewed publications emphasized that if fecal samples are to be collected, the **timing** of sample collection, transport, storage, and processing are all important to consider. Collecting the entire bowel movement may be important for some studies to ensure adequate

sample volumes when multiple outcomes (e.g., metagenomics, metabolomics, biomarkers) will be measured. However, doing so often presents logistical challenges like the need to collect and transport large sample volumes (3). Differences in collection and transport methods add variability to results (5) and if quick sampling and proper collection procedures are not in place, the data generated are not useful and possibly even misleading. Johnson et al. also suggested that if samples are not collected on site, fresh samples should be transported to the research facility quickly where study personnel can process, aliquot, and store them (3). Alternatives to transporting fresh samples include having participants process and freeze samples at home or using preservatives that maintain sample integrity without cold chain storage (5, 7-8). Johnson et al. noted that having participants process their own samples can be a challenge as the burden of collecting a whole bowel movement and aliquoting into cryovials or microcentrifuge tubes may be overwhelming, and an alternative is using tubes with a detachable scoop connected to the lid (3). However, even with that convenience, the requirement to process samples may remain a deterrent for potential participants and can limit the number of aliquots collected. Several publications suggested that using preservatives to store samples may preserve sample integrity when quick transport, processing and freezing is not possible (3, 5, 7-8). Several preservatives that can allow samples to be stored at ambient temperatures were described (3, 5) with 95% ethanol, RNA later and the OMNIgene gut kit showing high reproducibility and stability. However, some preservatives may interfere with certain measurements and 95% ethanol may have advantages over other methods in that respect when multiple analyses (e.g., DNA, rRNA, metabolites) are planned (3). Whatever the method, providing clear instructions on hygienic sample collection and ensuring ease of sample handling is critical (8).

In terms of **frequency** of sample collection, it was suggested that a "large" number of samples is needed to reach valid conclusions (4) and multiple measurements may be necessary to provide an accurate understanding of the individuals' microbiota (8). One publication suggested collecting multiple samples per time point or daily sampling throughout the study (3). Another suggestion was to collect samples at baseline, early, middle, and late points (7-8). Regardless of these recommendations, due to many staff- and budget-related constraints, most diet-gut microbiome studies collect a single stool sample per time point.

3.3.2 Metadata to collect

Summary of best practice recommendations

Four publications discussed best practices for metadata to record when collecting fecal samples (3-5, 8). The only point of agreement was that transit time should be measured and recorded (3-4, 8). If transit time cannot be directly measured, recording bowel movement frequency (3, 5) or Bristol stool scores (5) were recommended as alternatives.

Considerations and gaps

Two publications (3-4) discussed considerations relating to metadata to collect for biological samples. Johnson et al. pointed out that measuring and reporting transit time when possible is recommended given evidence that rapid transit times, in particular (e.g., due to diarrhea), can affect gut microbiome composition.

Aside from transit time, measuring fecal pH and moisture can be considered. Klurfeld et al. described both as potential examples of cost-effective and relatively easy measurements that can serve as general outcomes and aid in data interpretation.

3.3.3 Sequencing

Summary of best practice recommendations

Five publications discussed best practices for DNA sequencing (2-3, 5, 7-8). Four of them (2-3, 7-8) agreed that the sequencing method chosen should be based on the research questions, resolution needed to address study hypotheses, resource availability, features of the microbiome being studied, dietary intervention, and response variables. Other recommendations were to include non-template controls during library preparation to detect contaminant DNA (5), to use controls to address batch effects (7), and to incorporate mock communities to identify potential sources of bias (8).

Considerations and gaps

Seven publications (1-3, 5-8) discussed considerations for DNA sequencing. Multiple viable options for gut microbiome compositional and/or functional analysis such as shotgun metagenomic sequencing, 16S rRNA amplicon sequencing, fluorescence in situ hybridization (FISH), qPCR and DNA microarrays among others were described (8). Shanahan et al. urged making decisions on which method to use at the trial design phase, prior to participant recruitment and data collection and to keep the selected technique consistent across all aspects of sample processing and analysis. Consistency was described as absolutely crucial as different sequencing platforms and bioinformatics tools/parameters can considerably influence results (Swann et al. cites 92, 93, 96, 97 as references, 2, 8). A brief overview of considerations discussed regarding several common gut microbiome assessment techniques is provided below.

One of the most used microbial sequencing techniques is **16S rRNA amplicon sequencing**. Advantages of the 16S methodology are that it is relatively low-cost and quick (3, 5) and provides a snapshot of overall microbial community composition (7). Disadvantages include relatively low-resolution that does not accurately assign taxonomy beyond the genus level (3, 7-8), inability to differentiate between closely related organisms (3), restriction to

bacteria and archaea only (7), and potential to introduce bias based on the primers chosen (3). As a result, 16S rRNA amplicon sequencing is not desirable when a detailed assessment of taxonomy or community function is needed (3).

FISH and **qPCR** techniques are both 16S-based and can also be used to target specific bacterial groups, as well as provide quantitative data (e.g. to measure absolute abundance) (8). In addition, FISH is the only method that allows for direct visualization of histological localization of microbes in tissue (8). Unfortunately, like 16S amplicon sequencing, FISH and qPCR may fail to capture taxa below detection thresholds (8).

Whole metagenomic shotgun sequencing and **shallow shotgun sequencing** are alternatives to 16S- based techniques. The main advantages of the whole metagenomic sequencing are that it provides species (and even strain) level resolution (3, 5, 8), can measure microbial organisms other than bacteria and archaea (e.g. eukaryotic species and viruses) (3), is less prone to amplification bias (3), and can provide information on potential metabolic properties and functional shifts in addition to taxonomy (5-8). Disadvantages, however, include high cost (2-3, 6-8), high analytical and computational burden, and the need for special expertise to interpret the obtained data (2, 6-8). Additionally, databases used for computational profiling may lack genomes for a large fraction of the intestinal microbiota and data interpretation is complex, since presence of a gene does not necessarily indicate the gene is being expressed (8). Another limiting factor is that there are no set recommendations for minimum sequencing depth for metagenomics analysis (5) and it is generally constrained by the microbial "dark matter" (microbes not yet cultured in a laboratory setting) (7). Johnson et al. suggested shallow shotgun sequencing as a more cost-effective approach to whole genome sequencing that can also provide accurate resolution at the species level (3).

DNA microarrays were described as the most comprehensive pre-designed method for gut microbiota assessment (8). The method utilizes oligonucleotide probes immobilized onto a glass slide to hybridize to complementary nucleotide sequences and provides phylogenetic identification and quantification (8). DNA microarrays are highly reproducible and can be useful for defining complex microbial signatures relevant for a dietary treatment or enabling sensitive detection of probiotic species used in an intervention (8). The disadvantages of this method include providing incomplete coverage of the ecosystem (8), high subjectivity to cross-hybridization (2), potential difficulty detecting low abundance taxa (2), and limitation to identifying species that will hybridize to the probes provided on the slide (2).

Regardless of the methodology used, once the sequences have been identified, studies typically use the sequences to assign taxonomy. Shanahan et al. cautioned that taxonomic assignment using operational taxonomic units (OTUs) can make comparisons across studies difficult because each analysis will result in a unique set of OTUs (7). A more recent approach is to identify exact amplicon sequence variants (ASVs). ASVs give better resolution than OTUs and increase reproducibility as the same ASVs can potentially be identified across studies (7). In addition, ASVs retain all unique sequences, removing only those determined to represent sequencing error, which improves resolution and reproducibility (7).

Choi et al. described **metatranscriptomics** as a method that can provide information about gene expression, which can improve identification of important diet-gut microbiome-disease relations (1). Shanahan et al., however, pointed out a disadvantage of this method, which is that RNA has a short half-life, making timing of sample collection a major confounding factor (7). In addition, this method is technically difficult to execute and requires attention to specific sample collection and storage procedures (6).

Although not a sequencing method, **metabolomic phenotyping** is a method that can be useful for providing insight into the biochemical output of the gut microbiome and how these outputs impact host health (8). Suggested metabolites to consider in diet-gut microbiome studies include short chain fatty acids, secondary bile acids, products of amino acid breakdown (e.g., tryptophan metabolites) and polyamines among others (8). However, metabolite analysis, just like metagenomics methods, is constrained by the volume of the microbial "dark matter" (7).

3.3.4 Relative vs. absolute measures

Summary of best practice recommendations

Four publications (5-8) addressed best practices for relative and absolute abundance measures. The only notable point of agreement was the importance of maintaining consistency in methodology throughout the study (7-8). Two publications recommended measuring and reporting both relative and absolute measures, especially if DNA yields vary between samples (5,7). Another publication recommended using qPCR in probiotic studies to aid with understanding probiotic persistence in the gut.

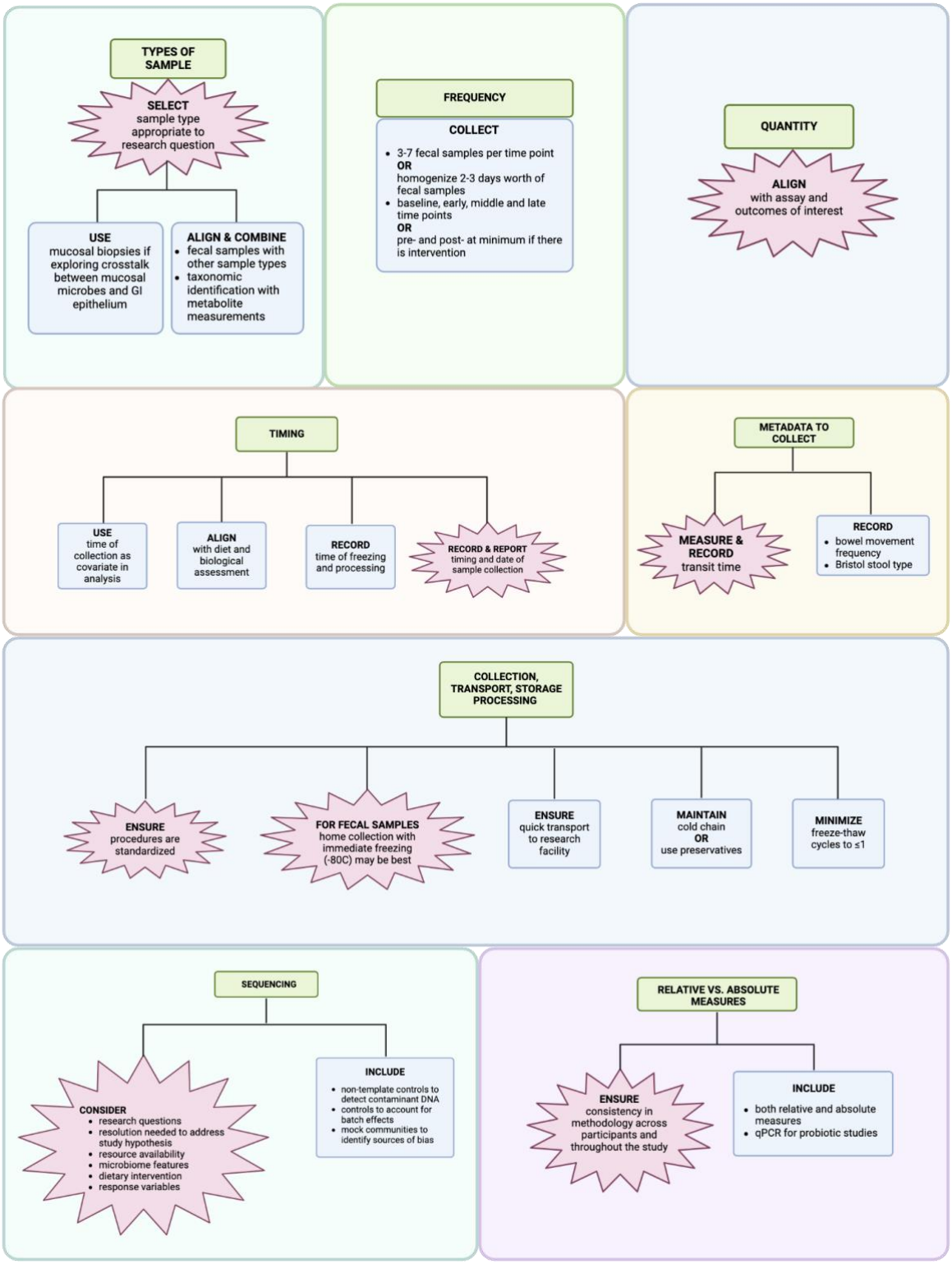
Considerations and gaps in knowledge

Four publications (2, 4, 7-8) discussed considerations for relative and absolute abundance measures. As discussed above, 16S amplicon sequencing and shotgun sequencing only provides relative abundance data. Without quantification it is unclear whether changes in relative abundance are driven by a change in absolute abundance or by collinear relationships within the community (7). Currently there are no efficient and low-cost methods for measuring absolute abundance within the entire community and the extent to which the total number of bacteria in the gut is important is somewhat unclear (4).

Table 2.3. Points of agreement and gaps in knowledge: Biological sample analysis

Element		References	Points of agreement	Gaps
Bio sample collection	*types of samples*	5-8	Sample types that are appropriate for the research question should be collected	The most accessible and commonly used sampling techniques (e.g., fecal samples) may not adequately capture the diet-microbiome interactions most relevant to host health outcomes.
	quantity	3, 5	Will vary based on assay/measurement of interest	
	frequency	3, 5-8	None	Optimal number of samples per time point and number of time points to include are undetermined.
	timing	3-5, 7-8	Important to record and report time of collection	Best approaches for incorporating collection timing, processing and freezing in analyses are undetermined.
	collection	3, 7-8	- Use standardized procedures - Home sampling with immediate freezing is best	Universal agreement on standardized procedures for sample collection and transport when immediate freezing is not possible is needed
	transport, storage, and processing	3, 5, 7-8	Maintain consistency in procedures throughout the study. Immediately freeze & store fecal samples at -80C for gut microbiota analysis	
Metadata to collect		3-5, 8	Transit time should be measured and recorded	- Impact of transit time on diet-gut microbiota interactions not fully characterized. - Whether indirect assessments of stool form (e.g., Bristol stool scale) are sufficient proxies for transit time and water content, and whether they can be used as a simple means of comparing dietary effects on fecal characteristics is unclear[4]
Sequencing		2-3, 5, 7-8	Should be based on research questions and align with resolution needed to address study hypotheses, resource availability, and features of microbiome being studied	No consensus on best sequencing protocols
Relative vs absolute measures		5-8	Consistency throughout study and among participants is important	-Availability of efficient and low-cost methods for measuring absolute abundance within the entire community are needed. -The extent to which the total number of bacteria in a community is important is unclear[4]

Figure 2.4: Best practice recommendations for biological sample analysis



3.4 Statistical analysis

A summary of the results for biological sample analysis element are provided in Table 2.4 and Figure 2.5.

3.4.1 Data transformation and normalization

Summary of best practice recommendations

Four publications (2, 5, 7-8) discussed best practices for data transformation and normalization. The notable point of agreement was that the data should be transformed in some way, but there was no agreeance on the specific methodology to use. One publication stated that proportional and non-normally distributed data should be transformed (7). Another suggested normalization and/or rarefaction for sequence count data (2) and the remaining two publications mentioned the importance of rarefaction as well (5, 8).

Considerations and gaps

Considerations and gaps for data transformation and normalization were discussed in two publications (1-2). Choi et al. emphasized that feature-level microbiome data, beta-diversity metrics, and intakes of foods and food groups measured using dietary records and FFQs are rarely normally distributed (1). As such, various data transformations may be needed to analyze diet and gut microbiome data depending on statistical approach and study hypothesis. However, Choi et al. also noted that transforming data is not always straightforward. For example, Hughes et al. highlighted that while log transformation of microbiome sequencing data is often recommended, this method requires a pseudo count for the many zeros in the data set, the choice of pseudo count can impact results and there is no consensus on what count should be used (2). Data normalization may also be required in some instances and a common technique is total sum scaling (TSS). The method divides the number of reads assigned to a specific taxon by the total

number of reads in the sample providing a measure of relative abundance (2). However, while TSS is straightforward, it may introduce bias (2) and controversy exists on whether normalization is required and, if so, the most appropriate normalization approach.

3.4.2 Model

Summary of best practice recommendations

Five publications (1-2, 5, 7-8) discussed best practices for statistical modelling in diet-gut microbiome research. The one point of agreement was models and tools designed specifically for microbiome analyses should be used (5, 7). One publication recommended treating both dietary and gut microbiome data as compositional data (1). One publication recommended using multivariate and mixed statistical models to deal with confounding effects (7), while another stressed the importance of non-parametric approaches (1). One publication recommended accounting for error due to zero inflation (i.e., the presence of many zeros in a dataset) (1), with another recommending Poisson and negative binomial distributions to handle zero-inflation (8). Two publications generally agreed that treating dietary data as an additional "ome" and applying three-dimensional modelling or "nutritional geometry" approaches is important (1, 7). Nutritional geometry is often defined as a modelling approach that considers how mixtures of nutrients and other dietary components affect host health rather than focusing on any one nutrient in isolation (59).

Considerations and gaps

Four publications (1-2, 4, 8) discussed considerations for statistical modelling. A comprehensive discussion of various statistical tests, metrics, and model fitting/performance tests suitable for diet-gut microbiome studies can be found in Hughes et al. and Choi et al (1-2). Briefly, applying multivariate approaches to dietary data analysis in diet-gut microbiome

research was discussed as a best practice. Choi et al. explained that this is largely because multivariate approaches have the potential to improve integration of diet and microbiome data, strengthen diet–microbiome signatures, and help control for variation in microbiome composition introduced by nutrition studies with microbiome-focused outcomes (1). An advantage of these approaches is that direct comparison between microbiome communities and dietary patterns can be made, thus improving integration of diet and microbiome data (1). Additional advantages of multivariate ecologically based approaches can be to reduce the problem of overfitting models that results from including too many variables such as many dietary components or microbial taxa (2) and reducing the issue of autocorrelation. In dietary data, autocorrelation can arise from foods commonly consumed together (e.g., salad and salad dressing) (1). Klurfeld et al. discussed another example of autocorrelation using diets high in protein and fat, which are necessarily low in carbohydrate (often digestible and non-digestible carbohydrate) making it almost impossible to attribute causality to an individual macronutrient (4). Similarly, within microbiome datasets, autocorrelation can arise from bacterial guilds and genome interaction groups that increase and decrease in concert and the compositionality of the data (i.e. an increase in abundance of one taxon will proportionally decrease the abundance of another) (1). Finally, application of emerging methods such as machine learning in diet-gut microbiome research was encouraged in two publications (1, 8). The methods were described as potentially helping to uncover various dietary patterns obtained from repeated evaluations from a large population, construct models, and make microbiome composition predictions by learning from training data (1, 8) and as complementing multivariate statistical approaches.

3.4.3 Type I error

Summary of best practice recommendations

Three publications (2, 5, 7) included best practices for addressing type I error, all agreeing that correction for multiple comparisons should be applied, and the method use reported. One publication also suggested utilizing stringent filtering methods when analyzing gut microbiome datasets to reduce type I error (7).

Considerations and gaps

Only one publication (7) discussed considerations for handling type I error. Shanahan et al. emphasized that care must be taken not to overinterpret the significance of changes in abundance of individual ASVs/OTUs and that presence of "rare microbes" (e.g., present in some but absent in the majority) may lead to an increased risk of type I error.

3.4.4 Diversity measures

Summary of best practice recommendations

Three publications (5, 7-8) suggested best practices for measuring diversity, focusing on metrics of microbial diversity specifically. All agreed that diversity metrics should be assessed in combination with other measures (e.g. structural stability over time, distinct phases of microbiota assembly, Principal Coordinates Analysis (PCoA), etc.). One publication also emphasized the importance of reporting the specific metric(s) used (7).

Considerations and gaps

Four publications (1, 5, 7-8) discussed factors to consider when measuring, analyzing and reporting microbiota or diet diversity measurements. Three publications agreed that high **microbiota diversity** and gene richness are generally considered desirable but do not equate to greater abundances of microbes performing beneficial functions and is not an absolute requirement for stability and resilience of the microbial ecosystem (5, 7-8).

Several publications also noted that diversity metrics can be applied to dietary data (1,7]). The approaches discussed included diet quality and diet pattern analysis:

Diet quality (Healthy Diet Indicator (HDI), Healthy Diet Score (HDS), Healthy Eating Index (HEI), Alternate Healthy Eating Index (AHEI), Mediterranean Diet Score (MDS)) assessments can be used in cohort, case-control, cross-sectional, and longitudinal study designs and may help account for dietary complexity and interactions between dietary factors (7). An advantage of these indices is that many have been validated by relating index scores to a specific health outcome (7) and facilitate comparisons across studies using the same index (1), both of which may be advantageous for diet-gut microbiome studies. An additional advantage can be that the indices typically reduce multivariate dietary data to a single continuous variable, which can make statistical analyses more straightforward. The main disadvantage of diet quality indices is that the majority require additional nutrient intake assessment (e.g., food records, 24-hr recall, FFQ, etc.) to calculate the final score (7).

Diet pattern analyses are also suitable for cohort, case-control, cross-sectional, and longitudinal study designs, are generally good at accounting for diet complexity and interactions between dietary factors and can be used as a covariate to determine whether the effects of a nutrient are independent of the overall dietary pattern (7). The disadvantages of these methods include that patterns are empirically derived from data and not from diet-health evidence and that the empirical process can include arbitrary decisions (e.g., food groups, number of factors/clusters to be retained) (7).

Choi et al. noted that dietary pattern analysis facilitates identification of diet-microbiome relationships in the context of dietary patterns and food groups, which is relevant because single nutrients are rarely consumed in isolation and food groups associate more strongly with the gut

microbiome compared to conventional nutrients (1). Food-based dietary patterns analysis avoids some limitations inherent in nutrient databases in which representation of food-derived biomolecules is lacking, and incorporates the concept of food synergy, that is foods may behave differently when consumed in combination rather than individually (1). Relatedly, Choi et al. also discussed applying metrics originally meant for ecological assessment to measure diet diversity (as described in the statistical modeling section above). For example, hierarchical structures used in different food and nutrient databases can provide tree-like, lattice or hierarchical structures that organize food data and inform analysis of dietary patterns using approaches analogous to UniFrac, which is commonly used for microbiome analysis (1). The approach has potential to allow for analysis of multiple levels of dietary patterns, foods, and nutrients along with food synergy somewhat simultaneously (1). Development of a metabolite-informed tree structure for foods that accurately represent the diversity of food components in a hierarchical way without dependence on expert-defined food grouping structures or hierarchies could further improve this approach (1). However, appropriate ways to implement ecological metrics in dietary data analysis and how to interpret the obtained results is still largely undefined (1) and work to define the optimal tree structure for this purpose is still needed (1).

3.4.5 Reporting and interpreting results

Reporting taxonomy

Summary of best practice recommendations

Best practices for reporting taxonomy were discussed in two publications (5, 7) both agreeing that the databases and versions used in bioinformatics pipelines should be reported.

Considerations and gaps

Three publications (1, 7-8) discussed factors to consider when reporting taxonomy. Swann et al. noted that reporting at the ASV/OTU or species level allows for granular reporting which is valuable given the metabolic variability of the microbiome but highlights inter-individual variability can complicate analyses as granularity increases (8). Reporting at higher taxonomic levels (e.g., genus, family, phyla) may overcome some of that variability allowing for identification of key trends but will also mask potentially important details because microbes within a taxonomic group can have different metabolic activities or differential responses to dietary substrates (8). It was also emphasized that taxonomy alone does not provide mechanistic insight into the effect of diet on microbial utilization of nutrients (7) given that metabolic functions are not always linked to taxonomic identity (7), the most highly differentially abundant taxa may not represent meaningful differences in function, and low-abundant taxa (which are routinely excluded from analysis) can influence function (1). Thus, reporting on taxonomy alone may not be sufficient for diet-gut microbiome studies and direct measurement of metabolites in combination with characterizing microbial profiles (taxonomy and genes or gene pathways) may be more informative for health outcomes (7). In addition, there is little consistency as to which classification or taxonomic levels are reported in diet-gut microbiome studies (7).

3.4.6 Reporting of other results

Summary of best practice recommendations

Three publications (5, 7-8) included best practices for reporting and interpreting results in diet-gut microbiome studies. No points of agreement were identified, as each of the three publications addressed a slightly different aspect of reporting. One publication highlighted the importance of discriminating biological and statistical significance while cautioning against overinterpreting results (5). Another publication suggested focusing on networks of co-abundant

organisms that respond to the same dietary substrate and considering substrate availability and intestinal region sampled when interpreting results (7). Finally, the third publication focused on the importance of reporting both positive and negative outcomes for transparency and confidence (8).

Considerations and gaps

Three publications (1, 4, 8) discussed considerations for reporting and interpreting results. Choi et al. discussed methods for reporting dietary data which included providing gram weight of foods consumed or normalized food weights that account for typically consumed serving sizes and also discussed methods for reporting ratios of nutrients to energy intake. Swann et al. focused on considerations for reporting gut microbiome-related outcomes, stating that the typical endpoints reported are alpha/beta diversity, taxa richness and relative abundance of individual taxa. Klurfeld et al. noted that almost all journals have online versions and offer online supplemental materials as an option, which allows detailed reporting of dietary data.

3.4.7 Data sharing

Summary of best practice recommendations

Three publications (5, 7-8) suggested best practices for data sharing in diet-gut microbiome research. The notable points of agreement included ensuring that sequencing data (5), raw datasets (7) and code relating to statistical tools used in the analysis (5, 8) are made publicly available.

Considerations and gaps

No specific considerations or gaps related to data sharing were discussed in any of the eight publications included in this review.

Table 2.4. Points of agreement and gaps in knowledge: Statistical analysis

Element		References	Points of agreement	Gaps
Diet transformation and normalization		2, 5, 7-8	Data should be transformed	- Standardized best practices for choosing the most appropriate transformation and normalization methods are needed - When log transformation is used the optimal pseudo count for zeros in the dataset is undetermined - Appropriate use of rarefaction and other normalization strategies remains controversial
Model	*general characteristics*	1-2, 5, 7-8	Models/tools designed specifically for microbiome analyses should be used	- While importance of utilizing gut microbiome specific models is recognized, there is no current consensus on the best statistical models for a given study design. - No consensus on most appropriate way to deal with zero inflation or compositionality in datasets.
	analyzing diet-microbiome interactions	1, 7-8	Both dietary and gut microbiome data are compositional and should be treated as such for analysis	Tools for treating dietary data as compositional and merging it with gut microbiome data for analysis are not well-developed.
Type I error		2, 5, 7	Correction for multiple comparisons should be applied and method reported	
Reporting taxonomy		5, 7	Reporting database(s) and version used is important	- Use of taxonomic assignment databases not standardized and can give different results - Taxonomy classification and reporting is inconsistent, and the level of taxonomy required for understanding effects of diet-microbiome interactions on health outcomes is undefined.
Diet and microbiota diversity measures		5, 7-8	*Diet:* None *Microbiota:* Diversity metrics should be assessed and reported in combination with other measures of microbial community characteristics	*Diet:* - Applying ecological metrics to diet may reveal dimensions of diet variation that previously haven't been explored. However, most appropriate ways to apply ecological metrics to diet unknown and multiple unanswered questions relating to implementing this approach and how to interpret results[1] *Microbiota:* -The essential diversity metrics to include in diet-microbiome studies have not been standardized
Interpreting and reporting results		5, 7-8	None	
Data sharing		5, 7-8	- Code relating to statistical tools used in the analysis should be made publicly available - Raw datasets should be made available to the public - Sequencing data should be made available in repositories	

Figure 2.5. Best practice recommendations for statistical analysis

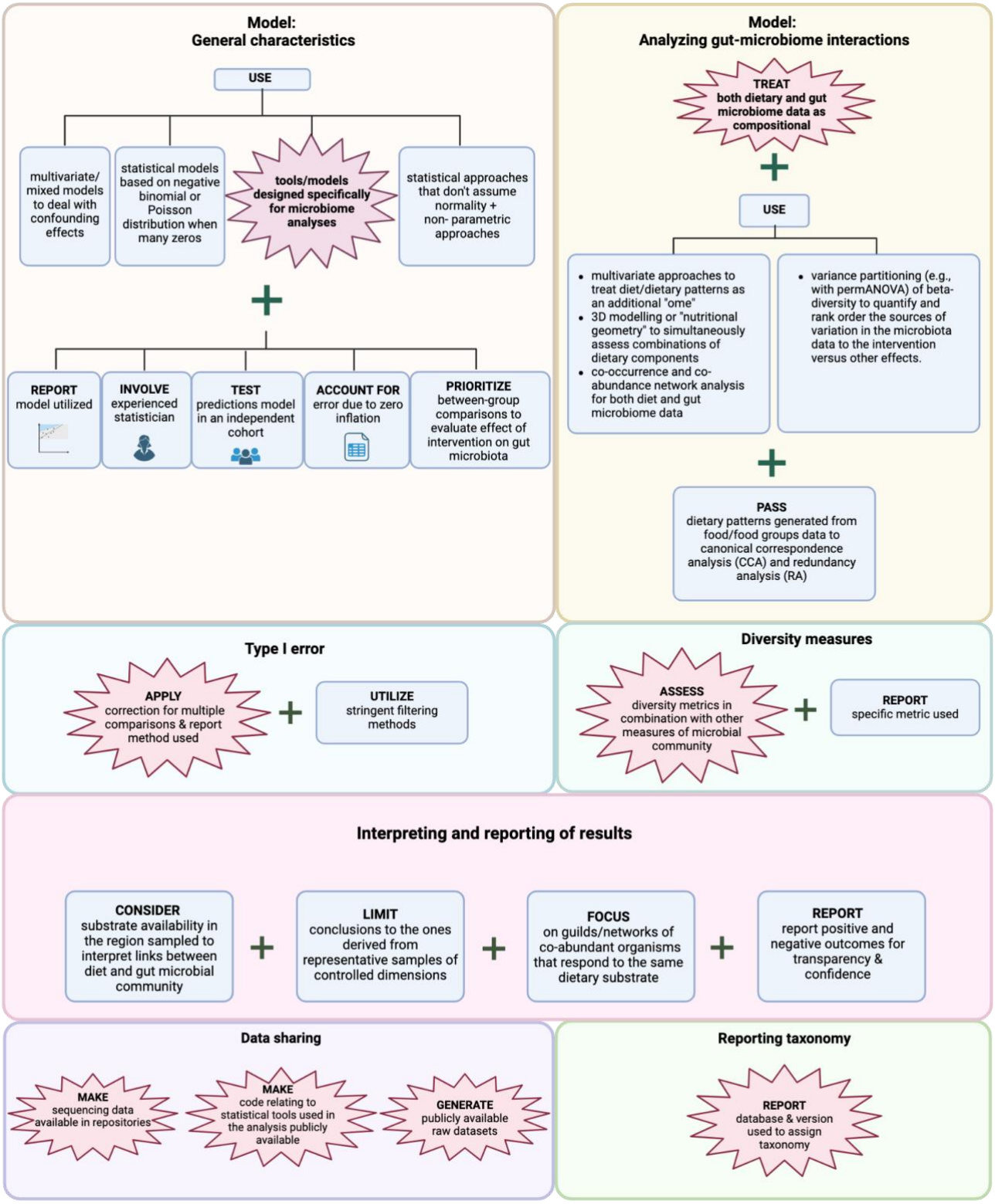


Figure legend

Figure content reflects points of agreement on best practices and additional considerations extracted during the umbrella review

Shapes



Explosion box: point of agreement



Rectangular box: best practice suggestions for which there were no points of agreement

Colors



Purple boxes: questions related to specific element/subelement



Blue boxes: best practice suggestions



Green boxes: names of sub-elements

Lines/arrows



Arrow: sub-element leads or contributes to another sub-element



Square bracket: multiple best practice recommendations apply to the same sub-element

4. Discussion

Several important decisions need to be made when conducting any human nutrition study. These decisions include development of research questions and aims, identifying the target population, intervention, comparator and outcomes (i.e. PICO criteria), selecting the appropriate study design within that context, and developing plans for administering interventions and measuring and analyzing study outcomes. Once the study is completed, methods and findings must be communicated transparently and in a way that facilitates reproducibility. Previous reports have discussed considerations for designing and communicating randomized controlled trials in human nutrition research (51, 60-61). In general, diet-gut microbiome studies should be designed, conducted and reported based on the same principles but must also consider design elements that are unique to the study of diet-gut microbiome interactions. In this umbrella review we consolidated current recommended best practices, considerations, and gaps in knowledge pertaining to the design, conduct and reporting of research aiming to determine effects of diet-gut microbiome interactions on human health related outcomes. Eight publications meeting all review criteria were identified and contributed data to the review. Notably, across all the elements and sub-elements of study design and research methodology for which data were extracted, no element or sub-element was discussed by all eight publications. Nonetheless, several points of agreement regarding best practices were identified. Below we discuss those best practices within the context of considerations and gaps related to the design and conduct of studies aiming to identify the role of diet-gut microbiome interactions in human health.

Study design and participant selection

Developing research questions and aims is integral to any human nutrition study. A challenge in diet-gut microbiome research highlighted by several of the included reviews is the

difficultly in demonstrating that a specific interaction between a dietary component and the gut microbiota impacts health. For example, health effects of certain dietary fibers may be attributable to direct effects of that fiber in the gastrointestinal tract rather than or in addition to any interaction with the gut microbiota. A diet-gut microbiota interaction could also impact human health through different pathways such as a microbiota-mediated biotransformation of a dietary component or through an impact of a dietary component on gut microbiota composition. Research questions and aims must therefore be developed with both the nature of the diet-microbiota interaction and mechanism of action on health outcomes in mind. If an aim is to convincingly demonstrate causal effects of a diet-gut microbiota interaction on human health, the inclusion of supporting mechanistic in vitro or animal studies needs to be considered.

Across the publications reviewed, there was general recognition that randomized-controlled trials are the gold standard for demonstrating causal effects of a nutrition intervention on human health (51) and are a best practice for determining effects of diet-gut microbiome interactions on health-related outcomes. However, there was not agreement on the most appropriate type of study design within the RCT framework. Crossover designs are appealing for diet-gut microbiome research because they can help control for the large inter-personal variability in the effects of diet on gut microbiota composition that may depend, in part, on baseline gut microbiota composition (62). Often, rapid baseline microbiota assessment, which could achieve the same goal in parallel arm studies by allowing subject stratification, is not feasible. Results of this review highlight a general lack of consensus on whether inter-individual variability is best addressed in the study design or elsewhere (e.g. statistically) and how to best account for that variability remains a gap. On the other hand, crossover designs may not be appropriate for outcomes that take a long time to develop and require a washout period, the

duration of which may be unknown for many gut microbiota-targeted nutrition interventions. Therefore, the suitability of a particular RCT design in diet-gut microbiome research must ultimately consider the impact of inter-individual variability along with the research question, study population, available resources and outcome variables.

An additional factor that both influences and is influenced by the study design is sample size. Utilizing the "largest sample size possible" was suggested as best practice in one publication (7) but doing so may not always be the most time or resource efficient approach to answer the research question. An identified challenge for sample size calculations in diet-gut microbiome studies is that the best way to measure microbiome responses to dietary interventions (e.g., taxa, metabolites or health markers) is not always clear and the gut microbiome features (if any) on which sample size calculations should be based are largely unknown. Several publications noted that changes in gut microbial community composition and functionality can be used as indicators of response in some cases (1, 8). However, these cases are limited given that the "healthy" gut microbiome remains undefined and widespread agreement on the compositional and/or functional changes having beneficial or detrimental effects on host health is lacking (63-64).To address this gap and to better inform effect sizes, sample size calculations and study designs in diet-gut microbiome research, further work on identifying gut microbiota biomarkers and related thresholds associated with health and disease risk is needed (63).

Defining gut microbiota biomarkers of health and disease would also help determine the optimal intervention durations for studies testing effects of microbiota-targeted nutrition interventions on host health. Papers included in this review noted that intervention durations required to demonstrate microbiota-mediated changes in host phenotype and to elicit long-term

or permanent changes in the gut microbiome are generally unknown. While 3-5 days may be sufficient duration for some dietary interventions to elicit changes in the gut microbiota (57), that duration is likely insufficient to meaningfully impact host heath, achieve gut microbiota stabilization or result in long-lasting impacts on the community. Identifying intervention durations required to sustain diet-mediated changes in the gut microbiome would help inform the designs of future diet-gut microbiome studies (65). To do so, existing and future RCTs should consider incorporating follow up assessments that allow for tracking the persistence of diet-mediated changes in the gut microbiome.

Additional aspects of study design considered in this review were participant selection and important participant metadata in diet-gut microbiome research. Across the papers reviewed, there was general agreement on several factors that should be considered when establishing participant inclusion and exclusion criteria (see Figure 2). Among those factors was use of medications and -biotics likely to influence the gut microbiome including antibiotics, prebiotics and probiotics. However, there was not agreement on appropriate pre-study washout periods from these products. Establishing optimal washout durations could help in standardization of diet-gut microbiome study designs. However, doing so can be challenging because the effects of many supplements and medications on the gut microbiome are unknown. Multiple papers included in this review also suggested considering habitual diet and baseline gut microbiome (3, 6, 8) in study inclusion/exclusion criteria but the specific guidance on how to best address these factors was lacking. Aside from -biotic and medication use, habitual diet and baseline gut microbiome, many other potential factors that could introduce variability into effects of a diet intervention on the gut microbiome were discussed. Accounting for all possible factors in any study design or data analysis plan is likely not feasible (i.e., too many covariates result in overfit

models). Therefore, at minimum, diet-gut microbiome studies should report the critical metadata identified in this umbrella review (see Table 2 and Figure 3) and consider measuring, controlling and/or reporting on other factors identified as potentially relevant covariates (see Supplemental Table 2).

Dietary data

A notable finding of this umbrella review was that many gaps related to the development and measurement of nutritional interventions in diet-gut microbiome research exist. A common theme among the included publications was that multiple different factors within the diet (e.g., nutritive and non-nutritive compounds, food matrix and structure, preparation method, etc.) could theoretically impact how a food, diet or dietary component interacts with the gut microbiota. Developing nutrition-based interventions and appropriate controls must therefore consider those factors and attempt to reduce any potential impact on study results by matching interventions or diets to the extent possible, excepting the dietary component under study. Measuring and reporting on all potential factors that may impact how an intervention interacts with the gut microbiota is likely unachievable or not practical for most studies involving whole diets and the level of detail needed to ensure reproducibility for some outcomes is unclear. However, developing a minimal reporting standard for various categories of nutrition-based interventions in diet-microbiome research would strengthen study designs and facilitate reproducibility. In the interim, intervention descriptions should be as detailed as possible and aim to provide the information listed in the Supplemental table 2.

Minimizing the effect of inter-individual variability in habitual dietary intake was also identified as important to consider in diet-gut microbiome studies but as lacking best practice recommendations. Two options for doing so are diet standardization and diet stabilization.

Standardizing intake by providing whole diets to study participants can help alleviate some of the concerns identified in the paragraph above but is expensive, not feasible over very long time periods, and could obscure intervention effects by affecting the gut microbiota. On the other hand, among the publications reviewed there was not clear consensus on how diet stabilization should be defined and best incorporated into a study protocol. If diet stabilization is to be widely adopted in gut-microbiome research, a standard definition of stabilization that is feasible to implement and verify is needed.

