EFFECT OF DIET QUALITY ON GASTROINTESTINAL INTEGRITY, INNATE IMMUNITY, AND CIRCULATING LIPOPROTEINS

A Dissertation

by

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ABSTRACT

Human nutrition has largely focused on energy intake and recommended macronutrients to prevent obesity and disease development. Increased gut permeability, through diet or obesity, is thought to play a causative role in chronic systemic inflammation leading to disease. Dairy milk and its fractions have been shown by others to reduce body weight gain and adiposity and improve intestinal integrity relative to control diets. Studies using accelerated disease models, i.e. high-fat diet feeding, have allowed for assessing specific mechanisms but may not physiologically relevant for human dietary patterns and disease. To assess the effects of dairy milk or fractions within a moderate fat macronutrient distribution, 60 weanling C57Bl/6 male mice were fed one of four experimental diets for 13 weeks: isolated soy protein (ISP), dried whole milk powder (DWMP), milk protein concentrate (MPC) or milk fat globule membrane (MFGM). Isocaloric and isonitrogenous diets provided 20% energy (EN) as protein, 50 EN% carbohydrate and 30 EN% fat. Mice (n=15/diet treatment) grew and consumed feed similarly. Gut permeability, plasma LPS, and whole blood cytokine concentrations were low for all groups. Overall, dairy proteins had similar effects on lipoprotein classes. Relative to ISP, dairy proteins increased triacylglycerol-rich lipoproteins (2.8% v.1.4%) and high-density lipoproteins (75.6% v. 70.0%) and decreased low density lipoproteins (LDL) (21.6% v. 28.5%, $p<0.05$). Small dense LDL were reduced 70% in mice fed diets containing dairy milk fractions ($p<0.005$). Consumption of dairy proteins appears to improve lipoprotein profiles in C57/Bl6 mice when consumed in a moderate fat diet.

A second study sought to if degree of dietary refinement altered gut health and lipoprotein biology. In a 14 week feeding trial, weanling C57Bl/6 mice (n=15/diet) were fed one of four diets differing in dietary protein source (soy or milk protein) and degree of refinement. All diets provided 20%EN PRO, 30% EN fat, and 50% EN CHO. Purified diets contained either isolated soy protein (ISP) or dried whole milk powder (DWMP) as the protein source, while non-purified diets contained either soybean meal (SPC), or dried whole milk powder (DMC) as protein sources. Mice fed purified diets were heavier than those fed non-purified diets, with ISP-fed mice being heaviest and having the most fat. At week 12, *in vivo* intestinal permeability was highest in ISP-fed mice (12.8 ng/mL), being significantly increased over DMC (8.96 ng/mL; $p<0.02$). Purified diet feeding resulted in increased apparent GI-p, regardless of protein source (12.1 ng/mL vs. 9.56 ng/mL; $p<0.004$). Measured cytokines were generally low and non-indicative of either tissue or systemic inflammation. Density distributions of NBD-stained lipoproteins showed that diets containing milk protein increased triacylglycerol-rich lipoproteins and high-density lipoproteins and decreased low-density lipoproteins ($p<0.04$). Compared to all other diets, mice fed ISP had a 30% increase in small dense LDL ($p<0.000$). In diets providing 30% energy as fat, degree of purification seemingly has a greater effect on body weight and intestinal integrity, while milk-containing diets resulted in improved lipoprotein profiles regardless of purification.

DEDICATION

This dissertation is dedicated to my parents, Greg and Phyllis Price. Thank you for supporting me through this crazy journey. You raised me to be independent, to work toward my goals, and to never give up. Thank you so much for encouraging me to pursue my dreams. I love you.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a dissertation committee consisting of Dr. Rosemary L. Walzem (committee chair), Dr. Suresh D. Pillai, and Dr. Nancy D. Turner of the Department of Nutrition and Food Science, Dr. Robert C. Alaniz of the Department of Microbial Pathogenesis and Immunology, and Dr. Stephen B. Smith of the Department of Animal Science.

The data analyzed in Chapter 2 (Figures 1-3) and Chapter 3 (Figures 3-5, Tables 7-9) were conducted by Dr. Sangeetha Baskaran. Dr. Xiuzhi Wu conducted analyses for data depicted in Chapter 3 (Figures 1, 3 and Tables 1-6). The GI Lab of Texas A&M University in the Department of Small Animal Clinical Sciences under Dr. Jorg Steiner (director) measured data depicted in Chapter 2 (Table 7, Dr. Yasushi Minamoto) and Chapter 4 (Figures 3-4, Tables 7-9, Ms. Amanda Blake). Dr. Luis Tedeschi ran statistical analyses on data presented in Chapter 2 (Tables 3-4). Dr. Stephen Smith and Dr. Jinhee Hwang assisted in dietary fatty acid analyses presented in Chapter 2 (Table 1), Chapter 4 (Table 1) and Appendix C.

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NOMENCLATURE

2-AG	2-Arachidonylglycerol
AAS	Amino Acid Score
ABCA1	ATP-Binding Cassette Transporter A1
ABI	Applied Biosystems
ACCI	Associates of Cape Cod, Inc.
ACTB	Actin, Beta
AI	Adequate Intake
AMDR	Acceptable Macronutrient Distribution Range
AOAC	Association of Official Agricultural Chemists
AP2	Adipocyte Protein 2
ApoA1	Apolipoprotein A1
AUC	Area Under the Curve
BA	Bile Acid
BCFA	Branched Chain Fatty Acids
BMI	Body Mass Index
BV	Biological Value
C/EBP-α	CCAAT/Enhancer Binding Protein alpha
CB	Cannabinoid
CB1	Cannabinoid Receptor 1
CB2	Cannabinoid Receptor 2

CD11d	Cluster of Differentiation 11d (aka Integrin $\alpha_D\beta_{2)}$
CD14	Cluster of Differentiation 14
CETP	Cholesteryl Ester Transfer Protein
CHD	Congestive Heart Disease
CRP	C-reactive Protein
C_T	Comparative Critical Threshold
CVD	Cardiovascular Disease
DIAAS	Digestible Indispensable Amino Acid Score
DMC	Dried Whole Milk Chow
DRI	Dietary Reference Intake
DWMP	Dried Whole Milk Powder
EAA	Essential Amino Acid
eCB	Endocannabinoid
EN	Energy
EU	Endotoxin Unit
FA	Fatty Acid
FAAH	Fatty Acid Amide Hydrolase
FAO	Food and Agriculture Organization of the United Nations
FITC-d	Fluorescein Isothiocyanate Dextran
FDA	U.S. Food and Drug Administration
GALT	Gut-Associated Lymphoid Tissue
GI	Gastrointestinal

GI-P	Gastrointestinal Permeability
GLP-1	Glucagon-like Peptide 1
GPR	G-couple Protein Receptor
H&E	Hematoxylin and Eosin
HDL	High Density Lipoprotein
HDL-C	High Density Lipoprotein – Cholesterol
IACUC	Institutional Care and Use Committee
iALP	Intestinal Alkaline Phosphatase
IL	Interleukin
ISP	Isolated Soy Protein
LCAT	Lechitin:Cholesterol Acyltransferase
LDL	Low Density Lipoprotein
LDL-C	Low Density Lipoprotein – Cholesterol
LPS	Lipopolysaccharide
MFGM	Milk Fat Globule Membrane
MGL	Monoacylglycerol Lipase
MPC	Milk Protein Concentrate
MPO	Myeloperoxidase
MUFA	Monounsaturated Fatty Acids
MyD88	Myeloid Differentiation Protein 88
NAPE-PLD	N-AcylPhosphatidlyEthanolamine-hydrolizing PhosphoLipase D
NFDM	Non-Fat Dry Milk

NFκB	Nuclear Factor Kappa Beta
NID	Natural Ingredient Diet
NPU	Net Protein Utilization
PCR	Polymerase Chain Reaction
PD	Purified Diet
PDCAAS	Protein Digestibility Corrected Amino Acid Score
PER	Protein Efficiency Ratio
PPAR-α	Proliferator Peroxisome Proliferator alpha
PPAR-γ	Proliferator Peroxisome Proliferator gamma
PUFA	Polyunsaturated Fatty Acids
RCT	Reverse Cholesterol Transport
RDA	Recommended Daily Allowance
RT-qPCR	Real-Time Reverse Transcriptase Quantitative PCR
SAA	Serum Amyloid A
SCFA	Short Chain Fatty Acids
SFA	Saturated Fatty Acids
SPC	Soy Protein Chow
SR-B1	Scavenger Receptor Class B, Member 1
T2DM	Type 2 Diabetes Mellitus
TEER	Trans-Epithelial Electrical Resistance
TICE	Trans-Intestinal Cholesterol Efflux
TLR	Toll-like Receptor

TRL	Triglyceride-rich Lipoproteins
TRPV1	Transient Receptor Potential Vanilloid Receptor 1
VLDL	Very Low Density Lipoproteins
WHO	World Health Organization
ZO-1	Zona Occludens Protein 1

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1. INTRODUCTION

1.1. Diet and Nutrition – Standards

Human nutrition has largely focused on what foods to eat and the proper macronutrient distributions to meet physiologic requirements and prevent overt nutrient deficiencies. As such, the United States Department of Agriculture established recommendations for macronutrients and food components based on population-based studies [1,2]. Availability of standardized dietary recommendations has not limited the U.S. population steadily increasing body weight, waist circumference and body mass index (BMI) [3]. Changes in macronutrient intakes, including percentage of calories consumed as fat, are considered causal for the obesity epidemic and increasing risk of metabolic diseases [4]. Caloric intake alone can have an impact on body weight regulation and immune cell function [5,6]. Food composition can also affect satiety, with protein content increasing satiety more than carbohydrate or fat [6]. Thus, it is important to correctly identify dietary components, including macronutrient distributions, protein quality, and fatty acid compositions, required to promote optimal health.

1.1.1. **Protein Quality**

U.S. Dietary Guidelines for Americans recommend 10-35% of calories from protein for adult men and women [1]. Recommended Daily Allowances (RDAs) for healthy individuals, based on nitrogen balance studies, are set at 0.8 g good quality protein/kg body weight/day.

A number of different methods to calculate protein quality are available. The simplest is the growth-based Protein Efficiency Ratio (PER), which measures the ability of a protein to support the growth of a weanling rat. It represents the ratio of weight gain to the amount of protein consumed. This method is useful for measuring growth, but not for maintenance, limiting its usefulness in fully developed animals/humans. This method also does not reveal why a protein is of higher or lower quality.

Values for Amino Acid Score (AAS) provide the first insight into protein quality. A chemical technique, AAS provides a relatively fast, consistent, and inexpensive measurement of protein composition. The test measures the essential amino acids (EAA) present in a protein and compares the values with a reference protein, typically milk or whole egg. Protein quality is evaluated based upon the most limiting indispensable amino acid. If the AAS value is greater than 1.0, the protein is considered to contain EAA in excess of human requirements.

Another important consideration in protein utilization is protein digestibility. While defined protocols exist to specify the diet context used in determinations of protein digestibility tabular values, the digestibility of protein and bioavailability of amino acids in mixed diets can be negatively impacted by the insoluble fiber and antinutritive factor content of diets. In a review of that topic by Gilani, et al.[7], it was noted that the digestibility of protein in traditional diets from developing countries such as India,

Guatemala, and Brazil are considerably lower compared to that of protein in typical North American diets (54% - 78% versus 88% -94%).

Protein digestibility is taken into account in determinations of protein *Biological Value* (BV) as it measures the amount of nitrogen retained in comparison to the amount of nitrogen absorbed. The Net Protein Utilization (NPU) score assesses the amount of digestible nitrogen that is used for tissue formation. Both the BV and the NPU methods utilize measures of nitrogen availability and digestibility to give an accurate appraisal of maintenance needs. The Protein Digestibility Corrected Amino Acid Score (PDCAAS) has served in food regulatory settings since 1989 to describe protein content or make claims[8] and comprises the AAS with an added digestibility factor. This method was adopted because it is based on human amino acid requirements, and so the values are more relevant for human needs than are those based on the amino acid needs of animals such as young growing rats, although in the original method, rats were used to assess digestibility.[9] Interestingly, because AAS is a component of PDCAAS, PDCAAS values greater than 1.0 are also considered indicators that the protein contains EAAs in excess of the human requirements. For this reason, in 1989 at a joint Food and Agriculture Organization (FAO) of the United Nations and World Health Organization (FAO/WHO) expert consultation [8], it was decided that proteins having values higher than 1.0 would be rounded down to 1.0. This decision proved contentious. In a more recent FAO expert consultation [10], the PDCAAS method was criticized for not crediting the extra nutritional value of high-quality proteins, overestimating nutritional quality of

products containing antinutritional factors, failing to adequately account for the bioavailability of amino acids, and overestimating the quality of poorly digestible proteins supplemented with limiting amino acids and of protein co-limiting in more than one amino acid. That report advocated adoption of the *Digestible Indispensable Amino Acid Score* (DIAAS), using the following equation:

$$\text{DIAAS} = \frac{\text{mg digestible dietary EAA in 1 g of dietary protein}}{\text{mg of same EAA in 1 g of the reference protein}} \times 100\%$$

where reference protein for infant formulas = human breast milk and reference for all other foods = pattern for young children (6 months – 3 years) as by the FAO report [10].

The ratio is calculated for each dietary EAA and the lowest value designated as the DIAAS indicator of protein quality. Digestibility is to be based on the true ileal digestibility of each amino acid, preferably determined in humans, a growing pig or, as a last resort, the growing rat. Detailed examples of the calculation for single foods and multiple ingredient dishes and diets can be found in the FAO report [10]. Mathai, et al., used pigs to develop DIAAS values for four animal and four plant proteins and used ratio truncation to 1.0 to calculate PDCAAS-like values from the same experimental data [9]. This group concluded that the PDCAAS method assumption that ileal digestibility of all amino acids can be predicted from the total tract digestibility of crude protein is incorrect and creates an insurmountable flaw in the PDCAAS method. The 2011 FAO/WHO expert consultation on dietary protein quality evaluation in human nutrition recommended adoption of DIAAS as the regulatory norm [10]. Protein composition and quality are important factors in experimental dietary design and dietary

recommendations. It is also important to consider the dietary matrix (lipid profiles and carbohydrate refinement) when determining practical protein quality implications.

1.1.2. **Fatty Acids**

Dietary fat, a source of energy, is classified based on its chemical structures. When describing specific fatty acids, the carbon chain length, number and position of double bonds are provided in addition to a common name. For example, stearic acid has 18 carbon atoms and no double bonds is chemically described as 18:0, while the chemical description of linoleic acid, a fatty acid with 18 carbon atoms and 2 double bonds, is 18:2, n-6. The n-6 indicates that the first double bond was located at the sixth carbon-carbon bond from the methyl carbon of the chain. A second important fatty acid is the n-3 family in which the first double bond is located at the third carbon-carbon bond from the methyl carbon of the fatty acid. The best known fatty acid of this family is docosahexaenoic acid, a fatty acid with 22 carbon atoms and 6 double bonds, is 22:6, n-3 In regulatory definitions, the term "fat" refers to a substance composed chiefly of triacylglycerols comprised of predominantly saturated fatty acids (SFA) that lack double bonds and which is solid or semi-solid at room temperature (23°C). Conversely, the term "oil" refers to a substance composed chiefly of triacylglycerols comprised of predominantly of monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) and which is liquid at room temperature [11]. The term "lipid" for purposes here includes both fats and oils, but excludes non-energy production cholesterol and cholesteryl esters.

An acceptable macronutrient distribution range (AMDR) is set at 20-35% of energy for humans, though adequate intake (AI) or dietary reference intakes (DRI) do not set values for lipid intake [12]. Due to negative associations with saturated fatty acid (SFA) intake and coronary heart disease [13], current recommendations are to limit SFA intake to less than 10% of daily caloric intake [1,14,15]. Monounsaturated fatty acids (MUFA) do not have RDA at present. Polyunsaturated fatty acids (PUFA), most commonly n-3 and n-6, do have adequate intake levels for human consumption. Linoleic acid, 18:2, is an essential fatty acid and precursor to arachidonic acid.[12] Omega-3 (n-3), PUFAs associated with improved neurological function and adequate growth in children, are also required in the diet. Human AIs for linoleic acid and the n-3 PUFA α-lenolenic acid are set at 12-17/g day and 1.1-1.6g/d, respectively (females-males), to prevent essential fatty acid deficiency [12].

1.1.3. **Dietary Fiber**

Human nutritional definitions of total fiber includes two components: dietary fiber and functional fiber [12]. In this context, dietary fiber is considered to be non-digestible carbohydrates and lignins from plant sources. Functional fiber includes any non-digestible carbohydrates that confer physiological effects to human health [12]. Total fiber intake is recommended to be 25 g/day [16], with approximately 50% arising from dietary fiber [12]. In FDA regulated nutrition facts labels, dietary fiber is classified as soluble or insoluble [16]. In literature produced for the purpose of consumer education and outreach, soluble fiber is cited for its association with blocking dietary cholesterol and fat

absorption and controlling blood glucose, whereas insoluble fiber is cited for its ability to improve intestinal motility and stool formation [16].

From a more structural chemistry perspective, dietary fiber refers to any substance that cannot be digested in the small intestine [17]. Chemically, dietary fiber can include non-digestible oligosaccharides, non-starch polysaccharides, lignins, tannins and more. Analytical methods were established by the Association of Official Agricultural Chemists (AOAC) and classified fibers as insoluble or soluble fiber, based upon their extraction at different pHs [18]. In animal feeds, the term neutral detergent fiber (insoluble fiber) is more commonly used [19]. Neutral detergent fiber (NDF), including cellulose, hemicellulose, and lignin, are extracted by boiling the sample and treating with α-amylase. Total dietary fiber, in this context, includes NDF and water-soluble nonstarch polysaccharides (i.e. galactans, β-glucans and pectins) [19].

1.2. Macronutrient Effects on Health

Dietary components directly affect body composition. An extensive study of changes in dietary macronutrient distributions determined energy from fat, more so than carbohydrate or protein, drove body fat gain in four strains of mice [20]. Thirty diets were formulated with varying levels of protein (5-30%), carbohydrate as sugar (5-30%), and fat (10-80%). After 12 weeks of experimental diets, incremental increases of % energy as protein with a constant 60% energy as fat were associated with increased body weight, lean mass and adiposity. When dietary fat content was held constant at 20% of

energy, incremental alterations in protein content (5-20% of energy) increased body mass measures [20]. However, protein content of 30% or higher was associated with decreased body mass and a leaning effect. Experimental diets of varying fat content showed a significant increase in energy intake and adiposity. Changes in sugar percentages did not affect energy intake or body fat gain. Consistent effects of diet across four mouse strains is a strength of this study; however, diets used casein, cellulose, cocoa butter, and a variety of vegetable oils in formulations [20]. Whether the results observed would be effected by different protein or fat sources remains to be seen.

In human studies, similar results have been noted between dietary quality and phenotypic outcomes. An inpatient randomized controlled trial of 10 males and 10 females evaluated the effect of processing on metabolic outcomes [21]. Energy intake was increased with ultra-processed diets and was associated with increased eating rates, body weight and fat mass gain, and reduced high-density lipoprotein cholesterol (HDL-C). Of interest, HDL-C was lower following raw diet phases and significantly lower than baseline or ultra-processed diets [21]. Similarly, a significant increase in spontaneous feed intake in an over-fed hen model resulted in body weight gain, lowered immune response to LPS stimulation and reduced heterophil activity [5].

1.2.1. **Protein Quality and Effects on Health**

Currently, contention exists for recommendations of plant-based vs. animal-based dietary proteins, their propensity to cause or prevent disease, and their overall

contributions to immune status and chronic disease prevention [22-27]. In a weanling pig model, Mathai, et al. evaluated the quality of common plant and animal proteins including milk protein concentrate, skim milk powder, soy, pea, and wheat [9]. Calculated PDCAAS and DIAAS scores did not differ for milk protein concentrate; however, statistical differences existed for the remaining protein sources (Table 1.1). Calculated DIAAS were highest for dairy proteins and greater than analyzed plant proteins. DIAAS was consistently lower than PDCAAS for all dairy proteins, while PDCAAS values were higher than DIAAS for all plant proteins analyzed. This phenomenon was also described by Galani Sarwar and Pearce[28,29] for proteins with antinutritives and poorly digested proteins, where PDCAAS values seemingly overestimated quality scores of plant proteins.

Table 1.1 Comparison of protein digestibility corrected amino acid scores (PDCAAS) and digestible indispensable amino acid scores (DIAAS) based on different requirement[1] #‡§

Ingredients	PDCAAS 1991‡	PDCAAS 1991, untruncated	PDCAAS 2013§	DIAAS	SEM	*P*
WPI	**99**[a]	**99**[b]	**97**[b]	**100**[a]	0.3	<0·0001
WPC	**100**[b]	**107**[a]	**107**[a]	**107**[a]	0.4	<0·0001
MPC	**100**[c]	**127**[a]	**121**[b]	**120**[b]	0.5	<0·0001
SMP	**100**[d]	**121**[a]	**112**[b]	**105**[c]	1.1	<0·0001
PPC	**75**[a]	**75**[a]	**71**[b]	**62**[c]	0.6	<0·0001
SPI	**93**[a]	**93**[a]	**86**[b]	**84**[c]	0.5	<0·0001
Soya flour	**98**[a]	**98**[a]	**93**[b]	**89**[c]	1.3	<0·0001
Wheat	**50**[a]	**50**[a]	**51**[a]	**45**[b]	1.3	0.013

WPI, whey protein isolate; WPC, whey protein concentrate; SMP, skimmed milk powder; PPC, pea protein concentrate; SPI, soya protein isolate.
Mean values within a row with unlike superscript letters are different (P < 0·05).
 # Values for PDCAAS were calculated from the total tract digestibility of crude protein in pigs and values for DIAAS were calculated from the ileal digestibility of amino acids in pigs.
‡ PDCAAS were calculated using the recommended amino acid scoring pattern for preschool children (2–5 years). The indispensable amino acids reference patterns are expressed as mg amino acid/g protein
§ PDCAAS and DIAAS were calculated using the recommended amino acid scoring pattern for a child (6 months to 3 years). The indispensable amino acid reference patterns are expressed as mg amino acid/g protein

[1] Reproduced with permission of The Licensor through PLSclear. British Journal of Nutrition, 117(4), Mathai, Liu, and Stein, Values for digestible indispensable amino acid scores (DIAAS) for some dairy and plant proteins may better describe protein quality than values calculated using the concept for protein digestibility-corrected amino acid scores (PDCAAS), 495, Copyright 2017

1.2.2. **Dairy Milk and Its Components**

Dairy milk is a high-quality animal-protein source consumed in developed and developing countries,[30] comprising approximately 10% of calories in the average US diet across all age groups[31]. In addition to its lipid and protein components, dairy milk contains bioactives, vitamins, and minerals.[31,32] Heat, biochemical and physical processing methods can impact milk protein nutrient value by releasing bioactive peptides. The processing type can improve or limit protein digestibility and amino acids availability and should be considered in evaluating its dietary inclusion.[30]

A prospective study of more than 120,000 men and women explored effect of diet and lifestyle factors on body weight gain.[33] Among dairy products, butter intake was highly correlated with weight gain, while whole-fat milk, cheese, and low-fat milk were not significantly correlated changes in body weight. A meta-analysis of dairy product consumption showed increased body weight when caloric intake was uninhibited, but dairy products promoted weight loss in a calorically restricted diet.[34] More interesting, increased body weight gain with dairy products in high caloric diets was not associated with alterations to body compositions, while dairy in caloric restriction reduced fat mass and increased lean mass[34]. Whey protein isolate, added to high-fat diets, did not affect body weight gain in C57Bl/6 mice; however, mice increased caloric intake and reduced total cholesterol and low-density lipoprotein cholesterol (LDL-C).[35] Addition of whey protein to low-fat diets lowered body fat mass but did not affect other metabolic

outcomes.[35] Thus, it is evident that diet context (caloric and macronutrient densities) play a role in food product effects on metabolic outcomes.

In addition to providing major macronutrients, vitamins, and minerals, foods contain many factors, including bioactive peptides, which can affect health. Glycoproteins found in milk, i.e.lactoferrin, and growth factors can influence gut inflammation via inherent anti-fungal properties and maintenance of epithelial cell proliferation and maturation.[36] Other peptides in bovine milk are widely cited for affecting immune responses: suppress or inhibit lymphocyte proliferation, enhance splenic antigens, and suppressing cytokine production following LPS challenge.[36]

The Coronary Artery Risk Development in Young Adults (CARDIA) study explored dairy intake, obesity and insulin resistance among 18-30 year olds [23]. A food frequency questionnaire assessed dietary diary intake, with the bulk being from milk, butter, cream, and cheese. Dairy consumption was associated with overweight status at baseline, but no association to insulin resistance was observed [23]. After 10 years, insulin resistance risk reduced with increased dairy intake in obese or overweight individuals [23]. Fiber intake and dietary protein content were also associated with insulin resistance risk. Risk status was not affected for lean individuals. Thus, the health status of individuals may affect the ability to process and use the nutritive properties of food components.

A human randomized trial investigated the effect of dairy on blood lipids and fecal fat excretion.[37] Experimental diets provided similar macronutrient distributions and differing levels of calcium depending on their inclusion of skimmed milk or cheese products. Milk and cheese diets lowered total cholesterol and LDL-C, but did not change HDL-C compared to control diets.[37] Fecal energy and fat excretion also increased significantly with dairy intake and was correlated to changes in blood lipids.[37] The authors of this study attributed the changes to dairy calcium. The effect of calcium source on body weight regulation was studied in a diet-induced obesity mouse model by Thomas, et al.[38] Diets including non-fat dry milk (NFDM) reduced body weight gain compared to soy-protein control diets with supplemented with equivalent or high calcium levels.[39] NFDM decreased energy consumption, fat deposition, and adipose inflammatory marker genes and increased glucose tolerance compared to either normal or high-calcium control diets.[39] In obese mice, diets containing NFDM reduced body weight and improved glucose control without altering alter plasma cytokines compared to soy-protein diets containing normal or high calcium.[40]

Milk fat globule membrane protein (MFGM), a bioactive fraction of dairy milk, is comprised of lipid and protein components.[41,42] When included in a high-fat diet, MFGM reduced gastrointestinal permeability in C57Bl/6 mice. After LPS injection, MFGM feeding prevented mortality and reduced pro-inflammatory cytokine expression.[43]

1.2.3. **Other Animal Proteins**

Eotaxin and IFN-g increased in whey powder and meal worm diets, while gluten meal increased IL-6 concentrations [44]. Rat pups, gavaged with whey protein extract containing native bioactive components, decreased MHC 1 &II labeling in ileal epithelial cells compared to PBS only [45]. The authors suggested whey extract induces TGF-B secreting cells which, in turn, may downregulate the immune response.

The Kuopio Ischaemic Heart Disease Risk Factor Study included 2641 Finnish men, half of which had a history of T2DM, CVD or cancer [46]. Four day diet records were correlated with mortality rates. Significant associations between red meat intake and increased mortality was observed in the highest quartile of red meat intake. No correlations were observed with red meat intake in the lower intake quartiles. Total protein intake was not significantly correlated with mortality [46]. The study did not include nitrate data and did not separate fresh red meat from processed meat products.

1.2.4. **Plant-based Protein Sources**

Plant-based proteins are often prescribed to human subjects in an effort to improve inflammatory profiles and metabolic outcomes [47]. A systemic review of 2500 human plant-based dietary trials reported lower inflammatory state following plant-based diets including reductions in C-reactive protein and interleukin-6 [47]. C57Bl/6 mice fed SBM decreased mTOR protein and activity possibly leading to lower energy levels [44]. Relative to diets containing fish meal or fish oil and wheat grain meal, zebrafish soybean meal

diets induced neutrophils in larval intestines and increased larval expression IL-1β, IL-8 [48].

Soy-protein diets, providing high levels of isoflavones, reduced body weight and inhibit adipose accumulation in mice [49]. In addition to increased fat mass, males increased muscle mass as %BW. A study investigating isoflavone effects in C57Bl/6 mice, genistein and daidzein reduced feed intake and feed efficiency compared to control. Mice fed diets containing daidzein had a higher body weight gain than those fed genistein, although both diets reduced body weight from control [50]. Hepatic lipids were not different from controls, but daidzein increased hepatic lipids compared to genistein.

1.2.5. **Dietary Fat and Fatty Acids**

For the past 40 years, governing agencies have recommended limiting the intake of saturated fatty acids to prevent negative health outcomes [13]. As such, a trend to limit full-fat dairy products resulted in human nutrition. However, a meta-analysis by Guo, et al., showed dairy intake at both low or high inclusion rates was not associated with mortality, cardiovascular disease(CVD) or congestive heart disease (CHD) [51]. Dairy fat, specifically, has been associated with prevention of obesity and metabolic syndrome development and improving blood lipids [13].

A meta-analysis evaluated clinical trials replacing SFAs with n-6 PUFA and the effect on lipoproteins and CVD [52]. The authors categorized trials as “adequately controlled” or

"inadequately controlled", an important distinction that limits the effect of confounding factors on data interpretation. Adequately controlled trials did not show a difference in CHD events nor was there a change in mortality with changes in dietary fatty acid composition [52]. A randomized control trial replaced SFA with PUFA in healthy males and females with women moderate hypercholesteremia [53]. PUFA replacement resulted increased fiber and protein intake with lower carbohydrate. The experimental group had a higher BMI, reduced serum cholesterol and triglycerides compared to controls [53].

Post-menopausal women with mild hypercholesterolemia were fed diets consisting of similar macronutrients distributions but differing in primary fatty acid sources [54]. The RCT included palmitic stearate, or oleate as 50% of fat content. Inflammatory markers, CRP, TNF-α, IL-6, SAA-1, were not affected by diet phases; however, after a washout period, stearate and oleate lowered LDL-C and HDL-C. High palmitate diets increased HDL-C. Across all diets, the secondary bile acids lithocholic and deoxycholic acid were negatively correlated with HDL-C, while LDL=C correlated positively with lithocholic acid production [54].

Huang, et al. sought to determine the effect of dietary fat on adipose inflammation and microbiota [55]. Mice fed high-fat diets containing milk-fat adipose inflammatory genes compared to diets containing lard or safflower oil; %BW gain also increased with milk-fat feeding, but failed to reach statistical significance. Safflower oil, a source of high PUFA, increased macrophage infiltration in gonadal adipose [55].

High-fat (60% kcal) diet feeding in C57Bl/6J mice delayed whole-gut transit with slowed colonic propulsion compared to mice fed diets containing 18% kCal as fat [56]. Altered microbiota led to an increase in plasma and stool endotoxin and TLR4 intestinal expression [56]. In a separate study, high-fat diets accelerated Crohn's disease progession and increased T-cell responses [57]. The marked change in intestinal inflammation was not linked to obesity or fat deposition.

