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Local and Systemic Effects of Flavonoid Intake with Submaximal Cycling Exercise: A Randomized Controlled Trial

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LOCAL AND SYSTEMIC EFFECTS OF FLAVONOID INTAKE WITH SUBMAXIMAL CYCLING EXERCISE: A RANDOMIZED CONTROLLED TRIAL

by

Stephanie Kung

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ABSTRACT

Local and Systemic Effects of Flavonoid Intake with Submaximal Cycling Exercise: A Randomized Controlled Trial

by

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Utah State University, 2021

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Submaximal endurance exercise is a known cause of elevated gastrointestinal permeability, injury, and inflammation, which may contribute to symptoms such as intestinal cramps and diarrhea. Preclinical and some clinical studies suggest that dietary flavonoids, a class of plant secondary metabolites, may regulate intestinal permeability, reduce chronic low-grade inflammation, and modulate the intestinal microbiota. The purpose of this study was to determine the local and systemic effects of flavonoid intake, in addition to cycling performance. A randomized, double blind, placebo controlled crossover trial was conducted with 12 recreational cyclists (eight men, four women) from August 2018 to May 2019 (ClinicalTrials.gov identifier: NCT03427879). Subjects were randomized to a treatment sequence and consumed a high or low flavonoid (490 or 5 mg), pre-workout beverage daily for 15 days. At the end of each intervention, a submaximal cycling trial (one hour at ~70% maximal oxygen capacity) was conducted in a controlled laboratory setting (23 °C). Plasma samples were collected pre- and post-

exercise (immediately, 1-h post, 4-h post). Primary outcomes were intestinal injury and permeability, assessed by within-subject comparisons of plasma intestinal fatty acid-binding protein and urinary sugar excretion. Due to the contribution of the microbiota to host metabolism, additional studies of the intestinal microbiome, short chain fatty acid excretion, and plasma metabolomics were conducted.

Food frequency questionnaires estimated total flavonoid intake at 635 ± 195 mg per week. Overall, subjects had low intestinal inflammation, and no differences in short chain fatty acids or alpha and beta diversity were found. The treatment tended to increase force applied during cycling ($p = 0.051$; effect size, $d = 0.16$). Despite observing exercise effects on biomarkers, no treatment effects were found for intestinal permeability or injury ($p > 0.05$). Circulating carbohydrates, lipids, and amino acids changed to varying degrees following exercise, suggesting that multiple fuel substrate pathways were utilized throughout exercise and recovery.

Sub-chronic supplementation with blueberry, cocoa, and green tea flavonoids did not significantly affect intestinal health in recreational cyclists. For high intensity exercise, a high flavonoid beverage performed as well as the control and did not alleviate exercise-induced intestinal permeability or injury.

(217 pages)

PUBLIC ABSTRACT

Local and Systemic Effects of Flavonoid Intake with Submaximal Cycling Exercise: A Randomized Controlled Trial

Stephanie Kung

Moderate to high intensity endurance exercise is a known cause of exercise-induced gastrointestinal syndrome, a condition often associated with intestinal complaints such as discomfort, cramps, and diarrhea. Oftentimes, elevated intestinal inflammation and permeability (a “leaky gut”) are also observed. Previous research has shown that flavonoids, natural compounds found in many fruits and vegetables, may mitigate these exercise-induced effects. In particular, certain cocoa powders contain high levels of flavonoids, and chocolate milk is a good source of protein and sugars for sustaining intense activity. Thus, the purpose of this study was to evaluate the effects of a dairy pre-workout beverage, with flavonoids from cocoa, blueberries, and green tea. We hypothesized that regular consumption of these flavonoids would alleviate exercise-induced gastrointestinal syndrome. A clinical trial was conducted with twelve subjects, who consumed the pre-workout once per day. After 14 days, subjects completed a one-hour cycling test to determine the effects of the beverage on performance and gastrointestinal health. Each participant underwent both the treatment and control in a randomized order, serving as his or her own control for comparison.

Our results showed that the cycling trial was strenuous and caused measurable changes in biomarkers of inflammation and intestinal injury, but ultimately, the flavonoid pre-workout did not differ from the control. These findings suggest that short-term

flavonoid supplementation from processed food ingredients (cocoa powder, freeze-dried blueberries, and powdered green tea) may not have protective effects on the gut during exercise-induced stress. However, the product was well-liked by the subjects and no negative effects were observed. Nutritional strategies for reducing incidence of exercise-induced gastrointestinal syndrome is a relatively new area of research, and these results can help shape future recommendations in sports nutrition for endurance athletes and coaches. This project contributes to current scientific knowledge regarding the interaction between flavonoid consumption and intestinal health. Furthermore, similar mechanisms are thought to drive related conditions such as exertional heat stress, ulcerative colitis, and inflammatory bowel disease. Due to the prevalence of a leaky gut and inflammation in not only endurance exercise but also these other conditions, more research—especially well-controlled human clinical trials—is warranted.

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Stephanie Kung

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LIST OF ABBREVIATIONS

ARE = Antioxidant response element

COX = Cyclooxygenase

ELISA = Enzyme-linked immunosorbent assay

eNOS = Endothelial nitric oxide synthase 3

FFQ = Food frequency questionnaire

FMD = Flow mediated dilation

GC-MS = Gas chromatography-mass spectrometry

GI = Gastrointestinal

HFB = High flavonoid beverage

HFD = High flavonoid diet

HR = Heart rate

IBS = Irritable bowel syndrome

I-FABP = Intestinal fatty acid-binding protein

IL = Interleukin

LC-MS = Liquid chromatography-mass spectrometry

LFB = Low flavonoid beverage

LFD = Low flavonoid diet

LOX = Lipoxygenase

LPH = Lactase-phlorizin hydrolase

LPS = Lipopolysaccharide

NF-κB = Nuclear factor kappa-light-chain-enhancer of activated B cells

NHANES = National Health and Nutrition Examination Survey

NO = Nitric oxide

Nrf2 = Nuclear factor erythroid 2-related factor 2

NSAIDs = Non-steroidal anti-inflammatory drugs

PAR-Q = Participation readiness questionnaire

PCR = Polymerase chain reaction

PLA2 = Phospholipase A2

PSI = Physiological strain index

RCT = Randomized controlled trial

ROS = Reactive oxygen species

RPE = Rating of perceived exertion

rRNA = ribosomal ribonucleic acid

SCFA = Short chain fatty acids

TCA = Tricarboxylic acid cycle

TLR4 = Toll-like receptor 4

TMS = Trimethylsilyl

TNF-α = Tumor necrosis factor alpha

TTE = Time to exhaustion

TT = Time trial

VO_2 max = Maximal oxygen uptake

ZO-1 = Zonula occludens-1

CHAPTER I

INTRODUCTION

Bill Rodgers, four-time winner of both the Boston and New York City marathons, said that "more marathons are won or lost in the porta-toilets than at the dinner table." The "runner's trots" (acute exercise-induced diarrhea) and other gastrointestinal (GI) complaints commonly occur during long distance running and other endurance sports [1]. For example, in one ultramarathon, 85% of athletes reported experiencing debilitating GI symptoms, resulting in reduced nutritional intake during the race and recovery period [2]. These GI symptoms are thought to be driven by the physiological changes that occur during high-intensity exercise, during which core temperature steadily rises and oxygen availability in the intestinal tissues decreases [1,3,4]. Other contributors to GI distress may include mechanical (e.g., posture, repetitive movement), dietary (e.g., high fat, fiber, or fluid intake), and neuroendocrine (e.g., cortisol secretion) factors [4].

Furthermore, intense exercise greater than 70% of maximal oxygen uptake (VO_2max) and core temperatures above 39.5 °C consistently result in elevated GI permeability and inflammation, both of which may contribute further to the etiology of GI distress [1,5,6]. Gastrointestinal permeability refers to the movement of small molecules across the GI epithelium, the largest interface between the external environment and internal milieu; the intestinal epithelium is a selectively permeable membrane that regulates the absorption of water, ions, and nutrients while excluding pro-inflammatory agents [7]. While mild GI symptoms are an inconvenience and may impact athletic performance, exercise-induced intestinal injury and elevated permeability may

result in the passage of exogenous toxins, microorganisms, and antigens that can further compromise an athlete's overall health by triggering a systemic inflammatory response [8].

Currently, effective nutritional interventions are limited, though many different products, including bovine colostrum [9], probiotics [10], glutamine [11], nitrate [12], and carbohydrates [13], have been investigated. A growing body of work suggests that flavonoids, a diverse class of secondary metabolites abundant in fruits and vegetables, may have the potential to alleviate exercise-induced GI inflammation and permeability [14]. Findings from *in vitro* and animal studies suggest that flavonoids can modulate intestinal permeability via the intestinal microbiota and tight junction protein complexes, while downregulating pro-inflammatory pathways [15–23]. A variety of flavonoids have also been effective in preventing or treating cell culture and animal models of inflammatory bowel disease (IBD), a condition which also is driven by gut inflammation and barrier dysfunction [24–27].

However, observations from cell culture and animal studies do not always translate to the same effect in the human context, and few human clinical trials have been conducted [14]. In the Polyphenol Study, a randomized controlled trial (RCT) previously conducted at Utah State University, dietary polyphenols (a larger class of molecules that includes flavonoids) were demonstrated to have a protective effect on the intestinal barrier. Chronic administration of a high flavonoid diet (HFD) reduced gut permeability and inflammation in obese and overweight humans (unpublished data)—a population that displays elevated intestinal permeability and chronic low-grade inflammation [7,28]. As intestinal permeability has also been observed to increase with age, another crossover

trial provided a high polyphenol diet to a population of older subjects (≥ 60 years) and reported an improvement in intestinal permeability in subjects with high baseline permeability [29]. We hypothesize that these reductions in GI permeability will translate to endurance athletes, who are also known to experience an impaired GI barrier as discussed previously.

Although previous clinical trials have evaluated flavonoid supplementation in combination with exercise, study endpoints were generally markers of oxidative stress, and evaluations of GI permeability or injury were not included [30–35]. There were, however, two recent clinical trials conducted with healthy subjects, investigating polyphenol intake with exercise-induced GI permeability and inflammation as primary endpoints. First, Szymanski et al. demonstrated that 3-day supplementation with the polyphenol curcumin reduced markers of GI damage and the inflammatory response following 60 minutes of moderate-intensity running in hot environmental conditions [36]. Second, two weeks of moderate flavonoid intake reduced intestinal permeability both at rest and after 45 minutes walking at ~60% VO_2max, though not following a 2.5 hour run at ~70% VO_2max [37]. Taking the findings from pre-clinical studies together with these recent human studies, dietary flavonoids are a promising intervention to address the effects of exercise in the GI tract.

Advancements in high-throughput technology and data processing has enabled scientific inquiries with "-omics" techniques, allowing investigators to gather more detailed information regarding individuals' microbiomes and metabolomes. Metabolomics is a comprehensive analysis used to detect and identify all small molecules within a biofluid or tissue, and can provide a picture of the real-time metabolic response

to a combination of external and internal stimuli [38]. The GI tract is colonized by a dynamic community of commensal microorganisms—the gut microbiota—that performs many essential functions for the host such as vitamin synthesis and nutrient metabolism [14,39]. The intestinal microbiome is a key mediator in the effects of the diet. Microbiome analysis attempts to characterize the diversity and abundance of these microorganisms within a sample, as well as compare the community structures between samples [40]. In the lower GI tract, flavonoids are extensively metabolized to a variety of intermediates and products, and even dependent on the microbiota to be transformed to more bioavailable and bioactive metabolites [41,42]. Conversely, flavonoid intake can also shape the composition of the intestinal microbiome—dietary changes can begin to alter the intestinal community in as little as one day [42,43]. Furthermore, the effects of dietary flavonoid intake may be highly individualized. Recent microbiome analyses have revealed a significant effect of inter-individual variation in responding to dietary interventions [14,29]. Systemic effects of flavonoid intake are mediated by metabolites (requiring metabolomics analysis), which depend in part on the functions of each individual's intestinal microbiota (requiring microbiome analysis) [44]. As a result, a study of the physiological effects of flavonoid intake would greatly benefit from including supplementary microbiome and metabolomics analyses.

The overall hypothesis of this project is that sub-chronic supplementation of flavonoids will ameliorate exercise-induced gut injury, permeability, and inflammation. Because of the close interaction between the gut microbiome and human health, we conducted secondary analyses on the effects of flavonoid supplementation on the gut

microbiome, fecal short chain fatty acid (SCFA) content, and plasma metabolomics profile. The project objectives were as follows:

1. Formulate a dairy-based, pre-workout beverage that includes flavonoids from blueberry, cocoa, and green tea at a level similar to those provided in prior clinical studies where reductions in gut permeability were previously demonstrated.
2. Conduct a RCT with trained cyclists to determine the effect of sub-chronic consumption of the flavonoid sports nutrition beverage on gut permeability, injury, inflammation, and performance during an exercise time trial.
3. Determine the effect of chronic consumption of the flavonoid sports nutrition beverage compared to a low flavonoid control on microbiome composition, SCFA production, and the plasma metabolome.

The overall goal of our research is to investigate the effects of bioactive compounds on intestinal health. To this end, it is important to understand the local and systemic effects of dietary flavonoids. The results from this project will further our understanding of the interactions among dietary flavonoids, the gut microbiota, and the GI barrier. If proven effective, such a product could potentially benefit athletes and other individuals displaying elevated GI permeability, which is also commonly reported in exertional heatstroke, septic shock, irritable bowel syndrome, celiac disease, food allergies, obesity, and inflammatory bowel disease [45–50]. Research in these areas is ongoing, as several of these conditions still lack an effective nutritional treatment [45,46,51]. Since fluid bovine milk is well-suited for exercise nutrition and generally liked among athletes, this presents an opportunity to provide a dairy-based product specifically designed to address exercise-related intestinal changes [52]. The dairy

industry, nutraceutical companies, and cocoa, tea, and blueberry producers also have opportunity to benefit from this research. Finally, research findings could extend knowledge of sports nutrition specifically for endurance athletes, as addressing changes in gut permeability, injury, and inflammation is a relatively new concern in the field.

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CHAPTER II

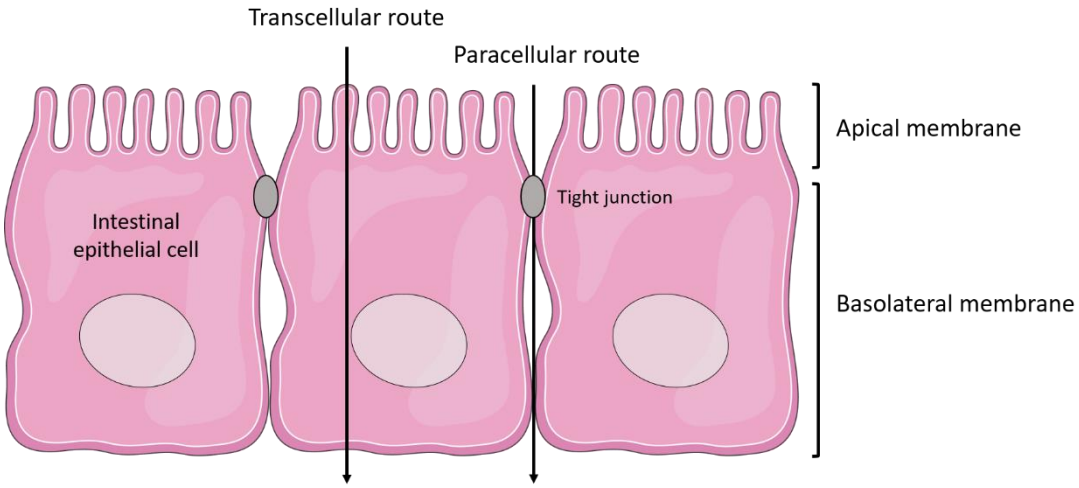
LITERATURE REVIEW

1. The Gastrointestinal Barrier

The intestinal epithelium is a selective barrier that separates luminal contents (molecules, enzymes, and microorganisms) from internal circulation and consists of a single layer of absorptive enterocytes, secretory goblet cells, peptide- and hormone-secreting enteroendocrine cells, and Paneth cells [1,2]. Adjacent cells are connected together at the apical (luminal) surface by tight junctions, which are protein complexes comprised of the transmembrane proteins occludin, claudin, tricellulin, and junctional adhesion molecules (JAMs) [2–4]. Occludin and claudin are anchored to the cytoplasmic zonula-occludin proteins, which are bound to cytoskeletal actin and myosin filaments [2].

The intestinal barrier plays an important role in regulating the passage of nutrients and small molecules from the intestinal lumen to internal circulation [1]. To enter hepatic and systemic circulation, luminal contents either pass through intestinal cells (transcellular route) or the interstitial space between cells (paracellular route) (Figure 2-1) [3]. Disruption of this selective barrier can result in unregulated translocation of exogenous antigens, toxins, and pathogens, potentially causing local and systemic inflammation [2]. A “leaky gut” or increased intestinal permeability, may be caused by several factors, including epithelial damage to the mucosal lining or altered tight junction protein expression [1,5].

Figure 2-1. Structure of the intestinal epithelial membrane and the transcellular and paracellular routes of transport.



2. Endurance Exercise and GI Distress

Intensive endurance exercise is known to cause intestinal barrier dysfunction, and approximately 30-50% of individuals experience GI symptoms during exercise and competition [6]. Gastrointestinal complaints such as abdominal cramps, diarrhea, vomiting, and nausea are commonly experienced by endurance athletes [7]. In one study, following a bout of high-intensity exercise, symptomatic athletes experienced greater intestinal permeability than asymptomatic athletes did [8]. Interestingly, the two groups did not differ in baseline gut permeability measured at rest [8]. On the other hand, increased gut permeability has also been observed even in the absence of GI symptoms, which suggests that even asymptomatic athletes may be at risk of exercise-induced intestinal injury [9].

2.1 Splanchnic Hypoperfusion

Changes in two physiological factors are thought to cause this dysregulation in intestinal permeability during exercise: splanchnic (abdominal) circulation and core body

temperature [10,11]. During exercise, up to 80% of splanchnic blood flow is reduced and redirected to support active tissues (i.e., cardiac, pulmonary, and skeletal muscle tissue) [10]. Splanchnic blood flow has been shown to decrease 30-60% after 30 minutes of exercise at 60-70% VO_2max [11]. Gut hypoperfusion and ischemia are consequences of prolonged, inadequate mesenteric blood flow, leading to low oxygen and glucose availability [7,12]. These effects are greater at higher exercise intensities and environmental temperatures, and may also be affected by age, prandial state, and training status [6,11,13]. Depending on the duration and intensity of exercise, mucosal erosions and ischemic colitis have also been reported [7]. Even in mild or less severe cases, occurrence of increased intestinal permeability has the potential to hinder athletic performance and the subsequent recovery period [7].

2.2 Core Temperature

Secondly, elevated GI permeability is believed to be strongly influenced by changes in core temperature [9,14]. During exercise, localized gut hyperthermia occurs as core body temperature gradually rises, which causes hypoxia and cellular metabolic stress in the splanchnic capillary beds [15]. Enterocytes located at villus tips are particularly vulnerable to hypoxia as there is generally lower oxygen availability due to the countercurrent exchange in the villi [7,16]. At elevated temperatures of 37-42.5 °C, both *in vitro* and pre-clinical studies have shown that the intestinal barrier is compromised [3,17–20]. When rats were heat stressed at 42.5 °C, Lambert et al. observed epithelial cell sloughing, vacuolization, and loss of microvilli [18]. In a systematic review of sixteen clinical studies, Pires et al. reported that beyond the threshold temperature of 39 °C, increased intestinal permeability was always observed following an exercise test [14].

Exercise tests in controlled clinical settings at moderate to high intensities (≥ 70% VO_2max) have been shown to elevate core temperature to 38.2-39.6 °C [21]. In outdoor competition, peak core temperatures as high as 41.7 °C have been reported [22]. Beyond the critical thermal maximum of 41.6 °C in humans, sloughing and apoptosis of intestinal cells occur, leaving gaps in the barrier, and without rapid intervention, can result in multi-organ failure and death [3].

2.3 Exercise and Endotoxemia

In some cases of elevated intestinal permeability, paracellular gaps may become large enough to permit translocation of intestinal microorganisms or their cellular components—particularly lipopolysaccharide (LPS) [13]. LPS is typically present at high levels in the intestinal lumen, due to the large population of Gram negative bacteria that reside there [23]. However in systemic circulation, LPS is a major activator of the inflammatory cytokine response, recruiting leukocytes and promoting the production of inflammatory cytokines, nuclear factors, and heat shock proteins [14]. Furthermore, the presence of LPS in interstitial fluid and at the basolateral side of the intestinal epithelium can induce tight junction permeability by promoting expression of toll-like receptor 4 (TLR4), the pattern recognition receptor for LPS, which is responsible for activating the Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) inflammatory signaling cascade [23]. In this way, leakage of LPS due to external stresses like exercise may result in a prolonged inflammatory response [23]. Higher circulatory levels of anti-LPS immunoglobulins have been reported in competitive triathletes, potentially due to frequent exposure to LPS from intestinal Gram negative bacteria during training and exercise [24]. Several studies have reported exercise-induced permeability and

endotoxemia (elevated LPS in circulation) in athletes following competition [24–27]. In exertional heat stroke, the resulting defects in the intestinal barrier are thought to lead to LPS leakage, activation of the inflammatory response, and multiple organ failure [25].

2.4 Nutritional Strategies to Mitigate GI Distress

Though exercise-induced gut permeability is a common occurrence, there are few nutritional strategies that have been shown to prevent it, and this may be due in part to the fact that the mechanisms are still not well understood [1]. Most recommendations are general in nature, such as limiting the intake of high fat, fiber, and protein foods and hyperosmotic beverages immediately preceding exercise [6,7,11,28]. During a triathlon, incidence of GI symptoms was modestly associated with morning intake of carbohydrates, caffeine, and total calories [29]. Dietary recommendations are also dependent on the individual's training habits, dietary preferences, sport, and genetics [28]. Some promising strategies have been proposed, though research is still ongoing and most nutritional interventions have yielded mixed results.

First, adequate hydration is essential, as dehydration can further exacerbate exercise-induced symptoms [11,13]. Strategic carbohydrate consumption also appears to reduce GI symptoms, injury, and inflammation, especially when coupled with "gut training" to increase individual tolerance to consuming carbohydrates during exercise [30–32]. For example, ingestion of multiple transportable carbohydrates (e.g., 2:1 glucose to fructose ratio) versus a single carbohydrate reduces osmotic pressure in the gut and may alleviate symptoms [6,30,33]. On the other hand, Sessions et al. found that ingestion of a carbohydrate gel during a 60-minute running trial at 70% VO_2max increased markers of intestinal injury and inflammation compared to a non-carbohydrate control [34].

Another nutritional intervention is to consume compounds such as L-citrulline and glutamine that increase nitric oxide (NO) production in the gut to stimulate vasodilation and counteract the exercise-induced reduction in splanchnic perfusion [6,26,35,36]. However, these studies have also yielded mixed results, and GI complaints also appear to associate with the consumption of vegetables containing dietary nitrate [6,33,37]. Other potential nutritional strategies under investigation include probiotics and bovine colostrum supplementation [38–43].

3. Effect of Flavonoids on Human Health and Exercise

3.1 Polyphenols and Flavonoids

Polyphenols are a large class of plant secondary metabolites characterized by one or more aromatic benzene rings with two or more hydroxyl functional groups [44]. These compounds are produced in plants for a variety of purposes including pigmentation, growth, protection from ultraviolet radiation, and microbial or insect resistance [44]. Common dietary sources of polyphenols include fruits, vegetables, coffee, and tea, with the average individual consuming anywhere from 100 to 2000 mg total polyphenols daily [45,46]. Diets rich in polyphenols are associated with a reduction in the risk for several non-communicable diseases, presumably due to the antioxidative, anti-inflammatory, and antimicrobial properties of polyphenols, and epidemiological data has shown that populations consuming diets high in fruits and vegetables have lower risk of chronic and degenerative age-associated diseases [5,45,47–51]. Furthermore, a number of studies have suggested that plant polyphenols such as procyanidins, (-)epigallocatechin gallate, quercetin, and (-)-epicatechin may protect epithelial tissue integrity by regulating tight junction protein expression, assembly, and disassembly [5,52–56].

Within polyphenols are four main compound classes: lignans, stilbenes, phenolic acids, and flavonoids [44]. Common dietary flavonoids and food sources are listed in Table 2-1. In particular, flavonoids, which comprise the major group within polyphenols, have been of interest in health and nutrition research because of their non-nutritive bioactive properties—health-promoting effects beyond basic nutritional needs [45,57]. Flavonoids are characterized by two aromatic rings connected by a 3-carbon bridge (C_6-C_3-C_6) and further categorized in to six sub-classes: flavonols, flavanols, flavanones, flavones, anthocyanins, isoflavonoids [55]. Flavanols and anthocyanins are the most abundant polyphenols in the human diet [45].

Polyphenols are chemically classified as radical scavenging molecules and metal ion chelators, but due to their low bioavailability and low levels in plasma and tissues, their health effects *in vivo* are more likely via modulation of cellular metabolism and signaling [46]. Maximum plasma levels of polyphenol metabolites in comparison to those of endogenous phenols and antioxidants (e.g., ascorbate, p-hydroxyphenyl lactate, p-hydroxyphenyl pyruvate, tyrosine, homogentisic acid, α-tocopherol) (~0.1-22 μM versus 160-380 μM, respectively) also suggest that it is unlikely that polyphenols themselves are significant contributors to total antioxidant capacity as direct antioxidants [58]. One possible exception is in the intestinal lumen where flavonoids are present at higher concentrations and may act directly to chelate redox-active metal ions and scavenge free radicals [59]. Findings from cell culture and pre-clinical studies suggest that flavonoids (both purified compounds and polyphenolic plant extracts) have protective effects on the intestinal barrier, enhance tight junction complex formation, and reduce intestinal inflammation [53,60–63].

Table 2-1. Common dietary flavonoids and food sources [44,64].

Flavonoid	Example compounds	Dietary sources
Simple Flavonoids		
Flavan-3-ols	Catechin, Epicatechin, Gallocatechin, Epigallocatechin, Proanthocyanidins	Green tea, apple, red wine, cocoa
Flavones	Luteolin, Apigenin	Herbs and spices; parsley, capsicum pepper, celery
Flavonols	Kaempferol, Quercetin, Myricetin	Ubiquitous in plant foods; onions, leeks, broccoli, cherry tomato, apple, berries, beans, tea and red wine
Flavanones	Hesperetin, Naringenin, Eriodictyol, Tangeritin	Citrus fruits and herbs (oregano)
Isoflavones	Genistein, Daidzein	Soybeans, soy-based foods
Anthocyanins	Delphinidin, Pelargonidin, Cyanidin, Malvidin, Petunidin	Red, blue, and purple fruits and berries; wine, blackcurrant, cherry
Complex Flavonoids		
Proanthocyanidins (Condensed Tannins or Flavanol Polymers)	Procyanidins, Prodelphinidins, Propelargonidin	Chocolate, stone fruit, grapes, strawberries, nut skins, beer, wine, legumes

3.2 Effects on GI Permeability: Tight Junctions

The potential regulatory pathways for the effects of polyphenols at the intestinal epithelium were discussed in depth by Yang et al. [5]. First, dietary flavonoids such as anthocyanins and quercetin have been shown to inhibit NF-κB signaling, a key transcription factor involved in many inflammatory pathways, and tumor necrosis factor alpha (TNF-α), a pro-inflammatory cytokine [5]. In addition to the transcription of pro-inflammatory cytokines, chemokines, cell adhesion molecules, and acute phase proteins, NF-κB activation appears to disrupt the intestinal barrier; inhibition of NF-κB signaling by pyrrolidine dithiocarbamate resulted in increased expression of the tight junction proteins occludin and zonula occludens-1 (ZO-1) [5]. Epicatechin, a flavan-3-ol

commonly found in cocoa, can prevent TNF-α barrier disruption through NF-κB inhibition [56]. Anthocyanins have also been shown to inhibit membrane permeabilization by TNF-α and activate cellular kinases like p38-MAPK which regulate tight junctions [65,66]. In another study, anthocyanin supplementation in mice mitigated high fat diet-induced intestinal permeabilization and increased tight junction protein expression via production of the intestinal hormone GLP-2 [65]. Other potential mechanisms of polyphenols acting at the intestinal epithelium include regulation of protein kinase pathways, reduction of reactive oxygen species (ROS), and suppression of cellular enzymes like protein phosphatase 2A and Rho GTPases—all of which have downstream effects on tight junction permeability [5,66].

3.3 Effects on GI Permeability and Inflammation

Polyphenols and polyphenol-derived metabolites have been shown to be likely regulators of intestinal permeability in preclinical models, but overall, human clinical data regarding flavonoid supplementation to mitigate intestinal inflammation and permeability is limited [62,67]. Curcumin, a polyphenolic compound found in turmeric, may have beneficial effects on exercise-induced inflammation and intestinal permeability in hot environmental conditions (20 °C vs. 37 °C) [68,69]. Short term, 3-day supplementation with 500 mg/day of curcumin reduced measures of intestinal injury and thermal strain during one hour of sub-maximal treadmill running in hot conditions (37 °C) in non-heat acclimated subjects [69]. Typically, plasma I-FABP, an indicator of intestinal injury, is elevated following exercise, but this effect was blunted with curcumin supplementation compared to placebo (58% vs. 87% increase from pre-exercise values, respectively) [69]. Physiological strain index (PSI), an indicator of exertional heat stress

risk, is determined from core temperature and heart rate, and the PSI was significantly lower with curcumin supplementation than placebo during the last 20 minutes of the exercise test [69].

Previously, a study conducted at USU (the Polyphenol Study) demonstrated the efficacy of chronic consumption of dietary flavonoids in reducing measures of gut permeability and inflammation in human subjects (unpublished data). A randomized, double-blind, crossover trial was conducted with two six-week treatment periods separated by a washout period. Overweight and mildly obese subjects were recruited for this study, as this population is known to exhibit chronic low-grade intestinal inflammation [1,70]. A well-controlled whole foods diet was provided to participants who consumed either 340 mg total flavonoids per 1000 kcals on the high flavonoid diet (HFD) or 10 mg per 1000 kcals on the low flavonoid diet (LFD). The two macronutrient-matched diets differed in only flavonoid content. The results in Table 2-2 show that the HFD reduced gastrointestinal permeability compared to the LFD as determined by the urinary sugar permeability test relative to mannitol excretion. Furthermore, fecal levels of calprotectin, myeloperoxidase, and eosinophil protein X were also significantly lower with the HFD treatment, which suggests that dietary flavonoids may reduce intestinal inflammation. (Calprotectin, a peptide released by activated neutrophils, is a biomarker for gut inflammation that is stable even at room temperature and resists proteolytic degradation [2]. Both myeloperoxidase, an enzyme secreted by neutrophils, and eosinophil protein X are also sensitive biomarkers for inflammation [71].)

Table 2-2. Effects of HFD and LFD on markers of intestinal permeability and inflammation (unpublished data).