Whether a diet is stabilized or standardized, an accurate measurement of the potential microbiota-modulating compounds in the diet is necessary. Unfortunately, accurate assessment of dietary intake is difficult (66), an issue that is not unique to diet-gut microbiome research. However, a major gap within diet-gut microbiome research is that no single existing database appears to capture all information on dietary factors that may impact diet-microbiome interactions. This issue complicates comparisons across studies and can impact reproducibility. As stated by Johnson et al., a shared food ontology that can harmonize dietary data collected in different global regions and by different tools may be one approach to addressing this gap. Further adoption of the emerging ecologically-based statistical approaches discussed below may also help.

Measuring adherence to an intervention and any provided diet is crucial for interpreting results. One method for measuring adherence while overcoming inaccuracies in diet measurement is measuring nutrient intake biomarkers. Unfortunately, few established biomarkers for foods commonly used in diet-gut microbiome studies exist. For example, no established or reliable biomarkers of dietary fiber, the most common type of intervention used in diet-gut microbiome studies, are currently available. Future research aiming to refine existing biomarkers

of food intake and using novel methods of monitoring dietary adherence using cost-effective technologies will be essential for advancing understanding of diet-microbiome relationships.

Biological sample collection

Publications included in this review generally lacked agreement on concrete best practice recommendations for the biological sample collection and analysis in diet-gut microbiome studies, providing vague, non-specific and in some cases inconsistent recommendations. Most diet-gut microbiome studies rely on fecal sample collection for practical purposes. This can aid comparability across studies but, as noted by several of the included publications, may not fully capture diet-microbiome interactions most relevant to host health. The continued development of non-invasive techniques for measuring diet-microbiome interactions throughout the gastrointestinal tract will help to advance understanding of those interactions in human health and potentially help identify important interactions that may be missed by the current reliance on fecal samples.

While sample type and quantity were described as dependent on the research question and outcomes of interest, there was general agreement that more frequent sample collections are better (3, 5-8). Frequency, however, needs to be balanced with feasibility, which will depend on sample type (e.g., collection frequency will be limited in studies using biopsies). Ultimately, the optimal number of samples per time point and number of time points to include in diet-gut microbiome studies has not been established. In addition, transit time was recognized as an important piece of biological sample metadata to collect but the impact of transit time on the gut microbiome and diet-gut microbiome interactions is not yet fully characterized.

Sample transport and storage conditions must also be considered. Publications included in this review agreed that consistency and use of standardized procedures within a study is a best

practice. Unfortunately, standardizing procedures within a study does not necessarily facilitate between-study comparisons if different procedures are used in different studies. It is well recognized that results of studies relying on fecal samples can be influenced by how samples are collected, transported and stored (30). Likewise, differences sample are processing (e.g., DNA extraction method), sequencing protocols and bioinformatics pipelines can introduce variability that may impact study results, their interpretation and comparison of results across studies (67-75). These issues, combined with different collection and storage protocols, undoubtedly contribute to inconsistencies within the evidence base. In general, there is a strong need for agreement regarding best practices for sample collection, processing and analysis and readers are referred to several recent reviews on the topic (35-36, 38-39, 47, 54, 76-77). A critical and emerging area to address is how to best incorporate measures of absolute abundance into sequencing protocols that generate compositional data. Doing so will enable detection of changes in microbial cell-counts which are likely to differ from changes in relative abundance (76-77). Ultimately, the sample collection, processing and analysis procedures used in any diet-gut microbiome research study will to some extent be based on research questions and providing the resolution needed to address study hypotheses within available resources and logistical constraints. However, improving standardization of sample collection, processing and analysis procedures will facilitate advancement and reproducibility in the field.

Statistical analysis

Many current diet-gut microbiome studies rely on 16S rRNA amplicon sequencing or, increasingly, shotgun sequencing. Both methods generate sparse (i.e., many zeros) and compositional (i.e, proportions) datasets that violate the assumptions of parametric statistical models. This has led to the re-purposing of statistical approaches developed for other types of

sequencing data and the development of new statistical approaches to analyze microbiome datasets. As with sample collection and analysis, the choice of statistical approach, from how the data are normalized and transformed to the statistical model used, can impact study results (78-84). Despite promising and ongoing developments in statistical modeling for diet-gut microbiome research, significant gaps in knowledge exist. For example, while half of the reviewed publications (2, 5, 7-8) indicated that compositional microbiome data should be transformed in some way, the ideal transformation method is unclear. Further, the reviewed publications agreed on the importance of utilizing gut microbiome specific models for statistical analysis but there was no agreement on the best statistical models for a given study design or most appropriate ways to deal with zero inflation and compositionality. Standardizing analytical approaches for different types of microbiome data and study designs would facilitate between-study comparisons but is complicated by the frequent introduction of new, often improved modelling options. Presently, one potential option for increasing confidence in study results is to compare results generated from multiple different microbiome-specific statistical models and applying appropriate p-value adjustments to reduce false positive rates and control false discovery rates.

Like microbiome data, diet data can be considered compositional and treating it as such has potential to improve integration of these two data types. An emerging technique in the field is applying ecological approaches to dietary analysis to facilitate integration with compositional microbiome data (85-87). Further development of these methods and others, including development of statistical techniques for mediation analysis that can incorporate multiple highly multivariate model inputs, will likely facilitate new insights into diet-gut microbiome interactions and their impact on host health.

Finally, publications included in this review noted that the level of taxonomy required for understanding effects of diet-gut microbiome interactions on health outcomes is undefined and the microbiome taxonomies and diversity metrics reported are often inconsistent across studies. These issues underlie the importance of reporting databases and versions used in bioinformatics pipelines and making raw data publicly available (2-3, 7). Doing so enables high-quality meta-analyses and improves reproducibility. However, developing standardized reporting metrics for microbiome taxonomy and diversity would facilitate comparisons between studies while minimizing the need to re-analyze data. Given that most journals publish online supplemental tables and figures, results can include multiple diversity metrics and all taxa included in differential abundance analyses so that both null and positive findings are transparently reported.

Strengths/limitations

This umbrella review has several strengths and limitations that should be considered. Strengths include the use of standard methods to develop the review protocol (88) and pre-registration. The narrow focus on publications related to design of studies assessing diet and the gut microbiome allowed the review to capture recent discussions on best practices unique to diet-gut microbiome research. However, a limitation to that narrow focus is we did not extract data from publications focused solely on the design of microbiome or nutrition studies, respectively. Thus, the extracted data may not fully capture all relevant best practices and considerations for conducting microbiome or nutrition studies per se. An additional limitation is only one database was searched and relevant publications could have been missed. The search results, however, were supplemented with the manual screening of reference lists from included publications and other related reviews, as well as co-author input. This review only included publications that focused on adult and free-living populations and therefore may not capture unique aspects of

study design and research methodology for other populations. Finally, this review only included papers published in the past ten years. That criterion may have excluded relevant literature but likely also minimized the inclusion of obsolete recommendations and considerations which is important in this rapidly evolving field.

5. Conclusions

Accumulating evidence indicates that diet-gut microbiome-host interactions influence human health and disease. However, identifying direct causal links between diet-gut microbiome interactions and human health is challenging and many inconsistencies in the evidence base exist. This umbrella review identified many issues that researchers must consider when designing, implementing and reporting diet-gut microbiome studies and that best practice recommendations to guide decisions are lacking for many of those considerations.

A recurring theme of the included publications was that best practices will depend on the research question, specific aims, outcomes and feasibility of the study. While true, addressing several gaps would help facilitate the establishment of broadly applicable best practices for RCTs focused on diet-gut microbiome interactions in human health that are ultimately needed to improve reproducibility and advance the field. Several of those gaps are not unique to diet-microbiome research. These include issues that challenge nutrition science in general such as difficulty in accurately assessing dietary intake, an absence of validated intake biomarkers for many nutrients and diet components, and incomplete food and nutrient databases. Other gaps relevant across the field of gut microbiology are largely related to standardizing best practices for sample collection, processing, transport and storage, sequencing protocols and bioinformatics workflows, and optimal statistical approaches for dealing with sparse and compositional data. Also not unique to diet-gut microbiome research is overcoming inter-individual variability across

human gut microbiomes and a need to define a healthy microbiome, if possible. The latter would assist in identifying meaningful biomarkers, effects and effect sizes. Gaps more unique to the study of diet-gut microbiome interactions in human health include improving and harmonizing existing food and nutrient databases to account for currently underrepresented microbiota-modulating dietary components; determining the dietary components that are absolutely critical to match between nutrition interventions and controls; improving understanding of intervention durations required to elicit durable changes in the gut microbiome; defining appropriate washout periods from various microbiota-modulating factors (e.g., diet, medications, etc.); and further developing and refining statistical models for integrating diet and gut microbiome data.

Future research and expert discussions are needed to address those gaps and establish and evolve best practices in the field. In the interim, the best practices and considerations in this review, and the publications from which those data were extracted, can provide a guide for designing and conducting diet-gut microbiome studies. Research teams leading those efforts should strive to be interdisciplinary to ensure existing and new best practices can be adopted and implemented. Additionally, studies should be reported transparently following guidelines for reporting clinical trials (89) and microbiome studies (90). To the extent possible, that reporting should include making study data, and especially sequencing data, publicly available to facilitate re-analysis and high-quality meta-analysis as databases and methods continue to improve.

6. Supplemental Materials

Supplemental Table 2.1: Best Practices for study design and participant selection

Element		Best practice	Reference
Research questions and aims		Should be hypothesis driven and all aspects designed to answer a question regarding a specific diet-microbiome interaction.	7
		Should consider mechanisms, interventions, and outcomes.	6
		Clearly state questions/aims at the outset.	8
Trial design		Should be guided by the hypothesis.	7
		Trial design must consider dietary intervention, resources available, and responses being measured.	2
		Utilize randomized controlled trial design if possible.	2, 4, 6, 8
		Favor longitudinal studies with a crossover design over parallel designs.	3, 5
		Parallel group design may be more suitable for probiotic studies.	6
		Standardized diets should be implemented if possible.	2
		Combining human and animal models may be more informative than either approach alone.	4
		Include both microbiota- and host-specific assessments if aim is to demonstrate an intervention can confer health benefits to the host via interactions with the microbiota.	8
		Primary outcomes should be determined by the central research question. Metric should be dependent on the type of intervention and its intended effect (is likely to be based on biochemical and chemical traits rather than the microbiota).	8
Blinding		Blinding should be used when possible. Double-blinding (participants and researchers) is considered the gold standard. Triple blinding (participants, researchers and data analysts) may be best for probiotic clinical trials.	3, 8, 6 6
		Report if blinding was used and if successful.	5
Duration	*run-in*	≥3-5 days*	3
	intervention	Will depend on the primary outcome measure and, for probiotic studies, the specific strain utilized. 4-16 weeks common for probiotic studies.	6
		2-3 weeks so that gut microbiota can stabilize to intervention diet.	5
	washout	Determine based on follow-up assessments and ensure duration is sufficient to prevent carryover effects.	3, 6
		≥2 weeks	4, 6
		≥4 weeks	5
Effect size		Should be based on clinically relevant effects or standardized health recommendations where possible. Biological significance should be considered.	2, 5
Sample size calculations		Utilize largest sample size possible**	7
		400-500 for case-control and cross-sectional studies.	3

		Should be hypothesis-driven and may be based on biochemical, chemical, and physiologic traits rather than microbiota.	8
		Should consider intrinsic human gut microbiome variation.	5
Statistical analysis plan (SAP)		Statistical hypothesis and general features of primary variable analysis must be in place before submission for ethical approval. Detailed plan should be finalized before recruitment and sample collection.	7-8
		- Review and update after blind review of data and communicate to all relevant parties before blinding break. - If microbial-related variable is the primary/secondary endpoint, analysis methods cannot be changed after unblinding	8
Interindividual variability		Minimize impact by increasing sample size if appropriate and feasible. Otherwise, collect repeated samples to address temporal variability and reduce within-person noise.	3, 7
		Utilize participant baseline microbiome as control if feasible.	5
Inclusion criteria* **		Define age range of interest.	8
		Consider and/or match groups based on baseline dietary patterns and ability to maintain habitual diet if possible.	6, 8
		Sequence gut baseline microbiota to enroll predicted responders if feasible.	3, 8
		Case-control designs: maintain temporal, geographical, and demographic consistency in participant recruitment.	5
Exclusion criteria (factors to consider)	*demographics/ anthropometrics*	BMI, age, ethnicity	5
	consumption of -biotics	Probiotic use	5-6, 8
		Prebiotic use	6, 8
		Fermented foods (in some cases)	6
	washout for -biotics consumption	3–4 weeks	6
		4-6 weeks	5
	medication use	Antibiotics	5-6
		Antifungal/antiviral, other drugs that may affect gut microbiota (e.g. Metformin)	5
	washout for medication use	3-4 weeks	6
		3-6 months	5
	other	Comorbidities/pre-existing conditions (e.g. T2DM)	5-6
		Weight loss programs, special diets (e.g., vegan, paleo, gluten-free, etc.), plan to change exercise level/intensity, recent long-distance travel, baseline microbiota composition.	8
	demographics/ anthropometrics	Sex/gender and age	3, 5-6, 8
		Ethnicity	3, 5-7
		Race	6
		Cultural identification	5
		Anthropometric data	5, 7
		Health status	7-8
		Health history and diet-related GI symptoms	6
		Recent illness, allergies	3

Metadata to collect	*overall and GI health-related factors*	Primary disease and severity	7
		Comorbidities	5, 7
	supplement and medication use	Medication use	3, 6-8
		Supplement use/history	3, 6
		Probiotics	3, 7-8
		Prebiotics	3, 8
		Antibiotics	3, 7
	diet	Habitual diet	3, 5-6, 8
		Fiber intake	8
		Coffee consumption	4
		Alcohol consumption and water source	3
	lifestyle-related factors	Lifestyle factors	6-7
		Exercise/physical activity	3-4, 6, 8
		Stress and smoking	4
		Recent circadian disruptions	8
	geography/ environment	Geographic location/environment	5-6
		Urban/rural environment, birth location, immigration history	3
		Living structure, socioeconomic environment	5
	other	Recent or planned weight loss/gain	3
		Socio-environmental conditions (crowding, family composition and size), pets in the house	8
Inclusion of diverse population		When possible, be inclusive of all populations and utilize race/ethnicity as covariates in analysis.	6

Legend
Abbreviations: BMI, body mass index; T2DM, type 2 diabetes mellitus
Expert group opinion:
* Length of run-in will likely depend on study design and purpose of the run-in. A few days for run-in and washout periods may be appropriate for functional food/probiotic intervention studies where probiotic presence in the colon is the main outcome. For other studies this duration will likely be inadequate and for crossover studies specifically, washout should be at least the same length as intervention. Regardless of the selected duration, justification should be provided.
** Utilizing largest sample size may not be feasible due to associated cost. Sample size needed to answer the question of interest should be used. This can be determined via power analyses based on primary outcome variable.
*** Sex may be another important factor to consider in inclusion criteria.

Supplemental Table 2.2. Best Practices for dietary data

Element		Best practice	Reference
Intervention design and development	*diets*	Provide foods from metabolic kitchen, weigh all eaten and uneaten foods if possible. Otherwise, have subjects record everything eaten.	4
		Ensure participants maintain weight stability, unless study aim is related to weight loss.	5
		Control total amounts of dietary fiber, resistant starch, and fructans and other oligosaccharides.	4-5
	foods	Consider dietary components that may act as microbiota modulators (e.g., macronutrient composition, certain micronutrients, non-nutritive compounds, polyphenols, probiotics, etc.).	8
	supplements	Probiotics: - Ensure intervention is based on in vitro/preclinical and/or human studies and pharmacokinetics. Mechanism of action should align with primary outcome. - Preserve in a live and robust state for entire study duration.	6
		Ensure compliance with specific characterization requirements of known microbial modulators when using probiotics, prebiotic fibers, and polyphenols as intervention.	8

	dose	For probiotic interventions: - Determine based on previous work (e.g., clinical data) and gut survivability. - Measure viability at start and end of study, add midpoint measurement for longer-term studies.	6
	placebo/ control	Incorporate placebo or control whenever possible.	2-3, 6, 8
		For interventions of isolated dietary fibers use microcrystalline cellulose.	8
		Whenever possible ensure placebo/control is indistinguishable from true intervention (e.g., physical appearance, taste profile, size) and has minimal effect on gut microbiota composition and/or activity. - When not possible, present control as separate intervention and not true control.	2, 6, 8 2
		Ensure dietary intake/dietary pattern is equivalent among randomized groups.	5-6
Assessing adherence/ compliance	*biomarkers*	Measure relevant biomarkers when possible.	5-8
		Relevant biomarkers: - quantity of probiotic strains in fecal samples if intervention is probiotic - urine alkyl resorcinol for whole grains - urine/fecal ferulic acid or serum dihydroferulic acid for rye bran/whole grain wheat intake - polyphenol metabolites (details reported elsewhere)[91] - breath hydrogen	8 6
		No established/reliable biomarkers currently available for fiber. May be relevant to measure fecal SCFAs or pH for fermentable fiber.	4, 8
		Blood samples should be timed to match pharmacokinetics of metabolite production. - To capture potential effect of fermentable fiber on peak SCFAs concentrations blood samples can be timed for ~6 hours after consumption of intervention food.	5
	ensuring compliance	Should be assessed/monitored and methods reported.	5-6
		Collect uneaten intervention product and, if all meals are provided, uneaten foods/beverages to determine true intake.	3, 5
		Have participants provide all items consumed to researchers so that food composition can be measured directly if consuming their own foods.	3
		Collect multiple food records (when diets are not fully provided).	3, 5
		Clearly explain study meal plans and ask participants whether they are willing to consume all foods as part of the screening process.	3
		Define acceptable levels of compliance a priori and exclude non-compliant subjects.	3, 8
		Consider overall diet (e. g. global dietary parameters like diet quality and diet pattern) in addition to focusing on single nutrients or food categories if relevant for outcomes of interest.	1, 4, 7

Describing diet/intervention		Provide as much detail as possible. - For any intervention: trade names of the products used. - For controlled feeding studies: brand names and amounts of all foods consumed.	4
		- Divide foods into groups that have particular relevance to the gut microbiome based on fiber type or phytocompound composition known or suspected to affect the microbiome.	3
		-Quantify dietary constituents relevant to diet-microbe interactions if possible (may be limited to those that are readily quantifiable in human diets and have been measured comprehensively across the entire food supply).	7
		Probiotics and synbiotics: identify phenotypic and physiological characteristics (from *in vitro* data). Report safety, purity, potency, viability, and stability. Name strains in accord with International Code of Nomenclature. Strains should be deposited in an internationally recognized culture collection.	6, 8
		Parabiotics: Report method of inactivation. Postbiotics: Characterize metabolites and inanimate microbes.	6
		Isolated fibers/resistant starch and prebiotics: describe fiber source, CHO/sugar composition, purity, degree of polymerization, particle size, average molecular weight and its distribution range, glycosidic bonds, solubility, viscosity. Confirm resistance to digestion.	4, 8
		Isolated polyphenols: describe source, degree of polymerization, chemical structure, oligo-/polymer content and degree of polymerization.	8
Diet standardization		Select best dietary approach based on specific study characteristics (will vary). Ensure provided diets are consumed at the same time and location whenever possible.	3
		Provide diet the week prior to and throughout intervention, if possible. Otherwise, measure and report dietary intake.	6
		-Instruct participants to stabilize habitual diet rather than standardizing diet across participants. This can be done either by asking participants to maintain their normal diet (simple approach) or by assigning each subject to a specific constrained diet based on their own recent dietary intake data (more complex approach). -Account for variability in dietary intake at baseline.	3, 8
		Closely monitor foods/nutrients associated with outcome of interest and/or known to impact gut microbiome.	5
Methods	*emerging methods*	Utilize technology and biochemical markers of intake as available. Emerging methods includes mobile and wearable technologies.	3
	commonly used methods	Select method based on nature of study, participant population, feasibility, strengths/limitations related to research question. Review specific best practice guidelines reported elsewhere.[58]	6-8
		Combine multiple 24-hr recalls (AMPM method via ASA24) or 3–4-day food records (include 1 weekend day and use weighed records if possible) and FFQs.	3, 6
		Collect data in a way to allow for analysis of food choices and dietary patterns/quality as well as nutrient totals.	1, 3, 7
		Employ strategies to reduce under-reporting and/or other recall errors: provide clear instructions prior to completion of diet recording, utilize food models. Perform comprehensive cross-checking of collected data.	7

		- At a minimum, participants recording their intake should be trained using detailed examples showing the level of detail necessary to complete an accurate record (serving sizes, ingredient specificity, preparation methods, inclusion of commonly forgotten foods/additives). - Include detailed longitudinal analysis of food intake that accounts for the multivariate nature of dietary data and relationships between foods.	3
Database selection	*from nutrition-to microbiome-centered*	Report diet database utilized.	8
		- Avoid relying on existing nutrient composition variables as foods themselves are important when exploring diet-microbiome covariation. - Connect dietary data to food databases that contain extensive information about foods and food components and use machine learning approaches to compare with microbiome data.	3
Other considerations	*timing with microbiota analysis*	Collect 2-3 days of diet recalls/food records prior to sample collection period.	3
		Time dietary assessment so that it is immediately adjacent to biological sample collection.	7
	timing & location of meal/supplement consumption	Record and report.	3, 5-6
		Keep meal timing consistent between participants if possible (e.g., at study center).	3

Legend
Abbreviations: SCFA, short-chain fatty acids; CHO, carbohydrate; AMPM, automated multiple-pass; ASA24, automated self-administered 24-hr dietary assessment tool; FFQ, food frequency questionnaire.

Supplemental Table 2.3. Best Practices for sample analysis

Element		Best practice	Reference
Bio sample collection *	*types of samples*	Should be appropriate to research question (e.g., stool vs. mucosa, small vs. large intestine).	7-8
		Mucosal biopsies are the gold standard if aiming to explore crosstalk between mucosal associated microbes and/or the intestinal epithelium and tissue-specific immune responses.	8
		Samples for microbiota analysis should be aligned with other sample types (e.g., blood).	5, 7
		Direct measurement of metabolites in combination with characterizing microbial profile may be more informative for health outcomes than taxonomic identification alone.	7
	quantity	Will vary based on outcomes of interest.	5
		A few grams often enough for deep shotgun sequencing and metagenomics	3
	frequency	Repeated microbiome sampling may help account for intra-individual variability. Collect 3-7 fecal samples per time point if possible.	3
		Collection and homogenization of 2-3 days' worth of fecal samples is recommended.	5
		Ideal to include baseline, early, middle, and late points during the intervention.	7-8
		Pre and post time points are a recommended minimum if study includes dietary intervention.	6
	timing	Record and report time and date of collection.	3-5, 8
		Use time of collection as covariate in analyses.	3

		Align collection with diet and biological assessment periods.	3, 7
		Record amount of time between sample collection and processing/freezing**.	5
	collection	Ensure procedures are standardized.	3, 7
		When samples are not collected in the laboratory/research center, home sampling with immediate freezing is recommended.	3, 8
		Participants can collect whole bowel movement using a container and a hat, transfer small amounts to storage tubes.	3
	transport, storage, and processing	Ensure procedures are standardized and appropriate for the type of sample and outcome of interest.	3, 5
		For microbiota analysis: freeze fecal sample immediately after defecation and store at -80C.	3, 5, 8
		Minimize freeze-thaw cycles to ≤ 1.	5, 8
		Maintain cold chain or use preservatives.	7-8
		An alternative to snap freezing is using solutions that preserve nucleic acid material (e.g. ethanol, RNAlater, OMNIgene Gut kit, DNA/RNA shield (Zymo).	3, 5
		For whole stool collection: ensure quick transport** to research facility.	3
		Mix fecal samples well if homogenization is required.	5
Metadata to collect		Transit time should be measured and recorded.	3-4, 8
		Record bowel movement frequency.	3, 5
		Record Bristol stool type.	5
Sequencing		Should be based on research questions and align with resolution needed to address study hypotheses, resource availability, and features of microbiome (e.g., taxonomic, or functional resolution) being studied. Dietary intervention and response variables are especially important to consider.	2-3, 7-8
		- Non-template controls should be included during library prep to detect contaminant DNA. - Use mechanical cell lysis prior to DNA isolation.	5
		If large numbers of samples need to be processed in batches or after a different length of storage, use controls to account for batch effects, and consider batch effects during statistical analysis.	7
		Use of mock communities to identify potential sources of bias.	8
Relative vs absolute measures		Ensure consistency in methodology across participants and throughout the study.	7-8
		Measure and report both, especially if DNA yields vary between samples.	5, 7
		qPCR especially applicable to probiotic studies to measure fecal content of intervention strain(s).	6

Legend
Abbreviations: RNA, ribonucleic acid; DNA, deoxyribonucleic acid
Expert group opinion:
*If one wants to assess metabolites, transcripts, etc. the best practices for sample collection and storage may differ from those required for optimal microbiome sequencing
**Specific recommendations are provided elsewhere[11]

Supplemental Table 2.4. Best Practices for statistical analysis

Element	Best practice	Reference
Data transformatio	Proportional and non-normally distributed data should be transformed (e.g., centered-log ratio).	7
	Sequence count data often require normalization and/or rarefaction. Choice of method depends on data characteristics such as library size.	2

n and normalization		Rarefaction recommended for amplicon analysis (usually at least 10,000 reads/samples).	5
		Apply rarefaction or use read count as an offset in statistical models if sequencing across samples is uneven.	8
Model	*general characteristics*	Utilize models/tools designed specifically for microbiome analyses. Report model utilized.	5, 7
		Use multivariate and mixed models to deal with confounding effects. Involvement of experienced statistician is important.	7
		Statistical approaches that don't assume normality are essential and non-parametric approaches are needed. Non-parametric methods often better suited to microbiome taxonomy and beta diversity analyses.	1
		Between group comparisons should be prioritized to evaluate effects of an intervention on the microbiota.	8
		Prediction models: - Testing a model in an independent cohort allows for broader assessment of the model (vs. internal cross validation) in the broad population. - Which method to use depends on the nature and complexity of the data as well as the desired format of prediction.	2
		Need to account for the error due to zero inflation.	1
		When many zeros, statistical models based on negative binomial or Poisson distribution should be used for reliable identification of taxa that differ in abundance between groups.	8
	analyzing diet-microbiome interactions	- Both dietary and gut microbiome data are compositional. Consider adjusting for compositionality or only analyzing relative changes. - Adopting multivariate approaches for diet allows diet and dietary patterns to be treated as an additional "ome" for integration with other multi-omic data sets like microbiome sequencing data and fecal metabolomics. - Once dietary patterns have been generated from food or food-group level data, then they can be passed to canonical correspondence analysis (CCA) and redundancy analysis (RA) to explore if the constraint of microbiome beta diversity distances by diet explains any amount of the variation in microbiome composition. - Co-occurrence networks and co-abundance network analysis using both diet data at the food or food group level and microbiome features at the species or genera level with enough data density has the potential to reveal relations between dietary intake and specific members of the microbial community.	1
		Variance partitioning, e.g., with permutational ANOVA (permANOVA) of beta-diversity provides a useful measure to quantify and rank order the sources of variation in the microbiota data to the intervention versus other effects.	8
		3D modelling or "nutritional geometry" to simultaneously assess combinations of dietary components may prove useful in deciphering some of the complexity of interactions in diet-microbiome studies in humans.	7
Type I error		Apply correction for multiple comparisons (e.g., Bonferroni, Benjamini-Hochberg, etc.) and report method used.	2, 5, 7
		Utilize stringent filtering methods.	7
Reporting taxonomy		Report database and version used to assign taxonomy.	5

Diversity measures	Specific metric should be reported.	5
	Consider assessing diversity metrics in combination with other measures of microbial community (e.g. structural stability over time, distinct phases of microbiota assembly, Principal Coordinates Analysis (PCoA), etc.).	5, 7-8
Reporting of results	Limit conclusions to the ones derived from representative samples of controlled dimensions, and do not overinterpret results. Recognize biological relevance vs statistical significance. Variance explained in PCoA should be reported to determine biological relevance.	5
	Interpreting links between dietary intake and intestinal microbial community must consider substrate availability in the region sampled.	7
	Focusing on guilds or networks of co-abundant organisms that respond to the same dietary substrate may be informative.	7
	Generate publicly available raw datasets. This will provide scope for meta-analysis of data regardless of original analysis techniques and metrics used for taxonomic levels reported.	7
	Positive and negative outcomes should be reported for transparency and confidence.	8
Data sharing	Sequencing data should be made available in repositories such as the National Center for Biotechnology Information (NCBI), Sequence Read Archive (SRA), and GitLab.	5
	Code relating to statistical tools used in the analysis should be made publicly available where possible.	5, 8

Legend

Abbreviations: ANOVA, analysis of variance

1) Choi Y, Hoops SL, Thoma CJ, Johnson AJ. A guide to dietary pattern-microbiome data integration. J Nutr 2022; 152(5):1187-1199.

2) Hughes RL, Marco ML, Hughes JP, Keim NL, Kable ME. The role of the gut microbiome in predicting response to diet and the development of precision nutrition models – part I: Overview of current methods. Adv Nutr 2019; 10(6):953-978.

3) Johnson AJ, Zheng JJ, Kang JW, Saboe A, Knights D, Zivkovic AM. A guide to diet-microbiome study design. Front Nutr 2020; 7:79. doi: 10.3389/fnut.2020.00079

4) Klurfeld DM, Davis CD, Karp RW, Allen-Vercoe E, Chang EB, Chassaing B, Fahey GC Jr, Hamaker BR, Holscher HD, Lampe JW, Marette A, Martens E, O'Keefe SJ, Rose DJ, Saarela M, Schneeman BO, Slavin JL, Sonnenburg JL, Swanson KS, Wu GD, Lynch CJ. Considerations for best practices in studies of fiber or other dietary components and the intestinal microbiome. Am J Physiol Endocrinol Metab 2018; 315:E1087–E1097. http://doi.org/10.1152/ajpendo.00058.2018

5) Marques FZ, Jama HA, Tsyganov K, Gill PA. Guidelines for transparency on gut microbiome studies in essential and experimental hypertension. Hypertens 2019; 74:1279-1293. https://doi.org/10.1161/HYPERTENSIONAHA.119.13079

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7) Shanahan ER, McMaster JJ, Staudacher HM. Conducting research on diet-microbiome interactions: A review of current challenges, essential methodological principles, and recommendations for best practice in study design. J Hum Nut Diet 2021; 34(4):631-644. doi: 10.1111/jhn.12868

8) Swann JR, Rajilic-Stojanovic M, Salonen A, Sakwinska O, Gill C, Meynier A, Fanca-Berthon P, Schelkle B, Segata N, Shortt C, Tuohy K, Hasselwander O. Considerations for the design and conduct of human gut microbiota intervention studies relating to foods. Eur J Nutr 2020; 59(8):3347-3368. doi: 10.1007/s00394-020-0223

Supplemental table 2.5: JBI Critical Appraisal Checklist for Text and Opinion Papers

yes/no/unclear/not applicable (N/A)	Klurfeld	Johnson	Swann	Choi	Shanahan	Marques	Hughes	Mohr
Is the source of the opinion clearly identified?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Does the source of opinion have standing in the field of expertise?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Are the interests of the relevant population the central focus of the opinion?	N/A	N/A	N/A	N/A	N/A	Yes	Yes	Yes
Is the stated position the result of an analytical process, and is there logic in the opinion expressed?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Is there reference to the extant literature?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Is any incongruence with the literature/sources logically defended?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

7. References

Articles included in the present review:

1. Choi Y, Hoops SL, Thoma CJ, Johnson AJ. **A guide to dietary pattern-microbiome data integration**. J Nutr 2022; 152(5):1187-1199.
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8. Swann JR, Rajilic-Stojanovic M, Salonen A, Sakwinska O, Gill C, Meynier A, Fanca-Berthon P, Schelkle B, Segata N, Shortt C, Tuohy K, Hasselwander O. **Considerations for the design and conduct of human gut microbiota intervention studies relating to foods.** Eur J Nutr 2020; 59(8):3347-3368. doi: 10.1007/s00394-020-0223

Other references:

9. Rinninella E, Tohumcu E, Raoul P, Fiorani M, Cintoni M, Mele MC, Cammarota G, Gasbarrini A, Ianiro G. **The role of diet in shaping human gut microbiota.** Best Pract Res Clin Gastroenterol 2023, 62-63:101828. doi: 10.1016/j.bpg.2023.101828. Epub 2023 Feb 19. PMID: 37094913.
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Chapter 3

Effects of walnuts on biomarkers of inflammation, blood lipids, as well as gut microbiota, microbial- and walnut-derived metabolites and gut inflammation in at risk overweight/obese men and postmenopausal women

Abbreviations: ACVD, atherosclerotic cardiovascular disease, ANCOVA, analysis of covariance; BA, bile acid; BMI, body mass index; CA, cholic acid; CCL2, chemokine C-C motif ligand 2; CDCA, chenodeoxycholic acid; CI, confidence interval; CPN, calprotectin; CVD, cardiovascular disease; DCA, deoxycholic acid; DHC, dihydrocoumarin; DHF, dihydroflavone; EA, ellagic acid; EDTA, ethylenediamine tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; ESHA, Elizabeth Stewart Hands and Associates; ESI, electrospray ionization; FXR, farnesoid X receptor; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; GDCA, glycodeoxycholic acid; GI, gastrointestinal; GLCA, glycolithocholic acid; GPCR-1, G-coupled protein receptor 1; GUDCA, glycoursodeoxycholic acid; IBD, inflammatory bowel disease; ICAM-1, intercellular adhesion molecule 1; IL-6, interleukin 6; IL-8, interleukin 8; IsoUroAG, isourolithin A glucuronide; LCA, lithocholic acid; LDL-C, low-density lipoprotein cholesterol; MD, Mediterranean diet; MeOH, methanol; NAFLD, non-alcoholic fatty liver disease; NGAL, lipocalin-2; OUT, operational taxonomic unit; oxLDL, oxidized low-density lipoprotein; PAI-1, plasminogen activator inhibitor 1; PVDF, polyvinylidene fluoride; RA, relative abundance; RT, room temperature; SCFA, short-chain fatty acid; SG, specific gravity; SQL, structured query language; TCA, taurocholic acid; TC, total cholesterol; TCDCA, taoruchenodeoxycholic acid; TDCA, taurodeoxycholic acid; TG, triglycerides; TLCA, taurolithocholic acid; TUDCA, tauroursodeoxycholic acid; UCD, university of California, Davis; UDCA, ursodeoxycholic acid; UM-A, urolithin metabotype A; UM-B, urolithin metabotype B; UM-0, urolithin metabotype 0; UPLC-MS/MS, ultra-performance liquid chromatography-mass spectrometry; Uro-A, urolithin A, Uro-B, urolithin B; Uro-C, urolithin C; UroAG, urolithin A glucuronide; UroBG, urolithin B glucuronide; US, United States; VCAM-1, vascular adhesion molecule 1.

Abstract

This study investigated the effects of daily walnut consumption on established cardiovascular health biomarkers as well as gut inflammation and gut microbiome. Thirty generally healthy overweight and obese adults (BMI >25 kg/m^2) between the ages of 45 and 75 years old with borderline elevated low-density lipoprotein cholesterol (LDL-C; 130-189 mg/dL) participated in this randomized crossover study. Participants were initially randomized to consume 56 g of walnuts or follow a no-nut diet for 3 weeks. A 1-week run-in and a 3-week washout period was incorporated into the study design, after which they consumed the alternate diet intervention. A significant decrease in total cholesterol (TC) and LDL-C levels was observed after walnut consumption compared to the no walnut controls ($p < 0.01$). No differences in blood inflammatory markers or oxidized low density lipoprotein (oxLDL) were detected but there was a significant decrease in fecal levels of gut inflammatory markers, lipocalin-2 (NGAL and) and calprotectin (CPN), after walnut consumption compared to the no-nut diet ($p < 0.05$). Walnuts also increased relative abundance of *Roseburia,* while decreasing *Bifidobacterium* and *Alistipes* genera ($p < 0.05$) in fecal samples. Fecal levels of LCA secondary bile acid were also lower in the walnut group compared to controls ($p < 0.05$). Participants exhibited a differential capacity to produce various urolithin metabolites, which allowed for clustering according to the previously reported metabotypes, with 70% of participants belonging to UM-A, 13% belonging to UM-B and 10% belonging to UM-0 metabotypes. Based on findings that walnut consumption modulates the gut microbiome and the observed correlation analyses suggests that urolithin metabolites may be contributing either directly or indirectly to favorable cardiovascular and gut health effects exerted by walnut consumption, as they were correlated with changes in blood

lipids and levels of fecal primary/secondary bile acids. The study was registered at www.clinicaltrials.gov as NCT05321277.

Keywords: tree nuts, microbiome, inflammation, bile acids, urolithins, metabotypes

1. Introduction

Heart disease remains the leading cause of death in the United States (US). Approximately 655,000 Americans die from it each year averaging one death every 36 seconds [1]. Both genetic predisposition and environmental factors contribute to pathogenesis of cardiovascular disease (CVD), with the environmental component partially mediated by diet and gut microbiota composition/functionality [2-3].

Walnuts are a big part of the heart-healthy Mediterranean diet (MD), and daily consumption may be beneficial for prevention of coronary heart disease [4]. The modulation of blood lipid levels through dietary interventions focused on consumption of walnuts and healthy fats has been well established [5-11]. Evidence of modulation by walnuts of vascular function, blood pressure and chronic inflammation is limited or inconsistent [6-10, 12-14].

Plasminogen activator inhibitor 1 (PAI-1) is a major inhibitor of fibrinolytic system and is tightly linked to inflammatory processes in the body. Elevated levels of PAI-1 may create a prothrombotic state and contribute to development of CVD [15]. Colonic fibroblasts, endothelial cells, cardiomyocytes, adipose tissue and hepatocytes are some of the cells producing PAI-1. PAI-1 levels in the colon have been shown to be promising markers of intestinal inflammation and neoplasia, while circulating PAI-1 levels are positively associated with hypertension, adipose, age and overweight/obesity [16]. Recent evidence demonstrates that urolithin metabolites produced as a result of interaction between walnut polyphenolic compounds and gut microbiota may have the potential to inhibit PAI-1 release by colonic fibroblasts *in vitro* [17]. It

has also been established that urolithins after being absorbed in the gastrointestinal (GI) tract appear in circulation where they persist and interact with peripheral tissues for up to 48 hrs [18-20]. This allows them to affect inflammatory processes in the periphery [21] and inhibit PAI-1 release from endothelial cells [22]. To our knowledge, the effect of walnut consumption on circulating PAI-1 levels in human subjects has not been investigated yet.

Intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) also play an integral role in development of inflammation in the body. These molecules facilitate attachment and trans endothelial migration of leukocytes, which may lead to formation of atherosclerotic plaques. Moreover, circulating forms of these molecules have been shown to be elevated in various inflammatory conditions [23]. Evidence demonstrates that walnut consumption has the potential to decrease ICAM-1 and VCAM-1 levels in the circulation and low doses of walnut-derived metabolite, urolithin A (UroA), decreased ICAM-1 in several recent *in vitro* trials [21, 24-25].