1.2.6. **Dietary Fiber Intake**

In addition to protein and fat considerations, fiber content and composition can affect metabolic outcomes. Broiler chickens, as an animal model of rapid weight, were fed diets containing two different dietary soluble fiber sources [58]. High pectin diets promoted body weight gain and feed intake, compared to a low-fiber chitosan-containing diet. Changes in body weight gain were accompanied with increased plasma cholesterol, HDL-C, and decreased duodenal bile acids [58], although these changes may be attributable to body weight gain independent of dietary composition. A brief review of control diets in metabolic research studies concluded most did not account for the effect of fiber amount or composition [59]. In the reviewed literature, crude fiber in commercial diets was highly variable, averaging 15-25% by weight. Additionally, fiber included many grain-based sources or cellulose, whose solubility and bioavailability could affected their respective study's outcomes.[59] As expected, dietary composition affects

intestinal health and microbiota, potentially leading to systemic inflammation or disease development.[60]

1.3. Intestinal Health

The gastrointestinal track is a highly complex, integrated system serving primary roles in digestion, immunity and barrier function. The GI system is the largest component of the immune system,[61] containing 70% of the body's immune cells[62] and serves as a primary interface between environment, diet, microbial communities and host. Dietary protein quality and amino acid composition is well documented to contribute positively to intestinal health and gut-associated lymphoid tissue (GALT)[63-65]. The addition of glutamine to culture media can prevent epithelial cell death and increase tight junction protein expression.[66,67] Animal models provided oral glutamine supplementation reduced apoptotic protein expression, improved overall growth in piglets after LPS challenge, and maintained mucosal integrity in a mouse model of intestinal obstruction.[66,68] Arginine and threonine increase intestinal IgA, while methionine and cysteine have been shown to reduce DSS-induced permeability and inflammation.[65]

Within the intestine alone, various cell types integrate to maintain the health of the host through nutrient absorption and transport, immune response to foreign antigen and responding to endogenous stimuli from the systemic side.[65,69,70] Epithelial cells and mucus produced serve as primary barriers to potential pathogens while more specific immune cell types continually patrol the intestinal tissue and gut-associated lymphoid

tissue to detect exogenous antigens.[71-73] While specific immune cells (i.e. dendritic cells, macrophages, mast cells, T cells and B cells) and associated receptors are primarily responsible for recognition of pathogens, various other systems play a role in innate immunity.[65,74,75]

Devkota and Chang explored dietary fat sources (milk fat and safflower oil) and changes in microbiota, bile acid production, and intestinal inflammation [76]. Milk-fat derived saturated fatty acids in a high-fat diet were positively correlated with increased taurcholate production; colitis scores also increased with the addition of *B. wadsworthia*, a minor component of healthy microbial communities [76]. The use of IL-10 knockout mice for the study limits the down-regulation of inflammatory responses. It is also important to note that dietary milk-fat did not induce colitis in the absence of *B. wadsworthia*.

The gastrointestinal tract can affect adipocyte differentiation, cannabinoids, endocannabinoids, and mitochondrial biogenesis. Intestinal adipocyte differentiation is regulated by proliferator peroxisome proliferator gamma (PPAR-γ), adipocyte protein 2 (AP2), and CCAAT/enhancer binding protein alpha (C/EBP-α). PPAR-γ affects adipogenesis by regulating mesenchymal stem cell differentiation[77] and has been shown to affect cholesterol efflux in macrophages.[78] Both AP2 and C/EBP-α promote adipogenesis by regulating pre-adipocyte differentiation and regulating gene expression in mature adipocytes.[79,80]

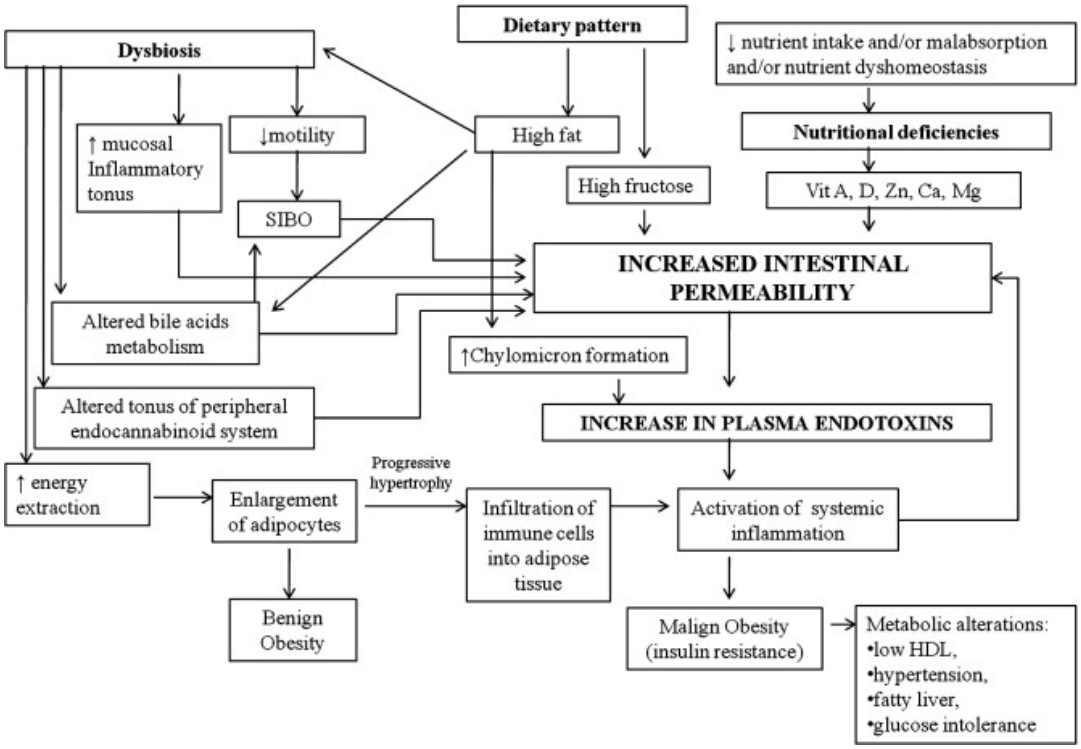
Cannabinoid (CB) receptors can influence gastrointestinal health and immune activation. Cannabinoid receptor 1 (CB1) has been shown to regulate gut motility and satiety, delaying gastrointestinal transit and leading to increased food intake.[81-83] CB2, originally isolated on macrophages, has also been found in the ileum and stomach of animal models.[82] CB2, mediates the immunosuppressive effects of eCBs [84,85]; pretreatment of endothelial cells with CB2 agonists successfully lowered neutrophil activation and reduced TNF-a release [86]. Transient receptor potential vanilloid receptor 1 (TRPV1) serves as a receptor for capsaicin and are involved in neuroinflammatory, vasodilation, and hypersecretion pathways.[82] PPAR-α as targets of endocannabinoinds, the G-coupled protein receptors (GPR55, GPR119, GPR41) are involved in cross-talk with CB1 [87]. GPR119 agonists are shown to decrease feed intake and reduce body weight gain.[88] Peroxisome proliferator-activated receptor alpha (PPAR-α), found in enterocytes and enteric neurons, can regulate satiety [87].

Encocannabinoids (eCB) are endogenously produced substrates which act on cannabinoid receptors. N-acylphosphatidlyethanolamine-hydrolizing phospholipase D (NAPE-PLD) synthesis is induced upon feeding.[89] Through its actions, NAPE-PLD can reduce feed intake and regulate body weight gain. Fatty acid amide hydrolase (FAAH) inactivates 2- arachidonylglycerol (2-AG), the major cannabinoid ligand, and plays a role in gut motility and inflammation.[87] Monoacylglycerol lipase (MGL), primarily hydrolyzes 2-AG. Its inhibition has been shown to improve intestinal integrity in a mouse gut inflammation model.[90]

1.4. Intestinal Permeability

As the host of the largest LPS mass in gut microbiota, the majority of systemic endotoxin enters through the gut [91]. Lipopolysaccharide (LPS), the primary component of gram-negative bacterial cell walls, binds to and activates the immune system [92,93]. Physical barriers, mucins, anti-microbial peptides, enzymes and LPS-binding protein play a role in limiting endotoxin access to epithelial cells [91]. Impaired gastrointestinal health is thought to play a causative role in chronic systemic inflammation leading to disease (Figure 1.1)[94,95]. Potentially considered a driver of organ failure curing critical illness, intestinal hyperpermeability can control systemic inflammation [96]. High-fat diets have been linked to increased plasma endotoxin [97], with endotoxin levels significantly higher than those associated with carbohydrate feeding [98]. Chylomicrons, formed in the post-prandial phase, increased endotoxin passage through intestinal epithelium [91].

Figure 1.1 Interplay Between Intestinal Permeability, Obesity, and Chronic Disease Risk[2]



Increased intestinal permeability was hypothesized by others to allow bacterial endotoxin inflammogens, e.g. LPS, passage into systemic circulation and so increase non-specific inflammatory tone and chronic disease risk [99]. Test diets in that study contained an extremely high fat content (60% kcal); while this macronutrient distribution may be mechanistically revealing, it may have little physiological relevance. At present, it is not clear whether diet directly increases "gut leakiness" or whether it alters

[2] Reprinted from Nutrition Research, 32(9), Teixeira, Collado, Ferreira, Bressan, and Peluzio, Potential mechanisms for the emerging link between obesity and increased intestinal permeability, 637-647, Copyright 2012, with permission from Elsevier.

microbiota metabolism in such a way that more LPS is produced.[67,95,99-101] This is an important detail, as LPS alone will increase intestinal tight junction permeability.[102] Thus, the presence of endotoxin in plasma is used as a marker of intestinal permeability;[103] as noted above, LPS is found in the cell membrane of gram-negative bacteria and is not made by vertebrates. Ideally, non-specific migration of LPS through the intestine does not occur, therefore eliminating the presence of plasma LPS. Clinically, increased plasma LPS concentrations are associated with obesity, dyslipidemia and chronic inflammation.[104] Perhaps as a component of adaptive immune processes, LPS from commensal bacteria may commonly transverse the gut wall, priming the innate immune system without triggering an active inflammatory response.[105-107]

Indole, a microbial degradation product, was shown to modulate intestinal permeability and toll-like receptor (TLR) expression.[108] Intestinal epithelial cells, treated with 1 mM indole, induced tight junction protein expression thereby reducing apparent intestinal permeability. TLR-3 and -9 increased with indole treatment in conjunction with increased production of cytokines, including IL-1 and IL-10. Thus, metabolites of protein digestion may directly influence epithelial cell health and intestinal integrity.

Components of the intestinal environment, including diet and microbiota composition, can affect intestinal integrity. Caco-2 cells, cultured in serum-free media, have unstable tight junctions.[109] Addition of milk whey protein to the media stabilized tight junctions

and reduced permeability as assessed by increased trans-epithelial electrical resistance (TEER). Microbiota can independently alter intestinal structures. In Caco-2 cell cultures, *L. plantarum* increased TEER and gene expression of tight junction proteins.[110] Among rats with diet-induced obesity, FITC-dextran permeability and TLR4 expression increased along with plasma endotoxin[100].

1.5. Hallmarks of Inflammation

Key genes and cytokines can provide insight into the inflammatory status for both animal model and human patients. The pro-inflammatory cytokines, TNF-α, NFκB, IL-1β, MIP-2, IL-12p70, and IL-6, are widely used markers of inflammatory status, being synthesized by macrophages upon activation.[111] Toll-like receptors (TLR), activated by specific ligands (i.e. cytokines, LPS), mediate the inflammatory cascade (nuclear factor kappa beta (NFκB)) transcription.[111] NFκB, in this role, serves as a mediator of innate and adaptive immunity, regulating the production or activation of macrophages, dendritic cells, neutrophils and inflammatory T-cells.[112]

Eotaxin, another inflammatory cytokine, is widely used as a marker of allergic response due to its primary role in activating eosinophils.[113]

IL-10, an anti-inflammatory cytokine, regulates the actions of the immune system. Through its actions, it down-regulates the inflammatory process under normal conditions and development of auto-immunity.[114] *In vitro*, IL-10 has also been shown to prime leukocytes, increasing TNF-α production after immune challenge.[115] Isolated human

peripheral blood mononuclear cells, pre-treated with IL-10, also reduced TNF-α gene expression and altered NFκB translocation.[116] Thus, circulating IL-10 regulates immune response by multiple mechanisms; baseline IL-10 values should be carefully considered when evaluating inflammatory responses to external stimuli.

Myeloperoxidase activity, a potent marker of neutrophil infiltration,[117,118] is increased with intestinal compromise via loss of barrier function and inflammation.[119,120] Guinea pig experimental ileitis marked the association between loss of intestinal integrity by loss of epithelial cells and villi structures with increased myeloperoxidase activity.[121] Intestinal alkaline phosphatase (iALP) has been characterized as a marker of mature enterocytes and normal epithelial cell turnover.[61] iALP serves in many roles, including modulating lipid absorption and reducing LPS-induced inflammation and intestinal permeability to bacterial antigens.[122] DSS-induced intestinal damage in mice was alleviated by treatment with exogenous iALP, reducing intestinal inflammation, MPO activity and histological scores.[123] Dietary components have an impact on iAP expression and production[61], decreasing activity with high-fat diet feeding.[100]

Milk sphingomyelin has been linked to gastrointestinal inflammation.[124,125] Sphingomyelin, derived from dairy milk, reduced abberant crypt foci in the presence of a 45% fat diet[124] and reduced obesity and inhibited systemic inflammation in a 60% fat diet.[125] Sphingomyelin reduced serum endotoxin and cytokine concentrations, with few changes to small intestine and colonic inflammatory gene expression.[125] Isolated

macrophages were cultured and stimulated with LPS. MFGM inhibited the inflammatory response to LPS in macrophages; ceramides and sphingosine associated with MFGM were determined to be the primary mechanism by which the inflammatory response was inhibited.[125]

Transgenic mice with intestinal specific blockage of MyD88 signaling and dnMyD88 overexpressed intestinal epithelial cells exhibited alterations to inflammatory signaling.[126] Cytokine release (NF-kB) in response to LPS challenge was delayed in mice lacking MyD88. In young animals, no evidence of intestinal inflammation was observed.As transgenic mice aged, intestinal inflammation increased, along with neutrophils, lymphocytes, and macrophage infiltration[126]. The authors determined intestinal inflammation was not due to changes in epithelial apoptosis or permeability. Colonic explants of human IBD patients released increased IL-1β and TNF-α compared to non-inflamed controls. When explants were challenged with bacterial antigens, including LPS, the magnitude of cytokine release was also greater than healthy controls, further underlining the importance of gastrointestinal integrity and inflammation[127].

Glinghammer, et al., studied the effect of dietary dairy intake using fecal waters in HT-29 cell (a human colon cancer line) cultures. Fecal water, collected from human feces, was introduced to culture media. A 40% increase in cell survival was associated with high dairy products in the diet; no changes in fecal water genotoxicity were observed. Dietary intake of the human subjects was assessed by questionnaire; those in the “dairy

product-rich" classification had increased dietary energy, protein, fat, calcium, and cholesterol.[22] The authors did not report the energy or nutrient composition of fecal waters, so it is unclear if or which changes in dietary patterns, beyond dairy inclusion, are responsible for the culture results. Adding MFGM to diets containing anhydrous milk fat as the lipid source provided 11% (w/w) sphingomyelin and prevented aberrant crypt foci formation in Fisher rats.[124] In conjunction, MFGM feeding increased plasma trigylcerides and fatty acids but did not change body fatness.

1.6. Lipoproteins

1.6.1. **Historical role in RCT and CVD risk factors**

LDL-Cholesterol (LDL-C) is widely associated with increased CVD risk and CHD disease progression; interventions to reduce LDL-C are associated with improved cardiovascular outcomes.[128,129] Small, dense LDL are associated with CVD[130] and adverse outcomes in those with previous CVD events, including coronary artery disease and stroke.[131] LDL-3, -4, and -5 were strong predictors of cardiovascular events in men with peripheral artery disease or abdominal aortic aneurysm.[132] Among men with ischaemic stroke, small dense LDL were also associated with increased 2-year mortality.[133]

Historically, HDL-Cholesterol was considered to be "good" due to its association with RCT and cholesterol elimination from the body. Clinically, in relation to heart disease, little thought was given beyond the measurement of relative abundance of cholesterol in

the HDL fraction of serum/plasma in relation to evaluation of vascular or metabolic health. Recently, two fundamental problems have been recognized in this clinical perspective. Key to this shifting perspective is a growing body of evidence related to the structure and subsequent functionality of HDL as well as recognition of functions in addition to RCT. Pivotal observations include those in subjects with HDL-Cholesterol levels within expected "healthy" ranges who, never the less, developed atherosclerosis or autoimmune type conditions.[134]

Through RCT, peripheral cholesterol is shuttled through plasma, the liver and biliary tract to be excreted in feces[69,135,136]. Nascent, immature HDL are formed by esterification of free cholesterol by the enzyme lecithin:cholesterol acyltransferase(LCAT). Cholesteryl esters within mature HDL can be transferred to low-density lipoproteins (LDL) or very-low-density lipoproteins (VLDL) by the action of cholesteryl ester transfer proteins (CETP) in exchange for triacylglycerol. Mature HDL binds to the scavenger receptor class B, member 1 (SR-B1) receptor on the liver, where its cargo of cholesterol, cholesteryl ester and/or LPS is metabolized into bile acids for excretion.

Basic studies show that HDL is a highly complex macromolecular structure whose components can vary greatly.[137] Through proteomic studies of native human HDL, more than 40 separate proteins were identified in HDL fractions.[138] The primary core protein of functional HDL, apolipoprotein A1 (apoA1),[138-140] specifically binds to LPS and may be an essential component of innate immune functionality in the intestine. Serum

amyloid A (SAA) is an acute-phase protein whose expression is induced upon immune challenge.[141] SAA, especially isoforms 1 and 2, binds lipids and forms a molecule of similar density to those formed with apoA1, thus also forming "HDL". When immature HDL are formed with the protein SAA instead of apoA1, HDL functionality is impaired and immune capabilities are greatly compromised[142-144]. Han, et al., showed SAA directly reduced HDL's anti-inflammatory actions on adipose for both humans and mice[145]. SAA reduced cholesterol efflux while increasing IL-1b and IL-6 adipocyte gene expression.[146] Scavenger receptor beta-1 (SRB1), primarily located in the liver, clears cholesterol and cholesteryl esters from circulation.[147]In SRB1 knockout mice, high HDL-C levels did not reduced susceptibility to diet-induced athersosclerosis.[148]

In studies of HDL subfractions, HDL-2b were better predictors of coronary heart disease than LDL or HDL-Cholesterol levels.[149,150] HDL-2b more predictive of positive outcomes than HDL-3's. Results from the Malmo Diet & Cancer study showed cardioprotective properties attributable to HDL-2b and pre-beta HDL, potentially due to improved cholesterol efflux capacity of the HDL.[131]

An observational study including 117,000+ Danes from the Copenhagen General Population study and the Copenhagen City Heart study showed clear associations between low HDL-Cholesterol and autoimmune disease, including Crohn's disease and celiac disease.[151] As expected, apoA1 was also significantly associated with

autoimmunity; neither C-reactive protein (CRP) nor plasma triglycerides were associated with disease occurrence.[151] The study did not report total cholesterol.

HDL functionality can be affected by dietary composition without measurable differences in protein or cholesterol components. Overweight, postmenopausal women fed diets containing whole-egg versus yolk-free eggs increased cholesterol efflux capacity. [152] As respected, dietary cholesterol and MUFAs increased in the whole egg treatment. However the dietary changes were not associated with alterations to serum cholesterol, subclasses (LDL, HDL), apoA1, CRP or SAA between diet groups. LCAT, CETP and PON activity were also the same between dietary treatments.

1.6.2. **Multiple Roles of Apolipoprotein A1**

At the core of HDL particles, apoA1 serves in a multiple of roles within the traditional role of RCT and beyond.[138-140] Myeloid differentiation protein-88 (MyD88), an adaptor protein of TLRs, plays a role in RCT. ApoA1, acting directly on TLR4, activates NFkB through MyD88-dependent pathways.[153] *In vitro* experiments using pre-dendritic cell primary cultures noted apoA1's ability to induce cytokine responses.[154] ApoA1 effectively decreased monocyte differentiation in a cytokine-dependent manner.[154]

ApoA1's conformation can alter its functionality. The ability of apoA1 to adopt different residue conformations can effect LCAT activity and the ability to esterify cholesterol without affecting cholesterol efflux or binding of LCAT to particles.[155]

The functional properties of apoA1 directly impact health and inflammatory status both *in vivo* and *in vitro.* ApoA1 global knockout mice reduced plasma cholesterol by 72% and HDL-C by 74% compared to wild-type mice when fed standard laboratory chow.[156] Similar reduction in To compensate for loss of apoA1, HDL mice from knockout increased ApoE protein content, comprising 25% of HDL protein content. ApoA2, apoA3, apoA4, and apoC were also increased in knockout mice HDL along with a marked reduction in cholestoryl ester.[156]

Transgenic mice with human apoA1 increased serum HDL-C concentrations but did not change total cholesterol or triglyceride concentrations compared to controls.[157] Despite increased HDL-C and apoA1 concentrations, transgenic mice did not have increase paraoxonase activity or lipid peroxidation protection. Using zymosan to induce sterile inflammation in a separate study with transgenic mice with human apoA1, total monocyte and neutrophil numbers were reduced but retained similar chemokines concentrations.[158]

In cell cultures, apoA1 affected the ability of immune cells to respond to LPS stimuli. Apolipoprotein A1 inhibited NF-kB activation associated with LPS challenge in cultured dendritic cells and complement activation in necrotic cells.[159] Murine macrophages pretreated with ApoA1 lowered the chemotactic response; this response led to limited monocyte recruitment in zymosan-challenged transgenic mice.[158]

1.6.3. **Influence of Intestinal HDL and apoA1 on CVD Risk Factors and Systemic Inflammation**

High density lipoproteins (HDL), most recognized for their role in mediating reverse cholesterol transport (RCT), have recently gained prominence for their role in innate immunity[69,160,161]. High-density lipoproteins actively bind LPS and aid in its clearance from peripheral tissues and plasma[69]. These lipoproteins are also able to detect of viruses and prevent parasitic infections through a variety of mechanisms. The phospholipid and cholesterol of lipoprotein molecules can neutralize parasite/viral components; additionally, the acute phase immune response can displace apoA1 with SAA, thus providing free apoA1 for binding of viruses[69,162].

Approximately 70% of plasma HDL is manufactured in the liver with the remaining 30% being produced by the intestinal epithelia[163]. Plasma HDL is well accepted to play a significant role in binding LPS and sequestering pathogens until they can be processed and disposed of by the liver[164]. The dual roles of HDL in LPS and cholesterol transport are highlighted in shared pathways of disposal[69].

Danielsen, et al., determined enterocyte apolipoprotein production by pig intestinal tissue explants[165]. Upon stimulations, explants showed rapid release of apoA1 into culture media, while apoB48 largely remained in tissues. Feeding did not affect lipoprotein production rates; however, jejunal segments of fed animals reduced apoA1 secretion to culture media.[165] Native HDL, isolated from human sera, effectively bound

to cholesterol-loaded enterocytes.[166] This interaction was inhibited by pre-treating the cells with anti-apoA1 and anti-apoA2 antibodies, underlining the importance of apolipoproteins in HDL function.

Within disease states, HDL and apoA1 can serve as markers of inflammatory states. Human subjects with active Crohn's disease, reduced plasma HDL-C and apoA1 were accompanied with increases in plasma SAA and CRP compared to non-inflamed CD patients and healthy controls.[167] Additionally, the active disease state was associated with increased carotid intima medium thickness, a marker used to track atherosclerosis progression. In two mouse strains modeling systemic inflammation, COX-2 and IL-10 knockouts, apoA1 mimetics effectively reduced intestinal inflammation.[168] The mimetics, given in drinking water, further highlight the and intestinal inflammation

In a study of 30 men, changes in gut permeability, lipoproteins and circulating inflammatory markers were studied.[169] Gut permeability was not associated to body weight or BMI, although adiposity was not reported. Intestinal and colonic permeability were each associated with plasma HDL-C. Inflammatory markers (CRP, CD14, IL-6, LPS-binding protein) remained within clinical normal rangers and were not associated with HDL-C[169]. Dietary intake records showed increased protein intake to be positively correlated with endotoxin, CRP and CD14.

To determine the interactions of apoA1, small intestine sections were excised from fast pigs and incubated with immunofluorescent antibodies.[170] ApoA1 was predominantly located in the brush border and scattered throughout the lamina propria.[170] Exposure to bile and taurocholate stimulated the release of apoA1 from the intestinal brush border. These results indicate a role of apoA1 in trans-intestinal cholesterol efflux (TICE). Combined with an earlier study[165], apoA1 clearly operates in roles other than reverse cholesterol transport. Given the GI tract accounts for 30% of lipidated HDL production[163], we hypothesize that prolonged gut dysfunction may be a primary source of dysfunctional HDL

2. EFFECT OF DAIRY MILK FRACTIONS ON GASTROINTESTINAL INTEGRITY IN C57BL/6 MICE

2.1. Introduction

Dairy milk, an animal protein source recommended as part of USDA's "MyPlate"[171], is a complex food product that provides vitamins and minerals, as well as oligosaccharides, protein, and lipids. Human clinical and epidemiological studies established associations with dairy intake and reduced colorectal cancer risk and improved body weight regulation[172-174]. Initially, such associations were ascribed to dairy calcium[173,175]; however, specifically designed animal studies provided evidence that dairy protein and lipid components served active roles in those positive outcomes[39,43,124,176]. Milk digestion gives rise to novel bioactive and protein lipid moieties[41,177-179]. Several previous studies using dairy protein sources showed non-fat dry milk (NFDM) reduced body fat gain and inflammatory responses compared to isolated soy protein in mouse models[39,180]. In a LPS challenge model, a high-fat diet containing milk fat globule membrane (MFGM) protected against gut leakiness and reduced pro-inflammatory cytokines[43]. MFGM reduced the incidence aberrant crypt foci in Fisher-344 rats, despite no change in cancer gene expression[124]. The potential for full-fat milk products to influence body weight gain, gut health, and inflammatory responses is poorly studied.

Obesity, a leading cause of metabolic disease, is thought to increase gut permeability, leading to increased endotoxemia and plasma cytokines[94,181,182]. The potential of whole

milk or its fractions to influence intestinal health markers is uncertain outside of accelerate disease models, Furthermore, current studies investigating the value of dairy or soy for propensity to promote or inhibit disease largely do so in the context of high-fat diet feeding[43,76,124,183]. Milk-derived saturated fats have been shown to promote colitis and microbial dysbiosis in an IL-10-/- mouse model with high-fat diet feeding[76]. However, no studies have addressed the effects of whole dairy milk or its fractions within the context of diets formulated to macronutrient distributions following standard mouse breeder chow and the Dietary Guidelines for Americans of 30% energy (EN) as fat, 20% EN as protein and 50%EN as carbohydrate[184-186]. Full-fat dairy as dried whole milk powder (DWMP) or its fractions, milk protein concentrate (MPC) and MFGM, were evaluated with isolated soy protein (ISP) serving as a plant-based protein comparator. Our study sought to determine the effects of whole milk or its fractions compared to a plant-based protein source in diets containing the similar caloric densities.

2.2. Materials and Methods

2.2.1. **Purified Diets - Animal Growth, in vivo Intestinal Permeability, and Motility**

The Institutional Animal Care and Use Committee (IACUC) at Texas A&M University approved all animal studies. Weanling (21-day-old) C57Bl6 male mice were obtained from Jackson Labs (Bar Harbor, ME) and housed individually with 12-hour (6 am on/6 pm off) light cycles. Mice were provided with a closed-formula, non-purified diet (Teklad Rodent Diet #8604, Harlan Laboratories, Indianapolis, IN) for seven days to adapt to local environmental conditions and measure growth rates. On day seven,

animals were sorted into four groups of five animals each, with similar starting body weights and growth rates. Groups were randomly assigned to a purified diet (PD) treatment comprised of isocaloric, isonitrogenous diets that differed in their protein sources: ISP, DWMP, MPC, and MFGM (Table 2.1, Appendix A-C). ISP and MFGM contained lard as primary lipid source, with saturated fats averaging 34% of fatty acid profiles (Appendix C). DWMP and MFGM contained dairy fat as the primary lipid source with saturated fats equaling 56% of total fatty acids. Animals were fed test diets ad libitum for 13 weeks.

At week 12, mice received an oral gavage of 600 mg/kg BW FITC-dextran with 5% Evans blue dye suspended in 5% arabic gum solution to assess in vivo intestinal permeability and motility [95,187,188]. Mice were then returned to individual cages with paper liners and fecal outputs monitored; whole gut transit time was defined as time from oral gavage until mice first excreted blue feces. Ability to exclude the oral FITC-dextran dose (Sigma-Aldrich, St. Louis, MO) was assessed by cheek bleed four hours post-gavage and plasma fluorescence measured in a microplate reader with excitation 485 nm and emission 535 nm. Plasma FITC concentrations were determined from a standard curve. Mice were euthanized by CO2 asphyxiation and exsanguinated by cardiac puncture. Whole blood was collected into heparinized tubes and plasma separated within two hours following centrifugation. Liver, thymus, spleen, gastrocnemius muscle (one leg), and retroperitoneal fat pads were collected, weighed,

and stored at -80°C; epididymal, subcutaneous and inguinal fat pad weights were recorded.

Gastrointestinal tracts were removed and lengths measured prior to division into intestinal regions of proximal and distal small intestine, cecum, and ascending and descending colon. Tissue sections were divided for histology, enzyme assays and gene expression analysis. Cecal contents were aliquoted for short-chain fatty acid and indole concentration analyses.

Table 2.1 Diet Composition

	ISP	DWMP	MPC	MFGM
Protein, % by weight	18.7	18.0	18.6	18.6
Carbohydrate, % by weight	46.9	46.4	47.8	47.1
Fat, % by weight	12.2	12.4	12.1	12.5
Cholesterol mg/kg	95	378	139	74
Cholesterol (% by weight)	0.01	0.04	0.01	0.01

Protein, % kcal from	20.1	19.5	19.9	19.8
Carbohydrate, % kcal from	50.4	50.3	51.1	50.2
Fat, % kcal from	29.5	30.2	29.1	30
Saturated Fat, % of total fat	33.8	58.6	35.4	55.5
MUFA, % of total fat	39.7	26.5	40.1	31.5
PUFA, % of total fat	26.5	14.9	24.5	12.9
Kcal/g	3.7	3.7	3.7	3.8

Values are calculated from ingredient analysis or manufacturer data

Ingredient	**g/kg**	**g/kg**	**g/kg**	**g/kg**
Isolated Soy Protein	210			
Dried Whole Milk Powder		390		
Milk Protein Concentrate			230	
Milk Fat Globule Membrane Protein				325
Casein		95		
DL-Methionine	2.5			3
L-Cystine	1.05	2.2	2.3	1.05
Sucrose	190	40	190	190
Corn Starch	188.13	175.58	189.28	178.68
Maltodextrin	100	100	100	100
Cellulose	140	140	140	110
Soybean Oil	20	20	20	20
Anhydrous Milkfat				27
Lard	100		95	
Mineral Mix, w/o Ca & P	17	17	17	17
Calcium Phosphate, dibasic	10.9		0.5	9.2
Calcium Carbonate	4.5	4.3		3.15
Vitamin Mix, AIN-93-VX	12.7	12.7	12.7	12.7
Choline Bitartrate	3.2	3.2	3.2	3.2
TBHQ, antioxidant	0.02	0.02	0.02	0.02

2.2.2. **Plasma Endotoxin Assays**

Bacterial endotoxin transfer into circulation was measured in plasma by LAL assay with Glucashield® buffer (Associates of Cape Cod, Inc. (ACCI), East Falmouth, MA). Diluted Plasma samples were diluted 1:10 in endotoxin-free water, plated into a 96-well microplate with equal volume of diluted lysate and incubated for 74 minutes at 37°C. The reaction was stopped by adding 50% acetic acid; absorbance read immediately at 405 nm. Sample concentrations were determined from a standard curve created from Control Standard Endotoxin (ACCI).