Endpoint	LFD	HFD	P-Value
Sucrose/Mannitol	0.0057 ± 0.0007	0.0031 ± 0.0003	0.009
Lactulose/Mannitol	0.022 ± 0.003	0.014 ± 0.002	0.007
Sucralose/Mannitol	0.055 ± 0.005	0.043 ± 0.003	0.03
Fecal Calprotectin (μg/g)	3.7 ± 0.4	1.7 ± 0.2	< 0.0001
Fecal Myeloperoxidase (μg/g)	51.8 ± 16.9	18.0 ± 7.5	0.01
Fecal Eosinophil Protein X (μg/g)	26.1 ± 10.	7.5 ± 1.6	0.04

3.4 Effects on Exercise: Performance and Recovery

Flavonoid supplementation may potentially enhance exercise performance by delaying onset of fatigue and also improve recovery following training [58]. The mechanisms are thought to be due to the action of flavonoids on several pathways related to oxidative stress and inflammation: enhancement of endothelial function and muscle perfusion by the NO pathway, activation of endogenous antioxidant systems via the Nuclear factor erythroid 2-related factor 2 (Nrf2) and antioxidant response element (ARE) pathway, inhibition of cyclooxygenase enzymes (COX1 and COX2) to activate anti-inflammatory pathways, and downregulation of NF-κB signaling [58].

3.4.1 Vascular Function

Findings from clinical studies have shown that polyphenol supplementation can improve flow mediated dilation (FMD), a marker for vascular function, through increased NO synthesis and availability [72–74]. Nitric oxide is a potent vasodilator, which affects cardiac output, tissue perfusion, and subsequently athletic performance [58]. As discussed previously, one of the nutritional strategies under investigation for mitigating exercise-related intestinal symptoms involves foods and supplements that increase NO production and availability in epithelial tissue, to increase tissue perfusion and improve

oxygen availability [26,33,35,36]. Polyphenols may reduce ROS such as superoxide, and limiting superoxide can increase NO availability [58]. In addition, polyphenols may also activate endothelial nitric oxide synthase 3 (eNOS) to increase NO production [58].

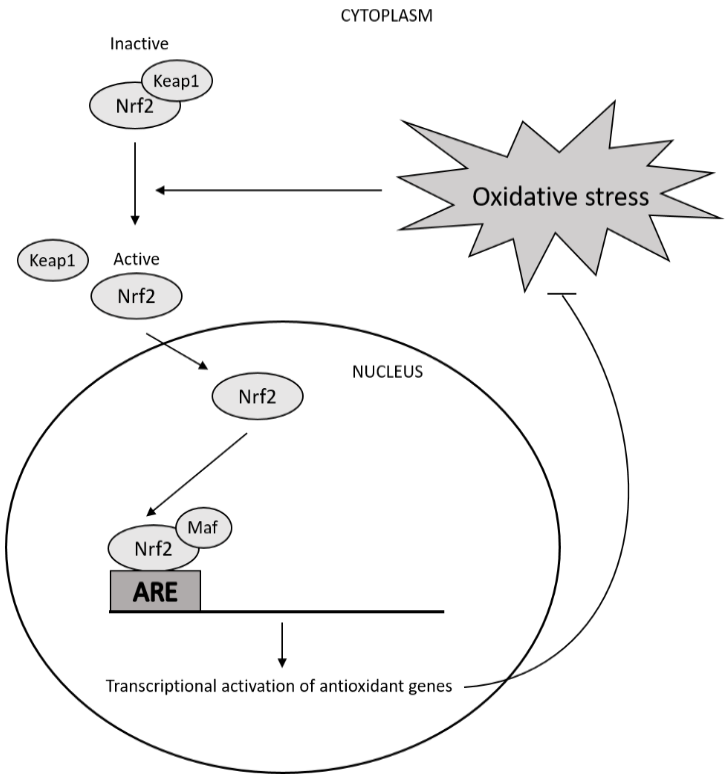
Several polyphenol-rich fruits have been investigated for their potential as ergogenic aids due to their effects on vascular function [75–77]. In a clinical trial with seven days of Montmorency cherry powder supplementation (463 mg polyphenols/day), overall 15-km cycling time trial (TT) performance was improved by 4.6% [75]. Resting tissue oxygenation index, a measure of muscle oxygenation, was significantly higher with the Montmorency cherry intervention relative to the placebo (70% vs. 69%). In another study, acute cranberry and grape seed polyphenol supplementation (600 mg) increased FMD at rest in elite athletes, but there was no effect of the polyphenol extract on 3-km cycling TT performance [76]. Finally, acute pomegranate extract intake (1000 mg) significantly increased blood flow and vessel diameter following repeated sprint ability testing on a cycle ergometer [77]. These findings suggest that polyphenols may increase muscle oxygenation through the NO-mediated pathways as discussed previously.

3.4.2 Oxidative Stress

Another potential effect of polyphenols on athletic performance is believed to be through modulation of redox status. Polyphenols can activate native antioxidant systems and downregulate ROS-generating enzymes such as NADPH oxidase [58]. During exercise, ROS are produced and increase with exercise intensity, and excess generation of ROS during high intensity, long duration exercise may slowly drain performance capacity as endogenous antioxidants are depleted [58,78]. This redox imbalance is believed to cause quicker fatigue due to vasodilation, reduced calcium sensitivity in active muscle

tissues, and increased central fatigue via the somatosensory cortex [58]. Dietary polyphenols are thought to activate the adaptive stress response by acting as oxidative stressors which modify and inhibit Keap1, an inhibitor of the transcription factor Nrf2 [79]. Upon degradation of Keap1, Nrf2 translocates to the nucleus to activate the ARE transcriptional pathway, which ultimately has downstream effects of promoting endogenous antioxidant enzymes like catalase and glutathione peroxidase (Figure 2-2). An upregulation in native antioxidants via Nrf2 and ARE signaling most likely occurs with chronic polyphenol supplementation longer than three days [58].

Figure 2-2. Regulation of the Keap1/Nrf2/ARE pathway via oxidative stressors such as ROS and polyphenols.



In one study, 30-day supplementation with lychee fruit polyphenols (~66 mg/day) increased submaximal running time to exhaustion [80]. During the study, participants maintained their regular exercise routines, and VO_2max did not change significantly over the 30-day period for the lychee treatment group. The lychee treatment significantly increased the subjects' anaerobic threshold, a common indicator of endurance capacity, while neither the placebo nor a vitamin C and E treatment had an effect on running performance. The authors proposed that the outcomes of the polyphenol treatment were likely due to the modulation of multiple redox signaling pathways rather than through direct free radical scavenging, which would have been the expected mechanism driving any effects of the vitamin treatment [80]. Several other studies have demonstrated that polyphenol intake may reduce exercise-related oxidative stress, though no performance changes were observed [81–85]. For example, dark chocolate consumption (2 weeks, 40 g twice daily) significantly reduced markers of oxidative stress (F_2-isoprostanes and oxidized low density lipoproteins) after an exhaustive endurance cycling trial [81]. However, no differences in time to exhaustion at 90% VO_2max were observed between the dark chocolate treatment and the cocoa liquor-free control.

3.4.3 Inflammation

Under normal conditions, the inflammatory response is a rapid, regulated, and coordinated response to external or internal perturbations; tissue damage, pathogen invasion, and exercise are common activators of inflammation [64]. The inflammatory response is characterized by a series of cascading events: release of pro-inflammatory molecules, recruitment of specific leukocytes, increased expression of oxidative enzymes, and production of anti-inflammatory mediators once the initial insult is under control

[64]. During exercise, skeletal muscle releases the cytokine interleukin (IL)-6, proportional to relative exercise intensity and duration [86,87]. Heat stress and increased GI permeability will further elevate IL-6 production [88]. IL-6 has been implicated in both pro- and anti-inflammatory pathways depending on the target tissues and is involved in energy substrate mobilization (gluconeogenesis and lipolysis), glucose uptake, and lipid oxidation [88,89]. Muscle-derived IL-6 is particularly important during endurance exercise for regulating muscle function and exercise capacity by mediating osteocalcin production, which acts on muscle tissue to increase exercise capacity [87]. Other circulating cytokines that increase following exercise include IL-1ra, IL-10, IL-8, and monocyte chemotactic protein (MCP-1) [89].

Polyphenols can act in several ways to reduce inflammation. First, polyphenols can inhibit NF-κB signaling, a key transcription factor involved in many inflammatory pathways [90]. Polyphenols can also downregulate expression of the pro-inflammatory enzymes cyclooxygenase (COX), lipoxygenase (LOX), and phospholipase A2 (PLA2), which ultimately reduces the release of several pro-inflammatory molecules: arachidonic acid, prostaglandins, and leukotrienes [90].

Polyphenol intake has been shown to mitigate inflammatory stress with a multitude of different clinical study designs: free-living and controlled diets, healthy subjects and those with chronic diseases, short- and long-term interventions [64]. In a meta-analysis by Peluso et al., 32 placebo-controlled clinical trials showed a decrease in circulating TNF-α as a result of chronic flavonoid supplementation [91]. In some exercise studies, a polyphenol intervention resulted in lower levels of IL-6 following exercise [83,91–94]. In one study, the effect of six-week blueberry consumption on oxidative

stress and inflammation was evaluated in a group of trained runners [84]. Following a 2.5-hour treadmill run at 72% VO_2max, the anti-inflammatory cytokine IL-10 and natural killer cell counts (related to anticancer activity) were elevated, while oxidative stress (urinary 5-OHMU and plasma F2-isoprostanes) was reduced.

In another study, investigators aimed to determine the effects of Cavendish bananas, mini-yellow bananas, a carbohydrate beverage, and water on inflammation and plasma metabolites prior to and following a 75 km cycling TT [31]. (Cavendish and mini-yellow bananas have similar carbohydrate content, but mini-yellow bananas have approximately 63% higher polyphenol content.) Investigators reported that COX2 mRNA expression was reduced in THP-1 monocytes cultured in plasma collected during the recovery period from the banana treatments, suggesting that plasma metabolites following banana ingestion can downregulate COX2, potentially mediating exercise-induced inflammation in monocytes. It should be noted that bananas also contain other components in addition to polyphenols (e.g., amino acids, dopamine, serotonin, vitamins), and the specific banana metabolites resulting in COX2 inhibition were unknown. Additionally, both banana treatments and the carbohydrate beverage reduced inflammatory markers (IL-6, IL-10, IL-1ra, leukocyte counts) to a similar extent, which is consistent with findings from previous studies regarding the immunomodulatory effects of carbohydrate intake during exercise.

4. The Gut Microbiota and Dietary Flavonoids

4.1 Introduction to the Gut Microbiota

The intestinal microbiota is a dynamic community of microorganisms that occupy the GI tract (primarily the colon) and includes bacteria, Eukarya, and Archaea [95]. These

microorganisms are thought to have co-evolved in a symbiotic relationship with their human hosts [95]. Within an individual, the diversity and composition of the gut microbiota are relatively stable by two to three years of age, but large shifts may still occur due to host and environmental perturbations such as dietary changes, illness, and antibiotics treatment; given time, the composition of the intestinal microbiota will return to normal [95]. The intestinal microbiota can be profiled using metagenomic sequencing of the 16S ribosomal ribonucleic acid (rRNA) gene [95]. Over 1,000 different species have been found colonizing the human intestinal tract, and collectively, they have a combined metagenome and metabolic capability 100-times greater than their human host [96]. Microbiota diversity varies substantially from one individual to another—though there are similarities in a few resident microorganisms and the functional genome, or microbiome [96]. The human gut microbiota primarily consists of bacteria from the phyla *Firmicutes* (49-76%), *Bacteroidetes* (16-23%), *Proteobacteria* (~8%), and *Actinobacteria* (~3%), and the proportions of these microorganisms also vary depending on the location within the GI tract [97].

An important part of the human intestinal ecosystem, the gut microbiota has been found to play a key role in several functions and processes. Some metabolic activities of the microbiota include the catabolism of indigestible carbohydrates and proteins, xenobiotic transformation, and vitamin synthesis [96]. Microbial degradation of these nutrients, xenobiotics, and drugs may increase or decrease the bioavailability and activity of these exogenous compounds [97]. The microbiota is also known to provide benefits to the host by reducing pathogen adhesion, supporting immune system development, and regulating intestinal permeability [95]. Additionally, the microbiota may also interact

with epigenetic modifications and plays a part in maintaining immune homeostasis [95]. Individuals with immune, inflammatory, or metabolic diseases often display gut dysbiosis, or an imbalanced microbial population [95].

One important function of the gut microbiota is aiding in the development of a mature intestinal epithelium and maintaining the integrity of the barrier [98]. In the GI tract, a mucus layer consisting of cross-linked mucin glycoproteins is secreted by specialized epithelial cells and aids in protecting the host from pathogen invasion; conversely, a compromised mucus layer results in increased pathogen susceptibility and localized inflammation [99]. Germ-free mice display thinner colonic mucus and epithelial cell dysfunction, but following colonization with conventional microflora, the integrity of the intestinal epithelium can be restored [99]. In one study, when dietary fiber was restricted, rapid and reproducible shifts were observed in the intestinal microbiota in mice [99]. Instead, certain strains of commensal bacteria that ordinarily rely on dietary fiber for energy shifted to metabolize mucin glycoproteins, degrading the protective mucus layer and increasing susceptibility to pathogen invasion [99]. In contrast, *Akkermansia* spp. are mucin-degrading commensal bacteria that are believed to help with maintaining the protective mucus layer by stimulating further mucus production and may also exhibit anti-inflammatory effects in the GI tract [100].

Due to the complex nature of the intestinal microbiota, more clinical studies are needed to elucidate how exercise stress impacts the microbiota and intestinal permeability. In a study by Karl et al., a strenuous 4-day cross-country ski-march induced a significant increase in intestinal permeability concomitant with a change in the intestinal microbiota [101]. The exercise regimen negatively impacted the microbiota by

reducing abundance of *Bacteroides* while increasing the abundance of potentially harmful microorganisms (*Peptostreptococcus*, *Staphylococcus*, *Peptoniphilus*, *Acidaminococcus*, and *Fusobacterium*) [101]. In contrast, Codella et al. have suggested that moderate exercise can positively shape the microbiota to improve chronic disease health outcomes due to the release of exercise-associated myokines, reduced intestinal transit time, overall weight loss, and increased fecal bile acids as a result of exercise [102]. In another study, exercise improved intestinal health in mice fed a high fat diet by modulating the microbiota, reducing COX-2 expression in the proximal and distal gut, and preserving normal intestinal villi morphology compared to sedentary animals [103]. Regular exercise is also positively associated with the presence of microbial species that promote barrier integrity (e.g., *Akkermansia muciniphila*, *Lactobacillus plantarum*, *Bacteroides fragilis*, *Bifidobacterium longum*, *Bifidobacteria bifidum*) and appears to increase overall microbial alpha diversity [104,105].

4.2 Short Chain Fatty Acid Production

Another function of the colonic microflora is the catabolism of indigestible carbohydrates to produce short chain fatty acids (SCFA) [106]. The three most abundant SCFA are acetate, propionate, and butyrate—typically present in the GI tract in a ratio ranging from 3:1:1 to 10:2:1 [107]. Ultimately, these SCFA have different metabolic fates: butyrate is primarily utilized as an energy source for colonocytes, propionate is absorbed through hepatic circulation by the liver, and acetate is metabolized by peripheral tissues such as the brain, muscle, pancreas, and adipose tissue [106]. Other aspects of the biological significance of colonic SCFA production are less clear and more research is still needed. Potentially, these exogenous SCFA produced by the colonic

microflora from undigested material result in greater dietary energy available to the host [108]. If so, excessive SCFA production may contribute to the development of obesity, as greater production of total SCFA has been observed in obese and overweight humans [108]. However, findings from observational studies also suggest that increased fiber intake, which subsequently increases SCFA production, results in improved health outcomes [108]. In fact, in colorectal cancer, the protective effect of fiber intake has been attributed to butyrate production by the intestinal microbiota [108].

Though not yet fully understood, microbial SCFA are increasingly being recognized as important signaling molecules between the microbiota and host [106]. Many of the microbiota-associated changes observed in human health may in fact be mediated through the action of SCFA. The SCFA-specific free fatty acid receptors 2 and 3 (FFAR 2/3) have been identified across various tissue and cell types, and the downstream effects of these receptors appear to be substrate- and tissue-dependent [106]. In the liver, acetate and butyrate are lipogenic while propionate is gluconeogenic and inhibits lipogenic effects of acetate [106]. Increased circulating SCFA is associated with lower adipogenesis and reduced lipid accumulation in adipocytes, resulting in smaller adipocytes [106]. Acetate can also stimulate leptin secretion in adipocytes, which is a hormone that regulates satiety [106]. Butyrate has received significant attention for its potential in protecting the intestinal barrier—it appears to regulate the tight junction protein complex by increasing expression and assembly of tight junction proteins like claudin-1, ZO-1, and occludin [106,109,110]. Butyrate may also have anti-inflammatory and anti-cancer properties [98]. In a mouse model of colitis, microbial-derived butyrate was shown to activate regulatory T cells via epigenetic modification of the *Foxp3* gene,

reducing development of colitis [111]. Interestingly, exercise can positively affect the presence of butyrate-producing bacteria in the gut like *Lachnospiraceae* and *Faecalibacterium prausnitzii* [104].

Finally, SCFA production can also shape the composition of the microbiota by reducing the growth of potentially pathogenic species [108]. A diet rich in fiber and carbohydrates was shown to lead to a greater production of SCFA and consequently, a reduction in intestinal and colonic pH, creating an environment unfavorable for the growth of many common human pathogens like *E. coli* and *Enterobacteriaceae* [112].

4.3 The Reciprocal Relationship Between Flavonoids and the Gut Microbiota

The relationship between dietary flavonoids and the intestinal microbiota is complex and bidirectional: dietary flavonoids can shape the diversity and composition of the microbiota and the microbiota is responsible for the metabolism and transformation of dietary flavonoids [113]. Only ~5-10% of flavonoids are absorbed in the small intestine, and those that are not absorbed in the small intestine are extensively metabolized by the colonic microflora [114]. The colonic microbiota can increase compound bioavailability through enzymatic catabolism into lower molecular weight metabolites, which can be either more or less bioactive than the parent compounds [114]. Consequently, the microbiota are likely mediators in the health effects exerted by dietary polyphenols [114].

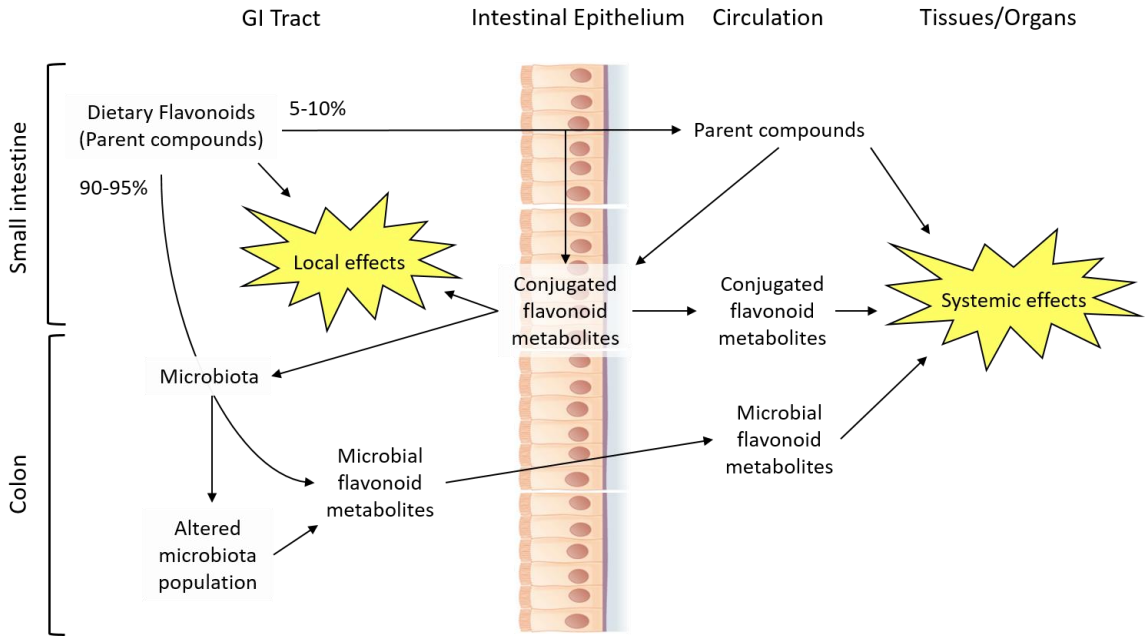
A schematic of the host and microbiota co-metabolism of dietary flavonoids is presented in Figure 2-3. In the small intestine, most flavonoids are deglycosylated by the brush border enzyme lactase-phlorizin hydrolase (LPH), and a small proportion are absorbed by epithelial cells and transformed to glucuronide, sulfate, and methylated conjugates through phase II metabolism [115,116]. Native polyphenols and their

conjugates can reach maximal levels in the plasma between 0.5-9 hours after ingestion [58,117]. In the colon, ingested flavonoids undergo ring fission and are extensively metabolized by the colonic microflora to phenolic acids, hydroxycinnamate, phloroglucinol, and other compounds [118]. In addition, phase II flavonoid-derived metabolites excreted through the enterohepatic pathway into the lower GI tract are subject to further biotransformation by the microbiota [118]. Microbial polyphenol metabolites appear in systemic circulation 6-48 hours after ingestion, and display significant variation among subjects, likely due to large inter-individual differences in intestinal microflora [58,119]. Additionally, microbial transformation has been found to be dependent on the degree of polymerization and structure of the polyphenol, as larger polymers may result in limited accessibility for microbial catabolic enzymes or reduced cellular uptake. For example, lower bioavailability was observed with procyanidin dimers and polymers compared to epicatechin monomers [120]. Nonetheless, given the highly adaptive nature of the microbiota to dietary changes, the gut microbiome may also be able to adapt to metabolize larger proanthocyanidin oligomers and polymers [121]. Microbial metabolism is a key step in making more bioactive metabolites available to tissues and organs beyond the intestinal tract. Without the action of the microbiota, not only would flavonoid metabolite absorption be severely limited, but so would the potential downstream systemic effects.

Since the microbiota can have a variety of effects on the human host, flavonoid-mediated modification of the microbiota population can affect a large array of health outcomes. Flavonoids have well-documented anti-microbial properties and can inhibit both pathogenic and beneficial microorganisms [122]. Conversely, specific flavonoids

can also act to increase the abundance of certain microbial species within the intestinal community, while other flavonoids may have both inhibitory and stimulatory effects on different strains [97,123,124]. These effects appear to be structure-dependent and aglycones may have greater inhibitory activity than their glycated counterparts [125]. Additionally, dietary flavonoids can also increase or inhibit bacterial adhesion, affecting the presence and diversity of species in the intestinal tract [123].

Figure 2-3. Host and intestinal microbiota co-metabolism of dietary flavonoids and their downstream effects.



Preclinical studies have demonstrated that polyphenol-rich extracts show potential to modulate *Akkermansia muciniphila* abundance and mucin protein expression, though more clinical data is still needed [65,126]. A polyphenolic cranberry extract ameliorated the obesogenic effects of a high fat/high sucrose diet (weight gain, visceral obesity, triglyceride accumulation, insulin resistance), reduced intestinal inflammation and metabolic endotoxemia, and increased the abundance of *Akkermansia* spp. in mice [127].

Similarly, supplementation with grape polyphenols in mice blunted the negative metabolic effects of a high fat diet while increasing the abundance of *Akkermansia* spp. [128]. In another rodent study, supplementation of a high fat diet with blueberries changed the microbiota composition while preserving ileal villus morphology and increasing Muc2 gene expression relative to a pair-fed, high fat diet group [129]. Additionally, the probiotic *Lactobacillus plantarum* also modulates the epithelial barrier; treatment of IL-10 knockout mice, which display colonic barrier dysfunction compared to wild type mice, with *Lactobacillus plantarum* significantly reduced the severity of colitis development and paracellular permeability [130]. In other rodent studies, supplementation with bilberry anthocyanin extract, apple procyanidins, and green tea catechins were shown to modulate the gut microbiota and improve markers of intestinal permeability [131–133].

5. References

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CHAPTER III

EFFECT OF FLAVONOID SUPPLEMENTATION ON EXERCISE-INDUCED INTESTINAL INJURY, PERMEABILITY, AND INFLAMMATION

Introduction

The gastrointestinal (GI) barrier is a selectively permeable membrane that facilitates water and nutrient uptake while excluding potentially harmful antigens and pathogens [1]. Intestinal permeability refers to the movement of luminal contents to the internal milieu through either the epithelial cells lining the GI tract or the interstitial space between neighboring cells, which is regulated by tight junction protein complexes [1]. Both external and internal stimuli can affect intestinal permeability, and elevated permeability is a common feature of several autoimmune, intestinal, and metabolic diseases and conditions [1].

In particular, high intensity endurance exercise causes transient, increased intestinal permeability [2]. Severe or prolonged exercise-induced intestinal permeability can be problematic and cause local and systemic inflammation—potentially hindering physical performance and recovery [3]. At rest, 20% of total cardiac output is directed to the splanchnic organs; during exercise, up to 80% of this blood flow is shunted to muscle and other peripheral tissues instead [4]. Prolonged hypoperfusion of visceral tissues causes localized hypoxia and mucosal injury, and reperfusion injury can occur due to reactive oxygen species (ROS) accumulation and localized inflammation [4]. Exercise intensity is thought to be one factor affecting intestinal permeability. In a study by Pals et al., participants ran for 1 hour at 40, 60, and 80% VO_2max, and a significant increase in

intestinal permeability relative to baseline was only observed after running at 80% of VO_2max [5]. Second, heat stress has also been demonstrated to cause epithelial cell injury and disrupt tight junctions both *in vitro* and *in vivo*; thus, the magnitude of change in core temperature is another important factor in exercise-induced intestinal permeability [2]. A systematic review of human exercise studies by Pires et al. reported a positive association between magnitude of core temperature change and increased intestinal permeability [4]. In studies where post-exercise core temperatures were above 39 °C, increased intestinal permeability was always observed [4].

Several dietary nutrients have been shown to regulate intestinal permeability [6]. Specifically, flavonoids (plant secondary metabolites abundant in fruits and vegetables) have been demonstrated to modulate tight junction permeability through several mechanisms *in vitro* [7]. Flavonoids can activate the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway which upregulates endogenous antioxidant capacity, and flavonoids also have anti-inflammatory activity through the downregulation of nuclear factor kappa B (NF-κB) signaling (see Chapter II, Section 3) [7–9]. Dietary flavonoids also exhibit other beneficial effects through NO-related mechanisms—including vasodilation, regulation of glucose uptake, and improved endothelial function [9,10]. Given these various biological activities and the positive health outcomes commonly observed with flavonoid-rich dietary patterns, flavonoids are thought to have great potential as protective agents for the intestinal barrier [11,12]. Furthermore, the vasoactive, antioxidant, and anti-inflammatory effects of flavonoid consumption may also enhance exercise performance and improve recovery by increasing muscle perfusion and delaying onset of fatigue [10].

Consequently, the purpose of this study was to determine the effects of sub-chronic (fifteen-day) consumption of a high flavonoid pre-workout beverage (HFB) on GI permeability and inflammation following a bout of high-intensity endurance exercise. The flavonoid content of the investigational product was formulated to provide a moderate dose of flavonoids in one serving (490 mg/day). Previous clinical studies investigating flavonoid supplementation and exercise performance span a range of doses from relatively low to several times higher (100-2000 mg/day); however, a review of these studies concluded that a dose of ~300 mg polyphenols consumed 1-2 h prior to exercise may be sufficient to improve endurance and repeated sprint performance [10]. Our primary endpoint was intestinal injury as indicated by plasma intestinal fatty acid-binding protein (I-FABP). Secondary endpoints included intestinal permeability (excreted urinary sugars), inflammatory response (circulating cytokines), and cycling performance (work output and cycling distance). Based on prior work, we hypothesized that the HFB would ameliorate exercise-induced intestinal injury and permeability relative to a low flavonoid control (LFB). Resilience to exercise-induced changes in GI permeability may also result in a reduction in acute exercise-associated inflammation and improved cycling performance.

Materials and Methods

Ethical approval

This study was conducted in accordance with the 2008 Helsinki Declaration for Human Research Ethics and approved by the Utah State University (USU) research ethics committee (IRB Protocol #9255). Participants were informed of all study requirements

and provided written informed consent prior to enrollment. This study was registered at ClinicalTrials.gov prior to study start (ClinicalTrials.gov Identifier: NCT03427879).

Participants

For a complete study recruitment flow diagram, see Appendix A, Figure A-1. Participants were recruited by word of mouth and through advertisements posted at local cycling businesses and gyms in Logan, Utah and at USU (Appendix A, Figure A-2). Seventeen cyclists were screened for study eligibility, and of those, fourteen subjects were enrolled and randomized to treatments. One subject dropped out due to scheduling conflicts, and one subject was withdrawn due to failure to adhere to the study protocol. Twelve recreational cyclists (female n = 4, male n = 8; mean ± SD: age 37 ± 11 years, body mass 78 ± 13 kg, height 1.74 ± 0.09 m, VO_2max 43 ± 6 ml/kg/min) completed both arms of the study.

Table 3-1. Participant characteristics.

Subject	**Age (years)**	**Body Mass (kg)**	**Height (cm)**	**BMI (kg/m^2)**	**VO_2max (ml/kg/min)**	**70% Power (W)**	**Cycling Experience (years)**
1	49	68.5	161.5	26.3	43.9	130	10
2	33	100.0	183.6	29.7	39.3	180	.
3	43	58.2	158.1	23.3	41.4	105	15
4	36	87.0	182.1	26.2	42.6	160	.
5	40	87.7	179.9	27.1	51.4	215	23
6	45	69.7	165.5	25.4	41.0	145	12
7	18	72.8	182.7	21.8	46.5	165	12
8	33	99.4	173.1	33.2	28.1	145	1
9	55	79.6	179.0	24.8	43.5	160	10
10	40	69.1	178.2	21.8	46.2	160	5
11	18	69.1	165.0	25.4	44.1	165	6
12	28	72.1	178.7	22.6	50.1	200	20
Mean ± SD	36.5 ± 11.4	77.7 ± 13.1	174.0 ± 9.0	25.6 ± 3.3	43.2 ± 5.9	160.8 ± 29.3	11.4 ± 6.7
Range	18-55	58.2-100	158.1-183.6	21.8-33.2	28.1-51.4	105-215	1-23

Inclusion/exclusion criteria

Eligibility and general health were assessed with a participation readiness questionnaire (PAR-Q) and health screening assessment. A complete list of study inclusion and exclusion criteria is provided in Appendix A, Table A-1. Briefly, key inclusion criteria were as follows: male or female of any race or ethnicity, age 18-55 years, training at least 3 times per week, participated in a cycling event in the past 12 months, and free of chronic disease and GI conditions. Exclusion criteria included a medical history of heart disease, hypertension, diabetes, Crohn's disease, IBS, colitis, celiac disease, inflammatory or autoimmune disease, allergies to pre-workout ingredients, and/or lactose intolerance. Participants were also excluded for chronic use of NSAIDs, flavonoid supplement consumption < 1 month prior, or antibiotic use < 3 months prior. For women, additional exclusion criteria included those who were pregnant, breastfeeding, or < 6 months postpartum.

Preliminary assessments

During the screening period, each participant completed a maximal aerobic capacity (VO_2max) test via expired gas analysis to determine power settings for the two subsequent cycling trials. This preliminary test also allowed participants to be familiarized with the Velotron cycle ergometer (SRAM LLC; Chicago, IL). The incremental graded VO_2max test began at 100 Watts (W) and increased by 25 W/min until either volitional exhaustion or rpm < 60. Expired gas samples were collected using a two-way valve mouthpiece (Hans Rudolph 700 series; Kansas City, MO), and oxygen consumption was recorded with a computerized on-line metabolic measurement system (Parvomedics TrueOne 2400; Sandy, UT). After the test and a ten-minute rest, a follow-

up assessment was performed to validate the predicted power (W) at 70% VO_2max for steady state exercise (fixed power). Power settings were adjusted in 5 W increments as needed to achieve 70% VO_2max for a minimum of 3 minutes before the final power setting for the exercise trials was determined.