Dietary walnut intake has also been demonstrated to contribute to changes in abundance of fecal microbiota and has been associated with favorable levels of short-chain fatty acids (SCFA) and secondary bile acids (BA) in feces [5, 26-30]. These microbial-derived metabolites can act as signaling molecules influencing lipid metabolism, immune responses and cardiometabolic disease risk through multiple mechanisms [31-33]. Changes in several physical and chemical properties of feces including pH, consistency and moisture, may be indicative of gut microbiota changes. Stool consistency, for instance, has been proposed to be a reliable indicator of changes in gut microbiota composition, richness, bacterial growth rates, and transit time [34-35]. In addition, a recent human trial demonstrated that stool moisture and pH after almond consumption were correlated with changes in gut microbiota composition and SCFA

production [36]. To our knowledge, the present study is the first human trial examining how these fecal characteristics are affected by daily walnut consumption.

Walnuts may also contribute to gut barrier integrity and affect gut inflammatory processes, which are some of the additional factors potentially linking gut microbiota and cardiometabolic health. Impaired gut barrier function and bowel wall edema have been reported in patients with heart failure [37-38]. When gut barrier function is impaired microbial products can leak into the lumen and consequently into the circulation leading to development of a pro-inflammatory state. This pro-inflammatory state correlates with CVD symptom severity and poorer outcomes [39-41]. Fecal CPN is currently the most widely used biomarker of gut inflammation [42]. It is the major protein in monocytes and macrophages that directly correlates with quantification of neutrophilic infiltrate in the gut mucosa [43]. NGAL is a relatively novel marker of intestinal inflammation that represents a broader cellular distribution compared to CPN [44]. This unique feature allows NGAL to serve as a complementary biomarker that may be indicative of epithelial cell disruption and/or damage as well as a marker of low-grade gut inflammation [43-45]. Walnut consumption has shown the potential to decrease intestinal inflammation in mice, likely due to urolithin metabolite production by the gut microbiota [46]. Urolithins have demonstrated anti-inflammatory effects in the gut *in vivo* and *in vitro* [47-48]. Effects of daily walnut consumption on fecal NGAL and CPN levels in humans has not been investigated yet.

CVD is a complex multi-factorial disease. Its pathophysiology arises from perturbations in multiple systems, including the gut microbiome. Taking into consideration the tight interrelations between host and gut microbes, investigating just one of these systems to understand the mechanisms through which dietary interventions may be affecting pathogenesis

of CVD is insufficient. Analysis of host and microbial metabolites along with the established CVD biomarkers is necessary for a complete understanding of how walnuts and other foods are exerting their cardioprotective effects. Therefore, the objectives of this study were to investigate the effects of daily walnut consumption on established CVD biomarkers (e.g. blood lipids), gut inflammation, gut microbiome and walnut- as well as microbial-derived metabolites (e.g. urolithins, SCFAs). This will help us contribute to generation of novel and testable hypotheses for further exploration.

2. Methods and materials

2.1 Study participants

Overweight and obese men and postmenopausal women between 45-75 years of age, BMI of ≥25.0 kg/m^2 and borderline elevated LDL-C levels (130-189 mg/dL) were recruited from the Davis – Sacramento metropolitan area. This study was approved by the University of California, Davis (UCD) Institutional Review Board and written informed consent of all participants was obtained prior to enrollment. The study was registered at www.clinicaltrials.gov as NCT05321277. Interested participants were screened to determine eligibility. Exclusion criteria included allergy to walnuts or other nuts, eating more than 25 grams of fiber per day, taking medications/supplements known to affect metabolism or gut microbiota composition, documented chronic diseases (diabetes, renal or liver disease, metabolic syndrome, active cancer, etc.). Individuals who smoked, followed extreme dietary or exercise patterns, were taking exogenous hormones and experiencing recent weight fluctuations were also excluded. As shown in Figure 2.1, thirty-two participants were enrolled in the study and randomized. One participant withdrew due to health reasons and another due to scheduling conflicts. The remaining thirty participants completed the study.

2.2 Study design

The study was powered with the focus on LDL-C changes in response to walnut consumption. To detect mean differences in LDL-C of 9 mg/dL with $\alpha = 0.05$ and 90% power, we required 25 subjects. Estimates were based on research by Holscher et al. in a similar population [30] and were consistent with several other crossover trials involving dietary walnut intervention [49-50]. The Holscher et al. study was a controlled full feeding trial, whereas we proposed a free-living study with subjects consuming walnuts provided by investigators. Therefore, there was expected to be slightly more variability inherent in the results. Therefore, we enrolled 30 subjects to account for potential attrition (20%) and incomplete participant data (dietary non-compliance, inability to produce all stool samples as required).

The study design was a randomized, controlled crossover trial spanning 10 weeks. The layout consisted of 1-week run-in period followed by 3 weeks of the first intervention, 3 weeks of washout period, and 3 weeks of the alternate intervention. Participants were asked to avoid all nuts and nut-derived products, fermented foods, pomegranates and berries and non-essential dietary supplements known to affect gut microbiota throughout the whole duration of the study. They were also asked to continue their normal diets as much as possible, while limiting high-polyphenol and fiber-containing foods and beverages (e.g. most fruits and vegetables, beans/peas/lentils/legumes, seeds, whole grains, cocoa and dark chocolate, tea/coffee, wine/beer/brandy/rum/whiskey/sake). Intervention food, walnuts, were prepackaged in snack-sized packets containing 28 g of shelled raw English walnuts each (donated by the California Walnut Board). Participants were asked to consume 2 packets per day for 3 weeks when on the walnut intervention and not to cook or bake with walnuts. This provided 360 kcals, 36 g of total fat (3 g saturated, 26 g polyunsaturated, 5 g monounsaturated), 4g of fiber and 8g of protein.

Participants came in for their study visits at the beginning and end of each of their intervention periods. They were asked to fast, avoid alcohol consumption and engaging in vigorous physical activity within the day before and the morning of their study visit. At each study visit, anthropometric measurements (weight, waist and hip circumference, blood pressure) and a fasting blood sample were collected. A lithium heparin tube was sent to UCD Health System (UCDHS) Pathology Laboratory for lipid panel analysis, while an additional sample was collected into EDTA tubes, aliquoted into microcentrifuge tubes and stored at -80°C for additional analyses. Participants also brought a first void urine sample (collected on the morning of the study visit) and a fecal sample (collected 24-48 hrs within the study visit as described below). A total of 4 food record clusters were collected throughout the study. Each record included 3 days out of the week that led up to the participant study visit (2 weekdays and 1 weekend day, with one of these being the day before the study visit). Food records were analyzed by Food Processor (SQL Version 10.2, ESHA Research, Salem, OR). Food records were used to estimate macronutrient/micronutrient intake, as well as assess compliance with the study diet. Compliance was also measured by the number of full and empty walnut packets returned.

2.3 Biological sample collection and analyses

Blood

At each study visit, 21 ml of blood was collected (3 ml into a lithium heparin tube and 18 ml into EDTA tubes) and centrifuged at 1,000g for 15 mins at 4°C. Lithium heparin tube was sent to HS Pathology Laboratory for lipid panel analysis. Plasma separated from the EDTA tubes was divided in smaller aliquots and stored at -80°C for future analyses.

Feces

Participants were given a fecal collection kit prior to each of their visits. The kit consisted of a fecal collection bowl with a hat, 4 fecal collection tubes with scoopers attached to their lids, nitrile gloves, Ziploc bags, a cooler, ice packs and a freezer-friendly container. The fecal sample had to be collected within 24-48 hrs of the study visit. Participants produced the sample into the collection bowl and scooped out appropriate amounts into each collection tube. They were instructed to scoop from all parts of the sample (beginning, middle and end) to obtain a representative subsample. They were instructed to immediately freeze the sample in the home freezer and transport the sample to the research facility as soon as possible using the provided cooler and ice packs. They were also asked to bring the remaining sample in the collection bowl for researchers to assess stool consistency based on the Bristol stool scale. Upon receiving the sample, it was stored at -80 until analysis. The date and time of collection were reported along with the Bristol stool scale scores.

Urine

Participants were provided with a urine collection cup and instructed to collect the first void urine on the day of each of their study visits. If needed to store, the urine sample was stored in the home fridge and then transported to the research facility in the provided cooler with ice packs. Once received, the urine was aliquoted into microcentrifuge tubes and stored at -80 for further analysis. Date and time of collection were recorded.

2.4 Established markers of cardiometabolic health

Blood lipid and lipoprotein levels were analyzed at the UCDHS Pathology laboratory. Plasma biomarkers of endothelial activation including VCAM-1 and soluble intercellular adhesion molecule 1 (sICAM-1), Interleukin (IL)-6 cytokine, and vascular reactivity molecule plasminogen activator inhibitor-1 (PAI-1) levels were analyzed by ELISA (R&D System,

Minneapolis, MN). Assays were performed in duplicate with all samples from the same subject run on the same plate. oxLDL was measured in plasma using a commercially available sandwich ELISA kit (catalog #10-1143-01, Mercodia, Uppsala, Sweden).

2.5 Physical and chemical characteristics of feces

Stool pH

Three grams of stool sample were weighed on an electronic scale. Six milliliters of distilled water was added to each sample. Samples were then vortexed for 30 secs and centrifuged at 3000 xg, RT for 15 min. pH meter electrode was then inserted into the separated liquid and the pH reading was recorded. All pH measurements were taken in duplicates at 25C.

Stool consistency

Stool consistency was assessed according to the Bristol stool scale. Participants were asked to score each of their samples. Ideally, participants were instructed to bring the whole bowel movement with them for the researchers to double-check the recorded score. However, because participants had already scooped out some of the sample and potentially had stored it in the home freezer, it was challenging for researchers to identify what the original sample consistency was. In addition, most participants expressed unwillingness to provide the whole bowel movement due to the feelings of aversion.

Stool moisture

Empty crucibles were baked in an oven at 105C overnight. One gram (+/-0.0005g) of sample was then weighed into the dry crucible and baked at 105C overnight again. The crucibles with the baked sample were then weighed the next day in 15 min increments until the constant weight was achieved. All samples were tested in duplicates. Percent fecal moisture was calculated using the following formula:

$$\textit{Percent moisture} = \frac{\text{(Wt of wet spl) - (Wt of last bake, spl only)}}{\text{(Wt of wet spl)}} \times 100$$

2.6 Ellagitannin metabolite analysis in plasma and urine

(method and sample analyses provided by collaborator, Dr. John Newman)

Chemical reagents

Urolithins A, B, C and D, along with the glucuronide conjugates of urolithins A and B, as well as ellagic acid, were quantified against authentic standards by ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) in the presence of internal standards after protein precipitation. All solvents were of LC/MS grade from Fisher Scientific (Santa Clara, CA).

Plasma sample preparation

Plasma (200µL) was enriched with 5µL of 4µM dihydroxycoumarin (DHC) and 4-nitrophenyl-glucuronide (4-NP-Gluc) in methanol (MeOH) used as analytical surrogates. Protein precipitation was achieved by mixing with 600µL of acetonitrile with 2% formic acid solution which was vortexed for one min, sonicated for 10 min, and chilled at -20°C for 10 min. Samples were then centrifuged for 5 min at 4°C at 14000g, and supernatants were transferred to 2mL glass vials enriched with 10µL of 20% glycerol keeper in MeOH, and solvent was removed by centrifugal vacuum evaporation using a GeneVac EZ-2 (SP scientific, Warminster, PA) with lamp off and aqueous setting for 1.5h. The samples were resuspended with 100µL of 0.2µM 5,7-dihydroxyflavone (DHF) in MeOH, mixed and filtered through 0.2µm PVDF 96-well filter plates (Agilent Technologies; Santa Clara, CA), for 5 min at 4°C and 1000g. Samples were stored for<3d at -20°C until analysis.

Urine sample preparation

Prior to processing, urine sample specific gravity was determined using an RHD-B501 benchtop digital clinical refractometer (LAXCO Inc., Mill Creek, WA). Samples with specific gravity (SG) >1.015 were diluted down this level with H_2O. Prepared samples (200µL) were then vortexed for 1 min and centrifuged for 10 min at 4°C at 14000g. The samples were filtered using 0.2µm PVDF 96-well filter plate (Agilent Technologies), centrifuged for 5 min at 4°C and 1000g. Filtratesub-aliquots of 20µL were then diluted with 70µL of H_2O with 0.1% formic acid and enriched with 5µL of MeOH containing 4µM DHC and 4-NP-Gluc and 5µL of 4µM DHF in MeOH. Samples were stored for<3d at -20°C until analysis.

Urolithin analysis

Targeted analytes were separated using a 3 x 100 mm, 2.7µm Poroshell 120 EC-C18 (Agilent, Santa Clara, CA) and detected by negative mode electrospray ionization on a 4000 QTRAP (Sciex, Redwood City, CA) operated in multi-reaction monitoring mode. Urolithin glucuronides were purchased from Toronto Research chemicals (Toronto, Canada), ellagic acid was purchased from TCI (Portland, OR) and all other urolithins and internal standards were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Iso-urolithin A-glucuronide was identified by its relative retention time to the other analytical targets previously reported [51] and quantified using the response factor of urolithin A glucuronide.

Data quality

Data quality was assessed by evaluating surrogate recoveries and pooled sample replicate precision for metabolites with concentrations >10x the limit of detection. In plasma, surrogate recoveries ranged from 81.2 ± 51% for DHC and 66.1 ± 32% 4-NP-Gluc. Pooled sample replicate (n =6) precision was <30% for all detected analytes above 1nM. In urine, surrogate recoveries ranged from 75 ± 23% for DHC and 90 ± 25% 4-NP-Gluc and replicate precision was

<30% for analytes above 200nM, with the exception of isoUrolithin A Glucuronide, which was 32%. As 24-hr collection was not performed, creatinine was measured for normalizing concentration of urine samples.

2.7 Fecal bile acid analysis

(method and sample analyses by Metabolon)

Lyophilized fecal samples were analyzed for 15 bile acids by LC-MS/MS according to Metabolon Method TAM178. Solutions and calibration standards were prepared according to method TAM178.

Method summary

Calibration samples were prepared at eight different concentration levels by spiking an acidified methanol solution with corresponding calibration spiking solutions. Calibration samples, study samples, and quality control samples were spiked with a solution of labeled internal standards and subjected to protein precipitation with an organic solvent (acidified methanol). Following centrifugation, an aliquot of the organic supernatant was evaporated to dryness in a gentle stream of nitrogen. The dried extracts were reconstituted and injected onto an Agilent 1290 Infinity / SCIEX QTRAP 6500 or 6500+ LC-MS/MS system equipped with a C18 reverse phase UHPLC column. The mass spectrometer was operated in negative mode using electrospray ionization (ESI). The peak area of each bile acid parent (pseudo-MRM mode) or product ion was measured against the peak area of the respective internal standard parent (pseudo-MRM mode) or product ion. Quantitation was performed using a weighted linear least squares regression analysis generated from fortified calibration standards prepared immediately prior to each run. LC-MS/MS raw data was collected using SCIEX software Analyst 1.7.3 and

processed using SCIEX OS-MQ software v3.1.6. Data reduction was performed using Microsoft Excel for Microsoft 365 MSO.

Sample analysis

Four levels of QC were prepared. Two of the levels (QC Low and QC High) were prepared in a surrogate matrix by spiking with stock solutions to obtain the appropriate concentrations for each level. The other two levels (QC Medium and QC ALOQ) were prepared in human feces either at endogenous levels or by spiking with stock solutions to obtain the appropriate concentrations for each level. Sample analysis was carried out in a 96-well plate format containing two calibration curves and eight QC samples per plate to monitor assay performance. Two batches were prepared and analyzed undiluted and at a 100-fold dilution. Accuracy was evaluated using the corresponding QC replicates in the sample runs. The QCs met acceptance criteria at all levels for all analytes. The measured concentration was compared to the LLOQ and ULOQ to determine if the sample is BLOQ or ALOQ. The measured concentration was normalized upon the mass of sample used for analysis (Equation 1 below) and reported as a normalized result:

Equation1

Normalized Conc. ($ngmg$) = Measured Conc. (ng/mg) × 10 mg/Sample Mass (mg)

2.8 Fecal short-chain fatty acids analysis

(method and sample analyses by Metabolon)

Fecal samples were analyzed for eight short-chain fatty acids: acetic acid (C2), propionic acid (C3), isobutyric acid (C4), butyric acid (C4), 2-methyl-butyric acid (C5), isovaleric acid (C5), valeric acid (C5) and caproic acid (hexanoic acid, C6) by LC-MS/MS (Metabolon Method

TAM135: “LC-MS/MS Method for the Quantitation of Short Chain Fatty Acid (C2 to C6) in Human Feces”).

Samples were spiked with stable labelled internal standards, homogenized, and subjected to protein precipitation with an organic solvent. After centrifugation, an aliquot of the supernatant was derivatized and injected onto an Agilent 1290/AB Sciex QTrap 6500 LC MS/MS system equipped with a C18 reversed phase UHPLC column. The mass spectrometer is operated in negative mode using ESI. The peak area of the individual analyte product ions was measured against the peak area of the product ions of the corresponding internal standards. Quantitation was performed using a weighted linear least squares regression analysis generated from fortified calibration standards prepared concurrently with study samples.
LC-MS/MS raw data was collected using SCIEX software Analyst 1.7.3 and processed using SCIEX OS-MQ software v3.1.6. Data reduction is performed using Microsoft Excel for Microsoft 365 MSO.

Sample analysis

The endogenous QC was prepared from a single lot of human stool. All analytes were at the endogenous level except hexanoic acid, which was spiked due to low levels in this lot. The other three levels of QC (Low, Medium, and High) were prepared by spiking phosphate buffered saline (PBS) with all analytes to achieve the appropriate concentrations for each level.
Sample analysis was carried out in a 96-well plate format containing two calibration curves and eight QC samples per plate to monitor assay performance. Two plates were prepared and analyzed. Accuracy was evaluated using the corresponding QC replicates in the sample runs. The QCs met acceptance criteria at all levels for all analytes. The quantitation limits (LLOQ and ULOQ) were based on a target weight of 100 mg. The result used for statistical analysis was a

corrected concentration based on the actual weight of the sample. This was calculated by Equation 1 below:

Corrected Conc. ($\mu g g$) = Measured Conc. ($\mu g / g$) × 100 mg/Sample Mass (mg)

2.9 Microbial 16S analysis

Gut microbiota analysis was performed by 16S rDNA sequencing (targeting the V3-V4 hypervariable regions) on the Element Aviti (Host Microbe Systems Biology Core, services, UC Davis, Davis, CA) after extracting bacterial DNA from fecal samples. Taxonomy was assigned using Silva 2022 version 138 through QUIIME 2. Jaccard and Bray Curtis diversity analyses were also performed using QUIIME 2.

2.10 Markers of gut inflammation

Both fecal NGAL and CPN were measured via commercially available ELISA kits (R&D Systems, Minneapolis, MN and Immunodiagnostik (IDK) Inc, respectively).

2.11 Statistical analyses

All data were checked for normality via Shapiro-Wilk normality tests and QQ-plots. Data that were not normally distributed were then log transformed. Baseline levels and difference from baseline was assessed via paired t-test. Differences between intervention assignment at endpoint were assessed via ANCOVA with baseline levels used as covariates. 16S bacterial abundancy data were analyzed with the use of mixed-effects ANOVA with treatment as the mixed effect and participant and period as the random effects. Bivariate correlations were conducted to assess the relations between variables. Pearson's correlations were used for normally distributed variables and Spearman correlation for nonnormally distributed variables. Bonferroni false discovery rate was used to adjust for multiple comparisons. Statistical significance was set at $p = 0.05$. Statistical analysis was performed using R statistical software.

3. Results

3.1 Baseline clinical characteristics

Baseline characteristics of the participants are listed in Table 3.1. At baseline, the mean BMI was 30 ± 5.7 kg/m^2, age mean was 59 ± 7.1 years old and mean LDL-C was 152.5 ± 21.7 mg/dL. There were three male and twenty-seven female participants. Average fiber consumption was 11.9 ± 4.5 g.

3.2 Dietary intake and compliance

According to food records, participants maintained their habitual diet throughout the study and abstained from polyphenol-, fiber- and probiotic-rich foods as directed. Participants consumed diets similar in macro- and micronutrient composition during both intervention periods with higher consumption of omega-3 and omega-6 fats in the walnut group (Supplemental Table 3.1). The diets were reflective of a typical American diet (45% carbohydrates, 16% protein, and 36-42% fat) that was high in saturated fat, high in sodium, and low in fiber, potassium, and magnesium. Participant compliance was 97% as measured by counting the returned empty walnut packets and with confirmation from the food records.

3.3 Anthropometrics and blood lipids

There was an increase in weight (mean difference from baseline = 0.60 kg, 95% CI: 0.23, 0.96; p=0.004) after walnut consumption and a decrease in weight (mean difference from baseline = -0.44, 95% CI: -80, -0.07, p = 0.02) after no walnut intake, which resulted in a statistically significant difference at endpoint between the two interventions (p = 0.0002). BMI followed the same pattern with a median increase of 0.25 kg/m^2 after 3 weeks of walnut intake and a median decrease of -0.13 kg/m^2 after3 weeks of no walnut intake with a statistically significant difference at endpoint (p = 0.0002).

TC significantly decreased after 3 weeks of walnut consumption with a mean change of -6.86 mg/dL, while it increased after no walnut intervention with the mean change of 10.86 mg/dL (p-value between groups at endpoint = 0.004). There was also a decrease in LDL-C of -8.79 mg/dL after walnut compared to no walnut intake (p-value at endpoint = 0.002). There were no differences between interventions at endpoint for blood pressure, waist/hip circumference, triglycerides (TG) and high-density lipoprotein cholesterol (HDL-C). The summary of these results can be viewed in Table 3.2.

3.4 Blood inflammatory markers and oxLDL

There was a significant increase in PAI-1 from baseline in both interventions but no significant differences between interventions at endpoint. There was also a significant decrease from baseline in ICAM-1 after 3 weeks of no walnut ($p = 0.001$; Table 3.3), but no differences between interventions at endpoint. There were no within- or between-intervention differences in VCAM-1, interleukin-6 (IL-6) and oxLDL.

3.5 Gut inflammatory markers and physicochemical characteristics of feces

There was a significant decrease in NGAL after walnut consumption with a mean change of -0.58 mg/kg (95% CI: -0.96, -0.20, $p= 0.0004$) from baseline. NGAL levels were also significantly lower after walnut intake at endpoint compared to no walnut intake ($p = 0.01$). The same pattern was observed for CPN, where levels significantly decreased from baseline after walnut intake (mean change -23.75 ug/mg, 95% CI: -45.88, -1.62), which were significantly lower at endpoint ($p = 0.02$) compared to no walnut intake.

There were no within- or between-intervention differences in either fecal moisture or consistency. There was a significant decrease in fecal pH after walnut consumption compared to baseline with a mean change of -0.28 ± 0.39 ($p = 0.0006$), while during the no walnut intake

period fecal pH significantly increased (0.19 ± 0.42; p = 0.02). This led to a significantly lower fecal pH at endpoint (p = 0.00002) after walnut consumption (6.85 ± 0.46) compared to the no walnut intervention (7.32 ± 0.41). The summary of the results in this section are provided in Table 3.4.

3.6 Bile acids

For most bile acids, there were no significant intervention effects (Table 3.5). However, at endpoint, the secondary bile acid lithocholic bile acid (LCA) was significantly lower after walnut consumption (561.52 ng/g, 95% CI: 404.83, 1121.82) compared to no walnut intake (1226.54 ng/g (709.82, 1852.83) with p-value = 0.01. In addition, there was a trend at endpoint (p = 0.1) for lower levels of the secondary bile acid deoxycholic (DCA) after walnut consumption (1005.52 ng/g, 95% CI: 387.23, 1863.01) compared to no walnut (1656.03 ng/g, 95% CI: 781.54, 2780.22).

3.7 Short-chain fatty acids

Results for the short-chain fatty acid analyses can be viewed in Table 3.6. Isobutyric acid was lower after walnut consumption (97.84 ug/g, 95% CI: 64.03, 128.39) compared to no walnut intake (149.10 ug/g, 95% CI: 105.91, 192.32) at endpoint (p = 0.007). 2-Methylbutyric, isovaleric and valeric acids were also lower after walnut consumption compared to no walnut intervention (p = 0.04, 0.03 and 0.006, respectively).

3.8 16S microbiota analysis

The mean (SD) of bacterial operational taxonomic units (OTU) was 120,162 (35,666), with a maximum of 202,892 counts and a minimum of 39,453 counts/sample. At the phylum level the sequences were dominated by Firmicutes, Bacteroidetes, and Proteobacteria (53.4%, 37.9%, and 4.5%, respectively). One hundred and thirty-five families were identified in total and

seven of them (Bacteroidaceae, Lachnospiraceae, Ruminococcaceae, Prevotellaceae, Oscillospiraceae, Rikenellaceae, Sutterellaceae) made up >75% of all families. Three hundred and thirteen genera were identified and sixty-eight of them represented approximately 90% of sequences. The most abundant genera included *Blautia, Bacteroides, Faecalibacterium, Prevotella, Phascolactobacterium, Akkermansia, Alistipes, Fusicatenibacter, Agathobacter and Anaerostipes.* Archaeal OTUs were observed in nine out of 30 participants. The mean ± SD archaeal OTU was 195 ± 365 counts with a maximum of 1,870 and a minimum of two counts/sample. Eukaryota OTUs were present in three participants with mean ± SD of 17 ± 14 counts with a maximum of 41 and a minimum of 2 counts/sample. Walnut consumption did not affect either Archaea or Eukaryota abundancies.

There were no differences in bacterial phyla relative abundancies (RA) between the walnut and no walnut groups at endpoint. Changes in relative abundance of several bacterial genera were observed, however. For instance, there was a significant increase in *Roseburia* genus relative abundance after 3 weeks of walnut consumption compared to no walnut intake (least squares mean 1.27% and 0.92% , respectively; $p = 0.01$). There was a decrease in relative abundance of the genera *Alistipes* and *Bifidobacterium* after walnut consumption compared to no walnuts ($p = 0.049$ and 0.03, respectively). There were no within- or between-intervention differences between other bacterial genera.

Interestingly *Gordonibacter*, the genus that has shown the ability to metabolize ellagitannins into urolithin metabolites [91], was detected in five participants pre- (mean RA = 0.01%) and in eleven participants after (mean RA = 0.02%) walnut intervention. For those where *Gordonibacter* was detected prior to walnut intake, four of these individuals retained *Gordonibacter* after the walnut intervention and one did not. Otherwise, seven participants out of

the eleven with *Gordonibacter* at walnut endpoint did not have *Gordonibacter* at baseline. Two participants' gut microbiota contained *Gordonibacter* at the beginning and end of the no walnut intervention (mean RA at both timepoints = 0.01%). Both of these participants were also a part of the five and eleven (mentioned above) that had *Gordonibacter* before and after walnut intervention. One of them was randomized into the walnut and then no walnut group, while the other received interventions on the reverse order. Another bacterial genus with the potential to metabolize ellagitannins, *Egerthella* [91], was detected in 14 individuals after walnut consumption with a mean relative abundance of 0.04%. This genus was maintained in 12 individuals at the same relative abundance (0.04%) after the no walnut intake period.

Bacterial diversity results can be viewed in Figures 2.3 through 2.7. Diversity was assessed via Jaccard (weighted and unweighted) and Bray-Curtis methods. The Bray-Curtis dissimilarity is based on occurrence data (abundance), while the Jaccard distance is based on presence/absence data (does not include abundance information). The principal coordinates analysis (PCoA) plots presented are organized either by intervention and timepoint or by patient. No clear separations appear between the walnut and no walnut interventions at endpoint at any of the plots. It can also be seen that each participant tends to be closer to itself at different time points (e.g. pre- vs. post-walnut) than it is to other participants at the same timepoint (e.g. both at post-walnut timepoint). This is also supported by the lack of differences between groups in relative abundance of bacterial phyla and most of the bacterial genera. The presented PCoA plots were briefly examined by a trained gut microbiome biostatistician and lack of differences between interventions was confirmed, with further testing needed to draw definitive conclusions. Results are displayed in Table 3.7 and Figure 3.2.

3.9 Ellagitannin metabolites

As previously reported, three urolithin phenotypes were detected [20]. Based on observations in at least one visit, ~70% of the study population showed evidence of urolithin (UroA) formation (Phenotype A), 10% also showed urolithin B (UroB) formation (Phenotype B) and 20% showed no detectable urolithins in their plasma (Phenotype 0) (Figure 3.8). %UroB at Peak in plasma of PhenotypeA individuals was 0-7%, while it was 66-1947% in Phenotype B individuals. %Uro B at Peak in urine of Phenotype A individuals was 0-14% and 26-250% in Phenotype B individuals. Distribution of %B at Peak in all individuals is presented in Supplemental Table 3.2.

Analysis by phenotype shows strong phenotype dependent interactions between treatment and response that are matched between plasma and urine samples. These are graphically summarized in Figure 3.9. However, phenotype assignments based on the criteria of Phenotype B being indicated by the mere presence of UroB or isourolithin-A (IsoUroA) appears inconclusive (Supplemental Table 3.3).

Using a principal components analysis in Figure 3.10 A-D of all post intervention positive data points, we see that there is a gradient of “phenotypes” based on the relative abundance of the urolithins, and the sheer magnitude of the response to walnut consumption in plasma is large. Finally, looking at a simple Pearson’s correlation matrix across all samples (excluding Phenotype 0) the urolithin metabolite associations in plasma are shown in Supplemental Figure 3.4.

Baseline characteristics discussed above were also described when subjects were clustered according to their urolithin metabotypes (Table 3.9 and 3.10). No statistical comparisons could be made due to the unequal distribution of metabotypes among participants

with metabotype B (UM-B) and metabotype 0 (UM-0) being represented by only four and five individuals, respectively. Changes in urolithin metabolite levels in plasma and urine after walnut consumption can be viewed in Table 3.11 and 3.12.

3.10 Correlation analyses

All correlation analyses were performed on the walnut arm only. Additional analyses in the no walnut arm will be performed for publication.

Plasma/urine urolithin metabolites and primary outcomes

There were no correlations between the blood lipids that changed after walnut consumption (TC and LDL-s) and any Uro metabolites in plasma. In urine, however, UroAG was negatively correlated with TC ($r = -0.54$, $p = 0.016$) and LDL-C ($r = -0.50$, $p = 0.03$) in UM-A individuals only. Interestingly, urine UroAG also had a low negative correlation with the primary bile acid CA ($r = -0.43$, $p = 0.021$), as well two secondary bile acids GUDCA ($r = -0.42$, $p = 0.027$) and TUDCA ($r = -0.38$, $p = 0.045$) and a moderate negative correlation with the secondary bile acid DCA ($r = -0.59$, $p = 0.001$) in all individuals. Only the correlation with DCA persisted when participants were clustered into the UM-A group ($r = -0.52$, $p = 0.023$).

While HDL-C and TG did not change with walnut consumption, we did observe a few correlations with Uro metabolites. For instance, HDL-C was positively correlated with plasma UroA ($r = 0.37$, $p = 0.049$) and negatively correlated with EA ($r = -0.38$, $p = 0.04$). Notably, plasma UroA also had a low negative correlation with the secondary bile acid GLCA ($r = -0.37$, $p = 0.043$) in all individuals, which persisted in UM-A group as well ($r = -0.47$, $p = 0.032$). In addition, HDL-C was positively correlated with urine IsoUroAG ($r = 0.42$, $p = 0.028$), while IsoUroAG was negatively correlated with the secondary bile acid DCA ($r = -0.44$, $p = 0.019$)

TG was positively correlated with plasma IsoUroAG (r = 0.48, p = 0.033) in UM-A individuals only but there were no correlations with any urine Uro metabolites. Plasma IsoUroAG also had a low negative correlation with the primary bile acids GCDCA (-0.44, p = 0.017) and GCA (r = -0.39, p = 0.035) as well as the secondary bile acid GLCA (r = -0.38, p = 0.037) in all individuals.

These and other correlations between blood lipids (Table 3.13 and 3.14) as well as fecal bile acids and plasma/urine Uro metabolites are presented in Tables 3.19 and 3.20.

Plasma/urine urolithin metabolites and secondary outcomes

There were correlations identified between any of the plasma/urine Uro metabolites and weight/BMI (Table 3.20).

Bacterial abundancies

There was only one correlation found between the bacterial RA and other outcomes. There was a low positive correlation between RA of *Roseburia* genus and NGAL levels (r = 0.44, p = 0.015) (Table 3.8).

Blood inflammatory markers

A low positive correlation was observed between plasma UroAG and VCAM-1 (r = 0.39, p = 0.033), as well as a moderate negative correlation between plasma UroAG and oxLDL in UM-A group only (r = -0.6, p = 0.004).

There was a low positive correlation between urine UroA and PAI-1 levels in all individuals (r = 0.4, p = 0.037). There was a low negative and a moderate negative correlation between urine UroA levels and oxLDL in all individuals (r = -0.42, p = 0.032) and UM-A group (r = -0.61, p = 0.0062), respectively. There were also low negative correlations between urine Urine UroB levels and IL-6, as well as oxLDL in all individuals (p = 0.018 and 0.043,

respectively). Urine UroAG and IsoUroAG was positively correlated with PAI-1 levels in all individuals, as well as UM-A group separately ($r = 0.55$ for both correlations, $p = 0.0026$ and 0.015, respectively). Similar correlation was observed between urine IroUroAG and PAI-1 levels in all individuals ($r = 0.49$, $p = 0.0084$). Urine Iso-UroAG and EA were negatively correlated (low correlation) with oxLDL in all individuals ($p = 0.031$ and 0.042, respectively). Summary of these results can be viewed in Table 3.15 and 3.16.

Gut inflammatory markers

There was a low positive correlation between urine urolithin-B glucuronide (UroBG) and CPN ($r = 0.46$, $p = 0.014$) in all individuals and a moderate correlation between the same variables ($r = 0.53$, $p = 0.021$) in UM-A group. No other correlations were identified. Results are displayed in Table 3.17.

4. Discussion

In this experiment we looked at the effects of daily walnut consumption on established CVD biomarkers, as well as gut microbiota-related outcomes, including microbial abundancies, metabolites and gut inflammatory markers. We observed an increase in weight and BMI after walnut consumption compared to the no walnut intervention, which is not in full alignment with the recent meta-analysis and systematic review by Guasch-Ferre et al. [9]. This review explored the effects of walnuts on blood lipids and other cardiovascular disease risk factors (included 24 controlled trials and a total of 1020 participants). They reported that no appreciable effects on weight and BMI were observed in individual trials with body change after walnut consumption ranging from -2.12 to 1.88 kg and for BMI between -1.15 and 0.92 [9]. While the between-group difference in weight and BMI at endpoint was statistically significant in our study, the observed changes (-0.6 kg weight gain and 0.25 kg/m^2 increase in BMI) are within the ranges of change

reported in other studies. This may also have been due to the fact that the daily walnut dose provided 360 kcals and participants may have incorporated them into their dietary pattern without replacing other snacks. However, the mean kcal consumption was not significantly different between the walnut and no walnut groups.

We also observed a significant decrease in TC (-6.86 mg/dL) and LDL-C levels (-8.79 mg/dL), which is consistent with Holscher et al. study [30] that the present trial was powered on. Holscher et al. saw a decrease in total cholesterol of -7 mg/dL and a decrease in LDL-C of -9 mg/dl after daily consumption of 42 g of raw walnuts for 3 weeks. Moreover, the Guasch-Ferre et al. [9] meta-analysis and systematic review reported that walnut-enriched diets resulted in -6.99 mg/dL reduction in TC and -5.51 mg/dL reduction in LDL-C. Regarding other blood lipids/lipoproteins, a systematic review by Garcia-Conesa found no changes in TC or HDL-C but reported that LDL-C and TGs were significantly lowered by ellagitannin-containing foods, nuts in particular [52]. Guasch-Ferre et al. reported that there was no shift in HDL-C after walnut-enriched diets compared to control but there was a significant decrease in TGs -4.69 mg/dL, however [9]. We did not see a statistically significant difference in either HDL-C or TG between the two groups at endpoint.

No notable results were observed for either blood inflammatory markers (VCAM-1, ICAM-1, IL-6, PAI-1) or oxLDL after walnut consumption in the present trial. This is consistent with Holscher et al. study, where they reported no differences between groups for ICAM-1, VCAM-1 and IL-6. The overall evidence on the effects of walnuts on blood inflammatory markers and oxLDL has generally been inconsistent to date [24]. For instance, Cofan et al. reported no changes in ICAM-1 and VCAM-1 but a significant decrease in IL-6 after 2 years of walnut consumption providing 15% of total kcals compared to the no walnut group [53]. The

proposed mechanism through which walnut consumption may affect inflammation and LDL-C oxidation is through gut microbial metabolism of ellagitannins (ETs) into various urolithin molecules. Urolithins are much better absorbed than the original ETs and EA. Therefore, they have been suggested to be responsible for the health effects attributed to the consumption of ET-rich foods that include pomegranates, berries, walnuts and other nuts, and many tropical fruits [54-55]. In fact, it has recently been demonstrated that low doses of UroA can decrease ICAM-1 *in vitro* [56-57], while Uro-A glucuronide (~5-15 μM) has the potential to significantly inhibit monocyte adhesion and endothelial cell migration [58]. These effects are generally associated with a moderate but significant downregulation of the levels of chemokine (C-C motif) ligand 2 (CCL2) and PAI-1 [58]. Evidence from *in vitro* studies shows that Uro-A can inhibit endothelial cell migration and is able to decrease the expression of CCL2 and interleukin-8 (IL-8) [58]. Uro-A and Uro-B have also been shown to reduce LDL oxidation *in vitro* [59]. Gonzalez-Sarrias reported a significant decrease in oxLDL after 3 weeks of ellagitannin-rich pomegranate extract consumption in healthy overweight/obese individuals over the age of 40, which is in conflict with our results [60]. The *in vitro* trials are not always translatable to humans due to discrepancies in the doses and the types of polyphenolic components tested (parent compound vs. circulating metabolites). The conflicting result compared to Gonzalez-Sarrias et al. study may be due to the differences in matrices in which the polyphenols were delivered (whole walnuts vs. PE capsule) and the dose of polyphenolics administered (capsules contained more concentrated amounts vs. walnuts).

We also observed a significant decrease in both NGAL and CPN after walnut consumption compared to the no walnut controls. This is in alignment with evidence showing that walnut consumption has the potential to decrease intestinal inflammation in mice [46].

Previous studies have demonstrated that NGAL is upregulated in ischemia-reperfusion processes, coronary heart disease and myocardial infarction [61-64] suggesting that it is a promising and useful biomarker of the severity and mortality of heart diseases [65]. Another study demonstrated that an increased NGAL expression has been detected in the mouse cardiomyocytes [65]. Moreover, hypoxia has shown the potential to induce mRNA expression and protein levels of NGAL in cardiac myocyte cell lines [66]. Furthermore, a study by Aigner et al. reported increased levels of NGAL after heart transplantation in rodents [67]. To our knowledge, there are no human trials investigating the effects of a dietary intervention on fecal NGAL levels in diseased or healthy individuals.