2.2.3. **Intestinal Enzyme Assays**

Myeloperoxidase (MPO) activity in descending colon sections was measured using a commercially available fluorometric MPO assay kit (ab111749, Abcam Inc., Cambridge, MA). Frozen tissue was homogenized in four volumes of assay buffer, plated in a black microplate and assayed following manufacturer's instructions. Microplate was read kinetically for one hour at Ex/Em 485/520 on a Synergy2 microplate reader using Gen5 Software (Biotek Instruments, Winooski, VT). MPO activity in samples was calculated from fluorescein generated in MPO standards (1 unit MPO activity = MPO required to generate 1 umol fluorescein/minute from oxidized aminophenyl fluorescein).

Intestinal alkaline phosphatase (iALP) activity in small intestine and colon was determined using SensoLyte pNPP ALP assay kit (Anaspec, Fremont, CA). Intestinal tissue was homogenized with lysis buffer (400 ul/50 mg tissue), centrifuged and

supernatant collected. Small intestine samples were diluted 1:100 or 1:200 with dilution buffer; colon samples were diluted 1:50. Standards and diluted samples were incubated with pNPP reaction mixture for 25 minutes and absorbance read at 405 nm. Sample concentrations were calculated based on a standard curve.

2.2.4. **Histology**

To investigate changes in structural development of the intestine, histological sections from distal small intestine were analyzed for general morphology. Fresh tissues were fixed immediately by submersion in Z-fix fixative solution (Anatech, Ltd., Battle Creek, MI) for 24-36 hours, rinsed with PBS and stored in 70% ethanol. Fixed tissues were embedded in paraffin and stained with hematoxylin and eosin (H&E) (TAMU College of Veterinary Medicine Histology Lab, College Station, TX). Stained sections were scanned at 20x magnification in a Hamamatsu C9600-12 slide scanner (Hamamatsu Photonics, Hamamatsu City, Japan). Manual analyses of scanned images for villus length/width, crypt depth/width and smooth muscle thickness were conducted using NDP.view2 software (Hamamatsu Photonics). For each sample slide, a minimum of 20 measurements were obtained per morphological characteristic and sample average determined. Average values were used in subsequent data analyses.

2.2.5. **Gene Expression**

Real-time reverse transcriptase (RT-qPCR) using the comparative C_T method ($\Delta\Delta C_T$) for relative quantitation was used for gene expression analysis (Table 2.2). Frozen proximal small intestine samples were homogenized in TRizol (ThermoFisher, Waltham, MA) with a tissue homogenizer and total RNA was extracted using RNeasy mini kit (#74104, Qiagen, Germantown, MD) following manufacturer's instructions. Total RNA was then subjected to DNase treatment using Ambion TURBO DNA-*free* (#AM1907, ThermoFisher, Waltham, MA) to remove genomic DNA. The quantity and quality of RNA were determined using an Eppendorf Biophotometer (Eppendorf, Hamburg, Germany); a minimum 1.8 260/280 absorbance ratio was considered acceptable. The RNA samples were normalized, and reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit protocol (Applied Biosystems (ABI), Foster City, CA). cDNA was used as a template for RT-qPCR amplification of target gene (Appendix D). RT-qPCR reactions were performed using the 7500 Real-Time fast PCR system with SYBR Green reagents (ABI). Comparative critical threshold (C_T) method was used to determine the relative mRNA levels. β-actin (ACTB) was used as an endogenous control. Intestinal samples from ISP-fed mice were used as calibrator for obtaining relative quantitative expression of genes for DWMP, MPC and MFGM-fed intestinal tissue samples. Fold differences of 2.0 or greater were considered significant.

Table 2.2 Analyzed Gene Targets

Gene Name	Gene Shortcode	Functional Group
actin, beta	ACTB	Housekeeping
apolipoprotein A1	APOA1	Inflammation/HDL
toll-like receptor 4	TLR4	Inflammation
toll-like receptor 5	TLR5	Inflammation
myeloid differentation primary response protein 88	MYD88	Inflammation
nuclear factor of kappa light polypeptide gene enhyancer in B-cells 1	NFKB1	Inflammation
tumor necrosis factor - alpha	TNFA	Inflammation
zona occludens protein 1 (aka Tight junction protein 1: TJP1)	ZO1	Tight Junction
fatty acid amide hydrolase	FAAH	eCB
monoacylglycerol lipase	MGL	eCB
N-acylphosphatidylethanolamine phosphlipase D	NAPE-PLD	eCB
cannabinoid receptor type 2	CB2	Cannabinoid
transient receptor potential cation channel, subfamily V, member 1	TRPV1	Cannabinoid
g protein-coupled receptor 119	GPR119	Cannabinoid
G protein-coupled receptor 41	GPR41	Cannabinoid
G protein-coupled Receptor 55	GPR55	Cannabinoid
fas cell surface death receptor	FAS	Cannabinoid/Lipogenesis
cannabinoid receptor type 1	CB1	Cannabinoid/Lipogenesis
adipocyte protein 2 (aka fatty acid binding protein 4: FABP4)	AP2	Adipocyte Differentiation
CCAAT/Enhancer Binding Protein	C/EBP	Adipocyte Differentiation
peroxisome proliferator-activated receptor gamma	PPARg	Adipocyte Differentiation
peroxisome proliferator-activated receptor alpha	PPARA	Adipocyte Differentiation
acetyl-CoA carboxylase	ACC	Lipogenesis
sterol regulatory element-binding protein 1	SREBP	Lipogenesis

2.2.6. **Cecal Metabolite Content**

Stable isotope dilution gas chromatography was used to measure short chain fatty acids (SCFA) and branched chain fatty acids (BCFA)[189]. In total, six fatty acids were measured: acetic acid, propionic acid, butyric acid, isovaleric acid, isobutyric acid, and valeric acid. Cecal samples were weighed, diluted 1:5 in 2N HCl and homogenized for 30 minutes at room temperature. Homogenized samples were then centrifuged and supernatant collected. 500 uL of supernatant was mixed with 10 uL internal standard and extracted using a C18 solid phase extraction column. After derivitization with *N*-tert-Butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA), samples were separated and quantified by GC-MS. The ratio of area under the curve (AUC) to the internal standard curve was used to quantify SCFA/BCFA concentrations. Concentrations were determined based on g cecal content used for extraction.

Frozen cecal samples were homogenized in 1:3 chloroform:methanol, centrifuged and supernatant collected for indole extraction[108]. The addition of distilled water followed by brief centrifugation allowed for separation of methanol and chloroform phases. LC-MS was performed on reconstituted samples following the protocol published by Jin, et al[190].

2.2.7. **Chow-fed Reference Mice**

It is largely accepted that chow-fed mice serve as "the gold standard" for laboratory mouse growth and health. An extensive literature search to define GI-P clinical values for healthy C57Bl/6 mice was inconclusive. To establish clinical values of healthy,

chow-fed animals, a separate cohort of 10 age-matched, 16 week C57Bl/6 male mice were purchased (Jackson Laboratories, Bar Harbor, ME). Mice were housed in TAMU facilities with standard 12-hour light/dark cycles and maintained on 4% Teklad Rodent Diet (Harlan, Madison, WI; Appendix A-C)) for two weeks to acclimate to local conditions. Mice received an oral dose of 600 mg/kg BW 4kDA FITC-dextran and were returned to cages. After 4 hours, mice were euthanized by $C0_2$ asphyxiation and exsanguinated by cardiac puncture. Plasma fluorescence and endotoxin were determined as described above.

2.2.8. **Statistical Analysis**

The effect of treatments on the "variables of interest" was determined with PROC MIXED of SAS (SAS Inst., Cary, NC), assuming initial body weight as covariate, "cohort" (1, 2 or 3) as a random factor to account for heterogeneity in the covariance structure. The restricted maximum likelihood was used to estimate the covariate parameters. Because degrees of freedom were different among treatments, the Kenward-Roger method was used to compute the denominator degrees of freedom. The least-square means method was used for multiple comparisons of treatment means after adjusting the P-value for Tukey. Significance was assumed when P-value was equal to or less than 0.05. For parameters lacking diet-cohort interaction (i.e. blood measures and gene expression data), one-way ANOVA and Tukey's HSD were used. Data are reported as means ± standard error of the mean unless otherwise noted.

2.3. Results

2.3.1. **Animal Growth and Development**

Starting body weight proved a reliable predictor of % body weight gain ($R^2 = 0.816$, $p<0.0001$). Cohort 2 (n=20) had the heaviest start weights, while cohort 3 (n=20) had the lightest start weight (Appendix E, $p<0.0001$). To account for the differences in initial body weight, a mixed model was created in SAS for data involving all cohorts. Final body weights differed by diet after 13 weeks on experimental diets. Across all cohorts (n=15/dietary treatment), MFGM-fed mice were heaviest, had the highest total weight gain, and % weight gain (Table 2.3). Feed intake was greatest in DWMP, significantly so compared to MPC (Table 2.3).

Table 2.3 Animal Growth and Feed Intake

	ISP	DWMP	MPC	MFGM
Start Weight	16.0 ± 1.24	16.9 ± 1.24	16.0 ± 1.24	16.7 ± 1.24
Final Weight	30.0 ± 1.49^{b}	30.3 ± 1.49^{b}	29.6 ± 1.49^{b}	32.3 ± 1.49^{a}
Total Weight Gain	13.7 ± 1.49^{b}	13.9 ± 1.49^{b}	13.2 ± 1.49^{b}	16.0 ± 1.49^{a}
Total Feed Disappearance	256 ± 7.09^{ab}	267 ± 7.09^{a}	252 ± 7.09^{b}	263 ± 7.08^{a}
Feed Efficiency	19.6 ± 1.89^{a}	20.3 ± 1.89^{a}	19.9 ± 1.89^{a}	17.5 ± 1.89^{b}

Data represents values from all cohorts; n=15 mice/diet group. Values are means (g) ± standard error of the mean; feed efficiency values are means (g feed/g gain) ± standard errors. Different letters signify differences between groups ($p<0.05$).

Organ weights and fat pads trended similarly, with MFGM mostly being the heaviest (Table 2.4). Liver and fat pad weights were heaviest in MFGM, significantly differing from DWMP and MPC. DWMP had the heaviest cecum, which was significant compared to MPC and MFGM. Organ weights expressed as % of final body weight

removed any significant diet effects (Appendix F). Dietary protein source did not alter small intestine ($p<0.77$) and colon lengths ($p<0.40$).

Table 2.4 Organ Weights

	ISP	DWMP	MPC	MFGM
Liver	$1.21 \pm 0.05^{a,b}$	1.10 ± 0.05^{b}	1.08 ± 0.05^{b}	1.34 ± 0.05^{a}
Cecum	$0.32 \pm 0.02^{a,b}$	0.39 ± 0.02^{a}	0.31 ± 0.02^{b}	0.28 ± 0.02^{b}
Gastrocnemius Muscle	0.16 ± 0.01	0.16 ± 0.01	0.15 ± 0.01	0.16 ± 0.01
Retroperitoneal Fat Pad	0.33 ± 0.06^{ab}	0.27 ± 0.06^{b}	0.30 ± 0.06^{b}	0.36 ± 0.06^{a}
Fat Pads: Inguinal, Subcutaneous, Epididymal (n=5)	3.19 ± 0.26^{ab}	3.50 ± 0.18^{b}	2.94 ± 0.06^{b}	3.98 ± 0.15^{a}
Total Fat Pads (n=5)	3.56 ± 0.30	3.85 ± 0.21	3.26 ± 0.05	4.40 ± 0.18
Spleen	0.081 ± 0.007	0.078 ± 0.008	0.080 ± 0.007	0.083 ± 0.007
Thymus (mg)	32.3 ± 1.93	32.7 ± 2.09	34.4 ± 1.94	33.2 ± 2.06

Data represents values from all cohorts; n=15 mice/diet group unless otherwise noted. Values are means (g) ± standard errors; thymus values are means (mg) ± standard errors. Different letters signify differences between groups ($p<0.05$).

2.3.2. *in vivo* Intestinal Permeability and Motility

Mice fed DWMP and MFGM had lower levels of FITC-dextran in the plasma than did mice fed ISP (Table 2.5). Mice fed DWMP had the highest intestinal integrity as indicated by the lowest FITC-dextran concentration in peripheral blood, with values close to statistical significance ($p<0.0545$). Mice fed ISP and MPC had the highest plasma concentration of FITC-dextran. We found low and similar plasma endotoxin concentrations in mice from all purified diet groups (Table 2.5). Plasma IgA was greatest

in MFGM-fed mice, being increased 80 ug/mL DWMP and MPC and 120 ug/mL over ISP.

2.3.3. **Intestinal Enzyme Assays**

Myeloperoxidase activity, a marker of neutrophil infiltration, was similar in mice fed dairy-protein containing diets (Table 2.5, $p<0.873$). However, MPO activity, increased >370% in distal colons of ISP-fed mice over dairy-fed mice ($p<0.006$). Intestinal alkaline phosphatase, a marker of intestinal cell turnover, was numerically lower in small intestines from DWMP- and MPC-fed mice, but did not differ statistically between diet groups ($p<0.118$). When soy/dairy protein sources were compared collectively, dairy-fed mice (n=15, 2.64 ± 0.21 mg/mL) had less alkaline phosphatase in small intestine tissues compared to ISP-fed mice (n=5, 4.49 ± 0.82 mg/mL, $p<0.027$).

Table 2.5 Markers of GI Integrity

	ISP	DWMP	MPC	MFGM	p-value
Transit Time (hr) n=5	3.24 ± 0.37	2.83 ± 0.39	3.14 ± 0.60	3.58 ± 1.42	0.930
Endotoxin (EU/mL) n=15	0.10 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.875
FITC-Dextran (ug/mL) n=5	0.19 ± 0.02	0.11 ± 0.01	0.19 ± 0.03	0.15 ± 0.02	0.0545
IgA (ug/mL) n=15	161 ± 18.2^{c}	212 ± 18.2^{b}	204 ± 18.2^{bc}	285 ± 18.2^{a}	0.000
MPO (uU/mL) n=5	36.3 ± 10.7^{a}	9.75 ± 2.59^{b}	8.16 ± 1.70^{b}	8.36 ± 1.23^{b}	0.006
ALP (mg/mL) n=5	4.49 ± 0.90	2.65 ± 0.38	2.28 ± 0.26	2.99 ± 0.26	0.118

MPO results are expressed as uU/mL, where 1 uU = 1 pmol/min activity.

2.3.4. **Histology**

H&E stained sections of distal small intestines were analyzed for villi length/width, crypt depth/width and smooth muscle thickness (Table 2.6). Numerically, dairy-fed mice had the longest villi and crypts, although the values did not reach statistical significance due to intergroup variability. In particular, histological sections from one animal were barely quantifiable, perhaps through compromise during the embedding process. Exclusion of that animal from statistical analyses, leaving only 4 mice in MFGM, resulted in statistical differences in crypt depths with MFGM- and MPC-fed mice having deeper crypts than ISP ($p<0.014$). Villus lengths/widths, crypt widths and smooth muscle thickness remained similar between diet groups. General observations of remaining histological sections did not reveal immune compromise and immunohistochemistry staining for CD3 was not different between diet groups.

Table 2.6 Small Intestine Morphology

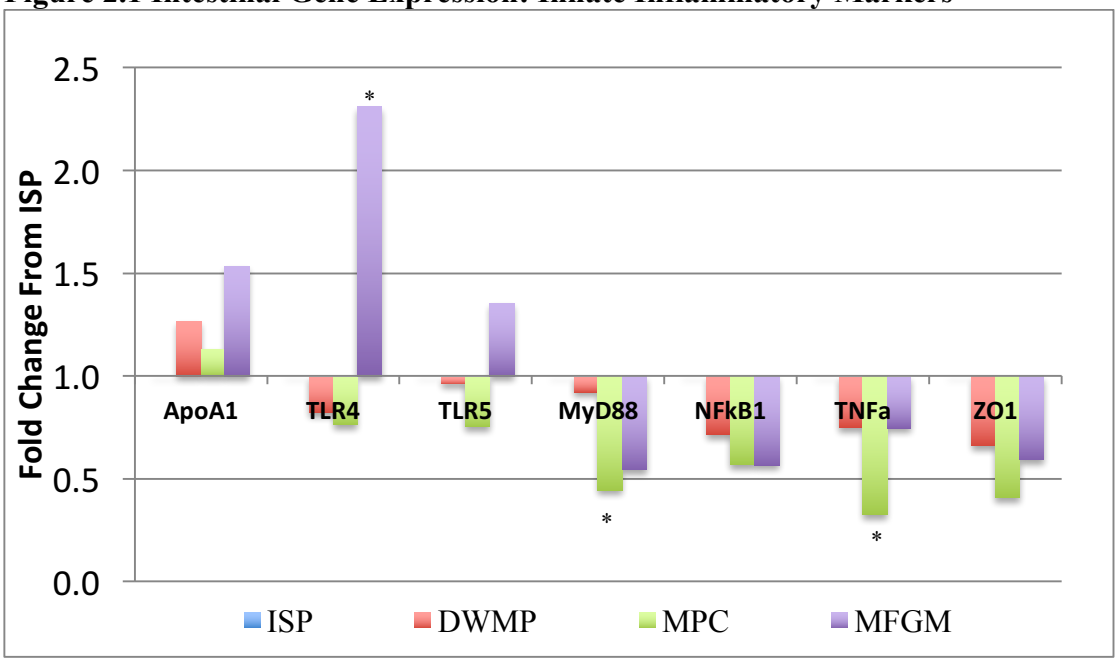
	ISP	DWMP	MPC	MFGM	p-value
Villus Length	234 ± 27.0	264 ± 29.8	269 ± 18.9	262 ± 41.8	0.760
Villus Width	64.0 ± 3.65	58.9 ± 4.55	66.2 ± 1.48	71.0 ± 1.41	0.104
Crypt Depth	75.7 ± 3.63	87.9 ± 4.27	89.9 ± 3.90	86.8 ± 5.89	0.077
Crypt Width	27.8 ± 1.93	27.3 ± 0.86	30.3 ± 0.62	28.9 ± 0.80	0.594
Smooth Muscle	33.5 ± 2.02	40.5 ± 3.74	32.9 ± 2.06	31.2 ± 3.54	0.189

Data represents values from cohort 3 only; n=5 mice/diet group. Individual data points were averaged to produce a single value per mouse for each measure. Values are means (μm) ± standard errors. Different letters signify differences between groups ($p<0.05$).

2.3.5. **Gene Expression**

Overall, fold changes in distal small intestine gene expression were modest and largely reflective of a healthy gut state. When dividing gene expression data into functional groups, innate inflammatory genes (ApoA1, TLR4, TLR5, MyD88, NFkB1, TNFα, ZO-1) were not indicative of an inflammatory state (Figure 1). Toll-like receptor 4 (TLR4) in MFGM-fed mice intestines increased 2.3-fold compared to ISP; however, the downstream genes (NFkB and TNFα) were not upregulated. Zona occludin-1, ZO-1 (also known as Tight Junction Protein 1, TJP1) was measured in small intestinal tissues; ZO-1 gene expression did not change between dietary treatment groups.

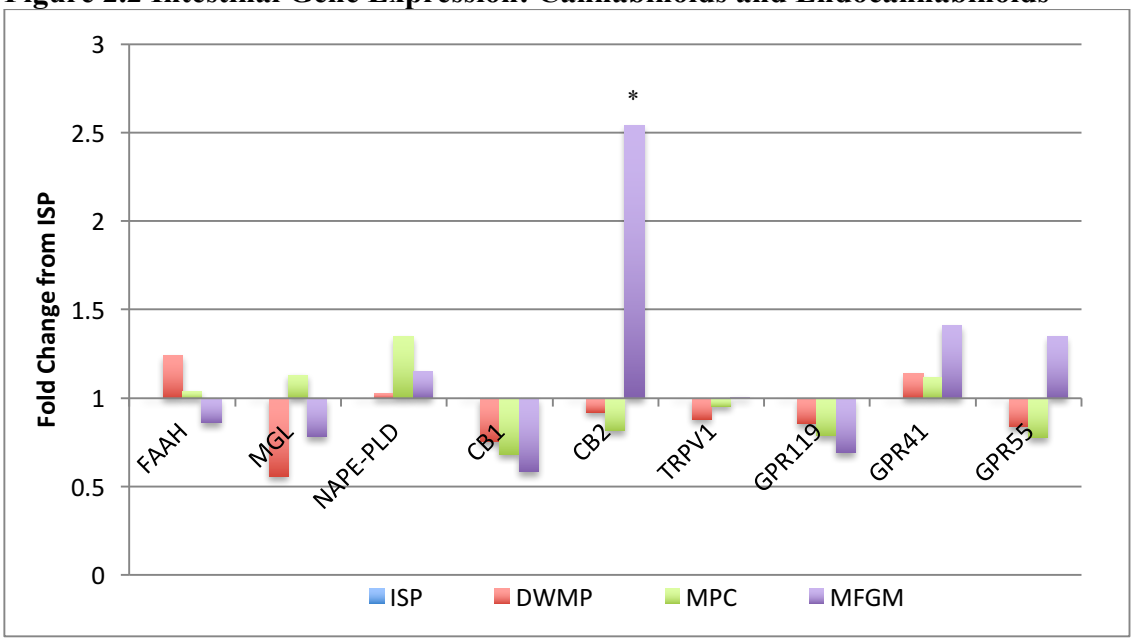
Figure 2.1 Intestinal Gene Expression: Innate Inflammatory Markers



Gene expression data were from small intestines of cohort 3 only; fold increases were considered significant if at least 2-fold greater than the ISP comparator. TLR4 was increased 2.3 fold in MFGM compared to ISP; however, downstream responses (NFκB and TNFα) were modestly downregulated. Tight Junction Protein (ZO-1) was not different between diet groups.

Intestinal cannabinoids (CB) and endocannabinoid (eCB) genes have been implicated in regulating appetite control and gut physiology[191-195]. As such, genes corresponding to these pathways were measured (Figure 2). eCB gene expression did not change with different dietary treatments; however, cannabinoid receptor 2 (CB2) increased 2.5-fold in small intestines of MFGM-fed mice compared to those of ISP-fed mice. Downstream CB2 receptor targets (TRPV1, GPR119, GPR41 and GPR55) were not different.

Figure 2.2 Intestinal Gene Expression: Cannabinoids and Endocannabinoids

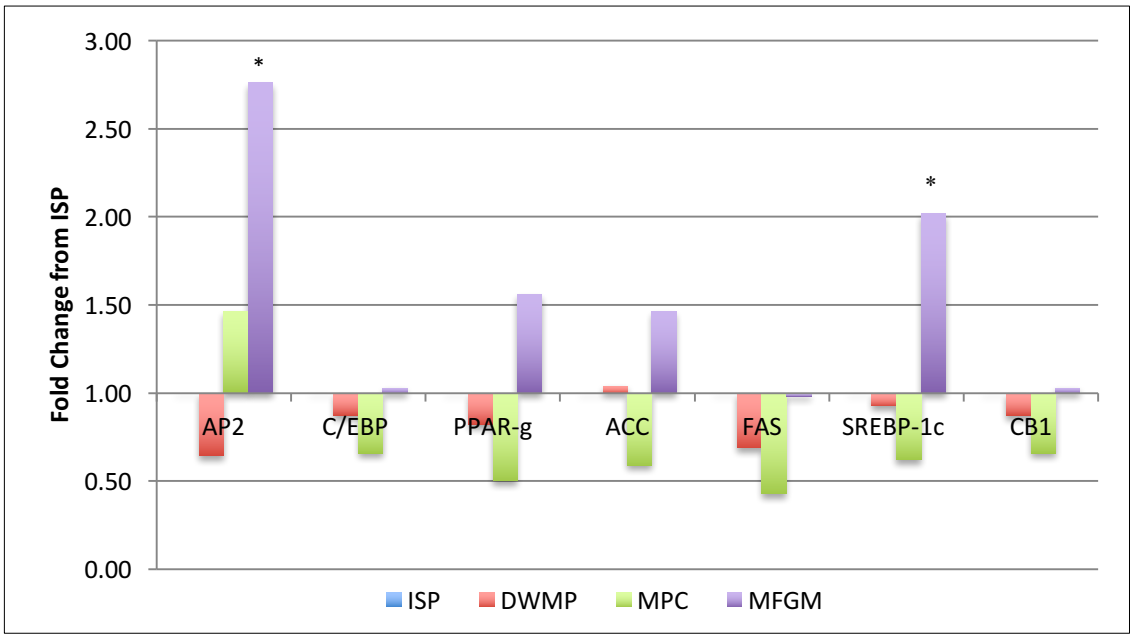


Gene expression data were from small intestines of cohort 3 only; fold increases were considered significant if at least 2-fold greater than the ISP comparator. CB2 was increased 2.5-fold in intestines from MFGM-fed mice compared to ISP. Other CB and eCB genes were unchanged.

While previous studies have focused on the effects of dietary dairy fat, the potential for dairy proteins to manipulate adipogenesis or lipogenesis is poorly defined. Therefore, expression of key genes in these pathways were evaluated (Figure 3). AP2 (adipocyte protein 2) was increased 2.76-fold in MFGM tissues, while SREBP-1c also increased 2-

fold in the same animals compared to ISP-fed mice. Upregulations of both genes indicate increased intestinal transport of fatty acids in MFGM-fed mouse intestines. The remaining adipocyte differentiation and lipogenesis genes were unaffected by changes in dietary protein.

Figure 2.3 Intestinal Gene Expression: Adipocyte Differentiation and Lipogenesis



Gene expression data from small intestines of cohort 3 only (n=5/diet); fold increases were considered significant if at least 2-fold greater than the ISP comparator. AP2 was increased 2.75-fold and SREBP-1c increased 2-fold in intestines from MFGM-fed mice compared to ISP. Other adipocyte differentiation and lipogenesis genes were unchanged.

2.3.7. **Cecal Metabolite Content**

When expressed as concentrations (i.e. uMol/g cecal content), cecal metabolites were largely similar across diet groups (Table 2.7). Notably, butyric acid concentrations were decreased in ceca of MPC-fed mice ($p<0.0003$). The remaining cecal fatty acids measured did not differ between diet groups. Potential shifts in relative distribution of SCFA/BCFAs were evaluated by calculating % of total and ratios between individual metabolites. Expressing individual SCFAs or BCFAs as % of total fatty acids to analyze relative abundance did not show any changes between diet groups ($p<0.09$). Cecal indole, cited as an anti-inflammatory in models of mucosal injury [189,196], did not differ between dietary treatments ($p<0.27$).

Table 2.7 Cecal Metabolite Content

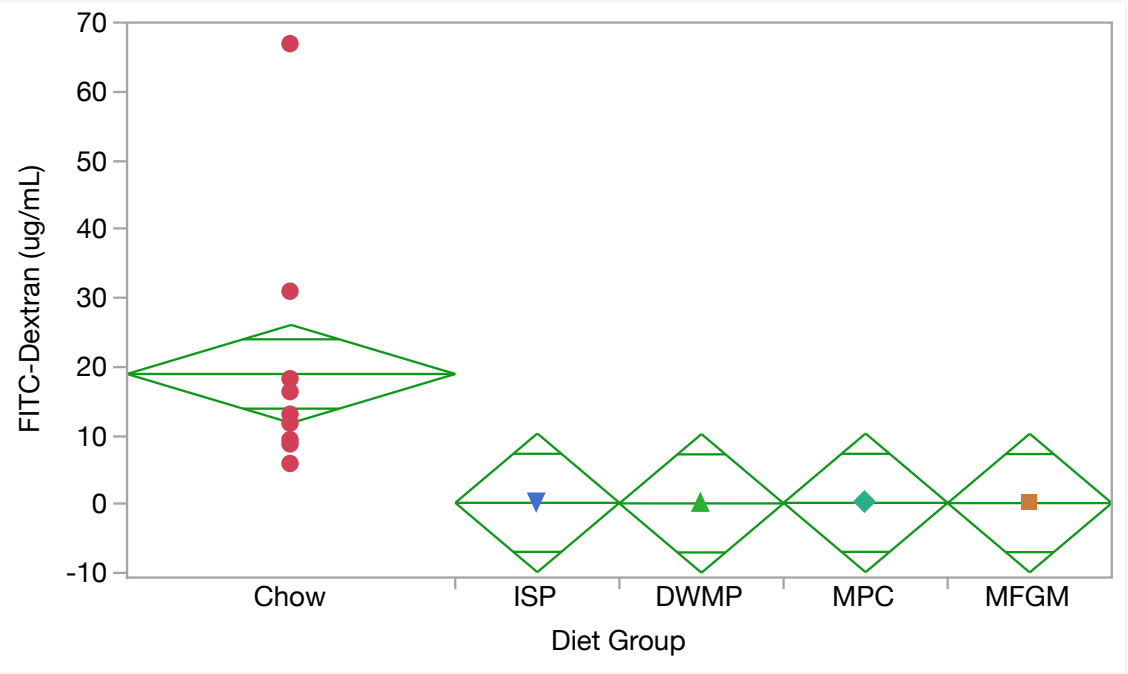
	ISP	DWMP	MPC	MFGM	p-value
Acetic Acid	23.8 ± 5.15	17.5 ± 5.14	18.3 ± 5.17	21.7 ± 5.14	0.066
Proprionic Acid	1.79 ± 0.42	1.60 ± 0.42	1.67 ± 0.44	2.37 ± 0.42	0.428
Butyric Acid	4.23 ± 0.32[a]	3.48 ± 0.32[a]	2.21 ± 0.33[b]	3.47 ± 0.32[a]	0.0003
Isobutyric Acid	0.26 ± 0.09	0.22 ± 0.09	0.25 ± 0.10	0.25 ± 0.09	0.836
Isovaleric Acid	0.11 ± 0.04	0.09 ± 0.04	0.08 ± 0.04	0.11 ± 0.04	0.107
Valeric Acid	0.25 ± 0.09	0.23 ± 0.09	0.20 ± 0.09	0.30 ± 0.09	0.129
Indole	159 ± 99.3	239 ± 101	90.4 ± 100	252 ± 97.4	0.274

SCFA values are expressed as average uMol/g cecal content ± standard error.
Indole values represent concentrations in nMol/g cecal content ± standard error.