Participants also performed a baseline intestinal permeability assessment with an overnight, 8-hour urine collection. For the test, participants fasted four hours following their evening meal. After 4 hours, participants emptied their bladder and drank a 130 ml sugar solution containing 10 g sucrose, 5 g lactulose, 1 g maltitol, and 1 g sucralose. (The intended sugar permeability test includes mannitol, but due to a supplier error, maltitol was provided instead.) For the next eight hours, urine was collected in a 3 L container with 50 µl of 10% thymol as a preservative. During this time, participants were only permitted to drink water. In the morning, participants voided into the collection jug and recorded the total volume. A subsample (50 ml) was aliquoted to another container, and samples were stored frozen for future analysis.

Investigational product

For each intervention, participants were provided with fifteen individual servings of pre-workout mix (containing all dry ingredients in Table 3-2), shelf-stable 2% white milk, and a shaker bottle. Instructions provided to the participants for preparing the pre-workout are included in Appendix A, Figure A-3. One package of pre-workout mix (64 g) was prepared with 240 ml milk and consumed once per day, two hours prior to exercise or the subject's typical exercise time. One serving of the high flavonoid beverage (HFB) provided approximately 490 mg total flavonoids, while the low flavonoid beverage (LFB) contained approximately 4.6 mg flavonoids due to three

modifications: alkalized cocoa, blueberry placebo powder, and omission of green tea powder. The green tea extract contained approximately 800 mg/g tea catechins (PureBulk, Inc; Roseburg, Oregon). High flavonoid cocoa powder (83 mg/g cocoa flavanols) and alkalized cocoa (10 mg/g flavanols) were provided by Barry Callebaut (Zurich, Switzerland). Freeze-dried blueberry powder (14.0 mg/g total anthocyanins) and blueberry placebo powder were supplied by the US Highbush Blueberry Council (Folsom, California).

Table 3-2. High flavonoid beverage (HFB) and low flavonoid beverage (LFB).

Ingredient	**HFB % (w/w)**	**Ingredient**	**LFB % (w/w)**
2% Milk	78.1	2% Milk	78.2
Sugar	8.6	Sugar	8.6
Maltodextrin	8.6	Maltodextrin	8.6
Blueberry powder	2.4	Placebo powder	2.4
Cocoa powder	1.6	Alkalized cocoa powder	1.6
Whey protein isolate	0.6	Whey protein isolate	0.6
Green tea powder	0.1	Green tea powder	0

Study endpoints

The primary endpoint of this study was I-FABP detected in the plasma, a non-invasive biomarker of intestinal injury and permeability [3]. I-FABP is a cytosolic protein expressed in mature small and large intestinal enterocytes and present only at low levels in the plasma unless released from injured or lysed cells [3]. Because circulating I-FABP is rapidly cleared and has a half-life of only eleven minutes, the change in I-FABP from baseline is an early marker for intestinal injury [13]. Circulating I-FABP correlates strongly with abdominal trauma, appearance of inflammatory markers, determination of gut ischemia with gastric tonometry, and the severity of morphologic epithelial intestinal damage [3,14]. (In gastric tonometry, a tonometer balloon is inserted with a catheter into

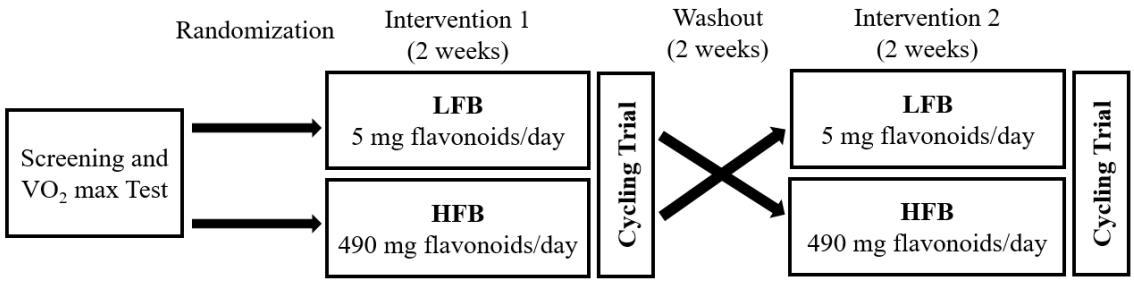
the gastric cavity to detect accumulated CO_2 in the gut mucosa due to underperfusion [3].)

The second endpoint was the urinary excretion of orally-ingested sugars (sucrose, lactulose, and sucralose)—a non-invasive method to determine GI permeability. Sucrose, which is digested by the enzyme sucrase in the small intestine, is an indicator of gastric permeability, lactulose is fermented by the microbiota in the colon and therefore indicates of small intestinal permeability, and sucralose is non-digestible, so recovered sucralose in the urine reflects overall GI permeability [1,15].

The plasma cytokines TNF-α, IL-6, and IL-10 were selected as secondary endpoints. The pro-inflammatory cytokine TNF-α increases the rate of epithelial cell turnover and activates opening of the tight junction, leaving gaps in the intestinal barrier [1,16]. IL-6 is secreted from skeletal muscle during exercise in response to heat stress and low energy availability [17]. It can also affect gastric emptying and plays an important role in attenuating intestinal permeability and mucosal injury caused by a dangerous rise in core temperature [18]. Lastly, IL-10 is an anti-inflammatory cytokine promoted by IL-6 that downregulates further production of pro-inflammatory cytokines [17].

Study design

Figure 3-1. Schematic overview of crossover study design with low flavonoid beverage (LFB) and high flavonoid beverage (HFB).



The study design was a randomized crossover trial, with a minimum fourteen-day washout between the two intervention periods (Figure 3-1). Following completion of screening and baseline assessments, each participant was randomly assigned a treatment order. With the exception of high flavonoid foods, flavonoid supplements, and NSAIDs, participants were free to consume their regular diet and maintain their usual training schedule. In addition, participants were instructed to abstain from alcohol and strenuous activity 24 hours prior to each urine collection and cycling trial. Gut permeability assessments using orally-administered sugar test probes and an overnight eight-hour urine collection were performed at baseline and on Day 12 of each intervention. On Day 15, a gut permeability assessment was also performed immediately following the trial with a six-hour urine collection. Meals consumed during these periods were recorded and replicated during the second intervention. Each intervention concluded with a one-hour cycling trial on Day 15, during which blood samples were collected pre-, post-, 1 h post-, and 4 h post-exercise. The schedule for clinic assessments and specimen collections is provided in Appendix A, Figure A-4. A pre-loaded time trial (TT) was selected for the study, as it has been shown to produce more reliable performance results in test-retest designs and also provides quantitative endpoints of work and distance [19]. In contrast, within-subject designs with fixed cycling intensities (e.g. 1 hour at 70% VO_2max) typically produce greater variation and are more prone to being affected by psychological factors [19].

Main exercise visit

On Day 15, two hours prior to the cycling trial, participants consumed their typical pre-race breakfast (recorded and replicated for Phase 2), the HFB/LFB pre-

workout, and an ingestible temperature sensor pill (CorTemp, HQ Inc.; Palmetto, FL). At the clinic, subjects emptied their bladders, body mass was measured, and a blood sample was taken. Subjects warmed up for five minutes at a self-selected pace prior to starting the cycling trial. The trial consisted of 45 minutes cycling at a fixed power (70% VO_2max), followed by a fifteen-minute "all out" time trial (TT), during which subjects were free to adjust the power setting and rode at maximal effort to complete as much work (J) as possible. Throughout the exercise test, heart rate and core temperature were recorded at five minute intervals, and a final core temperature measurement was taken five minutes after completion of exercise. Rating of perceived exertion (RPE) was recorded at minutes 5, 15, 25, and 35, and expired air was collected to determine VO_2 at 10, 20, 30, and 40 min. The visual RPE scale presented to subjects is included in Appendix A, Figure A-6. Subjects were provided with a small amount of room temperature water (0.5 ml/kg bodyweight) at 15 minutes. Following the trial, participants were seated and another blood sample was collected. Participants then ingested the gut permeability sugar solution and collected urine for six hours. During the first two hours, subjects were only permitted to drink water, and certain foods and beverages were restricted to minimize interference with the test. All meals and snacks were recorded in a food log and repeated during the second intervention. Two final blood samples were collected one and four hours post-exercise. Adverse events and symptoms experienced in the 24 hours following the exercise test were noted with brief questionnaire. A sample schedule for Day 15 is provided in Appendix A, Figure A-5.

Blood sampling and analysis

Seated venous blood samples were collected via antecubital venipuncture in K_2EDTA-treated Vacutainer tubes and centrifuged (1500 × *g*, 10 min, 4 °C). Plasma was aliquoted to 1.5-ml polypropylene microcentrifuge tubes and stored frozen at -80 °C until further analysis.

A commercial enzyme-linked immunosorbent assay (ELISA) kit was used for determination of plasma I-FABP in duplicate (Hycult Biotech; Uden, The Netherlands). High sensitivity ELISA kits were used to analyze TNF-α and IL-10 according to the manufacturer's instructions (Invitrogen; Carlsbad, CA). Plasma IL-6 cytokine analysis was conducted with an ELISA kit according to the manufacturer's instructions (Invitrogen; Carlsbad, CA).

Urine sugar analysis for gut permeability

Urinary concentrations of each of the sugar probes (i.e., recovery of the sugars in urine specimens) were determined with gas chromatography-mass spectroscopy (GC-MS) analysis following trimethylsilyl (TMS) derivatization. Due to the low concentrations of recovered sugars, urine samples were spiked with 7.5 ppm sucrose, 2 ppm sucralose, and 20 ppm lactulose prior to drying and derivatization to increase the signal to noise ratio for the measurement. For the standards, a synthetic urine matrix was prepared with 0.33 M urea, 0.12 M sodium chloride, 0.016 M potassium diphosphate (KH_2PO_4), 0.007 M creatinine, and 0.004 M sodium monophosphate ($NaHPO_4$). Additionally, inositol was added to all samples as an internal standard (50 µl, 1 mg/ml). Samples were prepared in duplicate, and 400 µl of urine or 800 µl of a 1:1 standard and synthethic urine mix were evaporated to dryness in a speed vacuum centrifuge. After

drying, 100 µl of *O*-methoxylamine (CH_3ONH_2) in pyridine (20.0 mg/mL) was added to each sample and mixed thoroughly. Samples were heated at 70 °C for 1 hour. After heating, samples were centrifuged at 13,000 x *g* for 5 minutes, and 50 µl of the supernatant was combined with 50 µl of *N*,*O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA). The samples were heated again for 30 minutes at 70 °C and then transferred to a glass sample vial for GC-MS analysis.

The GC-MS platform was a Shimadzu single quadrupole gas chromatograph mass spectrometer (GCMS-QP2010 SE) with a Zebron ZB-5MS plus (35 m × 0.25 mm diameter × 0.25 µm film thickness) capillary GC column (Phenomenex, Torrance, CA, USA). Helium was used as the carrier gas, and the column flow rate was 1.16 ml/min, with a split ratio of 5:1. The temperatures of the inlet, ion source, and transfer line were 250 °C, 230 °C, and 290 °C, respectively. The column oven temperature program is included in Table 3-3. Compound peak areas were normalized by the inositol internal standard, and sugar concentrations were determined from standard curves (Appendix A, Table A-2). Recovered sugar concentrations were corrected to the actual concentration by subtracting the spiked concentration. Corrected concentrations were multiplied by total urine volume to determine recovered mass which was used to determine percent urinary recovery from the initial amount of sugar ingested.

Table 3-3. Gas chromatography (GC) oven temperature program for sugar separation.

Rate (°C/min)	Temperature (°C)	Hold Time (min)
--	80	0
20	180	1
2.5	210	1
20	245	10
1	255	0
20	280	0

Sample size

A previous study demonstrated a treatment difference of 328.6 ng/ml for change in I-FABP following exercise, with patient standard deviation for repeated measurements estimated at 389.8 ng/ml [20]. Assuming a similar effect size of 0.84 ($\alpha = 0.05$), the probability is 82 percent that the study will detect a treatment difference with a total of fourteen subjects.

Statistical analysis

All data were checked for normality and homogeneity of variance, and data violating model assumptions were log-transformed prior to analysis. The TT distance, work, and cycling cadence were analyzed with paired t-tests. Cohen's d (*d*) was used to determine effect size for cycling work output, and a *d* value of 0.2, 0.5, and 0.8 indicate small, medium, and large effect sizes, respectively. Core temperature, heart rate (HR), VO_2, rating of perceived exertion (RPE), plasma I-FABP, IL-10, IL-6, and TNF-α were analyzed using a two-way repeated measures ANOVA. For IL-6 and TNF-α, statistical analyses were performed on raw instrumental values. When main or interaction (intervention × time) effects were significant, Tukey's HSD was used for post-hoc comparisons, unless otherwise specified. Negative values for sugar recovery were input as 0. A one-way repeated measures ANOVA was used to analyze sugar probe recovery with condition (baseline, HFB Day 12, LFB Day 12) as the independent factor. Intestinal permeability following the exercise trial on Day 15 was analyzed with a paired t-test. Correlation analyses (Spearman's ρ) were conducted to determine if final core temperature and change in core temperature correlated with primary outcomes (change in I-FABP, post-exercise I-FABP, % sucrose, % lactulose, % sucralose). No significant

treatment order effect (HFB-LFB, LFB-HFB) was observed. Data are expressed as means ± SEM, unless otherwise indicated. Statistical analyses were performed with JMP version 15.2.1 for Windows (SAS Institute, Cary, NC) with significance at $\alpha = 0.05$.

Results

Both the flavonoid and control pre-workout beverages were well-tolerated by the subjects, with no reported adverse effects.

Physiological responses and exercise performance

The exercise test elicited a similar response in HR, VO_2, and RPE regardless of the treatment (Table 3-4, Figure 3-2). There was a significant effect of exercise on HR ($p < 0.0001$), where the HR during the TT (t = 50, 55, 60) was significantly higher than the pre-load exercise (t = 5-45). Initial HR (t = 0) was significantly lower than all other time points. Likewise, RPE increased during exercise and was significantly elevated at 25 and 35 min compared to 5 min ($p < 0.0001$). Final RPE at 35 min was 3.9 ± 0.5 (LFB) and 4.0 ± 0.4 (HFB), where an RPE of 4 is "somewhat hard". VO_2 was significantly lower at 10 min compared to all other times ($p = 0.0003$), but not different between trials.

For the TT, no differences were observed for cadence (HFB: 93 ± 9 rpm, LFB: 93 ± 11 rpm) or total cycling distance (HFB: 19.3 ± 0.7 km, LFB: 18.9 ± 0.8 km). However, a trend for increased work output was observed for the HFB ($p = 0.0511$, Cohen's $d = 0.16$) (Figure 3-3B).

A significant effect of time ($p < 0.0001$) and time × treatment interaction ($p < 0.0001$) was observed for core temperature (Figure 3-4A). Core temperature from 10 min onwards for both conditions was significantly elevated compared to pre-exercise. Pairwise comparisons across condition at each time were not significantly different after

correcting for multiple comparisons. A small decline in core temperature was observed at 20 min when participants were provided water (0.5 ml water/kg bodyweight) after 15 min of exercise. The maximum core temperatures immediately following exercise were 38.53 ± 0.06 °C (HFB) and 38.51 ± 0.23 °C (LFB). Overall changes in core temperature from pre-exercise (t = -5) to 60 min exercise were 2.24 ± 0.23 °C (LFB) and 1.73 ± 0.13 °C (HFB) (paired t-test, p = 0.008) (Figure 3-4B).

Table 3-4. Perceptual and physiological measures: rating of perceived exertion (RPE) and VO_2 during 1-h cycling trial.

	5 min	**15 min**	**25 min**	**35 min**
RPE				
HFB	2.9 ± 0.3^{a}	3.2 ± 0.3^{ab}	3.8 ± 0.4^{b}	4.0 ± 0.4^{b}
LFB	2.7 ± 0.4^{a}	3.3 ± 0.4^{ab}	3.6 ± 0.5^{b}	3.9 ± 0.5^{b}
HFB-LFB	0.3 ± 0.2	-0.1 ± 0.2	0.3 ± 0.2	0.1 ± 0.3
	10 min	**20 min**	**30 min**	**40 min**
VO_2 (ml/kg/min)				
HFB	29.9 ± 1.4^{a}	30.2 ± 1.3^{b}	30.6 ± 1.4^{b}	30.5 ± 1.3^{b}
LFB	29.6 ± 1.4^{a}	30.2 ± 1.5^{b}	30.4 ± 1.4^{b}	30.4 ± 1.4^{b}
HFB-LFB	0.37 ± 0.58	-0.01 ± 0.46	0.23 ± 0.33	0.10 ± 0.25

Values with different superscripts differed significantly ($p < 0.05$).

Figure 3-2. Heart rate response during 1-h cycling exercise at 70% VO_2.

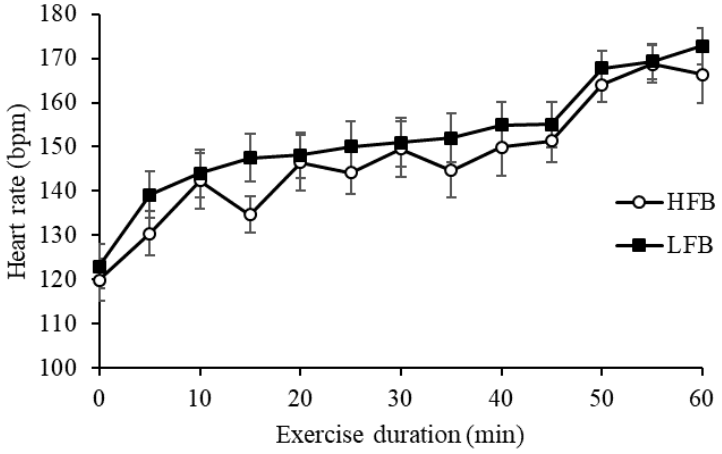
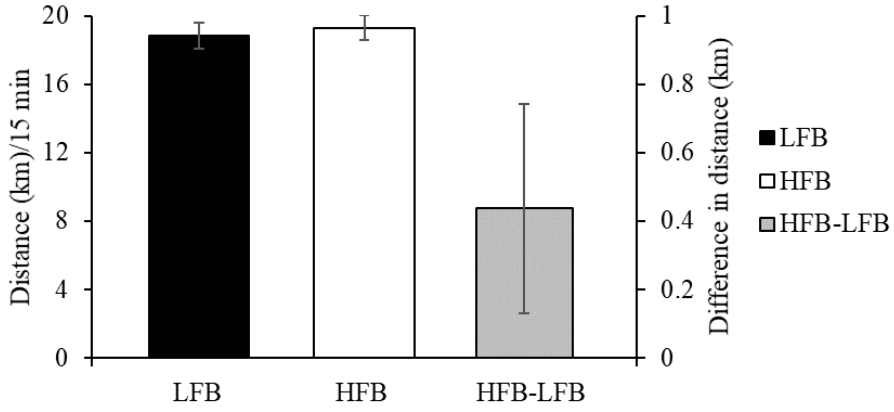


Figure 3-3. Cycling time trial performance. (A) Primary vertical axis: distance completed during the 15 min TT (LFB: 18.9 ± 0.8 km, HFB: 19.3 ± 0.7 km). Secondary vertical axis: within-subject treatment difference (0.4 ± 0.3 km, p = 0.181). (B) Primary vertical axis: work completed during the 15 min TT (LFB: 174.2 ± 11.3 kJ, HFB: 180.9 ± 12.3 kJ). Secondary vertical axis: within-subject treatment difference (6.6 ± 3.0 kJ, p = 0.0511).

(A)



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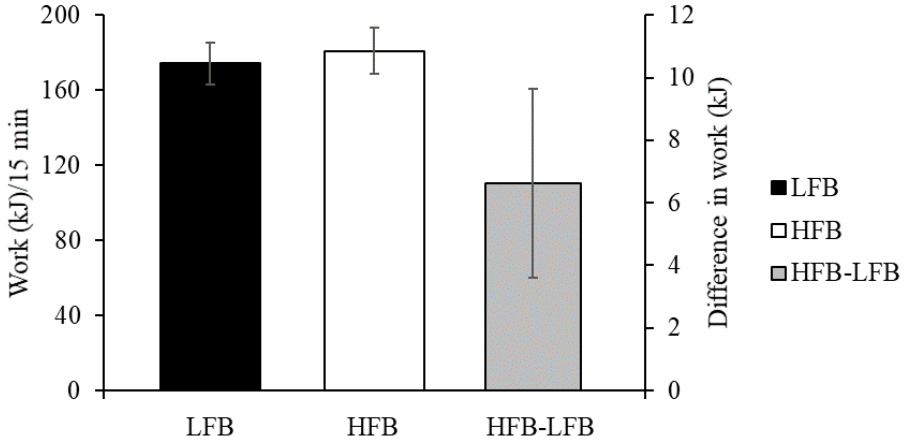
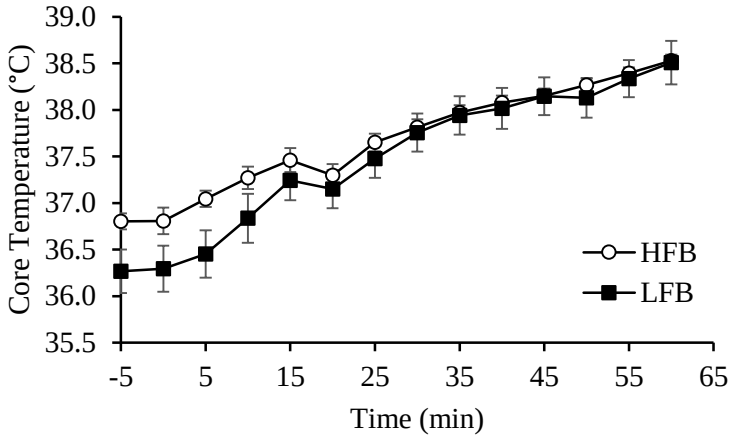
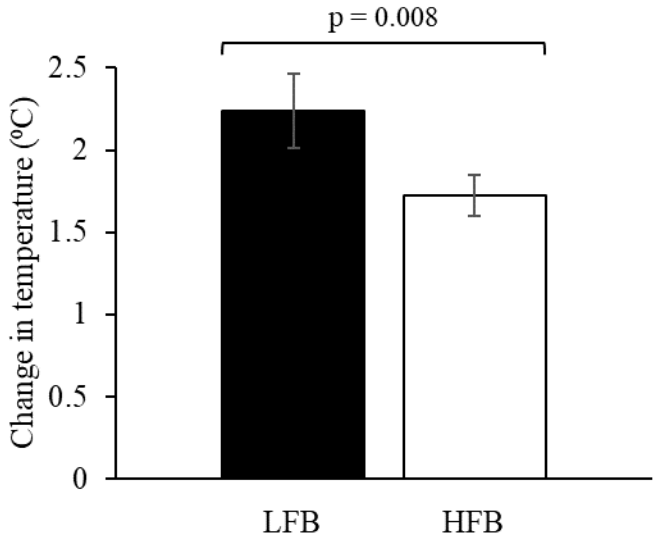


Figure 3-4. (A) Core temperature during exercise trial. (B) Overall change in core temperature during exercise: 2.24 ± 0.23 °C (LFB) and 1.73 ± 0.13 °C (HFB); paired t-test, p = 0.008.

(A)



(B)



Inflammatory markers

A main effect of time was observed for plasma IL-6 and IL-10 where both cytokines were significantly elevated post-exercise and after 1-h recovery (Table 3-5). Similarly, TNF-α was elevated immediately post-exercise compared to pre-exercise (Table 3-5). No significant effects were observed for treatment or time × treatment interaction.

Table 3-5. Plasma cytokines.

		Pre-exercise	Post-exercise		
			Immediate	**1-h**	**4-h**
IL-6 (pg/ml)	HFB	n.d.	0.76 ± 0.23*	0.22 ± 0.13*	n.d.
	LFB	n.d.	0.79 ± 0.38*	0.53 ± 0.20*	n.d.
	HFB-LFB	n.d.	-0.03 ± 0.42	-0.31 ± 0.19	n.d.
IL-10 (pg/ml)	HFB	1.49 ± 0.36	3.95 ± 0.69*	3.04 ± 0.44*	1.48 ± 0.28
	LFB	1.52 ± 0.38	3.76 ± 0.67*	2.69 ± 0.36*	1.86 ± 0.55
	HFB-LFB	-0.02 ± 0.36	0.20 ± 0.69	0.35 ± 0.44	-0.39 ± 0.28
TNF-α (pg/ml)	HFB	n.d.	0.28 ± 0.09*	n.d.	n.d.
	LFB	n.d.	0.31 ± 0.07*	n.d.	n.d.
	HFB-LFB	n.d.	-0.02 ± -0.06	n.d.	n.d.

Mixed effects, repeated measures 2-way ANOVA performed on raw instrumental values. n.d., not detected. *Significant difference from pre-exercise, $p < 0.0001$.

Gut injury and permeability

A significant effect of time was observed for plasma I-FABP ($p = 0.001$), but there was no significant treatment effect or treatment × exercise interaction (Figure 3-5). Plasma I-FABP was significantly elevated immediately post-exercise compared to all other time points. Mean change in I-FABP from pre- to immediately post-exercise did not differ between treatments: 300 ± 156 pg/ml (HFB) and 439 ± 162 (LFB) pg/ml. In addition, no significant correlation was found between final core temperature or change in core temperature and change in I-FABP.

Percent recovery of 8-h urinary sugars (sucrose, lactulose, and sucralose) at rest was not significantly different between condition (baseline, LFB, HFB) (Table 3-6). Similarly, percent recovery of 6-h urinary sugars was not significantly different between treatments following the exercise trial (Table 3-7). Since a post-exercise sample was missing for one subject, these analyses were conducted with $n = 11$. No significant correlation was found between final core temperature or change in core temperature and percent recovery of sucrose, lactulose, or sucralose.

Figure 3-5. Plasma I-FABP measured at t = 0, 1, 2, 5. Peak values of I-FABP immediately post-exercise (t = 1): 1161 ± 253 (HFB), 1177 ± 250 pg/ml (LFB). *Significant difference from pre-exercise (t = 0).

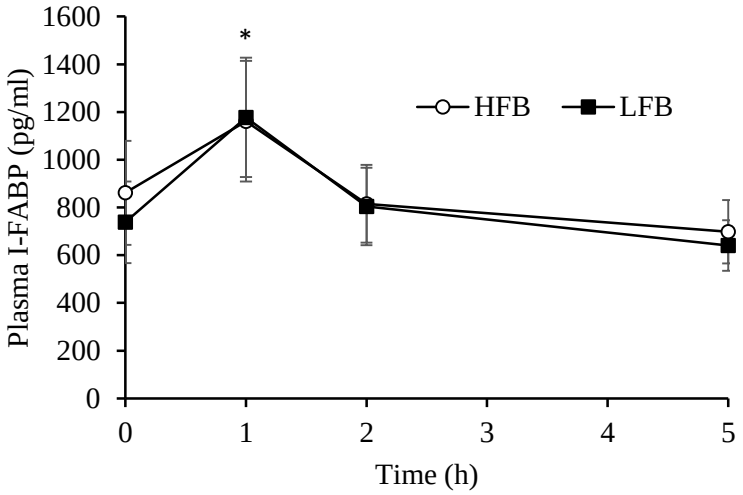


Table 3-6. 8-h Sugar probe excretion at rest, n = 12.

	Baseline	HFB (Day 12)	LFB (Day 12)	p-value
% Sucrose	0.035 ± 0.010	0.069 ± 0.023	0.056 ± 0.018	0.454
% Lactulose	0.424 ± 0.087	0.424 ± 0.081	0.324 ± 0.064	0.354
% Sucralose	1.206 ± 0.210	1.311 ± 0.264	1.237 ± 0.448	0.561

Table 3-7. 6-h Sugar probe excretion post-exercise, n = 11.

	HFB	LFB	HFB-LFB	p-value
% Sucrose	0.040 ± 0.008	0.066 ± 0.026	-0.026 ± 0.028	0.817
% Lactulose	0.293 ± 0.050	0.267 ± 0.085	0.026 ± 0.085	0.384
% Sucralose	0.775 ± 0.060	1.116 ± 0.386	-0.341 ± 0.404	0.994

Discussion

The purpose of the present study was to determine the effect of flavonoid supplementation on intestinal inflammation, injury, and permeability following moderate- to high-intensity cycling. On the HFB, recreational cyclists consumed ~490 mg of cocoa, blueberry, and green tea flavonoids daily for two weeks leading up to the cycling trial.

Contrary to our hypothesis, we observed no treatment effects on GI permeability, injury, or inflammation. There were no treatment differences between sugar probe recovery either at rest or post-exercise and no treatment effects on plasma I-FABP, IL-6, IL-10, and TNF-α before and following the exercise trial. Though there was no difference in cycling distance or cadence, HFB tended to increase cycling work during the TT.

The 1-hour cycling trials for both conditions were consistent in degree of exercise intensity, with a similar response in RPE and physiological measures of VO_2, HR, and peak core temperature. Interestingly, we observed a larger increase in core temperature for LFB due to a lower initial temperature, regardless of treatment sequence. To our knowledge, this difference in initial core temperature potentially due to two-week flavonoid supplementation has not been previously reported. However, the maximum core temperatures were not different, and since the general practice is to assess final core temperature in relation to epithelial integrity, we do not believe that this difference is a significant concern in the final outcome.

Both peak core temperature and exercise intensity (measured by $\%VO_2$) have a positive association with exercise-induced GI injury and permeability [4,5]. Several other cycling studies at similar intensity (70% VO_2max) and duration (one hour) reported increased GI permeability and injury [21–24]**.** In the present study, average maximal core temperature was 38.52 ± 0.12 °C, and we observed a significant increase in I-FABP across both arms from pre- to post-exercise. In a systematic review, Pires et al. reported that of participants with a final core temperature of 38.5 °C or below, ~36% showed increased permeability, and within the range of 38.6-39.0 °C, ~48% of participants showed augmented permeability [4]. Though the cycling protocol employed in the our

study was comparable in intensity and demonstrated acute GI injury, on average subjects had relatively lower peak core temperatures than those outlined by Pires [4], which may be one reason why no treatment effects on I-FABP and intestinal permeability were observed. When subjects were grouped into high and low peak core temperatures determined by values above and below the median (38.61 °C), no significant effect of high or low final core temperature was found for overall change in I-FABP or percent sugar recovery. Additionally, no correlations were found between final core temperature and primary outcomes. Perhaps if there was a larger effect of exercise, we may have observed a difference with the HFB alleviating these acute exercise effects. Additionally, 6- and 8-h urinary sugar probe excretion may not be as sensitive to modest exercise-induced changes on intestinal permeability, since relatively transient changes in permeability may recover quickly depending on intensity and duration of exercise [22]. Other studies have reported detecting exercise-induced changes in I-FABP and serum recovery of sugar probes that were not observed in urinary samples [22,25]. In the present study, exercise appeared to induce transient intestinal injury, since we only observed significant time effects for I-FABP immediately post-exercise, but not one or four hours later. Other exercise protocols ranging from 60-120% VO_2max have been shown to increase I-FABP by 50-250% immediately following exercise [26]. Since the extent of intestinal injury due to exercise was relatively moderate (~46% average increase in I-FABP from pre- to post-exercise), it is not surprising that a treatment effect on sugar probe permeability was not detected. Finally, other studies evaluating changes in I-FABP following moderate- to high-intensity exercise reported significant post-exercise treatment differences of ~300 pg/ml (curcumin supplement vs. placebo), 424 pg/ml (35

vs. 20 °C environmental temperature), 328.6 pg/ml (bovine colostrum vs. control), 401 pg/ml (ibuprofen vs. control), and 186.8 pg/ml (carbohydrate gel vs. placebo) [20,27–30]. A post-hoc power calculation was conducted for the post-exercise I-FABP response given the achieved sample size and estimated within-subject standard deviation of 380.8 pg/ml, and this study was powered to detect a treatment effect of ~480 pg/ml, but the actual mean difference observed was 16 pg/ml—much smaller than the sampling variation observed within subjects, and not likely a clinically meaningful difference had there been a true effect.

