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Effects of Dietary Polyunsaturated Fatty Acids on Colorectal Cancer and the Development of the Total Western Diet-2

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EFFECTS OF DIETARY POLYUNSATURATED FATTY ACIDS ON COLORECTAL CANCER AND THE DEVELOPMENT OF THE TOTAL WESTERN DIET-2

by

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ABSTRACT

The Effect of Dietary Polyunsaturated Fatty Acids on Colorectal Cancer and the Development of the Total Western Diet-2

by

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The Western diet is characterized by the consumption of foods high in omega-6 (n-6) fatty acids, leading to high ratios of omega-6 to omega-3 (n-3) fatty acids. This dietary pattern has been hypothesized to cause higher incidences of colorectal cancer (CRC) due to the pro-inflammatory nature of n-6 fatty acids. Using an azoxymethane and dextran sodium sulfate (AOM+DSS) induced inflammatory model, mice were fed identical diets, varying only in dietary polyunsaturated fatty acid (PUFA) concentration and n-6:n-3 ratio. They included 1.) AIN-93G, control diet, containing 7% (kcal) dietary PUFA 7:1 n-6:n-3 ratio, 2.) 2.5% dietary PUFA 1:1 n-6:n-3 ratio, 3.) 2.5% PUFA 20:1 n-6:n-3, 4.) 10% PUFA 1:1 n-6:n-3, and 5.) 10% PUFA 20:1 n-6:n-3. Mice fed the AIN-93G and the 2.5% PUFA with a 1:1 n-6:n-3 ratio resulted in significantly higher colon tumor multiplicity (p-value < 0.0001) compared to the other treatments. Additionally the ratio n-6:n-3 had a significant effect on tumor size. Mice fed diets with a PUFA ratio of 1:1 had significantly larger tumors than mice fed diets with a PUFA ratio of 20:1. Using colon length as a measure of inflammation, no significant differences were found between diet treatments. Results from our study suggest dietary PUFA profile influences the etiology of CRC.

Rodents are commonly used as pre-clinical animal models to study various human diseases. Typically, mice are fed a purified diet formulated to optimize the animal's growth and

health. However, these diets are considerably different than the average American diet. Hintze and colleagues developed the Total Western Diet (TWD), which matches both the macro- and micronutrient content of the average American diet, giving researchers a rodent diet more suitable for studying colon health and related diseases. The TWD-2 was designed to improve upon the TWD by incorporating the complex dietary components of whole-foods known to impact gut health. The TWD-2 is currently the only whole-food-based rodent diet that emulates both the macro- and micronutrients of the average American diet. Initiating studies using the TWD-2, in place of the AIN diets, will hopefully make the rodent a better model for human colorectal cancer research.

(77 pages)

PUBLIC ABSTRACT

The Effect of Dietary Polyunsaturated Fatty Acids on Colorectal Cancer and the Development of the Total Western Diet-2

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The Western diet is commonly consumed by industrialized societies and characterized by an increased consumption of vegetable oils rich in omega-6 (n-6) fatty acids. This results in a higher ratio of omega-6 to omega-3 (n-3) fatty acids in the diet. Omega-6 polyunsaturated fatty acids (PUFA) are believed to induce a pro-inflammatory response in the body. Therefore, this change in PUFA concentration and/or ratio of n-6:n-3 in the Western diet may contribute to colorectal cancer (CRC) risk. Five identical diets, varying only in PUFA concentration and n-6:n-3 ratio, were fed to mice dosed with a carcinogen and an inflammatory accent (AOM+DSS). The diets included: 1.) AIN-93G, control diet, containing 7% (kcal) dietary PUFA, 7:1 n-6:n-3 ratio, 2.) 2.5% dietary PUFA 1:1 n-6:n-3 ratio, 3.) 2.5% PUFA 20:1 n-6:n-3, 4.) 10% PUFA 1:1 n-6:n-3, and 5.) 10% PUFA 20:1 n-6:n-3. PUFA ratio had a significant effect on tumor size. Diets having an n-6:n-3 ratio of 1:1 resulted in significantly larger tumors than diets with an n-6:n-3ratio of 20:1. Mice fed either the AIN-93G or 2.5% 1:1 diet had the highest number of tumors compared to the other experimental diets. From these results, it appears that the dietary PUFA profile influences the etiology of CRC.

Studies investigating CRC commonly use rodent models to investigate human diseases. Typically rodents are fed diets formulated to promote growth and heath, however these diets are considerably different than the Western diet in terms of macro- and micronutrients. Diet is known to influence CRC incidence, which led to the development of the Total Western Diet (TWD) by Hinze and colleagues. The TWD is a rodent diet that uses purified ingredients to match the macro- and micronutrient composition of the average American diet. However, the

complex nature of whole-foods is also known to impact colon health, so the TWD was redesigned. The TWD-2 is the only whole-foods-based rodent diet that emulates the macro- and micronutrient consumption of the average American. Initiating studies using the TWD-2, in place of the AIN diets, will hopefully make the rodent a better model for human disease research.

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Sara Kellen

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CHAPTER 1

INTRODUCTION

The Western Diet

Dietary patterns have changed significantly since the Palaeolithic era, when human consumption patterns were characterized by an overall low caloric intake and diets were largely composed of lean meats, leafy green vegetables, nuts, and fruits (1). Today, in many developed industrialized societies, the Western diet is commonly consumed. The Western diet is characterized by an overall increase in energy intake, with a large portion of daily calories coming from processed meats, refined grains, and sweets. This eating pattern has been correlated to a number of health issues from metabolic syndrome to colon cancer (2-4). Immigrants who adopt the Western diet typically acquire the disease risks associated with this dietary pattern, which are often uncommon in their native culture (2). This skewed consumption pattern of the Western diet results in an increased overall intake of omega-6 fatty acids, due to increasedconsumption of vegetable oils, creating a higher ratio of omega-6 (n-6) to omega-3 (n-3) fattyacids in the diet (5). During the Palaeolithic era the ratio of n-6 to n-3 fatty acids was approximately 1:1, but current dietary patterns of the Western diet have resulted in a significantly unbalanced ratio of n-6 to n-3, and is estimated to be as high as 20-30:1 in some populations (5). This increase in n-6 consumption may be attributed to dietary recommendations that suggest to replace saturated fatty acids with polyunsaturated fats to lower total and LDL cholesterol levels (6, 7). Along with these dietary recommendations was the development of new food processing technologies that made large-scale production of vegetable oils more efficient and economical. This increase in n-6-rich vegetable oil production and the subsequent dietary recommendations are recognized as key factors responsible for the increase in n-6

polyunsaturated fatty acid (PUFA) intake in the Western diet (6, 7).

PUFA and Gut Inflammation

Omega-6 and omega-3 fatty acids consumed in the diet can be converted into signaling molecules in the body that have either a pro-inflammatory or an anti-inflammatory effect, respectively (8). Therefore, it is not surprising that increased levels of n-6 fatty acids have been shown to promote inflammatory diseases like Crohn's disease and ulcerative colitis (7, 9). Crohn's disease (CD) and ulcerative colitis (UC) are commonly classified under the more comprehensive term, inflammatory bowel disease (IBD). The cause of IBD is not well understood, but immunologic abnormalities, genetic predisposition, and environmental factors are all believed to contribute to the etiology of these diseases (10). These gut inflammatory diseases have been consistently associated with the etiology of colon cancer. The risk for colorectal cancer is believed to increase the longer IBD goes untreated (11, 12). The chronic inflammation associated with these diseases is thought to induce DNA modifications resulting in abnormal cell growth and an environment favorable for tumor development (13). Not surprisingly, the significant imbalance of the n-6 to n-3 ratio present in the Western diet is believed to influence the prevalence of CRC in Western societies. In a large cohort study, researchers found that rates of CRC were higher in women consuming a Western type diet (3).

Colorectal Cancer (CRC)

CRC is the third leading cause of cancer-related fatalities in the United States and is projected to cause over 50,000 deaths in 2014 (14). This disease primarily affects Caucasians, African Americans, and individuals over the age of 50 (14). Incidence rates of colorectal cancer are not uniform throughout the world. Most cases are localized in North America, Australia, New Zealand, and parts of Europe, indicating environmental factors greatly influence disease

prevalence. Epidemiological studies have recognized dietary habits and a sedentary lifestyle as the major environmental factors attributing to the increased risk of CRC (15). Along with environment factors and chronic gut inflammatory diseases, genetically inherited syndromes like familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer pose a significant threat for CRC occurrence. For insistence, several mutations identified in the tumor suppressor gene, adenomatosis polyposis coli (APC) have been shown to lead to the development of FAP in individuals. Researchers have identified a number of other genes shown to induce human colon carcinogenesis, including: *p53, CTNNB1, IGF12, TGFBR2, KRAS, BRAF,* and *TRP53* (16, 17).

Rodent Colorectal Cancer Models

To study colorectal cancer in depth, researchers commonly use rodents as a way to model human CRC. Rodents are relatively inexpensive and they allow scientists to better understand the mechanisms of the disease, as well as develop potential treatment options. In CRC studies, there are three common colitis-associated cancer models used today. They include: genetic, xenoplant, and chemical models, with each having its own benefits and drawbacks. In the genetic model, mice are genetically engineered to have mutations known to cause CRC. A common genetic model used by researchers is a knockout APC mouse. Through the introduction of a number of different mutations within the APC gene, tumor development similar to those found in patients with FAP will arise (16). In xenoplant models, tumor cells are subcutaneously or intravenously injected into genetically engineered immunodeficient mice, allowing further tumor growth to occur in the animal. This type of model, allows researchers to evaluate human tumor growth in vivo under different experimental environments (16). Carcinogen-induced colon cancer models have shown to be effective methods in creating tumor progression similar to that found in humans. The azoxymethane / dextran sodium sulfate (AOM/DSS) model is frequently used, as it

has proven to significantly increase the development of CRC and mimic human disease progression (16, 17). These two compounds work together effectively, as AOM causes tumor development and DSS induces inflammation. AOM is a chemical carcinogen that creates DNA-reactive adducts through several metabolic activation steps once in the body. These DNA adducts cause methylation of the nucleic acid guanine, resulting in DNA base pair mismatching. DSS is administered orally through drinking water and induces colitis in the animal through its cytotoxic effects on the epithelial lining of the colon (16). This CRC model is so effective because of how closely the cancer development in the animal resembles CRC development in humans (16).

Colon Carcinogenesis

Colorectal cancer is caused by a number of cellular dysfunctions and contributing environmental factors, which can cause this disease to take years to manifest. In CRC progression, normal epithelial cells lining the gastrointestinal tract will differentiate either through genetic predispositions or through environmental factors, leading to dysplasia (16). The earliest signs of neoplasia in the colon are dysplastic aberrant crypt foci (ACF). Through additional proliferation, ACF's form microadenomas, which can further evolve into macroadenomas and eventually lead to colon cancer (18, 19). In mice treated with AOM + DSS, tumors typically arise in the middle and distal portions of the colon creating nodular, polypoid, or tubular adenomas/adenocarcinomas, depending on tumor progression (19, 20).

Animal Diets

In many rodent models of human disease, animals are commonly fed a purified diet formulated by the American Institute of Nutrition (AIN). AIN developed these diets, AIN-76A and AIN-93G, to give scientists a nutritionally adequate diet to help promote growth and fertility

of the animal and give scientists a standard to use between research studies (21). However, when comparing these formulated diets to the average American diet using nutrient density, they vary significantly in both the macro- and micronutrient composition and in dietary complexity. Moreover, these diets are formulated with purified ingredients including sugar, cornstarch, casein, and soy or corn oil. Many researchers have proposed other dietary plans to better emulate the Western diet, including: the "cafeteria diet", the commercialized "Western diet", and the "New Western Diet" (22-24). While all these diets are more similar to American intakes as compared to the AIN diets, they still do not accurately emulate the average American consumption. The only formulated rodent diet that currently matches both the macro- and micronutrient profile of Americans is the Total Western Diet (TWD) developed by Hintze and colleagues (4). To get a representation of the typical American diet, data from the National Health and Nutrition Examination Survey (NHANES) *What We Eat in America* for the years 2007-2008 was used (25). The average daily intake reported for all nutrients was normalized to calories consumed and converted into a nutrient density measurement. Using a nutrient density approach accounts for differences in the metabolic rates between species.

When comparing the TWD to the AIN diets, significant differences in the macro- and micronutrient profiles of these two diets are apparent. The TWD contains fewer calories from protein and carbohydrates, and approximately twice the amount from fat (Table 1) (4). The TWD also contains approximately half the amount of complex carbohydrates and twice the amount of simple sugars compared to the AIN-93G diet. Additionally, the TWD contains only 60% of the dietary fiber found in the AIN-93G diet. Another notable difference between these two diets is the fatty acid profile. The TWD was designed to include multiple fat sources, thus giving it a more diverse fatty acid profile. Fat in the AIN diets comes from either soybean or corn oil, therefore resulting in a very simple fatty acid profile, lacking in complexity. A number of notable differences also occur in the micronutrient content between these diets (Table 2) (4). The impact

of these discrepancies between the AIN diets and the Western diet have been scrutinized recently as a number of studies have shown a correlation between diet, gut microbiome, and colon health (26-28). This connection between diet and gut health suggests a more biologically relevant dietary model is needed when investigating the influence of diet on CRC and the microbiome. To date, a whole-food based rodent diet, matching both the macro- and micronutrient levels of American consumption, has not been developed. In an effort to explore the impact diet has on gut microbiome and subsequent health issues, the Total Western Diet 2 (TWD-2) was formulated.

TABLE 1. Macronutrient composition of the AIN-76A, AIN-93G, and TWD from (4)

Macronutrient	AIN-76A[a]	AIN-93G[a]	TWD[a]
Carbohydrates			
Corn Starch	150.0	398.0	230.0
Maltodextrin	---	132.0	70.0
Sucrose	500.0	100.0	261.0
Cellulose	50.0	50.0	30.0
kcal (% of total)	67.7	60.1	54.5
Proteins			
Casein	200.0	200.0	190.0
L-cystine	---	3.0	2.85
DL-methionine	3.0	---	---
kcal (% of total)	20.8	18.8	15.4
Fats			
Soybean Oil	---	70.0	31.4
Anhydrous Milk Fat	---	---	36.3
Olive Oil	---	---	28.0
Lard	---	---	28.0
Beef Tallow	---	---	24.8
Corn Oil	50.0	---	16.5
Cholesterol	---	---	0.40
kcal (% of total)	11.5	17.2	34.5

[a] Values are g/kg diet for all components

TABLE 2. Micronutrient composition of the TWD compared to the AIN-93G from (4)

Micronutrient	AIN-93G	TWD
Minerals (mg/kg diet)		
Calcium	5000.0	2011.0
Phosphorus	3000.0	2757.0
Sodium	1019.0	7078.0
Potassium	3600.0	5333.0
Magnesium	507.0	589.0
Iron	35.0	31.0
Zinc	30.0	25.0
Copper	6.0	2.6
Selenium	0.15	0.2
Vitamins (unit/kg diet)		
Thiamin (mg)	5.0	3.5
Riboflavin (mg)	6.0	4.4
Niacin (mg)	30.0	50.6
Pyridoxine (mg)	6.0	3.9
Folate (mg)	2.0	1.3
Vitamin B_{12} (mg)	25.0	11.0
Vitamin A (IU)	4000.0	4300.0
Vitamin D (IU)	1000.0	391.0
Vitamin E (IU)	75.0	24.6
Vitamin K (µg)	750.0	189.0
Choline (mg)	1027.0	648.0

Gut Microbiome and Diet

Recently, research involving the role of the gut microbiome on disease incidence has made great advances. Emerging evidence suggests that an individual's gut microbiome greatly impacts overall health, playing a role in obesity, colon health, and even immune response (27, 29, 30). The types of microbiota found in the gut are believed to influence the development of these different health conditions. There are many factors that can affect the diversity of the microbiota including age, diet, antibiotic use, and other environmental factors (27). Recently, diet has been identified as a major factor influencing the microbiome, and the microbiota can change rapidly with a corresponding change in diet (28). Bacteria obtain energy through the fermentation of dietary components that are not completely digested and absorbed in the upper digestive tract of the host (26). In human diets, undigested carbohydrates that reach the colon can be fermented by the gut microbiota to produce short-chain fatty acids (SCFA) and bioactive plant metabolites.

SCFAs and plant metabolites have been associated with a decreased risk in cancer. The bioactive plant metabolites are able to turn on endogenous antioxidant genes to help prevent cellular damage (31, 32). The interaction between the metabolites and antigens produced by the gut microbiota, along with the metabolites and immune response produced by the host, play a significant role in influencing the overall health status of the colon (33).

Metabolomics of the Gastrointestinal Tract

Metabolomics is the identification and quantification of metabolites produced in the body. These metabolites are produced as a result of the metabolic activity of the host, as well as the metabolic activity of the microbes living in the gut. They can be analyzed from a variety of sources, such as urine, blood serum, and feces (34). Analyzing the metabolites present in fecal matter allows researchers to identify and quantify metabolites produced from the complex interaction between the individual's metabolic system and their gut microbiome (34, 35). Utilizing analytical techniques, such as nuclear magnetic resonance (NMR) or gas chromatography-mass spectrometry (GC-MS), allows scientists to categorize and measure changes in the metabolomic analytes in response to dietary changes and other environmental effects (35). A large majority of metabolites produced are organic acids, gases, and short chain fatty acids (36). Understanding the effect these metabolites have on the body and how they vary in response to dietary changes may help researchers determine the risk for different colon related diseases, like CRC.