2.3.8. **Chow-fed Reference Mice**

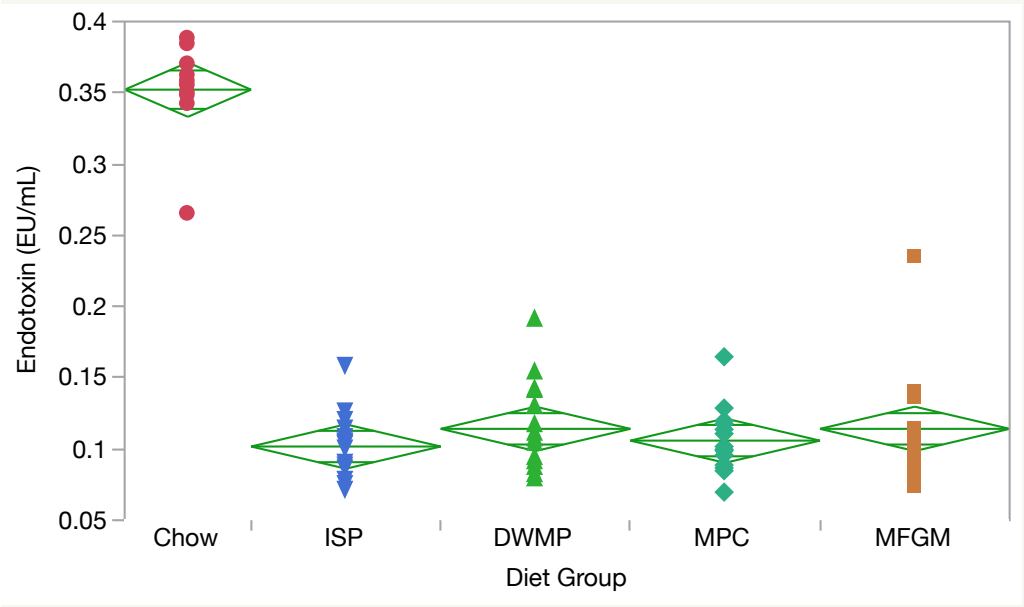
To determine reference values for age-matched reference mice, data collection followed those described for Trial 1. Plasma FITC-dextran fluorescence averaged 18.9±5.77 ug/mL, while plasma endotoxin concentrations were 0.35±0.01 EU/mL. When compared to Trial 1 (purified protein sources), plasma fluorescence increased 95-189-fold in reference mice over purified diets in conjunction with a 320% increase in endotoxin concentrations (Figure 4, 5). Plasma fluorescence to endotoxin (FITC-d/endotoxin) ratios increased 30.8-fold in reference mice (52.0 vs 1.69).

Figure 2.4 Purified vs Chow-fed – FITC-dextran permeability



Chow-fed mice (n=10) averaged 18.9±5.77 ug/mL FITC-d plasma fluorescence. Purified diets (n=60) averaged 0.16±0.01ug/mL FITC-d plasma fluorescence.

Figure 2.5 Purified vs Chow-fed - Endotoxin Concentrations



Plasma endotoxin content, measured by LAL assay, averaged 0.352±0.011 EU/ml for chow-fed mice (n=10) compared to average 0.109±0.004 EU/mL for purified diet-fed mice (n=60).

2.4. Discussion

Overall, our diets produced healthy mice with body weights on the upper limit of established growth curves from the breeder[91,197]. MFGM feeding resulted in heavier mice although total weight gain and % weight gain did not differ from the other diet groups. MFGM appears to preferentially promote fat deposition as evidenced by larger livers and retroperitoneal fat pads, as well as increased CB2, AP2 and SREBP-1c gene expression in small intestines[192,193,195]. Additionally, DWMP and MPC seem to promote leanness, having the lowest average liver and retroperitoneal fat pad mass. Larger cecal weights in DWMP may indicate a larger biomass since ceca weights remained heavier after correcting for final body weight ($p<0.010$). Gastrocnemius muscle, spleen and

thymus weights did not differ between diet groups, indicating normal muscle and immune organ growth for all mice.

Previous studies have hypothesized increased intestinal permeability may allow bacterial inflammogens in the form of lipopolysaccharide (LPS) passage into the systemic circulation and so increase non-specific inflammatory tone and chronic disease risk[99,198]. At present, it is not clear whether diet increases "gut leakiness" or whether it alters microbiota metabolism in such a way that more LPS is produced. This is an important issue as LPS alone will increase gut permeability[199,200]. Moreover, dairy components, specifically MFGM, have been shown to improve intestinal integrity and reduce intestinal permeability in LPS-challenged mice[43]. Although levels did not reach statistical significance, mice fed DWMP and MFGM had lower levels of FITC-dextran in the plasma than did mice fed ISP or MPC ($p<0.055$), possibly due to low sample size in our study. Low endotoxin results were not surprising as mice were healthy and had not been challenged by either poor quality diet or LPS dosing. This finding suggests that including dairy proteins and milk fat (DWMP and MFGM) in diets provides an acceptable macronutrient distribution. Whole-fat dairy products may subtly improve intestinal integrity as evidenced by decreased permeability and changes in intestinal CB2 gene expression. In addition to the association with GI-P[85,201], CB2 is highly associated with regulating innate immune function as a mediator of the immunosuppressive effects of eCBs[84,85]. Others have shown pretreatment of endothelial cells with CB2 agonists successfully lowers neutrophil activation and reduces TNF-a release[86]. Increased CB2

expression with MFGM feeding may prime intestinal tissues for an improved immune response in a challenge model. Further study is needed to elicit the mechanism by which dietary dairy fat strengthens intestinal integrity.

Microbial degradation products (SCFA/BCFAs and indole) varied little between dietary treatments. Butyrate concentrations have long been considered indicators of colon health, serving as a preferred energy source of colonic epithelial cells[202]; notably, butyric acid concentrations were highest in ceca from ISP- and MFGM-fed mice ($p<0.001$). Valeric acid, lowest in cohort 3, did not differ significantly between cohorts of different diet groups. Indole concentrations, a tryptophan metabolite hypothesized to improve tight junction proteins[108], did not change between ceca of mice from the four diets, remaining consistent with ZO-1 gene expression data.

While trying to determine probable associations between individual SCFA and BCFAs, we observed a linear relationship between isovalerate and isobutyrate. There was a 97% correlation across all diet groups and cohorts. This linear relationship has also been reported by Cardona, et al.[203] across four different species and multiple rearing/housing conditions and by Tjellström, et al.[204] in both healthy children and those diagnosed with Celiac disease. Cardona hypothesized that the linear relationship between isobutyrate and isovalerate was due to shedding of the intestinal epithelium rather than diet effects. Since this observation is consistent across species, rearing conditions and healthy/disease states, it is reasonable to assume the isobutyrate and isovalerate result from microbial

degradation of intestinal epithelium. In our study, no significant diet or cohort effect on the ratio of isobutyrate to isovalerate existed. We observed a very strong correlation (r^2=0.97), consistent to those previously reported. We also examined other SCFAs in relation to isovalerate:isobutyrate to determine if epithelial degradation products play a role in the degradation of dietary components to acetate, propionate, butyrate, and/or valerate. To create a correction for intestinal epithelial sources of BCFA, we calculated the average total of isobutyrate and isovalerate. After normalizing SCFAs by this correction factor, we observed no difference in diet groups for SCFA production.

Other markers of gut health, including MPO activity and histological analysis, showed marked changes with dietary protein source. Myeloperoxidase activity, a biomarker of neutrophil infiltration, increased more than 370% in colonic tissues of ISP-fed mice. MPO has been noted to mediate activation of neutrophils, the release of pro-inflammatory cytokines, and to increase in states of gastrointestinal compromise such as colitis[205,206]. In Fisher-344 rats, MFGM reduced aberrant crypt foci[124], a process associated with increased MPO activity. Studies noting inflammatory reductions with NFDM and MFGM feeding did not measure MPO activity[39,43]; given the significant reductions of plasma cytokines and morbidity/mortality rates in these studies, the possibility of dairy proteins to affect changes in intestinal neutrophil infiltration and the CB/eCB systems should be explored.

Remarkably, distal small intestines of MFGM-fed mice had longer, wider villi than both ISP- and DWMP-fed mice. Crypt depths were most shallow in ISP-fed mice, while DWMP-fed mouse intestines had the thickest smooth muscle. Prior studies indicated ileal smooth muscle thickness increases in association with obesity in rats[207]; however, our observed changes cannot be attributed to these causes as DWMP-fed mice were not obese nor did our diets contain varying fiber types which may attribute to changes in small intestine smooth muscle thickness[208]. Shorter, wider villi have been associated with disease and impaired nutrient absorption, while certain disease models resulting in lengthened villi are accompanied with marked loss of goblet cells[209]. Longer, wider villi without loss of goblet cells may provide increased surface area for nutrient absorption, consistent with the higher weight gains in MFGM-fed mice. Across all immunohistochemistry sections, staining for CD3, co-receptor of T-cells, was not grossly different and showed no evidence of immune compromise. Likewise, intestinal alkaline phosphatase, associated with mediating epithelial cell damage in the small intestine[122,123], remained unaffected by dietary changes in our study. Soy protein and supplemented with Ca^{2+} equivalent to levels found in non-fat dry milk promoted obesity in mice and adipose tissue inflammation[39]. In other animal models, increased dietary Ca^{2+} provoked naturally occurring necrotic enteritis and was associated with increased mortality[210]. DWMP contained the highest dietary calcium and calcium-to-phosphorus ratio (Appendix C). In our hands, DWMP's increased dietary calcium did not affect gastrointestinal health nor did it promote fat deposition relative to ISP. Thus, intestinal

integrity appears to be unaffected by isolated soy protein, dairy milk fractions, or varying Ca^{2+} levels in these diets.

Marked changes were observed in gastrointestinal permeability (GI-P) measures for chow-fed reference mice compared to those fed purified diets. Since all reference mice were healthy, the degree of increase in FITC-d permeability and plasma endotoxin was highly unexpected. As such, it is hard to determine whether the differences are due to dietary macronutrients, housing and transportation alterations or an unknown external challenge.

3. EFFECT OF DAIRY FRACTIONS ON LIPOPROTEINS, CIRCULATING INFLAMMATORY MARKERS, AND GENE EXPRESSION IN LIVER, MUSCLE AND ADIPOSE

3.1. Introduction

Diet and gastrointestinal integrity play pivotal roles in regulating lipoprotein biology[211]; alternately, HDL and their protein components are able to regulate inflammation[69,160,161]. Diets rich in either fat or simple carbohydrates are thought to promote obesity.[20] Inherent to such assertions is the assumption that protein is adequate and of equivalent quality. This may not always be true and differences in protein quality may be observed by how protein quality is reported and defined by PDCAAS or DIAAS values.[7,9] Obesity is a known contributor to dyslipidemia and impaired high density lipoprotein (HDL) function.[99,212-214] In addition, obesity is highly associated with altered gastrointestinal integrity and increased inflammatory tone.[95,215] We showed a strong association between fat pad accumulation and FITC-dextran intestinal permeability in C57Bl/6 mice fed diets with practical macronutrient distributions (Chapter 2).

Patients with gastrointestinal diseases, such as Crohn's disease, that compromise the epithelia barrier, have decreased HDL-cholesterol and lower apolipoprotein A1 concentrations.[167] Myeloid differentiation primary response protein 88 (MyD88) plays a role both in the inflammatory cascade and as an adaptor protein for apolipoprotein A1.[153] In these dual roles, MyD88 serves as a mediator between inflammation and reverse

cholesterol transport. As a regulator of cellular cholesterol, ATP-binding cassette transporter A1 (ABCA1), distributes cholesterol from macrophage or enterocyte mebranes to HDL's apoA1. Through intestinal ABCA1 knockout mouse models, Brunham, et al., showed 70% of lipidated HDL arises from the liver with the remaining 30% being produced by the intestinal epithelia.[163] Thus, the intestine is a critical component of HDL biology and systemic inflammation.

Given the role of the diet and gut health in systemic inflammatory tone and lipoprotein biology, we sought to determine the potential for full-fat dairy milk products to influence lipoprotein density profiles, liver gene expression, and inflammatory responses.

3.2. Materials and Methods

3.2.1. **Purified Diets - Animal Growth**

All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at Texas A&M University. Weanling (21-day-old) C57Bl6 male mice were housed and fed standard laboratory chow for one week prior to sorting to one of four isocaloric, isonitrogenous diets that differed in their protein sources: ISP, DWMP, MPC, and MFGM (See Chapter 2). At week 14 of experimental diet feeding, mice were euthanized by CO_2 asphyxiation and exsanguinated by cardiac puncture. Whole blood was collected into heparin tubes, placed on ice, and plasma separated within two hours following centrifugation. Liver, thymus, spleen, gastrocnemius muscle (one leg), and retroperitoneal fat pads were collected, weighed, and stored at -80°C for gene expression

analysis. For one cohort (n=5/diet), epididymal, subcutaneous and inguinal fat pad weights were recorded.

3.2.2. **Plasma Lipoprotein Distributions**

Plasma lipoproteins were analyzed following published methods.[214] Six uL plasma, stained with 10 uL C6 NBD ceramide (Cayman Chemical, Ann Arbor, MI), was diluted in 1284 uL 0.18M NaBiEDTA (TCI Chemicals, Portland, OR). Prepared samples were centrifuged in a MLA-130 fixed angle rotor (Beckman-Coulter, Indianapolis, IN) at 120,000 rpm for 6 hours at 5˚C using a Beckman Optima Max centrifuge (Beckman-Coulter); tubes were imaged in a dark room with a digital camera using a MH-100 metal halide continuous light source. A blue-violet filter centered at 407 nm and a yellow long-pass emission filter cut-on wavelength of 515 nm were used to match the spectrophotometric characteristics of C6 NBD ceramide. A gain of 1.0000, a target intensity of 30%, and an exposure time of 1 sec were selected. Images were converted to data by OriginPro2015 software (OriginLab, version 92E, Wellesley Hills, MA) and area under the curve (AUC) analyzed for density distributions. Average fluorescent intensity was plotted based on tube coordinate function and area under the curve quantified. Lipoproteins were divided into subfractions based on density.

3.2.3. **Gene Expression**

Real-time reverse transcriptase (RT-qPCR) using the comparative C_T method ($\Delta\Delta C_T$) for relative quantitation was used for gene expression analysis. Frozen tissue samples were

homogenized in TRizol (ThermoFisher, Waltham, MA) with a tissue homogenizer and total RNA was extracted using RNeasy mini kit (#74104, Qiagen, Germantown, MD) following manufacturer's instructions. Total RNA was then subjected to DNase treatment using Ambion TURBO DNA-*free* (#AM1907, ThermoFisher, Waltham, MA) to remove genomic DNA. The quantity and quality of RNA were determined using an Eppendorf Biophotometer (Eppendorf, Hamburg, Germany). The RNA samples were normalized, and reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit protocol (Applied Biosystems (ABI), Foster City, CA). cDNA was used as a template for RT-qPCR amplification of target gene (Appendix E). RT-qPCR reactions were performed using the 7500 Real-Time fast PCR system with SYBR Green reagents (ABI). Comparative critical threshold (C_T) method was used to determine the relative mRNA levels. β-actin (ACTB) was used as an endogenous control. Tissue samples from ISP-fed mice were used as calibrator for obtaining relative quantitative expression of genes for DWMP, MPC and MFGM-fed tissue samples. Target genes and their category of physiologic relevance (inflammation, lipo-/adipo-genesis, cannabinoid(CB) and endocannabinoid(eCB)) are listed in Table 3.1.

Table 3.1 Analyzed Gene Target and Physiologic Functional Category

Functional Category	Gene Shortcode	Gene Name	Tissue
Reference	ACTB	actin, beta	Liver, Adipose, Gastrocnemius Muscle
Inflammation	TLR4	toll-like receptor 4	Liver
Inflammation	TLR5	toll-like receptor 5	Liver
Inflammation	MyD88	myeloid differentiation primary response protein 88	Liver
Inflammation	NFκB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	Liver
Inflammation	TNFA	tumor necrosis factor - alpha	Liver
Inflammation, HDL	apoA1	apolipoprotein A1	Liver
Inflammation	CD11d	integrin subunit alpha D	Adipose
Lipogenesis	SREBP1c	sterol regulatory element-binding protein 1	Liver
Adipocyte Differentiation	PPARα	peroxisome proliferator-activated receptor alpha	Liver
Adipocyte Differentiation	PPARγ	peroxisome proliferator-activated receptor gamma	Liver, Gastrocnemius Muscle
Adipocyte Differentiation	AP2	adipocyte protein 2 (aka fatty acid binding protein 4: FABP4)	Liver
Adipocyte Differentiation	C/EBP	CCAAT/Enhancer Binding Protein	Liver

Table 3.1 Continued

Lipogenesis	FAS	fas cell surface death receptor	Liver
Lipogenesis	ACC	acetyl-CoA carboxylase	Liver
Cannabinoid	CB1	cannabinoid receptor type 1	
Cannabinoid	CB2	cannabinoid receptor type 2	Liver
Cannabinoid	TRPV1	transient receptor potential cation channel, subfamily V, member 1	Liver
Cannabinoid	GPR41	G protein-coupled receptor 41	Liver
Cannabinoid	GPR55	G protein-coupled Receptor 55	Liver
Cannabinoid	GPR119	g protein-coupled receptor 119	Liver
eCB	NAPE-PLD	N-acylphosphatidylethan-olamine phosphlipase D	Liver
eCB	FAAH	fatty acid amide hydrolase	Liver
Mitochondrial biogenesis	PGC-1α	PPAR gamma coactivator 1-alpha	Gastrocnemius Muscle

3.2.4. **LPS-Stimulated Cytokine Release Assay**

To investigate the inflammatory potential of peripheral monocytes after LPS challenge, we conducted a whole blood cytokine stimulation assay. LPS from two origins, *S. enteriditis* and *E. coli O111* (Sigma-Aldrich, St. Louis, MO), was used to evaluate

relative potencies of stimulated cytokine production. Fresh, heparinized whole blood (100 uL) diluted 1:10 with Russ-10 media was incubated with 100 ug LPS for 24 hours, centrifuged, and supernatant collected. The 24-hour incubation allowed complete detection of cytokine production, including initial response and resolution of the inflammatory response to LPS challenge. A multiplex magnetic bead-based assay (Millipore EMD, Billerica, MA) was used to measure *ex-vivo* cytokine production in peripheral blood. Whole blood cultures were centrifuged and supernatant stored at -80°C until immunoassay. A multiplex magnetic bead-based assay (Millipore) was used to measure cytokine concentrations in the supernatant. The cytokines of interest were TNF-α, IL-12p70, IL-10, IL-6, IFN-g, MIP-2 and IL-1β. Target cytokines were chosen due to their role in the inflammatory response pathway, being produced primarily by macrophages activated upon stimulation. Supernatant was diluted 1:6 with assay buffer to a final volume of 50 uL. Diluted sample (25 uL) was plated in a black microplate and assay proceeded as outlined in the manufacturer's instructions. After completion of the assay, fluorescence was measured in a Luminex plate reader and sample cytokine concentrations determined by xPonent software (version 3.1, Luminex Corporation, Austin, TX).

In a separate magnetic bead-based assay, eotaxin concentrations were determined in unchallenged plasma. Untreated plasma was diluted 1:2 with assay buffer and assayed according to kit protocols as above.

3.2.5. **Chow-fed Reference Animals**

As described in chapter 2, a separate cohort of 10 age-matched, 16 week C57Bl/6 male mice were purchased (Jackson Laboratories, Bar Harbor, ME) to establish reference values from unmanipulated mice. Mice were housed in TAMU facilities with standard 12-hour light/dark cycles and maintained on a nutritionally adequate, closed-formula diet containing 4% lipid by weight (4% Teklad Mouse/Rat Diet, Harlan, Madison, WI) for two weeks to acclimate to local conditions (Appendix A-C). Mice received an oral dose of 600 mg/kg BW 4kDA FITC-dextran and were returned to cages. After 4 hours, mice were euthanized by $C0_2$ asphyxiation and exsanguinated by cardiac puncture. Whole blood was collected into heparin tubes and processed as above. Lipoprotein density profiles and whole blood LPS-challenged cytokine release were determined as previously described.

3.2.6. **Statistical Analysis**

Diet-cohort interactions were analyzed with a mixed model effect as described in Chapter 2 (SAS, SAS Institute, Cary, NC). For parameters lacking diet-cohort interaction, including blood/plasma values and gene expression data, one-way ANOVA and Tukey's HSD were performed using JMP 14.0 software (SAS Institute, Cary, NC). Data are reported as means ± standard error of the mean unless otherwise noted. P-values <0.05 determined by Tukey-Kramer HSD were considered significant; therefore, specific p-values may not be included in text.

3.3. Results

3.3.1. **Animal Growth and Development**

As described in Chapter 2, growth rates and final body weights followed those expected for C57Bl/6 mice. Fat pads and livers were heaviest in MFGM-fed mice, being different from DWMP and MPC, while ceca were largest in DWMP (See Chapter 2). Gastrocnemius muscle, spleen and thymus weights did not differ.

3.3.2. **Plasma Lipoprotein Distributions**

Total lipoprotein mass (AUC) did not change with diet treatment (Table 3.2). Overall, purified dairy protein diets (DWMP, MPC, MFGM) had similar effects on lipoprotein classes. Among those common effects was increased TRL AUC, with MFGM being the highest and different from MPC and ISP (Tables 3.2 and 3.3). LDL (AUC) decreased in all dairy diets. Interestingly, HDL-3c (AUC), significantly increased in DWMP over MPC and MFGM. Given the similarities in total AUC, we evaluated the relative distributions of lipoprotein subclasses as % of total (Table 3.4). Consistent with AUC, %TRL increased with MFGM feeding and %LDL decreased in dairy diets compared to ISP. %HDL also increased in dairy-containing diets by an average of 6.8%. Values for FITC-dextran from Chapter 2, averaging 0.16 ± 0.02 ug/mL for all PDs, was significantly correlated with HDL AUC (FITC-Dextran (ug/mL) = 37.018259 - 0.0100031*Total HDL, $p<0.0003$).

Table 3.2 Lipoprotein Density Classes

	ISP	DWMP	MPC	MFGM	*P*	CHOW
TRL (AUC)	109 ± 34.7[c]	199 ± 34.8[ab]	150 ± 34.8[bc]	226 ± 34.8[a]	<0.0001	160 ± 14.3
LDL (AUC)	1865 ± 415[a]	1468 ± 415[b]	1338 ± 415[b]	1293 ± 415[b]	<0.0001	370 ± 16.6
HDL (AUC)	3728 ± 414	4040 ± 414	3870 ± 414	4023 ± 414	0.082	4023 ± 414
Total AUC	5759 ± 1047	5769 ± 1047	5297 ± 1047	5520 ± 1047	0.066	2600 ± 76.2
TRL (%AUC)	1.86 ± 0.31[c]	3.37 ± 0.31[ab]	2.77 ± 0.31[b]	3.99 ± 0.31[a]	<0.0001	6.20 ± 0.57
LDL (%AUC)	31.7 ± 3.01[a]	24.7 ± 3.01[b]	23.8 ± 3.01[b]	22.3 ± 3.01[b]	<0.0001	14.2 ± 0.43
HDL (%AUC)	66.2 ± 3.09[b]	72.0 ± 3.09[a]	73.3 ± 3.09[a]	73.8 ± 3.09[a]	<0.0001	79.6 ± 0.54

Table 3.3 Lipoprotein Density Subfractions as Area Under the Curve (AUC)

	ISP	DWMP	MPC	MFGM	*P*	CHOW
TRL	109 ± 34.7[c]	199 ± 34.8[ab]	150 ± 34.8[bc]	226 ± 34.8[a]	<0.0001	160 ± 14.3
LDL	1865 ± 415[a]	1468 ± 415[b]	1338 ± 415[b]	1293 ± 415[b]	<0.0001	370 ± 16.6
LDL-1	21.6 ± 4.03	26.6 ± 4.04	21.8 ± 4.04	27.5 ± 4.04	0.204	8.92 ± 2.68
LDL-2	51.0 ± 10.7	54.8 ± 10.7	48.6 ± 10.7	47.9 ± 10.7	0.584	14.8 ± 2.46
LDL-3	544 ± 173[a]	412 ± 173[ab]	399 ± 173[b]	358 ± 173[b]	0.004	80.9 ± 4.29
LDL-4	671 ± 138[a]	478 ± 138[b]	435 ± 138[b]	440 ± 138[b]	<0.0001	123 ± 6.11
LDL-5	577 ± 94.0[a]	490 ± 94.0[ab]	438 ± 94.0[b]	422 ± 94.0[b]	<0.0001	142 ± 8.06
HDL	3728 ± 414	4040 ± 414	3870 ± 414	4023 ± 414	0.082	4023 ± 414
HDL-2b	1758 ± 238	2014 ± 238	1835 ± 238	1907 ± 238	0.056	2071 ± 64.9
HDL-2a	1276 ± 135	1316 ± 135	1276 ± 135	1234 ± 135	0.077	776 ± 24.3
HDL-3a	503 ± 59.0	517 ± 59.0	514 ± 59.0	547 ± 59.0	0.493	331 ± 12.0
HDL-3b	131 ± 13.6	134 ± 13.6	127 ± 13.6	133 ± 13.6	0.835	79.8 ± 3.40
HDL-3c	77.1 ± 11.0[c]	95.9 ± 11.0[a]	79.0 ± 11.0[bc]	89.6 ± 11.0[ab]	<0.0001	44.2 ± 2.16
Total AUC	5759 ± 1047	5769 ± 1047	5297 ± 1047	5520 ± 1047	0.066	2600 ± 76.2

Values represent means ± standard error of area under the curve (AUC) for n=15/diet.
Different letters signify statistical differences by ANOVA and Tukey's HSD.

Table 3.4 Lipoprotein Density Profiles as Percentage of Total AUC

	ISP	DWMP	MPC	MFGM	*P*	CHOW
TRL	1.86 ± 0.31^{c}	3.37 ± 0.31^{ab}	2.77 ± 0.31^{b}	3.99 ± 0.31^{a}	<0.0001	6.20 ± 0.57
LDL	31.7 ± 3.01^{a}	24.7 ± 3.01^{b}	23.8 ± 3.01^{b}	22.3 ± 3.01^{b}	<0.0001	14.2 ± 0.43
LDL-1	0.36 ± 0.04	0.46 ± 0.04	0.42 ± 0.04	0.50 ± 0.04	0.180	0.34 ± 0.10
LDL-2	0.86 ± 0.07	0.95 ± 0.07	0.89 ± 0.07	0.86 ± 0.07	0.701	0.56 ± 0.09
LDL-3	8.98 ± 1.76^{a}	6.69 ± 1.76^{b}	6.79 ± 1.76^{b}	5.89 ± 1.76^{b}	0.0002	3.11 ± 0.12
LDL-4	11.4 ± 0.97^{a}	8.13 ± 0.97^{b}	7.75 ± 0.97^{b}	7.57 ± 0.97^{b}	<0.0001	4.73 ± 0.23
LDL-5	9.97 ± 0.48^{a}	8.35 ± 0.48^{b}	8.01 ± 0.48^{b}	7.57 ± 0.48^{b}	<0.0001	5.47 ± 0.24
HDL	66.2 ± 3.09^{b}	72.0 ± 3.09^{a}	73.3 ± 3.09^{a}	73.8 ± 3.09^{a}	<0.0001	79.6 ± 0.54
HDL-2b	31.2 ± 2.25^{b}	35.5 ± 2.25^{a}	34.2 ± 2.25^{ab}	34.7 ± 2.25^{a}	0.005	32.2 ± 0.70
HDL-2a	21.9 ± 1.34^{c}	23.4 ± 1.34^{bc}	24.4 ± 1.34^{ab}	25.3 ± 1.34^{a}	<0.0001	29.9 ± 0.40
HDL-3a	9.11 ± 0.89	8.91 ± 0.89	10.4 ± 0.89	9.82 ± 0.89	0.121	12.8 ± 0.40
HDL-3b	2.35 ± 0.29	2.37 ± 0.29	2.53 ± 0.29	2.43 ± 0.29	0.780	3.07 ± 0.09
HDL-3c	1.47 ± 0.32	1.73 ± 0.32	1.61 ± 0.32	1.63 ± 0.32	0.200	1.70 ± 0.08

Values represent means ± standard error of %total AUC for n=15/diet.
Different letters signify statistical differences by ANOVA and Tukey's HSD.

Dietary fatty acids are known to affect cholesterol metabolism[216]; therefore, we verified the impact of fat source on lipoprotein distributions in lard-containing diets (ISP, MPC) and those containing dairy fats (DWMP, MFGM). Diets containing lard increased %LDL with significant increases in the smaller diameter %LDL-3 and -4 subfractions (Table 3.5). Changes in total %HDL did not reach significance ($p<0.08$); however, the HDL-2b subfraction was significantly increased in diets with dairy fat sources. To determine the effect of protein source on lipoprotein distributions in diets containing lard, paired t-tests were conducted for ISP and MPC (data not shown). Total AUC was similar, but LDL-4 significantly lowered in MPC ($p<0.019$) and total LDL and LDL-5 being close to significant ($p<0.065$ and $p<0.055$, respectively). All three subclasses were significantly different when expressed as % of total. %TRL ($p<0.018$) and %HDL ($p<0.005$) increased while %LDL decreased ($p<0.002$) in MPC compared to ISP.

Table 3.5 Lipoprotein Density Profiles as percent of total AUC: Lard vs Dairy-fat Diets

	Lard containing* (n = 30)	Dairy-fat containing* (n = 30)	*p*
TRL	2.49 ± 0.20[b]	3.56 ± 0.25[a]	***0.001***
LDL	27.5 ± 1.34[a]	23.7 ± 0.76[b]	***0.017***
LDL-1	0.40 ± 0.02	0.47 ± 0.04	0.150
LDL-2	0.89 ± 0.04	0.90 ± 0.04	0.775
LDL-3	7.90 ± 0.65[a]	6.28 ± 0.44[b]	***0.045***
LDL-4	9.52 ± 0.51[a]	7.91 ± 0.24[b]	***0.006***
LDL-5	8.81 ± 0.29	8.16 ± 0.25	0.096
HDL	70.0 ± 1.30	72.7 ± 0.79	0.080
HDL-2b	32.6 ± 0.95[b]	35.4 ± 0.92[a]	***0.035***
HDL-2a	23.7 ± 0.59	23.9 ± 0.57	0.815
HDL-3a	9.76 ± 0.49	9.37 ± 0.34	0.514
HDL-3b	2.46 ± 0.14	2.39 ± 0.10	0.672
HDL-3c	1.54 ± 0.11	1.68 ± 0.09	0.335

Values represent means ± standard error of %total AUC. Different letters signify statistical differences by ANOVA and Tukey's HSD. *Lard diets include data from ISP and MPC; dairy diets include DWMP and MFGM.

Given the differences between ISP and MPC lipoprotein distributions, despite similar fat source, we analyzed the effect of dairy proteins compared to ISP. DWMP includes all proteins represented by the dairy fractions MPC and MFGM. Dairy proteins increased triacylglycerol-rich lipoproteins (Table 6, 3.4% v. 1.9%, $p<0.0005$) and total HDL (73.0% v. 66.2%, $p<0.0002$) and decreased LDL (23.7% v. 31.7%, $p<0.0001$). LDL-1 and LDL-2 did not differ by dietary protein source; however, small dense LDL (LDL-3, LDL-4, LDL-5) were reduced on average by 74% in dairy protein-fed mice compared to ISP-fed mice ($p<0.005$). HDL-2b and HDL-2a were significantly increased in dairy protein-fed mice ($p<0.05$) although HDL-3, present within the density range of 1.125-1.20 g/mL,[217] did not differ significantly. HDL-2a increased ~12% in mice fed MPC and MFGM ($p<0.05$). Dairy diets, analyzed by ANOVA without ISP to determine statistical differences between milk fractions, were not different by AUC or % of total for any subfraction. In paired t-tests, MPC reduced TRL, both AUC and %, compared to MFGM ($p<0.03$); however, no other lipoprotein classes were affected by dietary dairy fraction source. DWMP and MFGM were similar for all lipoprotein classes by paired t-tests.