Exercise modality is also known to affect the incidence of GI symptoms and stress; cycling induces relatively less inflammation, muscle damage, and oxidative stress than running [31]. In one study comparing GI permeability during running and cycling, running caused a larger increase in intestinal permeability [32]. This may be due to the mechanical impact and posture during running which is not as pronounced in cycling [33,34]. In previous work conducted with runners, we found that participants struggled to complete the designated running test in its entirety (1-h at 80% VO_2max), which resulted in the trials being cut short, and no significant changes in gut permeability were observed (unpublished data). Based on our collaborator's previous experience, cyclists recruited from the local community were expected to be capable of reliably completing the cycling protocol. Studies conducted elsewhere have also demonstrated measurable changes in gut permeability with cycling [21–24,32].

Another reason why we may not have observed an effect on intestinal permeability and injury is due to differences in flavonoid bioavailability. In the Polyphenol Study previously conducted at USU, we observed a significant reduction in

intestinal permeability and inflammation with a high polyphenol dietary intervention (340 mg flavonoids/1,000 kcal) provided to participants through a mixture of fruits, vegetables, juice, and chocolate (Chapter II, Section 3.3). In contrast, the investigational product in this study was prepared from a mixture of food ingredients with some degree of processing (e.g., drying, freeze-drying, extraction). While the flavonoid content of the blueberry, cocoa, and green tea powders were supplied by the manufacturers, some bioactivity may have been reduced during transit, storage, and any time prior to consumption. Bioavailability and bioaccessibility can vary widely from purified compounds, to extracts, to fresh whole foods [35]. Compounds in a food matrix can have either positive and negative effects in terms of flavonoid bioavailability [36]. Some polyphenols such as procyanidins may be less bioavailable when in a food matrix, while others may actually be preserved throughout the digestive tract and reach the small intestine in a greater quantity due to association with the food matrix [37]. Polyphenols ingested in combination or in the form of food along with other dietary components may have increased bioavailability or interact to exert synergistic effects [31,38]. Although an epicatechin (one of the primary flavan-3-ols in cocoa) supplement was shown to have a negative effect on aerobic and mitochondrial adaptations to cycle training in one study, others utilizing cocoa products showed neutral or positive effects on performance, inflammation, and oxidative stress [39–41]. While we cannot say for certain how the food matrix and processing affected bioavailability and bioefficacy of the investigational product, we acknowledge that this is a factor that may have impacted the results and one reason why the results differed from those of the Polyphenol Study.

Recently, Keirns et al. suggested that individuals who train regularly may be more resistant to a leaky gut than sedentary individuals when GI integrity is assessed at rest versus immediately post-exercise—that is, chronic exercise may improve GI integrity over time [42]. This may be another reason why we observed differences between the present study with active cyclists and the Polyphenol Study with sedentary, overweight/obese subjects. Part of the adaptive response to exercise and heat stress is the upregulation of heat-shock proteins (HSP) in muscle and cardiac tissue, which can help stabilize cytoskeletal proteins and tight junctions [43,44]. Prior heat stress conditioning may increase thermotolerance, cell survival, and resistance to barrier dysfunction, through HSP induction [44,45]. For example, an elevation in incubation temperature from 37 °C to 39 °C increased HSP and tight junction protein (ZO1 and occludin) expression after four hours in Caco-2 and HT29 intestinal cells [46]. On the other hand, it has not yet been demonstrated that chronic exercise can upregulate HSP expression in intestinal enterocytes [42]. More research is warranted, as the data currently available suggesting that chronic exercise can reduce intestinal permeability is limited to study populations with various metabolic conditions (insulin resistance, type II diabetes, and obesity) [42]. These studies also reported reductions in visceral fat mass and body weight over the course of the exercise program, which could be the reason for improvements in intestinal permeability through reduced circulating inflammatory cytokines [42]. Nevertheless, others have suggested that flavonoid supplementation may have a greater protective effect in untrained individuals; the effects of flavonoid supplementation on muscle mitochondrial biogenesis and performance, if any, may not be as great in trained versus untrained individuals, since untrained muscle tissue has a lower mitochondrial density

than trained [31]. Competitive athletes, regularly exposed to oxidative stress during training, have more active endogenous antioxidant and anti-inflammatory responses and increased muscular efficiency from years of consistent endurance training [47,48]. In one study, a fruit and vegetable flavonoid powder fed to highly trained cyclists for seventeen days did not have an effect on exercise-induced inflammation, but a similar dose of flavonoids was previously shown to have an effect in non-athletes [49]. The authors suggested that the intervention may not be as effective may be due to the higher level of fitness of the study population [49]. In another study, two-week flavonoid supplementation decreased intestinal permeability relative to placebo in an untrained population both at rest and after walking at ~62% VO_2max, while intestinal permeability was elevated in a group of trained runners following 2.5 h running at 69% VO_2max—though several other differences between the untrained and trained groups may have contributed to the results [50]. In the present study, subjects were trained, recreational athletes, with an average maximum aerobic capacity of 43.2 ± 5.9 ml/kg/min (range: 28.1-51.4 ml/kg/min). The cycling protocol was chosen to elicit final core temperatures above 38.5 °C, to increase the likelihood of observing changes in GI permeability [4]. Though we may have been more likely to observe an effect of flavonoid intake with untrained subjects, they would have been less likely to successfully complete this challenging exercise protocol. Also, it may not be as applicable to attempt to alleviate these exercise-induced GI changes in untrained individuals who do not exercise or compete regularly.

Physical exertion and hyperthermia are known to cause changes in plasma cytokine levels [16,51]. We observed relatively low levels of IL-6, IL-10, and TNF-α

despite the rigorous exercise protocol. As expected, plasma I-FABP and IL-6 increased in response to exercise and returned to baseline levels during the recovery period, regardless of the treatment. However, plasma IL-6 and TNF-α were relatively low even immediately following exercise and mostly undetectable at baseline and after 4 hours of recovery. Similarly, Osborne et al. found that I-FABP increased 140% from pre- to post-exercise, but no change in circulating pro-inflammatory cytokines or endotoxin following a 60-min trial at 35 °C (alternating between 40-70% VO_2max), with final core temperatures reaching 39.5 °C [30]. Other studies with a similar intensity (70% VO_2max) reported no changes in TNF-α (< 2 pg/ml pre- to post-exercise) and the plasma cytokines IL-10 and IL-6 (< 6 pg/ml) despite showing a significant increase in intestinal permeability and core temperature of 39.5 °C, respectively [52,53]. Short-term curcumin supplementation reduced the increase in I-FABP relative to placebo following a one-hour treadmill run at 65% VO_2max at 37 °C, but no significant interaction effects were observed between the treatment and time for IL-6, IL-10, and TNF-α [29]. In contrast, other studies have shown exercise effects on circulating cytokines, with varying effects of polyphenol ingestion. Two-week supplementation with the flavonoids quercetin and EGCG significantly reduced IL-6 and IL-10 but not TNF-α relative to placebo following a daily three-hour cycling protocol for three consecutive days [31]. McAnulty et al. found that six weeks of blueberry ingestion elevated the anti-inflammatory cytokine IL-10 to a greater extent than the control immediately following a 2.5 h run at ~72% VO_2max [54]. Based on these varied results, it is possible that shorter duration exercise at temperate conditions, such as in our study, does not elicit as large of a cytokine response as is more consistently

observed in hotter environmental temperatures, longer events, or non-controlled environments, such as a road marathon [55].

Interestingly, HFB supplementation appeared to result in increased work output with no difference in cadence or total distance during the fifteen-minute TT. Further post-hoc analyses showed that cycling power also tended to be greater in the HFB trial (HFB: 201 ± 14 W and LFB: 194 ± 13 W, $p = 0.051$, data not shown). Because cadence was not different between conditions, the greater power and work output for the HFB trial was likely the result of participants applying more force to the pedals. Given the achieved sample size and an estimated within-subject standard deviation of 9.3 kJ for repeated TT measurements [56], post-hoc power analysis ($\alpha = 0.05$, $\beta = 0.80$) showed that the study was powered to detect a minimum treatment difference of 11.7 kJ. However, the small effect size ($d = 0.16$) suggests that the observed difference in work (6.6 kJ) is not likely to have a meaningful impact on overall performance—as was demonstrated with the lack of an effect on total distance. If these performance effects are related to other factors that require a longer time to show measurable changes, then an effect may have been observed with a longer supplementation time. As mentioned previously, dietary flavonoids can activate endogenous antioxidant pathways via Nrf2 signaling to reduce oxidative stress and improve muscle perfusion through increased NO availability [8,10]. Presumably, these vascular and antioxidant improvements would have an effect on performance, perhaps through delaying onset of fatigue. A recent review found consistent evidence of reduced oxidative stress with both acute and chronic cocoa supplementation, but changes in exercise performance and recovery were inconsistent with some studies demonstrating improvements while others did not [57]. Some studies found no effect of

cocoa intake on inflammatory markers while performance improved [57]. Taub et al. reported increased VO_2max and maximum power with untrained subjects after three months' dark chocolate supplementation [40]. These improvements in cycling performance were observed alongside changes in skeletal muscle cellular metabolism and improved oxidative stress status (reduced protein carbonyls and increased glutathione) [40]. Another study with moderately active subjects reported greater distance covered during a two-minute cycling sprint with two weeks of dark chocolate intake, compared against white chocolate and no supplementation, and the authors theorized that the improved performance was due to lower oxygen cost (increased gas exchange threshold) with dark chocolate [41]. Other studies have found performance benefits with a variety of other dietary flavonoids and polyphenols. Morgan et al. observed ~4.6% faster 15 km TT performance with Montmorency cherry supplementation (257 mg anthocyanins/day for one week) relative to placebo in trained cyclists, which was thought to be driven by an improvement in muscle oxygenation due to NO availability and potentially reduced oxidative stress [58]. Several studies with both acute and chronic blackcurrant and pomegranate anthocyanin supplementation have also demonstrated improved performance in both trained and untrained subjects [9]. However, our hypothesis was that any observed performance improvements would be driven by a reduction in intestinal permeability or GI discomfort, and therefore, our primary endpoints were plasma I-FABP and urinary sugars; we did not measure markers of oxidative stress as this was beyond the scope of our study. Based on other studies and the effects of flavonoid ingestion on Nrf2 signaling, it is possible that we would have seen an attenuation in oxidative stress or

improvement in tissue oxygenation with HFB, which would help explain the slight performance benefits observed.

A limitation of this study was that there was no monosaccharide available for the intestinal permeability assessment. Larger molecules (e.g., lactulose, sucralose, and sucrose) travel paracellularly to enter systemic circulation from the intestinal lumen while smaller molecules (e.g., mannitol) are absorbed transcellularly [13,59]. As a result, larger molecules are indicators for changes in paracellular permeability while smaller test compounds control for gastric emptying, epithelial absorptive area, transit time, and renal function across individuals [60]. The urinary excretion ratio (the recovery of the larger probe relative to the smaller) is most often reported and provides a standardized measure of intestinal permeability at the region of the GI tract where the larger probe is absorbed [13]. Unfortunately, due to a supplier issue, a monosaccharide probe was not included in the intestinal permeability assessment. However, the crossover design of the study still allowed for meaningful within-subjects comparisons for percent recovery of the disaccharide probes, and some other studies have similarly reported percent recovery of disaccharide sugars [5,25,61–65]. Another limitation was that the study design restricted comparisons between different urine collections. The sugar excretion test is ideally performed fasted to minimize the contribution of dietary sugars, so urine samples collected at baseline and mid-intervention were fasted, eight-hour overnight collections. The collection immediately following the cycling trial was a six-hour collection (two hours fasted) in order to minimize participant burden and shorten the long clinic visit on Day 15. As a result, comparisons for intestinal permeability are only made among baseline and mid-intervention samples and between post-exercise samples.

Finally, another limitation is the potential for carryover effects from one intervention to the next. It was important to allow a sufficient length of time for any lingering effects of the prior treatment to dissipate prior to beginning the next arm, while still being short enough to encourage subject retention. Epithelial intestinal cells undergo complete turnover within two weeks [66]. Additionally, a two-week washout was used in the Polyphenol Study and no carryover effects were observed. For these reasons, a two-week washout period was selected for this study. In the statistical analysis, subjects were nested within treatment sequence, which accounted for whether the subject was randomly allocated to the AB or BA treatment order. No significant sequence effects were found in any of the analyses, suggesting that the two-week washout was sufficient to minimize potential carryover effects.

In conclusion, a randomized controlled crossover study was conducted to test the effects of a pre-workout high flavonoid beverage in reducing exercise-induced GI permeability and injury. During a one-hour, moderate intensity cycling test and TT, the HFB performed as well as a standard pre-workout mix. Despite the accumulating evidence suggesting potential for improvement of GI permeability, inflammation, and athletic performance with supplemental flavonoid intake, we found no differences between the treatment and placebo. Though there was potentially an effect of increased cycling work, the difference in means relative to error was small, and no difference in distance was observed. Due to the importance of addressing epithelial integrity and inflammation in not only exercise but also exertional heat stress and inflammatory diseases (e.g., inflammatory bowel disease, ulcerative colitis, type I diabetes), more work—especially well-controlled human clinical trials—is warranted. Future studies

should ideally include measurements of tissue perfusion or vascular effects (e.g., flow mediated dilation, tissue oxygenation index), mitochondrial metabolism (e.g., mitochondrial density, citrate synthase), and oxidative stress (e.g., glutathione, protein carbonyls, total antioxidant status).

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CHAPTER IV

EFFECT OF FLAVONOID SUPPLEMENTATION ON GUT MICROBIOTA PROFILE, SHORT CHAIN FATTY ACID EXCRETION, AND INTESTINAL INFLAMMATION

Introduction

Intestinal dysbiosis may occur throughout an individual's lifetime when the intestinal microbial community is shifted one way or another, or otherwise displays aberrant functioning compared to that of a healthy individual [1]. Though it is unclear whether intestinal dysbiosis is a cause or an effect, it is often observed in conjunction with inflammatory or metabolic pathologies [1–3]. For example, individuals with low microbial richness and diversity display more overall adiposity, insulin resistance, and dyslipidemia than those with greater microbial richness [4]. Low microbial diversity and dysbiosis are also observed in patients suffering from ulcerative colitis, irritable bowel syndrome, colorectal cancer, and inflammatory bowel diseases [2,5,6].

One contributing factor in shaping the gut microbial composition is the diet [7]. For example, high animal protein, carbohydrate, or prebiotic intake can all dramatically affect the species present in the GI tract [8]. Because of the impact of the diet on the gut microbiota and the gut microbiota on human health, it is important to investigate potential dietary strategies that can beneficially modulate the gut microbiota and possibly improve health outcomes later in life [1,9]. Dietary flavonoids, plant secondary metabolites abundant in fruits, tea, and coffee, have previously been demonstrated to help shape the

intestinal microbial population [8]. Due to low bioavailability in the small intestine, the majority of dietary polyphenols, which encompass flavonoids in addition to other phenolic compounds, transit to the large intestine and are metabolized by the colonic microflora [2]. Both parent polyphenols and their metabolites can inhibit or promote the growth of different microorganisms [10]. Polyphenols consist of thousands of structurally diverse compounds, and different compounds and doses can have varying effects on the microbiota [10]. Preclinical and some clinical studies have shown that dietary flavonoids can stimulate the growth of health-promoting microorganisms including *Lactobacillus* spp., *Bifidobacterium* spp., *Akkermansia* spp., *Roseburia* spp., and *Faecalibacterium* spp. [2,11–13]. Both *Lactobacillus* spp. and *Bifidobacterium* spp. are considered to have beneficial effects on intestinal health by reducing pathogen colonization and increasing SCFA production [11]. In one clinical trial, ingestion of 494 mg of cocoa flavanols/day for four weeks significantly increased *Lactobacillus* spp., *Enterococcus* spp. and *Bifidobacterium* spp. compared to a low flavanol control at 23 mg/day [14]. The cocoa flavanol treatment also significantly decreased numbers of the *C. histolyticum* group, and changes in the bifidobacteria and lactobacilli significantly correlated with a reduction in plasma C-reactive protein (CRP), a marker for inflammation [14]. In another clinical study, six-week consumption of a wild blueberry drink containing approximately 375 mg anthocyanins and 128 mg chlorogenic acid significantly increased *Bifidobacterium* spp. relative to the placebo [15]. In rats, mango polyphenol supplementation increased abundance of *Lactobacillus plantarum*, *Lactococcus lactis*, and *Clostridium butyrim*, along with an increase in the SCFAs butyrate and valerate [16]. Other *in vitro* studies have shown that cocoa and tea polyphenols can increase growth of commensal organisms

while inhibiting pathogens [17–19]. However, other clinical studies did not find an effect of polyphenols on gut microbial composition or SCFA production [20–22].

An interesting aspect of the interaction of between the intestinal microbiota and human health is short chain fatty acid (SCFA) production from non-digestible carbohydrates. The microbiota-produced colonic SCFA are believed to act as signaling molecules through interaction with specific G protein-coupled receptors and through the regulation of gene expression and disease pathogenesis via histone deacetylase inhibition [1]. Bacteroidetes are associated with acetate and propionate production while Firmicutes are the primary producers of butyrate [1]. Butyrate in particular is an important SCFA, because it plays a key role in the differentiation of colonic regulatory T cells, displays anti-inflammatory properties, and increases expression of claudin tight junction proteins [23–25]. Studies have shown that flavonoid supplementation can increase SCFA production, likely due to flavonoid-mediated changes in the microbiota [11]. For example, bilberry anthocyanin extract supplementation in rats increased abundance of butyrate producers (*Lactoacillus* and *Bacteroides*) while inhibiting the growth of harmful species (Verrucomicrobia and Euryarchaeota) [26]. (For additional discussion on SCFA, refer to Chapter II, Section 4.2.)

Microbial diversity and their collective, functional genome, which dictates the microbiome's metabolic capability, are closely tied with human health and disease. The gut microbiota can affect flavonoid activity by transforming the molecules to more bioavailable or bioactive metabolites, so the effects of flavonoid intake on human health may be partially mediated by the composition and metabolic activity of the gut microflora [3]. Moderate to high dietary flavonoid intake has the potential to positively

shape the gut microbiota, but the majority of the evidence is from animal and cell culture studies [2]. Similarly, preclinical studies suggest that intestinal SCFA can influence signaling of key processes, but clinical data is lacking [27]. As a result, it is important to further explore the relationship between flavonoid intake, the gut microbiota, and intestinal health in a controlled, human clinical trial. The present study extends previous work by exploring intestinal inflammation, microbiota composition, and SCFA excretion in response to supplemental flavonoid intake. Based on the literature suggesting that flavonoid intake can impact intestinal health, we hypothesized that an increase in dietary flavonoid intake of ~490 mg/day would change the proportions of intestinal bacterial taxa in a group of healthy volunteers, and that changes observed in the microbiome would also be reflected in altered SCFA excretion. Additionally, we hypothesized that elevated flavonoid intake would also result in a reduction in intestinal inflammation.

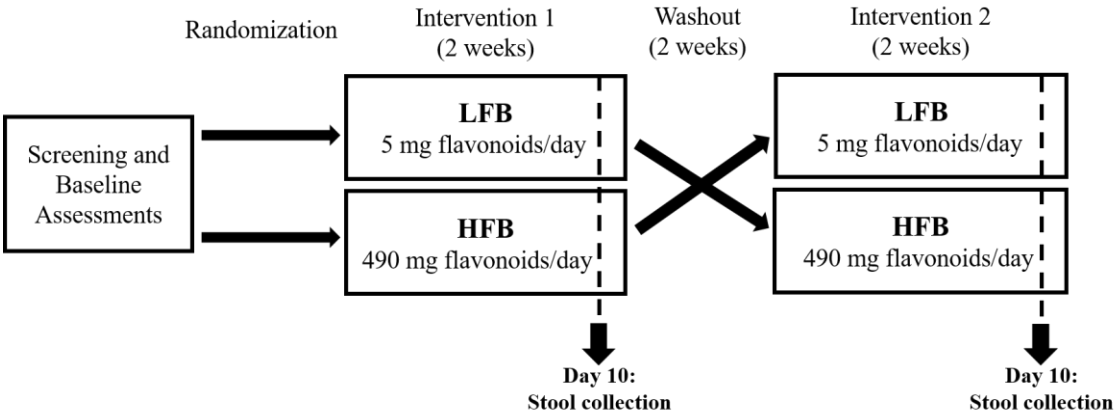
Materials and Methods

Study design

This study was conducted in conjunction with the clinical trial as described in Chapter III. Briefly, 12 recreationally-active cyclists (female n = 4, male n = 8; mean ± SD: age 37 ± 11 years, body mass index 25.6 ± 3.3 kg/m^2) completed this randomized, controlled crossover study conducted from August 2018 through May 2019 at Utah State University (USU). This study was conducted in accordance with the 2008 Helsinki Declaration for Human Research Ethics and approved by the USU research ethics committee (IRB Protocol #9255). Participants were informed of all study requirements and provided written informed consent prior to enrollment. A schematic of the study design and endpoint collection is provided in Figure 4-1.

A complete list of inclusion and exclusion criteria is provided in Appendix A, Table A-1. Exclusion criteria included chronic NSAID use, antibiotics treatment within 3 months of the study, or flavonoid supplement consumption within 1 month of the study. Additionally, those with a medical history of heart disease, hypertension, diabetes, Crohn's disease, IBS, colitis, celiac disease, inflammatory or autoimmune disease were also excluded from the study.

Figure 4-1. Schematic overview of crossover study design.



A high flavonoid beverage (HFB, 490 mg total flavonoids) or macronutrient-matched low flavonoid control (LFB, 5 mg flavonoids) was consumed once daily for fifteen days. Each serving of the investigational product (64 g powder) was prepared in a shaker bottle with 240 ml 2% white milk. During the intervention, participants were instructed to abstain from a provided list of high flavonoid foods, flavonoid supplements, and NSAIDs. Stool samples were collected once after 9-11 days of supplementation to determine fecal calprotectin, microbiota composition, and total SCFA. Participants collected stool samples in pre-labelled, freezer-safe containers, and samples were stored at -20 °C until the next clinic visit (within four days). Upon receipt at the clinic, samples

were homogenized by stirring and transferred to 50 ml tubes and stored at -80 °C until further analysis.

Flavonoid food frequency questionnaire

Prior to the first screening visit, participants were asked to self-administer a flavonoid food frequency questionnaire (FFQ) adapted from Somerset and Papier [28]. Subjects indicated their average consumption frequency of eight beverages, three cocoa products, seventeen fruits, and thirty vegetables (see Appendix B, Figure B-1). Each food serving size was converted to mass (g), and flavonoid content (mg) per 100 g of each food or beverage was determined from the USDA Database for Flavonoid Content of Selected Foods [29]. Reported food intake frequency was converted to weekly frequency for each food/beverage: never or < 1 time/mo = 0.7; 1-3 times/mo = 0.7; 1 time/wk = 0.43; 2-4 times/wk = 1.7; 1 time/day = 5; 2-3 times/day = 15; 4-5 times/day = 31.5; and > 6 times/day = 48. Weekly intake frequency of each item was multiplied by the flavonoid content to determine total weekly flavonoid intake.

Fecal calprotectin

Fecal calprotectin, a protein released by activated neutrophils or during cell death, is a reliable biomarker of intestinal inflammation [30]. Fecal calprotectin was determined in duplicate with a commercial ELISA kit following the manufacturer's instructions (Hycult Biotech; Uden, The Netherlands).

Fecal SCFA content

All samples and standards were prepared in duplicate. A series of standards (0.04-5 mM) containing acetic, propionic, butyric, isobutyric, valeric, isovaleric, and caproic acids were prepared in deionized water. Ethyl butyric acid was used as an internal

standard and prepared at 1 g/L in 250 g/L metaphosphoric acid. Approximately 0.1 g of stool was added to 0.9 ml deionized water. After mixing, 200 µl of the internal standard was added, and samples were centrifuged at 10,000 × *g* for 20 min. The supernatant was removed and filtered with a 0.2 µm syringe filter into a glass vial. Samples were stored at 4 °C prior to GC analysis. Analysis was performed with a Shimadzu GC2010 gas chromatograph coupled to a flame ionization detector (FID) (Shimadzu Scientific Instruments; Columbia, MD). The column was a Zebron ZB-FFAP (30 m × 0.53 mm diameter × 1.00 µm film thickness) capillary GC column (Phenomenex, Torrance, CA, USA). Hydrogen was the carrier gas, and the column flow rate was 15.94 ml/min, with a 5:1 split ratio. The temperatures of the injection port and FID were 200 °C and 350 °C, respectively. The column oven temperature program used for compound separation is shown in Table 4-1. Compound peak areas were normalized by the internal standard and concentrations were determined from the standard curves.

Table 4-1. GC oven temperature program for SCFA analysis.

Rate (°C/min)	**Temperature (°C)**	**Hold Time (min)**
--	60	1
17	260	8

Stool microbiota composition

Three replicates from each stool collection were prepared for 16S rRNA sequencing. Fecal DNA was extracted in duplicate using the DNeasy PowerSoil kit, following the manufacturer's instructions (Qiagen; Carlsbad, CA). Following extraction, 4 µl of 5 M NaCl was added to each sample. Samples were centrifuged at 10,000 × *g* for 5 min, and the supernatant was decanted and residual liquid was removed in a speed

vacuum evaporator. Precipitated DNA was re-suspended in 10 mM Tris. DNA was quantified via absorbance, and samples were normalized to 5 ng/µl in Tris-EDTA buffer. Polymerase chain reaction (PCR) amplification of the V3-V4 hypervariable region of the 16S rRNA gene was completed in triplicate with Platinum Hot Start PCR Master Mix (Invitrogen; Carlsbad, CA) and DNA primers (IDT; Coralville, IA). Unique index adapters (barcodes) were added to the DNA amplicon with a second round of PCR amplification. PCR reaction products were purified, quantified with Quant-iT PicoGreen dsDNA Assay Kit (ThermoFisher Scientific, Waltham, MA), and pooled at a final concentration of 1 ng/µl. The library pool was spiked with 5% PhiX as an internal control and prepared with MiSeq v3 reagents. High through-put sequencing of paired-end 300 base pair (bp) reads was completed in a single run on the Illumina MiSeq platform at the Center for Integrated BioSystems at USU.

The raw 16S rRNA amplicon sequencing dataset was processed with Quantitative Insights Into Microbial Ecology (QIIME2) [31]. Filtering, dereplication, chimera detection, and merging paired-end reads was completed with the Divisive Amplicon Denoising Algorithm (DADA2) R package [32]. An amplicon sequence variant (ASV) abundance table was generated, and the QIIME feature-classifier classify-sklearn command was used with a classifier pre-trained for the V3-V4 region and the most recent release of the Silva database [33].

Observed, Chao1, and Shannon alpha diversity indices were analyzed and plotted with MicrobiomeAnalyst [34]. Principal coordinates analysis (PCoA) plots were generated with unweighted UniFrac distances and visualized with EMPeror [35].

Statistical analysis

Data that did not meet assumptions of normality (FFQ and calprotectin) were analyzed with the non-parametric Wilcoxon signed-rank test, with false discovery rate (FDR) adjustments for multiple comparisons as needed. Since calprotectin values were below the assay detection limit, instrumental values were used for statistical testing. Mean flavonoid intake determined from the FFQ was compared to the National Health and Nutrition Examination Survey (NHANES) reference dataset as reported by Kim et al. [36]. Individual and total SCFA were analyzed with paired t-tests, with FDR adjustments for multiple comparisons. Microbiome differential analysis was performed with the DESeq2 method, with FDR adjustments for multiple comparisons. Group significance for beta diversity was determined with permutational multivariate analysis of variance (PERMANOVA), with a p-value < 0.01 considered statistically significant. Data are expressed as means ± SEM, unless otherwise indicated. Statistical analyses were performed with QIIME2, MicrobiomeAnalyst, and JMP version 15.2.1 for Windows (SAS Institute, Cary, NC) with significance at $\alpha = 0.05$, unless otherwise stated. Additional output for statistical analyses are provided in Appendix B.

Results

Participant characteristics

Twelve participants completed both arms of the crossover study (Table 4-2). The investigational product and control beverages were well-tolerated by subjects with no adverse events reported during the duration of the study (six weeks total).

Table 4-2. Participant baseline characteristics: age, body mass, body mass index (BMI), systolic blood pressure (SBP), diastolic blood pressure (DBP), resting heart rate (HR), blood glucose.

	Men	**Women**	**Combined**
n	8	4	12
Age (years)	37 (18-55)	35 (18-45)	37
Body mass (kg)	79.6 (68.5-100)	74.1 (58.2-99.4)	77.8
BMI (kg/m^2)	25.0 (21.8-29.7)	26.8 (23.3-33.2)	25.6
SBP (mmHg)	117 (107-136)	111 (104-114)	115
DBP (mmHg)	77 (67-90)	70 (66-74)	75
Resting HR (bpm)	58 (40-76)	63 (53-72)	60
Blood glucose (mg/dL)	92 (86-103)	82 (75-86)	89

Average weekly flavonoid consumption

Average intakes of all individual flavonoid compounds are reported in Appendix B, Table B-1. The estimated total consumption of the five classes of flavonoids is reported in Table 4-3. The pre-study FFQ estimated that the participants' average weekly consumption of total flavonoids was 635 ± 195 mg, with the highest subgroups being flavan-3-ols, flavonols, and anthocyanidins. The average weekly intake of dietary flavonoids of US adults aged 19+ years estimated from the NHANES 2007-2010 data reported by Kim et al., is also included in Table 4-3 [36]. After adjustments for multiple comparisons, flavan-3-ol and total flavonoid intake were significantly lower than those from the NHANES dataset ($p < 0.05$).

Table 4-3. Average weekly consumption of flavonoids.

Flavonoid Class	**Mean ± SEM (mg)**	**Range (mg)**	**US Adult Average (mg)**
Flavonols	136.5 ± 21.9	35.3 – 266.3	111.3 ± 2.8
Flavones	11.9 ± 2.5	3.6 – 29.4	8.4 ± 0.7
Flavanones	80.5 ± 14.7	4.6 – 193.0	85.4 ± 3.5
Flavan-3-ols	296.3 ± 169.3*	5.5 – 2031.2	1108.8 ± 59.5
Anthocyanidins	109.6 ± 35.4	8.0 – 452.6	80.5 ± 4.9
TOTAL	634.7 ± 194.6*	98.1 – 2321.9	1400.7 ± 62.3

*Significantly different from US Average [36] at $p < 0.05$.