Effect of Diet on Fatty Acid Profiles

Dietary fat plays an essential role in the body by providing a significant source of calories, influencing the body's inflammatory response, and playing a critical role in the dynamics of various cellular membranes (37). Maintaining membrane fluidity is crucial for cells

to function properly. The relative proportion of saturated and unsaturated fatty acids present in the membrane aid in the maintenance of this fluidity (37). Studies have shown dietary fatty acids are incorporated into membranes throughout the body in proportions relative to those found in the diet. Therefore, changes in dietary fat consumption can lead to shifts in the distribution of fatty acids in cell membranes (38-40). Researchers have examined the distribution of fatty acids in response to a number of diseases (38, 40, 41). In a study investigating CRC, the fatty acids in the red blood cell (RBC) membranes of CRC and control patients were compared. Researchers found CRC patients had significantly higher ratio of n-6:n-3 fatty acids and lower amounts of n-3 fatty acids in their RBC membranes. Researchers believe this difference was caused by an altered lipid metabolism in CRC patients (41).

Hypotheses

PUFA and Colorectal Cancer

In response to previous research pertaining to the potential effect of dietary PUFA on gut inflammation, a preliminary study by Snow et al. was carried out to investigate the dietary PUFA concentration and ratio effects on a lipopolysaccharide (LPS) inflammation model (42). Results from this study found that mice fed a diet high in both total PUFA and ratio of n-6 to n-3 fatty acids, had an increased systemic inflammatory response. Given this information, the potential link between dietary PUFA concentration and n-6:n-3 ratio was studied further. In the current study, we specifically examined the effect of high (10% kcal) and low (2.5% kcal) PUFA content along with high (20:1) and low (1:1) ratios of n-6 to n-3 fatty acids on gut inflammation and the subsequent impact on colorectal cancer risk using an AOM + DSS mouse model of CRC.
Based on the preliminary data, we hypothesized that a diet with high PUFA concentration and high n-6:n-3 ratio would increase tumor multiplicity and size compared to the low PUFA, low n-6:n-3 diet and the control AIN diet. The specific aims for this study include:

1. Determine the impact of five different experimental diets on colon cancer susceptibility using an AOM + DSS inflammation model of colorectal carcinogenesis. The experimental diets were as follows:

   Diet 1: AIN-93G basal diet: 7% energy from PUFA, 7:1 n-6 to n3 ratio (Control)

   Diet 2: AIN-93G basal diet: 2.5% energy from PUFA, 1:1 n-6 to n-3 ratio

   Diet 3: AIN-93G basal diet: 2.5% energy from PUFA, 20:1 n-6 to n-3 ratio

   Diet 4: AIN-93G basal diet: 10% energy from PUFA, 1:1 n-6 to n-3 ratio

   Diet 5: AIN-93G basal diet: 10% energy from PUFA, 20:1 n-6 to n-3 ratio
2. Compare the serum cytokines levels for TNF-α, INFγ, IL-17, IL-10, and IL-6, along with CRP, in mice fed experimental diets in both the control and cancer treatment groups.
3. Determine the effect of the experimental diets on fecal metabolites and the fatty acid profile of red blood cell and colon mucosal membranes.

Total Western Diet-2

To date, a whole-food based rodent diet that appropriately matches American eating patterns both on the macro- and micronutrient level has not been developed. The specific objective of this project is to effectively develop a functioning whole-food based rodent diet. Data from the NHANES survey *What We Eat in America* for the years 2007-2008 (25) and the *Retail Food Commodity Intakes: Mean Amounts of Retail Commodities per Individual*, 2001- 2002 (43) was used in the formulation of the TWD-2. Initiating studies using the TWD-2, in place of the AIN diets, will hopefully make the rodent a better model for colorectal cancer and microbiome research.

CHAPTER 2

EFFECTS OF DIETARY POLYUNSATURATED FATTY ACIDS ON COLORECTAL CANCER

Abstract

The Western diet is characterized by consuming a diet high in red and processed meats, refined grains, and sugars. This type of eating pattern results in an increased consumption of omega 6 fatty acids and a shift to a higher ratio of omega 6 (n-6) to omega 3 (n-3) fatty acids. Eicosanoids derived from n-6 fatty acids have been shown to induce a pro-inflammatory response in the body; while n-3 derived eicosanoids have an anti-inflammatory effect. The aim of this study was to determine if high dietary concentrations of total polyunsaturated fatty acids (PUFA) or the unbalanced ratio of n-6:n-3 found in Western diet would promote the development of colorectal cancer (CRC) using the AOM + DSS induced inflammatory model. Five different diets, which varied only in the dietary PUFA concentration and n-6 to n-3 ratio, were fed to mice. The diets used were 1.) AIN-93G control diet, 7% (kcal) dietary PUFA 7:1 n-6:n-3 ratio, 2.) 2.5% dietary PUFA 1:1 n-6:n-3 ratio, 3.) 2.5% PUFA 20:1 n-6:n-3, 4.) 10% PUFA 1:1 n-6:n-3, and 5.) 10% PUFA 20:1 n-6:n-3. The investigated outcomes included tumorigenesis, systemic inflammation, fatty acid analysis of red blood cell and colon mucosal membranes, and fecal metabolite analysis. Mice fed the AIN-93G and the 2.5% PUFA with 1:1 ratio of n-6 to n-3 diets had significantly higher colon tumor multiplicity ($p < 0.0001$) than mice fed the other diets. The dietary n-6:n-3 ratio but not the PUFA concentration had a significant effect on tumor size. Mice fed diets with a PUFA ratio of 1:1 had significantly larger tumors than mice fed diets with a PUFA ratio of 20:1 (p-value = 0.027). Diets with higher PUFA concentrations appeared to influence the systemic inflammatory response of TNF-α, IL-17, and IL-10. The fecal metabolite

profile was affected by both the dietary PUFA ratio and concentration, as well as AOM treatment. Our results suggest both the PUFA concentration and PUFA ratio influence the etiology of CRC. Specifically, higher ratios of n-6:n-3 dietary PUFA appear to be protective against AOM + DSS induced tumorigenesis.

Introduction

The essential fatty acids, linoleic acid (LA, C18:2n-6) and α-linolenic acid (ALA, C18:3n-3), must be obtained from the diet, as they cannot be synthesized *de novo*. In Western populations, the consumption of these two fatty acids has changed dramatically in the last 100 years. This change has resulted in an increase in the n-6:n-3 ratio, which studies suggest may result in detrimental health effects through excessive eicosanoid signaling (6, 7). Once in the body, LA and ALA are further elongated and desaturated through the delta-6-desaturase/delta-5-desaturase (D6D/D5D) enzymatic pathway, producing their respective highly unsaturated fatty acids: arachidonate (AA, C20:4n-6), eicosapentaenoate (EPA, C20:4n-3), and docosahexaenoate (DHA, C22:6n-3) (44). The average American diet contains substantially more LA than ALA. Consequently, this difference is reflected in the tissue PUFA content, with Americans exhibiting a greater proportion of AA compared to EPA in their adipose tissue (45). AA and EPA can be further converted to eicosanoid signaling molecules through one of two enzymatic pathways, cyclooxgenase (COX) or lipoxygenase (LOX), both of which are upregulated during the inflammatory state. The process by which these enzymes recognize and activate the n-6 or n-3 fatty acids appears to be nonspecific, suggesting the fatty acids are released based on their relative proportions in the membrane (37). Metabolites derived from AA are considered to have a stronger inflammatory response compared to the n-3, EPA and DHA, derived metabolites (37). The long chain omega-6 derived fatty acid, AA, has been shown to produce higher levels of

prostaglandin E2 (PGE_2) and leukotriene B4 (LTB_4) (39, 46). In colorectal cancer, PGE_2 has been shown to cause hypermethylation of DNA, reducing the expression of a number of tumor suppressor genes (47). LTB_4 influences the cellular microenvironment by acting as a growth factor and has been found at higher concentrations in some cancerous tissues (46). The prostaglandins and leukotrienes produced from omega-3 derived fatty acids through the LOX/COX pathway, LTB_5 or PGE_3, have been shown to have anti-inflammatory effects (37, 39). Production of PGE_3 will decrease the production of PGE_2, thus preventing this prostaglandin from promoting cell proliferation. PGE3 has also been shown to down-regulate the expression of the enzyme COX-2 required for PGE_2 production (48). Investigations have found that by incorporating EPA into the diet in its free fatty acid form results in less tumor multiplicity compared to mice fed a diet containing no EPA (39).

Another factor contributing to the development of CRC is an abnormal increase in a systemic response of the following cytokines: IL-6, TNF-α, IL-10, IL-17, and INFγ (49-51). Elevated levels of IL-6, IL-17, and TNF-α have been measured in individuals with chronic gut inflammatory conditions. Elevation of these cytokines promotes carcinogenesis through overactive signaling causing further pro-inflammatory responses, increased cell proliferation, and tumor growth (49, 50). IL-6 will stimulate production of C-reactive protein (CRP), an acute-phase protein, inducing a pro-inflammatory response in the body (52). The role of INF□ in the etiology of cancer is somewhat controversial. INFγ was believed to act in an anti-inflammatory/anti-tumorigenic way by increasing tumor immunogenicity through the upregulation MHC class I genes (53). Current thinking is that this cytokine actually decreases tumor immunogenicity through the down-regulation of tumor antigen expression. Without this expression, tumors do not stimulate an immune response, allowing further growth and

development (51). IL-10 plays an important role in regulating inflammation corresponding to colon cancer, through controlling cell growth and preventing tumor development (54).

Individuals with IBD or colorectal cancer have a number of factors affecting the etiology of these diseases. Not only do they have an increase in inflammatory cytokine and eicosanoid signaling, but studies have shown they also exhibit changes in their gut microbiome as compared to healthy individuals (33). Through 16s sequencing, researchers were able to compare bacterial phyla present in patients with CRC and healthy individuals. Their findings showed healthy individuals had a statistically higher amount of *Bacteroidetes* and statistically lower amount of *Proteobacteria*. At the genus level, CRC patients had a larger population of five different genera compared to healthy individuals including: *Porphyromonas, Escherichia/Shigella, Enterococcus, Streptococcus,* and *Peptostreptococcus* (33). A separate study suggests the members of the *Fusobacterium* genus may play a role in CRC, as this type of bacterium has been found at higher concentrations in tumor tissue (55). The bacteria present in the gut will produce metabolic substrates that may be beneficial or harmful to the host. For example, certain bacteria can produce metabolites such as acetaldehyde, hydrogen sulfide, or secondary bile acids that are believed to contribute to inflammation and tumor progression (33). One specific metabolite that has been shown to have a protective effect against CRC is butyrate. Butyrate has been shown to reduce oxidative damage to DNA and decrease CRC progression by inducing apoptosis and inhibiting tumor cell growth. Patients with CRC have been found to have less butyrate-producing bacteria compared to healthy individuals (33).

To examine the potential role of PUFA on gut inflammation and its role in the progression of CRC, plant derived dietary PUFAs, LA and ALA, were used to formulate diets differing in PUFA concentration and n-6:n-3 ratio. To date, plant derived dietary PUFA and the ratio of n-6 to n-3 has not been investigated in colorectal cancer rodent models. Considering that plant derived dietary PUFA accounts for 99% of the daily PUFA intake in Americans, it is critical

to understand the effects of these fatty acids on gut inflammation and ultimately their role in colorectal cancer in order to develop appropriate intervention strategies relevant to American diets (56). To investigate the effects of dietary PUFA on inflammation, gut metabolites, and CRC occurrence, mice were subjected to an AOM + DSS inflammatory challenge and fed diets that varied only in the percent of PUFA and ratio of n-6 to n-3 fatty acids. While the experimental diets represented some of the highest and lowest dietary PUFA concentrations and n-6:n-3 ratios reported, the control AIN-93G, allowed us to observe the effect of an intermediate level of dietary PUFA concentration and n-6:n-3 ratio, having 7% PUFA and n-6:n-3 ratio of 7:1. The experimental endpoints measured were tumor development and inflammation, serum cytokine levels, fatty acid profiles of red blood cells and colon mucosa, and fecal metabolite profiles.

Methods

Experimental Design

Five-week-old male C57BL/6J mice were purchased from Jackson Laboratory and allowed to acclimate for one week at Utah State University's Laboratory Animal Research Center. At six weeks of age, mice were randomly assigned to one of five experimental diets (Harlan Teklad) for a feeding period of 16 weeks: 1.) AIN-93G control diet, 2.) 2.5% (kcal) dietary PUFA 1:1 n-6:n-3 ratio, 3.) 2.5% PUFA 20:1 n-6:n-3, 4.) 10% PUFA 1:1 n-6:n-3, and 5.) 10% PUFA 20:1 n-6:n-3. The fatty acid profiles of these experimental diets are depicted in table 3.

TABLE 3. Fatty acid composition of experimental diets

	Target Values		Measured Values [a]								
Diet	PUFA %	Ratio	16:0	18:0	18:1	18:2	18:3	SFA	MUFA	PUFA	Ratio
1	7.0	7:1	1.2	0.5	2.5	5.7	0.8	1.7	2.5	6.4	7:1
2	2.5	1:1	2.8	2.8	8.4	1.5	1.4	5.6	8.4	2.8	1:1
3	2.5	20:1	2.4	1.7	10.2	2.4	0.2	4.1	10.2	2.5	16:1
4	10.0	1:1	2.2	1.1	3.7	5.3	4.8	3.3	3.7	10.0	1:1
5	10.0	20:1	2.7	0.8	4.5	8.5	0.4	3.5	4.5	8.8	24:1

[a] Values are expressed as percent total dietary energy.

Mice were then randomly distributed into the following treatment groups, AOM injection and DSS treated drinking water (n = 20) for the cancer outcome or saline injection and regular drinking water (n = 6) for the sham or control outcome. Sham treated animals were individually housed and the mice in the cancer treatment were co-housed. Animals had access to food and drinking water ad libitum. At seven weeks of age, cancer outcome mice were subcutaneously injected with a single, 10 mg/kg dose of AOM and administered drinking water containing 1% DSS for four weeks to facilitate tumorigenesis (57). Control mice received a subcutaneous injection of 10 mg/kg of saline and standard drinking water was administered throughout the study. At 16 weeks of age, all mice were euthanized via CO2 asphyxiation and cardiac puncture. Serum was collected using serum collection spin tubes (Sarstedt AG & Co) and flash frozen using liquid nitrogen. For metabolomic analysis, feces from the colon were collected and flash frozen using liquid nitrogen. The Institutional Animal Care and Use Committee (IACUC) of Utah State University approved all study procedures. The protocol number was 2217.

Tumor Analysis

The lower bowel, from below the cecum to the rectum, of AOM + DSS treated mice was removed to assess the impact of experimental diets on tumor multiplicity. Colons were washed with saline, split longitudinally, laid flat on filter paper, and stored in 70% ethanol at 4°C. To determine tumor multiplicity and size, colons were stained with methylene blue to aid in the

characterization of tumors by light microscopy. To determine tumor size, calipers were used to measure the length, width, and height. Using these measurements the volume was calculated using the equation $V= \pi/6$ (L x W x H).

Systemic Inflammation

Known cytokines and acute phase proteins involved with gut inflammation and IBD were measured using custom multiplex ELISA assays purchased from Milliplex. The following serum cytokines: INF-γ, IL-6, IL-10, IL-17, and TNF-α, in-junction with the acute phase protein, C-reactive protein (CRP), were measured. Protocols suggested by the manufacturer and a Bio-Plex 200 instrument equipped with the Bio-Plex Manager 5.0 software were used to analyze data.

GC-MS Analysis of Fecal Metabolites

The sample preparation protocol and GC-MS temperature program were adapted from Hintze et al. (58). Fecal pellets were broken up in 1 mL of 70% ethanol (v/v) + 1 mg/mL D4-succinate (Sigma-Aldrich, Co). D4-succinate was used as the internal standard. Samples were heated at 90°C for 5 min and then centrifuged at 5,000 x g for 5 min. Supernatant was removed and dried down under vacuum. Dried samples were suspended in 40 μL of 40 mg/mL *O*-methoxylamine hydrochloride in pyridine and incubated for one hour at 30°C. Following incubation, 40 μL of N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) was added to each sample and incubated for 30 min at 37°C with vortexing every 5 min. A 1 μL sample was injected into the gas chromatograph at a 10:1 split ratio with an inlet temperature held at 250°C. The temperature program started at 70°C for 1 min, followed by a 40°C/min-ramp to 110°C, with a 2 min hold at 110°C. A second ramp of 5°C/min-ramp was carried to a final temperature of 350°C (58). Helium was used as the carrier gas and separation was performed on a ZB-5 MSI

column (35 m length, 0.25 mm diameter and 0.25 μm film thickness). A GC-2010 (Shimadzu) gas chromatograph fitted with a GC-MS QP2010S (Shimadzu) mass spectrometer was used in this analysis. Compounds were identified using SpectConnect, Automated Mass Spectral Deconvolution and Identification System (AMDIS) software and the National Institute of Standards and Technology (NIST) mass spectral library. Peak areas were normalized to the internal standard and metabolite values were expressed as a ratio.

Fatty Acid Analysis of Red Blood Cells and Colon Mucosa

To analyze fatty acids present in colon mucosa and red blood cell (RBC) samples, direct fatty acid methyl ester (FAME) synthesis was conducted according to O'Fallon et al. and samples were analyzed using a GC2010 (Shimadzu) (59). Due to the small amount of RBC's available, solvents used for RBC preparation were diluted by factor of 10. A 1 μL sample was injected into the gas chromatograph at a 10:1 split ratio with an inlet temperature held at 250°C. The temperature program started at 153°C for 2 min, followed by a 2.3°C/min-ramp to 174°C, with a hold time of 20 sec. A second temperature increase of 2.5°C/min-ramp to a final temperature of 210°C, which was held for 5 min. Separation was performed on a DB-23 column (30 m length, 0.25 mm diameter and 0.25 μm film thickness) using helium as the carrier gas.