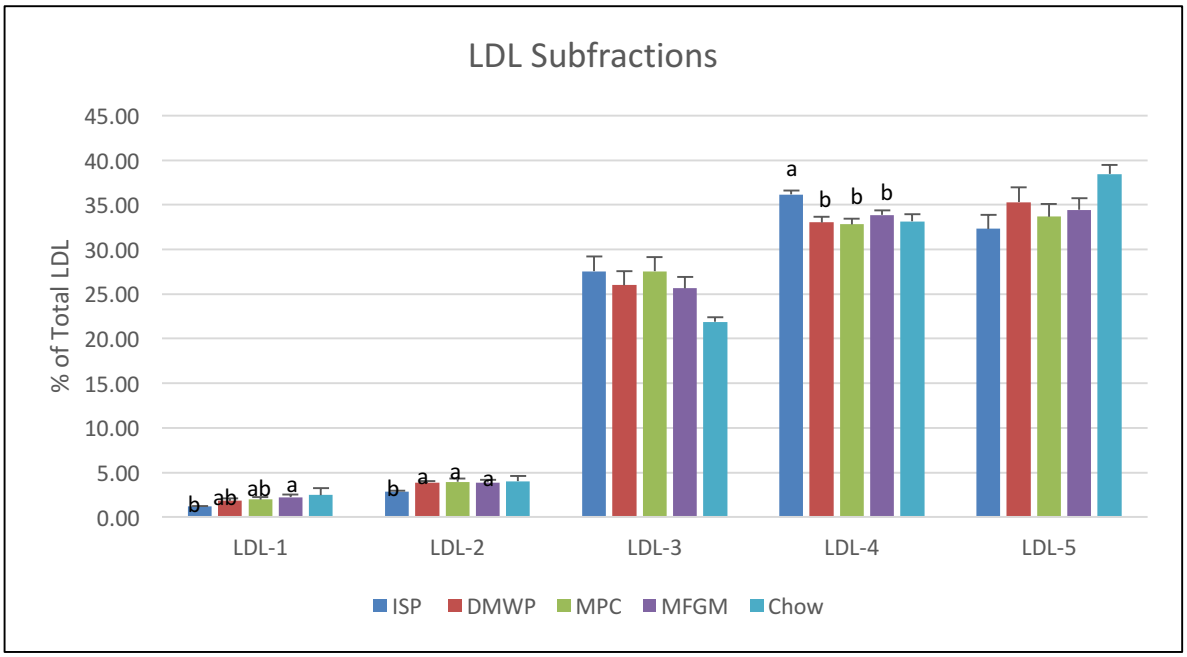
Table 3.6 Lipoprotein Density Profiles as percent of total AUC: ISP vs Dairy Protein Diets

	ISP (n = 15)	Dairy (n = 45)	*p*
TRL	1.86 ± 0.31[b]	3.38 ± 0.31[a]	<0.0005
LDL	31.7 ± 3.01[a]	23.6 ± 3.01[b]	<0.0001
LDL-1	0.36 ± 0.04	0.46 ± 0.04	0.082
LDL-2	0.86 ± 0.07	0.90 ± 0.07	0.670
LDL-3	8.98 ± 1.76[a]	6.46 ± 1.76[b]	<0.005
LDL-4	11.4 ± 0.97[a]	7.82 ± 0.97[b]	<0.0001
LDL-5	9.97 ± 0.48[a]	7.98 ± 0.48[b]	<0.0001
HDL	66.2 ± 3.09[b]	73.0 ± 3.09[a]	<0.0002
HDL-2b	31.2 ± 2.25[b]	34.8 ± 2.25[a]	<0.02
HDL-2a	21.9 ± 1.34[b]	24.4 ± 1.34[a]	<0.04
HDL-3a	9.11 ± 0.89	9.71 ± 0.89	0.379
HDL-3b	2.35 ± 0.29	2.44 ± 0.29	0.680
HDL-3c	1.47 ± 0.32	1.66 ± 0.32	0.302

Values represent means ± standard error of %total AUC. Different letters signify statistical differences by ANOVA and Tukey's HSD. Dairy diets include DWMP, MPC, MFGM.

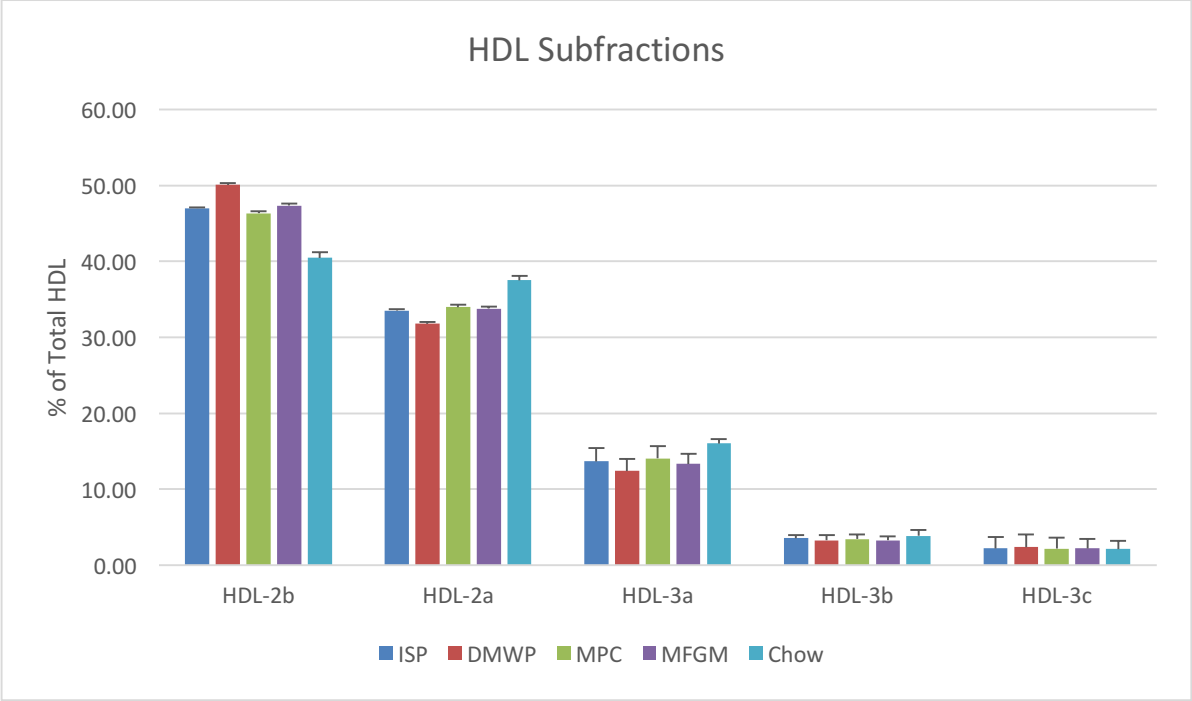
To determine the effect of diet on relative subfraction distributions, we analyzed each subfraction as percent of their respective density classes. LDL-1 and -2, as % of total LDL, were increased in dairy-fed mice, while LDL-4 was significantly reduced in dairy-fed mice (Figure 6). HDL subfractions were expressed as % of total HDL (Figure 7). HDL-2b was 3% higher in DWMP compared to ISP and MFGM and 4% higher than MPC, although these values failed to reach statistical significance. The remaining HDL subfractions were similar across diets.

Figure 3.1 LDL Subfractions as Percentage of Total LDL



Different letters represent statistical significance. Chow data is listed as reference values only and is not included in data analyses.

Figure 3.2 HDL Subfractions as percent of Total HDL



Differing letters represent statistical significance. Chow data is listed for reference only and is not included in data analyses.

3.3.3. **Gene Expression**

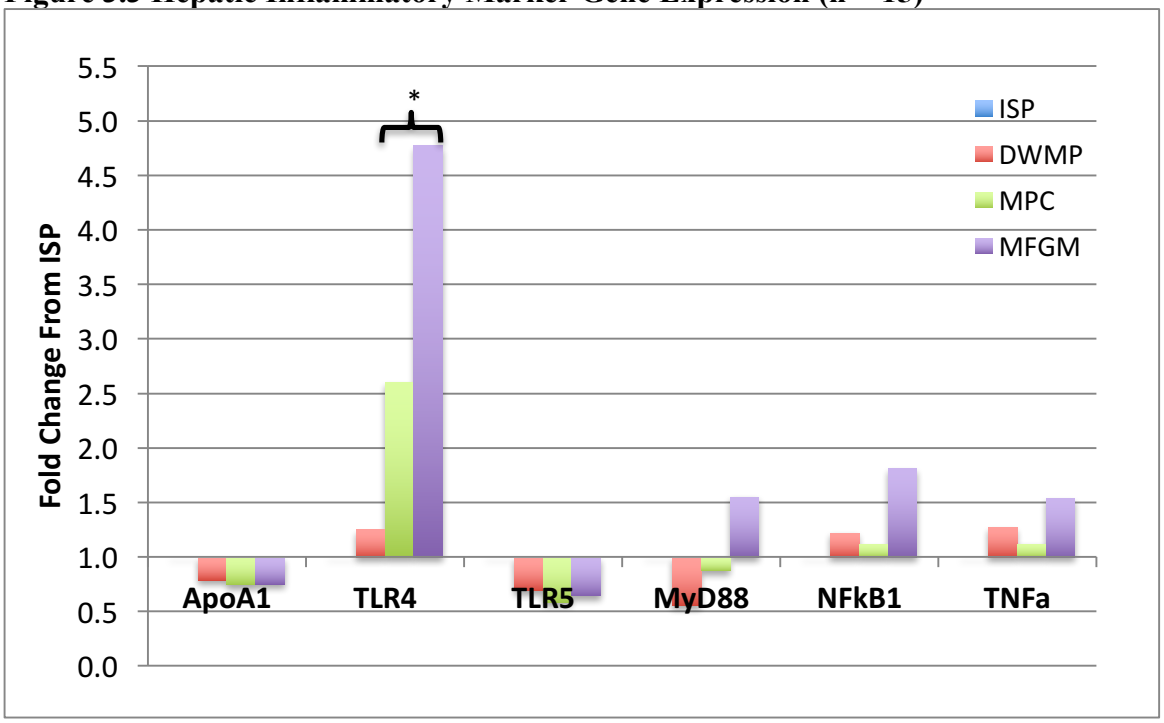
Changes in gene expression were evaluated by ΔCT values and fold changes through comparisons of outcomes from mice fed dairy-containing diets relative to those fed ISP. Few significant changes in gene expression were noted by ΔCT values for liver, adipose and gastrocnemius muscle (Table 3.7). Of note, liver fatty acid amide hydrolase (FAAH) was significantly increased in MPC, when different from MFGM. PPAR-γ was highest in livers from any of the dairy-fed mice, nearing significance ($p = 0.053$).

We next analyzed fold changes in livers of diary-fed mice using ISP-fed mice as a comparator using the $2^{-\Delta\Delta CT}$ method which produces fold change values that can be compared across treatments. Fold changes greater than 2 or less than 0.5 are considered significant. Within the genes indicative of inflammation, TRL4 increased 4.7-fold in MFGM and 2.6 fold in MPC relative to ISP (Figure 3.3). The remaining hepatic inflammatory genes, including ApoA1, were similar across diets. Among the genes indicative of lipoadipogenesis, PPAR-γ was down-regulated in all dairy diets, AP2 expression decreased in DWMP and FAS was down-regulated in MFGM (Figure 3.4). Within cannabinoid and endocannaboid gene expression, cannabinoid receptor GPR119 increased 189-fold in MFGM, while DWMP and MPC increased 2.7-and 3.3-fold, respectively, over ISP (Figure 3.5). Likewise, livers from MPC-fed mice had a 2.4-fold increase in CB1. All dairy diets showed down-regulation of GPCR41 gene expression. The remaining cannabinoid and endocannabinoid genes measured were similar across diet. The inflammatory marker gene, CD11D, was similarly expressed in adipose. PGC-1α and PPAR-γ expression in gastrocnemius muscle did not change.

Table 3.7 Gene Expression ΔCT values

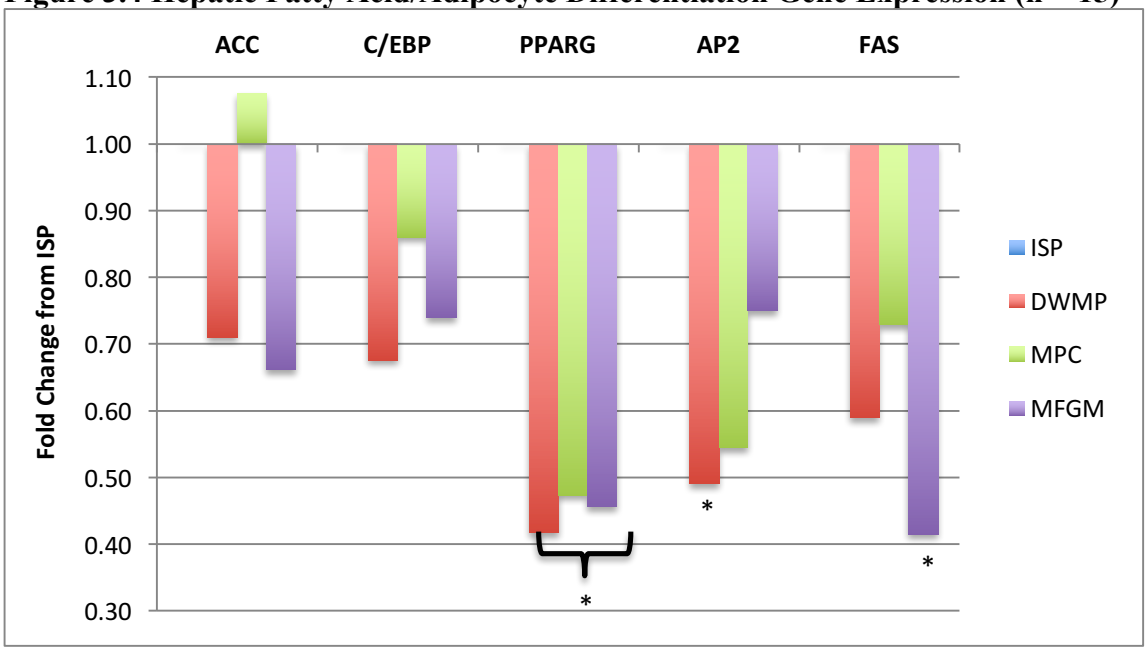
		ISP	DWMP	MPC	MFGM	p-value
Liver: Inflammatory	TLR4	5.76 ± 0.68	5.44 ± 0.50	4.37 ± 0.65	3.50 ± 1.06	0.1491
	TLR5	6.25 ± 0.28	6.77 ± 0.36	7.01 ± 0.34	6.87 ± 0.31	0.3700
	MyD88	6.28 ± 0.39	7.12 ± 0.74	6.47 ± 0.52	5.64 ± 0.53	0.3217
	NFκB1	6.14 ± 0.38	5.84 ± 0.42	5.98 ± 0.47	5.28 ± 0.56	0.5672
	TNF-α	10.36 ± 0.32	10.00 ± 0.40	10.19 ± 0.35	9.73 ± 0.45	0.6909
Liver: Lipids	apoA1	-3.94 ± 0.20	-3.59 ± 0.24	-3.52 ± 0.19	-3.53 ± 0.22	0.5387
	SREBP1c	2.90 ± 0.17	2.58 ± 0.24	2.91 ± 0.20	2.67 ± 0.17	0.5457
	PPAR-α	1.34 ± 0.25	1.38 ± 0.27	0.95 ± 0.33	1.26 ± 0.33	0.7386
	PPAR-γ	5.26 ± 0.51	6.52 ± 0.25	6.34 ± 0.30	6.39 ± 0.13	0.0532
	AP2	4.61 ± 0.58	5.64 ± 0.20	5.49 ± 0.62	5.03 ± 0.30	0.4090
	C/EBP	0.38 ± 0.23	0.95 ± 0.19	0.60 ± 0.24	0.82 ± 0.15	0.2473
	FAS	1.86 ± 0.38	2.62 ± 0.45	2.32 ± 0.26	3.13 ± 0.37	0.1139
	ACC	2.30 ± 0.27	2.79 ± 0.40	2.19 ±0.34	2.90 ± 0.31	0.3574
Liver: Cannabinoid & Endocannabinoids	CB1	15.84 ± 0.33	15.74 ± 0.57	14.56 ± 0.59	16.19 ± 0.43	0.1442
	CB2	11.72 ± 0.27	12.15 ± 0.15	11.81 ± 0.12	11.77 ± 0.15	0.3613
	TRPV1	12.22 ± 0.48	12.94 ± 0.53	11.92 ± 0.48	12.59 ± 0.55	0.5418
	GPR41	14.64 ± 0.94	16.62 ± 0.57	16.12 ± 0.65	15.97 ± 0.79	0.3265
	GPR55	11.49 ± 0.36	11.35 ± 0.44	11.49 ± 0.41	11.71 ± 0.55	0.9518
	GPR119	19.26 ± 1.37	17.82 ± 1.21	17.52 ± 0.93	11.69 ± 6.71	0.7930
	NAPE-PLD	9.94 ± 0.19	10.27 ± 0.23	10.63 ± 0.33	10.14 ± 0.15	0. 2602
	FAAH	4.38 ± 0.09^{ab}	4.40 ± 0.13^{ab}	4.69 ± 0.04^{a}	4.09 ± 0.06^{b}	0.0019
Adipose	CD11D	9.68 ± 0.76	9.22 ± 0.91	10.4 ± 1.15	9.94 ± 0.62	0.8173
Gastroc. Muscle	PGC-1α	3.23 ± 0.17	3.30 ± 0.15	2.86 ± 0.16	2.85 ± 0.18	0.1078
	PPAR-γ	6.71 ± 0.07	6.78 ± 0.76	6.46 ± 0.16	6.70 ± 0.14	0.3413

Figure 3.3 Hepatic Inflammatory Marker Gene Expression (n = 15)



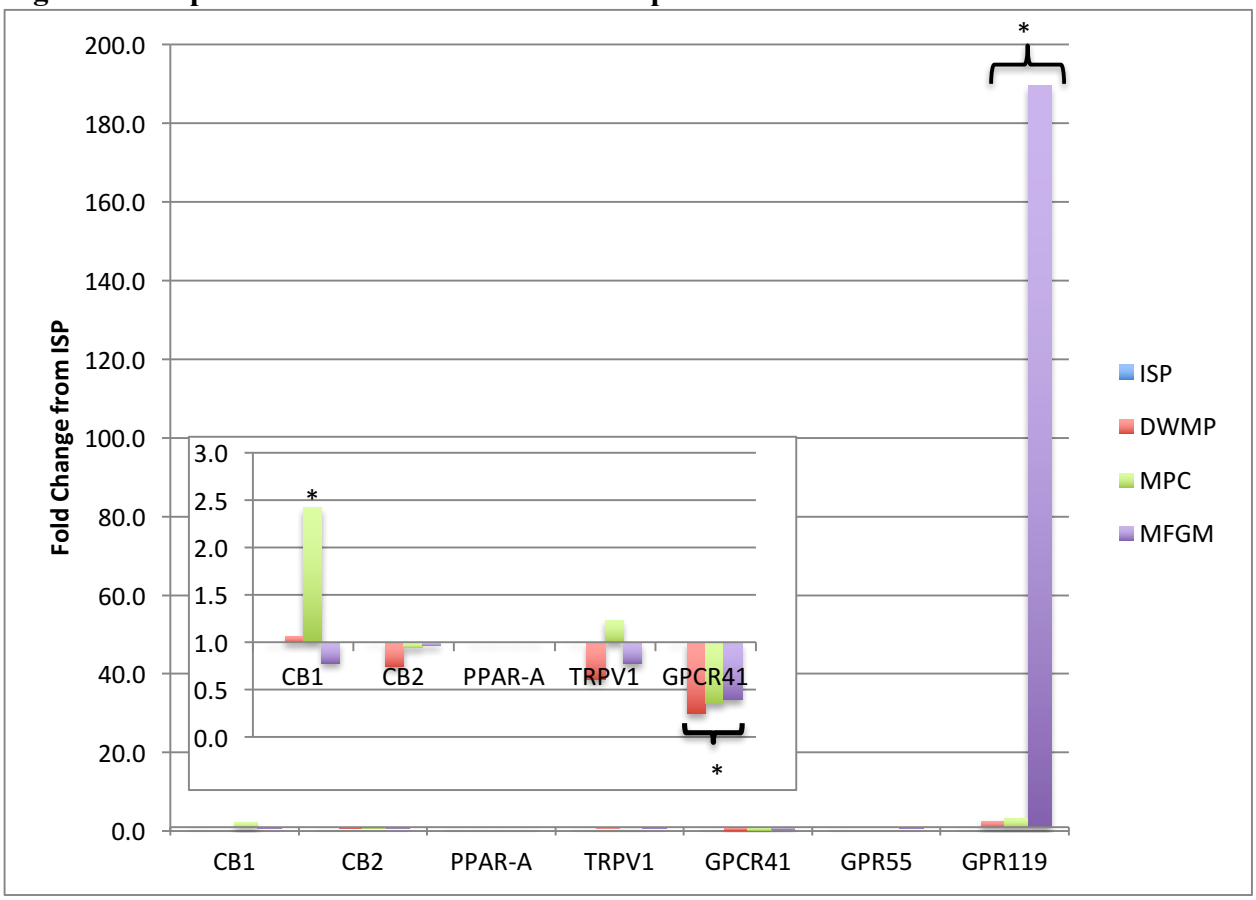
Data represent n=15/diet treatment. ISP served as a comparator using the $2^{-\Delta\,\Delta CT}$ method for fold change.

Figure 3.4 Hepatic Fatty Acid/Adipocyte Differentiation Gene Expression (n = 15)



Data represent n=15/diet treatment. ISP served as a comparator using the $2^{-\Delta\,\Delta CT}$ method for fold change.

Figure 3.5 Hepatic CB & eCB Marker Gene Expression



Data represent n=15/diet treatment. ISP served as a comparator using the $2^{-\Delta\,\Delta CT}$ method for fold change.

3.3.5. **LPS-Stimulated Cytokine Release Assay**

Baseline cytokine concentrations were low and indicative of a non-inflammatory state for any diet treatment. Statistical differences were observed in baseline TNF-α with MPC being increased more than 14 pg/mL over the other diets (Table 3.8). After challenge with LPS from *S. enteritidis*, TNF-α concentrations were highest in MFGM over DWMP and similar to MPC and ISP. MFGM also had the highest IL-10 concentration, being different from DWMP and ISP. To determine magnitude of cytokine release upon LPS challenge, change in concentrations from baseline were determined (Table 3.9). MFGM had the largest magnitude change in IL-10 following *S. enteritidis* challenge, being 7-fold increased over DWMP and 12-fold more than ISP. MFGM also mounted an increased IL-6 response, although statistical significance was not achieved ($p = 0.12$). Net change following stimulation values for TNFα in MPC-fed mice were not greater than those of mice in other diet droups. Increased sample numbers may be necessary to tease out statistical significance. Cytokine responses to *E. coli* challenge (concentrations and change from baseline) were similar across diets (Tables 3.8 and 3.9).

Table 3.8 Cytokine Concentrations

		ISP	DWMP	MPC	MFGM	Chow
Baseline (pg/mL)	**IFN-g**	0.00 ± 0.00	0.00 ± 0.00	0.04 ± 0.04	0.00 ± 0.00	0.00 ± 0.00
	IL-1β	0.55 ± 0.55	3.59 ± 3.59	5.08 ± 1.75	3.18 ± 1.52	5.12 ± 1.97
	IL-6	100 ± 52.9	41.9 ± 24.3	57.6 ± 18.9	35.4 ± 14.7	36.3 ± 26.8
	IL-12p70	8.81 ± 3.66	7.11 ± 3.67	4.42 ± 1.52	1.84 ± 1.23	12.4 ± 2.29
	MIP-2	112 ± 43.0	90.8 ± 39.1	94.6 ± 25.8	96.9 ± 25.2	166 ± 23.2
	TNF-α	0.43 ± 0.33^{b}	1.84 ± 1.84^{b}	18.2 ± 7.14^{a}	4.84 ± 1.67^{ab}	0.29 ± 0.29
	Eotaxin	4215 ± 238	4107 ± 172	3773 ± 201	3780 ± 211	---
	IL-10	0.21± 0.21	0.00 ± 0.00	0.00 ± 0.00	0.35 ± 0.28	7.24 ± 2.46
***S. enteriditis* challenge** (pg/mL)	**IFN-g**	0.00 ± 0.00	0.00 ± 0.00	0.24 ± 0.20	0.08 ± 0.05	1.61 ± 1.26
	IL-1β	242 ± 51.2	199 ± 59.4	123 ± 30.6	292 ± 59.1	143 ± 16.3
	IL-6	828 ± 208	926 ± 225	476 ± 129	1191 ± 247	1081 ± 141
	IL-12p70	10.8 ± 4.50	6.11 ± 2.39	6.28 ± 2.15	3.66 ± 1.50	17.6 ± 5.86
	MIP-2	935 ± 201	1492 ± 552	638 ± 144	1252 ± 228	753 ± 85.6
	TNF-α	11.3 ± 4.94^{ab}	3.91 ± 2.90^{b}	18.7 ± 3.15^{a}	22.8 ± 3.63^{a}	97.8 ± 12.0
	IL-10	1.44 ± 0.82^{b}	2.09 ± 1.18^{b}	6.07 ±2.91^{ab}	15.3 ± 5.27^{a}	168 ± 16.2
***E. coli O111:B4* challenge** (pg/mL)	**IFN-g**	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.21 ± 0.21	0.41 ± 0.41
	IL-1β	216 ± 19.2	255 ± 29.0	227 ± 50.2	152 ± 28.4	154 ± 21.7
	IL-6	2484 ± 313	3118 ± 934	3083 ± 1410	1978 ± 497	3010 ± 646
	IL-12p70	14.1 ± 5.32	13.5 ± 4.98	16.7 ± 10.3	2.38 ± 1.60	13.6 ± 2.46
	MIP-2	1720 ± 305	2578 ± 619	1792 ± 288	1899 ± 365	2292 ± 577
	TNF-α	66.2 ± 7.31	37.6 ± 11.8	59.6 ± 8.30	44.9 ± 9.65	26.6 ± 4.28
	IL-10	99.0 ± 24.4	76.0 ± 15.8	97.3 ± 28.8	99.6 ± 24.5	91.3 ± 12.2

Chow-fed animals are listed for references only; they are not included in statistical analyses. Kit sensitivity is to 0.13 pg/mL.[218]

Table 3.9 Cytokine Concentrations: Change from Baseline after LPS Challenge

		ISP	DWMP	MPC	MFGM	Chow
S. enteriditis **challenge** (Change from Baseline, pg/mL)	**IFN-g**	0.00 ± 0.00	0.00 ± 0.00	0.20 ± 0.21	-0.08 ± 0.13	1.61 ± 1.26
	IL-1β	225 ± 50.1	183 ± 57.8	118 ± 30.6	289 ± 59.2	138 ± 16.1
	IL-6	728 ± 219	825 ± 226	418 ± 127	1156 ± 253	1045 ± 135
	IL-12p70	1.30 ± 0.97	-0.93 ± 1.84	1.86 ± 1.75	2.26 ± 2.78	5.38 ± 4.89
	MIP-2	760 ± 180	1308 ± 494	543 ± 146	1155 ± 225	587 ± 73.9
	TNF-α	9.34 ± 4.17	1.94 ± 3.34	0.55 ± 9.71	18.0 ± 3.84	97.5 ± 11.9
	IL-10	1.23 ± 0.65[b]	1.95 ± 1.11[b]	6.07 ± 2.91[ab]	15.0 ± 5.22[a]	161 ± 15.0
E. coli O111:B4 **challenge** (Change from Baseline, pg/mL)	**IFN-g**	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.21 ± 0.21	0.41 ± 0.41
	IL-1β	216 ± 19.3	250 ± 37.2	224 ± 50.7	152 ± 28.4	149 ± 22.0
	IL-6	2334 ± 251	3061 ± 904	3001 ± 1403	1937 ± 480	2974 ± 641
	IL-12p70	0.89 ± 4.38	3.50 ± 2.80	12.0 ± 9.77	-0.26 ± 1.76	1.31 ± 2.64
	MIP-2	1570 ± 279	2451 ± 623	1710 ± 293	1852 ± 367	2126 ± 564
	TNF-α	65.6 ± 7.21	35.1 ± 12.3	37.9 ± 11.3	44.2 ± 9.83	26.3 ± 4.27
	IL-10	98.7 ± 24.5	76.0 ± 15.8	97.3 ± 28.8	99.6 ± 24.5	83.9 ± 11.9

Chow-fed animals are listed for references only; they are not included in statistical analyses.

3.3.6. **Chow-fed Reference Mice**

As detailed in Chapter 2, chow-fed reference mice showed increased gastrointestinal permeability (GI-P) and plasma endotoxin concentrations. These unexpected findings were accompanied by altered lipoprotein profiles. Mice fed non-purified diet (chow), remarkably, reduced total lipoprotein mass (total AUC) was halved when compared to any purified diet (Table 3.2, Figure 3.6). Chow-fed reference mice had the highest %TRL and total %HDL as well as the lowest % LDL (Table 3.4, Figure 3.7). LDL subfractions -2, -3, -4, and -5 were reduced in chow-fed mice while having the highest %HDL-2a, -3a, and -3b plasma lipoprotein content. Compared to the purified diets (PD), ISP, DWMP, MPC, and MFGM, mice fed non-purified diet (chow), had the highest percent triglyceride-rich lipoproteins (TRL) and %HDL as well as the lowest % LDL (Table 3.4). Despite marked increases in plasma endotoxin and %HDL of chow-fed mice, no correlations between the two values were observed (Endotoxin (EU/mL) = 0.0942991 + 3.7455e-6*Total HDL, R^2 =0.008).

Similar to PD-fed mice, baseline cytokines for chow-fed mice were low or undetectable (Table 3.8) and largely did not differ from cytokine concentrations of purified diet-fed mice. IL-10 increased 7-fold over all PDs; IL-6 concentrations were 58% of those in MPC and 65% of ISP. As with PDs, we challenged blood monocytes with LPS from *E. coli* and *S. enteritidis*. When challenged with LPS from *S. enteritidis*, IL-10 and TNF-α showed a marked response in whole blood of mice fed laboratory chow compared to purified diets (Table 3.9). IL-10 increased 130, 80, 26 and 11-fold over ISP, DWMP,

MPC and MFGM, respectively; TNF-α averaged a 33-fold increase in chow-fed whole blood. Whole-blood challenge by LPS from *E. coli* elicited a cytokine response similar to those observed in purified-diet fed mice. As the mice were obtained to provide reference values for native mouse diets compared to mice fed PDs, statistical analyses were not performed. Cytokine responses of chow-fed mice were mixed compared to PDs, mostly falling in the average range of all PDs, and possibly indicating all values were within clinical normals.