Fecal calprotectin

No significant difference between the treatments was observed in fecal calprotectin ($p > 0.05$). Mean fecal calprotectin for both conditions was below the assay detection limit of 16 μg/g.

Fecal SCFA

There was no significant difference between HFB and LFB for individual or total SCFA (Table 4-4). The most abundant fecal SCFA were acetate, propionate, and butyrate, present in a ratio of ~3:1:1, respectively. The total SCFA on the HFB and LFB were 115.1 ± 11.3 mM/g and 107.9 ± 9.7 mM/g, respectively.

Table 4-4. Individual and total SCFA composition of fecal samples (mM/g wet fecal content), n = 12.

SCFA (mM/g)	**HFB**	**LFB**	**HFB-LFB**	**p-value***
Acetic acid	63.3 ± 6.4	59.2 ± 6.5	4.1 ± 6.7	0.8789
Propionic acid	21.1 ± 2.2	20.7 ± 2.3	0.4 ± 2.3	0.8789
Butyric acid	21.9 ± 3.3	19.5 ± 2.1	2.4 ± 3.4	0.8789
Isobutyric acid	2.3 ± 0.3	2.2 ± 0.4	0.1 ± 0.3	0.8789
Valeric acid	2.6 ± 0.3	2.6 ± 0.3	0.1 ± 0.3	0.8789
Isovaleric acid	3.1 ± 0.4	3.0 ± 0.6	0.1 ± 0.4	0.8789
Caproic acid	0.8 ± 0.3	0.7 ± 0.2	0.1 ± 0.4	0.8789
TOTAL	115.1 ± 11.3	107.9 ± 9.7	7.2 ± 12.4	0.8789

Values expressed are means ± SEM. *FDR-adjusted p-value.

Intestinal microbiota composition

The average number of reads per sample was 82,944. After normalization (rarefying to the minimum library size and total sum scaling), the rarefaction curves showed adequate sequencing depth for all samples (Appendix B, Figure B-2). In total, 18 phyla were detected in the samples, with five phyla above one percent relative abundance: *Firmicutes* (81%), *Bacteroidetes* (10%), *Actinobacteria* (4.6%), *Tenericutes*

(2.4%), *Verrucomicrobia* (1.3%). No significant differences were observed between HFB and LFB for the relative abundance at the phylum level (Table 4-5). Likewise, the relative abundance of *Firmicutes* to *Bacteroidetes* was not significantly different between conditions (Appendix B, Tables B-5 and B-6).

Significant differences were found for two taxa: c_gammaproteobacteria ($p = 0.029$) and g_Eubacterium_ruminantium_group ($p = 0.025$) (Table 4-6). Higher abundance of Eubacterium ruminantium was observed with LFB whereas higher abundance of Gammaproteobacteria was observed in HFB samples. No differences were observed at the order, family, or species levels. Over 200 groups were detected at the genus level, and six potentially beneficial microorganisms (*Bacteroides*, *Faecalibacterium*, *Bifidobacterium*, *Roseburia*, *Akkermansia*, *Prevotella*) that have been reported to change with flavonoid intake were further examined with post-hoc within-subject tests, but no significant effects on relative abundance were observed (Table 4-7).

No significant treatment differences in Chao1, Observed, and Shannon metrics of alpha diversity were observed at any taxonomic level (Figure 4-2).

Within-subject comparisons based on unweighted UniFrac distance showed that there were no significant differences in beta diversity between the HFB and LFB samples (Appendix B, Table B-10). Unweighted UniFrac distances were visualized with PCoA to show the distance between samples in three-dimensional space. PCoA plots showed no clear separation between treatment groups (Figure 4-3A), and samples from each participant appeared to cluster together regardless of treatment (Figure 4-3B).

Table 4-5. Five most abundant phyla detected in samples (relative abundance).

Phylum	**HFB (%)**	**LFB (%)**	**HFB-LFB**
Firmicutes	78.61 ± 3.64	82.92 ± 2.69	-4.31 ± 3.30
Bacteroidetes	10.99 ± 2.64	9.83 ± 2.26	1.16 ± 2.44
Actinobacteria	5.78 ± 1.68	3.49 ± 0.84	2.29 ± 1.53
Tenericutes	2.79 ± 1.60	1.99 ± 1.60	0.80 ± 1.80
Verrucomicrobia	1.22 ± 0.93	1.43 ± 0.80	-0.21 ± 0.64

No significant differences with DESeq2 analysis.

Table 4-6. Relative percent abundance of significantly different features.

Taxa		**HFB (%)**	**LFB (%)**	**HFB-LFB**	**p-value***
Class	Gammaproteobacteria	0.32 ± 0.17	0.08 ± 0.02	0.24 ± 0.16	0.0285
Genus	*Eubacterium ruminantium*	0.04 ± 0.02	0.19 ± 0.14	-0.15 ± 0.12	0.0252

Differential analysis with DESeq2 algorithm. *FDR-adjusted p-value.

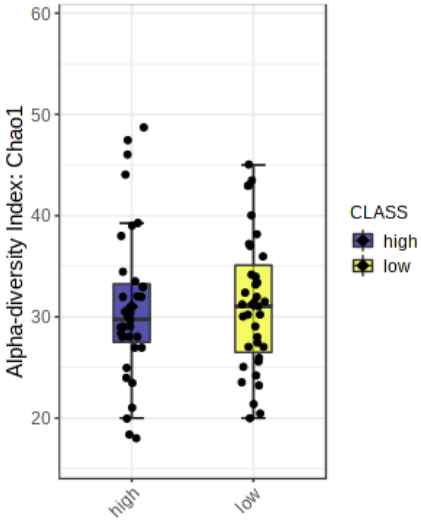
Table 4-7. Relative abundance of six taxa of interest.

Genus	**HFB (%)**	**LFB (%)**	**HFB-LFB**
Bacteroides	6.87 ± 2.08	5.69 ± 1.74	1.18 ± 1.87
Faecalibacterium	4.42 ± 1.18	3.51 ± 0.88	0.91 ± 1.26
Bifidobacterium	4.93 ± 1.58	2.70 ± 0.80	2.23 ± 1.46
Roseburia	1.82 ± 0.65	1.13 ± 0.34	0.69 ± 0.65
Akkermansia	1.22 ± 0.93	1.43 ± 0.80	-0.21 ± 0.64
Prevotella	0.20 ± 0.13	1.73 ± 1.07	-1.52 ± 1.07

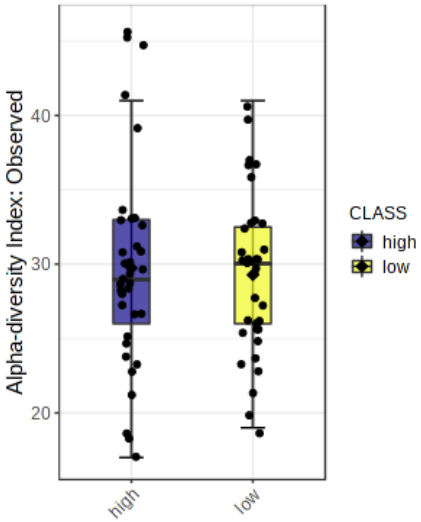
No significant differences with DESeq2 analysis.

Figure 4-2. Alpha diversity indices at the family level for (A) Chao1, (B) Observed species, and (C) Shannon.

(A)



(B)



(C)

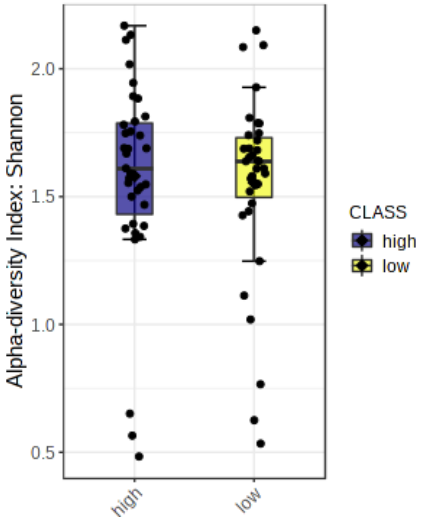
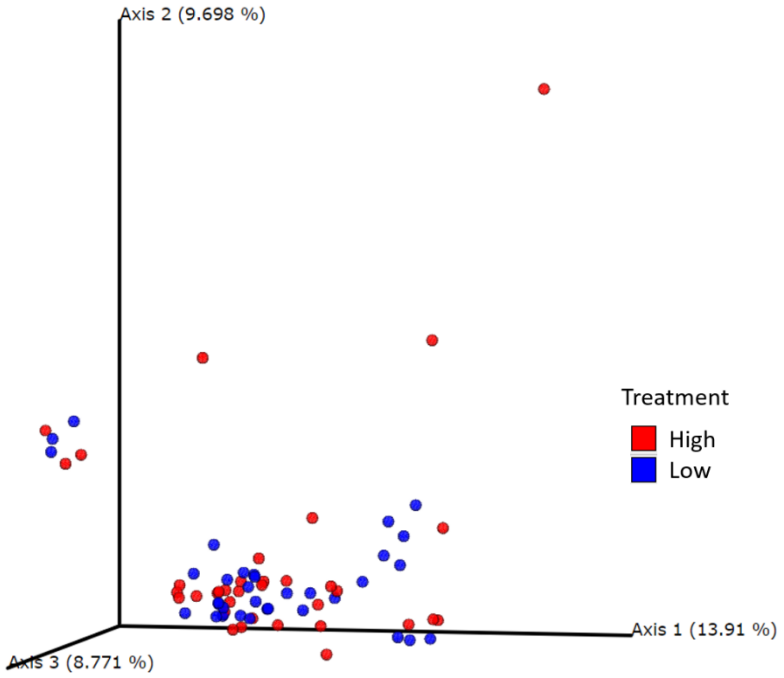
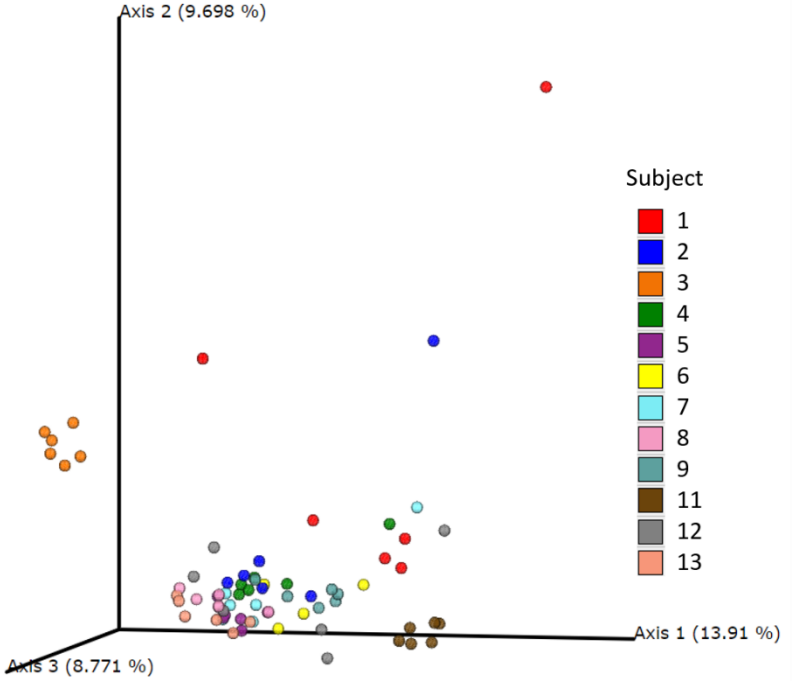


Figure 4-3. Principal coordinate analysis (PCoA) visualization of unweighted UniFrac distance colored by (A) treatment and (B) subject. Axes represent the percentage of variance explained by each coordinate dimension.

(A)



(B)



Discussion

In the present study, we observed no effect of sub-chronic, moderate (~490 mg/day) supplemental flavonoid intake on measures of intestinal health in a randomized controlled crossover trial with twelve healthy recreational cyclists. Surprisingly, daily consumption of a mixture of blueberry, cocoa, and green tea flavonoids did not result in significant differences in fecal SCFA, gut microbiota profile, or intestinal inflammation as compared to a low flavonoid control. The research participants were free from chronic disease and intestinal disorders, as was required by the study inclusion criteria. Participants were recreational cyclists of differing levels of experience and fitness, exercising at minimum three hours weekly. Though fecal calprotectin (a common biomarker of inflammatory intestinal pathologies) may not display measurable differences in the absence of disease, we expected to see an effect of the intervention on microbial composition and metabolism (SCFA content) due to the adaptable and responsive nature of the intestinal ecosystem to dietary intake [8].

Although individual flavonoid consumption is highly variable, the average US adult consumes an estimated 200-250 mg total flavonoids daily [37]. Overall, our subjects' estimated average intake of flavonoids reflected that of the US population for four of five classes of flavonoids. The estimated weekly intake of flavan-3-ols for our study participants (296 ± 169 mg) spanned a large range (6-2031 mg/week), and was significantly lower than that of the average American (1108 ± 60 mg) [36]. A significant dietary source of flavan-3-ols is brewed tea, and only three of twelve participants reported any amount of tea consumption, which may explain the difference from the NHANES dataset [36,37]. Due to this difference in flavan-3-ol intake, estimated total

flavonoid intake (635 ± 195 mg/week) was also significantly lower than that of the average American (1401 ± 62 mg/week).

Our subjects' estimated daily intake of total flavonoids was approximately 91 mg/day, ranging from ~14-332 mg/day among subjects. The HFB provided approximately 490 mg/day of anthocyanins and flavan-3-ols, an increase in flavonoid intake of ~1.5-35 times. If the subjects' dietary intake were closer to that of the NHANES data (200 mg/day), this would be an increase of about 2.5 times. The NHANES data was collected through 24-hour dietary recalls, administered by a trained interviewer. Although informative and convenient, the FFQ is a subjective assessment that is generally limited by the subjects' accuracy (e.g., correctly estimating serving sizes) and recall bias [38]. Additionally, the FFQ adapted for this study was developed by Somerset and Papier to be representative of the major flavonoid dietary sources in the Australian diet [28]. Though both Australian and American diets fall within the Western dietary pattern, this may have also contributed to the differences observed between the estimated mean intakes from this study and the NHANES data. The limitations inherent in these dietary assessments are a challenge for accurately determining the influence of diet on the intestinal microbiota [39]. Other studies evaluating the effects of flavonoid intake on intestinal microbiota have used flavonoid doses ranging from 494-750 mg, but did not provide an estimate of subjects' habitual flavonoid intake [13,14,20,21].

Overall fecal SCFA (HFB and LFB samples combined) were compared to those from participants of the Polyphenol Study (previously described in Chapter II, Section 3.3)—both analyses were conducted with the same methodology in the Ward lab at USU. Interestingly, SCFA levels from the present study were comparable to samples collected

from subjects during the low flavonoid intervention and significantly higher than those from the high flavonoid intervention of the Polyphenol Study (Appendix B, Table B-11). In the Polyphenol Study, the high flavonoid dietary intervention reduced total and individual SCFA recovered in the fecal content compared to the control, whereas in the present study, there were no differences between the treatment and control. Another study also reported no changes in SCFA following four weeks' consumption of apple fiber juice (7.5 g fiber), boysenberry polyphenol juice (750 mg polyphenols), or the combination thereof [20]. Dietary fiber is fermented by the colonic microflora to produce straight chain fatty acids while branched chain amino acids are typically metabolized into branched chain fatty acids. In states of gut dysbiosis, straight chain fatty acids often are reduced while branched chain fatty acids are elevated. While SCFA are generally believed to be beneficial to health, the significance of fecal SCFA content and whether changes in SCFA content are a cause or effect are still unclear. For example, a positive association was observed with fecal SCFA excretion, measures of gut dysbiosis, and cardiometabolic disease risk in a recent observational study of 441 adults [40]. On the other hand, exercise has been observed to increase SCFA excretion in lean but not obese individuals, and another study reported higher SCFA excretion in elite professional athletes compared to healthy controls [41,42]. Though determining the effect of exercise on intestinal microbiota and metabolism (SCFA) was not within the scope of this study, the potential effects of regular exercise training on microbial metabolism may have played a part in the subjects' lack of response to the intervention. A limitation of the study is that fecal SCFA excretion may not be representative of colonic production, as SCFA are absorbed and utilized by epithelial cells and other tissues, and absorption

depends on factors such as the expression of SCFA transporters and intestinal transit time [40]. As a result, an additional measurement of circulating SCFA may help in future studies to determine SCFA production, absorption, and excretion.

The gut microbiota varies considerably from one individual to another—microbial alpha diversity and SCFA content has been shown to vary with age, leanness, and exercise status [41–43]. Alpha diversity is a measure of the microbial diversity in a sample, which can be represented with species richness (i.e., the number of unique features in the sample) and/or evenness (i.e., distribution) [34]. Chao1 and observed alpha diversity are metrics of species richness, and the Shannon diversity index takes into account the evenness of species in addition to the overall number [34]. In the present study, supplemental flavonoid intake did not affect species richness or evenness relative to the control. Beta diversity compares the microbial community structure between multiple samples [44]. In particular, UniFrac is a metric used to represent the proportion of shared phylogenetic branches between samples, in which more similar communities have UniFrac scores closer to zero, while more divergent or independent samples have scores closer to one [44]. Within-subject comparisons of the two study conditions on UniFrac distance showed that there was not a differential effect of the study intervention on the relatively stable microbiome of each individual. These results were supported by the PCoA plots of beta diversity—samples tended to cluster by subject, regardless of treatment. Other studies have reported similar findings (minimal changes in intestinal microbiota following a dietary intervention), especially in shorter duration trials [22,45–47]. So, while microbiota compositional shifts due to diet have been reported to occur in as little as one day, an individual's microbiome profile is relatively stable over time and

likely shaped by a multitude of factors, including habitual dietary intake [48]. In a cross-sectional study, Wu et al. reported that an individual's overall microbiome profile had a greater association with long-term dietary patterns than short-term dietary changes [48]. In addition, a ten-day controlled-feeding study showed that despite producing measurable changes in microbial composition, the dietary intervention did not overcome inter-individual variation in beta diversity [48].

The gut microbiome is a key factor in the bioavailability and transformation of dietary flavonoids, so it is not surprising that flavonoid intake may have varied effects among individuals depending on their microbial community composition [49,50]. In some studies, a polyphenol or flavonoid treatment was shown to differentially affect sub-groups of participants. For example, six-week supplementation of freeze-dried blueberry powder (~392 mg anthocyanins) resulted in a trend of increased alpha diversity in older women (age: 65-77 years, n = 6) but not in younger women (age: 21-39, n = 11) [51]. Another study in overweight and obese adults found that 12-week supplementation with epigallocatechin-3-gallate and resveratrol impacted gut microbiota composition in men but not women [52]. Among the male participants, supplementation tended to reduce *Faecalibacterium prausnitzii* and significantly lowered Bacteroidetes, and increased post-prandial fat oxidation was significantly correlated to baseline Bacteroidetes abundance [52].

Interestingly, independent of factors such as age, sex, ethnicity, and general health, the human gut microbiome appears to naturally cluster in a few distinct patterns, or enterotypes, rather than on a continuous spectrum [53]. The existence of different enterotypes or metabotypes (nutrient-metabolizing phenotypes) may explain why human

clinical data has been inconclusive despite strong preclinical evidence; in some studies, the sample size may have been insufficient to overcome natural inter-individual variation among subjects. In fact, the potential effects of different gut microbiota metabotypes stratifying the human response to polyphenol supplementation has been previously demonstrated and is an on-going interest in polyphenol research [54–57].

In a study evaluating four weeks of pomegranate juice intake, no differences were observed in the microbiota composition from baseline to post-intervention, but the authors reported a correlation of specific intestinal microorganisms with changes in fecal phenolic metabolites [22]. The intestinal microbiota has a large functional metabolic capability to metabolize a variety of substrates and generate a variety of products [7]. Potentially, a beneficial change in host phenotype, as a result of the metabolism of the intestinal microbiome, may occur without requiring significant concomitant change in microbiota composition—especially in an adult with a stable and diverse microbiome. It may only be a matter of providing the right substrates in adequate amounts to observe health benefits, though this would likely require a much longer period (weeks to months) to see measurable changes in host phenotype [39].

Overall, our subjects had low levels of fecal calprotectin (< 16 μg/g), suggesting that they had very low intestinal inflammation regardless of the treatment. Mean fecal calprotectin was well below clinical levels—values lower than 50 μg/g are considered normal, and abnormal levels are 120 μg/g or higher, which are most often observed with serious intestinal conditions such as inflammatory bowel disease, Crohn's disease, and ulcerative colitis [58]. The flavonoid pre-workout drink did not appear to have an effect with subjects displaying low intestinal inflammation, which may be a reason why the

results from this study differed from previous work. In cases of low inflammation, a recent meta-analysis found that green tea supplementation did not have a significant effect on the inflammatory marker serum C-reactive protein (CRP) [59]. An important consideration when comparing findings from different studies is the study population and whether subjects have pre-existing conditions or are otherwise generally healthy. In many animal models, intestinal conditions or other pathologies are often the result of genetic modification, chemical induction, a selective diet (e.g., high fat), or some combination thereof. Approximately half of the clinical trials investigating the effects of polyphenol intake have been conducted in healthy subjects versus those with risk factors or disease [57]. In addition, another characteristic of our study population is that they were active and trained regularly. Exercise has been reported to have a positive effect on alpha diversity and the intestinal microbiota, and a six-week cycling protocol was found to increase *Akkermansia* abundance in a group of overweight women [60,61]. Elevated levels of the commensal microorganisms *Akkermansia*, *Lactobacillus*, and *Bifidobacterium* have been found in multiple observational studies to be associated with regular exercise [62].

Another reason why the observed results may have differed from previous studies is due to the amount of control over potentially confounding factors. Preclinical models are conducted in genetically identical animals, and experiments can be controlled for environmental, dietary, and other study conditions. For dietary intake, clinical studies can vary widely—from a controlled diet to a partially restricted diet to general diet counseling. Other clinical studies may be conducted in controlled settings, such as a hospital or nursing facility. The form of the dietary intervention (whether a supplement,

extract, or whole food) is also expected to have an effect; bioavailability of flavonoids differ depending on the food matrix as well as processing conditions [63]. In the Polyphenol Study as well as others evaluating dietary polyphenols intake, all study meals were provided for the treatment and control arms, reducing the potential variation introduced by diet-related factors [64,65]. An effective minimum dose, whether in supplement, extract, or whole foods, has yet to be established for flavonoids. Effective doses of dietary flavonoids versus those from a supplement or extract will likely differ. Higher fruit, vegetable, cocoa, coffee, and tea consumption will result in higher habitual polyphenol intake [66]. Whether or not additional flavonoid intake will have an effect in combination with a background diet high in dietary flavonoids is also unknown. Most clinical trials, including the present study, restrict high polyphenol foods and supplements during the course of the study. A few studies have evaluated the effect of supplemental flavonoid intake without imposing diet restrictions, and in one study, positive flavonoid-mediated effects were still observed [22,67].

A limitation of the present study which was mentioned earlier is the sample size. The analyses presented here were conducted in conjunction with the study as described in Chapter III, which was statistically powered for the primary endpoints of intestinal permeability and injury. While the appropriate within-subject comparisons were conducted, a larger sample size may have been necessary to detect significant changes in intestinal microbiota, given the large inter-individual variation known to exist [39]. This variability demonstrates the value of a crossover design for microbiome-related studies, and a strength of this study is the randomized, controlled, crossover design allowing for within-subject comparison.

In conclusion, we investigated the effects of a high flavonoid supplement containing blueberry, green tea, and cocoa in a randomized, controlled clinical trial. The purpose for conducting fecal SCFA, intestinal microbiome, and fecal calprotectin analyses was to determine the localized effects of supplemental flavonoid intake on intestinal health. We found no differences in a group of twelve generally healthy, amateur cyclists. We did not observe consistent changes among subjects or a significant change within a subject, but our results are consistent with previous work suggesting that short-term supplementation does not overcome inter-individual variation. Instead, long-term dietary habits appear to have the greatest impact on the microbial community. Our understanding of the interaction between the gut microbiota and human health is still lacking, and this relationship may even differ from one individual to the next. It is important to determine how flavonoid and polyphenol intake can affect gut health, which can influence future nutritional guidelines and further understanding of personalized nutrition.

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CHAPTER V

FLAVONOID SUPPLEMENTATION AND PLASMA METABOLOMICS

Introduction

Metabolomics is a comprehensive analysis used to identify and quantify all detectable small cellular metabolites (substrates, intermediates, products) present in a biological system, providing a high-resolution snapshot of the active biochemical processes at a given point in time [1,2]. Compared to the traditional use of individual biomarkers, metabolomics analyses are thought to better capture the biological impact of genetic or environmental changes, such as with disease, physical exercise, or a nutritional intervention [3]. Initially, metabolomics analyses were "targeted", involving the quantitation of specific molecules of interest using either an internal or external standard [2]. In the last decade, rapid technological advances in high-throughput methods, instrument sensitivity, and data processing have enabled the development of "untargeted" metabolomics to detect all compounds present in a sample—potentially hundreds or thousands of molecules in a single analysis [2].

In general, the three most commonly used analytical platforms for metabolomics are gas chromatography-mass spectrometry (GC-MS), liquid chromatography-MS (LC-MS), and nuclear magnetic resonance (NMR) spectroscopy [4]. Of these, MS techniques are more sensitive and more frequently employed than NMR [5,6]. In comparison to LC-MS, GC-MS has a greater number of standardized protocols, open-access availability to mass spectral deconvolution software, and a larger catalogue of published retention indices and reference spectra for compound identification. Thus, GC-MS-based

techniques are well-suited for resolving co-eluting compounds in complicated biological samples and continues to be the gold standard for metabolomics [2]. The drawback of GC-based metabolomics analyses is that it requires that analytes are volatile—meaning that samples containing non-volatile compounds (including the primary metabolites found in human plasma) require chemical derivatization prior to analysis [2]. Fortunately, many options for derivatization are available, and trimethylsilylation, the most commonly used method, can be performed under mild conditions with relatively high yield [5]. This derivatization replaces the acidic proton in carboxylic acids, amines, thiols, and hydroxides with a trimethylsilyl (TMS) group, and following derivatization, GC-MS analysis can detect a wide range of metabolites including sugars, amino acids, lipids, alcohols, catecholamines, and xenobiotics [2,5]. Generally, GC-MS is preferable for the separation and identification of sugars and hydroxyl acids, but for larger metabolites, including flavonoids and complex lipids, LC-MS methods are better suited for analysis [2]. As a result, these complementary methods are ideally utilized together to obtain greater coverage in metabolomics analyses [2].

Alongside advances in the capabilities of metabolomics profiling, research groups have begun to explore the effects of exercise on the human metabolome [1,6]. Large shifts in over 200 metabolites have been observed with exercise, particularly with longer duration and higher intensity exercise [1,3]. During exercise, cellular metabolism ramps up to supply up to ten times more adenosine triphosphate (ATP) than at rest to meet increased workload demands [6]. Depending on substrate availability, this involves the mobilization and utilization of glycogen, triglyceride, and amino acid stores [6]. Studies have consistently reported significant changes in lipids (e.g., plasma medium and long

chain fatty acids, fatty acid oxidation products, ketone bodies, and triglycerides), amino acids, bile acids, and tricarboxylic acid cycle intermediates with exercise [1,6].

An ongoing interest in sports nutrition research is in dietary supplements that complement and support exercise performance and recovery. Among these dietary supplements of interest are fruit-derived flavonoids for their potential ergogenic effects (for further discussion, see Chapter II, Section 3.4) [7]. Flavonoids are a diverse class of plant secondary metabolites, and the number of research publications involving these phytochemicals has increased rapidly in the last two decades [8]. This is due in part to the observation that diets abundant in flavonoid-containing fruits and vegetables have consistently been associated with a reduced risk of cardiometabolic disease and overall mortality [9–12]. The molecular basis for these effects was initially attributed to the high free-radical scavenging activity of flavonoids *in vitro* [13]. However, polyphenols (a class of phytochemicals of which flavonoids are the largest subgroup) are highly metabolized through a coordinated effort by both host and microbiota [14]. In general, with the exception of flavan-3-ols, native flavonoids are glycated, which results in low absorption in the small intestine and increases flavonoids exposed to the colonic microflora [15]. Except in the proximal intestinal tract where native polyphenols may reach high enough concentrations to potentially act as radical scavengers, the current consensus is that dietary flavonoids are not acting as direct antioxidants *in vivo* [13]. Instead, a single compound can be transformed into an array of different metabolites, and these metabolites are considered to be the main bioactive agents driving the physiological effects observed as a result of flavonoid consumption, and may even display greater bioactivity than their precursor parent compounds [7,16–18]. In a cell culture model,

various flavonoid metabolites (protocatechuic acid, 4-hydroxybenzoic acid, vanillic acid, benzoic acid-glucuronide, benzoic acid-sulfate, protocatechuic acid-3-glucuronide) were shown to reduce LPS-induced TNF-α production to a greater extent than their precursor flavonoids [18].

Microbiome-mediated effects have been found to be highly variable among individuals, likely due to the large microbial diversity from person to person [13]. However, the human intestinal microbiome is also characterized by functional redundancy, where different members can fill the same function (e.g., butyrate production) within the microbial community [19]. Characterization of the functional "metabolome" produced by the microbiome may be more important than the effects of inter-individual variation in microbiome diversity and abundance. Metabolomics analysis over a series of time points may allow better understanding of the dynamic changes occurring as a result of a nutritional intervention. Significant changes in microbiome-derived phenolic compounds have been detected in circulation following flavonoid intake. Several studies conducted by Nieman et al. have explored the effects of fruit and fruit-derived flavonoid consumption on the exercise metabolome [1,7,20–23]. In one that study utilized both ultrahigh performance LC-tandem MS (UHPLC-MS/MS) and GC-MS, elevated serum levels of the gut phenolic metabolites hippurate and 4-methylcatechol sulfate were detected after a 3-day intensive exercise workload with 17-day blueberry and green tea intake (equivalent to 3 cups of fresh blueberries and 1.3 cups of brewed green tea) [20]. However, no differences in oxidative stress or inflammation were observed between the polyphenol treatment and control [20]. In a 75-km cycling trial comparing banana consumption versus a 6% carbohydrate drink, GC-MS-based

metabolomics analysis showed that 56 out of 103 metabolites changed significantly with exercise, and only one metabolite (dopamine) was different between the banana treatment and carbohydrate control [21]. In another study comparing banana, pear, and a water control, cycling time improved 5.0% and 3.3% on the banana and pear treatments, respectively [22]. Banana and pear intake improved perceptual measures of performance (energy, focus, and overall well-being) and reduced post-exercise measures of inflammation (IL-10, cortisol, total leukocytes) [22]. Furthermore, UHPLC-MS/MS-based metabolomics showed that banana and pear ingestion elevated carbohydrate, fruit-specific, and phenolic compounds, while attenuating the exercise-induced metabolite response in lipid mobilization and oxidation pathways [22]. In a follow-up study, two different varieties of banana (Cavendish and mini-yellow) were compared with a 6% carbohydrate drink and water during a 75-km cycling trial [23]. (Mini-yellow bananas have a similar carbohydrate profile but 63% higher polyphenol content than Cavendish bananas [23].) Both banana treatments resulted in significant UHPLC-MS/MS-detected metabolite differences in amino acid and xenobiotic pathways from the water and carbohydrate controls; the water-only trial also resulted in a significantly greater exercise perturbation in the metabolite profile as compared to the carbohydrate and banana trials [23].