Statistical Analysis

JMP statistical software, version 11.0 (SAS Institute Inc.) was used to conduct the statistical analysis on all experimental endpoints. One-way and two-way analysis of variance (ANOVA) was used to evaluate statistical differences between experimental diets and investigated endpoints. One-way ANOVA was used to determine statistical differences between all experimental diets, including the control and two-way ANOVA was used to determine if

statistical differences were occurring between the main effects of PUFA concentration and PUFA ratio. Tukey-Kramer HSD post hoc analysis was used to test for pair-wise comparisons via one-way ANOVA and Student's t-test was used for paired comparison for the two-way ANOVA. All experimental endpoints were calculated as mean ± standard error, unless otherwise noted. SAS statistical software (version 9.4) was used for the principal component analysis. Statistical significance was predefined as a p-value < 0.05.

Results

Effect of Treatment and Weight Gain

Weights of the control and AOM + DSS treated mice in each diet were tracked throughout the feeding portion of the experiment. Diet treatment had no significant effect on weight gain in the sham treated mice. However, the AOM + DSS treated mice fed the AIN-93G diet had significantly higher weight gain than those fed the 2.5% 20:1, 10% 1:1, or 10% 20:1 diets (p-value < 0.05) (Figure 1). There was no significant difference in food consumption between any of the experimental diets. Over the course of the experiment, seven deaths occurred within the AOM + DSS treated mice (n= 2 – AIN-93G, n= 1 - 2.5% 20:1, n= 3 - 10% 1:1, and n= 1 - 10% 20:1). Deaths occurred either early on or late in the feeding portion of the study. Early deaths were caused by AOM toxicity, while the deaths that occurred late in the feeding study, were likely due to advanced tumor development resulting in obstruction of the bowel. At euthanization a number of AOM + DSS treated mice had a prolapsed anus likely caused by advanced tumor development in the distal colon.

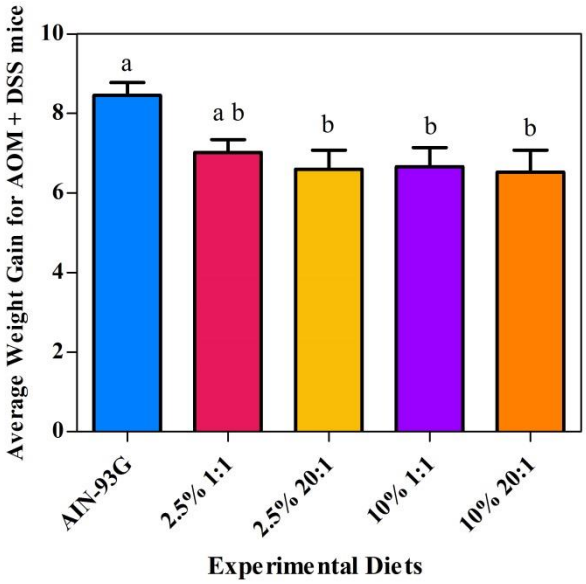

FIGURE 1. Weight gain of the AOM + DSS treated mice. Different lettered columns indicate a significant difference between experimental diets (p-value <0.05), which were determined by one-way ANOVA and Tukey-Kramer HSD post hoc analysis. Values for are expressed as mean ± standard error.

Effect of Experimental Diets on Tumor Multiplicity and Size

To assess cancer progression, tumors were identified based on the nomenclature accepted in the consensus report by Boivin et al. (18). There was a significant increase in tumor multiplicity (p-value < 0.0001) in mice fed either the 2.5% 1:1 or AIN-93G diets compared to mice fed any of the other experimental diets (Figure 2A). However, when tumor multiplicity was normalized to colon length, there was not a significant difference between diet treatments (Figure 2C). There was a significant main effect of larger tumor size for the n-6 to n-3 ratio but not PUFA concentration. Animals fed diets with a PUFA ratio of 1:1 had significantly larger tumors than animals fed diets with a PUFA ratio of 20:1 (p-value= 0.012) (Figure 2F). There was a significant difference between experimental diets on mouse colon lengths (p-value = 0.027), however when individual treatments were compared via post hoc analysis, there were no significant differences.

There was a significant interaction between, PUFA concentration and PUFA ratio, on colon length (p-value = 0.017) (Figure 2G).

Effect of Experimental Diets on Serum Cytokines

The effect of the experimental diets on the systemic pro-inflammatory response was determined by measuring serum levels of the following cytokines: INF-γ, IL-6, IL-10, IL-17, TNF-α, and C-reactive protein (CRP) in AOM + DSS treated mice. Levels of IL-10 and IL-6 were significantly higher in mice fed the AIN-93G diet than those fed the 10% 20:1 diet (p-value= 0.03 and p-value= 0.004, respectively) (Figure 3A & 3D). Levels of TNF-α were significantly higher in mice fed the 2.5% 1:1 diet compared to those fed the 10% 1:1 diet (p-value= 0.03) (Figure 3B). IL-17 was significantly higher in the serum of mice fed the AIN-93G, 2.5% 1:1, and 2.5% 20:1 diets compared to mice fed the 10% 20:1 or 10% 1:1 diets (p-value < 0.001) (Figure 3C). C-reactive protein was significantly higher in the serum mice fed either 10% 1:1 or 10% 20:1 diets compared to mice fed the AIN-93G diet (p-value = 0.001 and 0.03, respectively) (Figure 3E). There were no significant differences in INF-γ, Figure 3E). The ratio of n-6 to n-3 had no effect on serum cytokine or acute phase protein levels. However, the percentage of dietary PUFA had a significant effect on the levels of IL-10, IL-17, and TNF-α. Animals fed diets with a PUFA concentration of 2.5% had elevated serum levels of these specific cytokines compared to animals on the 10% PUFA diets (p-value < 0.05) (Figure 4).

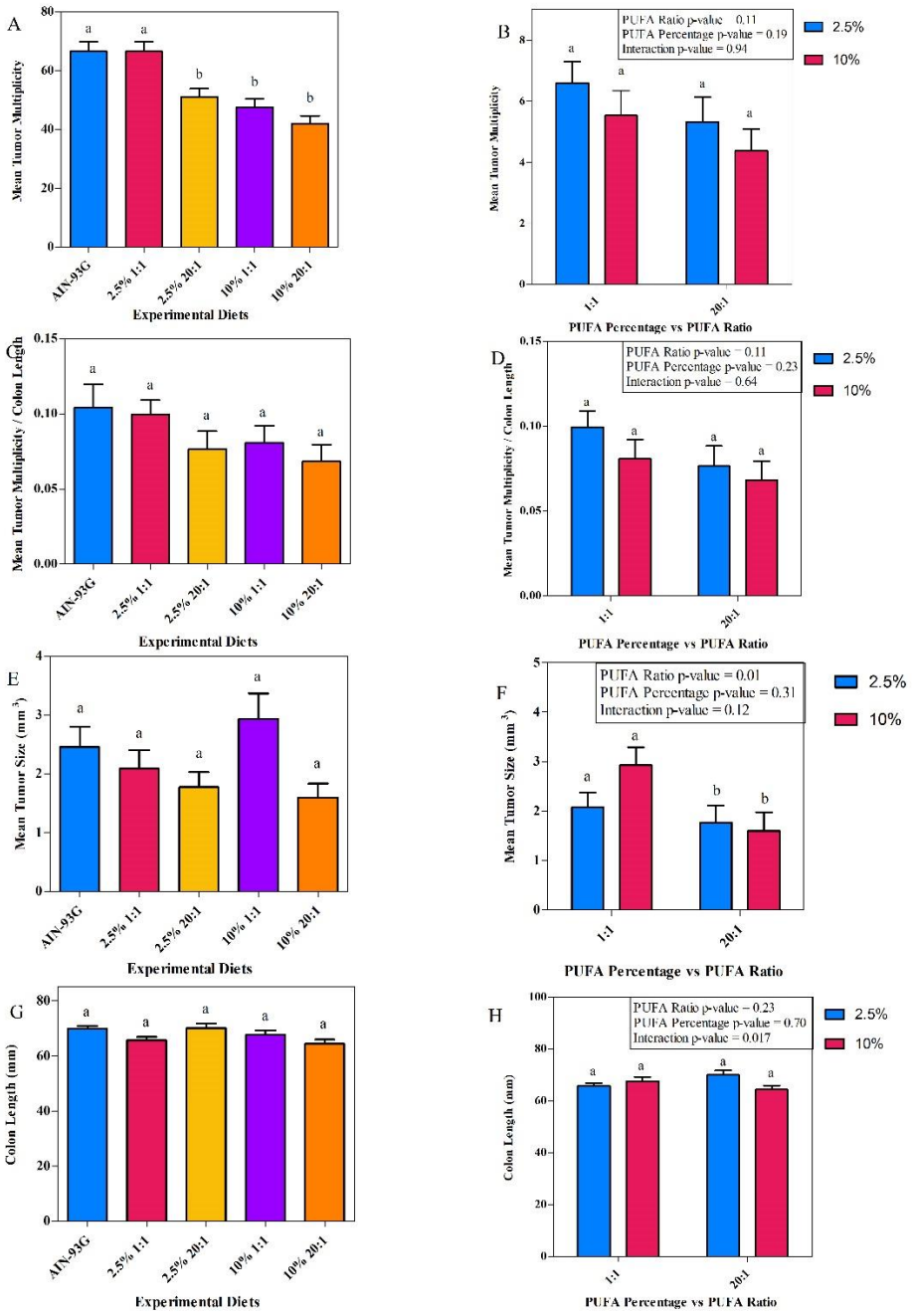

FIGURE 2. Tumorigenic response of mice fed experimental diets and treated with AOM + DSS. Effects of experimental diets on (A & B) tumor multiplicity, (C & D) mean tumor multiplicity/colon length, (E & F) mean tumor size and (G & H) average colon length. Values are expressed as mean ± standard error. Different lettered columns indicate a significant difference between factors (p-value <0.05), which were determined by one-way ANOVA (A), (C), (E), and (G) and Tukey-Kramer HSD post hoc analysis or two-way ANOVA (B), (D), (F), and (H) and Student's *t*-test.

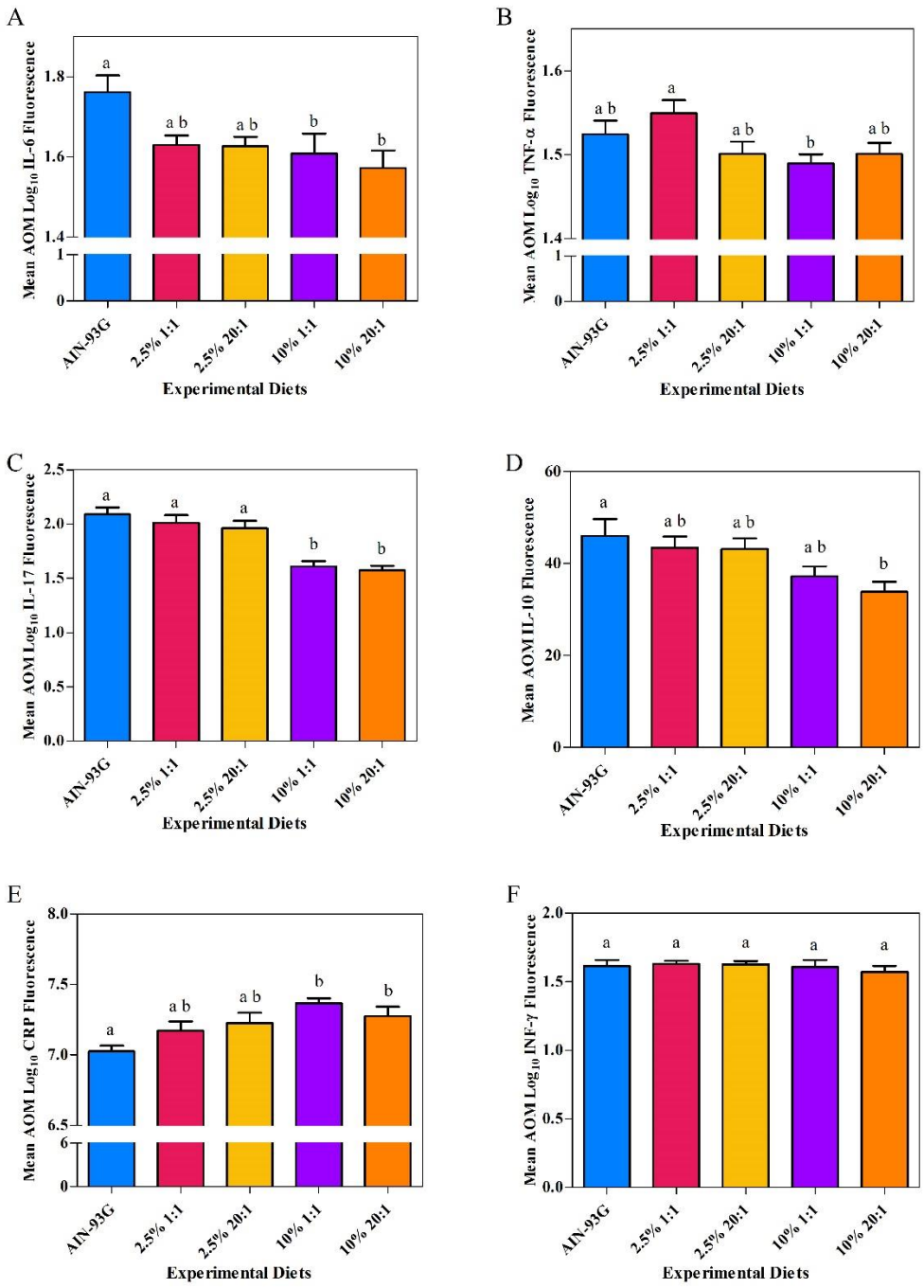

FIGURE 3. Effect of dietary PUFA on systemic inflammation after AOM + DSS treatment. Data for: IL-6 (A), TNF-α (B), IL-17 (C), CRP (E), and INF-γ(F) are log_{10} transformed mean cytokine or acute phase protein fluorescence ± standard error. IL-10 (D) is mean cytokine fluorescence ± standard error. Different lettered columns indicate a significant difference between factors (p-value <0.05), which were determined by one-way ANOVA and Tukey-Kramer HSD post hoc analysis.

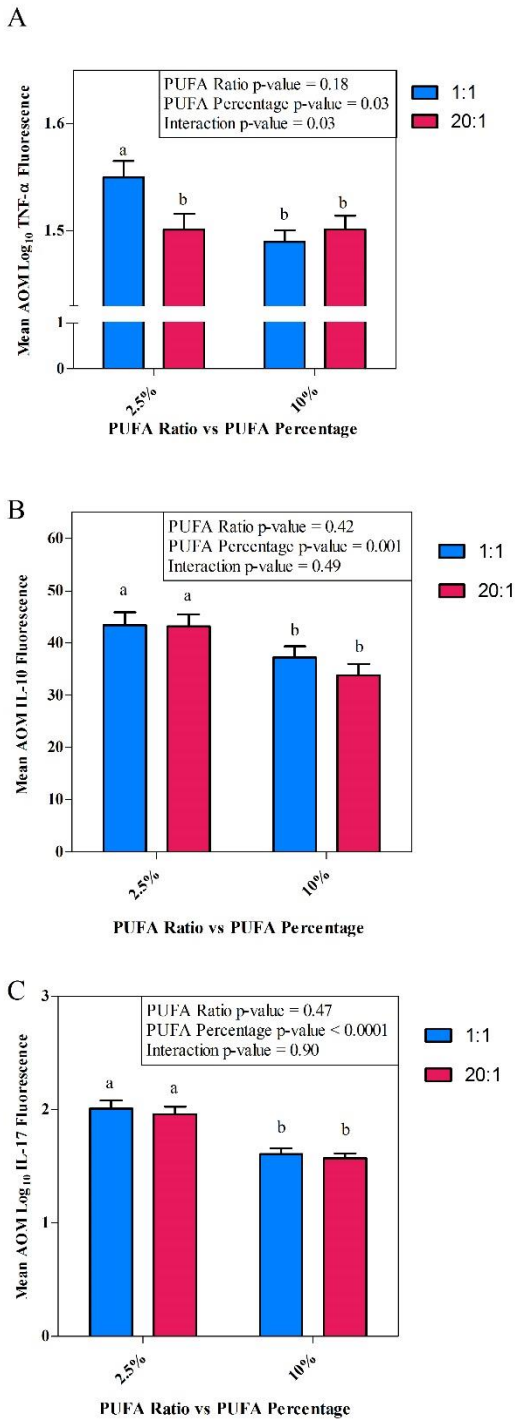

FIGURE 4. Effect of dietary PUFA ratio and PUFA percentage after AOM + DSS treatment. TNF-α (A) and IL-17 (C) are $\log_{10}$ transformed mean cytokine fluorescence ± standard error. IL-10 (B) is mean cytokine fluorescence ± standard error. Different lettered columns indicate a significant difference between factors (p-value <0.05), which were determined by two-way ANOVA and Student's *t*-test.