CPN is found in various body fluids in proportion to the degree of any existing inflammation and its concentration in feces is about six times that of plasma [68]. Measurement of fecal CPN is a useful surrogate marker of gastrointestinal inflammation. It has a high negative predictive value in ruling out inflammatory bowel disease (IBD) in undiagnosed, symptomatic patients and a high sensitivity for diagnosing the disease [68]. Fecal CPN as intestinal wall inflammation marker has been associated with dietary patterns, metabolic disorders, behavioral habits such as a tendency to depression and increased alcohol consumption, as well as carotid atherosclerosis [69]. To our knowledge, this is the first trial investigating the effects of dietary components on fecal CPN levels in healthy human subjects.

The effect of walnut consumption on gut inflammation in the current study is likely due to the production of urolithin metabolites from ellagitannins by gut microbes. Urolithins have demonstrated anti-inflammatory effects in the gut *in vivo* and *in vitro* [47-48]. Another potential mechanism for reduction of gut inflammatory markers is shifts in fecal primary/secondary BA pool composition. For instance, we observed a statistically significant decrease in the secondary

bile acid, LCA, after walnut consumption compared to no walnut controls. Levels of another secondary bile acid, DCA, appeared to also be lower after walnut consumption compared to no walnuts but no statistically significant differences were detected. Our results are partially consistent with Holscher et al. where they saw a significant reduction in both LCA and DCA after walnut consumption [30]. BAs are signaling molecules and metabolic regulators that activate farnesoid X receptor (FXR) and G protein-coupled bile acid receptor-1 (GPCR-1) to regulate lipid, glucose, and energy metabolism [70]. The bidirectional regulation of bile acids and the gut microbiota determine the rate of bile acid synthesis, the bile acid pool size, and the composition of the circulating bile acid pool [70]. The liver-gut-heart axis regulates lipid metabolism, inflammation, and the pathogenesis of metabolic diseases such as atherosclerotic CVD (ACVD), non-alcoholic fatty liver disease (NAFLD), diabetes, and obesity [70].

The gut-to-liver axis plays a critical role in BA synthesis and metabolism [71]. The gut microbiota metabolizes primary BAs to secondary BAs, which in turn control gut bacterial overgrowth [71]. In the postprandial state, the duodenum releases cholecystokinin to stimulate gallbladder contraction and secretion of bile into the GI tract for emulsification of fats [70]. Most BAs (~95%) are reabsorbed in the ileum. A small amount of BAs enter the colon, where gut bacterial bile salt hydrolases (BSHs) de-conjugate T/GCA and T/GCDCA [72-73]. Subsequently, bacterial 7α-dehydroxylase removes a 7-hydroxyl group from CA and CDCA to form the secondary bile acids DCA and LCA, respectively [70]. These secondary BAs are highly insoluble and toxic and are mostly excreted in the feces [70]. Some DCA is passively reabsorbed in the colon and secreted into the circulating BA pool [70]. Most LCA is excreted in feces; a small amount of LCA circulated to the liver is rapidly sulfur-conjugated by sulfotransferases and excreted in urine [70]. In humans, a small amount of CDCA is converted to UDCA by bacterial

7α/β-hydroxysteroid dehydrogenase [70]. Isomerization of CDCA to UDCA converts the highly hydrophobic CDCA to the highly soluble UDCA [70]. In humans, the bile acid pool consists of the hydrophobic bile acids CA, CDCA, and DCA in an approximate 40:40:20 ratio, and bile acids are conjugated to G and T in a ratio of 3:1 [70]. The gut bacterial enzymes BSH and 7α-dehydroxylase play a critical role in the regulation of bile acid synthesis, pool size, and homeostasis. Alteration of bile acid composition and pool size by gut bacteria causes dysbiosis and significantly impacts the host metabolism, as well as pathogenesis of metabolic liver and heart diseases [73-77].

In addition to fecal bile acids, we also assessed the effects of walnuts on fecal SCFA levels. We found that 2-methylbutyric, isobutyric, isovaleric and valeric acids were lower after walnut consumption compared to the no walnut controls. This is expected, as for SCFA level to increase, the dietary intervention needs to provide enough of the appropriate type of fiber and the microbes that can metabolize this specific type of fiber need to be present in the gut at the same time. These would be ideal conditions that probably were not created by the mere addition of walnuts to the participant diet in our study. In addition, 95% of SCFAs are absorbed by the host and the fecal levels are more a reflection of the host absorptive capacity vs. SCFA production by the gut microbes. In vitro trials have shown that *Roseburia* displays butyryl CoA:acetate CoA transferase activity and can ferment fiber to butyrate [78-80]. In contrast with this evidence, while we did observe a significant increase in RA of *Roseburia* after walnuts, levels of butyric acid did not change. This may be explained by the fact that we also saw a decrease in RA of *Bifidobacterium* genus, which produces acetate, the required metabolite for butyrate production through CoA:acetate CoA transferase enzyme activity [30]. Therefore, walnut consumption may have not resulted in an overall increase in butyrate levels. This is consistent with Holscher et al.

where they also observed an increase in *Roseburia* and a decrease in *Bifidobacterium* but no differences in predicted bacterial butanoate metabolism genes after walnut consumption compared to the no walnut controls [30].

A recent study by Bamberger et al. saw an increase in *Bifidobacterium* and a decrease in *Blautia* and *Anaerostipes* after 8 weeks of daily consumption of 43 g of walnuts [5]. Guetterman et al. showed an increase in RA of *Roseburia* and *Dialister* after 3 weeks of 42 g of walnut consumption [81]. Systematic review by Fitzgerald et al. concluded that walnut consumption increased relative abundance of *Fecalibacterium*, *Clostridium*, *Roseburia*, and *Dialister*, while decreasing the relative abundance of *Ruminococcus*, *Dorea*, *Oscillospira* and *Bifidobacterium* [82]. The genus *Clostridium* has been described as the main producer of LCA. Interestingly, while we did observe a significant decrease in LCA levels (as discussed above), we did not see any changes in RA of *Clostridium* genus between the two groups at endpoint. A more in-depth analysis of the gut microbial community (e.g. metagenomics) may reveal shifts undetected by 16S analysis.

Overall, our findings are consistent with what is reported by other walnut intervention trials, especially in terms of the increase in the RA of *Roseburia* and decrease in *Bifidobacterium* genera. The discrepancies between the reported results for other bacterial genera may stem from differences in study design (e.g. intervention duration, walnut dose, etc.) as well as the populations of interest.

Bacteria belonging to the *Gordonibacter* genus is known to be able to metabolize EA and has been shown to be correlated with urolithin metabolites in humans [83]. *Gordonibacter urolithinfaciens* (DSM27213) and *G. pamelaeae* specifically are needed to complete the hydrolysis [83]. Both belong to Coriobacteriaceae family that includes additional species that

metabolize other polyphenols into metabolites (e.g., producing equol from daidzein) [20]. Interestingly, bacterial genera other than *Gordonibacter* also need to be present for the completion of urolithin metabolite production. This includes tannase-producing genera like *Lactobacillus*, *Bifidobacteria* and *Roseburia* among others [84-85]. *In vitro* ETs have shown to promote the growth of *Akkermansia muciniphila* [86] which potentially also participates in ET metabolism.

This evidence has been supported by several human trials. For instance, a recent trial by Petersen et al. where participants consumed either walnut-enriched diet (WD), fatty acid-matched diet devoid of walnuts (WFMD) and the diet where oleic acid replaced alpha-linolenic acid (ORAD) found a significant increase in *Gordonibacter* after both WD and ORAD diets [87]. Relative abundance of Gordonibacter genus also increased in Garcia-Mantrana et al. study after only 3 days of 33 g walnut consumption [88]. Unfortunately, we were unable to statistically analyze *Gordonibacter* data in the present study because this genus was detected in only a few participants. But we did observe that *Gordonibacter* was detectable in only five participants pre-walnut intervention, while it appeared in eleven participants after the walnut intervention. Only two of these participants had *Gordonibacter* at the beginning and end of the no walnut intervention as well.

Regardless of not seeing the changes in bacterial genera capable of metabolizing walnut ellagitannins into urolithin metabolites with walnut consumption, we were able to detect the three urolithin metabotypes previously discussed in the literature [89]. A regular intake of ET-rich foods can potentially result in physiologically relevant concentrations of urolithins and their phase II derivatives in plasma. However, concentration of these metabolites in plasma varies considerably between individuals [89-90]. In addition, significant differences among subjects in

the ability to produce the final urolithins and the health benefits associated with consumption of ET-containing food products have been reported due to the various compositions of their gut microbiota [91]. In respect to the microbial metabolism, individuals can be stratified into three urolithin metabotypes: metabotype A – individuals producing only UroA (25%-80% of the volunteers), metabotype B, yielding IsoUroA and/or UroB in addition to UroA (10%-50%), and metabotype 0, which is not able to produce any urolithins (5%-25%) [89]; these phenotypes, or metabotypes, were equally observed in all the different studies and were independent of the ET-containing food ingested and the age and health status of the volunteers [89]. Notably, while UM-B has been reported to be associated with gut microbial perturbations and colorectal cancer (CRC) in patients [92], this metabotype is not usually prevalent in subjects with diseases not involving GI distress (e.g., prostate cancer, prostate hyperplasia, or chronic obstructive pulmonary disease) [89]. UM-B individuals in the present trial had higher levels of NGAL compared to the other two metabotypes after walnut consumption. CPN levels in UM-B individuals, however, were lower compared to UM-A but higher vs. UM-0 individuals. In contrast, a recent study has shown that the UM-B prevailed in healthy overweight (31%) versus normoweight (20%) subjects, supporting the association between a specific metabotype and the health status, particularly, the MetS and obesity [93]. Some other evidence, however, does suggest that the gut microbial ecology of UM-A could be "protective," while UM-B could be potentially prone to cardiometabolic risk [94-97]. In this regard, González-Sarrías et al. [98] described the improvement of cardiometabolic risk biomarkers in UM-B individuals after consuming an ET-rich pomegranate extract. In contrast, no statistically significant effects were observed in UM-A individuals or the whole group before clustering individuals according to their Ums [98]. In general, the relatively low number of volunteers with UM-0 (particularly in

based on urine samples) in different human studies has prevented drawing any statistically significant conclusion regarding the possible impact of this non-producer UM in the outcomes of the studies [94-99]. We were unable to test this statistically in the present study but upon visual inspection of the values, TC and TG levels appeared higher in UM-B individuals compared to the UM-A and UM-0 at baseline, as well as after walnut consumption. Interestingly, UM-B individuals in a recent Cortes-Martin trial exploring the effects of PE extract on gut microbiome composition/functionality were also reported to be at a higher cardiovascular risk [100]. Their serum TC, LDL-C, VLDL-C, oxLDL, ApoB, non-HDL-C, IDL-C, and small and large LDL-C levels were higher than those in the UM-0 and UM-A groups at baseline.

Garcia-Mantrana [88] reported that the gut microbiota associated with UM-B was more "sensitive" to their walnut intervention. *Blautia, Bifidobacterium*, and members of the Coriobacteriaceae family, including *Gordonibacter*, increased exclusively in UM-B subjects, while some members of the Lachnospiraceae family decreased in UM-A individuals [88]. *Coprococcus* and *Collinsella* increased in both UMs and higher acetate and propionate production resulted after walnuts intake [88]. This is not consistent with our results but, as mentioned above, we could not cluster our participants into the three metabotypes due to the uneven distribution. Interestingly, the participants in our study that gained *Gordonibacter* with walnut consumption were all categorized as UM-A.

In terms of specific urolithins, evidence shows that UroA inversely correlates with the severity of various MetS biomarkers [101]. It also can be considered as a potential candidate to use as an anti-cancer or anti-inflammatory agent in treatment interventions related to GI disorders [19, 47, 102]. Interestingly, in our study plasma UroA was positively correlated with HDL-C after walnut consumption in all individuals but not in UM-A participants separately,

while urine UroAG was negatively correlated with TC and LDL-C in UM-A group only. In the Cortes-Martin et al. trial correlations between urinary Uros and CVD biomarkers were observed in UM-B individuals [100]. TC, LDL-C, and non-HDL-C negatively correlated with urinary UroA excretion in UM-B individuals. However, no significant correlations were found between fecal Uro excretion and most CVD biomarkers [100]. These results may suggest that circulating plasma Uros could be responsible for the beneficial effects on CVD biomarkers [100].

We also explored correlations between various urolithin metabolites in plasma/urine and fecal levels of bile acids. In plasma, the most correlations between bile acids and urolithins were for UroB. It was negatively correlated with a few primary (CDCA, TCDCA, GCDCA, GCA, TCA) as well as secondary bile acids (GLCA, TDCA, TLCA, GDCA). Most of these correlations persisted when individuals were grouped into UM-A metabotype. Uro-A negatively correlated with just one secondary bile acid (GLCA, persisted in UM-A individuals analyzed separately), while UroAG negatively correlated with two additional secondary bile acids (TDCA and TUDCA, correlations were not detected in UM-A group separately). IsoUroAG was negatively correlated with one secondary bile acid (GLCA) in all individuals only. UroB, UroA and IsoUroA all negatively correlated with the secondary bile acid GLCA. This means that UM-B individuals may have an amplified effect on the levels of this bile acid, since they produce all three metabolites.

In urine most of the correlations between urolithins and bile acids were for UroD. It negatively correlated with five secondary bile acids (DCA, GUDCA, LCA, TLCA, and TUDCA) and three primary bile acids (CDCA, CA, GCDCA). UroA negatively correlated with three secondary bile acids (DCA, TDCA and TUDCA), while UroB negatively correlated with CDCA (primary) and GUDCA (secondary) only. IsoUroAG was negatively correlated with only one

secondary bile acid – DCA. UroA and UroB negatively correlated with different secondary bile acids, which may mean that UM-B individuals that can produce both metabolites may benefit more from ET-rich food consumption by being able to affect levels of multiple secondary bile acids. The recent Cortes-Martin et al. study did not measure plasma urolithin levels but they did assess urinary urolithin excretion [100]. They found that reduction of fecal DCA and LCA (secondary bile acids) was more prominent in UM-B individuals compared to UM-A, which may have contributed to the favorable changes in blood lipids/lipoproteins also observed in these individuals. CA and CDCA (primary bile acids), however, similarly reduced in both groups. They also observed an inverse correlation of UroA with overall fecal BA pool [100].

While more studies based on various urolithin metabotypes are warranted, it is important to keep in mind that establishment of the cut-off between high and low producers of urolithin metabolites is arbitrary [103]. It depends on many variables, including the GI motility, sample collection time, food matrix, the lag period between the last intake of (poly)phenolic precursor and the sample analysis, and the sensitivity of the analytical procedure. [94-95, 100]. Furthermore, the distribution of UM-A and UM-B is critically affected by aging [103]. The percentage of UM-0 was reported to be approximately constant (10%) in the range from 5 to 90 years of age in a Caucasian cohort (n = 839) [103]. In contrast, UM-A is more prevalent at an early age, decreasing from 85% up to 55% in adulthood, parallel to an increase of UM-B from 15% up to 45% [107]. From 35 to 40 years of age, the proportion of UM-A and UM-B (55%, and 45%, respectively) remains approximately the same [94, 104].

As in the case of other microbial-derived metabolites, a Uro production gradient within UM-A and UM-B individuals defines tentatively “high” and “low” producers of Uros [103]. However, as mentioned above, the criterion for establishing the boundary between high and low

Uro producers (also extrapolated to other microbial-derived metabolites such as equol, enterolignans, and hydroxyvalerolactones) is arbitrary [95]. This means that the possible associations between “high” or “low” Uros production and health issues may not be reproducible in all circumstances or conditions [103]. Therefore, whether a specific microbial-derived metabolite exerts biological activity, dependently or not of the associated microbiota, requires evaluating the effects upon direct administration of the metabolite [103].

In conclusion, consumption of 56 grams of walnuts daily for 3 weeks had a favorable effect on TC and LDL-C. It also resulted in lower levels of fecal gut inflammatory markers, which has never been shown in generally healthy individuals before. Moreover, walnut consumption led to shifts of gut microbial genera, including an increase in *Roseburia* and a decrease in *Bifidobacterium* genera. Walnuts also lowered levels of fecal lithocholic secondary bile acid, which has shown the potential to be positively associated with colonic inflammation and cancer [105]. In addition, study participants produced beneficial microbial-derived urolithin metabolites and presented with differential levels of these metabolites in plasma and urine, which allowed clustering them into various urolithin metabotypes. In general, these results contribute to the body of evidence that walnuts are a positive addition to one’s diet, but more human trials are needed to confirm that some individuals may benefit more from walnut consumption than others based on the composition and metabolic capacity of their gut microbiome.

5. References

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6. Tables and figures

Table 3.1. Participant baseline characteristics[1]

Characteristic	Mean ± SD
Age, *years*	59 ± 7.1
Sex, *M/F*	3/27
Weight, *kg*	81.9 ± 14.6
BMI, kg/m^2	30.0 ± 5.7
Waist circumference, *cm*	97.4 ± 11.9
Hip circumference, *cm*	112.0 ± 13.3
Systolic blood pressure, *mmHg*	116 ± 13
Diastolic blood pressure, *mmHg*	73 ± 9
Total cholesterol, *mg/dL*	234.3 ± 26.2
LDL cholesterol, *mg/dL*	152.5 ± 21.7
HDL cholesterol, *mg/dL*	62.2 ± 14.2
Triglycerides, *mg/dL*	108.9 ± 42.6
Fiber consumption, *g*	11.9 ± 4.5

[1] Values are mean ± SD, n = 30. BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TG, triglycerides.

Table 3.2. Anthropometric measurements and clinical characteristics after 3 weeks of walnut vs. no walnut consumption[1]

Variable	Treatment	Baseline	3 wk	Δ 3 wk - baseline	P_{within}	$P_{between}$	P_{order}
Weight, *kg*							
	Walnut	80.05 (72.60, 86.33)	80.45 (72.75, 87.55)	0.60 (0.23, 0.96)	0.004**	**0.0002*****	NS
	No walnut	79.95 (72.30, 87.97)	78.15 (71.95, 88.00)	-0.44 (-0.80, -0.07)	0.02*		
BMI, kg/m^2							
	Walnut	28.1 (25.8, 31.6)	28.5 (25.6, 32.2)	0.25 (0.11, 0.39)	0.002**	**0.0002*****	NS
	No walnut	28.0 (25.6, 32.3)	28.0 (25.5, 31.9)	-0.13 (-0.27, -0.0006)	0.04*		
WC, *cm*							
	Walnut	96.97 (12.8)	98.83 (12.2)	1.86 (5.16)	NS	NS	NS
	No walnut	98.09 (11.4)*	97.59 (11.7)	-0.5 (3.43)	NS		
HC, *cm*							
	Walnut	111.4 (13.2)	112.5 (12.9)	1.15 (3.9)	NS	NS	NS
	No walnut	112.7 (12.4)	111.4 (14.0)	-1.24 (3.9)	NS		
Systolic BP, *mmHg*							
	Walnut	117.1 (13.5)	117.5 (11.7)	0.4 (7.1)	NS	NS	NS
	No walnut	116.8 (12.5)	118.6 (12.4)	1.8 (9.7)	NS		
Diastolic BP, *mmHg*							
	Walnut	73.0 (9.6)	73.4 (8.5)	0.4 (6.5)	NS	NS	NS
	No walnut	73.7 (8.6)	73.8 (8.9)	0.1 (4.8)	NS		
TC, mg/dL							
	Walnut	240.03 (26.42)*	233.17 (28.30)	-6.86 (16.89)	0.04*	**0.004****	NS
	No walnut	230.96 (24.46)	241.83 (28.52)	10.86 (23.07)	0.02*		
LDL-C, mg/dL							
	Walnut	155.17 (22.50)*	146.38 (23.04)	-8.79 (14.96)	0.004**	**0.002****	NS
	No walnut	146.07 (19.79)	155.21 (22.77)	9.14 (20.92)	0.03*		
HDL-C, mg/dL							
	Walnut	62.72 (15.36)	63.9 (16.48)	1.17 (6.05)	NS	NS	NS
	No walnut	60.79 (15.42)	62.93 (15.45)	2.14 (7.08)	NS		
TG, mg/dL							
	Walnut	112 (71, 137)	99 (80, 136)	2.07 (-11.14, 15.28)	NS	NS	NS
	No walnut	120 (81, 138)	105 (81, 150)	-3.59 (-18.95, 11.78)	NS		

[1]Data are presented as means ± SD if normally distributed or median (IQR) if non-normally distributed, n = 30. Change from baseline was assessed via paired t-tests following a log10 transformation if necessary. Values at the 3-week endpoint were compared between treatments via ANCOVA test using baseline values as a covariate. P-values marked with * indicate statistical significance ($p < 0.05$). P-values marked with ** indicate statistical significance ($p < 0.01$). P-values marked with *** indicate statistical significance ($p < 0.001$). BMI, body mass index; WC, waist circumference; HC, hip circumference; BP, blood pressure; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides.

Table 3.3 Blood inflammatory markers and oxLDL after 3 weeks of walnut vs. no walnut consumption[1]

Variable	Group	Baseline	3 wk	Δ 3 wk - baseline	P_{within}	$P_{between}$	P_{order}
PAI-1, *ng/ml*							
	Walnut	4.78 (3.33, 7.85)	7.55 (4.35, 10.76)	0.14 (0.03, 0.26)	0.02*	NS	**0.003****
	No walnut	5.59 (3.13, 7.89)	6.28 (4.19, 8.52)	0.08 (0.01, 0.16)	0.03*		
ICAM-1, *ng/ml*							
	Walnut	243.26 (49.24)	241.65 (55.71)	-1.60 (28.53)	NS	NS	NS
	No walnut	250.13 (58.24)	233.48 (51.69)	-16.65 (25.05)	0.001***		
VCAM-1, *ng/ml*							
	Walnut	693.05 (141.19)	701.84 (159.04)	8.79 (105.48)	NS	NS	NS
	No walnut	707.71 (131.13)	701.05 (128.73)	-6.66 (96.72)	NS		
IL-6, *pg/ml*							
	Walnut	2.21 (1.59, 3.34)	2.35 (1.34, 3.04)	-0.01 (-0.49, 0.46)	NS	NS	NS
	No walnut	2.63 (1.61, 3.49)	2.35 (1.61, 3.02)	-0.29 (-0.67, 0.08)	NS		
oxLDL, *U/L*							
	Walnut	88.55 (25.31)	84.60 (23.12)	-3.95 (19.08)	NS	NS	NS
	No walnut	85.16 (24.20)	91.23 (32.00)	6.06 (27.79)	NS		

[1]Data are presented as means ± SD if normally distributed or median (IQR) if non-normally distributed, n = 30. Change from baseline was assessed via paired t-tests following a log10 transformation if necessary. Values at the 3-week endpoint were compared between treatments via ANCOVA test using baseline values as a covariate. P-values marked with * indicate statistical significance ($p < 0.05$). P-values marked with ** indicate statistical significance ($p < 0.01$). P-values marked with *** indicate statistical significance ($p < 0.001$).
PAI-1, plasminogen activator inhibitor-1; ICAM-1, intercellular adhesion molecule 1; VCAM-1, vascular cell adhesion molecule; IL-6, interleukin 6; oxLDL, oxidized low-density lipoprotein.

Table 3.4 Physicochemical characteristics of fecal samples and fecal levels of gut inflammatory markers after 3 weeks of walnut and vs. walnut consumption[1]

Variable	Group	Baseline	3 wk	Δ 3 wk - baseline	P_{within}	$P_{between}$	P_{order}
NGAL, *ug/g*	***Walnut***	1.23 (0.71, 1.43)	0.70 (0.39, 0.94)	-0.58 (-0.96, -0.20)	0.00004***	**0.01***	NS
	No walnut	1.40 (0.62, 2.43)	1.31 (0.79, 1.79)	-0.24 (-0.63, 0.15)	NS		
CPN, *ug/g*							
	Walnut	25.87 (13.81, 45.69)	17.42 (8.48, 27.33)	-23.75 (-45.88, -1.62)	0.005**	**0.02***	NS
	No walnut	30.20 (12.29, 48.12)	29.93 (19.36, 52.26)	-2.88 (-31.97, 26.22)	NS		
pH							
	Walnut	7.13 (0.31)	6.85 (0.46)	-0.28 (0.39)	0.0006***	**0.00002*****	NS
	No walnut	7.14 (0.39)	7.32 (0.41)	0.19 (0.42)	0.02*		
% Moisture							
	Walnut	72.22 (7.73)	71.53 (6.30)	-0.69 (7.94)	NS	NS	NS
	No walnut	74.44 (6.95)	73.87 (5.50)	-0.57 (5.92)	NS		
Consistency							
	Walnut	4.0 (2.0, 4.0)	4.0 (4.0, 4.0)	0.16 (-0.5, 0.8)	NS	NS	NS
	No walnut	4.0 (2.2, 5.0)	4.0 (4.0, 5.0)	0.4 (-0.2, 1.0)	NS		

[1]Data are presented as means ± SD if normally distributed or median (IQR) if non-normally distributed, n = 30. Change from baseline was assessed via paired t-tests following a log10 transformation if necessary. Values at the 3-week endpoint were compared between treatments via ANCOVA test using baseline values as a covariate. P-values marked with * indicate statistical significance ($p < 0.05$). P-values marked with ** indicate statistical significance ($p < 0.01$). P-values marked with *** indicate statistical significance ($p < 0.001$).
NGAL, lipocalin-2; CPN, calprotectin.

Table 3.5 Fecal bile acid concentrations after 3 weeks of walnut vs. no walnut consumption[1]

BAs, *ng/g*	**Walnut**	**No walnut**	**p**
CDCA	10.15 (2.88, 30.97)	8.52 (3.45, 60. 07)	NS
CA	5.99 (0.97, 16.26)	3.84 (1.54, 36.80)	NS
DCA	1005.52 (387.23, 1863.01)	1656.03 (781.54, 2780.22)	0.10
GCDCA	2.77 (1.09, 8.48)	2.52 (1.40, 4.55)	NS
GCA	3.11 (1.07, 7.86)	2.44 (1.22, 4.32)	NS
GDCA	2.80 (1.02, 7.19)	3.02 (1.98, 5.19)	NS
GLCA	0.12 (0.03, 0.17)	0.16 (0.08, 0.33)	NS
GUDCA	0.36 (0.11, 0.62)	0.28 (0.12, 0.99)	NS
LCA	561.52 (404.83, 1121.82)	1226.54 (709.82, 1852.83)	**0.01***
TCDCA	1.62 (0.67, 4.51)	1.07 (0.59, 2.38)	NS
TCA	1.20 (0.66, 3.75)	0.64 (0.39, 1.46)	NS
TDCA	1.35 (0.44, 3.51)	0.83 (0.35, 1.66)	NS
TLCA	0.13 (0.05, 0.45)	0.09 (0.05, 0.41)	NS
TUDCA	0.07 (0.03, 0.15)	0.06 (0.03, 0.14)	NS
UDCA	30.90 (2.53, 31.20)	38.50 (0.79, 42.63)	NS

[1]Data are presented as median (IQR), n = 30. Values at the 3-week endpoint were compared between treatments via ANCOVA test using baseline values as a covariate. P-values marked with * indicate statistical significance ($p < 0.05$).

Cholic Acid (CA), Chenodeoxycholic Acid (CDCA), Deoxycholic Acid (DCA), Lithocholic Acid (LCA), Ursodeoxycholic Acid (UDCA), Glycocholic Acid (GCA), Glycochenodeoxycholic Acid (GCDCA), Glycodeoxycholic Acid (GDCA), Glycoursodeoxycholic Acid (GUDCA), Taurocholic Acid (TCA), Taurochenodeoxycholic Acid (TCDCA), Taurodeoxycholic Acid (TDCA), Taurolithocholic Acid (TLCA), Tauroursodeoxycholic Acid (TUDCA), and Glycolithocholic Acid (GLCA).

Table 3.6 levels of fecal SCFAs after 3 weeks of walnut vs. no walnut consumption[1]

SCFAs, *ug/g*	**Walnut**	**No walnut**	**p**
2-Methylbutyric acid	69.85 (44.09, 95.67)	113.08 (78.22, 158.86)	**0.04***
Acetic acid	1542.33 (1182.62, 2906.21)	2038.84 (1691.61, 2995.01)	NS
Butyric acid	642.48 (420.79, 1095.94)	839.93 (519.81, 1198.30)	NS
Hexanoic acid	24.28 (3.03, 80.54)	30.54 (5.15, 114.80)	NS
Isobutyric acid	97.84 (64.03, 128.39)	149.10 (105.91, 192.32)	**0.007****
Isovaleric acid	90.60 (50.61, 113.74)	130.90 (97.96, 171.48)	**0.03***
Propionic acid	698.32 (439.11, 1002.53)	872.22 (669.91, 945.43)	NS
Valeric acid	125.28 (99.36, 202.29)	185.49 (146.88, 275.71)	**0.006****

[1]Data are presented as median (IQR), n = 30. Values at the 3-week endpoint were compared between treatments via ANCOVA test using baseline values as a covariate. P-values marked with * indicate statistical significance ($p < 0.05$). P-values marked with ** indicate statistical significance ($p < 0.01$).
SCFAs, short-chain fatty acids.

Table 3.7 Percent relative abundance of bacterial phyla/genera in fecal samples after 3 weeks of walnut vs. no walnut consumption[1]

Phyla and genera	Walnut	No walnut	SE	P
Firmicutes	52.82	53.97	1.0	0.44
Blautia	9.43	9.22	0.57	0.24
Faecalibacterium	4.27	4.06	0.32	0.92
Phascolactobacterium	3.73	4.00	0.39	0.74
Ruminococcus	2.14	2.38	0.26	0.89
Fusicatenibacter	1.43	1.61	0.17	0.46
Clostridium	1.37	1.43	0.23	0.95
Agathobacter	1.30	1.38	0.19	0.46
Roseburia	1.27	0.92	0.14	**0.01***
Anaerostipes	1.15	0.98	0.12	0.80
Coprococcus	0.74	0.54	0.13	0.59
Dorea	0.65	0.65	0.05	0.79
Streptococcus	0.69	0.51	0.19	0.96
Lachnospira	0.57	0.58	0.08	0.52
Bacteroidetes	38.79	36.93	1.0	0.15
Bacteroides	24.29	23.37	1.57	0.57
Alistipes	2.25	3.04	0.35	**0.049***
Parabacteroides	2.09	2.11	0.18	0.21
Actinobacteria	1.01	1.25	0.2	0.15
Bifidobacterium	0.92	1.13	0.22	**0.03***
Collinsella	0.18	0.26	0.03	0.06
Verrucomicrobia	2.00	2.59	0.6	0.40
Akkermansia	1.95	1.76	0.88	0.55
Proteobacteria	4.59	4.51	0.6	0.76

[1]Data are presented as least square means, n = 30. Values at the 3-week endpoint were compared between treatments via mixed effects ANOVA with treatment as the mixed effect and participant and period as the random effects. P-values marked with * indicate statistical significance ($p < 0.05$).

Table 3.8 Correlations between bacterial genera and variables that differed between groups at endpoint[1]

	Roseburia		***Alistipes***		***Bifidobacterium***	
	r	**P**	**r**	**P**	**r**	**P**
Blood lipoproteins						
TC, *mg/dL*	-0.14	0.47	-0.18	0.37	0.07	0.74
LDL-C, *mg/dL*	-0.14	0.48	-0.15	0.44	0.12	0.61
Bile acids						
Lithocholic acid, *ng/ml*	-0.12	0.53	-0.0012	0.99	0.053	0.81
Short-chain fatty acids						
2-Methylbutyric acid, *ug/g*	-0.18	0.35	0.05	0.80	-0.075	0.73
Isobutyric acid, *ug/g*	0.04	0.83	-0.093	0.63	-0.16	0.46
Isovaleric acid, *ug/g*	-0.12	0.52	0.039	0.84	-0.17	0.43
Valeric acid, *ug/g*	0.13	0.49	-0.34	0.068	-0.33	0.12
Gut inflammatory markers						
NGAL, *mg/kg*	**0.44**	**0.015***	0.24	0.21	0.12	0.57
CPN, *ng/mg*	-0.035	0.85	0.11	0.58	-0.038	0.87

[1]Bivariate correlations were conducted to assess the relations between variables. Pearson's correlations were used for normally distributed variables and Spearman correlation for nonnormally distributed variables. P-values marked with * indicate statistical significance ($p < 0.05$).

TC, total cholesterol; LDL-C, low-dencity lipoprotein cholesterol; NGAL, lipocalin-2; CPN, calprotectin.

Table 3.9 Anthropometrics and clinical characteristics of participants clustered by urolithin metabotype at baseline[1]

Characteristic	All individuals (n=30)	UM-A (n=21)	UM-B (n=4)	UM-0 (n=5)
Age, *years*	59 (7.1)	59.8 (7.7)	56.5 (7.8)	57.6 (2.8)
Sex, *M/F*	3/27	2/19	1/3	0/5
Weight, *kg*	81.9 (14.6)	84.8 (15.3)	80.5 (11.8)	70.8 (8.4)
BMI, kg/m^2	30.0 (5.7)	31.0 (6.2)	29.4 (4.3)	26.4 (2.7)
WC, *cm*	97.4 (11.9)	99.8 (12.4)	93.8 (9.9)	90.6 (7.6)
HC, *cm*	112.0 (13.3)	115.0 (13.8)	107.0 (13.2)	104.0 (8.8)
sBP, *mmHg*	116 (13)	117 (14)	112 (7)	113 (11)
dBP, *mmHg*	73 (9)	74 (10)	72 (8)	69 (9)
TC, *mg/dL*	234.3 (26.2)	236.0 (29.2)	239.0 (22.3)	221.0 (7.9)
LDL-C, *mg/dL*	152.5 (21.7)	156.0 (24.3)	152 (12.1)	140 (12.0)
HDL-C, *mg/dL*	62.2 (14.2)	64.4 (14.4)	59 (19.4)	55.6 (3.4)
TG, *mg/dL*	108.9 (42.6)	101 (36.6)	140 (55.5)	127 (46.3)

[1]Data are presented as untransformed mean (SD).
BMI, body mass index; WC, waist circumference; HC, hip circumference; sBP, systolic blood pressur; dBP, diastolic blood pressure; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides.

Table 3.10 Blood lipid/lipoprotein, oxLDL, blood inflammatory and gut inflammatory markers at baseline and after 3 weeks of walnut vs. no walnut consumption. Participants are clustered by urolithin metabotypes[1]

		All individuals (n=30)		UM-A (n=21)		UM-B (n=4)		UM-0 (n=5)	
Variable	**Treatment**	**Baseline**	**3 wk**	**Baseline**	**3 wk**	**Baseline**	**3 wk**	**Baseline**	**3 wk**
TC, *mg/dL*									
	Walnut	240.0 (26.4)	233.2 (28.3)	243.0 (26.3)	237. 2 (27.8)	237.1 (26.5)	239.0 (30.4)	229.0 (29.4)	213.1 (25.0)
	No walnut	231.0 (24.5)	241.8 (28.5)	235.0 (27.9)	242.1 (30.5)	226.1 (15.6)	248.1 (24.1)	221.1 (8.68)	236.2 (27.3)
LDL-C, *mg/dL*									
	Walnut	155.2 (22.5)	146.4 (23.0)	159.2 (23.7)	150.3 (23.8)	150.4 (16.4)	146.3 (20.1)	143.3 (19.1)	131.1 (19.0)
	No walnut	146.1 (19.8)	155.2 (22.8)	149.2 (22.7)	156.2 (24.1)	142.3 (5.2)	158.0 (17.3)	139.4 (12.5)	151.1 (24.9)
HDL-C, *mg/dL*									
	Walnut	62.7 (15.4)	63.9 (16.5)	64.2 (16.0)	65.8 (17.6)	59.2 (19.2)	59.8 (18.9)	59.8 (11.2)	59.4 (10.3)
	No walnut	60.8 (15.4)	62.9 (15.4)	62.6 (16.5)	64.1 (16.2)	56.5 (20.1)	62.3 (21.2)	56.8 (4.1)	59.4 (7.8)
TG, *mg/dL*									
	Walnut	112.4 (47.3)	114.5 (51.9)	104.2 (48.7)	104.0 (41.1)	132.2 (39.0)	168 (89.3)	130.1 (46.8)	114.2 (37.2)
	No walnut	121.3 (50.7)	117.7 (48.7)	118.2 (54.6)	114.1 (51.0)	139.0 (41.1)	128.2 (46.1)	122.0 (47.2)	126.3 (48.9)
PAI-1, *ng/ml*									
	Walnut	7.0 (6.5)	8.6 (5.7)	6.3 (5.5)	8.9 (5.5)	10.6 (13.4)	10.3 (9.1)	7.3 (2.2)	6.2 (3.1)
	No walnut	6.4 (4.7)	7.7 (5.1)	6.3 (4.5)	7.4 (5.4)	8.4 (8.3)	7.8 (6.6)	5.5 (0.3)	8.8 (3.3)
ICAM-1, *ng/ml*									
	Walnut	243.3 (49.2)	241.6 (55.7)	250.1 (50.5)	251.1 (61.0)	209.1 (28.4)	222.3 (17.9)	244.0 (53.5)	218.1 (46.3)
	No walnut	250.1 (58.2)	233.5 (51.7)	259.2 (58.9)	242.2 (55.1)	248.0 (38.3)	218.3 (25.7)	216.1 (65.6)	209.1 (48.0)
VCAM-1, *ng/ml*									
	Walnut	693.0 (141.2)	701.8 (159.0)	723.1 (155.0)	747.2 (165.0)	623.1 (51.8)	640.0 (51.6)	624.2 (87.2)	560.1 (72.2)
	No walnut	707.7 (131.1)	701.0 (128.7)	748.3 (129.0)	735.3 (131.1)	649.0 (94.3)	662.2 (61.2)	584.4 (63.5)	590.0 (88.6)
IL-6, *pg/ml*									
	Walnut	2.7 (1.8)	2.7 (2.1)	3.2 (1.9)	3.0 (2.3)	1.4 (0.4)	1.6 (0.4)	2.1 (1.3)	2.7 (1.8)
	No walnut	2.9 (1.7)	2.6 (1.7)	2.8 (1.6)	3.0 (1.4)	2.6 (1.2)	1.6 (1.0)	3.4 (2.7)	2.0 (2.6)
oxLDL, *U/L*									
	Walnut	88.5 (25.3)	84.6 (23.1)	88.7 (23.9)	86.7 (22.9)	81.0 (24.5)	75.8 (27.5)	94.1 (35.3)	82.7 (24.2)
	No walnut	85.2 (24.2)	91.2 (32.0)	88.1 (26.7)	87.3 (27.6)	78.0 (22.2)	87.8 (12.4)	78.4 (12.4)	110.0 (54.4)
NGAL, *ng/mg*									
	Walnut	1.4 (1.0)	0.8 (0.6)	1.3 (1.1)	0.8 (0.6)	1.3 (0.8)	1.1 (0.8)	1.9 (0.8)	0.7 (0.3)

	No walnut	1.5 (1.1)	1.3 (0.8)	1.7 (1.2)	1.4 (0.9)	1.6 (1.2)	1.2 (1.2)	1.2 (0.7)	1.2 (0.3)
CPN, *ng/mg*									
	Walnut	48.1 (64.8)	24.4 (22.9)	39.0 (49.4)	26.2 (25.8)	46.7 (36.0)	22.9 (16.4)	87.9 (122.0)	18.1 (13.7)
	No walnut	52.8 (69.1)	50.0 (65.6)	54.0 (72.9)	47.6 (54.1)	31.4 (20.1)	20.6 (13.3)	65.0 (84.3)	83.2 (119.0)

[1]Data are presented as untransformed mean (SD).

TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides; PAI-1, plasminogen activator inhibitor-1; ICAM-1, intercellular adhesion molecule 1; VCAM-1, vascular cell adhesion molecule; IL-6, interleukin 6; oxLDL, oxidized low-density lipoprotein; NGAL, lipocalin-2; CPN, calprotectin.

Table 3.11 Urine urolithin levels before and after walnut and no walnut intervention[1]

	Walnut		No walnut		$p_{between}$	p_{order}
	pre	**post**	**pre**	**post**		
UroA	6.24 (2.74, 29.3)	3710.00 (589.02, 12200.01)	3.38 (2.12, 14.05)	3.28 (1.99, 15.70)	5.8e-11***	NS
UroB	NA	NA	NA	NA	NA	NA
UroC	9.24 (7.71, 11.22)	201.03 (26.40, 656.02)	8.39 (7.46, 9.23)	8.69 (7.57, 11.00)	1.8e-08***	NS
UroD	11.15 (9.83, 15.57)	13.70 (12.30, 16.82)	10.85 (9.77, 11.65)	11.20 (9.31, 13.10)	NS	NS
UroAG	283.01 (166.22, 1622.53)	62900.01 (45600.23, 191000.03)	328.10 (121.22, 697.23)	335.11 (208.19, 967.34)	8.87e-12***	NS
UroBG	498.54 (230.55, 1197.51)	1020.10 (400.23, 4320.22)	391.55 (229.54, 1129.13)	641.10 (265.42, 1740.10)	NS	NS
IsoUroAG	85.40 (50.10, 221.25)	1070.34 (701.20, 2280.43)	65.40 (30.73, 148.50)	94.70 (42.90, 224.13)	5.70e-11***	NS
EA	667.51 (556.83, 935.02)	776.10 (652.30, 1020.33)	632.30 (548.50, 698.43)	654.34 (535.40, 893.11)	NS	NS

[1]Data are presented as untransformed median (IQR), n = 30. Differences between groups were tested via ANCOVA with baseline levels used as covariates. *** Indicates $p < 0.001$.
Uro-A, urolithin A; Uro-B, urolithin B; Uro-C, urolithin C, Uro-D, urolithin D, Uro AG, urolithin A glucuronide; Uro-BG, urolithin B glucuronide; IsoUro-AG, Isourolithin A glucuronide; EA, ellagic acid.

Table 3.12 Plasma urolithin levels before and after walnut and no walnut intervention[1]

	Walnut		No walnut		$p_{between}$	p_{order}
	pre	**post**	**pre**	**post**		
UroA	0.06 (0.04, 0.12)	1.53 (0.29, 3.02)	0.06 (0.04, 0.10)	0.07 (0.04, 0.09)	2.996e-10***	NS
UroB	NA	NA	NA	NA	NA	NA
UroC	0.13 (0.09, 0.22)	5.08 (0.28, 19.85)	0.36 (0.12, 0.67)	0.14 (0.09, 0.19)	1.661e-08***	NS
UroD	1.28 (1.24, 1.4)	1.34 (1.30, 1.44)	0.17 (0.12, 0.20)	1.29 (1.24, 1.35)	NS	NS
UroAG	1.27 (0.87, 4.60)	625.50 (127.0, 1585.0)	1.08 (0.90, 4.41)	1.43 (1.03, 3.24)	3.333e-13***	NS
UroBG	0.96 (0.52, 2.76)	3.29 (0.90, 7.11)	1.96 (0.83, 4.07)	2.22 (1.06, 4.31)	0.03*	NS
IsoUroAG	0.99 (0.91, 1.11)	5.60 (2.54, 12.05)	1.04 (0.94, 1.13)	1.02 (0.92, 1.16)	1.86e-15***	NS
EA	91.70 (77.3, 147.01)	110.50 (84.75, 152.25)	101.20 (74.42, 213.50)	115.01 (85.65, 170.25)	NS	NS

[1]Data are presented as untransformed median (IQR), n = 30. Differences between groups were tested via ANCOVA with baseline levels used as covariates. *** Indicates $p < 0.001$.
Uro-A, urolithin A; Uro-B, urolithin B; Uro-C, urolithin C, Uro-D, urolithin D, Uro AG, urolithin A glucuronide; Uro-BG, urolithin B glucuronide; IsoUro-AG, Isourolithin A glucuronide; EA, ellagic acid.

Table 3.12 Correlations between plasma Uros and changes in blood lipids/lipoproteins after 3 weeks of walnut consumption[1]

Urolithin metabolite *uM*	**TC**				**LDL-C**				**HDL-C**				**TG**			
	All individuals (n=30)		***UM-A (n=21)***		***All individuals (n=30)***		***UM-A (n=21)***		***All individuals (n=30)***		***UM-A (n=21)***		***All individuals (n=30)***		***UM-A (n=21)***	
	r	**P**	**r**	**P**	**r**	**P**	**r**	**P**	**r**	**P**	**r**	**P**	**r**	**P**	**r**	**P**
Uro-A	0.0049	0.98	-0.29	0.21	-0.13	0.49	-0.28	0.23	**0.37**	**0.049***	0.32	0.18	0.20	0.31	0.35	0.13
Uro-B	-0.007	0.97	-0.096	0.69	-0.16	0.42	-0.25	0.28	0.31	0.1	**0.49**	**0.029***	0.24	0.2	**0.44**	**0.052**
Uro-C	0.039	0.84	-0.051	0.83	-0.09	0.64	-0.12	0.62	0.22	0.25	0.23	0.34	0.12	0.52	0.13	0.58
Uro-D	0.21	0.27	0.29	0.21	0.18	0.36	0.27	0.25	-0.076	0.69	-0.04	0.87	-0.14	0.46	-0.18	0.45
Uro-AG	0.081	0.68	-0.22	0.34	-0.052	0.79	-0.22	0.36	0.33	0.082	0.17	0.48	0.12	0.55	0.27	0.25
Uro-BG	0.14	0.46	-0.044	0.85	-0.025	0.9	-0.14	0.55	-0.046	0.81	-0.11	0.64	0.032	0.87	0.17	0.48
IsoUro-AG	-0.029	0.88	-0.41	0.072	-0.11	0.57	-0.38	0.1	0.052	0.79	0.041	0.86	0.15	0.44	**0.48**	**0.033***
EA	-0.29	0.13	-0.2	0.4	-0.19	0.31	-0.15	0.54	**-0.38**	**0.04***	**-0.46**	**0.044***	0.20	0.3	0.15	0.53

[1] Bivariate correlations were conducted to assess the relations between variables. Pearson's correlations were used for normally distributed variables and Spearman correlation for nonnormally distributed variables. P-values marked with * indicate statistical significance ($p < 0.05$).

Uro-A, urolithin A; Uro-B, urolithin B; Uro-C, urolithin C, Uro-D, urolithin D, Uro AG, urolithin A glucuronide; Uro-BG, urolithin B glucuronide; IsoUro-AG, Isourolithin A glucuronide; EA, ellagic acid; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides

Table 3.14 Correlations between urine Uros and changes in blood lipid/lipoprotein changes after 3 weeks of walnut consumption[1]

Urolithin metabolite, *uM*	TC				LDL-C				HDL-C				TG			
	All individuals (n=30)		***UM-A (n=21)***		***All individuals (n=30)***		***UM-A (n=21)***		***All individuals (n=30)***		***UM-A (n=21)***		***All individuals (n=30)***		***UM-A (n=21)***	
	r	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**
Uro-A	0.13	0.53	-0.26	0.29	-0.014	0.94	-0.15	0.54	0.30	0.12	0.17	0.49	0.0084	0.97	0.072	0.77
Uro-B	-0.094	0.63	NA	NA	-0.20	0.3	NA	NA	-0.013	0.95	NA	NA	0.13	0.53	NA	NA
Uro-C	0.15	0.46	-0.13	0.6	0.062	0.75	-0.051	0.84	0.32	0.09	0.3	0.21	-0.057	0.77	0.019	0.94
Uro-D	-0.073	0.71	-0.12	0.62	-0.19	0.33	-0.18	0.47	0.25	0.19	0.39	0.097	0.19	0.33	0.25	0.3
Uro-AG	-0.06	0.76	**-0.54**	**0.016***	-0.2	0.32	**-0.5**	**0.03***	0.28	0.15	0.14	0.56	0.15	0.45	0.38	0.11
Uro-BG	0.057	0.77	-0.17	0.5	-0.028	0.89	-0.29	0.23	0.034	0.86	0.051	0.84	-0.018	0.93	0.24	0.32
IsoUro-AG	0.17	0.4	-0.19	0.43	0.014	0.94	-0.20	0.42	**0.42**	**0.028***	0.34	0.15	0.033	0.87	0.21	0.38
EA	-0.036	0.86	0.12	0.63	-0.12	0.54	0.091	0.71	-0.085	0.67	0.022	0.93	0.1	0.61	-0.087	0.72

[1] Bivariate correlations were conducted to assess the relations between variables. Pearson's correlations were used for normally distributed variables and Spearman correlation for nonnormally distributed variables. P-values marked with * indicate statistical significance ($p < 0.05$).

Uro-A, urolithin A; Uro-B, urolithin B; Uro-C, urolithin C, Uro-D, urolithin D, Uro AG, urolithin A glucuronide; Uro-BG, urolithin B glucuronide; IsoUro-AG, Isourolithin A glucuronide; EA, ellagic acid; TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides

Table 3.15 Correlations between urine Uros and blood inflammatory markers and oxLDL after 3 weeks of walnut consumption[1]

Urolithin metabolite, *uM*	**ICAM-1,** *ng/ml*		**VCAM-1,** *ng/ml*		**PAI-1,** *ng/ml*		**IL-6,** *pg/ml*		**oxLDL,** *U/L*	
	All	***UM-A***	***All***	***UM-A***	***All***	***UM-A***	***All***	***UM-A***	***All***	***UM-A***
UroA	0.12 (0.54)	0.12 (0.63)	0.24 (0.22)	-0.025 (0.92)	**0.4 (0.037)***	0.28 (0.24)	-0.012 (0.95)	0.092 (0.71)	**-0.41 (0.032)***	**-0.61 (0.0062)****
UroB	-0.053 (0.79)	NA	-0.012 (0.95)	NA	0.04 (0.84)	NA	**-0.44 (0.018)***	NA	**-0.38 (0.043)***	NA
UroC	-0.21 (0.28)	-0.36 (0.13)	0.063 (0.75)	-0.25 (0.31)	0.26 (0.19)	0.18 (0.46)	-0.0025 (0.99)	012 (0.61)	-0.085 (0.67)	0.054 (0.83)
UroD	-0.093 (0.64)	-0.17 (0.48)	-0.074 (0.71)	-0.16 (0.5)	0.21 (0.3)	0.21 (0.39)	-0.16 (0.41)	0.025 (0.92)	-0.32 (0.1)	**-0.55 (0.015)***
UroAG	-0.012 (0.95)	-0.058 (0.84)	0.11 (0.57)	-0.32 (0.18)	**0.55 (0.0026)****	**0.55 (0.015)***	-0.088 (0.65)	-0.088 (0.72)	-0.22 (0.27)	-0.31 (0.2)
UroBG	0.19 (0.33)	0.31 (0.19)	-0.098 (0.62)	-0.016 (0.95)	0.17 (0.39)	0.39 (0.097)	-0.36 (0.062)	-0.12 (0.62)	-0.15 (0.46)	0.012 (0.96)
IsoUroAG	-0.033 (0.87)	0.02 (0.93)	0.076 (0.7)	0.047 (0.85)	**0.49 (0.0084)****	0.37 (0.12)	-0.036 (0.86)	0.02 (0.94)	**-0.41 (0.031)***	-0.35 (0.15)
EA	0.097 (0.62)	-0.075 (0.76)	-0.017 (0.93)	-0.053 (0.83)	-0.17 (0.38)	-0.12 (0.56)	-0.35 (0.068)	-0.31 (0.19)	**-0.39 (0.042)***	-0.41 (0.081)

[1]Bivariate correlations were conducted to assess the relations between variables. "All" n = 30, "UM-A" n = 21. Pearson's correlations were used for normally distributed variables and Spearman correlation for nonnormally distributed variables. P-values marked with * indicate statistical significance ($p < 0.05$). P-values marked with ** indicate statistical significance ($p < 0.01$).

Uro-A, urolithin A; Uro-B, urolithin B; Uro-C, urolithin C, Uro-D, urolithin D, Uro AG, urolithin A glucuronide; Uro-BG, urolithin B glucuronide; IsoUro-AG, Isourolithin A glucuronide; EA, ellagic acid; PAI-1, plasminogen activator inhibitor-1; ICAM-1, intercellular adhesion molecule 1; VCAM-1, vascular cell adhesion molecule; IL-6, interleukin 6; oxLDL, oxidized low-density lipoprotein.

Table 3.16 Correlations between plasma Uros and blood inflammatory markers after 3 weeks of walnut consumption[1]

Urolithin metabolite, *uM*	**ICAM-1,** *ng/ml*		**VCAM-1,** *ng/ml*		**PAI-1,** *ng/ml*		**IL-6,** *pg/ml*		**oxLDL,** *U/L*	
	All	**UM-A**	**All**	**UM-A**	**All**	**UM-A**	**All**	**UM-A**	**All**	**UM-A**
UroA	-0.026 (0.89)	-0.15 (0.5)	0.22 (0.25)	-0.14 (0.54)	0.32 (0.09)	0.13 (0.57)	0.15 (0.43)	0.25 (0.28)	-0.12 (0.52)	-0.26 (0.25)
UroB	0.1 (0.6)	-0.83 (0.14)	0.2 (0.29)	-0.0085 (0.97)	0.16 (0.4)	0.039 (0.87)	-0.086 (0.65)	0.19 (0.42)	-0.31 (0.095)	**-0.49 (0.027)***
UroC	-0.12 (0.54)	-0.33 (0.14)	0.14 (0.44)	-0.27 (0.23)	0.15 (0.44)	0.044 (0.85)	0.1 (0.59)	0.2 (0.38)	0.15 (0.43)	0.12 (0.59)
UroD	0.21 (0.28)	0.23 (0.32)	0.11 (0.56)	0.19 (0.41)	-0.093 (0.63)	-0.29 (0.21)	-0.093 (063)	0.099 (0.67)	-0.0094 (0.96)	0.068 (0.77)
UroAG	0.24 (0.2)	0.23 (0.32)	**0.39 (0.033)***	0.27 (0.24)	**0.36 (0.052)**	0.16 (0.49)	0.19 (0.31)	0.36 (0.11)	-0.31 (0.099)	**-0.6 (0.0043)****
UroBG	0.17 (0.37)	0.13 (0.57)	0.088 (0.64)	-0.0052 (0.98)	0.18 (0.35)	0.2 (0.38)	-0.34 (0.066)	-0.22 (0.33)	-0.14 (0.48)	0.0013 (1.0)
IsoUroAG	0.29 (0.12)	0.39 (0.085)	0.31 (0.096)	0.17 (0.46)	0.26 (0.17)	0.25 (0.27)	0.093 (0.63)	0.3 (0.19)	-0.24 (0.2)	**-0.44 (0.05)**
EA	0.18 (0.34)	0.1 (0.66)	0.09 (0.64)	-0.055 (0.81)	-0.32 (0.081)	-0.37 (0.1)	-0.099 (0.6)	-0.11 (0.64)	-0.034 (0.86)	-0.0052 (0.98)

[1]Bivariate correlations were conducted to assess the relations between variables. "All" n = 30, "UM-A" n = 21. Pearson's correlations were used for normally distributed variables and Spearman correlation for nonnormally distributed variables. P-values marked with * indicate statistical significance ($p < 0.05$). P-values marked with ** indicate statistical significance ($p < 0.01$).

Uro-A, urolithin A; Uro-B, urolithin B; Uro-C, urolithin C, Uro-D, urolithin D, Uro AG, urolithin A glucuronide; Uro-BG, urolithin B glucuronide; IsoUro-AG, Isourolithin A glucuronide; EA, ellagic acid; PAI-1, plasminogen activator inhibitor-1; ICAM-1, intercellular adhesion molecule 1; VCAM-1, vascular cell adhesion molecule; IL-6, interleukin 6; oxLDL, oxidized low-density lipoprotein.

Table 3.17 Correlations between Uro levels and fecal gut inflammation marker levels after 3 wks of walnut consumption[1]

Urolithin metabolite, *uM*	**NGAL,** *ng/mg*				**CPN,** *ng/mg*			
	Urine		**Plasma**		**Urine**		**Plasma**	
	All	***UM-A***	***All***	***UM-A***	***All***	***UM-A***	***All***	***UM-A***
UroA	-0.099 (0.62)	-0.21 (0.29)	0.16 (0.39)	0.4 (0.074)	0.076 (0.7)	-0.096 (0.69)	-0.044 (0.82)	-0.18 (0.44)
UroB	-0.01 (0.96)	NA	0.035 (0.85)	0.24 (0.3)	0.23 (0.23)	NA	0.026 (0.89)	-0.081 (0.73)
UroC	0.067 (0.74)	0.12 (0.63)	0.13 (0.5)	0.26 (0.26)	-0.12 (0.53)	-0.36 (0.13)	0.14 (0.46)	0.19 (0.42)
UroD	-0.21 (0.28)	-0.25 (0.14)	-0.16 (0.39)	-0.27 (0.24)	-0.12 (0.55)	-0.13 (0.6)	0.21 (0.27)	-0.19 (0.4)
UroAG	-0.037 (0.85)	0.011 (0.96)	0.011 (0.95)	0.074 (0.75)	0.043 (0.83)	-0.14 (0.58)	0.0038 (0.98)	-0.19 (0.4)
UroBG	-0.038 (0.85)	-0.2 (0.4)	-0.00022 (1.0)	-0.078 (0.74)	**0.46 (0.014)***	**0.53 (0.021)***	0.27 (0.15)	0.28 (0.21)
IsoUroAG	0.031 (0.88)	-0.0061 (98)	0.25 (0.18)	0.36 (0.11)	-0.083 (0.67)	-0.2 (0.42)	-0.079 (0.68)	-0.17 (0.45)
EA	-0.16 (0.41)	-0.33 (0.17)	0.13 (0.5)	0.37 (0.1)	0.17 (0.38)	0.27 (0.27)	-0.22 (0.25)	-0.21 (0.36)

[1]Bivariate correlations were conducted to assess the relations between variables. "All" n = 30, "UM-A" n = 21. Pearson's correlations were used for normally distributed variables and Spearman correlation for nonnormally distributed variables. P-values marked with * indicate statistical significance ($p < 0.05$).

Uro-A, urolithin A; Uro-B, urolithin B; Uro-C, urolithin C, Uro-D, urolithin D, Uro AG, urolithin A glucuronide; Uro-BG, urolithin B glucuronide; IsoUro-AG, Isourolithin A glucuronide; EA, ellagic acid; NGAL, lipocalin-2; CPN, calprotectin.

Table 3.18 Correlations between plasma Uros and levels of fecal bile acids after 3 wks of walnut consumption[1]

	UroA		**UroB**		**UroC**		**UroD**		**UroAG**		**UroBG**		**IsoUroAG**		**EA**	
	All	***UM-A***	***All***	***UM-A***	***All***	***UM-A***	***All***	***UM-A***	***All***	***UM-A***	***All***	***UM-A***	***All***	***UM-A***	***All***	***UM-A***
CDCA	-0.16 (0.39)	-0.039 (0.87)	**-0.36 (0.049)***	-0.23 (0.32)	-0.063 (0.74)	0.21 (0.36)	0.039 (0.84)	0.14 (0.56)	-0.34 (0.064)	-0.38 (0.085)	-0.2 (0.28)	0.1 (0.66)	-0.25 (0.18)	-0.19 (0.4)	-0.13 (0.49)	-0.068 (0.77)
CA	-0.31 (0.095)	0.048 (0.84)	-0.29 (0.11)	-0.17 (0.47)	-0.18 (0.34)	0.32 (0.16)	0.079 (0.68)	0.29 (0.2)	**-0.47 (0.0095)****	-0.16 (0.48)	-0.094 (0.62)	0.28 (0.22)	**-0.36 (0.05)**	-0.13 (0.57)	-0.15 (0.43)	-0.26 (0.25)
DCA	-0.22 (0.25)	-0.022 (0.93)	-0.33 (0.073)	0.0013 (1.0)	-0.058 (0.76)	0.097 (0.67)	0.069 (0.72)	0.32 (0.16)	-0.16 (0.41)	0.13 (0.59)	-0.27 (0.15)	0.048 (0.84)	-0.033 (0.86)	0.41 (0.065)	0.12 (0.53)	0.29 (0.19)
GCDCA	-0.27 (0.14)	-0.23 (0.31)	**-0.68 (0.000053)*****	**-0.61 (0.0022)****	-0.16 (0.4)	-0.061 (0.79)	0.013 (0.95)	0.14 (0.54)	**-0.42 (0.02)***	**-0.55 (0.0096)****	-0.34 (0.067)	-0.13 (0.58)	**-0.44 (0.017)***	**-0.48 (0.027)***	-0.23 (0.22)	-0.17 (0.47)
GCA	-0.25 (0.19)	-0.18 (0.44)	**-0.59 (0.00054)*****	**-0.54 (0.013)***	-0.15 (0.42)	-0.0078 (0.98)	-0.18 (0.34)	-0.066 (0.78)	-0.33 (0.072)	-0.36 (0.11)	-0.34 (0.067)	-0.24 (0.54)	**-0.39 (0.035)***	-0.29 (0.2)	-0.35 (0.058)	-0.33 (0.14)
GDCA	-0.19 (0.31)	-0.15 (0.51)	**-0.55 (0.0019)****	**-0.6 (0.0051)****	-0.075 (0.69)	-0.04 (0.86)	0.05 (0.79)	0.11 (0.64)	-0.31 (0.096)	**-0.43 (0.054)**	-0.19 (0.31)	0.025 (0.92)	-0.28 (0.13)	-0.32 (0.16)	-0.24 (0.2)	-0.25 (0.27)
GLCA	**-0.37 (0.043)***	**-0.47 (0.032)***	**-0.39 (0.032)***	-0.32 (0.15)	-0.19 (0.32)	-0.15 (0.51)	-0.17 (0.38)	-0.14 (0.53)	-0.29 (0.12)	-0.29 (0.2)	-0.11 (0.57)	0.28 (022)	**-0.38 (0.037)***	-0.28 (0.22)	-0.083 (0.66)	-0.21 (0.36)
GUDCA	-0.25 (0.19)	0.045 (0.85)	-0.31 (0.095)	0.069 (0.77)	-0.31 (0.098)	-0.076 (0.74)	0.026 (0.89)	0.38 (0.094)	-0.24 (0.2)	0.018 (0.94)	**-0.4 (0.03)***	-0.12 (0.6)	-0.32 (0.084)	0.12 (0.61)	0.023 (0.91)	0.16 (0.5)
LCA	-0.29 (0.12)	-0.13 (0.57)	-0.34 (0.066)	-0.29 (0.2)	-0.3 (0.11)	-0.3 (0.18)	0.22 (0.23)	**0.42 (0.055)**	-0.1 (0.59)	0.16 (0.5)	-0.082 (0.67)	0.12 (0.6)	-0.0022 (0.99)	0.3 (0.18)	-0.13 (0.49)	0.16 (0.5)
TCDCA	-0.01 (0.96)	0.052 (0.82)	**-0.42 (0.021)***	**-0.42 (0.057)**	-0.16 (0.38)	-0.18 (0.45)	-0.0078 (0.97)	0.036 (0.88)	-0.14 (0.46)	-0.21 (0.36)	-0.23 (0.22)	-0.14 (0.54)	-0.18 (0.34)	-0.2 (0.37)	-0.27 (0.14)	-0.25 (0.27)
TCA	-0.11 (0.57)	-0.043 (0.85)	**-0.53 (0.0033)****	**-0.53 (0.015)***	-0.22 (0.24)	-0.17 (0.46)	-0.06 (0.75)	-0.0013 (1.0)	-0.17 (0.38)	-0.21 (0.36)	-0.26 (0.16)	-0.16 (0.49)	-0.23 (0.22)	-0.17 (0.45)	-0.26 (0.17)	-0.24 (0.29)

TDCA	-0.21 (0.27)	-0.099 (0.67)	**-0.48 (0.0082)****	**-0.57 (0.0076)****	-0.18 (0.33)	-0.17 (0.47)	0.036 (0.85)	0.16 (0.5)	**-0.37 (0.045)***	-0.39 (0.081)	-0.14 (0.44)	0.029 (0.9)	-0.3 (0.1)	-0.27 (0.23)	-0.05 (0.79)	-0.037 (0.87)
TLCA	-0.21 (0.26)	-0.17 (0.45)	**-0.4 (0.03)***	**-0.55 (0.011)***	-0.12 (0.52)	-0.049 (0.83)	-0.052 (0.79)	0.014 (0.95)	-0.31 (0.1)	-0.37 (0.099)	-0.12 (0.54)	-0.0052 (0.98)	-0.27 (0.15)	-0.2 (0.38)	-0.22 (0.24)	-0.12 (0.6)
TUDCA	-0.27 (0.15)	-0.048 (0.84)	-0.24 (0.21)	-0.14 (0.54)	-0.15 (0.44)	0.12 (0.61)	0.13 (0.49)	0.29 (0.2)	**-0.41 (0.024)***	-0.25 (0.28)	-0.07 (0.71)	0.15 (0.51)	-0.2 (0.28)	-0.086 (0.71)	-0.032 (0.87)	-0.023 (0.92)
UDCA	-0.099 (0.6)	-0.016 (0.95)	-0.079 (0.68)	0.057 (0.81)	-0.14 (0.47)	-0.14 (0.55)	-0.049 (0.8)	-0.015 (0.95)	-0.21 (0.26)	-0.14 (0.54)	-0.2 (0.29)	-0.11 (0.65)	-0.16 (0.38)	-0.076 (0.74)	0.28 (0.13)	0.21 (0.35)

[1]Bivariate correlations were conducted to assess the relations between variables. “All” n = 30, “UM-A” n = 21. Pearson’s correlations were used for normally distributed variables and Spearman correlation for nonnormally distributed variables. P-values marked with * indicate statistical significance ($p < 0.05$).P-values marked with * indicate statistical significance ($p < 0.05$). P-values marked with ** indicate statistical significance ($p < 0.01$). P-values marked with *** indicate statistical significance ($p < 0.001$).

Uro-A, urolithin A; Uro-B, urolithin B; Uro-C, urolithin C, Uro-D, urolithin D, Uro AG, urolithin A glucuronide; Uro-BG, urolithin B glucuronide; IsoUro-AG, Isourolithin A glucuronide; EA, ellagic acid; Cholic Acid (CA), Chenodeoxycholic Acid (CDCA), Deoxycholic Acid (DCA), Lithocholic Acid (LCA), Ursodeoxycholic Acid (UDCA), Glycocholic Acid (GCA), Glycochenodeoxycholic Acid (GCDCA), Glycodeoxycholic Acid (GDCA), Glycoursodeoxycholic Acid (GUDCA), Taurocholic Acid (TCA), Taurochenodeoxycholic Acid (TCDCA), Taurodeoxycholic Acid (TDCA), Taurolithocholic Acid (TLCA), Tauroursodeoxycholic Acid (TUDCA), and Glycolithocholic Acid (GLCA).

Table 3.19 Correlations between urine Uros and levels of fecal bile acids after 3 wks of walnut consumption[1]

	UroA		**UroB**		**UroC**		**UroD**		**UroAG**		**UroBG**		**IsoUroAG**		**EA**	
	all	***UM-A***	***all***	***UM-A***	***all***	***UM-A***	***all***	***UM-A***	***all***	***UM-A***	***all***	***UM-A***	***all***	***UM-A***	***all***	***UM-A***
CDCA	-0.29 (0.13)	-0.24 (0.32)	**-0.39 (0.041)***	NA	-0.11 (0.56)	0.19 (0.44)	**-0.46 (0.014)***	**-0.49 (0.031)***	-0.22 (0.26)	-0.1 (0.68)	-0.09 (0.65)	0.19 (0.43)	0.03 (0.88)	0.031 (0.9)	**-0.41 (0.031)***	-0.38 (0.11)
CA	**-0.47 (0.012)***	-0.16 (0.5)	-0.23 (0.24)	NA	-0.28 (0.16)	0.14 (0.58)	**-0.39 (0.04)***	-0.41 (0.079)	**-0.43 (0.021)***	-0.11 (0.65)	0.11 (0.56)	0.28 (0.24)	-0.17 (0.4)	0.062 (0.79)	-0.19 (0.34)	-0.23 (0.34)
DCA	**-0.49 (0.0083)****	-0.4 (0.091)	-0.035 (0.072)	NA	**-0.57 (0.0016)****	**-0.49 (0.036)***	**-0.45 (0.017)***	**-0.47 (0.045)***	**-0.59 (0.001)****	**-0.52 (0.023)***	-0.036 (0.86)	0.2 (0.41)	**-0.44 (0.019)***	-0.2 (0.41)	-0.33 (0.087)	-0.2 (0.41)
GCDCA	-0.28 (0.15)	-0.3 (0.21)	**-0.37 (0.05)**	NA	-0.23 (0.25)	-0.047 (0.85)	**-0.39 (0.038)***	-0.32 (0.18)	-0.22 (0.27)	-0.058 (0.81)	-0.085 (0.67)	0.29 (0.23)	-0.081 (0.68)	-0.047 (0.85)	-0.22 (0.25)	-0.025 (0.92)
GCA	-0.22 (0.26)	-0.13 (0.6)	-0.22 (0.25)	NA	-0.22 (0.25)	0.011 (0.97)	-0.32 (0.099)	-0.21 (0.39)	-0.13 (0.5)	0.056 (0.82)	0.048 (0.81)	0.41 (0.082)	-0.029 (0.88)	0.075 (0.76)	-0.24 (0.22)	-0.056 (0.82)
GDCA	-0.24 (0.21)	-0.3 (0.22)	-0.24 (0.21)	NA	-0.26 (0.18)	-0.15 (0.53)	-0.27 (0.16)	-0.19 (0.23)	-0.19 (0.33)	-0.11 (0.65)	-0.047 (0.81)	0.43 (0.068)	-0.11 (0.57)	0.055 (0.82)	-0.24 (0.21)	-0.13 (0.59)
GLCA	-0.07 (0.72)	0.15 (0.53)	-0.17 (0.39)	NA	-0.31 (0.11)	-0.16 (0.51)	-0.23 (0.24)	-0.27 (0.26)	-0.18 (0.36)	-0.037 (0.88)	0.054 (0.79)	**0.46 (0.05)**	-0.053 (0.79)	0.12 (0.62)	0.23 (0.24)	0.16 (0.5)
GUDCA	-0.36 (0.063)	-0.18 (0.46)	**-0.39 (0.043)***	NA	-0.37 (0.056)	-0.018 (0.94)	**-0.48 (0.01)***	-0.35 (0.15)	**-0.42 (0.027)***	-0.18 (0.45)	-0.2 (0.32)	-0.16 (0.52)	-0.12 (0.55)	0.022 (0.93)	-0.035 (0.86)	-0.066 (0.79)
LCA	-0.25 (0.2)	-0.15 (0.54)	-0.3 (0.13)	NA	**-0.39 (0.041)***	-0.26 (0.27)	**-0.38 (0.049)***	-0.38 (0.11)	-0.35 (0.066)	-0.28 (0.25)	0.0016 (0.99)	0.19 (0.42)	-0.27 (0.17)	-0.025 (0.92)	-0.36 (0.064)	-0.14 (0.57)
TCDCA	-0.14 (0.49)	-0.18 (0.45)	-0.069 (0.73)	NA	-0.14 (0.46)	-0.042 (0.87)	-0.23 (0.23)	-0.14 (0.56)	0.02 (0.92)	0.13 (0.6)	-0.091 (0.64)	0.16 (0.51)	0.16 (0.4)	0.3 (0.21)	-0.3 (0.12)	-0.13 (0.61)
TCA	-0.2 (0.31)	-0.26 (0.27)	-0.086 (0.67)	NA	-0.24 (0.22)	-0.13 (0.59)	-0.29 (0.14)	-0.22 (0.37)	-0.086 (0.66)	-0.0097 (0.97)	0.021 (0.92)	0.23 (0.35)	0.075 (0.7)	0.094 (0.7)	-0.28 (0.15)	-0.18 (0.45)

TDCA	**-0.42 (0.028)***	**-0.49 (0.037)***	-0.15 (0.45)	NA	-0.31 (0.1)	-0.24 (0.32)	**-0.37 (0.05)**	-0.42 (0.072)	-0.32 (0.097)	-0.23 (0.34)	-0.029 (0.88)	0.23 (0.35)	-0.23 (0.24)	-0.013 (0.96)	-0.27 (0.16)	-0.29 (0.23)
TLCA	-0.31 (0.11)	-0.39 (0.1)	-0.14 (0.49)	NA	-0.22 (0.26)	-0.082 (0.74)	**-0.43 (0.024)***	**-0.54 (0.017)***	-0.24 (0.21)	-0.17 (0.48)	0.023 (0.91)	0.3 (0.21)	-0.19 (0.33)	0.015 (0.95)	-0.34 (0.079)	-0.37 (0.12)
TUDCA	**-0.41 (0.031)***	-0.26 (0.29)	-0.23 (0.24)	NA	-0.24 (0.22)	-0.056 (0.82)	**-0.4 (0.035)***	**-0.46 (0.047)***	**-0.38 (0.045)***	-0.23 (0.34)	0.21 (0.29)	0.21 (0.39)	-0.14 (0.48)	-0.007 (0.98)	-0.25 (0.2)	-0.27 (0.26)
UDCA	-0.34 (0.076)	-0.35 (0.15)	0.027 (0.89)	NA	**-0.42 (0.027)***	**-0.46 (0.047)***	-0.093 (0.64)	-0.11 (0.66)	-0.31 (0.11)	-0.26 (0.27)	-0.14 (0.48)	-0.061 (0.8)	-0.16 (0.42)	-0.082 (0.74)	0.31 (0.11)	0.15 (0.55)

[1]Bivariate correlations were conducted to assess the relations between variables. “All” n = 30, “UM-A” n = 21. Pearson’s correlations were used for normally distributed variables and Spearman correlation for nonnormally distributed variables. P-values marked with * indicate statistical significance ($p < 0.05$).P-values marked with * indicate statistical significance ($p < 0.05$). P-values marked with ** indicate statistical significance ($p < 0.01$). P-values marked with *** indicate statistical significance ($p < 0.001$).

Uro-A, urolithin A; Uro-B, urolithin B; Uro-C, urolithin C, Uro-D, urolithin D, Uro AG, urolithin A glucuronide; Uro-BG, urolithin B glucuronide; IsoUro-AG, Isourolithin A glucuronide; EA, ellagic acid; Cholic Acid (CA), Chenodeoxycholic Acid (CDCA), Deoxycholic Acid (DCA), Lithocholic Acid (LCA), Ursodeoxycholic Acid (UDCA), Glycocholic Acid (GCA), Glycochenodeoxycholic Acid (GCDCA), Glycodeoxycholic Acid (GDCA), Glycoursodeoxycholic Acid (GUDCA), Taurocholic Acid (TCA), Taurochenodeoxycholic Acid (TCDCA), Taurodeoxycholic Acid (TDCA), Taurolithocholic Acid (TLCA), Tauroursodeoxycholic Acid (TUDCA), and Glycolithocholic Acid (GLCA)

Table 3.20 Correlations between urolithin metabolites after walnut consumption and participants' weight and BMI[1]

	Plasma				Urine			
	Weight		**BMI**		**Weight**		**BMI**	
	r	***p***	***r***	***p***	***r***	***p***	***r***	***p***
UroA	0.058	NS	-0.23	NS	0.11	NS	-0.24	NS
UroB	0.098	NS	0.21	NS	-0.06	NS	-0.23	NS
UroC	0.21	NS	0.21	NS	0.05	NS	0.19	NS
UroD	-0.21	NS	0.13	NS	0.05	NS	0.01	NS
UroAG	0.12	NS	0.13	NS	0.03	NS	-0.17	NS
UroBG	0.15	NS	0.19	NS	0.08	NS	0.10	NS
IsoUroAG	0.16	NS	0.28	NS	0.08	NS	0.02	NS
EA	-0.48	**0.007****	0.09	NS	-0.34	NS	0.05	NS

[1]Bivariate correlations were conducted to assess the relations between variables. "All" n = 30, "UM-A" n = 21. Pearson's correlations were used for normally distributed variables and Spearman correlation for nonnormally distributed variables. P-values marked with ** indicate statistical significance ($p < 0.01$).

Uro-A, urolithin A; Uro-B, urolithin B; Uro-C, urolithin C, Uro-D, urolithin D, Uro AG, urolithin A glucuronide; Uro-BG, urolithin B glucuronide; IsoUro-AG, Isourolithin A glucuronide; EA, ellagic acid.