Fruit-derived polyphenols appear to have a beneficial effect on exercise performance and recovery, and metabolomics studies have shown potential in determining phenolic and other metabolite responses to flavonoid intake [3,7]. While some exercise studies have incorporated metabolomics analyses, more work is still needed to better understand the interaction between flavonoid consumption and the

exercise metabolome [3]. A few different modes of exercise have been employed (cycling, running, swimming) with different supplementation schemes (acute and chronic), ranging from "whole food" interventions to purified polyphenols [3]. In the present study, we investigated changes in plasma metabolites following 15-day consumption of either a high- or low-flavonoid pre-workout beverage (HFB and LFB, respectively) in plasma samples from the randomized, controlled crossover trial described in Chapter III. Plasma samples were collected before exercise, following exercise, and during the subsequent recovery period to determine metabolite shifts over time with either treatment. The purpose of this study was to conduct an untargeted GC-MS-based metabolomics analysis to investigate the effect of flavonoid ingestion on the plasma metabolome, in order to better direct future recommendations for exercise nutrition. We hypothesized that flavonoid metabolites would be detected in the plasma following HFB consumption, and that plasma metabolites associated with exercise (e.g., glucose, lactate, energy metabolism intermediates, and lipids) would demonstrate the greatest extent of change from baseline to immediately post-exercise.

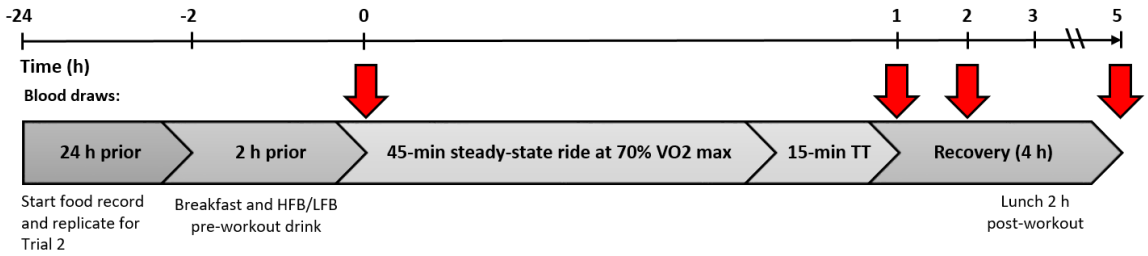
Materials and Methods

Research design

This study was conducted as a part of the randomized clinical trial as described previously in Chapter III. The study was approved by the USU institutional review board (IRB Protocol #9255). Participants were informed of all study requirements and provided written informed consent prior to enrollment. A complete list of inclusion and exclusion criteria is provided in Appendix A, Table A-1.

In brief, twelve cyclists completed this crossover study with two fifteen-day treatment arms separated by a two-week washout period. During the intervention, subjects consumed a pre-workout beverage containing blueberry, cocoa, and green tea flavonoids (~490 mg total flavonoids) or a low flavonoid control (< 5 mg flavonoids) daily. On Day 14, participants recorded their evening meal in a provided food journal and continued recording all foods and beverages consumed through the end of the final sample collection the following day. Participants were instructed to replicate all food and beverage intake for the second intervention. Additionally, participants were instructed to abstain from alcohol and strenuous activity on Day 14. On Day 15, research participants completed a 1-hour cycling trial (45-min at 70% VO_2max + 15-min TT) and provided four blood samples: pre-exercise, post-exercise, 1-h post-exercise, and 4-h post exercise (Figure 5-1). Seated venous blood samples were collected via antecubital venipuncture in potassium ethylenediaminetetraacetic acid (K_2EDTA)-treated Vacutainer tubes and centrifuged (1500 × *g*, 10 min, 4 °C). Plasma was aliquoted to 1.5-ml polypropylene microcentrifuge tubes and stored frozen at -80 °C until further analysis.

Figure 5-1. Schematic of study design and endpoint collection. HFB = high flavonoid beverage; LFB = low flavonoid beverage; TT = time trial.



Investigational product

The pre-workout supplement was designed to provide for the metabolic demands of high intensity endurance exercise [24,25]. A general recommendation for endurance exercise is 1-2 g carbohydrate (CHO) and 0.15-0.25 g protein (PRO) per kilogram of body weight consumed prior to activity [26]. The macronutrient composition of fluid bovine milk is particularly well-suited for this purpose, and dairy-derived protein is ideal for sports nutrition due to its ease of digestion and absorption, branched chain amino acid content, and protein quality [27]. As a result, the investigational product—prepared from blueberry, cocoa, and green tea powders and 2% milk—is formulated to supply 66 g CHO and 9 g PRO per serving.

One serving of the high flavonoid beverage (HFB) provided approximately 490 mg total flavonoids, while the low flavonoid beverage (LFB) contained approximately 4.6 mg flavonoids due to three modifications: alkalized cocoa, blueberry placebo powder, and the omission of green tea powder. The green tea extract contained approximately 800 mg/g tea catechins (PureBulk, Inc; Roseburg, Oregon). High flavonoid cocoa powder (83 mg/g cocoa flavanols) and alkalized cocoa (10 mg/g flavanols) were provided by Barry Callebaut (Zurich, Switzerland). Freeze-dried blueberry powder (14.0 mg/g total anthocyanins) and blueberry placebo powder were supplied by the US Highbush Blueberry Council (Folsom, California).

Plasma metabolomics

Methods for sample extraction and derivatization for gas chromatography-mass spectrometry (GC-MS) metabolomics profiling were adapted from Fiehn [2]. Sample extractions were prepared in duplicate. A method and derivatization blank was prepared

alongside the samples, but without the addition of plasma. For sample cleanup and extraction, 30 µL of plasma was mixed with 10 µL of 200 ppm ribitol (internal standard) and 1 mL of an acetonitrile, isopropanol, and water mixture (3:3:2, v/v/v) at -20°C. The mixture was shaken for 5 min, centrifuged at 14,000 relative centrifugal force (RCF) for 2 minutes. Next, 450 µL of supernatant was removed to a fresh container and evaporated to dryness in a centrifugal vacuum evaporator. The dried aliquot was re-suspended in 450 µL nitrogen-degassed acetonitrile/water (1:1, v/v), thoroughly mixed, and centrifuged at 14,000 RCF for 2 minutes. The supernatant was removed, evaporated to dryness, and stored at -20°C.

A reagent blank was prepared alongside the samples (without the addition of plasma) beginning from this point forward. Additionally, a quality control mixture of 25 sugars, amino acids, lipids, and other metabolites was prepared as well (Appendix C). Samples were derivatized with 20 µL methoxyamine hydrochloride in pyridine (20 mg/ml). Following the addition of methoxyamine, samples were shaken for 1.5 hours. Next, 80 µL of a mixture of N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) and fatty acid methyl esters (FAME) were added to each sample. Samples were then shaken for another 30 min and then submitted to GC-MS analysis.

The GC-MS platform was a Shimadzu single quadrupole gas chromatograph mass spectrometer (GCMS-QP2010 SE) with a Zebron ZB-5MSi plus (30 m × 0.25 mm diameter × 0.25 µm film thickness) capillary GC column (Phenomenex, Torrance, CA, USA). Helium was used as the carrier gas, and the column flow rate was 1.18 ml/min, with splitless injection and 5.5 min solvent cut time. The temperatures of the inlet, ion

source, and transfer line were 250 °C, 230 °C, and 290 °C, respectively. The column oven temperature program used for compound separation is shown in Table 5-1.

Table 5-1. GC oven temperature program for plasma metabolomics.

Rate (°C/min)	Temperature (°C)	Hold Time (min)
--	60	1
10	325	10

Compound peaks were selected by visual comparison of the chromatograms from the samples and method and reagent blanks, and features unique to the plasma samples were selected for identification. Peaks were deconvoluted with the automatic mass spectral deconvolution and identification system (AMDIS), identified with mass spectral and FAME retention index library comparison, and confirmed with the National Institute of Standards and Technology (NIST) mass spectral database. A quality control (QC) standard mixture of selected sugars, amino acids, lipids, and other small metabolites was also used for identification of these compounds. Peak areas were determined, and multiple peaks representing the same compound—isomers and those formed from incomplete TMS derivatization—were grouped into single features. Compounds determined to be artifacts (e.g., polysiloxanes from moisture introduced during derivatization, phthalates from plastics, derivatization reagent by-products, and EDTA from the collection tubes) and FAME were omitted from the final compound table.

Statistical analysis

The final peak intensities table was uploaded to MetaboAnalyst 5.0 and analyses were completed using the online platform [28]. Missing values were assumed to be below the limit of detection and were imputed with 1/5 of the compound minimum value.

Compound peak areas were normalized by the ribitol internal standard, log-transformed, and auto-scaled (mean-centered, divided by the standard deviation of each feature). A two-way repeated measures ANOVA was conducted to determine differences in normalized, scaled metabolite abundance, with the FDR adjusted q-value to take into account the multiple comparisons across metabolites. Statistical significance was set at $q < 0.05$. Log2-transformed within-subject fold changes immediately post-exercise, 1 h post-exercise, and 4 h post-exercise (relative to pre-exercise) were determined from the ribitol-normalized peak intensities. Post-hoc testing with Tukey's HSD was conducted for metabolites with a significant main effect; post-hoc tests were performed with JMP version 15.2.1 for Windows (SAS Institute, Cary, NC) with significance at $\alpha = 0.05$.

Results

Participant characteristics

Twelve subjects (female n = 4, male n = 8; mean ± SD: age 37 ± 11 years, body mass 78 ± 13 kg, height 1.74 ± 0.09 m, VO_2max 43 ± 6 ml/kg/min) completed both arms of the study. Both trials elicited a similar response in physiological and perceptual measures (Table 5-2).

Physiological and perceptual responses to exercise

The exercise test elicited a similar change in heart rate (HR), rating of perceived exertion (RPE), and core temperature regardless of the treatment (Table 5-2). Expired air samples collected during the steady state exercise confirmed that cyclists were cycling at ~70% VO_2max. Performance measures (time trial cycling distance and cadence) were not significantly different between trials (Table 5-2).

Table 5-2. Cycling trial: physiological and perceptual measures

	HFB	**LFB**	**HFB-LFB**
Final core temperature (°C)	38.53 ± 0.06	38.51 ± 0.23	0.02 ± 0.20
HR_{max} (bpm)	174 ± 4	175 ± 5	-1 ± 2
RPE	4.0 ± 0.4	3.9 ± 0.5	0.08 ± 0.3
TT distance (km)	19.3 ± 0.7	18.9 ± 0.8	0.4 ± 0.3
Cadence (rpm)	93 ± 3	93 ± 3	0 ± 2

HR = heart rate, bpm = beats per minute, RPE = rating of perceived exertion, TT = time trial, rpm = revolutions per minute.

Untargeted plasma metabolomics

Several hundred features were detected in each sample, but many were determined to be artifacts (contaminants or extraction/derivatization reagents), indistinguishable from the background signal, or unidentifiable due to lack of a suitable library match. Manual inspection of chromatograms yielded 92 metabolites in the plasma samples that differed from the derivatization and reagent controls. Data cleaning involved condensing metabolites with multiple peaks, removing artifacts, and peak alignment of the compound table across all 96 samples. A total of 62 metabolites were tabulated, with 42 identified metabolites and 20 unknown compounds. We identified eight carbohydrates, sixteen amino acids, twelve lipids, two cofactors and vitamins, two energy metabolites, and one xenobiotic. Annotated metabolites are listed in Table 5-3, along with their FAME retention indices (RI), retention times (RT), compound identifiers, and associated metabolic pathways.

Principle component analysis (PCA) showed that the samples grouped together according to time, with less distinction between treatments (Figure 5-2). The PC1, PC2, and PC3 axes explained 17.1%, 14.6%, and 10.2% of the total data set variance, respectively. There appeared to be a larger separation for the metabolome at time 5 than

at all other times, and a slight separation with the pre-exercise metabolome (time 0). Metabolites immediately post-exercise (time 1) and 1-h post exercise (time 2) appeared to have some separation but were grouped more closely together than other times.

The heatmap generated with Euclidean distance measure and Ward clustering algorithm is shown in Figure 5-3. Sample groups A-H and sub-groups 1-16 were designated based on the clusters generated. Groups and sub-group designations are also included in Table 5-3 along with the general classification of metabolite super and sub pathways. Group A metabolites include the ketone body 3-hydroxybutyrate along with four long chain fatty acids (14:0, 16:0, 18:1, 18:2n6); overall, these lipids were lower pre-exercise compared to immediately post-exercise, with mixed responses at later times. Carbohydrates and energy metabolism-associated compounds (group B) were elevated immediately post-exercise and lower 1-h and 4-h post-exercise. A few different metabolite classes are represented in group C with relatively higher abundance immediately post-exercise and 1-h post-exercise for myo-inositol and citrate. Seven unknown compounds were grouped in D with the lipids palmitelaidic acid, α-tocopherol, and cholesterol with most metabolites being more abundant at times 1 and 2 compared to 0 and 5. Interestingly, metabolites in groups E and F appear to be consistently elevated at time 5, 4-h post-exercise. A few different metabolite classes are represented in Group G, and these metabolites appear to be most abundant pre-exercise. Finally, Group H consists of eleven amino acids and related compounds, in addition to an unknown, carbohydrate, and vitamin/cofactor metabolite.

The two-way repeated measures ANOVA (log-transformed and auto-scaled values) showed that 56 of 62 total features were significant with respect to time (FDR-

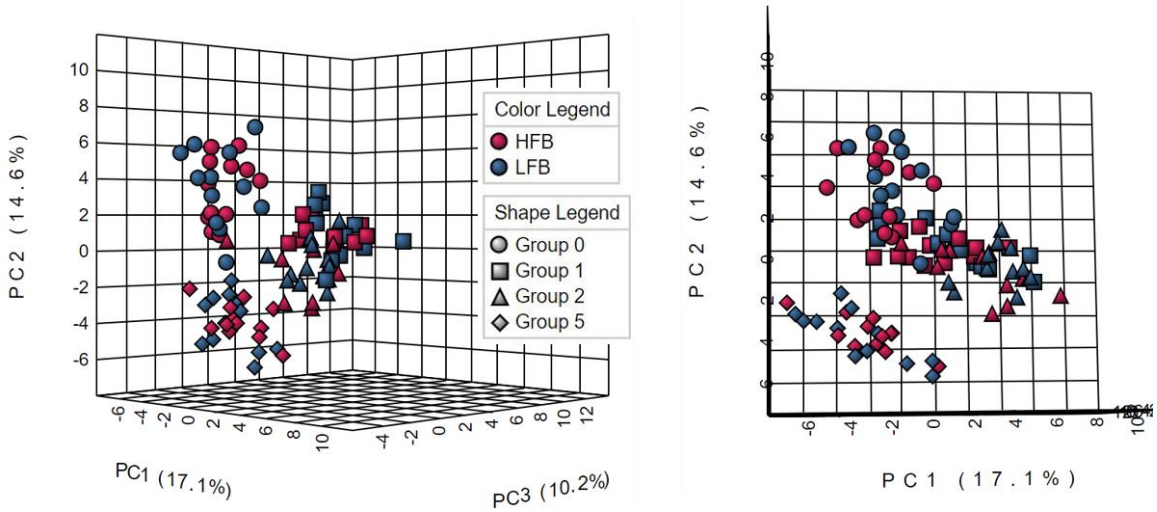
adjusted q-value < 0.05) (Appendix C, Table C-1). Metabolites without significant time effects were aspartate, benzoate, glycolic acid, 2-hydroxy-3-methylpentanoic acid (HMVA), methionine, and unknown 9. No significant treatment effects or treatment by time interactions were observed for any of the metabolites.

Of particular interest were select metabolites from three super pathways expected to change most with exercise: compounds involved in glycolysis and energy metabolism, lipids and ketone bodies, and amino acids. The response over time for a few selected compounds are shown in Figure 5-4. Since only the time effect was significant, values plotted are HFB and LFB combined.

The log2-transformed mean fold changes from pre-exercise for these metabolites are reported in Tables 5-4 through 5-6. Increases and decreases in abundance relative to pre-exercise is reflected in values > 0 and < 0, respectively. Since only the main effect of time was significant, differences determined from post-hoc tests are indicated for overall fold changes and not for either treatment individually.

Figure 5-2. Three-dimensional PCA of untargeted metabolomics analysis colored by treatment (A) and time (B).

(A)



(B)

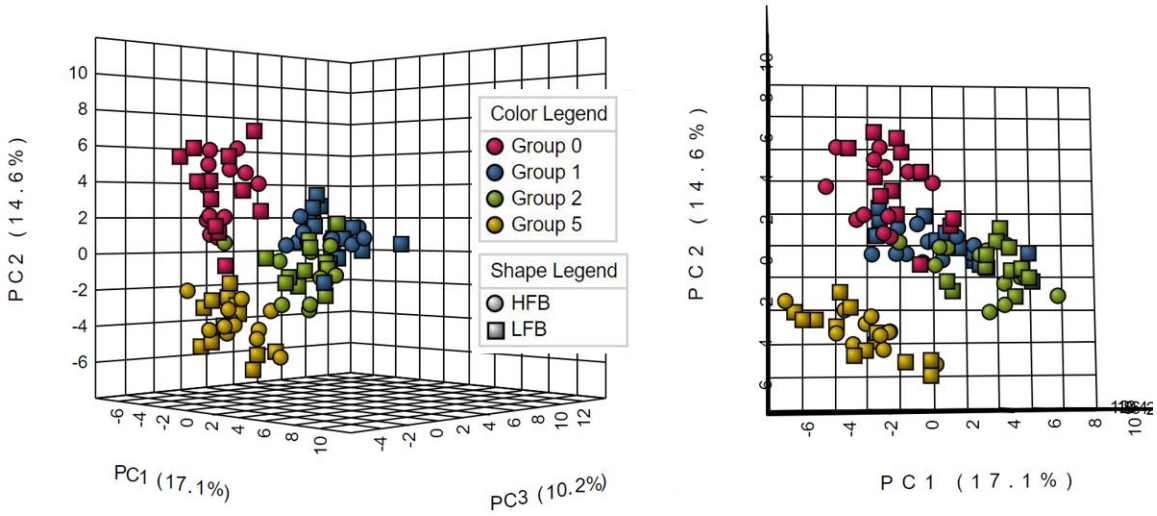


Figure 5-3. Heatmap visualization of plasma metabolites grouped by time, then treatment. Samples are arranged in columns. Darker red indicates higher abundance and darker blue indicates lower abundance.

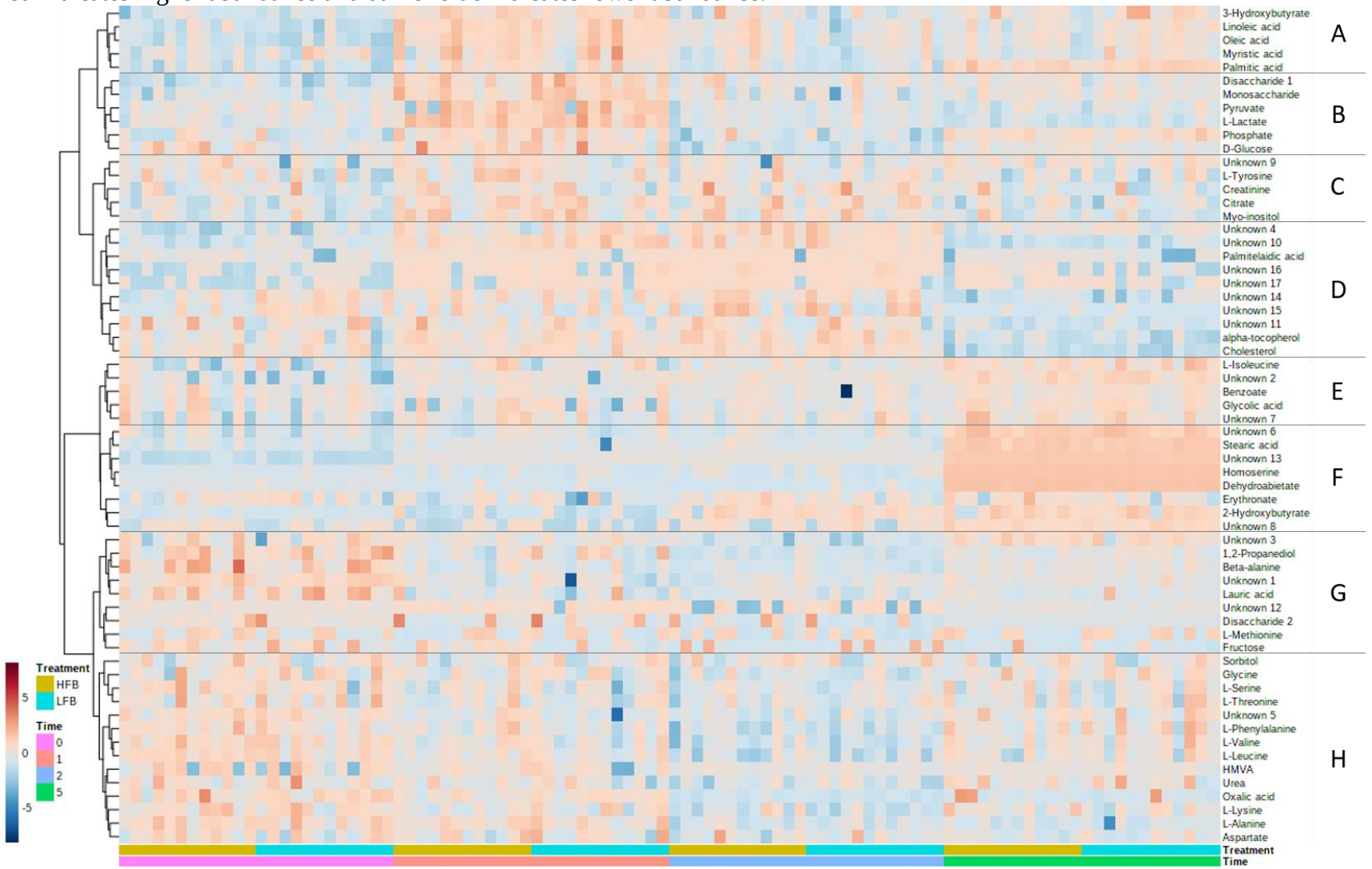


Table 5-3. Identified plasma metabolites: retention index (RI), retention time (RT), Human Metabolome Database (HMDB) number, PubChem identifier, Kyoto Encyclopedia of Genes and Genomes (KEGG) identifier, super and sub pathway association.

Metabolite	RI	RT	HMDB	PubChem	KEGG	Group	Sub-group	Class/Super pathway	Sub pathway
3-Hydroxybutyrate (BHBA)	1169	8.420	HMDB0000357	441	C01089	A	1	Lipid	Ketone bodies
Linoleic acid C18:2n6	2212	20.390	HMDB0000673	5280450	C01595	A	2	Lipid	Long chain fatty acids
Oleic acid C18:1	2217	20.442	HMDB0000207	445639	C00712	A	2	Lipid	Long chain fatty acids
Myristic acid C14:0	1847	16.870	HMDB0000806	11005	C06424	A	2	Lipid	Long chain fatty acids
Palmitic acid C16:0	2044	18.850	HMDB0000220	985	C00249	A	2	Lipid	Long chain fatty acids
Unknown disaccharide 1	1983	18.259				B	3	Carbohydrate	Disaccharides and oligosaccharides
Unknown monosaccharide	2058	18.977				B	3	Carbohydrate	Monosaccharides
Pyruvate	1063	6.843	HMDB0000243	1060	C00022	B	4	Carbohydrate	Glycolysis, gluconeogenesis, pyruvate metabolism
L-Lactate	1076	7.041	HMDB0000190	61503	C00186	B	4	Carbohydrate	Glycolysis, gluconeogenesis, pyruvate metabolism
Phosphate	1285	10.095	HMDB0001429	57424078	C00009	B	4	Energy	Oxidative phosphorylation
D-Glucose	1932 1950 2006	17.745 17.937 18.486	HMDB0000122	5793	C00221	B	4	Carbohydrate	Glycolysis, gluconeogenesis, pyruvate metabolism
Unknown 9	1864	17.047				C	5		
L-Tyrosine	1896	17.390	HMDB0000158	6057	C00082	C	5	Amino acid	Tyrosine metabolism
Creatinine	1571	13.790	HMDB0000562	588	C00791	C	6	Amino acid	Creatine metabolism Biogenic amine
Citrate	1839	16.790	HMDB0000094	311	C00158	C	6	Energy	Tricarboxylic acid cycle
Myo-inositol	2127	19.623	HMDB0000211	-	C00137	C	6	Carbohydrate	Galactose metabolism
Unknown 4	1469	12.537				D	7		
Unknown 10	2160	19.926				D	7		
Palmitelaidic acid trans-16:1	3934	32.240	HMDB0012328	5282745	-	D	7	Lipid	Long chain fatty acids
Unknown 16	3599	30.050				D	7		
Unknown 17	3925	32.165				D	7		

Table 5-3 continued. Identified plasma metabolites: retention index (RI), retention time (RT), Human Metabolome Database (HMDB) number, PubChem identifier, Kyoto Encyclopedia of Genes and Genomes (KEGG) identifier, super and sub pathway association.

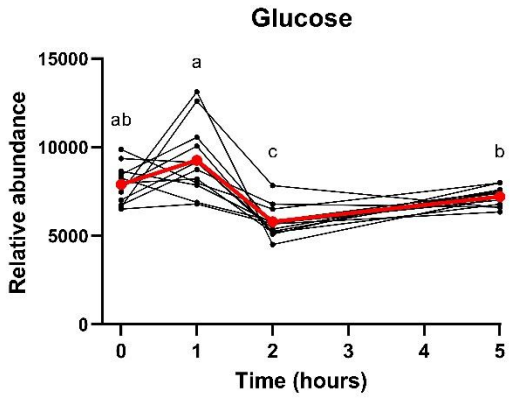
Metabolite	RI	RT	HMDB	PubChem	KEGG	Group	Sub-group	Class/Super pathway	Sub pathway
Unknown 14	2785	24.933				D	8		
Unknown 15	2820	25.191				D	8		
Unknown 11	2248	20.691				D	8		
α-tocopherol	3168	27.515	HMDB0001893	14985	C02477	D	8	Cofactors and vitamins	Tocopherol metabolism
Cholesterol	3198	27.707	HMDB0000067	5997	C00187	D	8	Lipid	Sterol
L-Isoleucine	1184	8.634	HMDB0000172	6306	C00407	E	9	Amino acid	Leucine, isoleucine, valine metabolism; Glucogenic/ketogenic
Unknown 2	1422	11.930				E	9		
Benzoate	1253	9.630	HMDB0001870	243	C00539	E	10	Xenobiotic	Benzoate metabolism
Glycolic acid	1090	7.249	HMDB0000115	757	C03547	E	10	Lipid	Hydroxy fatty acid
Unknown 7	1696	15.253				E	10		
Unknown 6	1553	13.567				F	11		
Stearic acid C18:0	2241	20.666	HMDB0000827	5281	C01530	F	11	Lipid	Long chain fatty acids
Unknown 13	2593	23.513				F	11		
Homoserine	1687	15.147	HMDB0000719	12647	C00263	F	11	Amino acid	Methionine, threonine, isoleucine
Dehydroabietic acid	2410	22.077	HMDB0061925	-	-	F	11	Lipid	-
Erythronate	1578	13.874	HMDB0000613	2781043	-	F	12	Carbohydrate	Aminosugar metabolism
2-Hydroxybutyrate	1138	7.960	HMDB0000008	11266	C05984	F	12	Amino acid Lipid	Glutathione metabolism Ketone bodies
Unknown 8	1705	15.341				F	12		
Unknown 3	1433	12.028				G	13		
1,2-Propanediol	1018	6.183	HMDB0001881	1030	C02912	G	13	Carbohydrate	Glycolysis, gluconeogenesis, pyruvate metabolism
Beta-alanine	1436	12.113	HMDB0000056	239	C00099	G	13	Nucleotide Amino acid	Pyrimidine metabolism, uracil containing
Unknown 1	1152	8.173				G	13		
Lauric acid C12:0	1663	14.727	HMDB0000638	3893	C02679	G	13	Lipid	Free fatty acids

Table 5-3 continued. Identified plasma metabolites: retention index (RI), retention time (RT), Human Metabolome Database (HMDB) number, PubChem identifier, Kyoto Encyclopedia of Genes and Genomes (KEGG) identifier, super and sub pathway association.

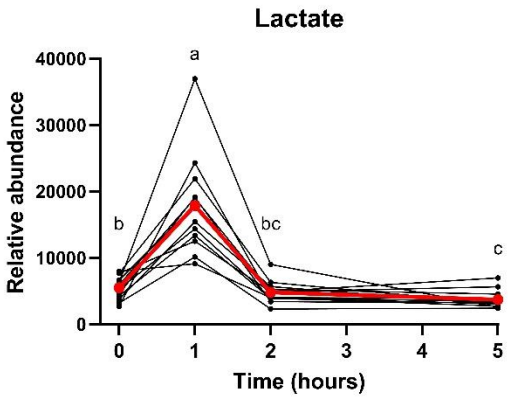
Metabolite	RI	RT	HMDB	PubChem	KEGG	Group	Sub-group	Class/Super pathway	Sub pathway
Unknown 12	2378	21.810				G	14		
Unknown disaccharide 2	2758	24.753				G	14	Carbohydrate	Disaccharides and oligosaccharides
L-Methionine	1416	11.838	HMDB0000696	6137	C00073	G	14	Amino acid	Methionine, cysteine, SAM, and taurine metabolism; Glucogenic
Fructose	1904 1918	17.455 17.61	HMDB0000660	439709	C02336	G	14	Carbohydrate	Fructose, mannose, and galactose metabolism
Sorbitol	1874	17.166	HMDB0000247	5780	C00794	H	15	Carbohydrate	Fructose, mannose, and galactose metabolism
Glycine	1317	10.528	HMDB0000123	750	C00037	H	15	Amino acid	Glycine, serine, threonine metabolism; Glucogenic
L-Serine	1264 1370	9.803 11.253	HMDB0000187	5951	C00065	H	15	Amino acid	Glycine, serine, threonine metabolism; Glucogenic
L-Threonine	1302 1398	10.323 11.626	HMDB0000167	6288	C00188	H	15	Amino acid	Glycine, serine, threonine metabolism; Glucogenic/ketogenic
Unknown 5	1494	12.856				H	16		
L-Phenylalanine	1554 1639	13.58 14.585	HMDB0000159	6140	C00079	H	16	Amino acid	Phenylalanine metabolism; Glucogenic/ketogenic
L-Valine	1099 1226	7.386 9.233	HMDB0000883	6287	C00183	H	16	Amino acid	Leucine, isoleucine, valine metabolism; Glucogenic
L-Leucine	1163 1280	8.343 10.016	HMDB0000687	6106	C00123	H	16	Amino acid	Leucine, isoleucine, valine metabolism; Ketogenic
2-Hydroxy-3-methylpentanoic acid (HMVA)	1189	8.769	HMDB0000317	10796774	-	H	16	Lipid/Amino acid	Hydroxy fatty acid; Product of ketogenic amino acid metabolism
Urea	1247	9.515	HMDB0000294	2447	C00086	H	16	Amino acid	Urea cycle; Arginine and proline metabolism
Oxalic acid	1141	8.015	HMDB0002329	971	C00209	H	16	Cofactors and vitamins	Ascorbate and aldarate metabolism
L-Lysine	1937	17.809	HMDB0000182	5962	C00047	H	16	Amino acid	Lysine metabolism; Ketogenic
L-Alanine	1115	7.621	HMDB0000161	5950	C00041	H	16	Amino acid	Alanine and aspartate metabolism; Glucogenic
L-Aspartic acid	1539	13.402	HMDB0000191	5960	C00049	H	16	Amino acid	Alanine and aspartate metabolism; Glucogenic

Figure 5-4. Plasma metabolites pre- and post-exercise. Overall mean is plotted in red, black lines are individual subjects. Since treatment effects were not significant, overall means are plotted for HFB and LFB combined, at each time. Different letters indicate significantly different groups (Tukey's HSD).