Fatty Acid Profile of Red Blood Cells

The fatty acid profiles of red blood cells (RBC) from mice on each of the five experimental diets, and from both the AOM + DSS and sham treatment were compared. Figure 5 depicts the fatty acids that were present at >1% in the RBC membranes. Animals fed the 10% PUFA diets had a significantly higher level of C18:2n-6 in the RBC membranes compared to those fed the 2.5% PUFA diets (p-value <0.0001). Interestingly, the AIN-93G diet, which contained only 7% PUFA resulted in significantly higher percentages of C18:2n-6 (p-value < 0.005) than those on the 10% PUFA diets (Figure 5). While the relative proportion of dietary fatty acids in the RBC membranes were influenced by the concentration of PUFA, the n-6 and n- 3 derived fatty acids: C20:4n-6, C20:5n-3, C22:2n-6, C22:5n-6, and C22:6n-3 were significantly affected by the ratio of PUFA in the diet (p-value < 0.0001). It should be noted, C18:3n-3 did not contribute >1% of the total fatty acids present in RBC membranes for any of the experimental diets, but higher percentages of the n-3 derived highly unsaturated PUFAs, EPA and DHA were observed (Figure 5). Compared to the AOM + DSS treated mice, shams had a higher percent of C18:0, C18:1n-9, C18:2n-6 and C20:3n-6 (p-value < 0.0001, p-value = 0.04, p-value < 0.0001, and p-value = 0.01, respectively).

Fatty Acid Profile of Colon Mucosa

The effect of the experimental diets on the fatty acid profile of colon mucosa from the AOM + DSS initiated mice was also analyzed. Animals fed the 10% 20:1 diet or the AIN-93G diet had a significantly higher level of C18:2n-6 in their colon mucosa compared to those fed the 2.5% PUFA diets. Significantly higher amounts of C18:1n-9 were measured in animals on the 2.5% PUFA diets compared to the 10% PUFA diets. Approximately 80% of the fatty acids present in the colon mucosa were C16:0, C18:1n-9 or C18:2n-6 (Figure 6), while none of the highly unsaturated n-6 or n-3 derived fatty acids were observed at percentages greater than 1%.

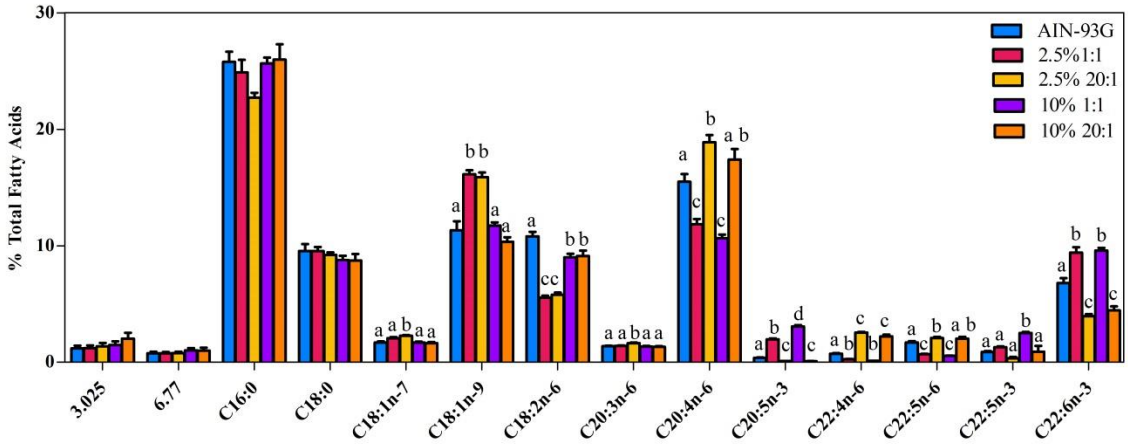

FIGURE 5. Fatty acid profile of red blood cells from mice fed experimental diets. Only fatty acids present at >1% of total fatty acids are shown. Values are expressed as percent of total fatty acids ± standard error. Different lettered columns indicate a significant difference between factors (p-value <0.05), which were determined by one-way ANOVA and Tukey-Kramer HSD post hoc analysis.

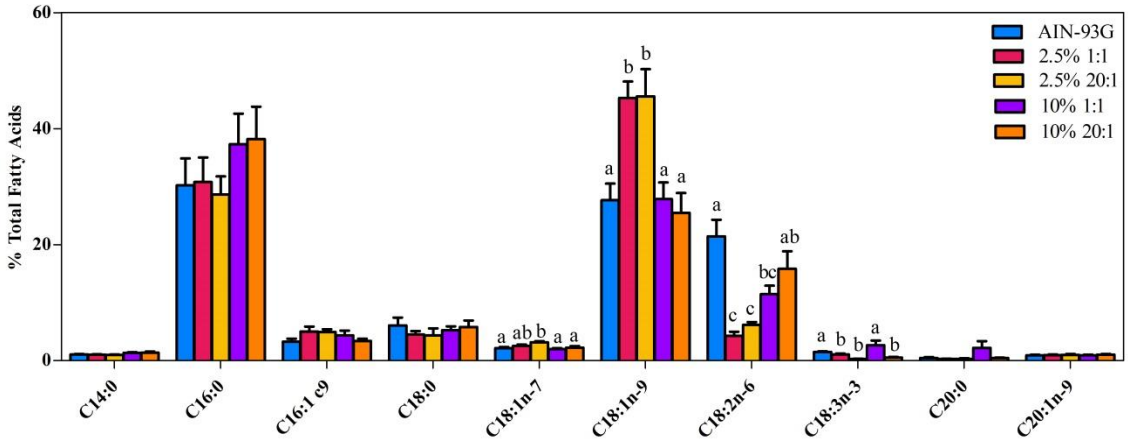

FIGURE 6. Fatty acid profile of colon mucosa from mice fed experimental diets. Only fatty acids present at >1% of total fatty acids are shown. Values are expressed as percent of total fatty acids ± standard error. Different lettered columns indicate a significant difference between factors (p-value <0.05), which were determined by one-way ANOVA and Tukey-Kramer HSD post hoc analysis.

Fecal Metabolome Analysis

GC-MS analysis of fecal samples from both the sham and AOM + DSS treated animals detected 98 compounds, of which 57 were positively matched to the NIST library (Tables 4 & 5 and Tables 8 & 9). A majority of these metabolites belong to the following classes: amino acids, organic acids, fatty acids, nucleic acids, and organic molecules. Of the 98 detected compounds, 41 were considered "unknowns" due to the inability to positively match retention indexes (RI) to the NIST database (Table 6 & 7 and Tables 10 & 11). Statistical analysis using one-way ANOVA revealed the experimental diets had a significant effect on five metabolites from the sham mice and eight metabolites from the AOM + DSS treated mice (Table 4 & 5). When analyzing the data by treatment group, sham or AOM + DSS, treatment had a significant effect on 15 metabolites. AOM + DSS treated mice had a significantly higher amount of the following metabolites: acetic acid, L-proline, L-aspartic acid, D-(-)-Rhamnose, benzeneacetic acid, butanedioic acid, and N-acetyl-D-glucosamine (p-value < 0.05). Sham mice had significantly higher amounts of: dopamine, docosanoic acid, 5-methyl-pyrimidine, adenine, and tetracosanoic acid (p-value < 0.05). The principal component analysis (PCA) of the positively identified metabolites showed distinct separation of the AOM + DSS treated and sham mice (Figure 7 & 10). There was distinct separation of experimental diets based on the dietary PUFA ratios in both the PCA plots for the sham and AOM + DSS treated mice (Figure 8 &9). In the sham treated mice, the 10% 1:1 diet was closely associated to a majority of the metabolites, while the AIN-93G diet was clearly separate from the detected metabolites (Figure 8). However, in the AOM + DSS treated mice, AIN-93G was clustered closely with a majority of metabolites (Figure 9). Plotting individual mice within each experimental diet (Figure 10) we see separation between AOM + DSS and sham treated mice, along with some minor clustering of mine within similar experimental diets.

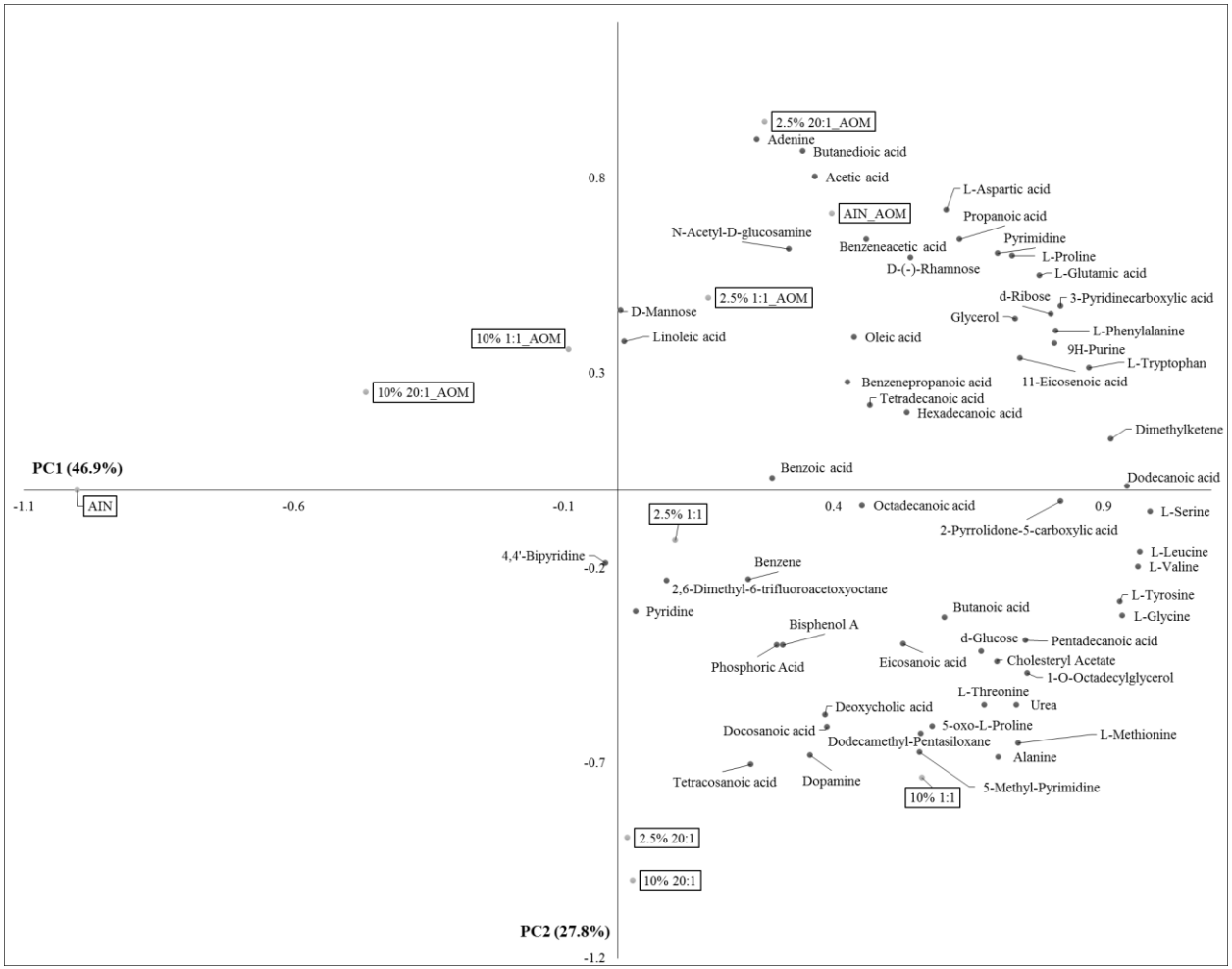

FIGURE 7. Principal component analysis for fecal metabolites found in mice fed different experimental diets. Principal components (PC) were determined based on the variation between the average amounts of each individual metabolite detected in response to different experimental diets. The experimental diets were plotted with respect to the PC's determined from the metabolites. The fecal samples were analyzed using GC-MS and metabolites were identified using AMIDS and NIST mass spectral library. SAS was used to develop the PCA plots.

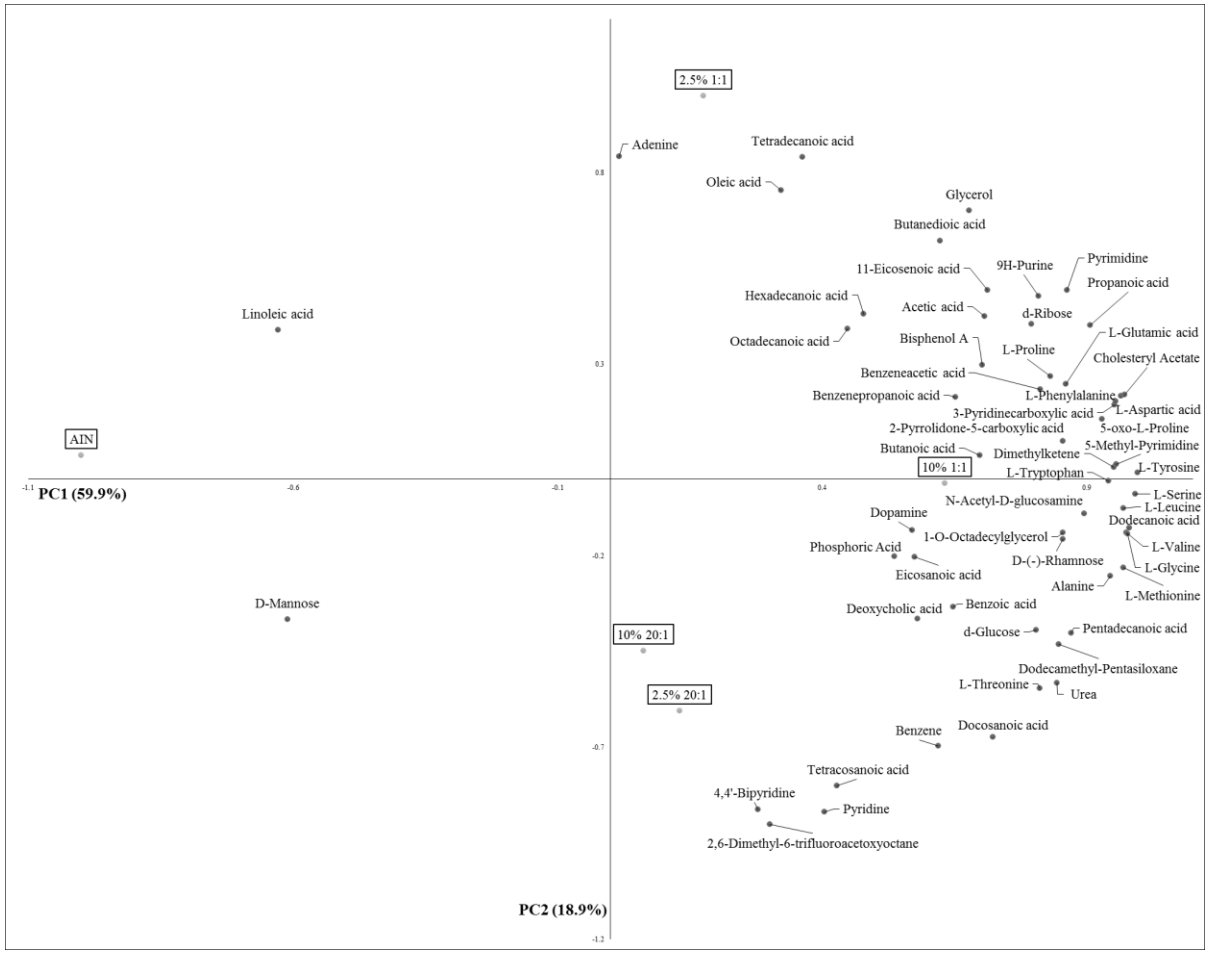

FIGURE 8. Principal component analysis for fecal metabolites found in sham treated mice fed different experimental diets. Principal components (PC) were determined based on the variation between the average amounts of each individual metabolite detected in response to different experimental diets. The experimental diets were plotted with respect to the PC's determined from the metabolites. The fecal samples were analyzed using GC-MS and metabolites were identified using AMIDS and NIST mass spectral library. SAS was used to develop the PCA plots.