Figure 3.6 Total AUC for Chow-fed Mice compared to PDs

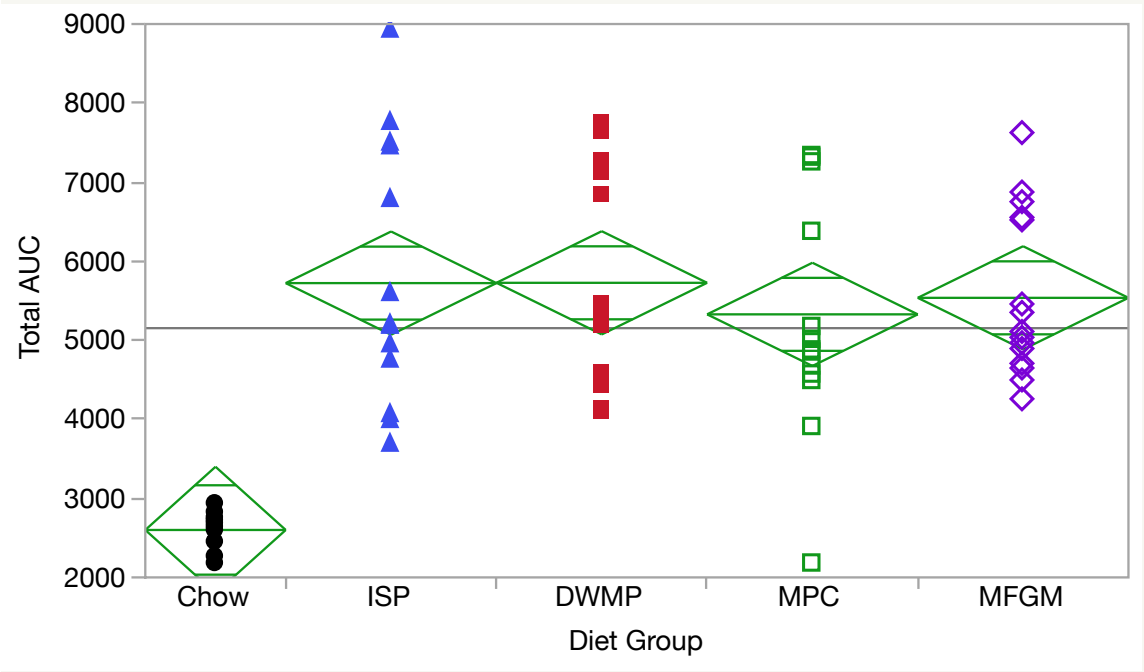
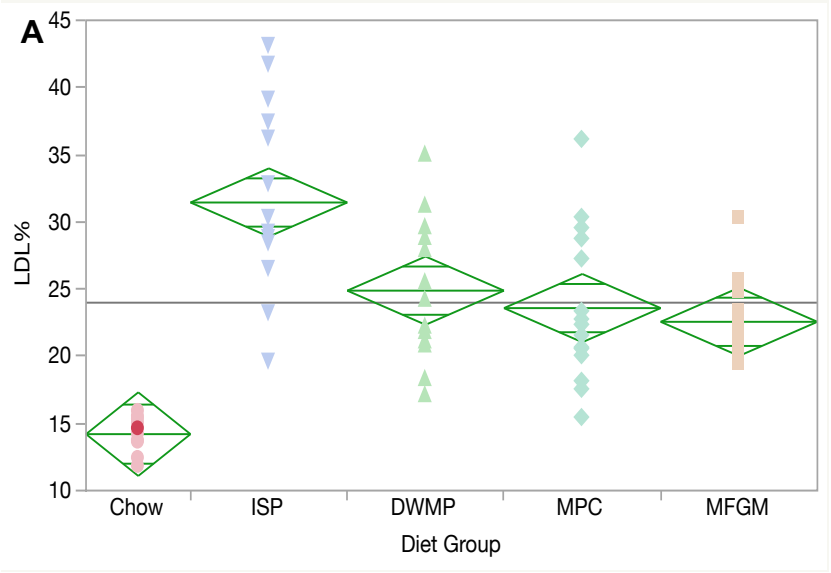
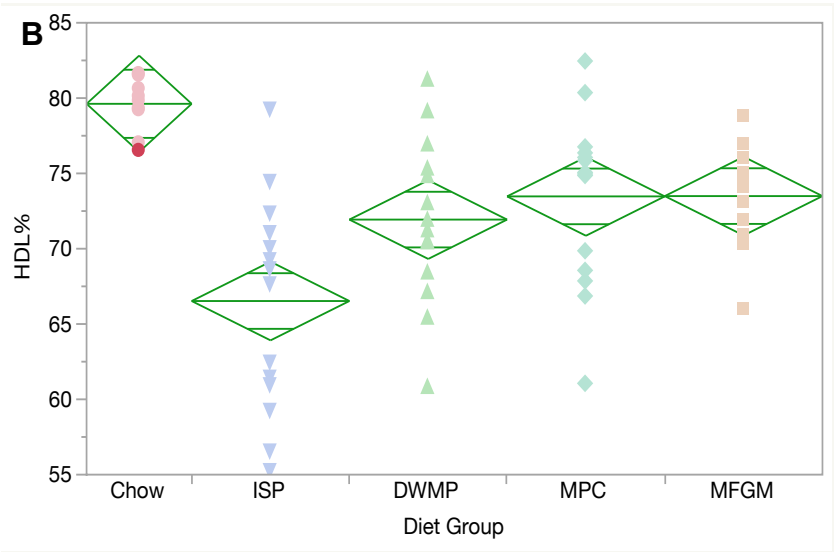


Figure 3.7 LDL and HDL (% of AUC) for Chow-fed Mice compared to PDs





Panel A: Distribution of LDL as % of Total AUC. Chow-fed mice reduced %LDL by 11.6% compared to PDs (14.2% vs. 25.6%).
Panel B: Distribution of HDL as % of Total AUC. Chow-fed mice increased %HDL by 8.4% compared to PDs (79.6% vs. 71.4%).
Not pictured: %TRL increased 3.2% in chow-fed mice (6.2%) compared to PDs (3.0%)

3.4. Discussion

Mice fed diets containing dairy proteins improved lipoprotein profiles and MFGM increased the immune response to LPS challenge in whole blood. While mice had similar lipoprotein mass (AUC), relative distributions of subclasses varied by diet. ISP increased LDL over any dairy-containing diet, especially in the small, dense LDL (-3, -4, -5). Small, dense LDL are associated with increased risk of CVD and are particularly linked to atherosclerosis development.[219] It appears that the dietary fat source plays a larger role in TRL than does dietary protein. TRL (AUC and % of total) were increased in MFGM over MPC and ISP, although relative distributions were small and under 4% for all diets. Dairy-protein diets promoted increased %HDL, with HDL-2b and -2a contributing the bulk of the HDL subclass. The HDL-2b and -2a subfractions are largely associated with improved lipoprotein profiles and reduced CVD risk due to their anti-inflammatory properties.[220] Interestingly, HDL-3c (AUC), which may have pro-or anti-inflammatory roles,[130,220] significantly increased in DWMP over MPC and MFGM. Determining the particle details (i.e. protein components) is necessary to determine the functionality of this subclass. As expected with similar HDL mass (AUC), Apoa1 liver gene expression was similar across diet groups.

Across all PDs, total HDL (AUC) was significantly correlated with FITC-d (FITC-Dextran (ug/mL) = 37.018259 - 0.0100031*Total HDL, $p<0.0003$). Brenhum, et al., showed 30% of lipidated HDL is formed in the gut.[163] This is consistent with the association between compromised intestinal integrity and lowered HDL-C and apoA1

found by van Leuven, et al.[167] Our methodology separates lipoproteins by flotation densities.[221,222] Lipoproteins in the density range d=1.125-1.210 g/mL primarily contain cholesteryl ester. Thus, the AUC identified by our method arises primarily from cholesteryl ester stained with NBD-ceramide.

In conjunction with altered lipoprotein profiles, the interaction between intestinal permeability and HDL mass raises interesting questions regarding the interaction of dietary protein source on lipoproteins. We observed marked differences in lipoprotein distributions with diets containing dairy proteins regardless of fat source. In our hands, dairy-protein containing diets lowered LDL, raised HDL and reduced FITC-dextran permeability. Lard in the MPC diet did little to dilute the effects of dairy, raising an interesting question about the interactions of specific dairy fractions. When comparing PDCAAS (protein digestibility corrected amino acid score), all dietary protein sources are equivalent at 1.0. However, when we consider the Digestible Indispensable Amino Acid Score (DIAAS), MPC is 36% higher than soy protein isolate (120% vs. 84%)[9]. In the same study, skim milk protein (SMP), presumed to be the same as DWMP but at a lower fat content, is 105%; no values were available in literature for MFGM. DIAAS incorporates ileal digestibility and does not truncate scores, as in PDCAAS formulations, perhaps providing a more realistic biological value of proteins. Amino acid profiles of composed diets did not provide any obvious discrepancies explaining lipoprotein effects (Appendix B). Total amino acids and branched-chain amino acids were similar across diets. ISP doubled arginine and glycine content compared to dairy-containing diets, but

these do not explain the changes in lipoprotein distributions. Glutamine was not reported due to its conversion to glutamic acid during protein hydrolysis of the amino acid analysis protocol. Specifically designed studies are needed to tease out the mechanistic roles of dairy fractions or its components to influence lipoprotein biology and HDL functionality.

CD11d in white adipose is known to be upregulated in diet-induced obesity and strongly associated with increased macrophage infiltration and inflammation of adipose.[223,224] Since our mice did not reach an obese level of fat accumulation, it is not surprising that our mice did not exhibit inflammatory signaling in adipose (CD11d). Similarly, PPAR-γ and PGC1-α expression in gastrocnemius muscles denote similar energy expenditure regulation and mitochondrial biogenesis.[225,226]

Liver gene expression was consistent with the normal growth rates of our mice. TLR4 increased in mice fed MFGM and MPC; however, none of the downstream signaling targets (MyD88, NFκB1, TNF-α) were upregulated suggesting that the presence of TLR4 stimulus was not sufficient to activate the inflammatory process. Baseline cytokines were also low in these mice. It is possible that components of MFGM (i.e. degree of fatty acid saturation or presence of sphingomyelin) in our diets trigger TLR4 at a rate not exhibited in DWMP. Sunshine and Iruela-Arispe showed TLR4 activation due to be membrane rigidity can be associated with changes to lipid compositions.[227] The increase in TLR4 with MPC feeding may be due to similar reasons.

The observed increases in liver CB1 and GPR119 with dairy feeding were not expected due similar motility and feed intake (Chapter 2). Livers of MPC-fed mice increased CB1 2.4-fold, while all dairy diets increased GPR119 signaling compared to ISP. CB1 is known to delay gut transit and increase food intake.[81-83] Interestingly, MPC-fed mice spontaneously decreased food intake and had the lowest weight gain (Chapter 2) despite these increases. GPR119 agonists stimulate the release of glucagon-like peptide 1 (GLP-1), which is associated with increased satiety, reduced feed intake and weight lost.[88] As with CB1 in MPC-fed mice, gene expression of GPR119 in livers of dairy-fed mice did not follow the expected outcomes. MFGM, with the most weight gain and highest feed intake, increased GPR119 gene expression the most compared to ISP.

Plasma cytokine analyses provided baseline measures of inflammatory tone as well as the ability of monocytes to respond to LPS-challenge. IL-10, measured for its role as an anti-inflammatory cytokine, is known to down-regulate the inflammatory response1,2. It has also been characterized as a key player in auto-immunity3. Baseline values, obtained from un-challenged samples, did not differ by diet group. This was expected due to the healthy status of our animals; further, this indicates our diets were not inducing an inflammatory state. Eotaxin, widely used as a marker of allergic response due to its primary role is in activating eosinophils4, did not change with dietary treatment.

Interestingly, stimulation by *E. coli* and *S. enteritidis* challenge elicited different responses. After *E. coli* stimulation, no differences were observed across diet groups. An increase in cytokine production after stimulation compared to baseline does indicate that our immune systems were not compromised; all diet groups seem to be capable of recognizing LPS and mounting an inflammatory response. However, the lack of significance between diet groups indicates a healthy response, not being impaired by dietary protein source. With *S. enteritidis* stimulation, we did see a marked increase in overall cytokine productions. Mice fed dairy proteins may be able to mount a more pronounced IL-6 and TNF-α response to LPS stimuli (presumably by peripheral macrophages) and dispose of pathogens; unfortunately, our sample size did not provide adequate power to reach statistical significance for these cytokines. Of importance, IL-10 was significantly increased in MFGM compared to DWMP and ISP. Higher levels of IL-10 in MFGM indicate these mice may better regulate their response to LPS stimulus, ramping down immune cell recruitment after initial inflammatory response. Additionally, these results highlight the diverse roles of dairy milk protein sub-fractions and their selective enhancement of the immune response.

Relevant clinical values for mice fed standard chow without further intervention are essential to interpreting results from mice fed purified diets. Mice fed non-purified chow diets (reference mice) had the highest triglyceride-rich lipoproteins and HDL as well as lowest percent LDL. Since mouse chow had significantly increased carbohydrate content (presumably high fiber) and lower fat, it is not unexpected that these mice had

lower plasma LDL and increased HDL. Additionally, the permeable nature of chow-fed mice guts may induce HDL biogenesis and directly influence increased HDL expression. With the increased circulating endotoxin and cytokines compared to purified-diet fed animals, it is probable that dietary components and apparent GI-P play a role in priming the immune system to handle non-commensal bacterial challenges. Whether dietary ingredients, degree of refinement, or macronutrient distributions are responsible for the discrepant results could not be determined. Thus, it is difficult to compare data from chow-fed mice with those fed purified diets due to variations in macronutrient compositions and altered carbohydrate refinement.

4. EFFECT OF DIET QUALITY ON GROWTH PERFORMANCE, INNATE IMMUNITY AND GI HEALTH IN C57BL/6 MICE

4.1. Introduction

Contention exists for recommendations of plant-based vs. animal-based dietary proteins, their propensity to cause or prevent disease, and their overall contributions to immune status and chronic disease prevention[22-27]. Dietary recommendations to increase fiber consumption and shift to plant-based proteins are often given with the aim of improving various health parameters including adiposity and gut health[47,228]. Obesity is known to be associated with increased gut permeability and risk of metabolic disease development[94,181]. Changes in gut permeability and endotoxin concentration has implicated in obesity and increase plasma cytokines[95,100]. High-fat diet feeding, frequently used to accelerate disease occurrence and weight gain in animal models, causes loss of intestinal integrity and inflammation[229,230].

In chapter 1, I showed improved intestinal health markers in mice fed purified diets containing dried whole milk powder compared to isolated soy protein as a protein source. Improved intestinal health markers included longer villus height, lower FITC-dextran permeability and reduction of colonic myeloperoxidase activity. In addition, diets containing dairy milk components improved lipoprotein profiles by lowering small, dense LDL and increasing HDL -2b and -2a. In an age-match cohort of chow-fed reference mice, FITC-dextran permeability, plasma HDL, and endotoxin concentrations

were positively correlated, being some 18-fold higher than age-matched mice fed purified diets (Chapters 1&2). These unexpected increases in gastrointestinal permeability (GI-P), plasma endotoxin and HDL prompted us to question the validity of using age-matched mice as comparators. Additionally, the stark difference in standard laboratory diet composition compared to purified diets raised further questions as to the validity of directly comparing mice (Appendix B – D). Given the differences in macronutrients and ingredient complexities between standard chow and purified diets, we designed natural ingredient diets matching protein and macronutrients of ISP and DWMP, but following carbohydrate sources of laboratory chow diets. The present study investigates whether dietary protein source (soy vs. dairy) and degree of carbohydrate purification (purified diet vs. natural ingredient diet) provoked different responses in gut-associated health outcomes.

4.2. Materials and Methods

4.2.1. **Animal Growth**

All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at Texas A&M University. Weanling (21-day-old) C57Bl6 male mice were obtained from Jackson Labs (Bar Harbor, ME) and housed 5/cage with 12-hour (6 am on/6 pm off) light cycles. Mice were provided with a closed-formula, non-purified diet (Teklad Rodent Diet #8604, Harlan Laboratories, Indianapolis, IN) for 14 days to adapt to local environmental conditions and measure growth rates. On day 14, animals were sorted into four groups of 15 animals each. All mice had similar starting growth rates

and body weights. Groups were randomly assigned to a diet treatment comprised of isocaloric, isonitrogenous diets that differed in their protein sources and degree of carbohydrate refinement: isolated soy protein (ISP), dried whole milk powder (DWMP), soybean meal natural ingredient chow (SPC), and dried whole milk natural ingredient chow (DMC) (Table 4.1)[231]. The purified diets (PDs) ISP and DWMP included cellulose as the sole fiber source. The natural ingredient diets (NIDs) SPC and DMC utilized a variety of carbohydrate and fiber sources including soybean meal (SPC only), wheat, wheat middlings, corn and corn gluten meal. Mice were housed 3/cage with five cages/diet and fed experimental diets *ad libitum* for 12 weeks.

Mice were euthanized by CO_2 asphyxiation and exsanguinated by cardiac puncture. Whole blood was collected into heparin tubes and plasma separated within two hours following centrifugation. Body lengths (tip to tail) were measured; livers, spleens, ceca, and fat pads (flank subcutaneous, epididymal, mesenteric, retroperitoneal) were collected, weighed, and stored at -80°C. Gastrointestinal tracts were removed, measured, and divided for histology, cytokine release, and myeloperoxidase assays.

Table 4.1 Diet Composition

	ISP	DWMP	SPC	DMC
Protein, % by weight	18.6	18.0	18.8	18.6
Carbohydrate, % by weight	46.9	46.4	45.0	49.7
Fat, % by weight	12.2	12.4	12.4	12.4
Cholesterol mg/kg	95	378	95	376
Cholesterol, % by weight	0.01	0.04	0.01	0.04
Protein, % kcal from	20.0	19.5	20.5	19.3
Carbohydrate, % kcal from	50.4	50.3	49.1	51.7
Fat, % kcal from	29.5	30.2	30.4	29
Saturated Fat, % of total fat	33.8	58.6	33.9	54.8
MUFA, % of total fat	39.7	26.5	37.2	29.1
PUFA, % of total fat	26.5	14.9	28.8	16.1
Kcal/g	3.7	3.7	3.7	3.8

Values are calculated from ingredient analysis or manufacturer data

	Purified Diets (PD)		Natural Ingredient Diets (NID)	
	ISP	DWMP	SPC	DMC
Isolated Soy Protein	210.0			
Soybean Oil	20.0	20.0	4.0	4.42
Lard	100.0		100.0	
Dried Whole Milk Powder		390.0		387.45
Casein		95.0		
Sucrose	190.0	40.0		
Corn Starch	188.13	175.58		
Maltodextrin	100.0	100.0	30.0	30.0
Cellulose	140.0	140.0		
Wheat			200	105
Wheat Middlings			100	100
Corn			294	295
Soybean Meal			196	
Corn Gluten Meal			50.0	68.0
Neutral Detergent Fiber (%)*	14.0%	14.0%	11.4%	8.4%
Isoflavones (ppm)†	558	3.00	922	7.00

***NDF calculated by Envigo nutritionists**

†Isoflavones measured by NestlePurina Analytical Laboratories

4.2.2. *in vivo* Intestinal Permeability and Motility

To assess *in vivo* intestinal permeability and mobility after 10 weeks of experimental diets, mice received an oral gavage of FITC-dextran and Evan's blue dye as previously described (Chapter 1). A solution of 600 mg/kg BW dose of 4 kDa FITC-dextran (Sigma-Aldrich, St. Louis, MO) with 5% Evans blue dye suspended in 5% arabic gum was delivered by oral gavage[95,187,188]. Plasma obtained by a cheek bleed four hours post-gavage was used to measure plasma fluorescence at excitation 485 nm and emission 535 nm. Plasma FITC concentrations were determined from a standard curve. Mice were monitored in individual cages with paper liners; whole gut transit time is defined as time from oral gavage until mice excreted first blue feces.

Plasma endotoxin was measured by LAL assay with Glucashield® buffer (Associates of Cape Cod, Inc. (ACCI), East Falmouth, MA). Plasma samples were diluted 1:10 in endotoxin-free water, plated into a 96-well microplate with equal volume of diluted lysate and incubated for 82 minutes at 37°C. The reaction was stopped by adding 50% acetic acid and absorbance read immediately at 405 nm. Sample concentrations were determined from a standard curve created from Control Standard Endotoxin (ACCI).

4.2.3. Colonic Myeloperoxidase

Myeloperoxidase (MPO) activity in colon was measured using a commercially available fluorometric assay kit (ab111749, Abcam Inc., Cambridge, MA). Frozen tissue was homogenized in four volumes of assay buffer, plated in a black microplate and assayed

following manufacturer's instructions. The microplate was read kinetically for one hour at Ex/Em 485/520 on a Synergy2 microplate reader using Gen5 Software (Biotek Instruments, Winooski, VT). MPO activity in samples was calculated from fluorescein generated in MPO standards (1 unit MPO activity = MPO required to generate 1 umol fluorescein/minute from oxidized aminophenyl fluorescein). Results are expressed as uU/mL, where 1 uU = 1 pmol/min activity.

4.2.4. **Histology**

To investigate changes in structural development of the intestine, histological sections from distal small intestine were analyzed for general morphology. Fresh tissues were fixed immediately by submersion in Z-fix fixative solution (Anatech, Ltd., Battle Creek, MI) for 24-36 hours, rinsed with PBS and stored in 70% ethanol. Fixed tissues were embedded in paraffin and stained with hematoxylin and eosin (H&E) (TAMU College of Veterinary Medicine Histology Lab, College Station, TX). Stained sections were scanned at 20x magnification in a Hamamatsu C9600-12 slide scanner (Hamamatsu Photonics, Hamamatsu City, Japan). Manual analysis of scanned images for villus length and crypt depth were conducted using NDP.view2 software (Hamamatsu Photonics). A minimum of 20 measurements per stained slide were obtained for each morphological marker and averaged for use as a mouse average in subsequent data analyses (n=15/diet treatment).

4.2.5. **Whole Blood and Ex-vivo Tissue Explant Cytokine Release**

Cytokines in peripheral whole blood and intestinal tissue explants were measured using a multiplex magnetic bead-based assay (Millipore EMD, Billerica, MA)[184,232]. 20 uL whole blood was diluted with 80 uL Russ-10 media, centrifuged and supernatant collected. Intestinal tissue explants (1 cm portions each of small intestine and colon) were incubated for 24 hours in 1 mL Russ-10 media supplemented with 10% heat-inactivated fetal bovine serum, centrifuged and supernatant collected for cytokine assays. The cytokines of interest were TNF-a, IL-10, IL-6, and IL-1b. Culture supernatants (blood and intestinal tissues) were assayed as outlined in the manufacturer's instructions. Fluorescence was measured in a Luminex plate reader and sample cytokine concentrations determined by xPonent software (version 3.1, Luminex Corporation, Austin, TX).

LPS-challenge of whole blood for monocyte response at 12- and 24-hour incubations was conducted to assess time-course of cytokine release. As with baseline measures, 20 uL whole blood was diluted in 80 uL Russ10 media with 100 ug of 1:1 *Salmonella enteritides:Eschericia coli B107* mixture. Microplates were incubated at 37C with 5%CO_2 for 12 or 24 hours. Plates were centrifuged and supernatant collected for cytokine concentration analysis as detailed above.

4.2.6. **Lipoprotein Density Profiles**

Plasma lipoproteins were analyzed as previously described[214]. Briefly, 6 uL plasma, stained with 10 uL C6 NBD ceramide (Cayman Chemical, Ann Arbor, MI), was diluted in 1284 uL 0.18M NaBiEDTA (TCI Chemicals, Portland, OR). Dilutions were centrifuged in a MLA-130 fixed angle rotor (Beckman-Coulter, Indianapolis, IN) at 120,000 rpm for 6 hours at 5˚C using a Beckman Optima Max centrifuge (Beckman-Coulter); tubes were imaged with a digital camera using a MH-100 metal halide continuous light source. A blue-violet filter centered at 407 nm and a yellow long pass emission filter cut-on wavelength of 515 nm were used to match the spectrophotometric characteristics of C6 NBD ceramide. A gain of 1.0000, a target intensity of 30%, and an exposure time of 1 sec were selected. Images were converted to data by OriginPro 2015 software (Version 92E, OriginLab, Wellesley Hills, MA) and area under the curve (AUC) analyzed for density distributions.

4.2.7. **Fecal Metbolites: Caloric Density, Nitrogen, Sterols, Fatty Acids, and Bile Acids**

Individual fecal samples, collected at week 10, were lyophilized for sterol, fatty acid, and bile acid composition analysis.[189,233] Fecal concentrations were used to calculate total analytes in feces based on fecal weights; total excretion values (ug/48h) served as baseline to calculate proportional abundance (% of total analyte). Cage litter (n=5/diet) was collected over 7 days at week 11; feces and orts (feed particles <2mm in cage litter) were separated and weighed. Feces from a 48-hour collection window were manually

ground to less than 1 mm diameter and frozen prior to bomb calorimetry and fecal nitrogen analyses. From the same litter collections, cage orts were isolated and stored for bomb calorimetry. Nitrogen analysis was conducted using a Rapid N-cube (Elementar, Mt. Laurel, NJ). Whole diet pellets were ground by hand and sifted to <1 mm diameter particles; dietary particles were analyzed similar to fecal and ort samples. Direct calorimetry (Parr 6300 Calorimeter, Parr Instrument Co., Moline, IL) determined fecal, ort, and diet energy densities.[234]

4.2.8. **Statistical Analysis**

Statistics were performed by ANOVA and Tukey's HSD with significance of $p<0.05$ using JMP 12.1 software (SAS Institute, Cary, NC). Data are reported as means ± standard error of the mean unless otherwise noted.

4.3. Results

4.3.1. **Animal Growth**

Observed weight gains of mice (n=15/diet) fell within published growth curves for C57Bl/6J males[91] (Figure 4.1). Overall, purified diet (PD) feeding increased total BW gain by 4 g (59.7% BW gain vs 42.4%, $p<0.0001$), while protein source did not independently affect body weight gain (avg 10.5±0.69 g, $p<0.255$). Beginning day 13 on experimental diets, SPC weighed less than DWMP ($p<0.029$); this difference persisted through the end of the study. On day 20, both ISP and DWMP differed significantly from SPC but not DMC ($p<0.010$). Beginning day 30, diets with soy protein began to show divergent weight gains with ISP being the heaviest and SPC being the lightest (30.2 g vs 26.0g $p<0.0009$); dairy protein diets did not differ from each other nor from their carbohydrate refinement counterpart. ISP were heaviest at termination having gained the most weight throughout the feeding trial as both total BW gain and %BW gain (% of total gain from initial body weight), being different from DWMP, SPC & DMC (Figure 4.2). Carcass lengths, measured from nose to tip of tail, averaged 17.7±0.05 cm and did not differ by diet ($p<0.075$).