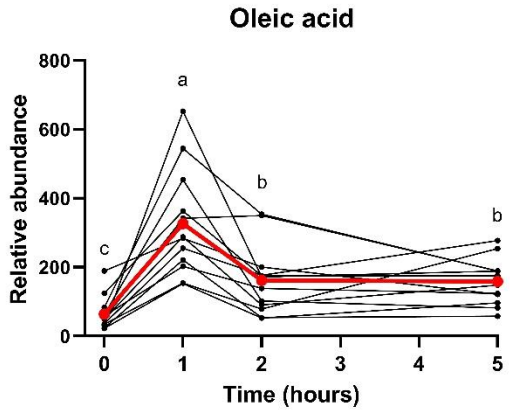
(A)



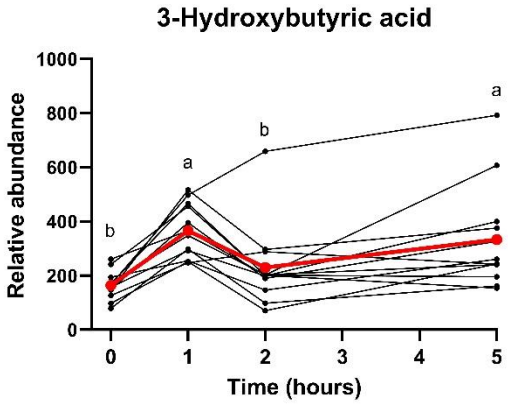
(B)



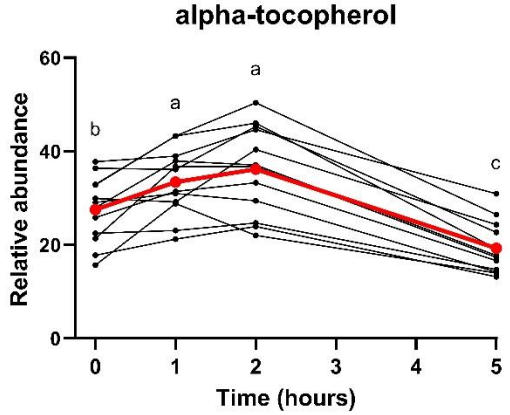
(C)



(D)



(E)



(F)

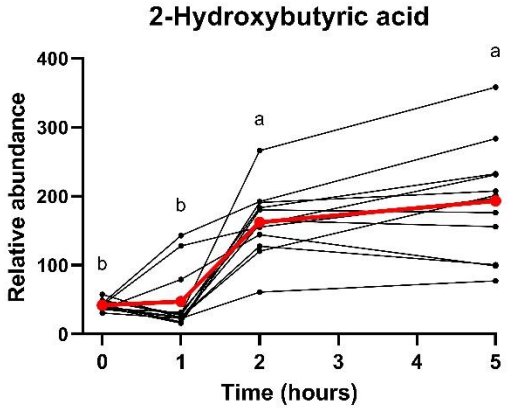


Figure 5-4 continued. Plasma metabolites pre- and post-exercise. Overall mean is plotted in red, black lines are individual subjects. Since treatment effects were not significant, overall means are plotted for HFB and LFB combined, at each time. Different letters indicate significantly different groups (Tukey's HSD).

(G) (H)

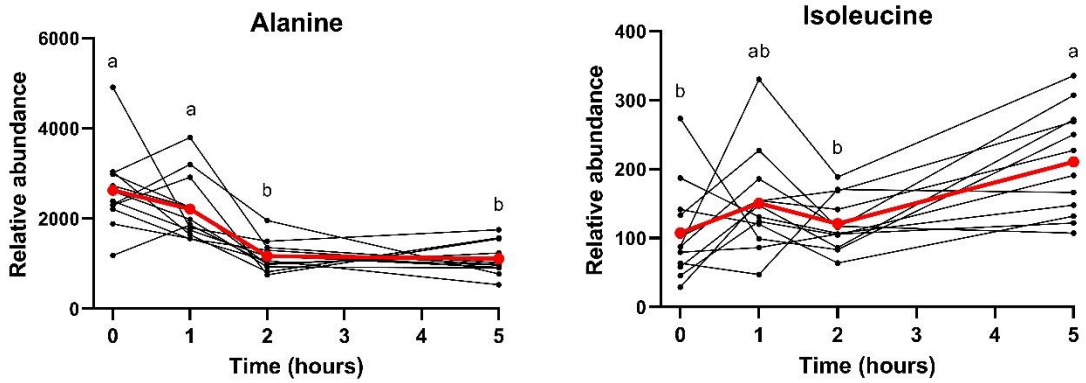


Table 5-4. Fold changes from baseline (immediately, 1 hour, and 4 hours post-exercise) in glycolysis and energy metabolites.

	Sub pathway	Time	Overall	HFB	LFB	HFB-LFB
Glucose	Glycolysis, gluconeogenesis, pyruvate metabolism	Post-ex	0.25	0.10	0.34	-0.24
		1 h post-ex	-0.40	-0.60	-0.26	-0.34
		4 h post-ex	-0.08	-0.24	0.04	-0.28
Lactate	Glycolysis, gluconeogenesis, pyruvate metabolism	Post-ex	1.76	1.66	1.75	-0.08
		1 h post-ex	-0.14	-0.23	-0.12	-0.11
		4 h post-ex	-0.50	-0.63	-0.48	-0.16
Pyruvate	Glycolysis, gluconeogenesis, pyruvate metabolism	Post-ex	0.92	0.63	1.04	-0.41
		1 h post-ex	-0.18	-0.22	-0.16	-0.06
		4 h post-ex	-0.23	-0.34	-0.20	-0.13
Citrate	Tricarboxylic acid cycle intermediate	Post-ex	0.47	0.42	0.50	-0.08
		1 h post-ex	0.35	0.27	0.40	-0.13
		4 h post-ex	0.04	0.00	0.08	-0.08

Data are log2-transformed values. Post-hoc testing: overall values lsmeans significant differences from pre-exercise determined by Tukey's HSD are highlighted.

Table 5-5. Fold changes from baseline (immediately, 1 hour, and 4 hours post-exercise) in lipid-related metabolites.

	Sub pathway	Time	Overall	HFB	LFB	HFB-LFB
Lauric acid (12:0)	Medium chain fatty acid	Post-ex	-0.61	-0.49	-0.89	0.40
		1 h post-ex	-0.60	-0.50	-0.89	0.39
		4 h post-ex	-0.58	-0.56	-0.73	0.17
Myristic acid (14:0)	Long chain fatty acid	Post-ex	0.72	0.76	0.55	0.20
		1 h post-ex	0.06	0.15	-0.27	0.41
		4 h post-ex	0.27	0.12	0.22	-0.10
Palmitic acid (16:0)	Long chain fatty acid	Post-ex	0.81	0.74	0.80	-0.06
		1 h post-ex	0.22	0.21	0.15	0.06
		4 h post-ex	1.15	1.00	1.22	-0.22
Stearic acid (18:0)	Long chain fatty acid	Post-ex	0.24	0.27	-0.01	0.28
		1 h post-ex	0.23	0.22	0.17	0.05
		4 h post-ex	1.79	1.71	1.81	-0.11
Oleic acid (18:1)	Long chain fatty acid	Post-ex	2.67	2.26	2.83	-0.57
		1 h post-ex	1.49	1.29	1.48	-0.19
		4 h post-ex	1.66	0.95	1.99	-1.04
Linoleic acid (18:2)	Long chain fatty acid	Post-ex	2.34	1.98	2.51	-0.52
		1 h post-ex	1.02	0.93	0.87	0.06
		4 h post-ex	1.43	0.72	1.76	-1.04
3-Hydroxybutyrate (BHBA)	Ketone body	Post-ex	1.24	1.33	1.08	0.25
		1 h post-ex	0.39	0.59	0.06	0.53
		4 h post-ex	0.99	0.83	0.96	-0.13
α-tocopherol	Vitamin	Post-ex	0.38	0.28	0.39	-0.11
		1 h post-ex	0.47	0.38	0.48	-0.10
		4 h post-ex	-0.43	-0.44	-0.54	0.10
2-Hydroxybutyrate	Glutathione metabolism	Post-ex	-0.21	-0.37	-0.31	-0.05
		1 h post-ex	1.95	1.73	1.99	-0.26
		4 h post-ex	2.10	1.81	2.15	-0.33

Data are log2-transformed values. Post-hoc testing: overall values lsmeans significant differences from pre-exercise determined by Tukey's HSD are highlighted.

Table 5-6. Fold changes from baseline (immediately, 1 hour, and 4 hours post-exercise) in selected amino acids.

	Sub pathway	Time	Overall	HFB	LFB	HFB-LFB
Alanine	Glucogenic amino acid	Post-ex	-0.16	-0.22	-0.25	0.03
		1 h post-ex	-1.10	-1.19	-1.11	-0.08
		4 h post-ex	-1.18	-1.20	-1.35	0.15
Glycine	Glucogenic amino acid	Post-ex	-0.07	-0.05	-0.13	0.08
		1 h post-ex	-0.31	-0.25	-0.41	0.17
		4 h post-ex	0.01	0.00	-0.03	0.03
Serine	Glucogenic amino acid	Post-ex	-0.31	-0.39	-0.37	-0.03
		1 h post-ex	-0.48	-0.58	-0.44	-0.14
		4 h post-ex	-0.24	-0.40	-0.20	-0.20
Valine	Glucogenic amino acid	Post-ex	-0.31	-0.28	-0.37	0.09
		1 h post-ex	-0.71	-0.69	-0.75	0.06
		4 h post-ex	-0.23	-0.29	-0.21	-0.09
Isoleucine	Glucogenic and ketogenic amino acid	Post-ex	0.91	0.87	0.51	0.36
		1 h post-ex	0.68	0.58	0.43	0.15
		4 h post-ex	1.50	1.17	1.37	-0.19
Phenylalanine	Glucogenic and ketogenic amino acid	Post-ex	-0.14	-0.15	-0.16	0.01
		1 h post-ex	-0.43	-0.49	-0.40	-0.09
		4 h post-ex	-0.03	-0.16	0.06	-0.22
Tyrosine	Glucogenic and ketogenic amino acid	Post-ex	0.26	0.18	0.30	-0.12
		1 h post-ex	0.12	-0.06	0.22	-0.28
		4 h post-ex	-0.01	-0.21	0.11	-0.32
Leucine	Ketogenic amino acid	Post-ex	-0.54	-0.70	-0.46	-0.24
		1 h post-ex	-1.37	-1.63	-1.34	-0.29
		4 h post-ex	-0.53	-0.90	-0.41	-0.49
Lysine	Ketogenic amino acid	Post-ex	-0.34	-0.60	-0.31	-0.29
		1 h post-ex	-1.09	-0.91	-1.46	0.56
		4 h post-ex	0.30	-0.28	-0.67	0.38

Data are log2-transformed values. Post-hoc testing: overall values lsmeans significant differences from pre-exercise determined by Tukey's HSD are highlighted.

Discussion

In the present study, we investigated the plasma metabolome before and after a bout of cycling exercise. A well-controlled exercise trial was conducted in a laboratory

setting during which participants cycled for 45 minutes at a fixed intensity (70% VO_2max), followed by a 15-minute time trial. Food intake beginning in the evening prior to the trial through the end of the recovery period was recorded by the participant and replicated during the second arm. Study aims were two-fold: to investigate the plasma metabolome pre- and post-exercise and to determine if flavonoid-derived metabolites can be detected from pre-workout flavonoid intake. In the discussion that follows, samples collected immediately (time 1), one hour (time 2), and four hours (time 5) post-exercise are considered early, intermediate, and late recovery phase samples, respectively [6].

The PCA and heatmap visualization showed metabolome differentiation with respect to time and not treatment, despite our hypothesis that the HFB and LFB would differentially impact metabolism. Metabolite profiles clustered distinctly according to time with the greatest separation in the late recovery phase than at any other time—several metabolites like homoserine only appeared at time 5. This may be due to the longer length of time between times 2 and 5, as well as the result of lunch and snacks consumed during this time. In terms of food intake, the participants consumed breakfast and the HFB/LFB two hours prior to sampling at time 0, a standardized sugar solution after sampling at time 1, and lunch/snacks were allowed between times 2 and 5. Breakfast, lunch, and snacks were individually-determined and replicated in the second trial. Nonetheless, individual differences in food intake are not likely to have changed the overall trends observed in the plasma metabolome as a whole, since food intake does not impact metabolic flux to the same extent as a bout of exhaustive endurance exercise [29].

The heatmap generated with the clustering algorithm grouped the metabolites into eight clusters based on similarities in their responses. Reviewing the associated metabolic

super and sub pathways, metabolites involved in similar pathways clustered together in the analysis. Overall, these metabolite patterns are consistent with the findings from a systematic review of exercise metabolomics studies: early phase post-exercise increases in carbohydrates and energy metabolism metabolites (lactate, pyruvate, TCA intermediates), a large elevation in lipids, and a mixed response in amino acids [6].

Large fold changes were observed in lactate, pyruvate, and citric acid immediately following exercise. Lactate, as a product of anaerobic glycolytic metabolism, has historically been used as an indicator of endurance capacity at a given exercise intensity [30]. High levels of pyruvate, in addition to lactate, accumulate as a result of glycolysis and anaerobic metabolism [6]. The time course profile of lactate and pyruvate (elevated immediately post-exercise, followed by a decline to baseline after an hour of recovery), reflect the responsiveness of these metabolic pathways to the energy demands of exercise. Several different pathways contribute to glucose flux: glycogenolysis and gluconeogenesis in the liver increase glucose availability while glucose uptake increases in muscle tissues through insulin-independent mechanisms [31,32]. Following cessation of exercise, glucose uptake continues to be elevated during recovery, which reduces circulating glucose and may explain the reduction in plasma glucose observed after one hour of recovery [33]. The TCA intermediate citrate was elevated during the early and intermediate phases of recovery but returned to baseline after four hours of recovery. Other than citrate, we did not detect any other TCA cycle intermediates (e.g., malate, aconitate, fumarate, succinate, and α-ketoglutarate), though they would be expected to respond similarly.

Endurance exercise elicits a strong response in circulating free fatty acids and ketone bodies as alternative fuel sources to glucose [6]. During prolonged moderate intensity exercise, lipids are mobilized for energy production, and lipid metabolism contributes a considerable amount to energy production through β-oxidation and ketone body formation [29]. Nieman et al. found that metabolites involved in lipolysis and lipid oxidation are strong predictors of the metabolic response to intensive endurance exercise [34]. Similarly, all 37 fatty acids reported in the systematic review by Schranner et al. increased following exercise [6]. In the present study, the greatest fold changes of all metabolites were observed in lipid-related compounds. In particular, long chain free fatty acids (palmitic, stearic, oleic, and linoleic) and the ketone body 3-hydroxybutyrate displayed large fold increases and sustained responses to exercise. This may demonstrate that lipolysis and lipid oxidation was increased as a result of exercise and continued to be pronounced during the late recovery phase. Prior data for responses of ketone bodies is somewhat mixed, but they more often appear to increase following exercise. Ketone bodies are synthesized from ketogenic amino acids and acetyl CoA as alternative fuel for the brain and muscle tissues during long-duration exercise when carbohydrates become limited [35]. Other lipid metabolites (triacylglycerols, dicarboxylate and monohydroxy fatty acids, acylcarnitines, and other ketone bodies) were not detected or identified in the analysis.

The patterns and directions of change for amino acids following exercise are not as consistent across studies in comparison to lipids, but in general, lower amino acid concentrations are reported following exercise [6]. Amino acids are in constant flux through multiple and even opposing metabolic pathways, depending on their metabolic

potential and sub pathway association. As glycogen stores are depleted and β-oxidation pathways become saturated during prolonged exercise, glucogenic and ketogenic amino acids may be transformed to glucose and ketone bodies, respectively, to provide the necessary substrates for continued energy metabolism [6,36]. The majority of the detected amino acids decreased relative to baseline with the exception of isoleucine, which was elevated ~3.7-fold at time 5. In the heatmap visualization, most proteinogenic amino acids clustered together in group H while isoleucine was in group E. This is somewhat surprising since isoleucine is a branched chain amino acid (BCAA) along with leucine and valine, but the reason for the difference in response to exercise is unclear. Aside from isoleucine, the fold changes of the amino acids were relatively small. Carbohydrate intake, such as with the pre-workout HFB and LFB, will reduce the rate of gluconeogenesis and ketogenesis, which also reduces amino acid demand [6]. Thus, smaller responses in amino acid mobilization and depletion would be expected with food intake.

Following exercise, two different antioxidant-related metabolites were elevated. Antioxidants are necessary to maintain cellular redox balance and prevent oxidative damage to DNA, lipids, and proteins [37]. There was a strong response of 2-hydroxybutyrate at the intermediate and late recovery phases where the metabolite increased 4-fold and 4.6-fold, respectively. Elevated lipid β-oxidation, NADH/NAD+ imbalance, and oxidative stress following intensive endurance exercise causes 2-hydroxybutyrate to increase [38]. Glutathione is a major endogenous antioxidant that is primarily responsible for reducing lipid peroxidation, and 2-hydroxybutyrate is a byproduct of supplementary glutathione synthesis via homocysteine [37,39]. The

presence of 2-hydroxybutyrate at these later times suggests continued and elevated glutathione demand despite the cessation of exercise several hours prior, which demonstrates the longer lasting effects of submaximal endurance exercise on oxidative stress and lipid metabolism. In contrast, 2-hydroxybutyrate was elevated one hour following a sprint session (three 80-meter maximal sprints), but returned to baseline within 30 minutes [39]. Another contributor to the antioxidant response is α-tocopherol (vitamin E). An exogenous lipid-soluble antioxidant, α-tocopherol is notably elevated from baseline in the early and intermediate recovery phases (potentially due to exercise-associated lipolysis), but lower in the late phase, which may be due to increased cellular uptake [40]. Both the responses in 2-hydroxybutyrate and α-tocopherol suggest an increased demand for cellular antioxidants in the intermediate and late recovery stages—the antioxidant response to exercise remained elevated even four hours later.

These findings lead to a related discussion regarding the adaptive response to exercise and exogenous antioxidant supplementation. Exercise increases reactive oxygen species (ROS) production by several times, but the adaptive response to this stimuli (elevated ROS) includes the activation of endogenous antioxidant defense systems, resulting in the upregulation of enzymatic and non-enzymatic antioxidants [41]. Within skeletal muscle, exercise-induced metabolic stress results in additional positive adaptations by activating native anti-inflammatory mechanisms, protein turnover, mitochondrial biogenesis, and the heat shock protein response [42]. The exercise and hormesis theory presents the idea that both a lack of exercise and over-training can have negative effects, while some moderate volume of exercise with adequate recovery is beneficial [43]. With regular training, the adaptive response to exercise can increase

resting antioxidant enzyme levels and reduce oxidative stress following subsequent bouts of exercise [41]. Though it may seem advisable to decrease exercise-induced oxidative stress by increasing antioxidant intake, ingesting large dose antioxidant supplements during regular exercise training may be counterproductive. Large dose vitamin C and E supplementation has been reported to interfere with these cellular adaptations by scavenging ROS, thus blunting the ROS-activated adaptive response to exercise [42]. In fact, supra-physiological doses of vitamins C and E have been shown to cause negative effects and reduced performance. Vitamin C supplemented at 1 g/day reduced positive training adaptations to an 8-week exercise training program—supplementation reduced antioxidant enzymes (superoxide dismutase and glutathione peroxidase) and mitochondrial biogenesis in a group of sedentary men [44]. In contrast, flavonoid supplementation has not been shown to inhibit positive exercise adaptations to the same extent, and as opposed to vitamins C and E, circulating flavonoids and their metabolites do not behave as direct antioxidants [45]. For example, 30-day supplementation with lychee fruit extract containing catechins and proanthocynaidins improved running time to exhaustion and the subjects' mean anaerobic threshold, whereas a combination of vitamins C and E resulted in a reduction in VO_2max [46]. In another study comparing vitamin C (1 g/day) and blackcurrant juice (300 mg anthocyanins/day) intake, oxidative markers (catalase, superoxide dismutase, protein carbonyls) were elevated following vitamin C supplementation but not the blackcurrant or placebo [47]. Finally, in a randomized controlled crossover trial evaluating seven day supplementation of 150 g blueberries, 1250 mg vitamin C, and a placebo, only the blueberry treatment reduced lipid hydroperoxides following a treadmill run at 70% VO_2max at 35°C, while no benefit

was observed with vitamin C [48]. Both blackcurrants and blueberries are high dietary sources of anthocyanins, a sub-class of flavonoids [13].

In the present study, we did not observe any performance benefits or differences in antioxidant-related metabolites due to the HFB treatment in comparison to the LFB control. Flavonoids exhibit anti-inflammatory and anti-oxidative effects, likely through downregulation of NF-κB signaling and the activation of the Nrf2/ARE pathway, respectively (see Chapter II, Section 3.4) [49]. The number of publications on flavonoids and their effects on human health and disease continues to grow [50]. Still, specifics regarding optimal flavonoid intake (minimum effective dose, supplement timing with exercise, and length of supplementation required) to have measurable changes on various outcome measures are still unknown [3]. In studies where the dietary intervention was provided chronically, it is difficult to ascertain whether beneficial effects on exercise were due to the last dose or an effect due to a cumulative effect of chronic intake [49]. Since the intestinal microbiota plays an essential role in flavonoid bioavailability and transformation, more time may be required for the effects of dietary flavonoid intake to be reflected physiologically [16].

The metabolomics analysis did not reveal any host- or microbiota-derived flavonoid metabolites in the plasma. Previously, metabolites of cocoa flavan-3-ols, tea catechins, and berry anthocyanins have been identified in blood and urine [20,22,51–54]. These studies relied on the use of LC-MS, HPLC, and UPLC to separate and detect flavonoid and phenolic metabolites, with a few using a combination of both GC-MS and LC-MS. Gas chromatography is a reliable method for simultaneous measurement of sugars, amino acids, and medium to long chain fatty acids, as was shown in this study.

We observed strong effects of exercise on the metabolome but were unable to capture nutritional differences. Unfortunately, GC methods are not optimized for separating larger metabolites, and the use of multiple analytical methods may be justified in order to cover broader range of compounds—including phenolic and flavonoid metabolites. However, this is often constrained by cost and limited sample volume for conducting multiple analyses. Ideally, multiple extraction conditions, derivatization agents, and analytical platforms could be employed to ensure greater coverage of the spectrum of metabolites as each method can be optimized for a specific class of compounds.

Another challenge encountered in this study was with compound identification. While metabolomics is excellent for profiling global changes, a common challenge is that hundreds of features may be detected in a sample, with limited resources for compound identification [4]. For example, in another study, 238 metabolites were detected with a combination of LC-MS and GC-MS, of which 75 remained unidentified [55]. In this study, initial untargeted analysis yielded over 300 metabolites, which proved to be a major challenge in data cleaning and compound identification. An extensive catalog of purified standards is required for identification and quantification, but this requires significant dedication of time and resources [4].

Overall, metabolomics analysis of small molecules in biological samples is a powerful method for profiling the metabolic response to an intervention. In this study, untargeted GC-MS-based metabolomics following metabolite trimethylsilylation was used to profile the whole array of small molecular metabolites in plasma. The metabolic perturbation following a one hour, submaximal cycling trial had a different response during the early, intermediate, and late phases of recovery. Metabolites involved in

similar pathways, such as long chain fatty acids, responded in a similar manner. Multiple classes of compounds (sugars, free fatty acids, and amino acids) changed in different directions in response to endurance exercise, suggesting that multiple fuel substrate pathways are activated to varying degrees at different times in exercise and recovery. Changes in energy metabolites, sugars, lipids were consistent with previous literature. Though some metabolites like lactate and pyruvate responded rapidly to exercise and returned to baseline during the intermediate recovery phase, several others, many unidentified, were relatively low but increased significantly in the late recovery period. As a result, it may be interesting to explore at later times following exercise, and future work could include an additional measurement 24 hours post-exercise. This would be especially relevant for detecting more microbiome-derived metabolites, as they take longer to appear in circulation. In addition, the responses of the antioxidant-related metabolites 2-hydroxybutyrate and α-tocopherol are interesting, and it may be worthwhile in future work to include measures of endogenous antioxidant mechanisms such as glutathione peroxidase, superoxide dismutase, and oxidized/reduced glutathione. Finally, depending on the resources available, future studies may find that the benefits of utilizing multiple analytical platforms and methods could outweigh the costs.

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CHAPTER VI

CONCLUSION

The body of work on dietary flavonoids in health and nutrition continues to grow, and emerging clinical evidence is promising [1,2]. The primary activities of flavonoids are anti-inflammatory, antioxidative, and vasoprotective—both in the GI tract and systemically [3]. Due to these potential bioactive effects, flavonoid intake is of interest in exercise nutrition research [4]. In the case of exercise-induced GI syndrome, transient changes in intestinal permeability, injury, and inflammation can negatively impact athletic performance and recovery [5]. Thus, the purpose of this project was to investigate the local and systemic effects of supplemental flavonoid intake in combination with submaximal cycling exercise. Two additional methodologies contributed to this project: studies of the intestinal microbiome and circulating metabolome.

Methods in culture-independent microbiome analysis have advanced rapidly over the last two decades, and new discoveries are continually made in how the intestinal microbiome shapes human health and disease [6]. Specifically, the gut microbiome plays an essential role in the biotransformation and biological effects of flavonoids [7]. For example, anthocyanins were originally believed to have very low bioavailability (less than 1%), but bioavailability was later estimated to be closer to 12% after finding over 30 different metabolites derived from one isotopically-labelled anthocyanin, many attributable to the action of the gut microbiota [8]. Inter-individual variation in the gut microbiome also plays a significant role in flavonoid metabolism. For example, microbiota-determined metabotypes are thought to differentially metabolize pomegranate

ellagitannins, resulting in different downstream health effects related to cardiovascular risk [9].

Metabolomics profiling is a powerful tool in nutrition research that provides a comprehensive analysis of the metabolic response to dietary intake [10]. Polyphenols are structurally diverse, and one challenge in comparing different studies is that the flavonoid intervention can vary from purified compounds, to processed ingredients, to whole foods. Despite this variation, metabolomics allows for characterization of host- and microbiota-derived metabolites, which can be correlated to observed outcomes. Peripheral tissues are not likely to be exposed to native flavonoids but to conjugated and transformed metabolites instead, and the effects of these metabolites have been found to be quite different from those of the parent compounds [8]. Typical levels of polyphenols in the GI tract are in the μM/mM range while their metabolites in circulation are expected to be in nM/μM range, which should be taken into account when comparing flavonoid doses in cell culture, animal, and clinical studies [11].

Overall, the subjects' estimated average flavonoid intake reflected that of the US population, though total flavonoids were significantly lower than the national average due to lower flavan-3-ol intake. Daily supplementation of 490 mg blueberry, green tea, and cocoa flavonoids, while similar to the amount provided in other studies, may have increased daily intake by 1.5 to 35 times. The subjects had low intestinal inflammation, which perhaps overshadowed the effects, if any, of supplemental flavonoid consumption. No effects of the flavonoid intervention were found on the intestinal microbiota or SCFA—suggesting that supplemental flavonoids may not have the same effects as flavonoids consumed in fresh, whole foods.

To evaluate the effects of sub-chronic supplemental flavonoid intake, we conducted a double-blind, randomized, controlled crossover trial with twelve recreationally-active cyclists (VO_2max 43.2 ± 5.9 ml/kg/min). Following submaximal cycling exercise, specific metabolite patterns were observed with respect to time, resulting in clustering of samples collected pre-exercise, post-exercise, 1-h post-exercise, and 4-h post-exercise. Metabolites like pyruvate and lactate changed immediately post-exercise, while long chain fatty acids had a larger response over a longer period of time, and circulating amino acids decreased to a smaller extent post-exercise. Antioxidant-related compounds like 2-hydroxybutyrate were elevated even after four hours of recovery, suggesting a large demand for antioxidants following exercise. With the HFB, we observed a trend for cyclists exerting greater force during the time trial, though no measures of intestinal injury, inflammation, and permeability or plasma metabolites were statistically significant with respect to treatment.

One potential reason why no treatment effects were observed may be that the bioactive effects of flavonoids are relatively modest, requiring longer supplementation periods or larger sample sizes to detect a treatment effect [12]. Additionally, previous studies recruited subjects with pathological conditions, presumably with significantly greater inflammatory stress and redox imbalance, where flavonoid supplementation may improve these measures to a greater extent [13]. Another consideration is the degree of exercise intensity and stress elicited by the study design. Gastrointestinal distress is most commonly observed in ultra-endurance events, and studies demonstrating an elevation in core temperature greater than 39 °C always reported elevated intestinal permeability [14,15]. Moreover, exercise-induced oxidative stress is generally considered to be

beneficial, resulting in positive adaptations through ROS-dependent mechanisms [16]. Thus, flavonoid supplementation may only be necessary for cases of extreme oxidative imbalance and strenuous exercise—when ROS production overcomes native antioxidant capacity, when perfusion is inadequate to meet demand, or when delaying fatigue is critical during competition. Consequently, future studies should consider utilizing more strenuous trials. Although this may prove to be a challenge for subject recruitment and retention, other studies have successfully implemented such trials, and it may provide the best and most practical insight into the effect of flavonoid intake on athletic performance.

A strength of this study was that it was a well-controlled, crossover clinical trial seeking to address an unmet need. The flavonoid pre-workout product was well-received by study participants. The aim of the study was appropriate and practical, with potential findings that could affect a large population of both amateur and professional athletes. Additionally, this study applied multiple methods to characterize the physiological response to the dietary intervention by utilizing traditional biomarkers as well as -omics techniques. Though we observed no apparent benefit of the flavonoid pre-workout, many factors may have influenced these results, and previous studies demonstrating effects have been conducted with curcumin, fruits, and juices [17–21]. Further study is warranted, alongside microbiome and metabolomics analyses, if resources allow.

Ultimately, dietary flavonoid intake through the consumption of varied fruits and vegetables may be the best recommendation. A diverse and well-balanced diet supplying natural plant bioactives through whole foods is preferable to supplementation with single antioxidants [3]. Unsurprisingly, the effects of a diet rich in whole fruits and vegetables, which also contain other important nutrients (vitamins, minerals, dietary fiber) in addition

to polyphenols, appear to differ from those of a diet supplemented with isolated flavonoids or extracts. The beneficial effects reported in epidemiological studies may be partially due to other food components or all components acting synergistically; flavonoids have been shown to interact with the food matrix during digestion, which can alternately increase or decrease their bioavailability [22–24].