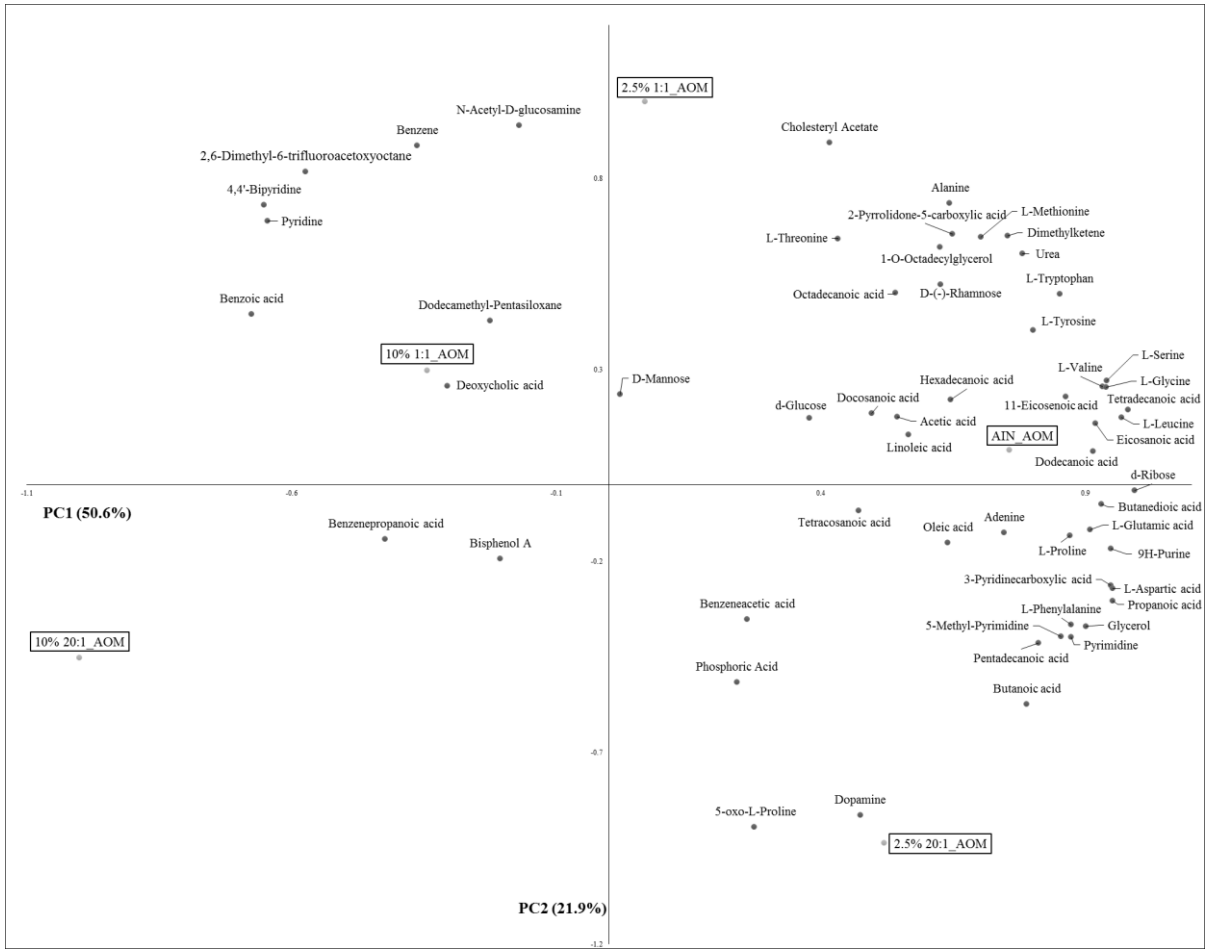

FIGURE 9. Principal component analysis for fecal metabolites found in AOM + DSS treated mice fed different experimental diets. Principal components (PC) were determined based on the variation between the averages amounts of each individual metabolite detected in response to different experimental diets. The experimental diets were plotted with respect to the PC's determined from the metabolites. The fecal samples were analyzed using GC-MS and metabolites were identified using AMIDS and NIST mass spectral library. SAS was used to develop the PCA plots.

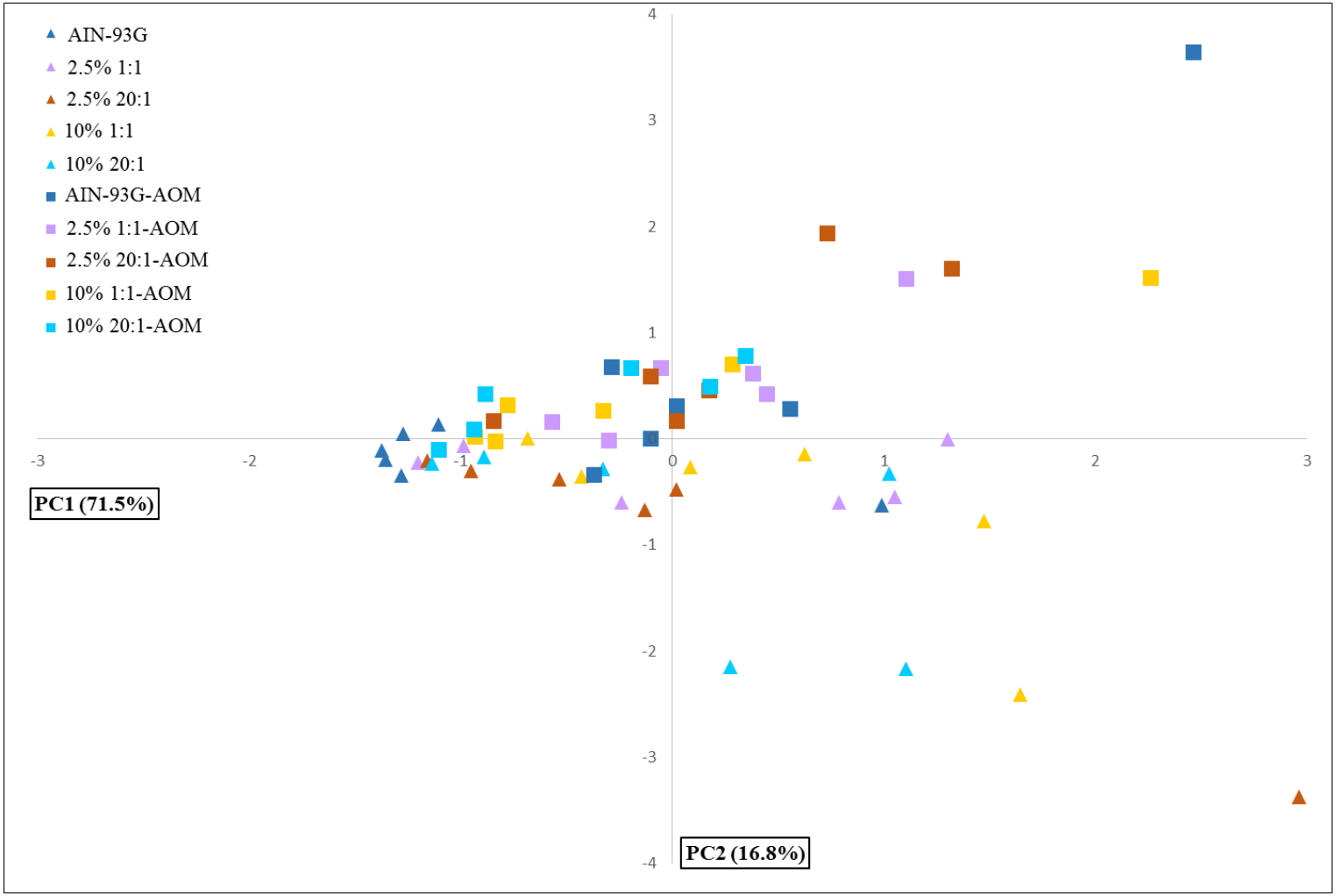

FIGURE 10. Principal component analysis for the fecal metabolomic profiles of mice fed different experimental diets. Principal components (PC) were determined based on the variation between each individual mouse's metabolomics profile. The fecal samples were analyzed using GC-MS and metabolites were identified using AMIDS and NIST mass spectral library. SAS was used to develop the PCA plots.

TABLE 4. Identified metabolites significantly different in sham mice

Metabolites	**LRI[2]**	**Diet Treatment – Sham**						
		AIN-93G[1]	2.5% 1:1[1]	2.5% 20:1[1]	10% 1:1[1]	10% 20:1[1]	SEM	p-value
Acetic Acid	1107	9.80^{ab}	23.00^{ab}	14.88^{ab}	32.14^{a}	8.14^{b}	5.51	0.027
Benzoic Acid	1522	0.49^{b}	0.85^{b}	7.20^{ab}	10.38^{a}	0.57^{b}	2.04	0.004
Benzeneprop-anoic Acid	1736	3.60^{b}	16.38^{b}	1.81^{b}	54.89^{a}	6.97^{b}	9.17	0.002
Oleic Acid	2211	242.32^{b}	1612.52^{a}	698.36^{ab}	440.57^{ab}	339.08^{b}	297.09	0.021
Octadecanoic Acid	2235	557.84^{b}	3329.22^{a}	2851.16^{ab}	1350.05^{ab}	961.49^{ab}	664.12	0.025

^{a,b,c,d} Different lettered superscripts within a row indicate significant difference between mean values (p-value < 0.05) determined by one-way ANOVA and Tukey-Kramer HSD post hoc analysis

[1] Values are expressed as mean ratio

[2] Linear retention index

TABLE 5. Identified metabolites significantly different in AOM + DSS mice

Metabolites	**LRI[2]**	**Diet Treatment – AOM+ DSS**						
		AIN-93G[1]	2.5% 1:1[1]	2.5% 20:1[1]	10% 1:1[1]	10% 20:1[1]	SEM	p-value
Hexadecanoic acid	2047	1644.42	2907.51	2720.59	1396.94	690.81	541.21	0.04
Oleic Acid	2211	634.67^{ab}	1270.62^{ab}	1758.82^{a}	330.86^{b}	169.97^{b}	290.16	0.003
Octadecanoic Acid	2235	1355.93^{bc}	2910.55^{a}	1894.96^{ab}	1070.40^{bc}	416.81^{c}	353.26	0.0006
Eicosanoic acid	2410	157.51	160.90	162.28	91.78	63.15	26.52	0.03
Docosanoic acid	2571	138.71^{a}	39.12^{b}	17.78^{b}	31.10^{b}	38.48^{b}	15.95	<0.0001
Cholesteryl acetate	2772	47.99	59.80	12.49	30.95	11.12	12.37	0.037
Butanoic Acid	1179	23.19	3.46	23.34	7.10	5.61	5.49	0.029
Tetracosanoic Acid	2724	27.50^{a}	2.18^{b}	4.33^{b}	3.18^{b}	8.16^{ab}	4.95	0.007

^{a,b,c,d} Different lettered superscripts within a row indicate significant difference between mean values (p-value < 0.05) determined by one-way ANOVA and Tukey-Kramer HSD post hoc analysis

[1] Values are expressed as mean ratio

[2] Linear retention index

TABLE 6. Unknown metabolites significantly different in sham mice

Unknown Metabolites	LRI	Diet Treatment - Sham						
		AIN-93G	2.5% 1:1	2.5% 20:1	10% 1:1	10% 20:1	SEM	p-value
Unknown 1	2831	2.072[ab]	3.415[ab]	9.639[ab]	13.866[a]	0.000[b]	3.21	0.027

[a,b,c,d] Different lettered superscripts within a row indicate significant difference between mean values (p-value < 0.05) determined by one-way ANOVA and Tukey-Kramer HSD post hoc analysis

[1] Values are expressed as mean ratio

[2] Linear retention index

TABLE 7. Unknown metabolites significantly different in AOM + DSS mice

Unknown Metabolites	LRI	Diet Treatment – AOM + DSS						
		AIN-93G	2.5% 1:1	2.5% 20:1	10% 1:1	10% 20:1	SEM	p-value
Unknown 2	2264	19.674[ab]	24.928[a]	5.656[ab]	0.000[b]	3.800[ab]	5.43	0.012
Unknown 3	2847	50.382[a]	0.000[b]	1.409[b]	12.486[ab]	1.773[b]	10.24	0.008
Unknown 4	3027	201.503	11.527	0.000	145.479	13.587	53.09	0.035

[a,b,c,d] Different lettered superscripts within a row indicate significant difference between mean values (p-value < 0.05) determined by one-way ANOVA and Tukey-Kramer HSD post hoc analysis

[1] Values are expressed as mean ratio

[2] Linear retention index

Discussion

Previous work by our group (unpublished data) and others (60) suggests that dietary PUFA concentrations and ratios play a role in gut inflammation and possibly the development of CRC. To further investigate these findings, experimental diets that were identical except for varying PUFA concentration and ratios of n-6 to n-3, were fed to mice. CRC development was assessed using the AOM + DSS model. Based on our preliminary data, we hypothesized that mice fed a diet with a low concentration of PUFA and a low n-6:n-3 ratio would be protected from CRC compared to mice fed a diet with a high concentration of PUFA and a high n-6:n-3 PUFA ratio. However, contrary to our hypothesis, animals fed either the AIN-93G or the 2.5% 1:1 diets had a higher colon tumor multiplicity compared to animals fed the other three

experimental diets. Yet, when tumor multiplicity was normalized to colon length, there was no longer a significant difference between diet treatments. Colon length was used to evaluate gut inflammation, as studies have shown colon length and gut inflammation have an inverse relationship (13, 57). Our data showed a significant interaction between the main effects of PUFA concentration and PUFA ratio on colon length (p-value = 0.017) (Figure 2H). However, there were no significant differences between experimental diets when compared via post hoc analysis. Our hypothesized inflammatory diet, 10% 20:1, did trend toward the shortest colons compared to mice fed the other experimental diets, but it also trended toward the smallest mean tumor size. In our study, the ratio of PUFA had a more significant impact on tumor size than the percent of PUFA, with diets having a PUFA ratio of 1:1 resulting in larger tumors compared to mice fed diets with a PUFA ratio of 20:1. Interestingly, animals fed the intermediate diet, AIN- 93G (7% PUFA and 7:1 n-6:n-3 ratio) had the highest number of tumors and the second highest tumor severity amongst the other experimental diets. This is relevant as the average American diet has a dietary PUFA concentration 8% and an n-6:n-3 ratio of 10:1 (61). In a similar study, it was reported that rats fed a high-fat diet containing a diverse fatty acid profile, similar to the Western diet, resulted in significantly higher tumor incidence, compared to rats fed a diet high in long chain n-3 fatty acids, EPA and DHA, in response to AOM (60). This study suggested that both the amount and the type of dietary fatty acid play a role in the etiology of CRC. Many studies investigating the effects of dietary n-6 and n-3 fatty acids in CRC use experimental diets containing the highly unsaturated 20- and 22-carbon forms of n-6 and n-3, like AA, EPA, and DHA (39, 60). In our study, the experimental diets used contained n-6 and n-3 fatty acids in their 18-carbon form, LA or ALA. We incorporated the 18-carbon forms of n-6 and n-3 in the experimental diets because approximately 99% of the dietary PUFA consumed by Americans is in this form (56). Using the 18-carbon forms of n-6 and n-3 fatty acids will allow the dietary fatty

acids to follow a similar desaturation pathway that occurs in humans and allow us to observe any changes that may occur in this pathway in response to AOM + DSS.

Previous work has demonstrated that membrane fatty acid profiles are reflective of the relative proportion of dietary fat consumed (38-40). Our study confirmed these findings, as dietary fat intake directly influenced the fatty acid profile of RBC membranes and colon mucosa. Previous studies have suggested the fatty acid distribution of certain membranes can influence disease occurrences, such as coronary heart disease and CRC (38, 40, 41). In the fatty acid profiles of the RBCs, the ratio of PUFA in the diet significantly influenced the fatty acid membrane composition for the highly unsaturated fatty acids: C20:4n-6, C22:4n-6, C22:5n-6, C22:5n-3, and C22:6n-3. Data from others would suggest the experimental diets that resulted in higher percentages of AA would produce higher levels of the pro-inflammatory eicosanoids, PGE_2 and LTB_4, in these animals (46). In a study, rats fed a diet high in n-6 fatty acids resulted in higher COX-2 expression and AA derived eicosanoids, compared to rats fed a low-fat or high n-3 fatty acid diet (60). Hawcroft et al. found a significant decrease in PGE_2 in mice fed a diet containing 5% EPA compared to a diet with no EPA (39). Animals in our study that were fed the high n-6:n-3 PUFA ratio diets showed higher percentages of AA in their RBC membranes. However, these diets did not result in an increased tumorigenic response. Measuring the levels of AA derived eicosanoids and COX-2 expression is needed to give us a better understanding of the inflammatory response to these experimental diets.

A study by Coviello et al. found patients with CRC had a significantly higher ratio of n-6:n-3 fatty acids and lower amount of n-3 and n-3 derived fatty acids incorporated into their RBC membranes compared to healthy individuals (41). Our results followed a similar trend, where the AOM + DSS treated mice had a higher n-6:n-3 ratio in their RBC membranes compared to the sham mice, however, this difference was not statistically significant. The fatty acid profile in the colon mucosa was quite different from the profile found in the RBC analysis, with a significantly

higher percentage of the unsaturated fatty acids: C14:0, C16:0, and C20:0, the mono-unsaturated fatty acids: C16:1c-9, C18:1n-7, C18:1n-9, and C20:1n-9, and the PUFAs: C18:2n-6 and C18:3n-3, in colon mucosa. None of the highly unsaturated fatty acids that were present in the RBC membranes were observed in colon mucosa at >1%. Changes in the distribution of fatty acids between RBC membranes and other tissues including, liver (40), adipose (62), and colon mucosa (63, 64) have been reported. In another study, similar results were reported when the fatty acid profile of intestinal polyps were evaluated. When they compared intestinal polyps to normal colon mucosa, a higher ratio of saturated fatty acids to PUFA tended to occur in polyps (63).

The systemic inflammatory response from these experimental diets did not support our proposed hypothesis. There was an inverse correlation between cytokine levels and PUFA concentration regardless of the n-6:n-3 PUFA ratio, for IL-10, IL-17, and TNF-α (Figure 4). Our data suggests there is a threshold in regard to the dietary PUFA concentration in promoting a pro-inflammatory response (Figure 3). Mice fed the AIN-93G control diet, containing 7% PUFA and a 7:1 n-6:n-3 ratio, had significantly higher serum levels of the cytokines IL-6, IL-17, and IL-10, in response to AOM + DSS induced inflammation, compared to the hypothesized inflammatory diet (10% PUFA and 20:1 n-6:n-3 ratio) (p-value = 0.01, p-value = 0.007, and p-value < 0.0001, respectively). In a similar study, the systemic response was measured in mice in response to LPS. Mice that were fed the AIN-76A also had elevated serum levels of pro-inflammatory cytokines, similar to what we saw from our results (42). The experimental diets in this study and our study had a more diverse fatty acid profile compared to either the AIN-93G or AIN-76A diets, which use a single source of fat, either soybean or corn oil, respectively. This suggests the type of dietary fat can influence the inflammatory response. Not all cytokines induce an inflammatory response; IL-10 plays an important role in regulating inflammation in the body. In fact, knockout IL-10 animals have been shown to spontaneously develop symptoms of IBD (50). In our study, mice fed the 10% 20:1 diet had significantly lower serum levels of IL-10 compared to mice fed

the AIN-93G diet. Lower serum levels of IL-10 could indicate why mice fed the 10% 20:1 diet tended to have more gut inflammation than mice on the AIN-93G diet.