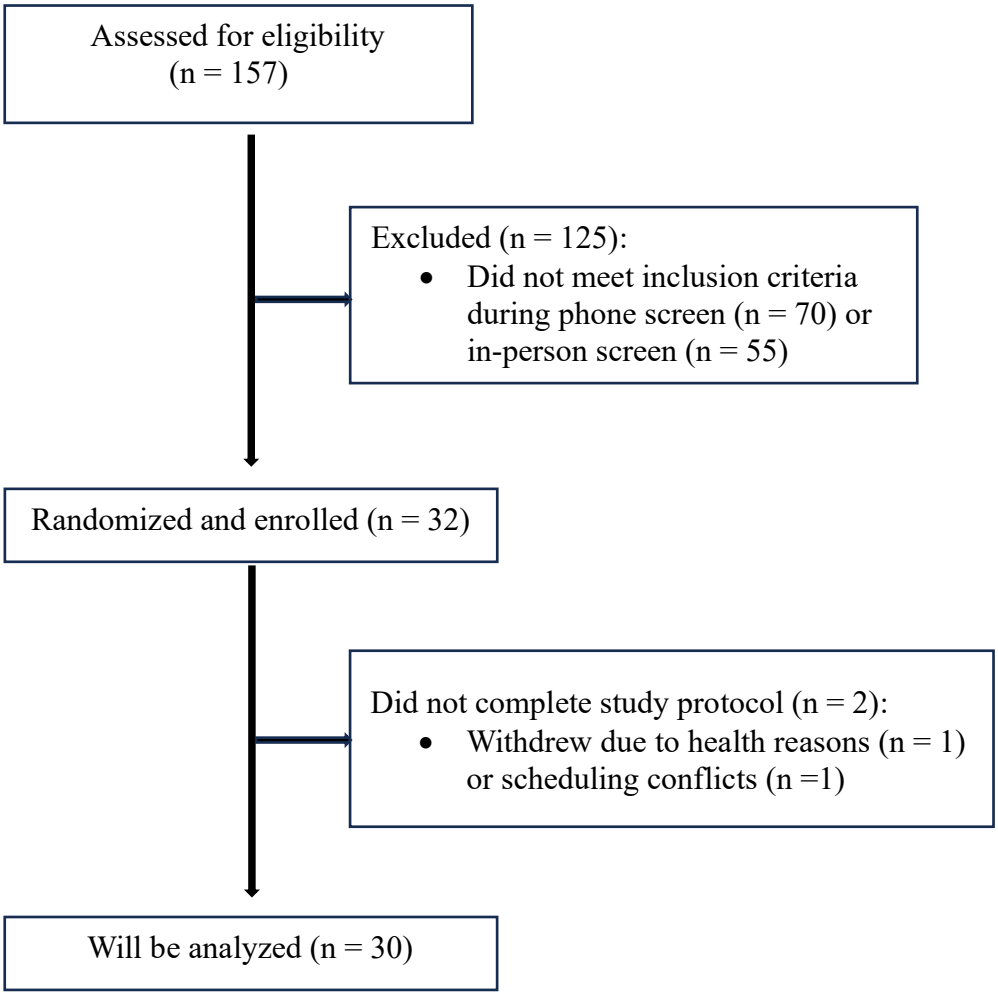


Figure 3.1 CONSORT flow diagram

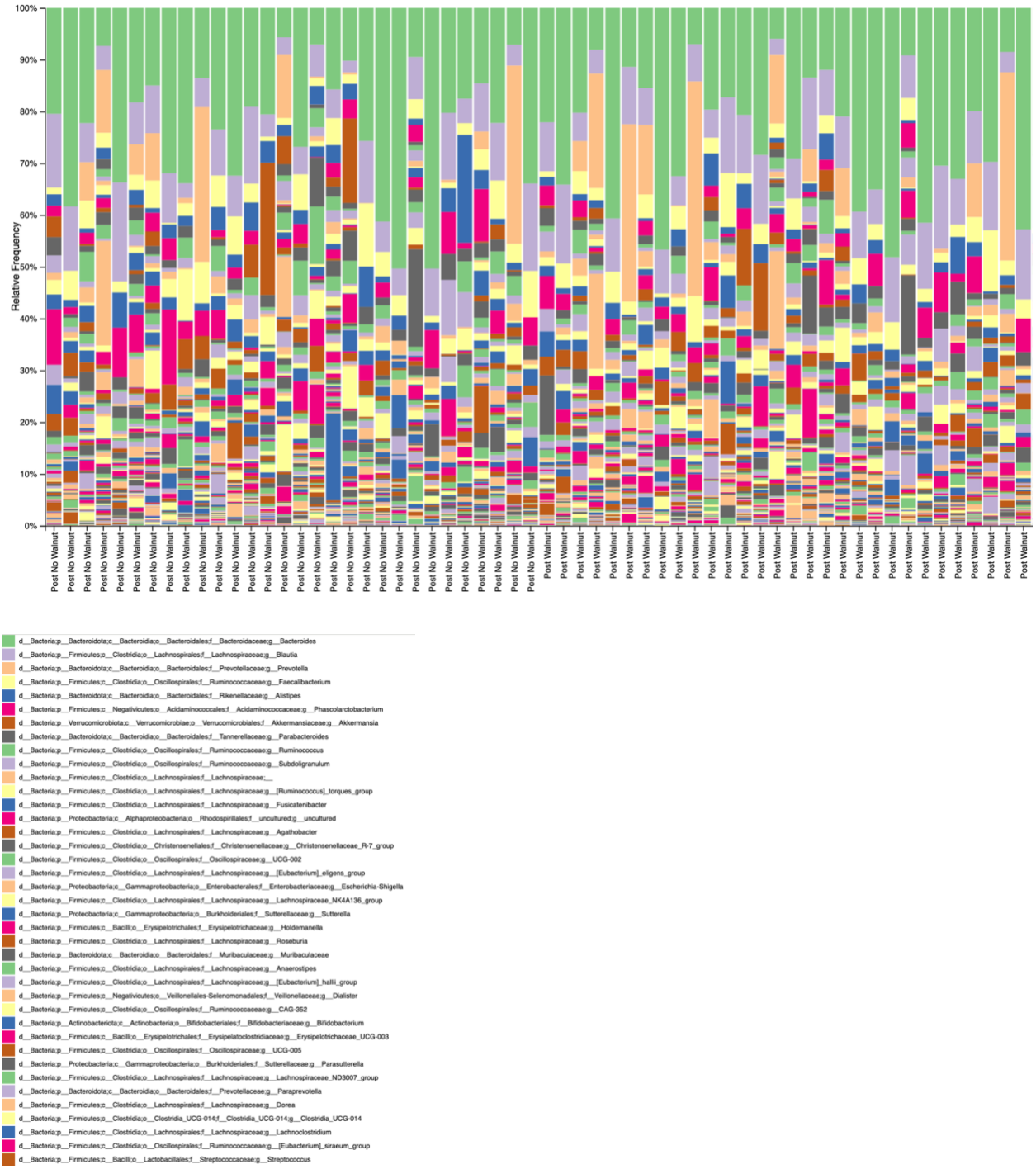


Figure 3.2 Bar plots of percentage bacterial relative abundancies after 3 wks of walnut vs. no walnut consumption, n=30.

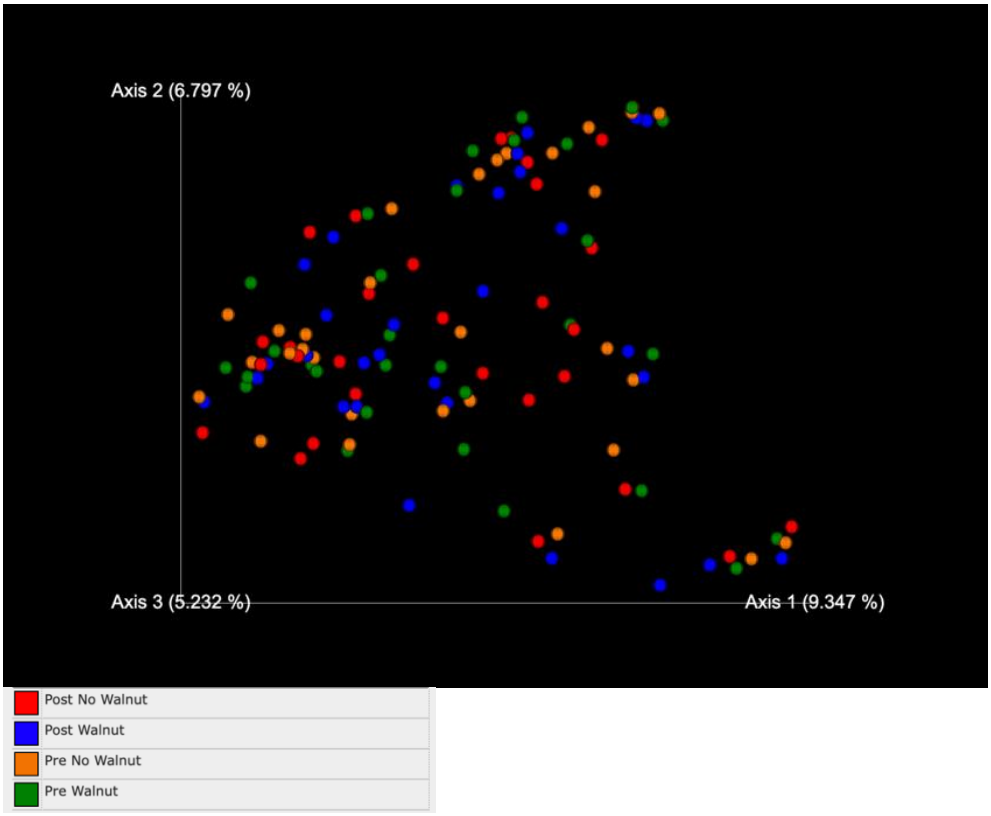


Figure 3.3 Bray-Curtis PCoA plot with data organized by intervention and timepoint

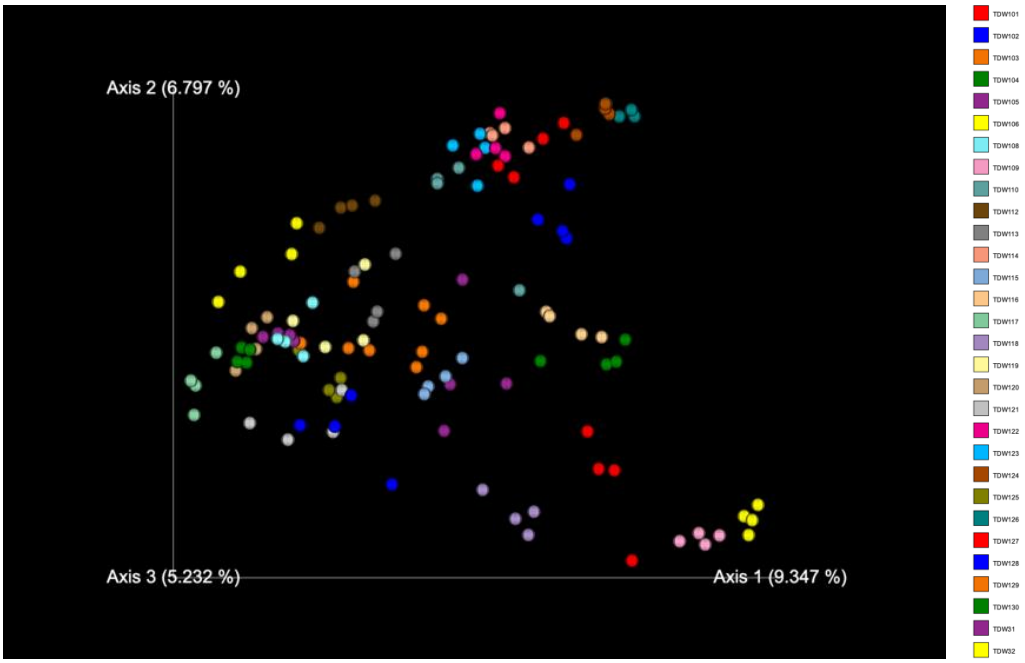


Figure 3.4 Bray-Curtis PCoA plot with data organized by participant

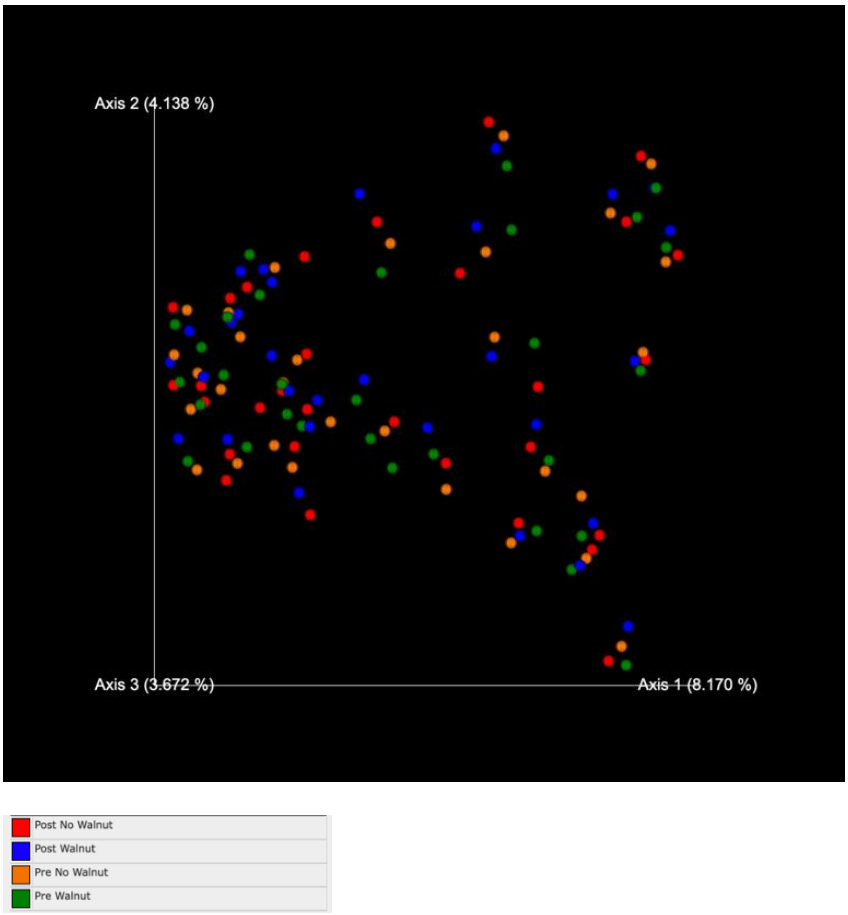


Figure 3.5 Unweighted Jaccard PCoA plot with data organized by intervention and timepoint

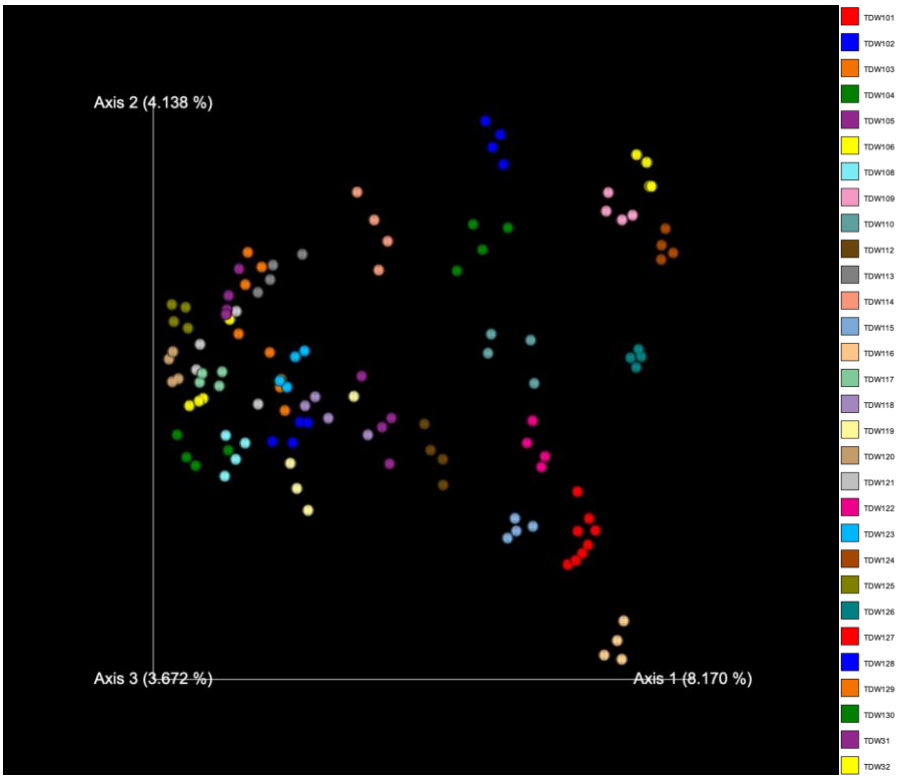


Figure 3.6 Unweighted Jacard PCoA plot with data organized by participant

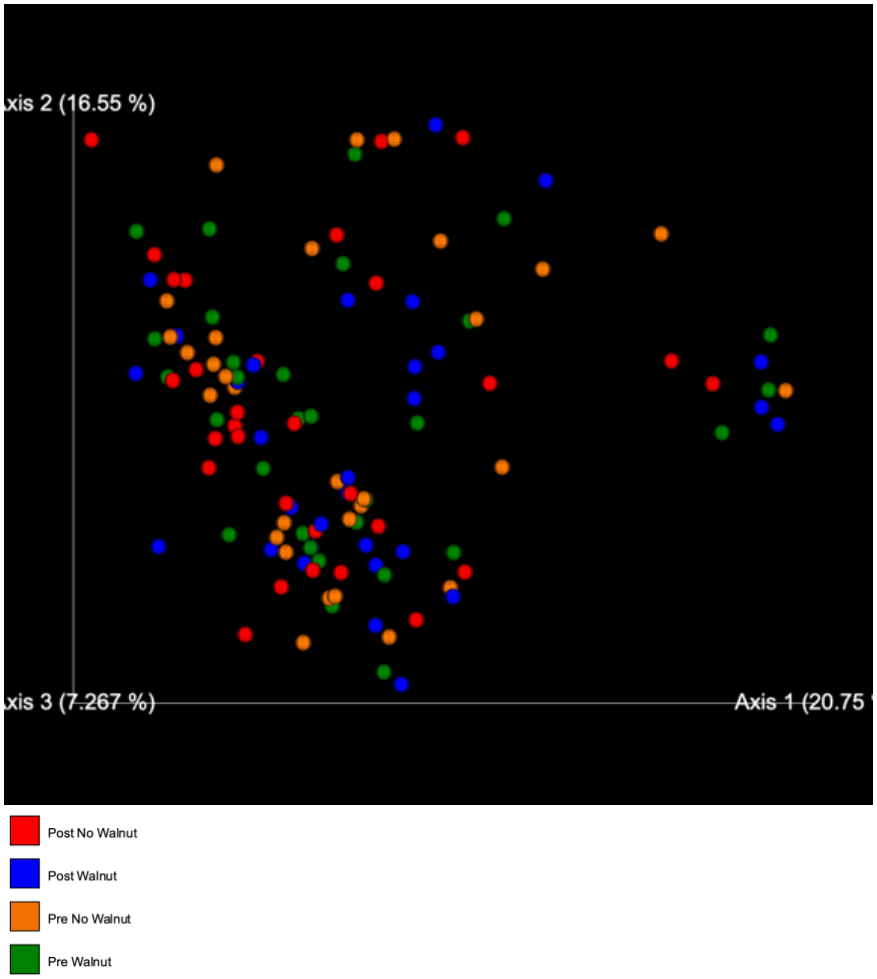


Figure 3.7 Weighted Jaccard PCoA with data organized by treatment and timepoint

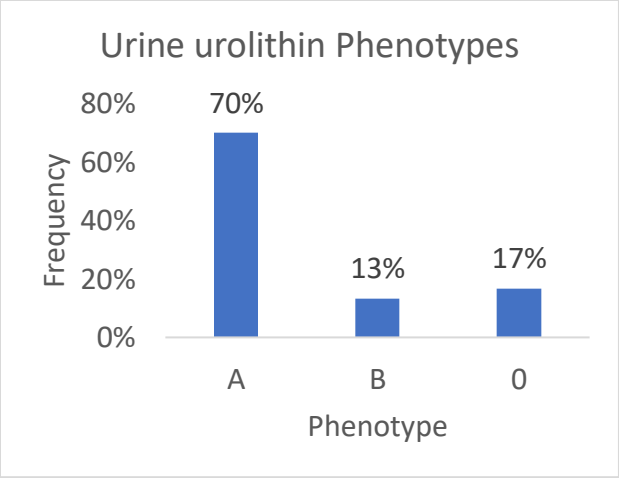


Figure 3.8 Urolithin phenotype distribution

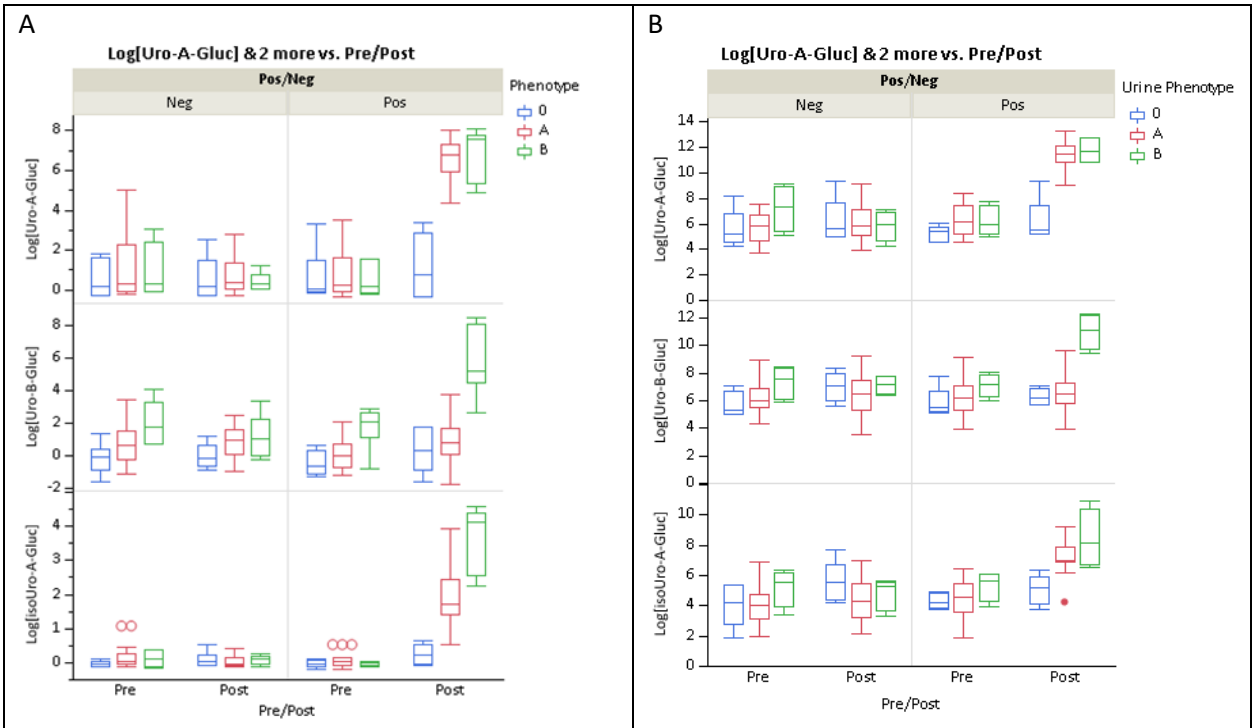
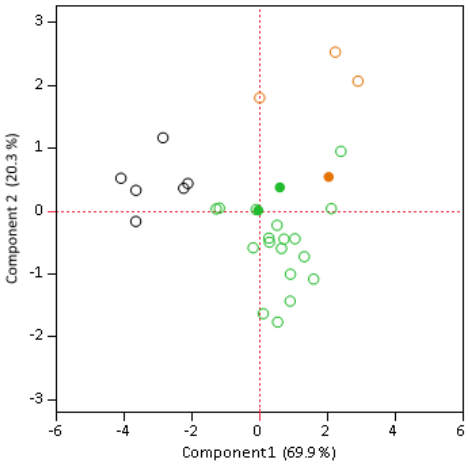
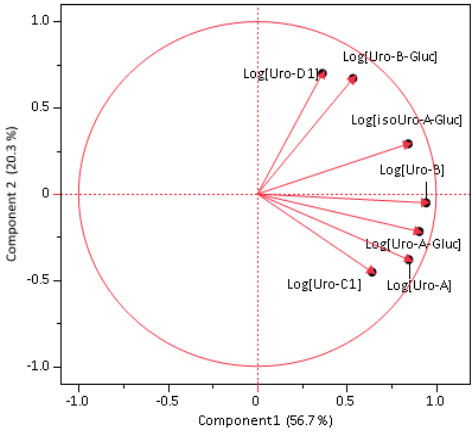


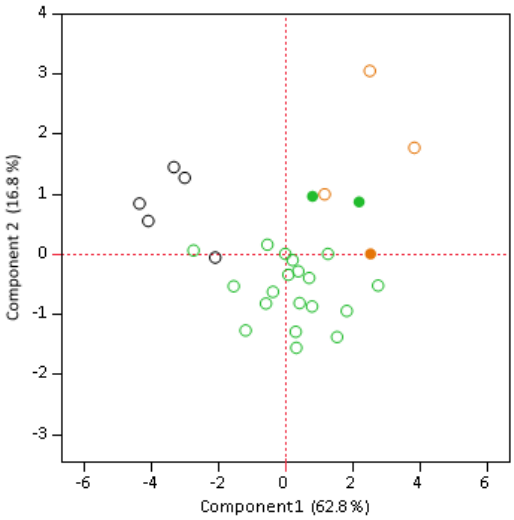
Figure 3.9 Urolithin responses by estimated phenotype using agnostic data dependent treatment group assignments for plasma (A) and urine (B). Neg –control treatment; Pos – walnut treatment; Pre – pre intervention; Post – post intervention



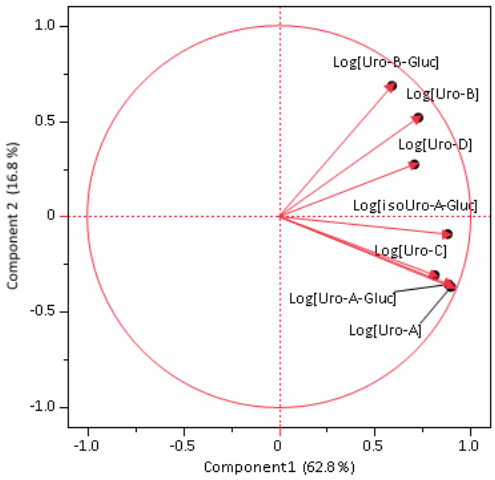
A. Plasma principle components scores plot. Phenotypes are indicated by color: Green = A; Orange = "B"Black = "0".



B. Plasma principle components loadings plot. Presence of urolinthins increases to the right, dominance shifts from Urolithin A to Urolithin B from bottom to top.



C. Urine principle components scores plot. Phenotypes are indicated by color: Green = A; Orange = "B"Black = "0".



D. Urine principle components loadings plot. Presence of urolinthins increases to the right, dominance shifts from Urolithin A to Urolithin B from bottom to top.

Figure 3.10 Urine and plasma urolithin principle component score plots

7. Supplemental materials

Supplemental Table 3.1 Intake of macronutrients and major micronutrients on walnuts and no walnuts[1]

Nutrient	Walnut	No walnut
Calories (kcal)	1890.7 ± 827.2	1735.2 ± 696.0
Protein (g)	74.0 ± 37.1	68.5 ± 32.4
Carbohydrates (g)	201.3 ± 92.0	207.6 ± 99.7
Total fiber (g)	18.3 ± 10.2	17.3 ± 11.9
Soluble fiber (g)	1.5 ± 1.7	0.8 ± 1.3
Insoluble fiber (g)	3.0 ± 3.0	1.7 ± 3.4
Sugar (g)	77.2 ± 50.5	81.0 ± 53.8
Fat (g)	89.3 ± 52.1	69.8 ± 35.2
Saturated (g)	26.4 ± 19.8	24.0 ± 13.4
Monounsaturated (g)	19.1 ± 13.4	16.6 ± 12.1
Polyunsaturated (g)	23.0 ± 25.3	9.1 ± 7.7
Trans fat (g)	0.4 ± 0.6	0.4 ± 0.5
Omega-3 (g)	3.6 ± 4.6	0.9 ± 0.9
Omega (6)	18.7 ± 20.7	7.3 ± 6.6
Cholesterol (mg)	245.4 ± 180.1	257.8 ± 392.4
Vitamin A (IU)	2175.5 ± 3534.9	2257.1 ± 2482.5
Vitamin D (IU)	100.3 ± 128.9	104.0 ± 136.8
Vitamin E (IU)	2.6 ± 2.4	2.5 ± 3.0
Vitamin K (mcg)	84.5 ± 218.0	67.7 ± 73.9
Vitamin C (mg)	58.8 ± 71.1	68.7 ± 109.8
Calcium (mg)	717.2 ± 516.9	653.3 ± 372.7
Iron (mg)	11.1 ± 7.1	10.6 ± 5.8
Zinc (mg)	7.3 ± 5.6	6.5 ± 5.6
Magnesium (mg)	201.8 ± 125.7	170.1 ± 109.2
Sodium (mg)	2850.2 ± 1888.1	2845.8 ± 1553.8
Potassium (mg)	2076.6 ± 1044.5	2030.3 ± 946.5
Caffeine (mg)	109.0 ± 129.1	113.4 ± 114.8
Alcohol (g)	3.2 ± 10.0	3.3 ± 11.0

[1]Values are mean ± SD. Food records from the day before each study visit and 2 days (1 weekend day and 1 weekday) during each treatment period were collected. The total set of twelve days of food records were analyzed using Food Processor (SQL, Version 10.2, ESHA Research, Salem, OR). Food records were used to estimate participants' macronutrient and micronutrient intake on the walnut and no walnut intervention arms.

Supplemental Table 3.2 %B at Peak in plasma and urine and corresponding metabotype of all individuals

Subject	%B at Peak in plasma	%B at Peak in urine	Metabotype
1	7%	3%	A
2	11%	8%	A
3	2%	1%	A
4	66%	26%	B
5	0%	0%	A
6	1%	0%	A
7	0%	2%	A
8	-	-	0
9	-	-	0
10	0%	0%	A
11	1%	3%	A
12	0%	0%	A
13	-	-	0
14	0%	0%	A
15	1947%	250%	B
16	0%	1%	A
17	0%	0%	A
18	6%	14%	A
19	268%	59%	B
20	1%	0%	A
21	0%	1%	A
22	-	-	0
23	5%	4%	A
24	0%	3%	A
25	400%	340%	B
26	0%	0%	A
27	2%	8%	A
28	1%	0%	A
29	0%	0%	A
30	-	-	0

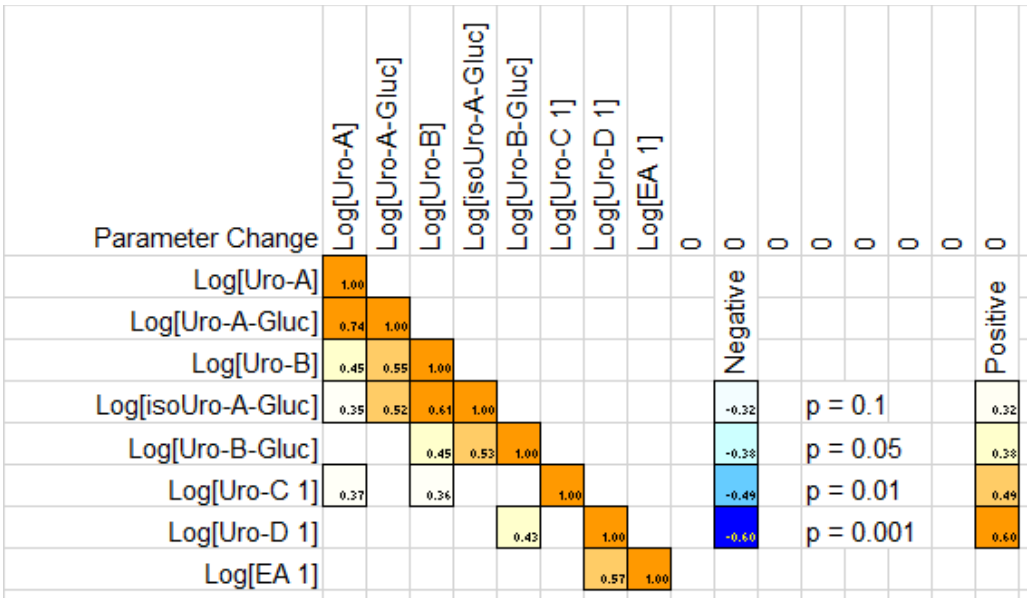
Supplemental Table 3.3 Urolithin levels in participant plasma and urine after clustering into urolithin metabotypes[1]

Urolithin metabolite, *uM*	**Plasma**			**Urine**		
	UM-A	***UM-B***	***UM-0***	***UM-A***	***UM-B***	***UM-0***
UroA	3.24 (2.93)	2.43 (2.47)	0.09 (0.07)	12819.44 (26247.56)	35391.50 (58627.73)	25.66 (51.62)
UroB	6.38 (4.67)	19.55 (13.87)	1.52 (2.42)	153.98 (193.67)[2]	5963.97 (7012.14)	12.26 (27.41)
UroC	26.92 (46.04)	0.12 (8.67)	0.14 (0.07)	991.23 (1968.67)	986.25 (569.48)	12.61 (7.75)
UroD	1.38 (0.13)	1.53 (0.13)	1.29 (0.07)	15.10 (5.17)	22.12 (11.49)	12.09 (1.22)
UroAG	1116.10 (851.53)	1327.25 (1448.52)	3.45 (5.13)	158403.68 (150587.99)	177800 (151059.02)	2408.80 (4914.80)
UroBG	12.95 (33.58)	1901.01 (2162.72)	0.98 (1.20)	1989.84 (3380.31)	100475.02 (94326.57)	1439.80 (1640.29)
IsoUroAG	15.56 (22.66)	44.31 (41.63)	1.13 (0.31)	2247.72 (2371.33)	16372.75 (26335.80)	666.34 (832.51)
EA	151.74 (115.31)	117.87 (66.13)	148.54 (119.36)	899.63 (537.94)	1037.50 (386.05)	1068.80 (776.56)

[1]Data are presented as untransformed means (SD).

[2] n = 11 (Urine UroB levels were detected in 11 individuals only)

Uro-A, urolithin A; Uro-B, urolithin B; Uro-C, urolithin C, Uro-D, urolithin D, Uro AG, urolithin A glucuronide; Uro-BG, urolithin B glucuronide; IsoUro-AG, Isourolithin A glucuronide; EA, ellagic acid; UM-A, urolithin metabotype A, UM-B, urolithin metabotype B; UM-), urolithin metabotype 0.



Supplemental Figure 3.4 Pearson's correlation matrix across all samples (excluding Phenotype 0) the urolithin metabolite associations in plasma.

Chapter 4

Effects of low- and high-polyphenol olive oil on biomarkers of inflammation and cytokine release from whole blood and PBMCs after stimulation with LPS in healthy adult subjects

Abbreviations: ALA, alpha linolenic acid; ApoA1, apolipoprotein A1; BMI, body mass index; CBD, cannabidiol; CEC, cholesterol efflux capacity; CHD, coronary heart disease; CI, confidence interval; CMP, comprehensive metabolic panel; CO, canola oil; COX-2, cyclooxygenase 2; CPT, cell preparation tube; CRP, C-reactive protein; CVD, cardiovascular disease; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; ET-1, endothelin 1; FDA, Food and Drug Administration; HDL, high density lipoprotein; HPOO, high polyphenol olive oil; HT, hydroxytyrosol; IFN- γ, interferon gamma 1; IL-1ß, interleukin 1b; IL-6, interleukin 6; IL-10, interleukin 10; IL-17, interleukin 17; IRAK, interleukin-1 receptor-associated kinase; LCAT, lecithin-cholesterol acyltransferase; LDL, low density lipoprotein; LPOO, low polyphenol olive oil; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1; MetS, metabolic syndrome; MUFA, monounsaturated fatty acids; MyD88, myeloid differentiation primary response 88; NF-kB, nuclear factor kappa B; NOX, nicotinamide adenine dinucleotide phosphate oxidase; OLE, oleuropein; OO, olive oil; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffered saline; PON-1, paraoxonase 1; PST, plasma separating tube; ROO, refine olive oil; RPMI, Roswell Park Memorial Institute; SH, sodium heparin; SST, serum separating tube; TC, total cholesterol; TG, triglycerides; TLR4, toll-like receptor 4; TMD, traditional Mediterranean diet; TNF-a, tumor necrosis alpha; TRAF-6, tumor necrosis 6 associated factor; T2DM, type 2 diabetes mellitus; UC, University of California; US, United States; VOO, virgin olive oil; WC, waist circumference.

Abstract

This study investigated the effects of consumption of two types of olive oil on biomarkers of cardiometabolic health in acute and short-term consumption settings. This chapter will focus on the influence of olive oil intake on circulating blood cytokines and on cytokine secretion from whole blood and peripheral blood mononuclear cells (PBMCs) with or without bacterial lipopolysaccharide (LPS) stimulation. Twenty-six generally healthy adults (BMI >18.5 kg/m^2) participated in this randomized crossover trial. Participants were randomized to consume 40 ml of low-polyphenol olive oil (LPOO) or high-polyphenol olive oil (HPOO) daily for 2 weeks to determine effects of short-term consumption, followed by a washout period and a 2 week intervention with daily consumption of the alternative olive oil. Assessment of metabolic responses were performed at the beginning and end of each intervention period at fasting measurement and following acute consumption (2hr and 4hr postprandial), after participants consumed their respective treatment. There were no notable differences between LPOO and HPOO treatments in levels of systemic cytokines and in cytokine secretion from stimulated or unstimulated whole blood. Short-term HPOO consumption resulted in lower IL-1ß levels secreted from LPS stimulated PBMCs compared to LPOO. There were no other effects of short-term consumption on any other cytokines for either type of olive oil. Differences were observed in cytokine release from PBMCs during the acute postprandial period. IL-6 levels spontaneously secreted from PBMCs were higher 2hrs after HPOO intake compared to LPOO, while TNF-a levels were higher 2hrs and 4hrs after HPOO vs. LPOO. In contrast, TNF-a levels secreted from LPS stimulated PBMCs were lower 4 hrs after HPOO consumption compared to LPOO. In conclusion, HPOO short-term consumption resulted in decreased levels of IL-1ß secreted from LPS-stimulated PBMCs only. Acute HPOO consumption attenuated TNF-a secretion from LPS

stimulated PBMCs. In contrast, TNF-a and IL-6 secretion spontaneously secreted from PBMCs was higher after HPOO consumption compared to LPOO. This trial was registered on clinicaltrials.gov as NCT05321277.

Keywords: olive oil; polyphenol; postprandial; inflammation; cytokine; LPS

1. Introduction

Cardiovascular disease (CVD) is the most common cause of death worldwide. Overweight and obesity are recognized as major risk factors for CVD [1]. Conditions commonly associated with obesity including dyslipidemia, endothelial dysfunction, and inflammation may also contribute to CVD [2, 3].

Expansion of the adipose tissue in overweight and obese states has been associated with inflammatory changes within adipose tissue, which contributes to chronic low-grade inflammation [4]. Low-grade inflammation is usually characterized by slightly elevated levels of circulating cytokines, chemokines, and acute phase reactants [4]. Increase in adipose tissue is commonly accompanied by infiltration of new inflammatory cells, macrophages in particular. In fact, evidence shows that macrophages comprise approximately 5-10% of total cells in lean adipose tissue vs. 60% in obese adipose tissue [5]. This process also plays a role in the development of atherosclerotic plaque where oxidized low-density lipoprotein (oxLDL) damages vascular endothelial cells, causing them to release monocyte chemoattractant protein 1 (MCP-1) [6]. This attracts monocytes to the site, which mature into macrophages, take up the oxLDL, and develop into foam cells, which release further pro-inflammatory cytokines, causing the plaque to grow [7]. Several other inflammatory molecules are associated with an increased risk for CVD, including C-reactive protein (CRP), interleukin-6 (IL-6), tumor-necrosis factor alpha (TNF-a) [7], and IL-1β [8]. TNF-a is a pro-inflammatory adipokine that has been positively associated

with obesity, insulin resistance and atherosclerosis [9]. It is highly expressed by macrophages from vascular stroma, M1 macrophages specifically, primarily in the VAT of obese patients [11-13]. This cytokine may inhibit lipoprotein lipase (LPL) activity and increase fatty acid mobilization from adipose tissue into the blood stream [14-15]. TNF-a affects synthesis of other pro-inflammatory adipokines through NF-kB activation**,** which results in expression of IL-6, chemokine (C-C motif ligand 2 (CCL2), and TNF-a itself [16-18]. IL-6 plays a role in inflammation, tissue injury and host defense [17]. Approximately one third of circulating IL-6 originates from adipose tissue [9] and an increase in IL-6 is positively associated with obesity, glucose intolerance, insulin resistance, T2DM and may serve as predictors of metabolic syndrome and CVD [9]. IL-1ß is produced by M1 macrophages. It may impair insulin signaling and increases lipolysis. In obese patients with hyperleptinemia circulating levels of IL-1ß antagonist, IL-RA, are seven times higher compared to nonobese patients [19-20].