Finally, responses to nutritional changes are highly individualized. In the field of sports nutrition, tailoring to personal preferences is often recommended, and individuals are known to respond differently to nutritional intake. Some practical considerations are differences in food preferences, sport-specific needs, training schedules, and seasons. With advances in bioinformatics and high-throughput data processing, there is potential to develop individualized dietary recommendations. Characteristics such as microbiome enterotypes may predict an individual's response to nutrition, which leads to differential metabolite production, potentially explaining the varied responses within human clinical trials. Future studies should investigate these characteristics that determine individual responses and work towards developing personalized recommendations for improving human health and athletic performance.

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APPENDICES

Appendix A. Exercise-Induced Intestinal Injury, Permeability, and Inflammation

Figure A-1. Study recruitment diagram.

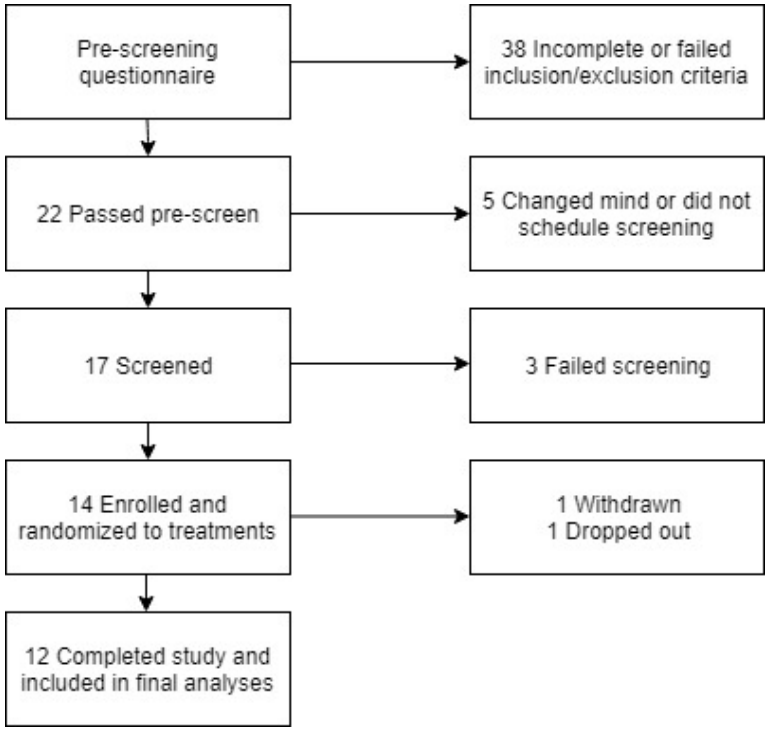


Figure A-2. Study recruitment materials.

Figure A-2 continued. Study recruitment materials.

Figure A-3. Guidelines provided to study participants for each intervention.

Instructions for Intervention:

Foods, supplements, and medication to avoid

Apples	Concord grapes	Red wine
Blueberries	Dark chocolate	Strawberries
Bilberries	Green, oolong, & black teas	Non-Dutch process (non-alkalized) cocoa
Blackberries	Raspberries	Flavonoid supplements (any kind)
Cranberries	Red cabbage	Non-steroidal anti-inflammatory drugs
Cherries	Plums	(e.g. aspirin, ibuprofen, Aleve, Tylenol)

Please check with us if you are unsure or have any questions.

You have been provided with the following:

- Blender bottle
- 14 servings of pre-workout mix
- A gallon of milk
- Intervention daily check-list

Notes:

- Please consume the pre-workout drink once a day during the intervention, approximately 2h prior to exercise. If you do not plan to exercise that day, take it 2h prior to your typical exercise time.
- Prepare the mix just before drinking (i.e., do not mix the dry powder and milk together until you are ready to drink it).

Directions:

1. Pour milk into the empty bottle with the metal mixer to the indicated fill line.
2. Empty the entire bag of dry powder into the bottle.
3. Taking care that the lid and opening are securely closed, shake to combine.
4. Enjoy! =)

Figure A-4. Schedule for clinic visits and assessments. For the food record: B – breakfast, L – lunch, D – dinner. One stool sample was collected during Days 9-11. On Day 15, blood draws were taken pre-, post-, 1 h post-, and 4 h post-exercise.

	Screening			**Intervention 1**														**Exercise Trial**	**Washout**	**Intervention 2**													**Exercise Trial**
Day	S1	S2	VO2 max Test	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	1-14	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Pre-workout drink				X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Body Weight	X																	X															X
Medical History	X																																
Blood Pressure	X																																
Stool Collection													X																X				
Sugar Probe Solution		X													X			X												X			X
Urine Collection		X													X			X												X			X
Food Record		L													L		D	B, L												L		D	B, L
Cycling Test			X															X															X
Core Temperature																		X															X
Blood Draw	X																	x4															x4

Figure A-5. Sample schedule for Day 15, the exercise trial.

Time	Cycling Trial
7:00 AM	Breakfast (recorded in food journal), HFB/LFB, and CorTemp pill
8:30 AM	Report to clinic with frozen urine and stool samples
8:40 AM	Blood draw and body weight measurement; HR monitor and adjust ergometer
8:55 AM	Warm-up at self-selected pace
9:00 AM	Exercise trial: 45 min 70% VO2max + 15 min time trial
10:05 AM	Blood draw; empty bladder, ingest sugar probe, and begin 6h urine collection
11:00 AM	Blood draw
12:00 PM	Lunch recorded in food journal
2:00 PM	Report to clinic for final blood draw
4:00 PM	End urine collection and freeze subsample
9:00 AM	Submit 24 hr adverse events/symptoms questionnaire

Figure A-6. Rating of perceived exertion (RPE) visual scale.

RPE	Description
0	complete rest
1	very, very easy
2	easy
3	moderate
4	somewhat hard
5	hard
6	
7	very hard
8	
9	
10	extremely hard (almost maximal)
———	exhaustion

Table A-1. Study inclusion and exclusion criteria.

Inclusion criteria
Male or female of any race or ethnicity between 18 to 55 years of age
Competed in a road race or triathlon in past 12 months
Free of chronic disease and GI conditions
Train at least 3 times per week, 1 hour at a time on average
Willing to prepare and consume provided pre-workout beverage daily
Willing to avoid consumption of high flavonoid foods/supplements and NSAIDs during study period
Willing to provide urine, stool, and blood samples

Table A-1 continued. Study inclusion and exclusion criteria.

Exclusion criteria
Age <18 or >55 years
Medical history of heart disease, hypertension, diabetes, Crohn's disease, IBS, colitis, celiac disease, inflammatory or autoimmune disease, and lactose intolerance
Uncontrolled hypertension: diastolic blood pressure >95 mm Hg or systolic blood pressure >160 mm Hg
For women: pregnancy, breast feeding or postpartum <6 months
Food allergies or restrictions to treatment/placebo beverages
Chronic use of NSAIDs
Consumption of flavonoid supplements <1 month prior to study start
Other conditions (medical, psychiatric, or behavioral) that may present a safety hazard to the participant or interfere with study participation, as determined by the principal investigators

Table A-2. Standard curves for sugar test probes.

Sugar	**Slope**	**Intercept**	**R^2**
Sucrose	0.0257	-0.0422	0.9996
Lactulose	0.0245	0.0355	0.9960
Sucralose	0.0249	-0.0017	0.9986

Appendix B. Gut Microbiota Profile, Short Chain Fatty Acid Excretion, and Intestinal Inflammation

Figure B-1. Adapted flavonoid FFQ self-administered by study participants.

Your 1-year dietary patterns

Your usual food intake: For each of the foods listed, check the box which shows your average intake over the past year. Pay particular attention to the amount for each food before you answer. Fill in only one circle per row.

Average over the whole year: ex. If you only eat cherries 4 times per week during summer (3 months), then your average intake over the year is once per week.

Vegetables	Never, or less than once per month	1-3 per month	1 per week	2-4 per week		1 per day	2-3 per day	4-5 per day	6+ per day
Avocado (½ cup or ½ fruit)									
Bean, green (½ cup)									
Beet (½ cup)									
Bell pepper (½ cup)									
Broccoli (½ cup)									
Brussels sprout (½ cup)									
Cabbage, green (½ cup)									
Cabbage, red (½ cup)									
Carrot (½ cup)									
Carrot juice (small glass)									
Cauliflower (½ cup)									
Celery (2-3 sticks)									
Eggplant (½ cup)									
Kale (½ cup)									
Lettuce, iceberg (1 cup)									
Lettuce, romaine (1 cup)									
Mushroom (½ cup)									
Olive (10 small)									
Onion, as garnish (1 slice)									
Onion, cooked (1 cup)									
Parsley garnish (1 sprig)									
Parsley salad (½ cup)									
Peas (½ cup)									
Rhubarb (½ cup)									
Swiss chard (1 cup)									
Spinach (1 cup)									
Sweet potato (½ cup)									
Tomato (2 slices)									
Tomato-based sauce (e.g. spaghetti) (½ cup)									
Zucchini (½ cup)									

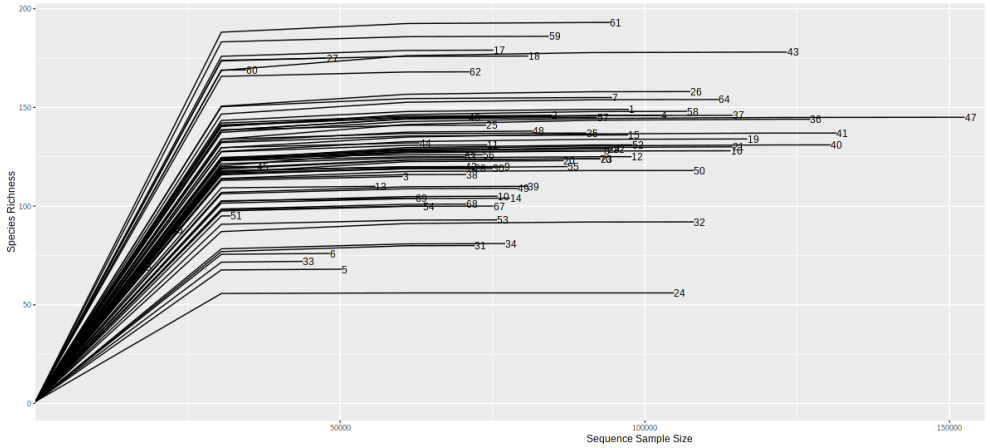
Figure B-1 continued. Adapted flavonoid FFQ self-administered by study participants.

Fruit	Never, or less than once per month	1-3 per month	1 per week	2-4 per week		1 per day	2-3 per day	4-5 per day	6+ per day
Apples (1 whole)									
Apple juice (small glass or 160 ml)									
Apricot (1 whole)									
Blueberries (½ cup)									
Cherries (½ cup)									
Grapes (½ cup)									
Grape juice (small glass or 160 ml)									
Grapefruit (½ fruit)									
Grapefruit juice (small glass or 160 ml)									
Lemon (1 slice)									
Lemon (juice from ½ fruit)									
Mandarin (1 whole)									
Mango (1 whole)									
Nectarine (1 fresh)									
Oranges (1 whole)									
Orange juice (small glass or 160 ml)									
Pears (1 whole)									

Beverages and Other	Never, or less than once per month	1-3 per month	1 per week	2-4 per week		1 per day	2-3 per day	4-5 per day	6+ per day
Chocolate, dark (5 squares or 20 g)									
Chocolate, milk (5 squares or 20 g)									
Cocoa (1 tsp)									
Coffee, brewed (small cup or 160 ml)									
Coffee, instant (1 tsp)									
Tea, black (small cup or 160 ml)									
Tea, green (small cup or 160 ml)									
Tomato juice (small glass or 160 ml)									
Vegetable juice (e.g. V-8) (small glass)									
Wine, red (glass or 160 ml)									
Wine, white (glass or 160 ml)									

Figure B-2. Rarefaction curves of raw (A) and normalized (rarefied to minimum library size and total sum scaling) data (B).

(A)



(B)

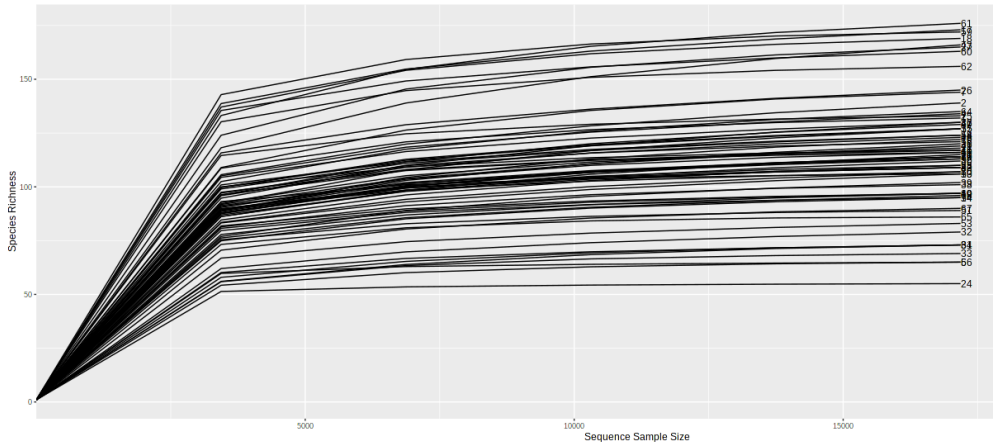


Table B-1. Estimated weekly intake of individual flavonoids.

Class	Flavonoid	Mean ± SEM (mg/week)
Flavonols	Isorhamnetin	7.94 ± 2.14
	Kaempferol	26.41 ± 5.34
	Myricetin	3.63 ± 1.41
	Quercetin	98.49 ± 16.79
Flavones	Apigenin	7.23 ± 2.16
	Luteolin	4.65 ± 0.86
Flavanones	Eriodictyol	0.93 ± 0.20
	Hesperetin	38.01 ± 7.54
	Naringenin	41.50 ± 9.15
Flavan-3-ols	(-)-Epicatechin	57.31 ± 11.42
	(+)-Catechin	22.87 ± 4.97
	(-)-Epicatechin 3-gallate	22.48 ± 16.20
	(-)-Epigallocatechin	36.87 ± 25.57
	(-)-Epigallocatechin 3-gallate	76.03 ± 54.14
	(+)-Gallocatechin	2.65 ± 1.90
	Theaflavin	1.46 ± 1.12
	Theaflavin-3, 3'-digallate	1.57 ± 1.20
	Theaflavin-3'-gallate	1.36 ± 1.04
	Thearubigins	73.65 ± 56.37
Anthocyanidins	Cyanidin	41.06 ± 15.15
	Delphinidin	17.88 ± 6.40
	Malvidin	29.23 ± 10.09
	Pelargonidin	0.05 ± 0.01
	Peonidin	8.59 ± 2.82
	Petunidin	12.81 ± 4.40

Table B-2. Wilcoxon signed rank sum test for flavonoid intake.

Parameter	Test Statistic	p-value	FDR-adjusted p-value
Flavonols	12.0	0.3804	0.5706
Flavones	13.0	0.3394	0.5706
Flavanones	-6.0	0.6772	0.8126
Flavan-3-ols	-36.0	0.0024	0.0144
Anthocyanidins	-1.0	0.9697	0.9697
Total	-34.0	0.0049	0.0147

Table B-3. Dependent t-test performed for fecal SCFA content.

Parameter	t ratio	p-value	FDR-adjusted p-value
Acetic acid	-0.60675	0.5563	0.8789
Propionic acid	-0.17074	0.8675	0.8789
Butyric acid	-0.69556	0.5011	0.8789
Isobutyric acid	-0.24649	0.8098	0.8789
Valeric acid	-0.22855	0.8234	0.8789
Isovaleric acid	-0.15598	0.8789	0.8789
Caproic acid	-0.3123	0.7607	0.8789
Total SCFA	-0.58098	0.5730	0.8789

Table B-4. Wilcoxon signed rank sum test performed for fecal calprotectin.

Parameter	Test statistic	p-value
Calprotectin	2.0	0.9097

Table B-5. *Firmicutes* to *Bacteroidetes* (F:B) ratio for each subject.

	HFB	LFB	HFB-LFB
101	2.15	8.05	-5.90
102	4.29	4.33	-0.04
103	133.40	141.46	-8.06
104	6.02	10.72	-4.70
105	2.50	3.25	-0.74
106	86.23	23.95	62.28
107	19.66	10.81	8.84
108	88.53	9.49	79.04
109	30.38	41.76	-11.38
111	3.83	35.89	-32.07
112	7.37	8.50	-1.13
113	6.90	2.73	4.17

Table B-6. Wilcoxon signed rank sum test performed for F:B.

Parameter	Test statistic	p-value
F:B	4.0	0.7910

Table B-7. Alpha diversity: Chao1

Taxa	Test statistic	p-value	FDR-adjusted p-value
Phylum	1.024	0.30995	0.91571
Class	0.8607	0.39306	0.91571
Order	0.29786	0.76682	0.91571
Family	-0.14726	0.88337	0.91571
Genus	0.23771	0.81282	0.91571
Species	0.10624	0.91571	0.91571
Feature-level	0.10624	0.91571	0.91571

Table B-8. Alpha diversity: Observed

Taxa	Test statistic	p-value	FDR-adjusted p-value
Phylum	0.99212	0.32518	0.9072
Class	0.90242	0.37049	0.9072
Order	0.86811	0.3888	0.9072
Family	0.30339	0.76258	0.93409
Genus	0.24628	0.80622	0.93409
Species	0.083003	0.93409	0.93409
Feature-level	0.083003	0.93409	0.93409

Table B-9. Alpha diversity: Shannon

Taxa	Test statistic	p-value	FDR-adjusted p-value
Phylum	1.1667	0.24744	0.91095
Class	0.56692	0.57265	0.91095
Order	0.65107	0.51722	0.91095
Family	0.30047	0.76474	0.91095
Genus	-0.37216	0.71101	0.91095
Species	-0.11229	0.91095	0.91095
Feature-level	-0.11229	0.91095	0.91095

Table B-10. PERMANOVA test for unweighted UniFrac distance, within-subject pairwise comparisons of HFB vs. LFB (n = 3).

Subject	pseudo-F	p-value	FDR-adjusted p-value
1	1.64	0.111	0.128
2	2.48	0.092	0.128
3	1.74	0.111	0.128
4	1.64	0.090	0.128
5	1.24	0.101	0.128
6	1.78	0.118	0.128
7	1.58	0.100	0.128
8	1.24	0.228	0.232
9	1.66	0.324	0.329
11	1.18	0.191	0.196
12	2.60	0.102	0.128
13	1.65	0.118	0.128

Table B-11. Results for independent t-tests comparing individual and total SCFA from the Cycling Study (CS) and the Polyphenol Study (PO).

SCFA (mM/g)	CS	LFD (PO)	CS vs. LFD p-value*	HFD (PO)	CS vs. HFD p-value*
Acetic acid	61.3 ± 5.5	56.2 ± 3.8	0.425	44.4 ± 2.6	0.027
Propionic acid	20.9 ± 1.9	17.6 ± 1.4	0.154	12.5 ± 0.9	0.008
Butyric acid	20.7 ± 2.2	16.0 ± 1.1	0.101	13.2 ± 1.0	0.020
Isobutyric acid	2.2 ± 0.3	1.7 ± 0.2	0.154	1.5 ± 0.1	0.067
Valeric acid	2.6 ± 0.2	1.9 ± 0.2	0.021	1.6 ± 0.1	0.008
Isovaleric acid	3.1 ± 0.5	2.4 ± 0.2	0.232	2.1 ± 0.1	0.116
Caproic acid	0.7 ± 0.2	0.9 ± 0.2	0.456	0.6 ± 0.1	0.643
TOTAL	111.5 ± 8.5	96.7 ± 5.9	0.154	75.8 ± 4.5	0.008

Note: Samples from HFB and LFB were combined together since they were not statistically different.

LFD = Low flavonoid diet, HFD = High flavonoid diet. Values expressed are means ± SEM. *FDR-adjusted p-value for multiple comparisons.

Appendix C. Plasma Metabolites

Table C-1. Two-way repeated measures ANOVA for plasma metabolites.

	Treatment (F.val)	Treatment (raw.p)	Treatment (adj.p)	Time (F.val)	Time (raw.p)	Time(adj.p)	Interaction (F.val)	Interaction (raw.p)	Interaction (adj.p)
1,2-Propanediol	2.270	0.146	0.968	20.154	2.21E-09	5.47E-09	0.230	0.875	0.967
2-Hydroxybutyrate	0.004	0.949	0.981	56.918	2.76E-18	2.45E-17	0.246	0.864	0.967
3-Hydroxybutyric acid	0.065	0.801	0.974	24.787	7.41E-11	2.30E-10	1.820	0.152	0.967
alpha-tocopherol	0.068	0.796	0.974	48.512	1.11E-16	8.61E-16	0.676	0.570	0.967
Beta-alanine	1.454	0.241	0.968	22.489	3.82E-10	9.87E-10	0.567	0.639	0.967
Cholesterol	0.400	0.534	0.968	47.041	2.22E-16	1.37E-15	0.215	0.885	0.967
Citrate	0.008	0.929	0.981	30.053	2.29E-12	9.45E-12	0.420	0.739	0.967
Creatinine	0.269	0.609	0.968	9.344	3.13E-05	4.61E-05	1.547	0.211	0.967
Dehydroabietic acid	0.397	0.535	0.968	362.820	6.18E-41	1.91E-39	0.869	0.462	0.967
D-Glucose	3.089	0.093	0.968	23.617	1.69E-10	4.77E-10	1.832	0.150	0.967
Disaccharide 1 RI:1983	0.027	0.872	0.976	28.157	7.68E-12	2.80E-11	1.449	0.237	0.967
Disaccharide 2 RI:2758	0.027	0.871	0.976	4.188	8.94E-03	1.11E-02	0.850	0.471	0.967
Erythronate	1.472	0.238	0.968	4.110	9.80E-03	1.17E-02	0.533	0.661	0.967
Fructose	0.005	0.943	0.981	3.689	1.61E-02	1.82E-02	1.542	0.212	0.967
Glycine	0.291	0.595	0.968	9.474	2.74E-05	4.14E-05	0.561	0.642	0.967
Homoserine	0.002	0.965	0.981	184.590	4.95E-32	1.02E-30	0.196	0.898	0.967
L-Alanine	0.204	0.656	0.968	29.373	3.51E-12	1.36E-11	0.168	0.918	0.967
Lauric acid	0.128	0.724	0.974	15.778	7.70E-08	1.65E-07	1.236	0.304	0.967
Linoleic acid C18:2	1.736	0.201	0.968	31.899	7.32E-13	3.24E-12	2.430	0.073	0.967
L-Isoleucine	0.452	0.508	0.968	9.283	3.33E-05	4.80E-05	0.452	0.717	0.967
L-Lactate	0.204	0.656	0.968	95.312	6.09E-24	9.44E-23	0.104	0.958	0.967
L-Leucine	0.094	0.762	0.974	23.052	2.54E-10	6.84E-10	0.636	0.595	0.967
L-Lysine	0.624	0.438	0.968	9.807	1.96E-05	3.03E-05	1.475	0.230	0.967
L-Phenylalanine	0.175	0.679	0.968	13.848	4.19E-07	7.64E-07	0.975	0.410	0.967
L-Serine	1.035	0.320	0.968	5.922	1.22E-03	1.57E-03	0.286	0.835	0.967
L-Threonine	0.091	0.765	0.974	3.049	3.46E-02	3.83E-02	0.206	0.892	0.967
L-Tyrosine	1.722	0.203	0.968	4.028	1.08E-02	1.24E-02	1.366	0.261	0.967
L-Valine	0.341	0.565	0.968	27.564	1.13E-11	3.90E-11	0.462	0.710	0.967
Monosaccharide RI:2058	0.048	0.828	0.976	16.871	3.06E-08	6.78E-08	0.691	0.561	0.967

Table C-1 continued. Two-way repeated measures ANOVA for plasma metabolites.

	Treatment (F.val)	Treatment (raw.p)	Treatment (adj.p)	Time (F.val)	Time (raw.p)	Time(adj.p)	Interaction (F.val)	Interaction (raw.p)	Interaction (adj.p)
Myo-inositol	0.167	0.687	0.968	40.172	6.86E-15	3.27E-14	0.161	0.922	0.967
Myristic acid	0.065	0.801	0.974	8.898	4.94E-05	6.96E-05	1.103	0.354	0.967
Oleic acid C18:1	1.093	0.307	0.968	44.751	6.69E-16	3.77E-15	2.148	0.103	0.967
Oxalic acid	0.023	0.882	0.976	17.712	1.53E-08	3.65E-08	0.785	0.507	0.967
Palmitelaidic acid	2.756	0.111	0.968	8.347	8.76E-05	1.21E-04	1.374	0.258	0.967
Palmitic acid C16:0	1.499	0.234	0.968	47.338	1.93E-16	1.33E-15	0.646	0.588	0.967
Phosphate	0.230	0.636	0.968	24.857	7.06E-11	2.30E-10	0.345	0.793	0.967
Pyruvate	1.205	0.284	0.968	12.297	1.74E-06	3.00E-06	0.387	0.763	0.967
Sorbitol	0.000	0.988	0.988	4.274	8.09E-03	1.02E-02	0.102	0.958	0.967
Stearic acid C18:0	3.887	0.061	0.968	60.558	6.29E-19	6.50E-18	0.593	0.622	0.967
Unknown 1 RI: 1152	0.329	0.572	0.968	11.877	2.59E-06	4.23E-06	0.640	0.592	0.967
Unknown 10 RI:2160	9.104	0.006	0.393	88.906	3.86E-23	4.79E-22	1.088	0.360	0.967
Unknown 11 RI:2248	0.251	0.621	0.968	14.348	2.68E-07	5.36E-07	0.534	0.660	0.967
Unknown 12 RI:2378	0.479	0.496	0.968	4.071	1.03E-02	1.20E-02	0.789	0.504	0.967
Unknown 13 RI:2593	0.594	0.449	0.968	833.830	2.20E-52	1.36E-50	0.187	0.905	0.967
Unknown 14 RI:2785	2.543	0.125	0.968	14.171	3.14E-07	6.08E-07	2.542	0.064	0.967
Unknown 15 RI:2820	0.730	0.402	0.968	8.022	1.23E-04	1.63E-04	2.639	0.057	0.967
Unknown 16 RI:3599	0.798	0.381	0.968	14.475	2.40E-07	4.95E-07	2.950	0.039	0.967
Unknown 17 RI:3925	0.319	0.578	0.968	8.276	9.44E-05	1.27E-04	1.307	0.280	0.967
Unknown 2 RI:1422	1.956	0.176	0.968	17.253	2.23E-08	5.12E-08	0.734	0.536	0.967
Unknown 3 RI:1433	0.324	0.575	0.968	14.049	3.50E-07	6.57E-07	0.088	0.967	0.967
Unknown 4 RI:1469	4.664	0.042	0.968	24.324	1.03E-10	3.03E-10	0.691	0.561	0.967
Unknown 5 RI:1494	0.823	0.374	0.968	10.494	9.87E-06	1.57E-05	1.110	0.352	0.967
Unknown 6 RI:1553	0.749	0.396	0.968	41.179	4.06E-15	2.10E-14	0.093	0.963	0.967
Unknown 7 RI:1696	1.550	0.226	0.968	4.107	9.84E-03	1.17E-02	0.315	0.815	0.967
Unknown 8 RI:1705	0.757	0.394	0.968	11.975	2.36E-06	3.96E-06	0.125	0.945	0.967
Urea	0.007	0.935	0.981	13.752	4.57E-07	8.10E-07	0.442	0.724	0.967

CURRICULUM VITAE

Stephanie Kung

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EDUCATION

Ph.D. in Food Science and Nutrition 2014-2021
Utah State University, GPA: 4.0

- Presidential Doctoral Research Fellow
- Graduate research and laboratory teaching assistant

B.S. in Chemical Biology, Nutritional Science Minor 2008-2012
University of California, Berkeley, GPA: 3.2

- Undergraduate research assistant in chemical ecology

EXPERIENCE

Utah State University 2014-2021
Clinical Study Coordinator

- Designed and coordinated a randomized controlled clinical trial, recruiting twelve athletes for a six-week crossover study
- Prepared research protocols and SOPs for clinic procedures and lab analyses
- Coordinated exercise testing, phlebotomy, and clinic visits with participants and research staff
- Specimen and data collection, processing, and analysis with ELISA, GC-FID, GC-MS, Illumina MiSeq

Graduate Research Assistant

- Training and mentoring undergraduate students and research assistants in laboratory safety and procedures
- Writing and updating lab SOPs

Teaching Assistant

- Food Chemistry lab course for undergraduate and graduate students for Fall 2015, 2016, 2019 semesters

Allied Laboratories 2012-2013
Clinical Science Laboratory Technician

- Accessioned and processed 450+ blood specimens daily for California Prenatal Screening Program analysis
- Performed daily instrument maintenance; prepared reagents, standards, and wash solutions

National Chung Hsing University 2011
Food Science and Biotechnology Research Intern

- Evaluated antioxidative activity of irradiated foods utilizing free-radical scavenging power, total polyphenols, total flavonoids, and reducing power assays

OTHER EXPERIENCE

USU Fall Student Research Symposium - Judge 2020
USU Spring Student Research Symposium - Judge 2018
IFTSA College Bowl Competition, Pacific Northwest Region 2018
Regional competition organization committee

- Drafted a budget and raised funds from sponsor companies, recruited judges, planned and hosted the regional competition

POSTER PRESENTATIONS

Kung, S, Hintze, K, Ward, R. 2020. Effect of a high flavonoid supplement on intestinal inflammation, SCFA, and the gut microbiome. Nutrition 2020 Annual Meeting.

Kung, S, Ward, R, Lefevre, M, Hintze, K. 2019. Effect of a high flavonoid pre-workout beverage on intestinal permeability and cycling performance. IFT & AACT's Utah Food and Candy Expo 2019.

Kung, S, Lefevre, M, Ward, R. 2016. Effect of a High Flavonoid Diet on Intestinal Short Chain Fatty Acid Production in Overweight and Obese Individuals. IFT & AACT's Utah Food and Candy Expo 2016.

COMPETITIONS

Dairy Product Development Competition (Idaho Milk Processors Association) 2015

- $10,000 grand prize awarded for Eureka! (a marinade-in-a-bag product featuring Greek yogurt whey)

Dairy Product Development Competition (Idaho Milk Processors Association) 2016

- $5,000 first prize for Pro2Go (a high protein frozen dairy dessert)

IFTSA College Bowl Competition – USU team member 2016

SKILLS

Laboratory

- Chromatography (GC, HPLC, TLC), GC-MS, UV-vis spectrophotometry, PCR, cell culture, ELISA, sample extractions, titrations

Computer

- Microsoft Office Applications (Word, Excel, Powerpoint), Windows OS, Agilent ChemStation, Shimadzu GC Solution, JMP statistical analysis, GraphPad Prism

COURSEWORK

- Biochemistry, Dairy Chemistry, Dairy Technology and Processing, Food Analysis, Food Chemistry, Food Proteins, Fundamentals of Nutrition Research, Nutrient and Gene Interactions, Product Development, Sensory Analysis

AWARDS

- Presidential Doctoral Research Fellowship (2014-2018)
- Build Dairy Program Tuition Award (2014-2019)
- Travel awards: School of Research and Graduate Studies (USU), Nutrition, Dietetics, and Food Science (USU), Build Dairy Program (Western Dairy Center)