The combination of metabolites produced by the gut microbiota and the metabolites and immune response produced by the host can influence colon health and the etiology of CRC (33). An examination of the PCA plots provides valuable insight into how the experimental diet affected the production of different gut metabolites. From the PCA analysis, the AOM + DSS treatment clearly influenced a separation between AOM + DSS and sham treated mice (Figure 7). This separation between AOM +DSS and sham treated mice can also be observed in the PCA plot of individual mice (Figure 10). Dietary PUFA ratio clearly influenced the separation between experimental diets and association with certain fecal metabolites. The sham mice fed the 10% 1:1 diet and the AOM + DSS treated mice fed the AIN-93G diet appeared to be more closely associated with a majority of metabolites compared to the other experimental diets (Figure 8 & 9). While the AIN-93G was closely associated to metabolites in the presence of AOM + DSS, without this induced inflammation the AIN-93G was clearly separated from the detected metabolites. Understanding how these metabolites affect colon health is unclear. Studies have shown that secondary bile acids are believed to have a negative effect on colon health as patients with CRC have been found to have higher levels deoxycholic acid (DCA) (65). Meanwhile, SCFA are believed to have a positive influence on colon health, with higher amounts of butyrate-producing bacteria found in healthy patients compared to individuals with CRC (33).

While the results of this study did not support the proposed hypothesis that a diet high in total PUFA with a high n-6:n-3 ratio would promote inflammation mediated CRC, the data collected presented some novel findings. Diets containing a 1:1 n-6 to n-3 ratio had significantly larger tumors, suggesting that once dysplastic cells were present, this diet advanced tumor growth. Both the type and amount of dietary fat has been shown to play a role in the post-initiation stages of colon carcinogenesis (66). The less prevalent tumor development and severity

associated with the 10% 20:1 diet may have been due to lower levels of pro-inflammatory cytokines associated with this treatment. Also of interest, mice fed the control AIN-93G diet had significantly higher serum levels of pro-inflammatory cytokines compared to mice in the other treatments. Again, it is important to note that all the experimental diets, including the control diet, only varied in the PUFA content and n-6 to n-3 ratio, and the results generated suggest both dietary components played a role in carcinogenesis.

In summary, mice fed diets with a PUFA ratio of 1:1 had significantly larger tumors compared to mice consuming diets with an n-6:n-3 ratio of 20:1 (Figure 2F). The serum cytokine levels for IL-10, IL-17, and TNF-α were significantly higher in mice fed diets containing 2.5% PUFA compared to mice fed the 10% PUFA diets (Figure 4). In regard to the fatty acid profiles, both the ratio and percent of PUFA influenced distribution of fatty acids in RBC membranes and colon mucosa membranes. The highly unsaturated fatty acids were significantly influenced by the dietary n-6:n-3 PUFA ratio, while C18:1n-9 and the essential fatty acid, C18:2n-6, were significantly influenced by the concentration of PUFA in the diet. Fecal metabolites appeared to be influenced by both the dietary PUFA concentration and n-6:n-3 ratio, as well as the AOM + DSS treatment. Our results suggest both the percent and ratio of PUFA play a role in the etiology of AOM+DSS induced CRC. Further research is needed to determine how these two factors interact and influence gut inflammation and the subsequent impact on CRC.

TABLE 8. Identified metabolites in sham mice, supplement

Metabolites	**LRI**[2]	**Diet Treatment – Sham**						
		AIN-93G[1]	2.5% 1:1[1]	2.5% 20:1[1]	10% 1:1[1]	10% 20:1[1]	SEM	p-value
Pryidine	1151	7.25	6.00	15.75	11.42	11.88	5.79	0.76
3-Pyridinecarboxylic acid	1285	9.30	25.63	19.13	29.25	23.71	7.44	0.39
4,4'-Bipyridine	1530	22.92	17.80	40.41	29.17	28.47	15.25	0.87
Propanoic Acid	1099	82.51	430.64	256.09	416.76	300.47	118.86	0.26
L-Valine	1115	399.80	1026.15	1079.18	1534.28	1175.13	394.29	0.39
Alanine	1546	71.34	191.95	219.29	254.54	239.91	73.20	0.43
5-oxo-L-Proline	1527	153.74	443.66	337.52	460.26	433.60	116.28	0.33
L-Proline	1293	29.03	89.82	57.52	146.61	55.76	30.16	0.094
L-Glycine	1136	422.33	937.51	974.96	1285.72	1060.38	253.46	0.21
L-Leucine	1162	345.97	942.07	947.79	1480.09	1007.72	344.72	0.27
L-Serine	1254	193.46	601.55	585.65	839.26	610.05	184.32	0.21
L-Methionine	1404	74.27	169.40	201.10	255.25	197.16	64.99	0.40
L-Aspartic Acid	1422	144.77	350.28	266.81	414.26	317.70	75.37	0.16
L-Glutamic Acid	1635	182.33	390.33	341.49	521.98	238.97	104.58	0.20
Phenylalanine	1638	58.47	265.78	199.71	278.68	218.91	95.18	0.51
L-Tryptophane	2196	23.70	61.23	55.43	99.20	63.61	20.93	0.19
L-Tyrosine	1890	236.51	738.40	686.92	962.45	707.22	262.29	0.42
L-Theronine	1291	312.66	512.24	773.91	849.68	855.83	258.52	0.50
D-Ribose	1713	264.26	718.55	573.07	558.24	478.49	221.27	0.69
D-(-)-Rhamnose	1759	11.38	64.96	83.74	65.45	55.82	27.66	0.45
D-Glucose	1934	381.77	1382.61	1973.64	1548.62	1811.88	582.89	0.36
D-Mannose	2018	118.57	83.98	99.94	104.46	93.22	62.32	0.99
Glycerol	1274	180.11	418.82	196.98	375.98	269.42	114.77	0.50
1-O-Octadecylglycerol	2612	1.33	83.96	65.96	174.33	148.27	46.57	0.10
Dimethylketene	1179	39.96	112.16	102.75	177.65	104.46	34.77	0.13
Dodecamethyl-Pentasiloxane	1182	3.74	17.19	25.29	31.27	32.87	12.86	0.50
Urea	1231	61.01	111.46	178.40	187.72	174.83	49.27	0.32
Phosphoric acid	1272	7.02	9.27	13.22	10.72	6.65	3.61	0.69
Dopamine	1358	57.73	190.16	128.35	198.11	329.44	94.65	0.37
Pryimidine	1335	78.02	153.39	111.37	155.57	108.44	51.54	0.80
Benzeneacetic Acid	1647	27.79	148.40	70.65	288.35	102.17	66.04	0.087
Dodecanoic Acid	1655	33.89	62.13	67.60	85.89	63.52	16.26	0.29
2-Pyrrolidone-5-carboxylic Acid	1536	146.67	259.18	259.88	390.44	188.06	88.15	0.38
9H-Purine	1815	33.93	105.80	77.85	84.82	62.29	33.64	0.64
Hexadecanoic acid	2047	488.53	3149.95	2438.74	1229.87	1199.92	653.07	0.054
Linoleic Acid	2205	245.14	158.10	69.63	165.53	153.97	72.77	0.58
Eicosanoic acid	2410	54.97	208.71	323.14	162.98	145.28	71.69	0.15
Docosanoic acid	2571	54.13	71.91	131.76	118.45	111.76	35.39	0.50
Cholesteryl acetate	2772	9.49	62.90	45.10	75.39	48.48	22.48	0.33
Deoxycholic Acid	3058	49.40	167.37	172.26	285.78	449.84	99.71	0.088
Benzene	1250	1.61	2.55	5.21	3.72	4.39	2.18	0.78
Butanedioic Acid	1307	0.00	5.03	2.65	3.22	0.00	2.11	0.40
Tetradecanoic Acid	1849	8.21	42.89	13.75	15.94	13.40	8.65	0.066
5-Methyl-Pyrimidine	1401	6.62	20.90	17.10	25.54	22.33	9.34	0.66
Adenine	1873	5.35	7.02	2.67	6.01	1.15	3.66	0.77
11-Eicosenoic acid	2388	0.00	124.53	17.60	104.38	97.42	39.96	0.13
Pentadecanoic Acid	1915	6.86	27.10	37.49	44.40	45.90	16.87	0.48
Butanoic Acid	1179	8.43	19.49	11.93	66.34	18.80	19.61	0.25
2,6-Dimethyl-6-trifluoroacetoxyoctane	1109	2.72	0.56	5.74	4.90	3.97	1.73	0.27
Bishenol A	2220	2.55	26.60	24.72	18.42	8.66	9.51	0.34
Tetracosanoic Acid	2724	16.46	6.65	47.05	38.54	25.60	13.90	0.27
N-Acetyl-D-glucosamine	2117	0.00	75.28	100.48	89.95	50.41	46.65	0.58

TABLE 9. Identified metabolites in AOM + DSS mice, supplement

Metabolites	**LRI[2]**	**Diet Treatment – AOM + DSS**						
		AIN-93G[1]	2.5% 1:1[1]	2.5% 20:1[1]	10% 1:1[1]	10% 20:1[1]	SEM	p-value
Pryidine	1151	8.61	14.16	5.90	16.38	12.57	4.22	0.42
3-Pyridinecarboxylic acid	1285	40.33	25.80	40.10	27.37	17.20	11.30	0.55
4,4'-Bipyridine	1530	28.82	38.99	17.74	35.85	37.28	10.99	0.64
Propanoic Acid	1099	1137.80	558.81	1170.78	379.87	177.09	424.76	0.37
Acetic Acid	1107	72.24	53.30	51.53	56.05	52.65	15.24	0.86
L-Valine	1115	1189.16	1133.25	1153.76	1022.51	684.20	271.56	0.68
L-Alanine	1546	171.55	172.88	118.48	129.12	99.95	32.35	0.42
5-oxo-L-Proline	1527	280.78	185.78	303.37	250.02	262.59	56.21	0.65
L-Proline	1293	148.56	136.20	181.68	135.84	77.15	42.27	0.54
L-Glycine	1136	982.96	939.25	951.21	880.70	687.54	147.19	0.64
L-Leucine	1162	1105.56	1025.89	1074.16	874.79	605.92	239.17	0.58
L-Serine	1254	697.37	662.20	665.33	595.41	422.59	142.57	0.67
L-Methionine	1404	156.31	158.78	122.92	118.82	104.74	31.46	0.68
L-Aspartic Acid	1422	927.79	603.09	920.31	432.23	366.23	265.86	0.43
L-Glutamic Acid	1635	546.26	533.56	646.09	392.80	312.17	142.71	0.50
L-Phenylalanine	1638	367.92	274.91	434.64	155.17	172.75	109.74	0.33
L-Tryptophane	2196	100.51	94.33	83.58	84.76	55.82	25.15	0.76
L-Tyrosine	1890	650.78	720.67	677.31	612.47	435.21	179.67	0.82
L-Theronine	1291	756.58	616.85	449.81	634.22	478.25	149.33	0.60
d-Ribose	1713	806.11	655.68	772.45	612.01	430.36	211.91	0.74
D-(-)-Rhamnose	1759	149.58	124.71	108.92	139.46	86.07	43.87	0.86
d-Glucose	1934	1047.18	1471.33	1562.71	1494.32	856.32	383.12	0.62
Mannose	2018	158.95	120.28	130.24	258.63	103.51	71.68	0.57
Glycerol	1274	402.88	317.42	482.81	279.52	218.11	97.63	0.36
1-O-Octadecylglycerol	2612	103.96	92.56	52.56	54.84	48.03	28.07	0.51
Dimethylketene	1179	150.25	154.95	126.22	131.29	91.72	32.37	0.67
Dodecamethyl-Pentasiloxane	1182	16.43	16.40	13.16	27.50	14.51	7.78	0.71
Urea	1231	138.89	135.65	125.36	128.95	112.43	24.04	0.94
Phosphoric acid	1272	6.59	4.93	9.82	9.48	5.65	2.30	0.45
Dopamine	1358	103.64	55.76	118.79	72.96	85.80	27.24	0.52
Pryimidine	1335	197.41	158.81	248.99	132.30	107.29	57.31	0.45
Benzoic Acid	1522	2.84	10.66	4.09	5.62	9.50	2.82	0.25
Benzeneacetic Acid	1647	476.56	235.05	322.64	331.83	366.15	125.05	0.75
Dodecanoic Acid	1655	77.37	76.47	78.82	49.63	44.49	15.22	0.33
Benzenepropanoic Acid	1736	20.73	39.02	33.04	16.43	43.73	12.66	0.50
2-Pyrrolidone-5-carboxylic Acid	1536	304.14	316.92	236.75	223.58	207.93	54.57	0.53
9H-Purine	1815	116.78	89.71	112.48	60.37	58.66	31.49	0.55
Linoleic Acid	2205	383.26	130.07	130.43	191.20	126.07	77.79	0.12
Deoxycholic Acid	3058	228.45	156.81	95.40	173.35	219.22	79.86	0.77
Benzene	1250	3.08	6.09	1.82	5.82	3.55	1.39	0.17
Butanedioic Acid	1307	51.59	33.86	39.83	25.70	23.45	14.41	0.65
Tetradecanoic Acid	1849	24.26	20.82	21.35	16.23	10.51	8.50	0.81
5-Methyl-Pyrimidine	1401	13.81	5.63	11.79	4.01	4.94	2.97	0.094
Adenine	1873	31.41	24.46	34.25	32.66	16.88	10.85	0.78
11-Eicosenoic acid	2388	203.93	111.23	98.49	73.32	37.88	48.12	0.19
Pentadecanoic Acid	1915	39.68	13.51	37.04	25.62	12.38	10.99	0.28
2,6-Dimethyl-6-trifluoroacetoxyoctane	1109	3.36	5.83	2.23	5.09	4.65	1.63	0.55
Bisphenol A	2220	6.26	1.24	10.47	22.39	7.12	7.01	0.30
N-Acetyl-D-glucosamine	2117	198.46	289.27	156.06	268.41	185.61	91.52	0.82

TABLE 10. Unknown metabolites in sham mice, supplement

Unknown Metabolites	LRI	Diet Treatment - Sham						
		AIN-93G	2.5% 1:1	2.5% 20:1	10% 1:1	10% 20:1	SEM	p-value
Unknown 1	1076	23.44	21.26	48.95	33.85	34.81	15.14	0.71
Unknown 2	1097	1.871	2.311	4.390	5.667	6.486	1.36	0.10
Unknown 3	1397	393.89	930.66	996.49	1578.34	928.15	386.07	0.34
Unknown 4	1590	7.148	8.227	0.821	16.506	28.759	9.88	0.34
Unknown 5	1480	0.000	10.921	2.110	10.683	14.531	6.04	0.39
Unknown 6	1290	0.000	165.369	72.628	206.189	148.831	0.98	0.44
Unknown 7	2431	8.290	40.041	66.045	41.838	61.475	20.58	0.32
Unknown 8	2068	2.972	28.433	48.103	24.311	30.292	14.93	0.35
Unknown 9	1265	0.723	9.370	5.300	10.184	12.304	4.20	0.34
Unknown 10	1318	4.250	15.756	6.581	11.446	25.139	6.24	0.17
Unknown 11	1862	17.987	71.277	67.828	87.146	157.946	41.66	0.24
Unknown 12	2440	5.773	38.098	68.618	49.440	106.108	29.24	0.20
Unknown 13	3188	363.69	1141.82	1222.37	1122.74	1376.75	360.00	0.34
Unkno wn 14	1122	0.391	0.074	1.014	0.732	0.915	0.39	0.44
Unknown 15	2197	9.61	12.71	14.80	21.64	6.56	6.62	0.57
Unknown 16	1156	2.437	1.740	11.405	3.889	9.980	2.96	0.088
Unknown 17	1212	2.945	5.129	9.268	7.688	13.284	2.68	0.099
Unknown 18	1957	1.851	6.929	5.806	6.754	2.863	2.24	0.38
Unknown 19	3294	1.553	18.566	2.092	6.896	7.937	7.02	0.45
Unknown 20	2021	0.335	4.729	5.119	2.791	0.883	1.70	0.20
Unknown 21	1291	2.512	0.823	2.490	3.147	0.475	1.65	0.73
Unknown 22	1682	1.363	1.726	0.000	2.550	0.163	1.08	0.43
Unknown 23	1086	0.360	1.164	1.189	1.709	1.819	0.92	0.81
Unknown 24	1754	0.000	1.054	0.432	5.220	0.000	1.69	0.18
Unknown 25	2264	0.437	2.082	2.740	5.674	0.000	2.97	0.68
Unknown 26	2955	26.769	0.000	10.357	24.429	34.999	16.37	0.58
Unknown 27	1938	386.033	975.765	911.513	1369.803	976.799	337.85	0.39
Unknown 28	2757	3.797	25.762	3.484	62.893	39.056	15.56	0.060
Unknown 29	2946	24.874	74.452	88.096	75.859	96.177	28.98	0.47
Unknown 30	2956	211.826	1324.562	733.643	1349.029	1265.731	417.17	0.26
Unknown 31	3047	137.191	419.843	351.234	397.996	524.824	135.55	0.37
Unknown 32	3085	153.549	807.565	574.533	916.394	1258.524	282.12	0.11
Unknown 33	1120	1.002	1.030	2.575	2.963	1.743	0.86	0.39
Unknown 34	2847	12.269	0.000	1.255	7.139	10.946	7.04	0.65
Unknown 35	3027	34.551	18.020	23.652	168.923	30.306	40.43	0.068
Unknown 36	1085	16.974	15.134	36.200	29.457	26.175	12.98	0.77
Unknown 37	1291	105.250	176.283	225.995	280.843	231.154	95.28	0.75
Unknown 38	1525	36.014	102.687	77.976	195.757	81.698	47.11	0.21
Unknown 39	1153	47.239	73.101	111.144	101.895	212.797	40.68	0.076
Unknown 40	2140	8.396	26.934	17.387	22.417	12.328	8.03	0.50
Unknown 41	1340	0.000	3.896	2.398	6.019	1.574	1.54	0.096
Unknown 42	2141	5.030	23.254	29.967	8.592	8.229	8.39	0.18