Epidemiologic evidence has found that plant-based diets rich in fruits and vegetables are inversely associated with CVD, however the cellular mechanisms of this protective relationship have yet to be confirmed [21]. The traditional Mediterranean diet (TMD) has been shown to provide cardioprotective effects [22-23]. Some of the main characteristics of TMD are high fruit, vegetable, legume, and grain intake; regular but moderate wine consumption; moderate fish consumption; low meat intake and low to moderate dairy product intake [24]. Olive oil (OO) is the main source of fat in the TMD. Monounsaturated fatty acids (MUFA) represent the major component of OO with oleic acid contributing to 55-83% of the total lipid composition [25]. In 2004, the Food and Drug Administration (FDA) approved a claim suggesting that consumption of approximately two tablespoons of OO daily may exert beneficial effects on coronary heart disease (CHD) risk, due to its MUFA content [26]. However, if MUFA content alone is

responsible for cardioprotective effects of OO, any type of MUFA-rich oil - rapeseed, canola, sunflower oil - would provide the same protection, which is in contradiction with recent evidence [27]. Studies that have directly compared OO with other MUFA-rich oils (i.e. flaxseed and sunflower oil) showed that OO consumption had a greater effect on LDL oxidation, lipoprotein concentration and LDL particle size [28-29]. This is likely due to the presence of polyphenols in OO, with higher amounts found in virgin (VOO) and extra virgin OO (EVOO). In fact, the European Food Safety Authority (EFSA) recently concluded that there is a cause-and-effect relationship between the consumption of OO polyphenols and protection of LDL particles from oxidative damage [30].

The composition of phenols in OO is rich and diverse, including such compounds as: alcohols hydroxytyrosol (HTyr) and tyrosol (Tyr); caffeic, gallic, p-coumaric, protocatechuic, sinapic, syringic and vanillic acids; flavonoids apigenin and luteolin; secoiridoids oleuropein (OLE) glucoside and aglycones of oleuropein; lignans (+)-pinoresinol and (+)-1-acetoxy-pinoresinol [31]. The main functional polyphenols in OO are HTyr and OLE. Several recent studies demonstrated that these polyphenolic compounds may provide protection from CVD by modulating key pathways related to inflammation, oxidative stress, homocysteine, cholesterol levels and cell adhesion [32-33]. In fact, Hernaez et al. [34] have recently shown that phenolic compounds in OO were able to increase cholesterol efflux capacity (CEC), which reflects the capacity of high-density lipoprotein cholesterol (HDL-C) to perform reverse cholesterol transport. Interestingly, besides an increase in CEC, higher levels of OO phenolic compound metabolites on HDL surface were also observed in this study. As suggested by Hernaez et al. [34], OO phenolic metabolites could be exerting a local antioxidant effect on HDL particles protecting apolipoprotein A-I (ApoA-I) and HDL lipids from oxidative modifications, making

ApoA-I more stable and the HDL particle more fluid and functional. This was also confirmed in the Virgin Olive Oil and HDL Functionality (VOHF) study [35], where consumption of polyphenol-enriched OO resulted in an increase in HDL-C levels and its oxidative status, an increase in HDL_2-subclass (inversely associated with cardiovascular risk [35]), as well as lecithin-cholesterol acyltransferase (LCAT) mass.

In addition, EVOO polyphenols are known to reduce energy harvesting in the gut by interacting with digestive enzymes [36-37] and modulate gut microbiota composition/functionality [38-41], as well as have anti-inflammatory effects. Once these compounds are metabolized and absorbed into the bloodstream they can interact with tissues and exert their beneficial effects. For instance, they are known to reduce expression of ROS generating enzymes/pathways, increase expression of antioxidant enzymes [42-47], and modulate expression of enzymes involved in cell immune responses [48-56]. One of the main anti-inflammatory mechanisms of action of phenolic compounds is through inhibition of eicosanoids biosynthesis (e.g. PGE2, leukotriene B4 (LTB4), thromboxane B2 (TXB2)) [57-58]. Phenolic compounds may also inactivate production of COX1 and 12-LOX pathway-derived arachidonic acid radicals [59-66].

Peripheral blood mononuclear cells (PBMCs) have been identified as carriers of systemic immune signals. Changes in their transcriptome have been used as biomarkers of several pathologies, such as inflammation, metabolic syndrome, cancer, etc. and are proposed to be used as markers of response to nutrition intervention [67]. A recent study conducted by D'Amore and colleagues [67] demonstrated that EVOO polyphenolic compounds may be participating in activation of several pathways involved in PBMC functions in healthy individuals as well as patients with metabolic syndrome (MetS). Acute intake (4 hr) of EVOO rich in polyphenols in

this study was able to improve glycemia and insulin sensitivity; upregulate CD28 signaling in T helper cells and Fcγ receptor-mediated phagocytosis in macrophage and monocyte pathways; suppress nuclear factor of activated T-cells (NFAT) and phosphoinositide 3-kinases (PI3K) signaling in B cells; as well as suppress both B and T cell receptors, NF-κB, IL1/3/8, receptor activator of nuclear factor k B (RANK), thrombin signaling cascades, lipopolysaccharide (LPS)-stimulated mitogen-activated protein kinases (MAPK) activation and nuclear factor erythroid 2-related factor 2 (NRF2)-mediated oxidative stress responses in healthy subjects [67]. Interestingly, these effects were less pronounced in individuals with MetS and after administration of EVOO low in polyphenols, which points to the importance of polyphenolic compounds and health status of the subject receiving EVOO [67].

The present study aimed to assess the effects of polyphenol-rich olive oil on HDL function, LDL oxidation and biomarkers of inflammation after acute and short-term consumption in healthy adults. We hypothesized that HDL function as measured by ex vivo CEC, LDL oxidation, and inflammatory cytokines will be lower following consumption of polyphenol-rich olive oil. This chapter will specifically focus on outcomes relating to levels of systemic cytokines and secretion of cytokines from whole blood and peripheral blood mononuclear cells (PBMCs) unstimulated or stimulated with bacterial lipopolysaccharide (LPS). Results relating to the remaining outcomes will be reported in a dissertation chapter of another graduate student in Steinberg laboratory.

2. Methods and materials

2.1 Study participants

Generally healthy male and female subjects within the range of 20-45 years of age and BMI ≥18.5kg/m^2 were recruited from the Davis and Sacramento metropolitan area. This study

was approved by the University of California (UC), Davis Institutional Review Board and written informed consent of all participants was obtained prior to enrollment. This study was registered at clinicaltrials.gov as NCT05321277. Interested participants were screened to determine eligibility. Adults unable to consent, individuals who are not yet adults (infants, children, teenagers), pregnant or lactating women, women planning to become pregnant in the next two months and prisoners were specifically excluded. Additional exclusion criteria included documented chronic disease, history of dyslipidemia or abnormal blood glucose levels (total cholesterol >240 mg/dL, fasting blood glucose levels >125 mg/dL), allergy to olives or gluten, smoker or living with a smoker, extreme dietary or exercise patterns, recent weight fluctuations (>10% in the last 6 months), taking prescription lipid medications or supplements that may affect lipoprotein metabolism (>1 g of fish oil/day, antioxidant supplements, CBD oil, etc.), taking exogenous hormones. As shown in Figure 2.1, twenty-six participants were enrolled in the study and randomized.

2.2 Study design

This was an 8-week, single site, randomized, crossover trial in generally healthy normal-weight, overweight and obese men and women. Subjects were recruited, screened, consented, and then enrolled in the study to consume in randomized order 40 ml of high-polyphenol olive oil (HPOO) or low-polyphenol olive oil (LPOO) daily for two weeks each, separated by a two-week washout period. Study assessments were performed before and after each two-week intervention period after an overnight fast and at 2hr and 4hr after acute consumption of olive oil, to evaluate post-prandial responses of cytokines.

Study procedures took place on the main UC Davis campus in the Ragle Human Nutrition Research Center. Prospective subjects who passed the initial phone screening were

consented and invited for an in-person screening at the Ragle Human Nutrition Research Center to have their height, weight, waist and hip circumference, and blood pressure measured. They were instructed to fast for 10-12 hr before the visit. Fasting blood samples were then collected for the lipid and comprehensive metabolic (CMP) panels. At this visit, subjects also filled out a short questionnaire about health history as well as physical activity habits to confirm eligibility. Subjects who qualified for the study were randomized to one of the treatment groups - 40 ml/day of HPOO or 40 ml/day LPOO for 2 weeks, followed by a 2-week washout period and a switch to the opposite treatment for 2 more weeks. Both types of OO used in the study were commercially available for human consumption and had appropriate certification. The HPOO was the Premium EVOO from Lucini Italia (www.lucini.com), while the LPOO was the ROO from Sciabica's California Olive Oil (www.sunshineinabottle.com). Both oils will be analyzed for polyphenol content and the results will be reported in the publication. Participants were instructed not to cook with the provided OO and use them as the finishing oils only (e.g. as salad dressing or dip for bread, on top of pasta after cooking it, etc.)

The first and the third study visit for female participants were scheduled on the 3rd through 5th days of their menstrual cycle to minimize the effect of sex hormones on blood lipid and cytokine levels. The olive oils were provided to the participants at the start of each intervention period in a glass bottle in a blinded manner, with no markings to distinguish the HPOO from the LPOO. Prior to starting the first intervention period all subjects underwent a 2-week run-in period to become accustomed to the study diet: a low-polyphenol diet with no nuts,

olive oil or other olive products. This diet was followed for the entire 8-week duration of the study.

A three-day food log (2 weekdays and 1 weekend day) was completed by each subject before each study visit. Food records were analyzed via Food Processor (SQL Version 10.2, ESHA Research, Salem, OR). Food records were used to estimate macronutrient intake, micronutrient intake, and compliance with the low-polyphenol diet. Compliance was also measured by weighing the returned bottles with the remainder of the olive oil (if any). The weight was then subtracted from the original weight of the bottle with olive oil.

2.3 Biological sample collection and analyses.

Participants arrived for the baseline visit (Day 1 of the first treatment period) after a 10-12 hr fast. Subjects were instructed to abstain from alcohol and excessively vigorous exercise on the day before the baseline visit. They were asked to standardize their evening meal before all the study visits such that a meal consisting of approximately the same foods was consumed each time. Anthropometric and blood pressure measurements from the screening visit were repeated. Before taking the blood pressure measurement, subjects were asked to sit quietly for 5 minutes with their feet flat on the floor with legs uncrossed. A properly sized cuff was then placed directly over the subject's arm (with no clothing underneath). During the measurement the subject's arm was supported so that the middle of the cuff was approximately at the level of the right atrium. Three blood pressure measurements were taken and recorded with 30-60 sec rest intervals in between. The average of the three measurements was calculated and used in the statistical analysis.

Before ingestion of either 40 ml of HPOO or LPOO with bread (time 0 hour), a urine sample was collected and venous blood draw was performed by a licensed nurse and

approximately 60 ml of blood was collected into vacuum evacuated collection tubes (PST for lipid panel, SH for whole blood LPS stimulation, EDTA for systemic cytokine assessment, CPT for PBMC isolation, SST for PON-1 activity/quantity).

Subjects then consumed the intervention food (OO with 2 slices of Trader Joe's white sourdough bread) within a 10-minute period, and the timing of the subsequent draws began with the first bite of food. Additional blood and urine samples were collected at 2 and 4hr postprandial following the same standardized protocol. Participants remained monitored for the duration of the acute period to ensure dietary compliance. The collected blood in PST and EDTA tubes was spun for 15 minutes x1800g at 4 ˚C immediately after collection, while SST tubes were allowed to clot for 30 minutes prior to aliquoting. CPT tubes were spun for 30 mins x 1500g at room temperature. Aliquots of plasma was stored at -80℃ until analysis. Peripheral blood mononuclear isolation was performed immediately following the blood draw. All study procedures were repeated at the end of each intervention period

(Day 14 of each intervention) and washout (Day 1 of the second intervention) period. Food logs, fasting urine and blood samples were collected.

2.4 Biomarkers of inflammation

PBMC isolation procedures: CPT tubes were centrifuged for 30 mins x 1500g at room temperature. Mononuclear cells were then collected using a sterile Pasteur pipet and transferred to a 15 ml conical falcon tube. Cold PBS was added to the isolated cells and the tube was inverted five times. The Falcon tube was then centrifuged for 15 minutes x 1000g at 4° C. The supernatant was carefully aspirated, 2 ml of cold PBS was re-added to the tube, and the PBMC cell pellet was resuspended. The suspension was then aliquoted in several Eppendorf tubes and centrifuged again for 10 minutes x 1000g at 4° C. The supernatant was aspirated out and the cell

pellet was resuspended in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum and 1% Penicillin-Streptomycin-Glutamine. A 20 uL aliquot was then transferred to another Eppendorf tube, mixed with trypan blue stain and the number of cells was counted with microscopy using a hemocytometer. The target concentration was $5x10^5$ cells/ml and the sample was diluted with additional RPMI 1640 media if needed to achieve this target number. Methods for PBMC stimulation with LPS were adapted from Schildberger et al. [68] and optimized based on the results of several preliminary experiments. Briefly, the cell suspension was aliquoted into a flat bottom 12-well place and incubated at 37 ˚C in a humidified 5%CO_2 incubator for 24 hr. LPS suspended in RPMI 1640 media (treatment; 100x solution for 100 ng/ml final LPS concentration) or media alone (control) were then added to each well in duplicates and incubated for another 24 hr (for a total of 48 hrs of incubation). Cell supernatants were then collected, centrifuged for 10 minutes x 1000g at 4˚C, aliquoted in Eppendorf tubes, and stored at -80˚C until analysis.

Methods for stimulation of whole blood with LPS were adapted from Thurm et al. [69] and optimized via several preliminary experiments. Briefly, blood samples collected into sodium heparin tubes for whole blood stimulated with LPS were aliquoted into two separate tubes in equal amounts (for treatment and control, respectively). 100x LPS solution (in RPMI 1640 media supplemented with 1% L-glutamine for a total 100 ng/ml LPS concentration) or RPMI media alone (for treatment and control, respectively) were then added to the respective tubes and incubated for 24 hr in a 5%CO_2 humidified incubator. The tubes were then centrifuged for 10 minutes x 1000g at 4° C and the supernatant aliquoted and stored at -80°C until analysis.

Levels of IL-1ß, IL-6 and TNF-a were analyzed from plasma using EDTA as an anticoagulant (systemic), whole blood stimulated/unstimulated with LPS, and isolated PBMCs

stimulated/unstimulated with LPS. The analysis was performed by the UC Davis IDDRC Biological and Molecular Analysis Core using Millipore Milliplex Human TH-17 Magnetic Panel (catalog # HTH17MAG-14K-03) via ELISA method following Millipore Luminex Protocol. The standard curves were constructed using Bioplex 200 and Bioplex Manager 6.1 software.

2.5 Statistical analyses

The primary outcome in this study was cholesterol efflux capacity (CEC). The study was powered based on three previously conducted human trials [70-72]. Power calculations were performed for 90%, $\alpha = 0.05$ and effect size of 5.39%. The required number of participants after accounting for 20% attrition was 22. The original plan was to include two groups (normoweight/overweight and obese) and enroll 22 participants in each group. Due to the difficulty recruiting subjects for the "obese" group, we have only one group, which was still powered in accordance with the original sample size calculation.

Secondary outcomes in this study were levels of circulating cytokines, and the secretion of cytokines from whole blood and PBMCs stimulated with LPS in the short-term (2 weeks) and acute (2hrs and 4hrs) consumption setting. The cytokines of interest include IL-1b, IL-6, and TNF-a. All data were checked for normality via Shapiro-Wilk normality tests and QQ-plots. Data that were not normally distributed were log10 transformed. Anthropometric, clinical and short-term OO consumption (2 weeks) cytokine data were tested with the linear mixed effects model to verify significance of interactions for intervention order. Intervention, order of intervention and interaction between treatment*order were used as fixed effects, while subjects were used as random effects. Comparisons within groups before/after OO consumption at all timepoints (0hr, 2hrs, 4hrs) were performed with a paired- t-test. Comparisons between the three timepoints

points withing groups after OO consumption were assessed via two-way repeated measures ANOVA, followed by a Bonferroni multiple comparison test. Differences between groups at each timepoint after OO consumption (0hr, 2hrs, 4hrs) were tested via ANCOVA with baseline levels used as a covariate. Statistical significance was set at $p = 0.05$. Statistical analysis was performed using R statistical software.

3. Results

3.1 Participant characteristics at baseline

Baseline characteristics of the participants are listed in Table 4.1. Briefly, the average age was 27 ± 6.1 years and there were 11 female and 15 male participants. The mean BMI was 25.9 ± 5.5 kg/m^2 and the waist to hip ratio was 0.8 ± 0.1. Out of 26 participants who completed the study protocol, 77% (11 male and 9 female) were normoweight or overweight and 23% (4 male, 2 female) were obese. Blood pressure and all lipid/lipoprotein levels were within normal limits.

3.2 Anthropometric measurements and blood lipid levels after short-term OO consumption (2 weeks) – 0hr timepoint

There was no weight change from baseline within groups and or between groups at endpoint. Waist circumference (WC) did not change after HPOO treatment, but significantly increased by 0.69 cm on average (95% CI: 0.02, 1.37) after LPOO treatment ($p = 0.04$). No difference in WC between groups at endpoint was detected, however. There was a decrease in diastolic blood pressure after two weeks of LPOO but not HPOO intake with the mean change from baseline of -2.85 mmHg (95% CI: -5.60, -0.09; $p = 0.04$). There was also an increase in HDL-C levels after two weeks of LPOO intake with the mean change from baseline of 2.44 mg/dL (95% CI: 0.35, 4.53; $p = 0.02$). However, between group differences at endpoint were not statistically significant for either diastolic blood pressure or HDL-C levels. In addition, there

were no differences within or between groups in hip circumference, systolic blood pressure or any other blood lipid/lipoprotein levels. There was an interaction for treatment order for TG levels but no differences between groups at endpoint were detected. A summary of these results is presented in Table 4.2.

According to food records, participants maintained their habitual diet throughout the study and abstained from polyphenol-rich foods as directed. Participants consumed diets similar in macro- and micronutrient composition during both treatment periods (Supplemental table 4.1). The diets were reflective of a typical American diet (43% carbohydrates, 18% protein, 39% fat), they were high in saturated fat, high in sodium, low in fiber, potassium, magnesium, and omega-3 fatty acids. Participant compliance was 85% as measured by returned olive oil bottle weights and, with confirmation from food records. On average in 2 weeks, participants consumed 398.1 ml of LPOO and 431.4 ml of HPOO.

3.3 Cytokine levels after short-term (2 weeks) OO consumption – 0hr timepoint

The only **within-group difference** (pre/post) at 0hr was for levels of IL-6 spontaneously secreted from PBMCs, where levels were lower after 2 weeks of HPOO compared to before HPOO with the mean change of -4.25 pg/ml (95% CI: -9.33, 0.81; p = 0.03). IL-6 levels were not different between groups, however.

The only **between-group difference** observed at 0hr after the consumption of two types of OO was in the levels of IL-1ß secreted from LPS stimulated PBMCs. IL-1ß levels were lower after HPOO (31.71 pg/ml, 95% CI: 14.88, 46.09) compared to the LPOO group (45.97 pg/ml, 95% CI: 21.84, 72.63), with a mean difference of -8.26 pg/ml (95% CI: -15.73, -0.79; p = 0.03; Table 4.3 and Figure 4.2**).**

3.4 Cytokine levels after acute OO consumption - 2hrs and 4hrs timepoints

There were several **within-group differences** observed at 2hrs and 4hrs. Comparisons at each time point before and after consumption of each type of OO, as well as comparisons between different timepoints within each group after OO consumption were performed.

For before- and after- comparisons at each time point, there were differences in TNF-a secreted from PBMCs at 4hrs only. In particular, TNF-a levels secreted from LPS stimulated PBMCs were lower after HPOO consumption compared to before consumption ($p = 0.004$; Table 4.5). In contrast, TNF-a levels spontaneously secreted from PBMCs at 4hrs were higher after HPOO compared to before HPOO intake ($p = 0.007$; Table 4.5).

In regard to differences between timepoints within each group after each type of OO consumption, there were differences in TNF-a levels in LPS stimulated whole blood only. After LPOO consumption levels were higher at both 2 hrs and 4hrs compared to 0hr (Table 4.6), while after HPOO the levels were higher only at 4 hrs compared to 0hr and 2 hrs (Table 4.7) ($p < 0.05$ for all comparisons). These changes did not yield statistically significant differences between groups, however (Table 4.8).

Several **between-group differences** at both 2hrs and 4hrs timepoints were observed. These differences were for PBMCs only. For instance, at the 2hr timepoint IL-6 and TNF-a levels spontaneously secreted from PBMCs were higher after HPOO compared to LPOO (Figure 4.3 and 4.4). Interestingly, both comparisons yielded marginally significant p-values at 0.048 and 0.049, respectively (Table 4.4). TNF-a spontaneously secreted from PBMCs was also higher 4hrs after HPOO compared to LPOO ($p = 0.014$; Table 4.5 and Figure 4.3), while TNF-a levels secreted from LPS stimulated PBMCs were lower at 4hrs after HPOO vs. LPOO ($p = 0.03$; Table 4.5).

4. Discussion

Evidence shows that regular consumption of high-polyphenol foods may decrease chronic disease risk mainly due to their ability to modulate low-grade inflammation [73-75]. Several mechanisms may be involved in the anti-inflammatory effects of these compounds, including modification of the signaling cascade and transcription network (e.g., interfering expression and activation of NF-kB), decrease of adhesion of immune cells to the endothelium, and improvement of endothelial function [75].

The non-glyceridic constituents of EVOO include approximately 30 phenolic compounds [76]. It has been reported that individuals consuming EVOO as a part of their regular diet may intake as much as 10-20 mg of phenolic compounds daily [37, 76]. These amounts of phenolics have been associated with a lower incidence of CVD [77]. Some EVOO phenolics have demonstrated the ability to inhibit eicosanoid production in animal/human cells *in vitro* [78-79], significantly decrease IL-1ß secretion [80] and modulate nitric oxide (NO) production in macrophage cell lines [81-82].

In this experiment we looked at the effects of HPOO vs. LPOO on systemic cytokines, as well as cytokines secreted from unstimulated or LPS stimulated whole blood and PBMCs. In summary, based on the obtained results, HPOO had an effect on cytokines secreted from LPS stimulated PBMCs. In particular, it decreased IL-1ß after short-term consumption (2 weeks) and IL-6 and TNF-a after acute consumption (2hr and 4hr). LPOO, in contrast, had no effects after short-term intake but its acute consumption did result in lower levels of IL-6 spontaneously secreted from PBMCs after 2hrs and 4hrs and TNF-a after 4hrs. There were no differences between the two types of OO in terms of their effect on systemic cytokines and cytokines secreted from whole blood. These results do not support our initial hypothesis that HPOO consumption will result in lower cytokine levels.

We did not observe any differences between groups in systemic cytokines and cytokines secreted from whole blood. This is in agreement with the results from a recent human trial by Zimmer et al., where three groups of participants at differing CV risk – healthy, hyperlipidemic, and post-myocardial infarction – consumed 25 g of extra-virgin olive oil (EVOO) daily for six months [83]. At endpoint, there was no change in plasma IL-6, TNF-a or IL-1ß [83]. The results obtained by Santangelo et al. support this conclusion as well [84]. In this study 11 participants with Type 2 Diabetes Mellitus (T2DM) consumed 25 g of refined olive oil (ROO) or HPOO for 4 weeks. No effects on circulating IL-6 and TNF-a cytokines were observed [84]. In a study by Rozati et al. 41 overweight/obese individuals aged ≥ 65 years consumed a typical American diet and were divided into 2 intervention groups: the control group received a mixture of corn, soybean oil and butter, while the treatment group was provided EVOO to replace all other oils in their diet for 3 months. No between group differences were found in T cell phenotype or cytokine production (IL-2, IFN- γ, IL-6, TNF-a) at endpoint [85]. This is partially supported by in vitro studies as well. For instance, a recent Miles et al. study showed that EVOO derived OLE and caffeic acid in 10^{-4} M concentrations decreased IL-1ß production by LPS stimulated whole blood cells (by 80% and 40%, respectfully), while there were no effects on IL-6 and TNF-a production [86].

Several other human trials did observe changes in circulating cytokine levels, however. Notably, these studies were done in populations with MetS and walnuts were consumed for longer periods of time (e.g. 60 days, 6 weeks, etc.). For instance, Khandouzi et al. compared the effects of 25 ml of daily ROO vs. canola oil (CO) for six weeks on circulating cytokines in individuals with at least one of the following: hypertension, dyslipidemia, or diabetes [87]. They observed a significant reduction in circulating IL-6 levels after ROO only [87]. The study by

Patti et al. tested daily consumption of 32 g of EVOO for 60 days in individuals with MetS on anthropometric measurements, hepatic steatosis, and plasma pro-inflammatory cytokines [88]. They observed a significant decrease in IL-6, IL-1ß, TNF-a and IL-17A, while IL-10 levels significantly increased [88].

Aside from the differences in methodology and populations of interest between studies, the discrepancies in the obtained results may also be due to the fact that phenolic compounds in the circulation are generally present in very small quantities [89] and the increase in the antioxidant capacity stays noticeable only one or two hours after intake [90]. In addition, polyphenolic bioavailability in vivo largely depends on their stability in the GI, and the extent of their metabolism and absorption [37]. For instance, before becoming available, EVOO polyphenolic compounds have to be released from the food matrix and undergo multiple modifications in the gut lumen [37]. Other factors affecting bioavailability may include polyphenolic compound concentration in the oil, as well as host enzymatic activity, habitual diet, genetics and gut microbiota composition and functionality [91-92].

EVOO phenolic compounds and their metabolites are present in plasma and tissues at concentrations 50-fold lower compared to endogenous antioxidants [93]. Data from human studies show that consumption of EVOO containing 250-750 mg of total phenols/kg, HTyr sulfate, HTyr acetate sulfate, homovanillic acid (HVA), HVA sulfate is present in the plasma in maximum concentrations at 1.3-4.1, 0.46-2.2, 0.17-0.65, and 0.12-0.53 uM, respectively [94]. Conjugates of phenolic metabolites have demonstrated lower radical scavenging capacity compared to the parental compounds and are quite inactive as antioxidants [95-96]. However, it is thought that polyphenols likely are bioactive via cell signaling as it has been demonstrated in vivo and not through systemic oxidative stress reduction [97]. Therefore, the effects may not be

prominent if assessed systemically. In addition, the beneficial effect of EVOO has been shown to be significantly improved with an antioxidant rich diet specifically [98]. For instance, a study by Perez-Martinez explored NF-kB activation in PBMCs after 4 weeks of either a Western diet (WD), a MedDiet enriched in VOO, and a high-CHO low-fat diet enriched in a-linolenic acid (ALA) in 16 healthy male volunteers [99]. They found that NF-kB activation increased after the WD compared to the MedDiet and the ALA-enriched diet but there were no differences between the latter two diets [99]. Therefore, olive oil might only be able to show its beneficial potential on circulating cytokines when taken concurrently with a MedDiet, not a typical American one [100-101].

Most of the observed effects in the present study were in the levels of cytokines secreted from stimulated or unstimulated PBMCs. Once LPS is added to PBMCs, it binds to Toll-like receptor 4 (TLR4), which activates the NF-kB pathway through the recruitment and activation of MyD88, IL-1R kinase (IRAK), TNFR associated factor 6 (TRAF-6), as well as NADPH oxidase (Nox) [102]. NF-kB is responsible for regulating the transcription of genes that are related to innate immunity and inflammatory responses, including the release of pro-inflammatory cytokines (e.g., IL-6, IL-1ß, TNF-a, etc.) [102]. In the present study a between-group difference was observed for IL-1ß cytokine secreted from LPS stimulated PBMCs after short-term consumption (2 weeks) but there were no acute intake effects (2hrs and 4hrs). Zimmer et al. reported a decrease in IL-1ß secretion from LPS stimulated PBMCs after 6 months of EVOO consumption in hyperlipidemic but not healthy adults [83], which is intriguing taking into consideration that participants in the present study were generally healthy. Camargo et al. studied 20 subjects with MetS and mean age of 56 years old (range 40-70) who consumed a high fat breakfast consisting of 60 g of white bread, 40 ml of VOO (either high or low in polyphenols)

and 60,000 IU of vitamin A per m^2 of body surface and measured changes in PBMC gene expression [103]. They showed that intake of high-polyphenol VOO decreased the expression of *IL1B*, *PTGS2* (encodes for prostaglandin-endoperoxidase synthase 2 (COX-2)), *IL6*, as well as several chemokine-related genes compared to the low-polyphenol oil postprandially [103].

TNF-a and IL-6 cytokine levels secreted from stimulated PBMCs in the present study were similar between the two intervention groups after short-term consumption (2 weeks), which is supported by the results from Zimmer et al. study [83]. We did see several acute intake (2hrs and 4hrs) effects, however. TNF-a levels secreted from LPS stimulated PBMCs were lower after HPOO compared to LPOO treatment at 4hrs. Interestingly, an opposite effect was observed for unstimulated PBMCs, where the spontaneously secreted TNF-a and IL-6 levels were higher after HPOO vs. LPOO at the 4hr timepoint. IL-6 levels were also higher at the 2 hr time point after HPOO but not LPOO.

Other studies also seem to produce similar conflicting results. For instance**,** a study by Jimenez-Gomez assessed PBMC cytokine gene expression and the systemic levels of cytokines in 23 generally healthy and young male subjects after a butter, OO and walnut breakfasts [104]. The butter breakfast resulted in an increase in TNF-a messenger RNA (mRNA) expression compared to the OO and walnut breakfasts in PBMCs [104]. Furthermore, they observed an increase in IL-6 mRNA after butter and OO but not after walnut breakfast. Interestingly, the systemic levels of these cytokines were not elevated after each treatment [104].

A recent in vitro study by Pojero et al. tested the effects of OO polyphenolic compounds, oleuropein (OLE) and hydroxytyrosol (HT), in nutritionally relevant concentrations on PBMCs regarding cell viability, frequency of leukocyte subsets and cytokine release [105]. OLE and HT were used either alone or as a pre-treatment before stimulating PBMCs with LPS. Both

polyphenols had no effect on cell viability and only limited effect on leukocyte subsets. They did not have any effect on the release of TNF-a or IL-6 cytokines either [105].

The lack of a clear pattern for cytokine release from the different types of cells tested in the present study may be explained by the fact that *in vitro* cytokine secretion is not necessarily reflective of their secretion *in vivo*. For instance, a recent study by Jansen et al. explored the relationships between *ex vivo* monocyte cytokine responses and *in vivo* cytokine responses in a large cohort of healthy volunteers (n = 110) using a short-lived LPS-induced systemic inflammation (LPS dose = 1 ng/kg). Baseline cytokine production was also assessed prior to LPS administration. No strong correlations were observed between baseline *ex vivo* cytokine production capacity and *in vivo* cytokine responses following LPS administration [106]. There have been several studies in small cohorts of healthy volunteers that reported that *ex vivo* cytokine production by LPS-stimulated whole blood also does not correlate with the *in vivo* cytokine response to LPS administration [107-108].

It has been hypothesized that tissue-resident macrophages and not circulating immune cells are mainly responsible for the *in vivo* cytokine response [109]. This may mean that cytokines measured in the blood are reflective of the extent of "spillover" from the tissues and whether *ex vivo* stimulations accurately reflect the *in vivo* immune status is largely unknown [106]. Other reports, however, suggest that while the primary cellular source of circulating cytokines is largely unknown, circulating monocytes are believed to serve as potent cytokine producers [110]. In sepsis, high levels of proinflammatory cytokines are observed in plasma but circulating leukocytes do not seem to be the major cellular source [111]. The capacity of leukocytes to produce cytokines upon adequate challenge has been used to estimate the entire functional capacity of the immune system [112]. Whole blood or isolated-cell stimulation

assay primarily assesses individuals' cytokine production by monocytes [113], a process that is potentially dictated by genetics [114]. Monocyte-derived cytokines are involved in immune responses, including activation of B cells, T cells and the enhancement of several neutrophil and macrophage/monocyte functions [115]. Monocyte-derived cytokines play an important role in host defense against bacterial infections [115]. Measuring cytokine secretion from LPS stimulated cells is commonly used as a model of immune responsiveness in non-infectious diseases as well [116]. A low stimulated cytokine production has been associated with increased mortality [117], metabolic syndrome and type 2 diabetes [118], lower muscle mass and strength [119] – consequences of the aging process. Monocytes from depressed individuals present an altered response to endotoxin stimulation as well [120]. Sex may also be a confounding factor [116, 121-123] but remains unclear [124].

In addition, it is important to keep in mind that *ex vivo* stimulation of whole blood and PBMCs is solely focusing on cytokine production, while the *in vivo* immune response is much more complex and involves multiple immunological and non-immunological cell types. In addition, *in vivo* response is regulated by feedback loops not only through other immunological organs [125] but also through gut microbiota [126].

Moreover, several earlier human trials concluded that spontaneous *ex vivo* cytokine production by PBMCs rarely occurs in healthy individuals [127-131] and the presence of spontaneous production *ex vivo* is usually preceded by *in vivo* stimulation [127]. Evidence also shows that in healthy subjects, a few cells produce more than one cytokine spontaneously and IL-6 was reported to be the cytokine most commonly produced [127]. Furthermore, IL-6 is one of the cytokines where spontaneous production by a given cell type tends to be significantly associated with a higher percentage of the same cell type producing the same

cytokine with stimulation [127]. There seems to be no predominant cell type secreting cytokines spontaneously but immunoregulatory cells often predominate in spontaneous TNF-a production [127].

It is well-established that TNF-α, IL-6, and IL-1ß are involved in the promotion and maintenance of inflammation. Despite the association of these cytokines with chronic disease and mortality, their increased levels are sometimes reported as indicators of healthy aging [132-125]. LPS increases the secretion of TNF-α, IL-6, and IL-1ß by human PBMCs [136-137] and monocytes [137-141]. However, data about the effects of olive oil components (e.g. polyphenols) on inflammatory cytokine production by human immune cells are extremely scarce [142]. In addition, clinical trials to date have not reached consensus on the effects of HPOO on inflammatory markers, as results greatly vary between studies. This may be due to the dose of OO provided as the absorption is dose-dependent [143]. The storage conditions are also very important, as the HPOO needs to be kept in a dark cool place. One of the main challenges for interpreting the results from studies using OO as the dietary intervention is lack of reporting of the chemical analysis of the OO used, as level of oxidation degradation is greatly affected by EVOO chemical composition and storage conditions [144]. In addition, the generally higher price of OO compared to other oils led to it being often tempered with by mixing it with different categories of OO or other vegetable oils [145].

Along these lines, one of the limitations of the present study is that we are yet to analyze the oils for their chemical composition, which may not be sufficiently accurate due to the prolonged OO storage. Another limitation is that the results may be more reflective of the Northern California college student than a general US population, since most of our participants were UC Davis graduate and undergraduate students. We also did not sufficiently adjust for

various menstrual cycle stages for our female participants. Evidence shows that the release of TNF-a was reduced in female subjects during the luteal phase compared to the follicular phase. In addition, compared to male subjects, secretion of TNF-a and IL-6 during the luteal phase was lower. Concertation of estradiol in plasma correlated with the secretion of TNF-a and IL-6 during the luteal phase [146]. While we did schedule the 1st and 3rd study visits for our female participants on their 1-5th days of their menstrual cycle, this may have not been enough to address the potential confounding factors. Further statistical analysis adjusting for the menstrual cycle phase may be necessary for publication. Additional analysis exploring the effects of biological sex on cytokine levels will also be performed.

This study had several strengths including its randomized crossover design and the 2-week washout period [89]. In addition, this study used repeated venipuncture instead of cannulation to obtain multiple blood samples from subjects. Thus, increases in cytokines observed in this study were not a localized inflammatory response to the cannula itself [147-148]. In addition, to our knowledge, this is the first study to assess the effects of OO consumption on cytokine secretion from whole blood and PBMCs in young and generally healthy adults, as most previous studies focused on older and/or at-risk populations.

In conclusion, we did not observe any significant between-group differences in systemic cytokines and cytokines secreted from unstimulated whole blood with two weeks of HPOO and LPOO consumption in healthy young adults. However, in LPS stimulated PBMC samples, IL-1ß secretion was lower after short-term HPOO consumption compared to LPOO. After acute consumption, IL-6 and TNF-a spontaneously secreted from PBMCs were increased 4hrs after HPOO but not LPOO consumption. In contrast, TNF-a secreted from LPS stimulated PBMCs was lower 4 hrs after HPOO intake compared to LPOO.

Adding HPOO to a diet makes a positive contribution to the intake of polyphenol-rich foods but may not necessarily modulate markers of inflammation. The results from primary outcomes of this study (CEC, ox-LDL, markers of endothelial function, etc.) in concert with the described cytokine observations may paint a better and more wholistic picture of the effects of OO polyphenols on cardiometabolic health, which will also assist with informing the direction for future studies.

5. References

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6. Tables and figures

Table 4.1 Baseline characteristics of participants[1]

Variable	
Age, *years*	27.0 ± 6.1
Sex, *M/F*	15/11
Waist circumference, *cm*	84.0 ± 14.3
Hip circumference, *cm*	98.3 ± 13.4
Waist/hip ratio	0.8 ± 0.1
Weight, *kg*	76.6 ± 22.6
BMI, kg/m^2	25.9 ± 5.5
Systolic blood pressure, *mmHg*	109.1 ± 10.4
Diastolic blood pressure, *mmHg*	70.2 ± 7.3
Total cholesterol, *mg/dL*	170.8 ± 29.4
HDL cholesterol, *mg/dL*	54.9 ± 13.1
LDL cholesterol, *mg/dL*	99.4 ± 24.4
TG, *mg/dL*	83.8 ± 48.9

[1]Values are mean ± SD, n = 26. HDL, high-density lipoprotein; LDL, low-density lipoprotein; TG, triglycerides.