Figure 4.1 Mouse Growth Curves from Daily Body Weights

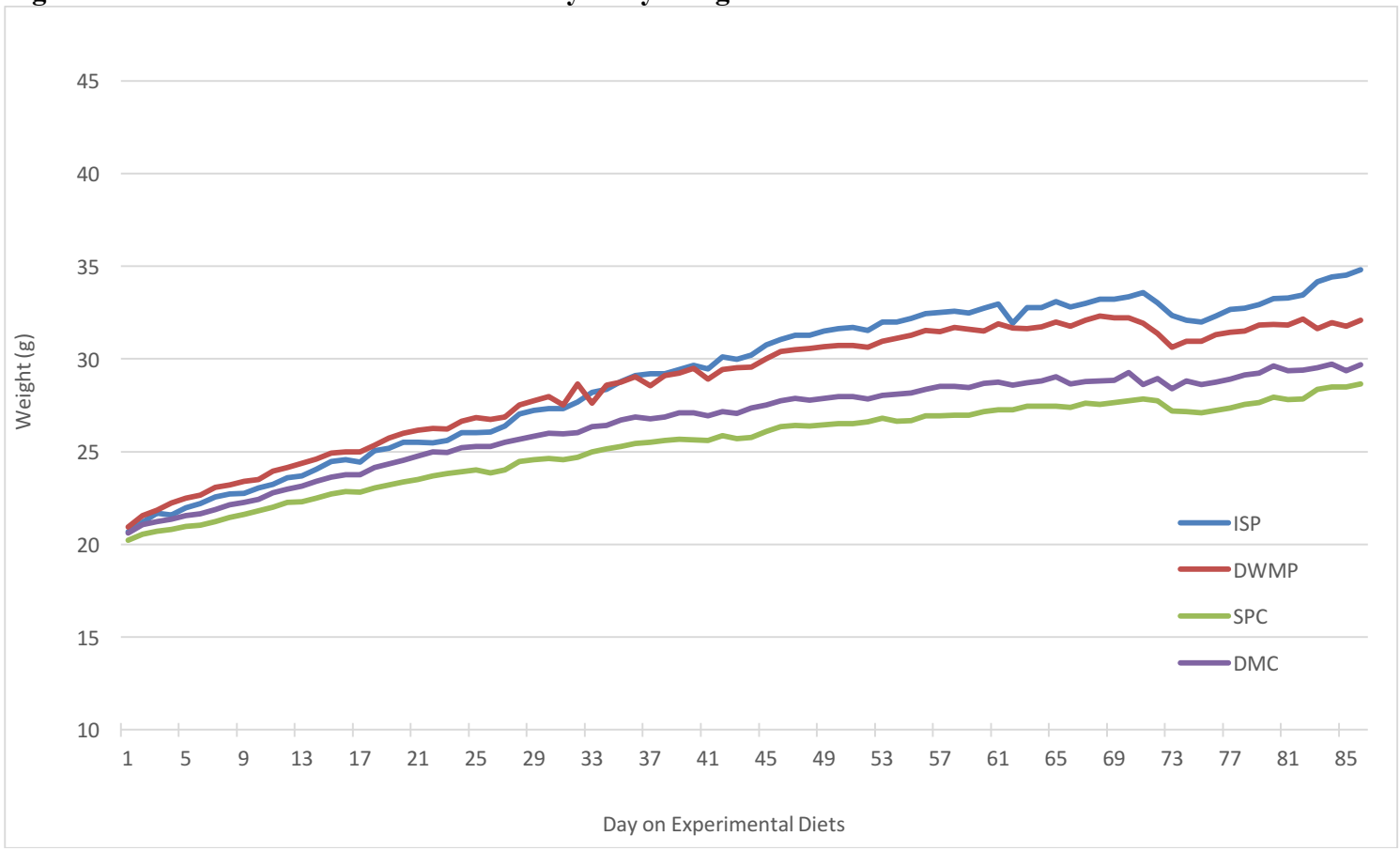
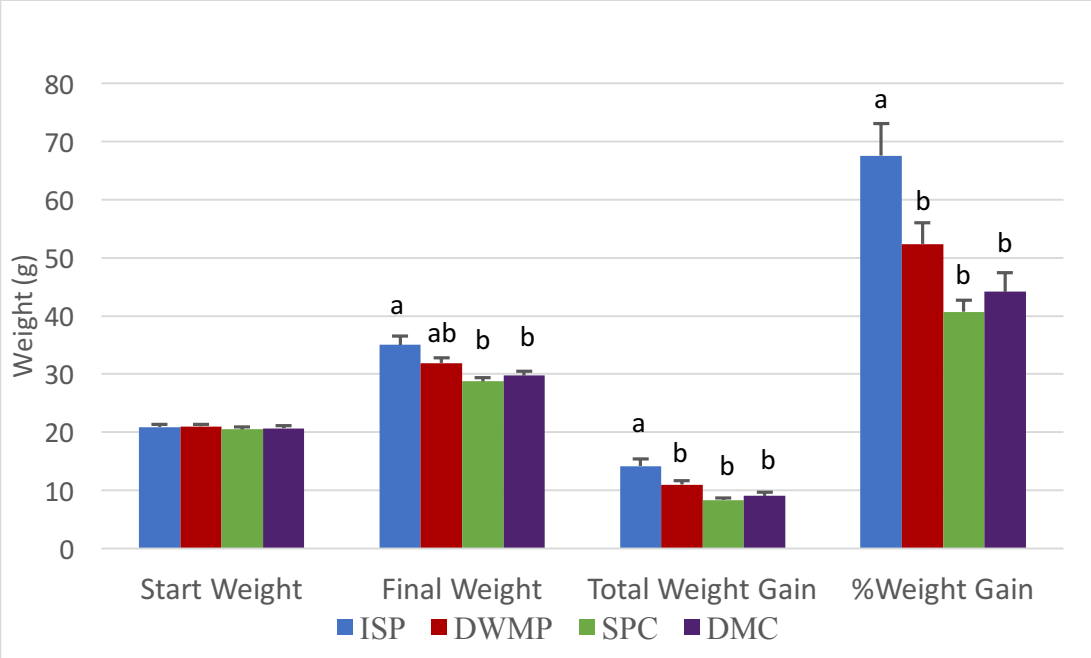


Figure 4.2 Body Weight Gain (n=15/diet)



Daily cage feed disappearance weights (n=5/diet) were measured in conjunction with daily body weights; DWMP cages had the highest feed loss by weight followed closely by SPC and nearing significance from the other diets (Table 4.2, $p<0.055$). Since this trend did not follow the observed weight gain in SPC mice, cage orts (food particles left at the bottom of the cage) were collected, weighed and caloric content measured by bomb calorimetry. Cage orts were highest in SPC, and corresponded significantly with total calories (Table 4.2). Caloric density of orts, as measured by bomb calorimetry, was not different between diet groups (Table 4.2). 48-hour fecal weights from the same cage collections were almost double in mice fed PDs (ISP, DWMP) compared to those fed NIDs (SPC, DMC) (avg 1.18 g vs 0.78, Table 4.3, $p<0.001$). Fecal output of DMC-fed mice was the least, being 75% of SPC and 56% of PDs (0.66±0.03g, 0.88±0.04g,

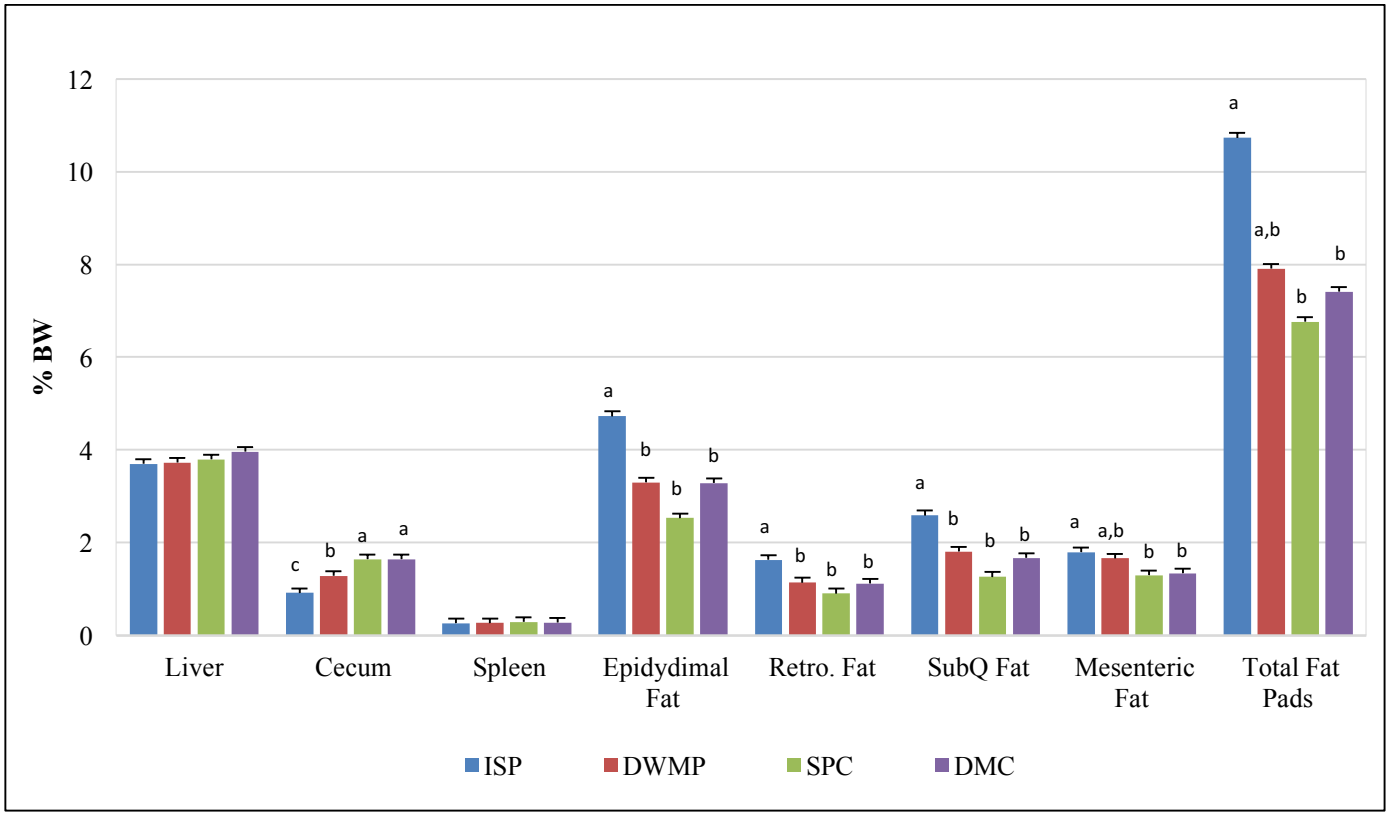
1.18±0.06g, respectively; $p<0.0001$). Orts and feces collected from cages (n=5/diet) were analyzed for caloric density. DMC had the most calorically dense orts, nearing significance when compared to the other diets (4.11±0.04 kCal/g sample vs average 3.88±0.01 kCal/g sample; $p<0.06$). When calculating calories in cage orts over 7d collection periods, orts from SPC-fed cages were increased 4.7-fold compared to other diets (29.8±2.32 kCal vs. 6.36±0.63 kCal, $p<0.0001$). Similarly, fecal caloric density was increased in SPC and DMC cages compared to PDs (avg 3.84±0.02 kCal/g sample vs 3.31±0.11; $p<0.0002$). Due to increased fecal output over the 7-day collection period, PD-fed mice excreted an average 8 kCal more than those fed NIDs ($p<0.0002$); DWMP had the highest fecal calories with DMC excreting the least over the one-week period (Table 4.2, $p<0.0006$)

Table 4.2 7 day Feed Disappearance and Cage Orts

	ISP	DWMP	SPC	DMC
7day Feed Disappearance (g)	68.0 ± 2.89^{a}	66.7 ± 2.59^{a}	63.3 ± 2.59^{a}	51.0 ± 2.59^{b}
Cage Orts (g)	1.63 ± 0.10^{b}	1.03 ± 0.12^{b}	7.62 ± 0.71^{a}	2.15 ± 0.14^{b}
Feces (g)	8.47 ± 0.90^{ab}	10.9 ± 0.84^{a}	6.54 ± 0.07^{bc}	5.53 ± 0.31^{c}
Orts Energy (kcal/g)	3.88 ± 0.07	3.83 ± 0.08	3.93 ± 0.08	4.11 ± 0.04
Fecal Energy (kcal/g)	3.51 ± 0.07^{a}	3.11 ± 0.17^{b}	3.81 ± 0.03^{a}	3.86 ± 0.04^{a}

At termination, livers, ceca, spleen and fat pads (subcutaneous at flank, retroperitoneal, epididymal and mesenteric) were collected and weighed. Livers were heaviest in ISP-fed mice (Figure 4.3, $p<0.035$), but differences did not persist when expressed as %BW($p<0.452$). Ceca of NID-fed mice were heaviest, while ISP-fed mice had the smallest ceca both as absolute weights and %BW($p<0.0001$). Spleen weights were similar across all diets ($p<0.600$). Fad pads were heaviest in ISP-fed mice ($p<0.001$); SPC-fed mice had the smallest fat pads but were not different from DWMP or DMC. Fat deposition to the dissected regions was proportional across all diets ($p<0.200$).

Figure 4.3 Organ Weights (percent of body weight)



Data represent averages of n=15/diet treatment. Different letters denote significance at p<0.05.

4.3.2. *in vivo* Intestinal Permeability and Motility

Gastrointestinal permeability (GI-P) and motility were more affected by carbohydrate refinement than protein source (Table 4.3). Plasma FITC-dextran fluorescence, an indicator of *in vivo* gut permeability, was low for all mice; fluorescence was highest in ISP-fed mice (0.013±0.00 ug/mL) and significantly higher than DMC-fed mice (0.009±0.00 ug/mL, $p<0.031$). Mice fed dairy-protein diets (DWMP, DMC) had numerically lower GI-P in both PDs (10.2 ng/ML vs 13.6 ng/mL, $p<0.11$) and NIDs (9 ng/mL vs 10.2 ng./mL, $p<0.000$). Gut motility, as assessed by time from oral gavage for Evans blue dye to appear in feces, was quickest in NID-fed mice, being 63% the time of PDs (4.15±0.15h vs 6.58±0.43h, $p<0.0001$). Plasma endotoxin, a marker of GI-P, was low and similar for all diet treatments ($p<0.768$).

Table 4.3 Gut function and health indicators, fecal collections

	ISP	DWMP	SPC	DMC	p-value
FITC-Dextran (ug/mL) n=12	0.013 ± 0.00[a]	0.011 ± 0.00[ab]	0.010 ± 0.00[ab]	0.009 ± 0.00[b]	0.007
Transit Time (hrs) n=15	6.96 ± 0.69[a]	6.22 ± 0.54[a]	3.96 ± 0.27[b]	4.35 ± 0.08[b]	0.000
FITC-Dextran:Transit Time n=12	0.040 ± 0.01	0.032 ± 0.00	0.039 ± 0.00	0.035 ± 0.00	0.400
48 h fecal collection (g) n=14	1.14 ± 0.05[a]	1.22 ± 0.06[a]	0.88 ± 0.04[b]	0.66 ± 0.03[b]	0.000
Fecal Nitrogen (%) n=5	1.52 ± 0.08[b]	1.76 ± 0.10[b]	2.99 ± 0.13[a]	2.98 ± 0.07[a]	0.000
Endotoxin (EU/mL) n=15	0.078 ± 0.02	0.097 ± 0.02	0.077 ± 0.02	0.096 ± 0.02	0.768

Endotoxin results are EU/mL; kit sensitivity = 0.005 EU/mL.

4.3.3. **Colonic Myeloperoxidase**

Colonic myeloperoxidase activity, a marker of neutrophil infiltration, increased in soy-containing diets. MPO activity increased 60% in ISP & SPC compared to dairy diets ($p<0.006$). Colons from SPC-fed mice had the highest MPO activity (85.8±16.6 uU/mL), being significantly different and increased 200% over DWMP (42.6±4.59 uU/mL, $p<0.023$).

4.3.4. **Histology**

H&E stained sections of distal small intestines were analyzed for villi length (Table 4.6). No differences in overall intestinal health were noted when scanning slides; all diets presented healthy morphology. SPC had the shortest villi, some 13% shorter than DMC and both PDs (338±9.24um vs 390±8.12um, $p<0.013$).

4.3.5. **Whole Blood and Ex-vivo Tissue Explant Cytokine Release**

Baseline cytokine concentrations in whole blood were low and not indicative of an inflammatory state (Table 4.4). There were statistically significant differences in blood IL-1β, with SPC being increased over the nearly undetectable levels in ISP (2.58 pg/mL vs 0.00 pg/mL, $p<0.039$). Tissue explants, likewise, showed statistical changes in cytokine release after 24 hours. Cell culture media collected after tissue explant incubation was not diluted for multiplex assay; therefore, cytokine concentrations are reported as pg analyte per cm tissue. In small intestine explants, DMC has the highest IL-1β and TNF-α concentrations, increased by 2.4 and 1.6-fold respectively ($p<0.0001$).

Small intestine explant cytokine release was similar for IL-6 and IL-10 for all diets ($p<0.406$). Within colonic tissue explants, ISP had the highest IL-1β concentration, being significantly increased 2.4-fold over DMC ($p<0.017$). Colonic cytokine release was similar across diets for IL-6, IL-10 and TNF-α ($p<0.354$).

Table 4.4 Cytokines: Whole Blood (baseline), Tissue Explants

		ISP	DWMP	SPC	DMC
Whole Blood (pg/mL)	**IL-1β**	0.00 ± 0.00^{b}	$1.97 \pm 0.49^{a,b}$	2.58 ± 1.06^{a}	$1.23 \pm 0.41^{a,b}$
	IL-6	0.02 ± 0.02	0.19 ± 0.13	0.01 ± 0.01	0.11 ± 0.10
	TNF-α	0.06 ± 0.06	0.38 ± 0.13	0.35 ± 0.19	0.15 ± 0.06
	IL-10	8.81 ± 3.66	1.33 ± 0.42	0.61 ± 0.17	0.51 ± 0.19
Small Intestine (pg/cm)	**IL-1β**	12.0 ± 1.78^{b}	13.3 ± 1.75^{b}	13.7 ± 1.38^{b}	32.4 ± 2.91^{a}
	IL-6	8028 ± 1660	9706 ± 1475	8724 ± 1763	12247 ± 2264
	TNF-α	2.16 ± 0.42^{b}	$2.74 \pm 0.37^{a,b}$	$2.59 \pm 0.52^{a,b}$	4.52 ± 0.78^{a}
	IL-10	14.0 ± 2.38	11.6 ± 1.19	9.73 ± 0.97	12.42 ± 1.91
Colon (pg/cm)	**IL-1β**	10.6 ± 1.86^{a}	$8.94 \pm 0.89^{a,b}$	$5.15 \pm 1.37^{a,b}$	4.48 ± 1.65^{b}
	IL-6	5541 ± 2073	11857 ± 2330	9996 ± 3343	12130 ± 2358
	TNF-α	1.44 ± 0.60	2.27 ± 0.66	3.94 ± 1.48	3.83 ± 0.91
	IL-10	12.5 ± 1.44	15.8 ± 1.60	12.1 ± 1.00	12.9 ± 1.87

Due to Luminex probe clogging during the plate reads and magnetic bead counts being below acceptable limits, cytokine release following LPS-challenge of whole blood could not be determined. Data obtained did not provide sufficient power to draw reliable conclusions. Sample numbers and bead counts are listed in Appendix H.

4.3.6. **Lipoprotein Density Distributions**

Total lipoprotein mass (total AUC) was similar for all diets ($p<0.215$); however, there were notable differences within distributions of subfractions (Table 4.5). TRLs were increased in dairy diets compared to soy-containing diets($p<0.0007$), while total LDL was increased in ISP ($p<0.024$). LDL-3 and -4 were highest in ISP, significantly increased over DWMP and all others, respectively ($p<0.003$). To evaluate relative proportions of lipoproteins, subfractions were expressed as % of total AUC (Table 4.6). Mice fed dairy milk proteins increased %TRL and %HDL and decreased %LDL compared to those on soy diets ($p<0.04$). ISP-fed mice had a 30% increase in small dense LDL (LDL-3, -4) and a 4.0% decrease in HDL-2b ($p<0.000$). The remaining HDL subfractions (-2a, -3a, -3b, -3c) were similar for all diets ($p<0.85$).

Table 4.5 Lipoprotein Density Profiles as Area Under the Curve (AUC)

	ISP	DWMP	SPC	DMC	*P*
TRL	302 ± 20.3[b]	433 ± 24.4[a]	365 ± 26.3[ab]	454 ± 37.5[a]	0.0027
LDL	3186 ± 129[a]	2699 ± 245[b]	2626 ± 110[b]	2595 ± 120[b]	0.0024
LDL-1	105 ± 2.38	113 ± 8.30	102 ± 2.51	108 ± 1.35	0.2891
LDL-2	157 ± 2.05[a]	200 ± 28.8[a]	671 ± 4.77[a]	780 ± 9.64[a]	0.4310
LDL-3	862 ± 41.2[a]	671 ± 77.6[b]	712 ± 45.1[ab]	686 ± 34.4[ab]	0.0030
LDL-4	1037 ± 59.0[a]	780 ± 78.5[b]	766 ± 36.2[b]	725 ± 39.4[b]	0.0001
LDL-5	1026 ± 45.2	935 ± 79.1	885 ± 36.3	906 ± 50.5	0.1528
HDL	5382 ± 207	5006 ± 270	5131 ± 178	5190 ± 144	0.7745
HDL-2B	3254 ± 116	3153 ± 180	3200 ± 96.2	3281 ± 91.3	0.8820
HDL-2A	1335 ± 84.5	1136 ± 91.8	1215 ± 61.6	1175 ± 58.6	0.4313
HDL-3A	344 ± 23.2[b]	307 ± 12.6[b]	299 ± 12.3[ab]	311 ± 9.54[b]	0.1716
HDL-3B	153 ± 9.15[ab]	143 ± 3.88[b]	138 ± 6.34[ab]	150 ± 8.59[ab]	0.5093
HDL-3C	296 ± 31.3	268 ± 25.5	279 ± 26.1	273 ± 23.3	0.9482
TOTAL	8869 ± 305	8138 ± 298	8123 ± 282	8239 ± 233	0.2147

Table 4.6 Lipoprotein subfraction as Percentage of Total AUC

	ISP	DWMP	SPC	DMC	*P*
TRL	3.45 ± 0.26^{b}	5.41 ± 0.36^{a}	4.45 ± 0.20^{ab}	5.49 ± 0.41^{a}	0.0001
LDL	36.0 ± 0.80^{a}	33.0 ± 2.77^{b}	32.3 ± 0.76^{ab}	31.4 ± 0.917^{b}	0.0012
LDL-1	1.20 ± 0.05	1.43 ± 0.15	1.27 ± 0.05	1.32 ± 0.03	0.2152
LDL-2	1.80 ± 0.07	2.55 ± 0.46	2.00 ± 0.08	2.08 ± 0.12	0.1827
LDL-3	9.77 ± 0.40^{a}	8.17 ± 0.91^{b}	8.69 ± 0.38^{ab}	8.29 ± 0.26^{ab}	0.0015
LDL-4	11.6 ± 0.35^{a}	9.49 ± 0.83^{b}	9.41 ± 0.26^{b}	8.74 ± 0.28^{b}	0.0001
LDL-5	11.6 ± 0.34^{a}	11.4 ± 0.75^{ab}	10.9 ± 0.35^{b}	11.0 ± 0.48^{b}	0.5393
HDL	60.6 ± 0.91^{b}	61.6 ± 2.88^{a}	63.2 ± 0.75^{ab}	63.1 ± 0.94^{ab}	0.0470
HDL-2b	36.8 ± 0.75^{b}	38.7 ± 1.78^{a}	39.5 ± 0.42^{a}	39.9 ± 0.58^{a}	0.0006
HDL-2a	15.0 ± 0.76	14.0 ± 1.11	14.9 ± 0.46	14.3 ± 0.66	0.8536
HDL-3a	3.85 ± 0.20	3.80 ± 0.16	3.70 ± 0.12	3.80 ± 0.12	0.8246
HDL-3b	1.72 ± 0.08	1.78 ± 0.08	1.71 ± 0.09	1.83 ± 0.10	0.8295
HDL-3c	3.29 ± 0.30	3.31 ± 0.32	3.39 ± 0.26	3.30 ± 0.26	0.9575

4.3.7. **Fecal Metabolites: Nitrogen, Sterol, Fatty Acid, and Bile Acid Content**

Fecal nitrogen, measured as an indicator of diet digestibility, was markedly increased in NIDs, being nearly double that of PDs (Table 4.7, $p<0.0001$). Fecal fatty acid, sterol and bile acid concentrations were analyzed as non-invasive markers of gastrointestinal health and microbial community activity. Fecal sterol and fatty acid concentrations were highest in NIDs, while bile acid concentrations were elevated in DMC alone (Table 4.7).

Table 4.7 Fecal Metabolites

	ISP	DWMP	SPC	DMC
Nitrogen (%)	1.52 ± 0.08^{b}	1.76 ± 0.10^{b}	2.99 ± 0.13^{a}	2.98 ± 0.07^{a}
Fatty Acids (ug/g feces)	15.4 ± 0.81^{b}	11.7 ± 1.04^{b}	45.7 ± 3.76^{a}	46.4 ± 2.19^{a}
Bile Acids (ug/g feces)	0.71 ± 0.03^{b}	0.73 ± 0.07^{b}	0.80 ± 0.04^{b}	1.26 ± 0.08^{a}
Sterols (ug/g feces)	2.32 ± 0.05^{b}	1.50 ± 0.08^{c}	5.51 ± 0.21^{a}	5.58 ± 0.11^{a}

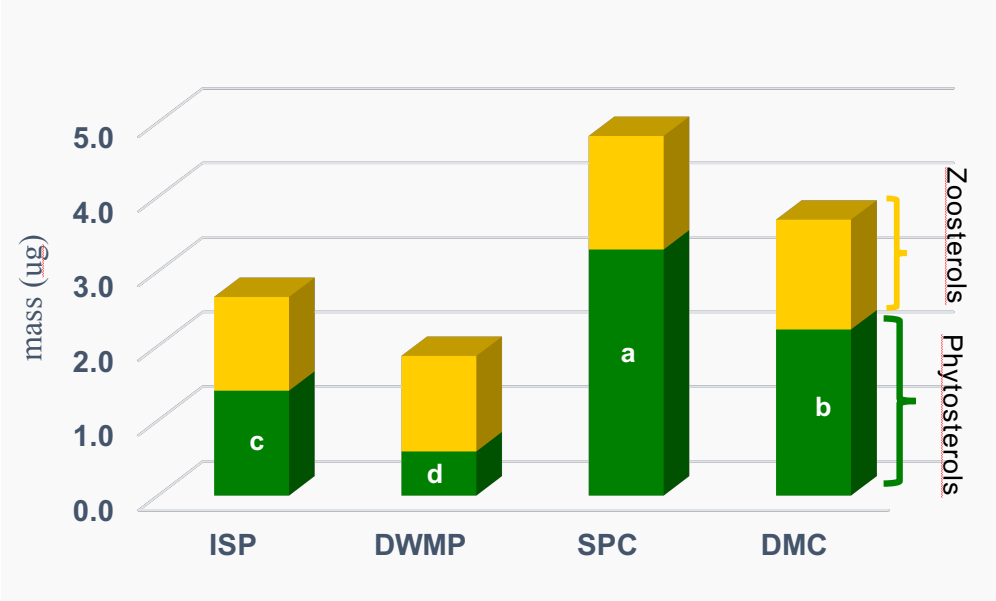
Fecal nitrogen (n=5 pooled cage samples/diet). Fatty acids, bile acids and sterols n=15 individual fecal samples/diet.

Sterol content of NID feces were double that of ISP and 3.7-fold higher than DWMP (Table 4.8, $p<0.000$). Phytosterols (brassicasterol, campesterol, stigmasterol, fusosterol, betasitosterol) & zoosterols (cholesterol, coprostanol, cholestenol, latherosterol) increased in NIDs (Figure 4.4, $p<0.01$); soy diets had 170% more phytosterols than dairy ($p<0.000$). Despite feces from NIDs having increased cholesterol concentrations (0.735±0.030 vs. 0.549±0.024 ug/mg feces, $p<0.0001$), %cholesterol was lower in NIDS by 26% ($p<0.000$).

Table 4.8 Fecal Sterol Concentrations (ug sterol/mg feces)

	ISP	*DWMP*	*SPC*	*DMC*
coprostanol-TMS	0.348 ±0.033^{c}	0.381 ± 0.041^{c}	0.788 ± 0.047^{b}	1.17 ± 0.037^{a}
cholesterol-TMS	0.595 ± 0.028^{bc}	0.502 ± 0.035^{c}	0.699 ± 0.049^{ab}	0.774 ± 0.033^{a}
cholestanol-TMS	0.059 ± 0.001^{d}	0.078 ± 0.003^{c}	0.101 ± 0.003^{b}	0.127 ± 0.004^{a}
brassicasterol-TMS	0.005 ± 0.000^{b}	0.005 ± 0.000^{b}	0.018 ± 0.001^{a}	0.018 ± 0.001^{a}
lathosterol-TMS	0.101 ± 0.007^{b}	0.069 ± 0.007^{c}	0.144 ± 0.009^{a}	0.152 ± 0.005^{a}
campesterol-TMS	0.376 ± 0.010^{c}	0.124 ± 0.007^{d}	0.942 ± 0.040^{a}	0.810 ± 0.024^{b}
stigmasterol-TMS	0.239 ± 0.009^{c}	0.072 ± 0.007^{d}	0.428 ± 0.017^{a}	0.364 ± 0.013^{b}
fusosterol-TMS	0.020 ± 0.001^{c}	0.005 ± 0.001^{d}	0.109 ± 0.005^{a}	0.094 ± 0.004^{b}
beta-sitosterol-TMS	0.542 ± 0.016^{c}	0.229 ± 0.015^{d}	0.668 ± 0.015^{a}	.779 ± 0.012^{b}
sitostanol-TMS	0.035 ± 0.006^{c}	0.036 ± 0.005^{c}	0.668 ± 0.015^{b}	0.779 ± 0.012^{a}
Total Sterols	2.32 ± 0.05^{b}	1.50 ± 0.08^{c}	5.51 ± 0.21^{a}	5.58 ± 0.11^{a}
Total Phytosterols	1.22 ± 0.04^{c}	0.47 ± 0.03^{d}	3.78 ± 0.16^{a}	3.36 ± 0.10^{b}
Total zoosterols	1.10 ± 0.05^{c}	1.03 ± 0.07^{c}	1.73 ± 0.09^{b}	2.22 ± 0.05^{a}

Figure 4.4 Fecal Sterol Excretion (ug/48 hr)



Data represent total fecal sterol output over 48 hours calculated from fecal weights and sterol concentrations (n=15/diet). Letters denote differences in phytosterols; total ug of zoosterols were similar across diets.

Feces from mice fed NIDs contained 3-fold increased fatty acid concentrations compared to PDs (Table 4.9, $p<0.000$). Palmitate was most concentrated in feces of DMC-fed mice, some 71% increased over SPC and 370% higher than PDs ($p<0.0001$). Palmitate made up the highest proportion of total FAs in dairy protein diets, accounting for 48% of total FAs in DWMP, 45% in DMC and 27% in soy-containing diets (ISP, SPC) ($p<0.001$). NIDs had the highest concentration of linoleate, α-linolenate, oleate with SPC being the highest in both and PDs having concentrations 14, 6 and 7-fold less than SPC, respectively ($p<0.001$). Similarly, stearate was most concentrated in SPC (13.9±1.39 ug/mg feces); deviating from the above trend, ISP fecal concentrations (8.3±0.47 ug/mg feces) were similar to DMC (10.5±0.56 ug/mg feces) and increased

over DWMP (3.8±0.40, $p<0.0001$). Arachidonate, gondoate, erucate, ducosonate, and nervonate were also increased in NIDs ($p<0.0001$), but concentrations were low and their sum accounted for less than 5% of total FAs present in the 48 hour fecal collections.

Table 4.9 Fecal Fatty Acid Concentrations (ug FA/mg feces)

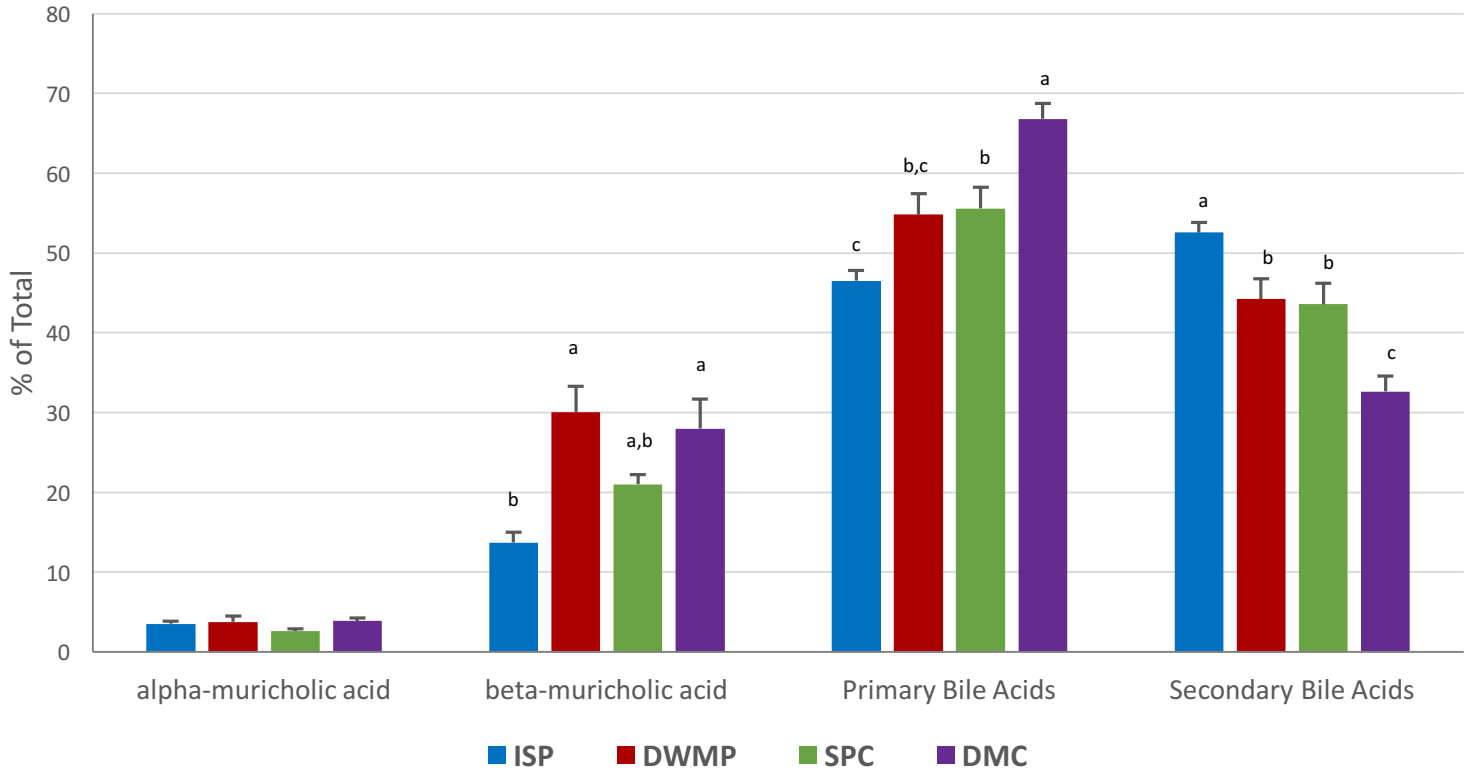
	ISP	*DWMP*	*SPC*	*DMC*
palmitate (16:0)	4.45 ± 0.20^{c}	5.64 ± 0.49^{c}	12.2 ± 1.11^{b}	21.0 ± 1.19^{a}
linoleate (18:2 n-6)	0.541 ± 0.026^{c}	0.403 ± 0.059^{c}	7.74 ± 0.649^{a}	5.74 ± 0.369^{b}
α-linolenate (18:3 n-3)	0.041 ± 0.002^{c}	0.041 ± 0.004^{c}	0.348 ± 0.029^{a}	0.261 ± 0.014^{b}
oleate (18:1 n-9)	1.15 ± 0.081^{c}	0.811 ± 0.067^{c}	8.20 ± 0.731^{a}	5.73 ± 0.352^{b}
cis-vaccenate (18:1 n-7)	0.260 ± 0.022^{b}	0.187 ± 0.015^{b}	1.30 ± 0.127^{a}	1.19 ± 0.048^{a}
stearate (18:0)	8.27 ± 0.469^{b}	3.80 ± 0.397^{c}	13.9 ± 1.39^{a}	10.5 ± 0.556^{b}
arachidonate (20:4 n-6)	0.178 ± 0.004^{c}	0.153 ± 0.006^{c}	0.432 ± 0.011^{a}	0.331 ± 0.030^{b}
gondoate (20:1 n-9)	0.192 ± 0.016^{c}	0.133 ± 0.010^{c}	0.412 ± 0.031^{a}	0.320 ± 0.013^{b}
erucate (22:1 n-9)	0.105 ± 0.009^{b}	0.075 ± 0.009^{b}	0.171 ± 0.013^{a}	0.187 ± 0.009^{a}
docosanoate (22:0)	0.335 ± 0.016^{c}	0.361 ± 0.030^{c}	0.643 ± 0.033^{b}	0.904 ± 0.029^{a}
nervonate (24:1 n-9)	0.083 ± 0.032^{b}	0.089 ± 0.011^{b}	0.281 ± 0.019^{a}	0.318 ± 0.016^{a}
Total Fatty Acids	15.4 ± 0.815^{b}	11.7 ± 1.04^{b}	45.7 ± 3.76^{a}	46.4 ± 2.19^{a}

Bile acid (BA) concentrations increased 1.5-fold in DMC feces compared to all others (Table 4.10, $p<0.000$). Fecal cholic acid concentrations increased 2-fold in DMC compared to all others while DMC deoxycholic acid (0.34) was numerically increased over ISP(0.30) and different from DWMP, SPC (0.26, $p<0.001$). ISP had the highest concentration of lithocholic acid, being different from DWMP (0.07 ug/mg feces vs 0.05, $p<0.048$). The murine specific primary bile acids, α-muricholic acid and β-muricholic acid, were highest in dairy diets (DMC, DWMP) compared to soy-containing diets (Figure 4.5, $p<0.003$). α-muricholic acid was present at 2-fold concentrations and β-muricholic acid increased 2.4 times in dairy diets compared to ISP and SPC. Primary BAs (cholic acid, chenodeoxycholic acid, α-muricholic acid, β-muricholic acid) were highest in dairy diets ($p<0.013$), while secondary BAs (lithocholic acid, deoxycholic acid) were increased in PDs with ISP containing 1.6 times that of DMC (Figure 4, $p<0.000$).