[1] Values are expressed as mean ratio

[2] Linear retention index

TABLE 11. Unknown metabolites in AOM + DSS mice, supplement

Unknown Metabolites	LRI	Diet Treatment – AOM + DSS						
		AIN-93G	2.5% 1:1	2.5% 20:1	10% 1:1	10% 20:1	SEM	p-value
Unknown 1	1076	39.83	39.64	27.12	44.97	37.36	11.24	0.85
Unknown 2	1097	2.573	2.564	1.069	2.117	2.231	1.04	0.84
Unknown 3	1397	1187.67	1158.03	1266.26	934.43	621.56	283.86	0.51
Unknown 4	1590	19.732	10.685	21.717	15.414	15.023	6.19	0.74
Unknown 5	1480	10.163	3.889	5.724	9.911	4.252	3.27	0.50
Unknown 6	1290	226.094	127.161	165.739	136.101	93.433	71.15	0.74
Unknown 7	2431	47.203	16.214	24.927	24.489	16.257	11.61	0.34
Unknown 8	2068	9.245	23.092	27.224	19.088	1.963	8.87	0.28
Unknown 9	1265	17.321	14.085	15.339	14.499	9.166	4.10	0.71
Unknown 10	1318	8.706	3.473	8.588	5.046	9.699	3.20	0.60
Unknown 11	1862	85.990	70.304	73.301	71.508	46.442	21.04	0.76
Unknown 12	2440	25.611	15.277	0.000	2.487	7.032	7.36	0.12
Unknown 13	3188	1063.52	820.39	607.22	661.13	565.00	240.75	0.59
Unknown 14	1122	0.832	0.875	0.000	1.395	0.615	0.38	0.16
Unknown 15	2197	0.623	2.874	0.379	3.706	0.531	0.97	0.064
Unknown 16	1156	3.976	4.578	1.116	3.765	4.613	1.99	0.72
Unknown 17	1212	6.504	7.745	5.348	4.675	6.186	1.60	0.71
Unknown 18	1639	25.70	41.04	66.50	23.57	14.05	15.97	0.19
Unknown 19	1957	5.335	8.011	6.178	5.950	5.643	2.01	0.89
Unknown 20	3294	27.401	19.796	15.550	11.940	5.087	7.66	0.34
Unknown 21	2021	4.647	4.045	3.970	3.249	3.654	1.80	0.99
Unknown 22	1291	17.506	4.536	8.001	5.169	5.456	3.78	0.12
Unknown 23	1682	6.629	6.677	11.264	2.817	2.796	3.18	0.33
Unknown 24	1086	2.670	1.044	1.064	1.021	0.599	0.57	0.12
Unknown 25	1754	9.485	3.189	1.355	0.692	4.365	2.40	0.11
Unknown 26	2955	0.000	0.000	233.149	51.833	0.000	106.81	0.48
Unknown 27	1938	1284.165	1094.035	1047.349	772.773	627.285	246.70	0.36
Unknown 28	2757	77.180	42.290	27.878	22.854	15.605	18.8	0.19
Unknown 29	2946	80.961	48.861	38.113	51.502	29.577	24.20	0.63
Unknown 30	2956	1483.341	1664.687	883.719	860.346	502.774	337.34	0.12
Unknown 31	3047	551.642	442.625	271.904	248.834	185.416	135.33	0.31
Unknown 32	3085	715.068	474.951	671.448	710.646	316.965	236.55	0.70
Unknown 33	1120	2.145	2.650	1.399	1.847	1.918	0.64	0.73
Unknown 34	1291	194.725	110.196	17.974	89.353	142.167	42.68	0.082
Unknown 35	1525	154.288	136.587	152.514	112.901	67.969	46.22	0.66
Unknown 36	1153	99.561	96.206	102.089	95.833	89.155	23.33	0.10
Unknown 37	2831	0.000	0.000	0.000	0.000	0.273	0.12	0.43
Unknown 38	2140	21.949	22.331	13.344	10.337	12.062	4.75	0.25
Unknown 39	1340	1.322	2.271	5.187	5.009	4.179	2.45	0.74
Unknown 40	2141	17.582	2.825	9.765	0.000	1.096	5.26	0.13

[1] Values are expressed as mean ratio

[2] Linear retention index

CHAPTER 3

THE DEVELOPMENT OF THE TOTAL WESTERN DIET-2

Abstract

Rodents are commonly used as models for studying various human diseases. Typically these animals are fed a standardized diet developed to optimize growth, however when comparing these diets to what the average American consumes, their nutrient densities are very different. Because diet is known to impact gut health and disease, Hintze and colleagues developed the Total Western Diet (TWD). By using data collected from the NHANES survey and translating it to a rodent diet using a nutrient density approach, they were able to create a standardized rodent diet that matches both the macro- and micronutrient content of the average American diet. However, the complex dietary components of whole-foods, like dietary fiber, can also impact gut health, so the TWD was redesigned to incorporate the use of whole foods in the formulation. Using the original TWD formulation and the USDA Food Commodities Database, the TWD-2 incorporates whole-foods while matching both the macro- and micronutrient content of the average American diet. The TWD-2 will help replicate the complex relationship between the gut microbiome and the human digestive system in rodents. Use of this diet can more accurately emulate the complexities of the human digestive system making the rodent a better model for human disease research.

Introduction

The influence of diet composition and the resulting gut microbiome is a relatively new area of research and is growing rapidly. Studies have demonstrated that diet has a major impact

on the gut microbiome of the host. In addition, a strong link between gut microbiome composition and colon health has been established (26-28). This strong relationship between diet, gut microbiome, and colon health points to a potential issue in results generated from studies using the current rodent diets to research colon diseases. When comparing the AIN rodent diets to the typical Western diet consumed by people in most developed nations there are several meaningful differences in the nutrient composition. Currently, the only rodent diet developed that accurately matches the nutrient composition of the average American diet is the Total Western Diet (TWD) (4). Unlike the AIN diets, the TWD gave researchers a formulated diet that replicated Western intakes. The compositional differences between the AIN and TWD diets suggest that the AIN developed diets may potentially skew research results when investigating interactions between diet and chronic disease. For example when comparing fiber levels, the TWD contains approximately half the amount of dietary fiber found in the AIN-93G diet. The research literature pertaining to dietary fiber and colorectal cancer suggests there is an inverse relationship between these two factors (31, 67). Individuals that consume diets rich in refined-gains had roughly a 40% increased risk of rectal cancer; where as individuals who consumed more whole-grains reduced their risk of rectal cancer by approximately 30% (67). Enzymes produced by bacteria in the colon are able to cleave the undigested insoluble fiber and release bioactive plant metabolites. These plant metabolites are able to turn on endogenous antioxidant genes which have been shown to protect DNA from oxidative damage through the sequestering of reactive oxygen species (32). Along with the dietary fiber content, the fatty acid profile of the AIN diets and the TWD are vastly different. This variation is significant as some studies have examined the effects of dietary fat intake on colon cancer and found that not only do the type of fatty acids effect cancer incidence, but the composition of fatty acids within the diet can impact

colorectal cancer occurrence as well (68, 69). Studies have also shown an inverse relationship between calcium and vitamin D intakes and CRC occurrence (70, 71). This is a quite relevant as there is more than twice the amount of calcium and almost three times more vitamin D in the AIN-93G than the TWD (Table 2).

Unlike the typical human diet, which is composed of diverse and highly complex foods, the AIN and TWD diets were developed using purified ingredients including: cornstarch, casein, sucrose. Additionally, cellulose is used as the single source of fiber (4, 72). With this type of diet formulation the majority of the nutrients are absorbed in the small intestine. By limiting the amount of nutrients that reach the large intestine, the gut microbiota composition will consequently reflect this lack of fermentable substrates. Previous research has shown this lack of complexity found in the AIN diets could potentially have a major impact on the gut microbiome of the test subjects and could therefore obscure research results when investigating the role of the Western dietary pattern in the development of colorectal cancer in humans (4).

In a study investigating how dietary factors affect the gut microbiota of individuals, the microbiome of two different diet groups, a Western Diet and a rural, well-balanced diet, were compared. Results from this study found a complete separation of the hierarchical clustering of bacteria between these two diet groups. In addition, the microbiome of the rural, well-balanced diet had a higher microbial richness and diversity (29). These results are important, as intestinal inflammation is believed to be associated with a decrease in amount and complexity of bacteria in the gut (73). Research has shown approximately 60% of the gut microbiota diversity is directly associated with an individuals diet and only 10% correlated to genetics (26). The dietary components that are not completely digested and absorbed in the upper digestive tract are a source of energy for the gut microbiota found in the colon (26). Short-chain fatty acids (SCFA)

including acetate, butyrate, and propionate are produced by bacteria as a byproduct of fermentation (31). The presence of butyrate and butyrate-producing bacteria has been shown to have a protective affect against CRC (33).

To better understand the correlations between the Western diet and human chronic disease, a different dietary model is needed to better replicate the actual human digestive experience when using rodent models. For that reason, a whole-food based rodent diet that emulates American dietary patterns was created, called the Total Western Diet 2 (TWD-2). Like the TWD, the TWD-2 was formulated using the average daily intake of all macro- and micronutrients reported by NHANES (25). Then, using the USDA commodity database, whole foods were selected that contributed more than 0.1% of the daily calories for the average American (43). Therefore, by using whole-food ingredients the TWD-2 matches the macro- and micronutrient composition of an average American diet. Through the use of whole-foods in the formulation, the TWD-2 will not only better emulate American eating patterns, but it will also allow the dietary components of whole-foods, like whole-grains, natural antioxidants, and the full portfolio of dietary fiber sources to influence the gut microbiota of rodents models. The TWD-2 will give researchers a diet that emulates all aspects of American dietary patterns. Use of this diet should make the rodent a better model for human chronic disease research.

Methods

Diet Formulation

The TWD formulation developed by Hintze et al., used the NHANES survey *What We Eat in America* for the years 2007-2008 and translated the average American's daily intake to a

rodent diet using a nutrient density approach (4, 25). The same methodology was used in the development of the TWD-2. The base diet was formulated to match the average American's macronutrient intakes reported by NHANES, which included 543.2 g/kg of carbohydrates, 167.8 g/kg of protein, and 165.4 g/kg of fat on a dry matter basis. Using the USDA *Retail Food Commodities*, food ingredients were added to the diet based on the percentage of calories they contributed to the American diet (43). Ingredients were classified as either carbohydrate or protein foods and added to the formulation to achieve 543.2 g/kg or 167.8 g/kg, respectively. For instance, according to the NHANES survey, sugar accounts for 36.4% of carbohydrate calories, so 543.2 g/kg total carbohydrates x 0.364 = 198 g/kg. Thus, approximately 198 g/kg of sugar was used in the formulation. All carbohydrate and protein food commodities were added to the diet using this methodology. Dairy foods contributed sources of both carbohydrates and proteins, so a combination of whey, casein, and lactose were used. Lean cuts of meat were used as protein ingredients to minimize the initial fat content. Lastly, to achieve a total fat content of 165.4 g/kg diet, initial fat content of the carbohydrate and protein ingredients were analyzed by GC and the fatty acid profile of the diet was matched to the NHANES data by adding a diverse blend of oils (Tables 12-14). Micronutrient analysis was done on a pilot batch of the TWD-2 to identify any vitamin or mineral deficiencies or excesses. A supplement was purchased and added to the diet, which ensured a micronutrient match of the average American diet.

Product Development

Ingredients selected from the *Retail Food Commodity Intake* database were purchased and prepared in Utah State University's Sensory Laboratory.

Table 12. Macronutrient profile of the Total Western Diet 2

Carbohydrate Foods	Ingredients	% Carbohydrate of US Diet	% Protein of US Diet	g/kg Carbohydrate in TWD-2	g/kg Protein in TWD-2	Estimated g/kg Fat in TWD-2	Total Amount of Ingredient (g)
Caloric Sweeteners	50% sugar 50% HFCS	36.4	0.0	204.3	0.0	0.0	242.0
Wheat	Wheat Flour	24.4	10.5	136.9	18.5	0.9	179.5
Potatoes	Potato Flour	6.9	1.9	38.6	3.2	0.08	46.4
Orange juice	Freeze dried Orange juice	6.8	1.5	38.3	2.6	0.4	43.1
Dairy Carb.	Lactose	6.3	0.0	35.1	0.0	0.0	35.1
Corn	Corn flour	5.2	1.4	29.3	3.6	0.8	38.4
Rice	Rice flour	3.3	0.8	18.3	1.4	0.2	22.9
Bananas	Freeze dried banana	2.0	0.0	11.3	0.0	0.1	12.8
Legumes	Dry roasted soybeans	1.7	1.9	9.5	11.5	3.3	29.2
Grapes	Freeze dried grapes	1.5	0.0	8.2	0.0	0.08	8.8
Apple juice	Freeze dried apple juice	1.3	0.0	7.3	0.0	0.04	7.6
Oat	Oat flour	1.1	0.8	6.2	1.4	0.4	9.5
Snap beans	Freeze dried canned beans	1.1	2.0	6.2	1.9	0.3	10.3
Apple	Freeze dried apple slices	0.9	0.0	5.3	0.0	0.04	5.5
Berries	Freeze dried strawberries	0.3	0.0	1.5	0.0	0.03	1.7
Protein Foods							
Dairy Protein	Casein	0.0	22.0	0.0	42.4	0.0	42.4
Beef	Freeze dried cooked ground beef	0.0	17.4	0.0	33.6	0.0	43.3
Chicken/turkey	Freeze dried cooked chicken tenders	0.0	18.5	0.0	35.6	4.1	44.6
Pork	Freeze dried cooked ground pork	0.0	9.9	0.0	19.1	4.7	24.4
Eggs	Dried egg whites	0.0	5.5	0.0	10.6	2.3	13.1
Fish	Freeze dried baked catfish	0.0	4.1	0.0	6.4	2.5	8.9
Peanuts	Roasted peanuts no salt	0.4	1.3	2.2	2.6	0.5	10.4
Tree nuts	Roasted cashews no salt	0.3	0.4	1.6	0.7	2.7	4.8
Fats							
Olive Oil		0.0	0.0	0.0	0.0	23.7	23.7
Soybean Oil		0.0	0.0	0.0	0.0	26.5	26.5
Corn Oil		0.0	0.0	0.0	0.0	13.9	13.9
Lard		0.0	0.0	0.0	0.0	23.7	23.7
Beef Tallow		0.0	0.0	0.0	0.0	21.0	21.0
Anhydrous Milk Fat		0.0	0.0	0.0	0.0	30.7	30.7
Cholesterol		0.0	0.0	0.0	0.0	0.3	0.3
			Totals	560.1	195.1	163.3	1024.5

Table 13. Mineral profile of the Total Western Diet 2

Carbohydrate Foods	Ingredients	Fiber (g)	Ca	P	Na	K	Mg	Fe	Zn	Cu	Se
Caloric sweeteners	50% sugar 50% HFCS	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Wheat	Wheat Flour	4.8	27.0	194.0	4.0	192.0	39.0	2.1	1.3	0.0	0.06
Potatoes	Potato Flour	2.7	30.0	78.0	26.0	465.0	30.0	0.6	0.3	0.0	0.0
Orange juice	Freeze dried Orange juice	0.7	41.0	63.0	4.0	737.0	41.0	0.7	0.2	0.0	0.0
Dairy Carb.	Lactose	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Corn	Corn flour	2.5	52.0	82.0	2.0	101.0	36.0	0.6	0.7	0.0	0.01
Rice	Rice flour	0.5	2.0	22.0	0.0	17.0	8.0	0.08	0.2	0.0	0.0
Bananas	Freeze dried banana	1.3	3.0	9.0	0.0	191.0	14.0	0.2	0.1	0.0	0.0
Legumes	Dry roasted soybeans	2.4	41.0	189.0	1.0	398.0	66.0	1.2	1.4	0.0	0.01
Grapes	Freeze dried grapes	0.4	7.0	5.0	1.0	89.0	2.0	0.1	0.02	0.0	0.0
Apple juice	Freeze dried apple juice	0.1	5.0	5.0	3.0	65.0	3.0	0.08	0.01	0.01	0.0
Oat	Oat flour	0.6	5.0	43.0	2.0	35.0	14.0	0.4	0.3	0.04	0.0
Snap beans	Freeze dried canned beans	3.7	56.0	31.0	360.0	155.0	19.0	1.29	0.3	0.06	0.0
Apple	Freeze dried apple slices	0.9	2.0	4.0	0.0	41.0	2.0	0.05	0.02	0.01	0.0
Berries	Freeze dried strawberries	0.4	3.0	5.0	0.0	29.0	2.0	0.08	0.03	0.01	0.0
Protein Foods											
Dairy Protein	Casein	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Beef	Freeze dried cooked ground beef	0.0	10.0	245.0	71.0	387.0	27.0	3.8	8.4	0.1	0.03
Chicken/turkey	Freeze dried cooked chicken tenders	0.0	18.0	240.0	106.0	299.0	31.0	1.5	2.6	0.08	0.03
Pork	Freeze dried cooked ground pork	0.0	12.0	163.0	52.0	268.0	17.0	0.7	1.6	0.03	0.03
Eggs	Dried egg whites	0.0	8.0	15.0	167.0	147.0	12.0	0.02	0.01	0.02	0.02
Fish	Freeze dried baked catfish	0.0	3.0	86.0	41.0	127.0	8.0	0.1	0.2	0.0	0.05
Peanuts	Roasted peanuts no salt	0.8	6.0	37.0	1.0	68.0	18.0	0.2	0.3	0.07	0.0
Tree nuts	Roasted cashews no salt	0.1	2.0	23.0	1.0	27.0	12.0	0.3	0.3	0.1	0.0
	Totals	21.9	333.0	1539.0	842.0	3838.0	401.0	14.1	18.3	0.5	0.2