Table 4.2 Anthropometric measurements and blood lipid levels before and after 2 weeks of LPOO and HPOO[1]

Variable	LPOO		P_{within}	HPOO		P_{within}	$P_{between}$	P_{order}
	Baseline	***2 weeks***		***Baseline***	***4 weeks***			
Weight, *kg*	76.8 ± 22.9	77.1 ± 23.3	NS	77.1 ± 22.8	77.1 ± 23.1	NS	NS	NS
Waist circumference, *cm*	83.1 ± 14.0	83.8 ± 13.9	**0.04***	84.0 ± 16.2	83.5 ± 13.7	NS	NS	NS
Hip circumference, *cm*	99.6 ± 14.2	99.0 ± 13.3	NS	98.5 ± 13.5	99.9 ± 13.3	NS	NS	NS
Systolic BP, *mmHg*	110.3 ± 10.9	109.2 ± 11.6	NS	110.3 ± 14.8	108.5 ± 14.1	NS	NS	NS
Diastolic BP, *mmHg*	73.5 ± 7.5	70.6 ± 7.4	**0.04***	71.4 ± 11.0	70.7 ± 9.7	NS	NS	NS
Total cholesterol, *mg/dL*	173.80 ± 32.26	171.56 ± 25.25	NS	177.36 ± 30.73	172.72 ± 25.70	NS	NS	NS
HDL cholesterol, *mg/dL*	54.20 ± 11.85	56.64 ± 12.61	**0.02***	56.16 ± 13.57	57.00 ± 12.64	NS	NS	NS
LDL cholesterol, *mg/dL*	99.80 ± 24.48	95.04 ± 24.22	NS	103.00 ± 24.75	98.24 ± 20.41	NS	NS	NS
TG, *mg/dL*	98.60 ± 53.96	92.92 ± 42.36	NS	91.12 ± 36.82	87.64 ± 37.42	NS	NS	**0.03***

[1]Data are untransformed means ± SD, n = 26. Change from baseline was assessed via paired t-tests following a log10 transformation if necessary. Values at the 2-week endpoint were compared between treatments via ANCOVA test using baseline values as a covariate. P-values marked with * indicate statistical significance ($p < 0.05$). BP, blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TG, triglycerides

Table 4.3 Cytokine levels before and after short-term (2 weeks) consumption of LPOO and HPOO - 0hr timepoint[1]

	LP		p_{within}	HP		p_{within}	$P_{between}$	p_{order}
	pre	**post**		**pre**	**post**			
IL-1ß								
Systemic	6.68 (5.86, 7.89)	6.63 (4.21, 9.25)	NS	5.05 (3.96, 7.83)	6.77 (4.22, 8.70)	NS	NS	**0.03***
WB stimulated	8689.05 (5644.49, 16477.72)	8647.00 (5467.42, 11457.95)	NS	8061.26 (6394.94, 12310.36)	9198.27 (6029.61, 13945.32)	NS	NS	NS
WB unstimulated	3.29 (1.94, 4.81)	3.39 (1.67, 6.33)	NS	3.40 (1.82, 7.38)	3.13 (1.89, 5.15)	NS	NS	NS
PBMC stimulated	38.04 (23.52, 54.64)	45.97 (21.84, 72.63)*	NS	38.80 (19.36, 51.91)	31.71 (14.88, 46.09)*	NS	**0.03***	NS
PBMC unstimulated	2.63 (1.06, 11.1)	2.65 (0.96, 5.89)	NS	4.79 (1.10, 6.91)	4.69 (0.85, 6.96)	NS	NS	NS
IL-6								
Systemic	10.46 (6.15, 21.94)	9.94 (5.04, 22.68)	NS	8.70 (5.65, 22.94)	12.84 (6.86, 23.32)	NS	NS	NS
WB stimulated	9187.58 (5499.52, 16457.87)	9404.51 (5515.36, 14446.84)	NS	9532.12 (5579.46, 13994.64)	8905.58 (5579.46, 17099.79)	NS	NS	NS
WB unstimulated	10.70 (4.24, 25.83)	10.44 (3.74, 28.37)	NS	12.36 (5.42, 29.40)	9.39 (5.10, 26.19)	NS	NS	NS
PBMC stimulated	3245.88 (2375.76, 6252.14)	3401.59 (2044.38, 6423.44)	NS	3973.27 (2122.38, 6145.79)	3425.66 (2248.64, 7354.44)	NS	NS	NS
PBMC unstimulated	3.56 (1.21, 9.14)	5.06 (2.23, 8.79)	NS	9.82 (2.32, 16.69)*	4.39 (2.32, 14.81)*	**0.03***	NS	NS
TNF-a								
Systemic	11.41 (8.64, 12.93)[a]	10.96 (9.33, 12.60)	NS	10.35 (9.02, 11.57)[b]	10.43 (8.83, 12.86)	NS	NS	NS
WB stimulated	4997.26 (2170.80, 7554.73)	4310.61 (3263.14, 6165.32)	NS	4814.41 (2413.48, 6266.45)	5256.58 (2930.78, 6797.27)	NS	NS	NS
WB unstimulated	12.00 (8.43, 17.01)	11.33 (7.95, 16.17)	NS	10.65 (7.31, 14.89)	11.58 (7.90, 14.10)	NS	NS	NS
PBMC stimulated	4119.14 (2359.61, 7711.72)	4804.49 (2569.59, 7356.39)	NS	4141.60 (1831.18, 6972.83)	3829.92 (2035.19, 5522.33)	NS	NS	NS

PBMC unstimulated	13.04 (6.16, 27.03)	13.88 (8.01, 17.07)	NS	15.33 (9.01, 28.51)	11.71 (6.08, 29.42)	NS	NS	NS

[1]Data are median (IQR), n = 26. Baseline values and mean change from baseline within groups was assessed via a paired t-test after log_{10} data transformation where necessary. Differences between groups at endpoint were evaluated via ANCOVA test with baseline values used as a covariate. *Indicates time point significantly different between treatments ($p<0.05$). LPOO, low-polyphenol olive oil; HPOO, high-polyphenol olive oil; WB, whole blood; PBMC, peripheral blood mononuclear cells.

Table 4.4 Cytokine levels before and after acute consumption of LPOO and HPOO - 2hr timepoint[1]

	LPOO		p_{within}	HPOO		p_{within}	$P_{between}$
	pre	**post**		**pre**	**post**		
IL-1ß							
Systemic	7.11 (5.49, 9.42)	6.67 (4.86, 8.22)	NS	6.68 (4.27, 9.12)	7.48 (5.28, 8.80)	NS	NS
WB stimulated	10668.32 (6215.50, 14626.83)	9966.37 (5932.73, 13846.49)	NS	8372.14 (6031.55, 11921.69)	9904.28 (7591.32, 14236.84)	NS	NS
WB unstimulated	2.94 (1.79, 5.81)	3.53 (1.62, 6.04)	NS	4.15 (2.24, 6.51)	3.21 (2.07, 7.05)	NS	NS
PBMC stimulated	37.85 (24.30, 76.62)	33.03 (16.12, 62.13)	NS	36.30 (14.57, 63.36)	31.96 (12.30, 39.99)	NS	NS
PBMC unstimulated	4.44 (1.28, 6.69)	2.09 (0.77, 4.17)	NS	5.87 (1.48, 9.07)	3.43 (1.10, 5.31)	NS	NS
IL-6							
Systemic	11.22 (7.84, 23.21)	10.50 (6.44, 20.53)	NS	9.39 (5.98, 24.66)	11.17 (5.25, 23.04)	NS	NS
WB stimulated	7922.22 (5666.13, 15140.34)	10702.21 (5743.95, 13446.09)	NS	7534.27 (5271.11, 13840.77)	9656.14 (5873.22, 15254.52)	NS	NS
WB unstimulated	10.39 (3.08, 25.88)	10.79 (3.03, 26.81)	NS	9.42 (4.74, 31.96)	13.72 (6.14, 31.08)	NS	NS
PBMC stimulated	3588.10 (2332.91, 5666.44)	3620.50 (1621.14, 5695.26)	NS	3670.01 (1515.52, 6152.93)	3370.91 (1669.34, 5709.70)	NS	NS
PBMC unstimulated	4.21 (2.18, 7.39)	4.50 (1.51, 7.46)*	NS	3.65 (0.99, 8.14)	5.76 (3.02, 9.66)*	NS	**0.048***
TNF-a							
Systemic	10.66 (9.05, 12.78)	9.65 (8.75, 13.06)	NS	10.34 (9.32, 11.74)	10.62 (8.74, 12.67)	NS	NS
WB stimulated	6667.44 (2197.11, 8304.82)	6592.27 (3437.62, 7256.40)	NS	5220.70 (2817.44, 7943.64)	4976.67 (3989.72, 6265.50)	NS	NS
WB unstimulated	11.90 (7.16, 14.74)	12.27 (8.39, 15.96)	NS	10.39 (7.58, 14.20)	11.45 (8.13, 15.83)	NS	NS
PBMC stimulated	4369.01 (2647.34, 6619.33)[a]	3505.78 (2448.40, 5843.94)	NS	3386.22 (2101.44, 5796.34)[b]	3023.95 (1911, 4936.13)	NS	NS
PBMC unstimulated	8.67 (5.10, 17.01)	9.94 (5.54, 13.41)*	NS	8.78 (4.36, 14.89)	11.46 (6.75, 22.30)*	NS	**0.049***

[1]Data are median (IQR), n = 26. Baseline values and mean change from baseline within groups was assessed via a paired t-test after log_{10} data transformation where necessary. Differences between groups at endpoint were evaluated via ANCOVA test with baseline values used as a covariate. *Indicates time point significantly different between treatments ($p<0.05$). LPOO, low-polyphenol olive oil; HPOO, high-polyphenol olive oil; WB, whole blood; PBMC, peripheral blood mononuclear cells.

Table 4.5 Cytokine levels before and after acute consumption of LPOO and HPOO - 4hr timepoint[1]

	LPOO		p_{within}	HPOO		p_{within}	$P_{between}$
	pre	**post**		**pre**	**post**		
IL-1ß							
Systemic	6.05 (4.68, 8.45)	6.98 (5.55, 9.61)	NS	6.08 (5.29, 8.19)	7.40 (4.84, 8.61)	NS	NS
WB stimulated	10364.54 (6140.12, 15995.43)	10082.88 (6684.04, 13494.59)	NS	9754.54 (7770.17, 13199.75)	9166.72 (7037.09, 15333.22)	NS	NS
WB unstimulated	3.65 (1.94, 5.90)	3.43 (1.54, 5.78)	NS	4.27 (2.01, 6.72)	3.58 (2.10, 6.20)	NS	NS
PBMC stimulated	40.10 (19.52, 64.11)	34.05 (15.65, 66.05)	NS	38.74 (20.01, 97.40)	37.44 (13.18, 51.13)	NS	NS
PBMC unstimulated	2.42 (0.38, 10.11)	1.96 (0.72, 7.95)	NS	1.78 (1.25, 7.42)	3.37 (1.57, 5.85)	NS	NS
IL-6							
Systemic	9.83 (4.89, 20.31)	9.10 (6.38, 24.29)	NS	10.60 (7.63, 22.71)	10.79 (4.82, 25.24)	NS	NS
WB stimulated	8149.12 (5821.21, 14208.01)	9030.56 (6259.94, 12288.60)	NS	9486.17 (6167.78, 12834.45)	9117.68 (5405.10, 12618.73)	NS	NS
WB unstimulated	11.21 (2.81, 28.19)	11.35 (4.32, 27.29)	NS	11.55 (4.19, 32.52)	8.51 (5.20, 26.77)	NS	NS
PBMC stimulated	4872.72 (2159.33, 6643.55)	4481.30 (2404.19, 5593.25)	NS	4968.02 (1960.03, 6134.44)	2774.77 (1436.01, 6312.30)	NS	NS
PBMC unstimulated	2.81 (1.63, 9.45)	4.78 (1.94, 9.66)	NS	4.16 (1.95, 11.32)	4.82 (3.46, 12.54)	NS	NS
TNF-a							
Systemic	10.72 (7.63, 11.73)	10.27 (8.67, 12.79)	NS	10.55 (7.86, 12.20)	11.66 (8.62, 13.07)	NS	NS
WB stimulated	6297.83 (3322.64, 7935.91)	5309.05 (3761.57, 8295.26)	NS	5420.93 (3761.64, 8295.33)	5882.58 (4077.33, 7744.45)	NS	NS
WB unstimulated	13.40 (7.98, 17.53)	12.28 (7.82, 17.72)	NS	11.79 (7.61, 15.64)	10.65 (7.54, 13.16)	NS	NS
PBMC stimulated	6141.03 (2547.73, 7743.33)	4829.34 (3528.97, 7369.93)*	NS	4971.42 (2305.44, 7778.65)	3706.84 (2511.35, 5810.28)*	**0.004****	**0.03***
PBMC unstimulated	9.62 (6.20, 14.81)	10.57 (5.19, 12.93)*	NS	11.99 (5.01, 16.30)	14.76 (7.51, 19.53)*	**0.007****	**0.014***

[1]Data are median (IQR), n = 26. Baseline values and mean change from baseline within groups was assessed via a paired t-test after $\log_{10}$ data transformation where necessary. Differences between groups at endpoint were evaluated via ANCOVA test with baseline values used as a covariate. *Indicates time point significantly different between treatments ($p<0.05$). LPOO, low-polyphenol olive oil; HPOO, high-polyphenol olive oil; WB, whole blood; PBMC, peripheral blood mononuclear cells.

Table 4.6 Cytokine levels at each timepoint after LPOO consumption[1]

	LPOO		
	0hr	**2hr**	**4hr**
IL-1ß			
Systemic	6.63 (4.21, 9.25)	6.67 (4.86, 8.22)	6.98 (5.55, 9.61)
WB stimulated	8647.08 (5467.42, 11458.00)	9966.37 (5932.73, 13846.49)	10082.88 (6684.04, 13494.59)
WB unstimulated	3.39 (1.67, 6.33)	3.53 (1.62, 6.04)	3.43 (1.54, 5.78)
PBMC stimulated	45.97 (21.84, 72.63)	33.03 (16.12, 62.13)	34.05 (15.65, 66.05)
PBMC unstimulated	2.65 (0.96, 5.89)	2.09 (0.77, 4.17)	1.96 (0.72, 7.95)
IL-6			
Systemic	9.94 (5.04, 22.68)	10.50 (6.44, 20.53)	9.10 (6.38, 24.29)
WB stimulated	9404.51 (5515.36, 14446.84)	10702.21 (5743.95, 13446.09)	9030.56 (6259.94, 12288.60)
WB unstimulated	10.44 (3.74, 28.37)	10.79 (3.03, 26.81)	11.35 (4.32, 27.29)
PBMC stimulated	3401.59 (2044.38, 6423.44)	3620.50 (1621.14, 5695.26)	4481.30 (2404.19, 5593.25)
PBMC unstimulated	5.06 (2.23, 8.79)	4.50 (1.51, 7.46)	4.78 (1.94, 9.66)
TNF-a			
Systemic	10.96 (9.33, 12.60)	9.65 (8.75, 13.06)	10.27 (8.67, 12.79)
WB stimulated	**4310.61 (3263.14, 6165.32)a**	**6592.27 (3437.62, 7256.40)b**	**5309.05 (3761.57, 8295.26)b**
WB unstimulated	11.33 (7.95, 16.17)	12.27 (8.39, 15.96)	12.28 (7.82, 17.72)
PBMC stimulated	4804.49 (2569.59, 7356.39)	3505.78 (2448.40, 5843.94)	4829.34 (3528.97, 7369.93)
PBMC unstimulated	**13.88 (8.01, 17.07)a**	**9.94 (5.54, 13.41)b**	**10.57 (5.19, 12.93)b**

[1]Data are median (IQR), n = 26. Data were compared using two-way repeated measures ANOVA with Bonferroni multiple comparisons test. Different superscript letters indicate timepoint significantly different within each group (multiplicity adjusted $p<0.05$). LPOO, low-polyphenol; WB, whole blood; PBMC, peripheral blood mononuclear cells.

Table 4.7 Cytokine levels at each timepoint after HPOO consumption[1]

	HPOO		
	0hr	**2hr**	**4hr**
	IL-1ß		
Systemic	6.77 (4.22, 8.70)	7.48 (5.28, 8.80)	7.40 (4.84, 8.61)
WB stimulated	9198.27 (6029.61, 13945.32)	9904.28 (7591.32, 14236.84)	9166.72 (7037.09, 15333.22)
WB unstimulated	3.13 (1.89, 5.15)	3.21 (2.07, 7.05)	3.58 (2.10, 6.20)
PBMC stimulated	31.71 (14.88, 46.09)	31.96 (12.30, 39.99)	37.44 (13.18, 51.13)
PBMC unstimulated	4.69 (0.85, 6.96)	3.43 (1.10, 5.31)	3.37 (1.57, 5.85)
IL-6			
Systemic	12.84 (6.86, 23.32)	11.17 (5.25, 23.04)	10.79 (4.82, 25.24)
WB stimulated	8905.58 (5579.46, 17099.79)	9656.14 (5873.22, 15254.52)	9117.68 (5405.10, 12618.73)
WB unstimulated	9.39 (5.10, 26.19)	13.72 (6.14, 31.08)	8.51 (5.20, 26.77)
PBMC stimulated	3425.66 (2248.64, 7354.44)	3370.91 (1669.34, 5709.70)	2774.77 (1436.01, 6312.30)
PBMC unstimulated	4.39 (2.32, 14.81)	5.76 (3.02, 9.66)	4.82 (3.46, 12.54)
TNF-a			
Systemic	10.43 (8.83, 12.86)	10.62 (8.74, 12.67)	11.66 (8.62, 13.07)
WB stimulated	**5256.58 (2930.78, 6797.27)a**	**4976.67 (3989.72, 6265.50)a**	**5882.58 (4077.33, 7744.45)b**
WB unstimulated	11.58 (7.90, 14.10)	11.45 (8.13, 15.83)	10.65 (7.54, 13.16)
PBMC stimulated	3829.92 (2035.19, 5522.33)	3023.95 (1911, 4936.13)	3706.84 (2511.35, 5810.28)
PBMC unstimulated	15.33 (9.01, 28.51)	11.46 (6.75, 22.30)	14.76 (7.51, 19.53)

[1]Data are median (IQR), n = 26. Data were compared using two-way repeated measures ANOVA with Bonferroni multiple comparisons test. Different superscript letters indicate timepoint significantly different within each group (multiplicity adjusted $p<0.05$). HPOO, low-polyphenol; WB, whole blood; PBMC, peripheral blood mononuclear cells.

Table 4.8 Cytokine levels after LPOO and HPOO consumption at each timepoint[1]

	LPOO			HPOO		
	0hr	**2hr**	**4hr**	**0hr**	**2hr**	**4hr**
IL-1b						
Systemic	6.63 (4.21, 9.25)	6.67 (4.86, 8.22)	6.98 (5.55, 9.61)	6.77 (4.22, 8.70)	7.48 (5.28, 8.80)	7.40 (4.84, 8.61)
WB stimulated	8647.08 (5467.42, 11458.00)	9966.37 (5932.73, 13846.49)	10082.88 (6684.04, 13494.59)	9198.27 (6029.61, 13945.32)	9904.28 (7591.32, 14236.84)	9166.72 (7037.09, 15333.22)
WB unstimulated	3.39 (1.67, 6.33)	3.53 (1.62, 6.04)	3.43 (1.54, 5.78)	3.13 (1.89, 5.15)	3.21 (2.07, 7.05)	3.58 (2.10, 6.20)
PBMC stimulated	45.97 (21.84, 72.63)*	33.03 (16.12, 62.13)	34.05 (15.65, 66.05)	31.71 (14.88, 46.09)*	31.96 (12.30, 39.99)	37.44 (13.18, 51.13)
PBMC unstimulated	2.65 (0.96, 5.89)	2.09 (0.77, 4.17)	1.96 (0.72, 7.95)	4.69 (0.85, 6.96)	3.43 (1.10, 5.31)	3.37 (1.57, 5.85)
IL-6						
Systemic	9.94 (5.04, 22.68)	10.50 (6.44, 20.53)	9.10 (6.38, 24.29)	12.84 (6.86, 23.32)	11.17 (5.25, 23.04)	10.79 (4.82, 25.24)
WB stimulated	9404.51 (5515.36, 14446.84)	10702.21 (5743.95, 13446.09)	9030.56 (6259.94, 12288.60)	8905.58 (5579.46, 17099.79)	9656.14 (5873.22, 15254.52)	9117.68 (5405.10, 12618.73)
WB unstimulated	10.44 (3.74, 28.37)	10.79 (3.03, 26.81)	11.35 (4.32, 27.29)	9.39 (5.10, 26.19)	13.72 (6.14, 31.08)	8.51 (5.20, 26.77)
PBMC stimulated	3401.59 (2044.38, 6423.44)	3620.50 (1621.14, 5695.26)	4481.30 (2404.19, 5593.25)	3425.66 (2248.64, 7354.44)	3370.91 (1669.34, 5709.70)	2774.77 (1436.01, 6312.30)
PBMC unstimulated	5.06 (2.23, 8.79)	4.50 (1.51, 7.46)*	4.78 (1.94, 9.66)	4.39 (2.32, 14.81)	5.76 (3.02, 9.66)*	4.82 (3.46, 12.54)
TNF-a						
Systemic	10.96 (9.33, 12.60)	9.65 (8.75, 13.06)	10.27 (8.67, 12.79)	10.43 (8.83, 12.86)	10.62 (8.74, 12.67)	11.66 (8.62, 13.07)
WB stimulated	4310.61 (3263.14, 6165.32)	6592.27 (3437.62, 7256.40)	5309.05 (3761.57, 8295.26)	5256.58 (2930.78, 6797.27)	4976.67 (3989.72, 6265.50)	5882.58 (4077.33, 7744.45)
WB unstimulated	11.33 (7.95, 16.17)	12.27 (8.39, 15.96)	12.28 (7.82, 17.72)	11.58 (7.90, 14.10)	11.45 (8.13, 15.83)	10.65 (7.54, 13.16)
PBMC stimulated	4804.49 (2569.59, 7356.39)	3505.78 (2448.40, 5843.94)	4829.34 (3528.97, 7369.93)*	3829.92 (2035.19, 5522.33)	3023.95 (1911, 4936.13)	3706.84 (2511.35, 5810.28)*

PBMC unstimulated	13.88 (8.01, 17.07)	9.94 (5.54, 13.41)*	10.57 (5.19, 12.93)*	15.33 (9.01, 28.51)	11.46 (6.75, 22.30)*	14.76 (7.51, 19.53)*

[1]Data are median (IQR), n = 26. Data were compared using two-way repeated measures ANOVA with Bonferroni multiple comparisons test. *Indicates timepoint significantly different between treatments (multiplicity adjusted $p<0.05$). LPOO, low-polyphenol olive oil; HPOO, high-polyphenol olive oil; WB, whole blood; PBMC, peripheral blood mononuclear cells.

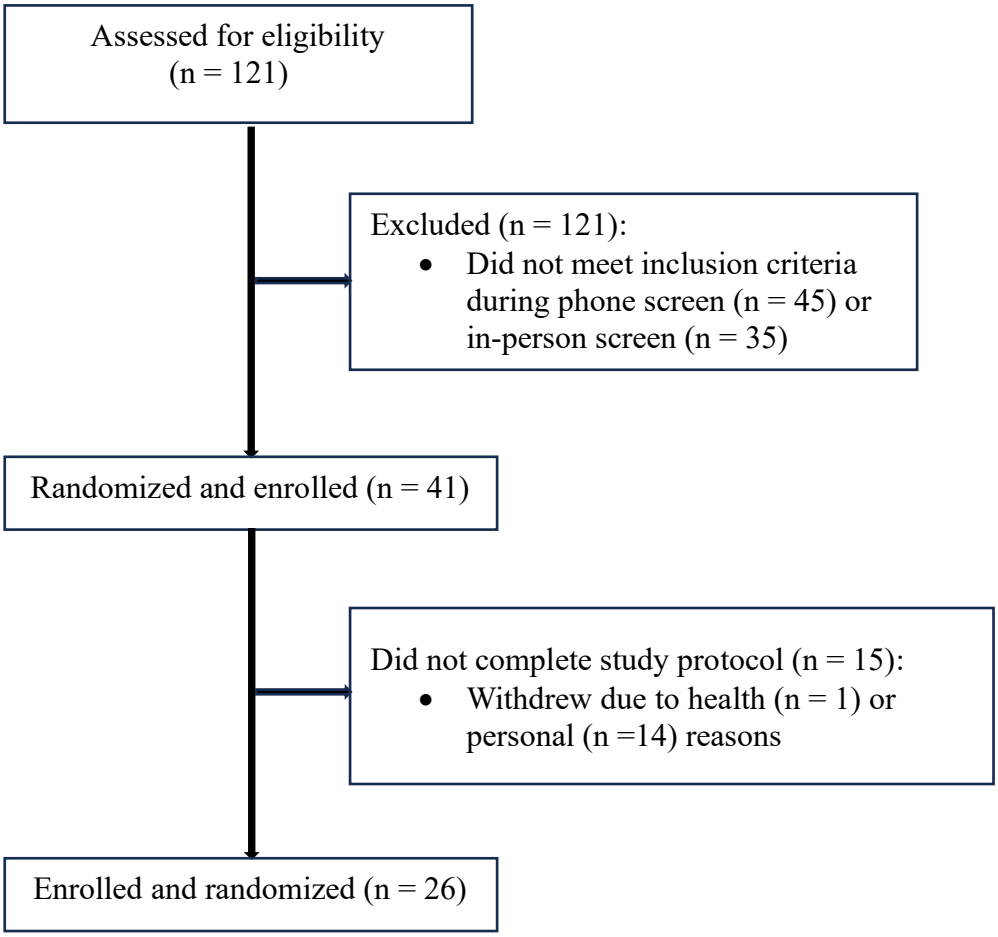


Figure 4.1 CONSORT flow diagram

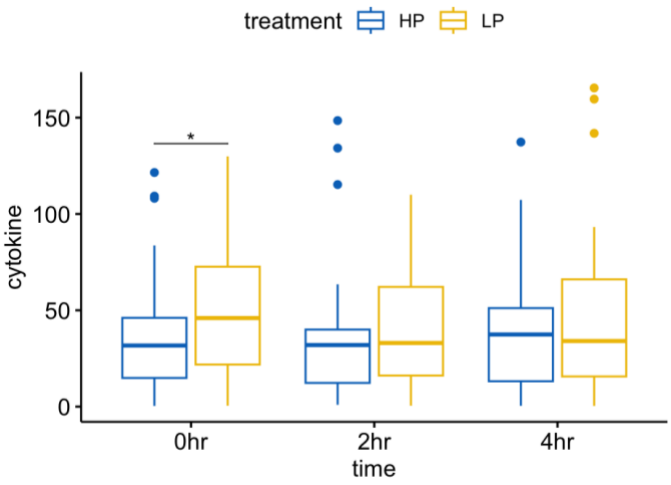


Figure 4.2 Change in IL-1ß (pg/ml) levels secreted from LPS-stimulated PBMCs at baseline, 2hrs, and 4hrs after consumption of LP and HP olive oils. Data is presented as untransformed median and IQR, n = 26. Data were compared via ANCOVA with baseline levels used as a covariate. Indicates timepoint significantly different between groups (multiplicity adjusted $p<0.05$). HP, high polyphenol; LP, low polyphenol.

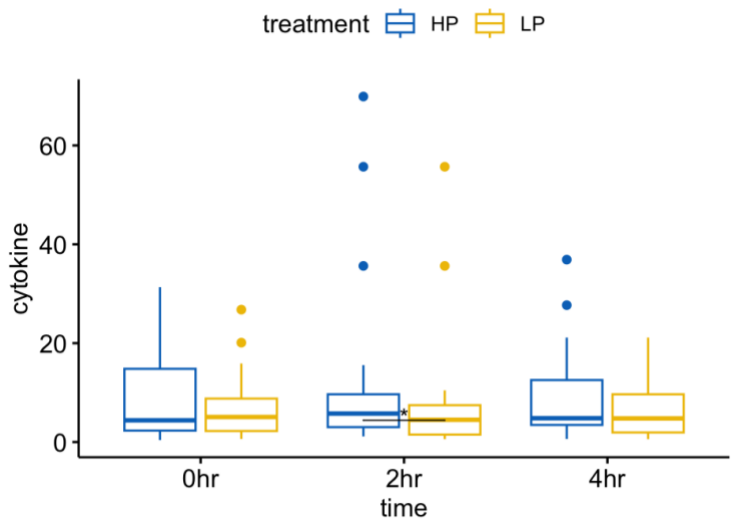


Figure 4.3 Change in IL-6 (pg/ml) levels spontaneously secreted from PBMCs at baseline, 2hrs, and 4hrs after consumption of LP and HP olive oils. Data is presented as untransformed median and IQR, n = 26. Data were compared via ANCOVA with baseline levels used as a covariate. Indicates timepoint significantly different between groups (multiplicity adjusted $p<0.05$). *Indicates timepoint significantly different between treatment groups (multiplicity adjusted $p<0.05$). HP, high polyphenol; LP, low polyphenol.

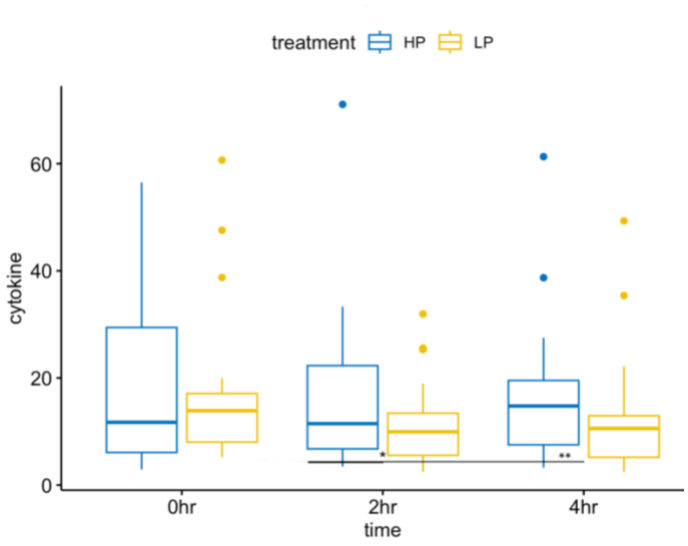


Figure 4.4 Change in TNF-a (pg/ml) levels spontaneously secreted from PBMCs at baseline, 2hrs, and 4hrs after consumption of LP and HP olive oils. Data is presented as untransformed median and IQR, n = 26. Data were compared via ANCOVA with baseline levels used as a covariate. Indicates timepoint significantly different between groups (multiplicity adjusted $p<0.05$). *Indicates timepoint significantly different between treatment groups (multiplicity adjusted $p<0.05$). HP, high polyphenol; LP, low polyphenol.

7. Supplemental materials

Supplemental table 4.1 Intake of macronutrients and major micronutrients on LPOO and HPOO[1]

Nutrient	LPOO	HPOO
Calories (kcal)	1909.6 ± 675.9	1866.5 ± 775.7
Protein (g)	92.7 ± 44.7	82.9 ± 51.5
Carbohydrates (g)	201.4 ± 88.2	204.8 ± 109.4
Fiber (g)	13.5 ± 9.2	14.3 ± 11.8
Sugar (g)	51.1 ± 35.6	43.2 ± 43.4
Fat (g)	82.5 ± 42.3	81.3 ± 45.3
Saturated (g)	25.4 ± 16.4	22.4 ± 14.0
Monounsaturated (g)	28.1 ± 22.8	28.5 ± 23.7
Polyunsaturated (g)	11.2 ± 7.6	10.9 ± 9.2
Trans fat (g)	0.7 ± 1.0	0.4 ± 0.5
Omega-3 (g)	1.1 ± 1.4	1.0 ± 1.9
Omega (6)	5.8 ± 5.3	5.4 ± 6.5
Cholesterol (mg)	251.8 ± 165.2	214.4 ± 179.9
Vitamin A (IU)	2572.2 ± 3825.4	2527. 1 ± 3712.2
Vitamin D (IU)	79.8 ± 98.7	70.9 ± 104.1
Vitamin E (IU)	5.3 ± 6.5	3.8 ± 4.2
Vitamin K (mcg)	30.2 ± 26.0	24.9 ± 32.1
Vitamin C (mg)	43.7 ± 58.0	32.3 ± 39.7
Calcium (mg)	582.2 ± 397.5	527.7 ± 459.9
Iron (mg)	11.3 ± 6.3	11.0 ± 8.2
Zinc (mg)	6.9 ± 6.4	5.6 ± 5.0
Magnesium (mg)	163.6 ± 95.4	136.3 ± 87.7
Sodium (mg)	2968.0 ± 1902.0	3024.4 ± 2134.2
Potassium (mg)	1702. 4 ± 1064.6	1446.5 ± 980.3
Caffeine (mg)	42.2 ± 63.9	48.5 ± 67.5

[1]Values are mean ± SD. Food records from the day before each study visit and 2 days (1 weekend day and 1 weekday) during each treatment period were collected. The total set of twelve days of food records were analyzed using Food Processor (SQL, Version 10.2, ESHA Research, Salem, OR). Food records were used to estimate participants' macronutrient and micronutrient intake on the LPOO and HPOO treatment arms. LPOO, low polyphenol olive oil; HPOO, high polyphenol olive oil.

Chapter 5
Conclusion

Chapters in this dissertation reviewed the literature on the effects of polyphenol- and lipid-rich foods on the gut-heart-brain axis, presented best practices, considerations and gaps for diet-gut microbiome research, as well as investigated the effects of two types of OO on cytokine secretion and the effects of walnuts on cardiometabolic and gut health of human subjects.

The literature review (Chapter 1) focused on the arising body of evidence in support of interconnections (or axis) between the gut microbiota, the heart, and the brain. One of the major ways one's lifestyle can affect this axis is through dietary practices. Various dietary components have shown the potential to affect the gut microbiota [1-7] resulting in the production of metabolites that can either act directly in the gut or get absorbed and penetrate into the bloodstream where they interact with various tissues and signaling pathways, consequently affecting the heart and the brain [6].

The overall focus of the dissertation was the Mediterranean diet and its specific foods, walnuts and olive oil. Research shows that there are several components in both that can potentially reach the distal gut and interact with the gut microbes.

Walnuts, for instance, contain fiber, fatty acids and ellagitannins. Evidence demonstrates that due to the cell wall structure, human bodies harvest 21% less energy from walnuts than what is predicted by the Atwater values [8], which means that some of the walnut components do indeed escape host digestion and are likely metabolized by the gut microbial communities. For instance, LA fatty acid can be metabolized by the *Roseburia* genus [9]. Interestingly, we observed an increase in percent relative abundance of *Roseburia* in our walnut study (Chapter 3) where participants consumed 56 g of walnuts daily for 3 weeks. This finding is supported by other human trials as well [10-12]. In terms of fiber in walnuts, there are not many studies examining the effects of whole walnut fiber on the gut microbiome. Most evidence so far is on

the effect of the walnut meal fiber in mouse models [13]. There has been no reliable biomarker of dietary fiber established yet and fecal pH and SCFA or breath hydrogen are recommended to be measured. While we did see a significant decrease in fecal pH after walnut consumption, the levels of SCFAs decreased. Other studies did observe an increase in SCFAs, however [14]. These discrepancies may be due to the fact that approximately 95% of SCFAs are absorbed by the host and fecal levels are a reflection of the host absorptive capacity rather than the SCFA production by the gut microbes. In addition, to see an increase in SCFA production as a result of dietary fiber intervention, the type of fiber and the sufficient amount of it that can be metabolized by the microbes already present in the gut is required. This may not be achieved by the mere addition of walnuts to the diet.

Ellagitannin compounds in walnuts also have the potential to interact with the gut microbes. They can be metabolized by the gut microbiota and urolithin metabolites are produced as a result. Several bacterial strains with the capacity to produce urolithins have been identified thus far – *Gordonibacter* and *Egerthella*. While in our walnut study we detected *Gordonibacter* in five out of 30 participants prior to walnut consumption, this number increased to eleven after the walnut consumption. We did not observe any changes in *Eggerthella*, however, which contradicts other trials [15]. Human subjects have also exhibited a differential ability to produce urolithin metabolites based on their gut microbiome composition. This led to discovery of three metabotypes of urolithin production – UM-A, UM-B and UM-0. Several studies show that individuals belonging to UM-B are usually more prone to conditions associated with GI distress but seem to benefit from consuming ET-containing foods the most [16]. Our walnut study results do support the evidence for the existence of several metabotypes but we were unable to

determine whether any specific metabotype had more prominent improvements after walnut consumption.

While the results we obtained in the walnut study are at least in partial alignment with what has been shown in other trials, interpretation of results is still challenging. For instance, it is still unclear what a "healthy" gut microbiome looks like, and which microbes are "beneficial" or "detrimental" to host health [17-18]. This leads to varying interpretation of results and makes drawing conclusions across studies challenging. Many studies report the changes they see in gut microbial composition but do not necessarily interpret what those changes may mean, or they interpret them in a way that supports their original hypothesis, thus labeling various bacteria as "detrimental" or "beneficial" when there may, in fact, be evidence in support of both. This emphasizes the need to describe gut microbes more as a part of the community as a whole. In addition, the environment, lifestyle and health status of the host should also be taken into consideration when interpreting shifts in gut microbiota composition.

Discrepancies in the reported resulted may also be due to differing research methodology and techniques used in human trials, as standardized practices for diet-gut mirobiome research are lacking. This issue was explored in detail in Chapter 2 that aimed to identify recommended best practices, define existing gaps, and collate considerations for the design of studies investigating diet-gut microbiome interactions in relation to human health outcomes. Several general points of agreement were identified but a recurring theme was that best practices will depend on the research aims, outcomes and feasibility. Multiple gaps were also identified, including underrepresentation of microbiota-modulating dietary components in food databases, lack of knowledge regarding intervention durations required to elicit lasting changes in the gut

microbiome, and the need to further develop and refine statistical approaches for integrating diet and gut microbiome data.

Regarding OO, while lacking fiber, it is rich in several prebiotic components including healthy fats and polyphenols, HTyr and OLE. While HTyr is mostly absorbed in the upper gut, OLE has the potential to persist all the way to the colon where it is metabolized to HTyr by Lactobacilli and Bifidobacteria genera [19-20]. These interactions result in modulation of the microbial community composition, which then affects the gut and overall health of the host [21]. Chapter 4 of the dissertation described an 8-week randomized crossover trial where generally healthy adults consumed two types of OO – high-polyphenol (HPOO) and low-polyphenol (LPOO). Blood samples were collected at the beginning and end of each intervention period (short-term consumption) as well as 2hrs and 4 hrs after consumption of both types of OO (acute consumption). The outcome variables the chapter focused on were the levels of circulating cytokines, as well as the secretion of cytokines in whole blood and from PBMCs stimulated with LPS. While there were no changes in circulating cytokines or cytokines secreted from the LPS-stimulated whole blood, we did observe several differences in cytokine secretion from PBMCs between HPOO and LPOO groups. Cytokine levels were different between groups at multiple timepoints but there was no clear pattern for either type of OO or any specific cytokine. For instance, while levels of IL-1b and TNF-a secreted from stimulated PBMCs were lower after HPOO at several timepoints, TNF-a levels were higher after HPOO compared to LPOO when PBMCs were not stimulated with LPS. Most of these changes were seen as a result of acute OO consumption (2 and 4 hrs) and not after the short-term consumption (2 weeks). We did not examine gut microbiota in this study, which could be a good next step for a future study.

In conclusion, while the diet-gut microbiome field keeps moving towards personalized nutrition recommendations, there are still many gaps in knowledge that need to be addressed. Evidence from the work in this dissertation contributes to the field by both providing several answers, as well as posing several further questions. Both are integral for reaching the final goal of prevention of chronic disease through personalized care and guidance.

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