Table 4.10 Fecal Bile Acid Excretion (ug BA/mg feces)

	ISP	*DWMP*	*SPC*	*DMC*
cholic acid	0.196 ± 0.015^{b}	0.133 ± 0.013^{b}	0.259 ± 0.039^{b}	0.415 ± 0.063^{a}
chenodeoxycholic acid	0.012 ± 0.001	0.013 ± 0.000	0.010 ± 0.001	0.015 ± 0.003
lithocholic acid	0.070 ± 0.003^{a}	0.049 ± 0.002^{b}	0.054 ± 0.006^{ab}	0.061 ± 0.009^{ab}
deoxycholic acid	0.301 ± 0.013^{ab}	0.255 ± 0.007^{b}	0.284 ± 0.011^{b}	0.340 ± 0.020^{a}
ursodeoxycholic acid	0.006 ± 0.000	0.006 ± 0.000	0.006 ± 0.000	0.007 ± 0.001
alpha-muricholic acid	0.026 ± 0.003^{b}	0.031 ± 0.008^{ab}	0.021 ± 0.002^{b}	0.050 ± 0.007^{a}
beta-muricholic acid	0.100 ± 0.012^{b}	0.246 ± 0.052^{ab}	0.166 ± 0.010^{b}	0.366 ± 0.071^{a}
Total Bile Acids	0.71 ± 0.03^{b}	0.73 ± 0.07^{b}	0.80 ± 0.04^{b}	1.26 ± 0.08^{a}
Total Primary Bile Acids	0.33 ± 0.02^{b}	0.42 ± 0.06^{b}	0.46 ± 0.04^{b}	0.85 ± 0.07^{a}
Total Secondary Bile Acids	0.37 ± 0.02^{ab}	0.30 ± 0.01^{c}	0.34 ± 0.01^{bc}	0.40 ± 0.03^{a}

Figure 4.5 Fecal Bile Acids (Percent of Total)



Data are diet averages (n=15) expressed as % of total bile acids.

4.4. Discussion

Overall, experimental diets produced healthy animals with growth curves following those expected for C57Bl/6 mice[91]. Soy-containing diets had divergent outcomes on body weight gain depending on degree of carbohydrate purification. At termination, ISP-fed mice were heaviest overall and SPC were lighter than either PD. Fat deposition increased in ISP-fed mice while SPC mice were leanest with total fat pads being 50% of ISP (Figure 4.2). Given the similar protein and fat compositions (Appendix B, C), we did not expect the outcomes in body weight gain and fat pad accumulation in soy-containing diets were highly surprising. Dairy-protein containing diets (DWMP, DMC) promoted normal growth and organs measured were largely unaffected by degree of purification. Livers were heaviest in ISP-fed mice, being significantly heavier than SPC ($P<0.035$); however, there were no differences in liver weights when expressed as %BW (Figure 4.2). Ceca were heaviest in NIDs regardless of protein source as both absolute weights and %BW.

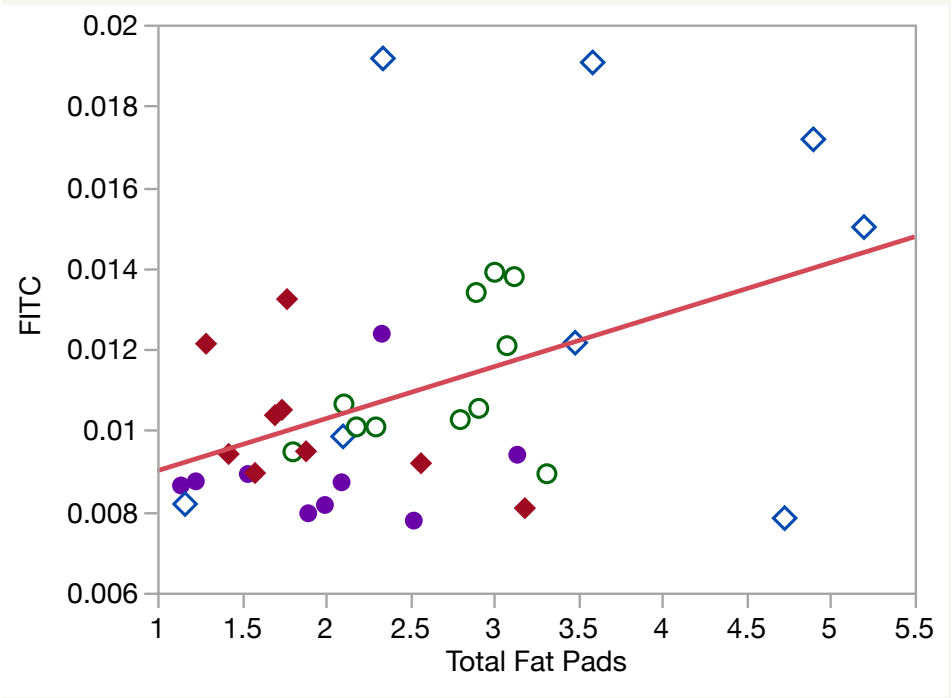
Prior studies by Thomas, et al., explored the role of dietary calcium in the development and reversal of obesity in mouse models[39,180]. While their studies showed increased fat deposition with diets containing isolated soy protein and supplemented Ca2+ equivalent to levels in non-fat dry milk, they lacked a matching NFDM diet with high Ca^{2+}. In our study, the soy diets (ISP, SPC) and DMC contained similar calcium levels (Appendix C); DWMP contained higher Ca^{2+}, being double that of the remaining diets. In our hands,

high dietary calcium in a full-fat dairy source did not provoke obesity. Likewise, NIDs with Ca^{2+} content equivalent to ISP resulted in leaner animals.

Given that SPC apparent food consumption (food disappearance) did not match the expected rate of gain (Figure 4.1, Table 4.2), we collected cage orts and measured energy densities via bomb calorimetry. Cages from SPC-fed mice had more orts, with food waste being 3.5-fold more than DMC and nearly 5 times more than either PD. However, caloric density of orts did not differ by diet ($p<0.065$), indicating mice were not selectively choosing individual diet components for consumption. Since orts did not differ in caloric densities, we can conclude mice were consuming the composed diets as provided. Therefore, dietary components were consumed consistently and should be considered for affecting changes mouse phenotypes.

GI-P, contributing to sub-acute inflammatory tone and incidence of obesity or metabolic disease,[99] was low for all mice but highest in ISP. As in Chapter 2, endotoxin levels were generally low. Plasma endotoxin increased in diary-containing diets, but failed to reach statistical significance ($p=0.7679$). FITC-d permeability was significantly correlated with fat pad accumulation across all diets (Figure 4.6, $p<0.007$, FITC = 0.00714+0.0488*fat pad g/100g BW).[231]

Figure 4.6 Association between Total Fat Pad Weight and FITC-dextran Intestinal Permeability



Correlation between fat pad weights (g) and FITC-dextran permeability (ug/mL) for all mice (n=60).

Degree of carbohydrate refinement appears to have a greater effect on motility, nitrogen utilization, and fecal lipids than protein source. NID motility was nearly half that of PDs, despite non-detergent fiber content being double that of PDs (Table 4.1). NIDs also had a minimum 2-fold increase in nitrogen (%), sterols (total mg over 48 hours) and fatty acids (ug/mg feces). Not surprisingly, proportion of phytosterols in feces was increased 16% and 28% in NIDs over PDs. Alternately, GI-P index (FITC:transit time ratio) revealed no differences between diets ($p<0.743$). Studies measuring both transit time and gut permeability have assumed consistent motility with 4-6 hours allowed for assessing

small intestinal permeability[235-238]; gut motility may affect apparent gut permeability measures and to our knowledge has not been studied directly.

Circulating plasma cytokines were numerically and statistically increased in soy-fed mice; however, cytokine levels for all mice did not reach levels indicating an inflammatory tone. High-fat diet and DSS-induced colitis models produced plasma cytokines 4-200 times higher than those observed in our mice[239-242]. Tissue explants did have a marked increase of inflammatory cytokines in small intestines of DMC-fed mice and IL-1βwas increased in colonic tissues from ISP-fed mice. Similar to plasma cytokines, those from tissue explants in overt disease models were 5-50 times more than those observed in the present study[127,232]. In conjunction with increased MPO activity, ISP-fed mice had significantly increased IL-1β release from colonic tissues while colons from DMC-fed mice were low for both parameters. Curiously, this was opposite of the general trend for our study. Across all diets, increasing MPO activity was negatively correlated with colonic IL-1β release ($p<0.03$). Others have shown that bacterial antigens modulate cytokine release in colonic explants[127]. It is possible that a challenge (ex: LPS administration) is necessary for colonic explants to exhibit cytokine release at clinically relevant concentrations.

Consistent with Trial 1 (Chapter 2)[243], diets containing milk proteins (DWMP, DMC) resulted in improved lipoprotein profiles compared to ISP. However, lipoproteins of soy-containing diets (ISP, SPC) differed by carbohydrate refinement. ISP increased %LDL,

specifically LDL-3 and LDL-4, while lowering %HDL with the bulk being lost in %HDL-2b. Small, dense LDL (LDL-3, -4, and -5) are associated with CVD risk[130] and were strong predictors of cardiovascular events in men with peripheral artery disease or abdominal aortic aneurysm[132]. Among men with ischaemic stroke, small dense LDL were also associated with increased 2-year mortality[133]. In studies of HDL subfractions, HDL-2b were better predictors of coronary heart disease than LDL or HDL-cholesterol levels[149,150]. SPC profiles largely followed the pattern of DWMP and DMC. Dairy also increased TRLs by 2% compared to ISP.

Carbohydrate refinement had a greater effect on fecal lipids than protein source. Feces from NIDs contained increased concentrations of fatty acids (340%), sterols (290%) and bile acids (141%). It is unclear whether these differences are due to motility, the complexities of the carbohydrate matrix, or some other factor. Although fecal fatty acid losses in NIDs were statistically increased, calculated loss for the 48h collection period was 0.15-0.35 calories. Assuming this rate is consistent, fecal fatty acid loss is not enough to account for the differences observed in weight gain.

Unsurprisingly, phytosterols accounted for the largest proportion of sterols in NID feces. Feces from mice fed soy-containing diets, likewise, had significantly higher phytosterols than did their dairy-containing counterparts. Zoosterol concentrations were also highest in feces of NID-fed mice. Total dietary sterols were equivalent for PDs and nearly doubled in NIDs (Table 4.7). DWMP and DMC contributed 0.02% cholesterol by weight

(242 and 213 mg cholesterol/kg feed, respectively), while ISP and SPC contained less than 0.01% cholesterol. Phytosterols comprised the largest proportion in ISP, SPC and DMC diets (78.3, 88.9, 74.2 %), whereas DWMP contained 59.4% zoosterols. Thus, dietary sterol sources cannot completely account for variations in fecal sterol compositions.

Bile acid concentrations were highest in feces of DMC-fed mice. ISP had the lowest concentration of primary bile acids while DWMP was lowest in secondary bile acids, although these differences failed to reach statistical significance. Among women with gallstones, high dietary cholesterol decreased bile acid production[244]. Breuniger, et al., showed associations between fecal bile acids and dyslipidemia development in a 1387 patient subset of the KORA FF4 study; the authors suggested fecal bile acids may be a biomarker of dyslipidemia[245]. The extent to which bile acids production and usage contribute to the changes in our study requires a carefully designed study**.**

5. CONCLUSIONS

5.1. Effect of dairy milk fractions on gastrointestinal integrity in C57Bl/6 mice

Overall, purified diets containing isolated soy or dairy proteins with macronutrients distributions meeting breeding mice and Dietary Guidelines for Americans recommendations, promoted healthy animal growth and gastrointestinal immune status. Subtle differences existed between dietary groups, but these differences largely did not indicate severe inflammatory tone or altered gastrointestinal morphology in mature animals. In this context, dairy milk should be considered a healthy, animal-based protein source.

Given the drastic increases in GI-P and plasma endotoxin of reference mice compared to purified diets, it is apparent that values alone may not provide a proper assessment of animal health status. Therefore, dietary-feeding trials designed to investigate the relationships among diet type/quality and gastrointestinal integrity are required. Specifically, diets with similar macronutrient compositions, but varying degrees of carbohydrate purification are needed to discover whether highly processed diets, as modeled by purified diet, represent a fundamental stress on gastrointestinal integrity.

5.2. Effect of dairy fractions on lipoproteins, circulating inflammatory markers, and gene expression in liver, muscle and adipose

Overall, purified diets containing isolated soy or dairy proteins within acceptable macronutrients distributions for both mice and those recommended by the Dietary Guidelines for Americans, promoted healthy animal growth and immune status. Subtle differences existed between dietary groups, but these differences largely did not indicate severe inflammatory tone or altered gastrointestinal morphology in mature animals. In this context, dairy milk should be considered a healthy, animal-based protein source. Consumption of dairy proteins appears to improve lipoprotein profiles in C57Bl/6 mice when consumed within the context of a US Dietary Guidelines macronutrient distribution.

5.3. Effect of diet quality on growth performance, innate immunity and GI health

Within the context of macronutrient distributions recommended by the US Dietary Guidelines[3], dietary protein and degree of diet purification appears to affect physiology in C57Bl/6 mice. In our study, diets containing dairy proteins reduced variability in growth and immune parameters while outcomes of soy diets were greatly affected by carbohydrate refinement. Plant-based proteins may have differential effects on weight gain and plasma lipoproteins that are influenced by degree of dietary carbohydrate purification. Macronutrient distributions alone may not be sufficient to assess dietary quality. Careful consideration of carbohydrate amounts and purification should be taken when designing dietary interventions/studies.

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APPENDIX A

DETAILED DIETARY INGREDIENTS FOR ALL DIETS

Ingredient (g/kg)	**ISP**	**DWMP**	**MPC**	**MFGM**	**SPC**	**DMC**
Isolated Soy Protein	210					
Dried Whole Milk Powder		390				387.45
Milk Protein Concentrate			230			
Milk Fat Globule Membrane Protein				325		
Soybean Meal (48%)					195.65	
Casein		95				
DL-Methionine	2.5			3	1.6	0.85
L-Cystine	1.05	2.2	2.3	1.05		
L-Lysine HCl, FG (78%)					2.3	
Sucrose	190	40	190	190		
Corn Starch	188.13	175.58	189.28	178.68		
Maltodextrin	100	100	100	100	30.0	30.0
Cellulose	140	140	140	110		
Wheat					200.0	105.0
Wheat Middlings					100.0	100.0
Corn					294.34	295.37
Corn Gluten Meal (60%)					50.0	68.0
Soybean Oil	20	20	20	20	4.0	4.42
Anhydrous Milkfat				27		
Lard	100		95		100.0	
Mineral Mix, w/o Ca & P (98057)	17	17	17	17		
Vitamin Mix, AIN-93-VX (94047)	12.7	12.7	12.7	12.7		
Mineral Mix, TSD (80318)					1.5	1.5
Vitamin Mix, TSD (81125)					3.0	3.0
Calcium Phosphate, dibasic	10.9		0.5	9.2		
Calcium Carbonate	4.5	4.3		3.15	6.2	3.9
Dicalcium Phosphate, FG (18.5% P, 21% Ca)					10.9	
Sodium Chloride					0.49	0.49
Choline Bitartrate	3.2	3.2	3.2	3.2		
TBHQ, antioxidant	0.02	0.02	0.02	0.02		
Ethoxyquin, antioxidant					0.02	0.02

APPENDIX B

DIET ANALYSES: AMINO ACIDS (% W/W)

	ISP	DWMP	MPC	MFGM	SPC	DMC	CHOW
Taurine	0.15	0.15	0.22	0.22	0.13	0.14	0.16
Hydroxyproline	0.00	0.00	0.14	0.00	0.00	0.01	0.01
Aspartic Acid	2.08	1.31	1.78	1.41	1.62	1.25	2.26
Threonine	0.68	0.74	1.13	0.81	0.66	0.68	0.89
Serine	0.82	0.80	0.82	0.92	0.78	0.78	0.98
Glutamic Acid	3.64	3.87	2.87	3.96	3.84	3.85	4.17
Proline	0.92	1.72	0.87	1.76	1.26	1.58	1.34
Lanthionine	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Glycine	0.79	0.36	0.43	0.35	0.77	0.51	1.23
Alanine	0.81	0.58	0.87	0.62	1.04	0.91	1.21
Cysteine	0.32	0.26	0.46	0.31	0.30	0.22	0.38
Valine	0.95	1.17	1.01	1.21	0.93	1.04	1.23
Methionine	0.48	0.44	0.62	0.52	0.47	0.47	0.38
Isoleucine	0.92	0.96	1.03	0.99	0.82	0.86	1.05
Leucine	1.52	1.72	1.81	1.84	1.86	2.03	1.91
Tyrosine	0.58	0.78	0.42	0.77	0.65	0.74	0.72
Phenylalanine	1.01	0.90	0.64	0.91	1.00	0.95	1.15
Hydroxylysine	0.02	0.03	0.02	0.02	0.05	0.05	0.05
Ornithine	0.01	0.00	0.00	0.00	0.01	0.01	0.02
Lysine	1.18	1.42	1.62	1.52	1.07	1.02	1.40
Histidine	0.47	0.50	0.36	0.52	0.47	0.46	0.59
Arginine	1.34	0.62	0.54	0.64	1.09	0.71	1.55
Tryptophan	0.28	0.28	0.34	0.26	0.22	0.24	0.31
TOTAL	**18.97**	**18.61**	**18.00**	**19.56**	**19.04**	**18.51**	**22.99**
Branched Chain Amino Acids	**3.39**	**3.85**	**3.85**	**4.04**	**3.61**	**3.93**	**4.19**

APPENDIX C

DIET ANALYSES: FATTY ACID PROFILES (% AUC)

FATTY ACID	*ISP*	*DWMP*	*MPC*	*MFGM*	*SPC*	*DMC*	*CHOW*
C6:0	0.00	0.06	0.00	0.15	0.00	0.22	0.00
C8:0	0.00	0.61	0.00	0.60	0.00	0.64	0.00
C10:0	0.07	1.28	0.12	2.26	0.00	2.23	0.04
C11:0	0.00	0.03	0.00	0.00	0.00	0.00	0.00
C12:0	0.08	2.94	0.14	2.85	0.08	2.78	0.06
C13:0	0.00	0.11	0.00	0.11	0.00	0.11	0.00
C14:0	1.21	9.86	1.54	9.25	1.17	9.37	0.83
C14:1	0.03	1.03	0.09	0.87	0.00	0.94	0.04
C16:0	21.5	29.9	22.2	27.7	21.9	28.6	15.2
C16:1	1.65	1.21	1.79	1.10	1.45	1.06	1.47
C18:0	10.6	11.3	11.3	12.4	10.5	10.6	3.7
C18:1T9	0.28	1.34	0.49	0.00	0.08	2.27	0.00
C18:1C9	31.9	22.2	32.0	25.5	30.9	20.8	20.4
C18:1C11	4.85	3.00	5.65	3.96	4.85	3.98	3.51
C18:2	24.3	12.8	21.6	11.3	26.2	14.7	46.9
C18:3	2.00	1.76	1.69	1.36	1.43	1.05	5.31
C20:0	0.19	0.22	0.20	0.20	0.19	0.18	0.17
C20:1	0.04	0.02	0.03	0.07	0.00	0.00	0.10
C20:2	0.57	0.08	0.55	0.10	0.58	0.17	0.34
C20:3	0.20	0.15	0.24	0.17	0.14	0.14	0.20
C20:4	0.51	0.00	0.46	0.00	0.54	0.05	0.00
C22:0	0.04	0.00	0.00	0.00	0.00	0.00	0.00
C22:1	0.00	0.00	0.00	0.00	0.00	0.08	0.85
C22:6	0.00	0.00	0.00	0.00	0.00	0.00	0.68
C24:0	0.00	0.02	0.00	0.00	0.00	0.00	0.10
C24:1	0.00	0.00	0.00	0.00	0.00	0.00	0.12
SFA	33.7	56.4	35.5	55.5	33.9	54.8	20.0
SC SFA	1.36	14.89	1.8	15.22	1.25	15.35	0.93
MUFA	39.7	26.5	40.1	31.5	37.2	29.1	26.5
PUFA	26.5	14.9	24.5	12.9	28.8	16.1	53.4

APPENDIX D

DIET ANALYSES: MINERAL CONTENT

	Unit	*ISP*	*DWMP*	*MPC*	*MFGM*	*SPC*	*DMC*	*CHOW*
Crude Protein	%	18.36	18.34	17.54	18.38	18.50	19.90	27.80
Moisture	%	5.40	4.80	---	---	4.30	6.70	3.90
Phosphorus	%	0.39	0.52	0.53	0.09	0.63	0.47	1.09
Potassium	%	0.30	0.46	1.07	0.13	0.74	0.8	1.26
Calcium	%	0.37	0.89	0.55	0.12	0.48	0.37	1.55
Magnesium	%	0.34	0.54	0.57	0.10	0.18	0.13	0.33
Ca:P	ratio	1.37	3.62	1.49	1.67	0.762	0.787	1.422
Sodium	ppm	3433	1373	2530	1634	276	1931	2430
Zinc	ppm	55.1	61.3	80.8	50.8	44	58	85
Iron	ppm	84.2	2455	571	373	56	38	59
Copper	ppm	21.7	6.69	17.0	11.3	12	14	19
Manganese	ppm	32.2	76.7	26.7	19.4	169	92	120
Sulfur	ppm	2339	1983	2371	3085	2247	2205	3051
Boron	ppm	2.10	1.13	1.25	1.03	8.82	0.46	14.85

APPENDIX E

QPCR PRIMER SEQUENCES

Gene Shortcode	**Primer Sequence**
ACTB	GGC TGT ATT CCC CTC CAT CG
APOA1	GCA CGT ATG GCA GCA AGA TG
TLR4	AGG AAG TTT CTC TGG ACT AAC AAG TTT AG
TLR5	GCC ACA TCA TTT CCA CTC CT
MYD88	CTA GGA CAA ACG CCG GAA CT
NFKB1	ACA CGA GGC TAC AAC TCT GC
TNFA	CCA CCA CGC TCT TCT GTC TAC
ZO1	TTT TTG ACA GGG GGA GTG G
FAAH	ACA GGC AGG CCT ATA CCC TT
MGL	CAG AGA GGC CAA CCT ACT TTT C
NAPE-PLD	GGG CGG CTC TCA CTT TCT A
CB2	TGA CAA ATG ACA CCC AGT CTT CT
TRPV1	CCT GCA TTG ACA CCT GTG
GPR119	GCC TTC GGA TGG CAT TTG TC
GPR41	TTT CTG AGC GTG GCC TAT CC
GPR55	CTA TCT ACA TGA TCA ACT TGG CTG TTT
FAS	TTC CAA GAC GAA AAT GAT GC
CB1	CTG ATG TTC TGG ATC GGA GTC
AP2	GAT GCC TTT GTG GGA ACC TG
C/EBP	GAG CCG AGA TAA AGC CAA ACA
PPARg	CTG CTC AAG TAT GGT GTC CAT GA
PPARA	TCG GCG AAC TAT TCG GCT G
ACC	TGT TGA GAC GCT GGT TTG TAG AA
SREBP	GAT CAA AGA GGA GCC AGT GC

APPENDIX F

CHAPTER 2 & 3: BODY WEIGHTS BY COHORT

	Start Weight	Final Weight	Total Weight Gain	% Weight Gain	Feed Intake
Cohort 1 (n=20)	16.1 ± 0.44^{B}	31.0 ± 0.52^{A}	14.9 ± 0.54^{B}	94.6 ± 5.22^{B}	264 ± 2.55^{A}
Cohort 2 (n=20)	18.5 ± 0.22^{A}	29.0 ± 0.48^{B}	10.5 ± 0.42^{C}	56.7 ± 2.36^{C}	248 ± 2.38^{B}
Cohort 3 (n=20)	14.4 ± 0.53^{C}	31.7 ± 0.59^{A}	17.3 ± 0.53^{A}	124 ± 7.67^{A}	268 ± 3.68^{A}

n=5	**Start Weight**	**Final Weight**	**Total Weight Gain**	**% Weight Gain**	**Feed Intake**
ISP – Cohort 1	16.8 ± 0.58^{abcd}	30.6 ± 0.88^{abc}	13.8 ± 0.66^{bcde}	82.3 ± 4.85^{bcd}	261 ± 3.59^{ab}
ISP – Cohort 2	17.9 ± 0.37^{abcd}	28.3 ± 0.64^{bc}	10.4 ± 0.72^{de}	58.2 ± 4.88^{cd}	246 ± 2.72
ISP – Cohort 3	13.8 ± 1.30^{d}	30.9 ± 1.52^{abc}	17.1 ± 0.63^{ab}	129 ± 15.6^{a}	260 ± 5.26^{ab}
DWMP – Cohort 1	16.4 ± 1.12^{abcd}	30.4 ± 1.15^{abc}	14.0 ± 1.17^{abcde}	88.2 ± 11.8^{bc}	271 ± 4.82^{a}
DWMP – Cohort 2	19.1 ± 0.35^{a}	28.9 ± 0.90^{abc}	9.82 ± 0.55^{e}	51.3 ± 2.04^{d}	257 ± 2.94^{ab}
DWMP – Cohort 3	14.6 ± 1.08^{cd}	32.1 ± 1.19^{abc}	17.5 ± 1.27^{ab}	124 ± 16.1^{a}	273 ± 6.39^{a}
MPC – Cohort 1	15.0 ± 0.84^{abcd}	29.8 ± 0.77^{abc}	14.8 ± 0.92^{abcd}	101 ± 11.0^{ab}	257 ± 4.73^{ab}
MPC – Cohort 2	18.2 ± 0.56^{abc}	27.9 ± 0.52^{c}	9.72 ± 0.60^{e}	53.9 ± 4.62^{d}	238 ± 3.33^{b}
MPC – Cohort 3	14.6 ± 0.84^{cd}	30.5 ± 0.24^{abc}	15.9 ± 1.02^{abc}	112 ± 15.1^{ab}	264 ± 9.41^{ab}
MFGM – Cohort 1	16.2 ± 0.97^{abcd}	33.1 ± 0.95^{ab}	16.9 ± 1.14^{ab}	107 ± 11.7^{ab}	267 ± 5.99^{a}
MFGM – Cohort 2	18.9 ± 0.32^{ab}	30.9 ± 1.29^{abc}	12.0 ± 1.17^{cde}	63.5 ± 5.91^{cd}	252 ± 5.80^{ab}
MFGM – Cohort 3	14.8 ± 1.24^{bcd}	33.4 ± 1.22^{a}	18.6 ± 1.13^{a}	132 ± 18.1^{a}	273 ± 8.14^{a}

APPENDIX G

CHAPTER 2 & 3: ORGAN WEIGHTS AS PERCENT OF BODY WEIGHT

	ISP	DWMP	MPC	MFGM	p-value
Liver	$3.99 \pm 0.07^{a,b}$	3.67 ± 0.14^{b}	3.62 ± 0.10^{b}	4.13 ± 0.11^{a}	0.0046
Cecum	$1.02 \pm 0.08^{a,b}$	1.28 ± 0.11^{a}	$1.11 \pm 0.04^{a,b}$	0.87 ± 0.05^{b}	0.0056
Gastrocnemius Muscle	0.47 ± 0.05	0.53 ± 0.02	0.54 ± 0.01	0.51 ± 0.02	0.2275
Retroperitoneal Fat Pad	$1.06 \pm 0.07^{a,b}$	0.90 ± 0.07^{b}	$0.93 \pm 0.04^{a,b}$	1.14 ± 0.06^{a}	0.0238
Fat Pads Inguinal, Subcutaneous, Epididymal (n=5)	3.38 ± 1.32	3.63 ± 1.39	3.21 ± 1.22	3.96 ± 1.50	0.1727
Total Fat Pads (n=5)	3.77 ± 1.47	3.99 ± 1.53	3.56 ± 1.35	4.37 ± 1.66	0.2294
Spleen	0.35 ± 0.07	0.23 ± 0.02	0.25 ± 0.01	0.27 ± 0.03	0.2596
Thymus	0.11 ± 0.01	0.10 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.939