Table 14. Vitamin profile of the Total Western Diet 2

Carbohydrate Foods	Ingredients	Thiamin	Riboflavin	Niacin	B6	Folate	B12	A (IU)	D (IU)	E (IU)	K	Choline	Cholesterol
Caloric sweeteners	50% sugar 50% HFCS	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Wheat	Wheat Flour	0.22	0.072	2.24	0.00	0.047	0.00	0.00	0.00	0.11	0.005	18.7	0.00
Potatoes	Potato Flour	0.11	0.024	1.63	0.00	0.012	0.00	0.00	0.00	0.12	0.00	18.3	0.00
Orange juice	Orange juice	0.33	0.11	1.47	0.00	0.11	0.00	737.0	0.00	0.15	0.0004	22.8	0.00
Dairy Carb	Lactose	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Corn	Corn flour	0.086	0.037	0.63	0.00	0.011	0.00	2.00	0.00	0.05	0.00	3.3	0.00
Rice	Rice flour	0.032	0.005	0.59	0.00	0.0001	0.00	0.00	0.00	0.03	0.00	1.3	0.00
Bananas	Freeze dried banana	0.023	0.031	0.36	0.00	0.002	0.00	32.0	0.00	0.05	0.0003	2.5	0.00
Legumes	Dry roasted soybeans	0.12	0.22	0.31	0.00	0.06	0.00	0.00	0.00	0.00	0.011	0.00	0.00
Grapes	Freeze dried grapes	0.043	0.027	0.14	0.00	0.002	0.00	47.0	0.00	0.09	0.0068	2.6	0.00
Apple juice	Apple juice	0.014	0.011	0.047	0.00	0.00	0.00	1.00	0.00	0.01	0.00	1.2	0.00
Oat	Oat flour	0.066	0.012	0.14	0.00	0.003	0.00	0.00	0.00	0.07	0.0003	2.8	0.00
Snap beans	Freeze dried canned beans	0.023	0.066	0.3	0.00	0.041	0.00	517.0	0.00	0.04	0.057	21.5	0.00
Apple	Freeze dried apple slices	0.007	0.01	0.035	0.00	0.001	0.00	21.0	0.00	0.00	0.0008	1.3	0.00
Berries	Freeze dried strawberries	0.005	0.004	0.074	0.00	0.005	0.00	2.00	0.00	0.08	0.0004	1.1	0.00
Protein Foods													
Dairy Protein	Casein & Whey	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Beef	Freeze dried cooked ground beef	0.042	0.21	6.80	0.44	0.007	0.0031	0.00	0.00	0.47	0.0016	110.7	90.0
Chicken/turkey	Freeze dried cooked chicken tenders	0.085	0.22	11.29	0.58	0.007	0.0004	65.0	6.00	0.0003	0.003	97.0	110.0
Pork	Freeze dried cooked pork	0.31	0.30	6.91	0.45	0.00	0.0005	0.00	0.00	0.00018	0.00	62.1	49.0
Eggs	Dried egg whites	0.001	0.33	0.11	0.005	0.002	0.00	0.00	0.00	0.00	0.00	1.1	0.00
Fish	Freeze dried baked catfish	0.008	0.035	0.88	0.065	0.004	0.0012	0.00	4.00	0.34	0.0009	0.00	23.0
Peanuts	Roasted peanuts no salt	0.045	0.01	1.4	0.027	0.015	0.00	0.00	0.00	0.00072	0.00	5.7	0.00
Tree nuts	Roasted cashews no salt	0.01	0.01	0.067	0.012	0.003	0.00	0.00	0.00	0.04	0.0017	2.9	0.00
	Totals	1.58	1.75	35.42	1.58	0.33	0.0052	1424.0	10.0	1.65	0.089	376.9	272.0

The leanest meats were selected to limit added fat. Meats were cooked in aluminum pans without additional oil and catfish was baked according to the manufactures direction Produce and meats were thinly sliced, frozen at-80°C overnight, and then freeze-dried at -80°C in a vacuum freeze-dryer. After freeze-drying, products were processed in a food processor and vacuum sealed in individual bags. Freeze-dried ingredients, added oils, juices, and other dry ingredients including a micronutrient supplement (see Table 17), were added according to the TWD-2 formulation (Table 12). After all ingredients were thoroughly mixed, the complete diet was freeze-dried again to obtain a water activity (a_w) of approximately 0.50, which is similar to a commercial rodent diet.

TWD-2 Fat Analysis - FAMES

To determine the initial fatty acid profile of the TWD-2, the diet was analyzed using direct fatty acid methyl ester (FAME) synthesis according to O'Fallon et al. and samples were analyzed using a GC2010 (Shimadzu) instrument (59). A 1 µL sample was injected into the gas chromatograph at a 150:1 split ratio with an inlet temperature held at 250°C. The temperature program started at 153°C for 2 min, followed by a 2.3°C/min-ramp to 174°C, with a hold time of 20 sec. A second temperature increase was performed with a 2.5°C/min-ramp to a final temperature of 210°C, which was then held for 5 min. Separation was performed on a DB-23 column (30 m length, 0.25 mm diameter and 0.25 µm film thickness) using helium as the carrier gas.

TWD-2 Fat Analysis - Soxhlet

Soxhlet extraction was carried out according to Mariod et al. with slight modifications (74). The TWD-2 was homogenized, placed into cellulose thimbles and soaked in petroleum

ether overnight. Fatty acid extraction was done with petroleum ether in a Soxhlet apparatus for 6 hours. Fat was recovered using a rotary evaporator and any remaining solvent was removed using 99.9% nitrogen.

TWD-2 Micronutrient Analysis

To determine the micronutrient content of the TWD-2, a pilot batch was sent to Medallion Laboratories (Plymouth, MN) for vitamin analysis. USU analytical labs performed the mineral analysis. From these analyses, a micronutrient supplement was formulated to ensure the TWD-2 matched the micronutrient composition of the average American diet.

Results

Initial Fat Content of TWD-2

The fat content of the selected carbohydrate and protein food commodities was analyzed via gas chromatography (Table 15). As expected from the diet formulation, an initial fat content of approximately 2% was calculated. The Soxhlet extraction confirmed these results (data not shown). Taking into account the 2% fat measured in the carbohydrate and protein food commodities, we were able to determine the amount and type of dietary fat needed using the same percentages from the oil blend used in the TWD formulation (Table 16).

TABLE 15. Initial fat analysis of TWD-2 via gas chromatography

Fatty Acid	TWD-2 (g/kg)	Standard Error
1.573	0.01	4.2E-4
C8:0	0.01	2.3E-4
C10:0	0.01	8.4E-4
C12:0	0.02	2.2E-3
C14:0	0.24	2.6E-2
C14:1c-9	0.04	5.1E-3
5.49	0.01	5.7E-4
5.71	0.01	1.3E-3
C15:0	0.04	3.7E-3
C16:0	4.06	2.6E-1
C16:1t-9	0.03	2.8E-3
C16:1c-9	0.35	3.7E-2
C17:0	0.10	9.1E-3
C17:1c[a]	0.50	1.1E-2
C18:0	1.88	1.3E-1
C18:1T	0.16	1.3E-2
C18:1n-9	8.74	2.5E-2
C18:1n-7	0.35	2.6E-2
C18:2n-6	4.28	1.2E-1
C20:0	0.09	7.0E-3
C18:3n-3	0.34	3.1E-3
C20:1n-12	0.01	2.6E-3
C20:1n-9	0.11	5.6E-3
C20:2n-6	0.04	4.2E-3
C22:0	0.10	1.6E-2
C20:3n-6	0.05	3.4E-3
C20:4n-6	0.21	1.3E-2
C20:5n-3	0.02	2.9E-3
C22:4n-6	0.06	5.1E-3
C22:3n-3	0.02	4.4E-4
C22:5n-3	0.03	2.4E-3
C24:0	0.06	8.8E-3
C22:6n-3	0.11	4.8E-3
Total Fat	22.08	

[a] Internal standard

TWD-2 Micronutrient Supplement

TWD-2 pilot batches were submitted for micronutrient analysis to determine the diet's initial composition (Medallion Labs and USU analytical labs). From these analyses a micronutrient supplement was developed and purchased (Harlan Teklad) (Table 17). Currently

there are three nutrients in the TWD-2 that exceed the average American consumption: niacin, vitamin D, and fiber. Both niacin and vitamin D quantities are insignificantly higher but the total fiber is approximately 1.5 times greater than the target amount. Analytical tests were not available for vitamin K and choline, so quantities were estimated through the use to the USDA nutrient database (75).

TABLE 16. Oil blend formulation for TWD and TWD-2

Oil Source	% Oil	TWD Formulation [a]	TWD-2 Formulation [a]
Olive Oil	16.9	28.0	23.7
Soybean Oil	19.0	31.4	26.5
Corn Oil	10.0	16.5	13.9
Lard	16.9	28.0	23.7
Beef Tallow	10.0	24.8	21.0
Anhydrous Milk Fat	21.9	36.3	30.7
Cholesterol	0.2	0.4	0.3
Total Fat	~100	165.4	139.8

[a] Values are all listed as g/kg

Discussion

The TWD-2 was successfully formulated and developed using whole-foods to match both the macro- and micronutrient intake of the average American diet. Fiber quantities were the only nutrient to exceed the reported intake according to the NHANES survey (25). This is important, as studies have shown individuals who consume higher amounts of dietary fiber are at a decreased risk for developing CRC due to the phenolic compounds produced by bacteria breaking down undigested fiber (31, 67). While our fiber content exceeded the data reported by NHANES, there is a potential for the NHANES data to be skewed since it is self-reported. In a redesign of the TWD-2, sources of fiber such as apple and grape skins could be removed in an effort to decrease the amount of fiber within the diet.

TABLE 17. Micronutrient analysis and supplement formulation

Micronutrients	TWD	TWD-2	Micronutrient Supplement
Minerals (mg/kg)			
Ca	2011	900	1111
P	2757	2300	457
Na	7078	4241	2837
K	5333	4700	633
Mg	589	500	89
Fe	31	15.1	15.9
Zn	25	15.7	9.3
Cu	2.6	1.7	0.9
Se	0.2	0.0	0.2
Vitamins (unit/kg)			
Thiamin-HCl (US) (mg)	3.5	2.2	1.30
Thiamin (EU) (mg)	3.5	1.73	1.77
Riboflavin (mg)	4.4	1.5	2.90
Niacin (mg)	50.6	53.4	0.00
Pryidoxine (mg)	3.9	3.09	0.81
Floate (mg)	1.3	0.82	0.48
B12 (mg)	0.011	0.008	0.003
A (IU)	4300	500	3800
D (IU)	391	400	0.00
E (IU)	24.6	15.8	8.80
K (mg)	0.189	0.089	0.10[a]
Choline (mg)	648	376.9	271.1[a]
Cholesterol (mg)	400	373	27
Other			
Fiber[b] (mg)	30000	49000	0.00

[a] Quantities were estimated using the USDA nutrient database
[b] This macronutrient was included in micronutrient analysis

Incorporating whole-foods in the TWD, allows undigested dietary components like dietary fiber and other undigested dietary components to interact with the gut microbiota. Studies have shown SCFAs and bioactive plant metabolites produced from undigested dietary fiber have a protective effect against CRC. Plant metabolites are able to turn on endogenous antioxidant genes which help to prevent cellular damage (32), while SCFA, specifically butyrate, is able to induce apoptosis and help prevent further tumor cell growth (33). The presence or absence of these metabolites can impact the individual's colon health and could subsequently impact their risk for

CRC development. Metabolites produced through the breakdown of undigested food, are a major element missing from the purified diets developed by AIN. From the published research we suspect the whole-food dietary component of the TWD-2 will impact the gut microbiome of rodents, making them a more accurate model for human CRC studies (26-28).

Freeze-drying of the individual food commodities was chosen as our method for controlling moisture content, since this method has been shown to be effective at preserving foods while maintaining the nutrient integrity (76). The target water activity of the TWD-2 was determined based on the water activity of the commercialized rodent diet, 0.50. Achieving a water activity of less than 0.6 prevents growth of molds, yeast, and pathogenic microorganisms (77). Another factor affecting microbial growth is pH level. To limit growth, pH levels should be below 4.6 to ensure pathogenic microorganisms are not present (77). The TWD-2 should be stored at -4° C prior to feeding or -80° C if stored for extended periods of time. Storing the finished diet pellets at these temperatures will increase the products shelf life by preventing microbial growth, extraneous moisture absorption, and slowing or preventing fat oxidation (78).

Future work on the TWD-2 includes a comprehensive feeding study to examine the effects of the TWD-2, TWD, and AIN-93G on weight gain, the gut microbiome, systemic inflammation, and tumorigenesis in response to AOM + DSS. We hypothesize that mice fed the TWD-2 or TWD diets will result in more tumorigenesis, due to its suboptimal micronutrient content. We also hypothesize the gut microbiome of mice fed the TWD-2 will be more reflective of the gut microbiome of the average American, and will be significantly different from the TWD and AIN-93G diets due to the use of whole-foods in the diet composition.

CHAPTER 4

SUMMARY

Dietary PUFA and CRC

In response to published research and a preliminary study, we investigated whether a diet with a high PUFA concentration and a high n-6 to n-3 PUFA ratio would induce a larger tumorigenic response compared to a diet with a low PUFA concentration and low n-6 to n-3 PUFA ratio, in response to AOM + DSS induced inflammation. To determine CRC outcome, we measured tumor multiplicity, tumor size, colon length, systemic inflammation, fecal metabolites, and the fatty acid profiles of colon mucosa and red blood cell membranes. From the experimental diets selected, we had hypothesized the diet with 10% energy from PUFA and an n-6 to n-3 ratio of 20:1 would result in an increased CRC response. From the measured endpoints, this hypothesized tumorigenic diet did not increase the CRC response in mice treated with AOM + DSS. However, our results did suggest that both the dietary PUFA content and the n-6 to n-3 ratio played a role in the development of CRC in response to AOM + DSS. The PUFA ratio played a more significant role in tumorigenesis, while the PUFA concentration played a more significant role in the systemic inflammatory response. Although the published research suggests the Western diet, which is characterized by a high PUFA content and high n-6:n-3 ratio would promote CRC, the results from our study suggest diets with a high n-6:n-3 PUFA ratio have a protective effect against CRC in response to AOM + DSS induced inflammation. It was also interesting to note that mice fed the control diet, AIN-93G, had elevated serum levels of pro-inflammatory cytokines, higher tumor multiplicity, and tended to have some of the largest tumors. These results from mice fed the AIN-93G diet, which contains approximately 7% energy from PUFA and an n-6 to n3 ratio of 7:1, suggest this intermediate level of PUFA content and n-6 to n-3 ratio had a greater impact on CRC development than the other experimental diets in response to

AOM + DSS. This is interesting, as according to the NHANES survey *What We Eat in America* for the years 2011-2012, the average American consumes a diet that contains approximately 8% energy from PUFA and an n-6:n-3 ratio of approximately 10:1 (61). With components of the experimental diets used in this study having similarities to the average American diet, understanding how the PUFA concentration and n-6:n-3 play a role in the etiology of CRC is important. Data from the other measured endpoints, systemic inflammatory response, fecal metabolomic analysis, and the fatty acid profiles of RBC and colon mucosal membranes, showed that the dietary PUFA content and/or n-6 to n-3 PUFA ratio influenced their respective outcomes. The dietary PUFA content greatly influenced the measured serum cytokine levels, while both the dietary PUFA content and n-6:n-3 PUFA ratio impacted the fecal metabolites produced and the fatty acid profiles of RBC and colon mucosal membranes. In conclusion, we were able to determine a diet with a high PUFA concentration and high n-6:n-3 PUFA ratio does not induce a more tumorigenic response to AOM + DSS compared to our other treatments. We found diets with a low n-6:n-3 PUFA ratio induced significantly larger tumors compared to diets with a high n-6:n-3 PUFA ratio. Moreover, mice fed the control AIN-93G diet, had an increased CRC outcome, compared to mice fed the other experimental diets.

Development of the TWD-2

Diet has been shown to greatly influence the gut microbiome and subsequently colon health (26, 28, 29). Diets currently used in rodent studies, such as the AIN diets (21), are formulated using purified ingredients. Because these rodent diets do not incorporate whole-foods into their formulations, they are missing the complex components like dietary fiber, bioactive plant metabolites, and SCFAs known to impact gut health (31-33, 67). Knowing the impact of whole-foods on gut health, it was evident that a new diet, which incorporates the use of whole foods in the formulation, needed to be developed. To understand how the TWD-2 impacts the gut

microbiome and CRC, a mouse feeding study using the TWD-2, TWD, and AIN-93G will be carried out. Through this study, we expect to better understand how the whole-food component of the TWD-2 affects colorectal carcinogenesis in response to AOM + DSS induced inflammation. We also hope this study will directly influence a change within the scientific research community by demonstrating the importance of incorporating whole-foods into rodent diets used in studies pertaining to colon health. Incorporating the TWD-2 into dietary studies will likely make the rodent a better human model, potentially allowing researchers to better understand the etiology of CRC.

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