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EFFECT OF DIETARY SHORT AND MEDIUM CHAIN FATTY ACIDS IN
MURINE LETHAL SEPSIS MODEL

by

David Son Dang

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Nutrition, Dietetics and Food Sciences

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2021

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ABSTRACT

Effect of Dietary Short and Medium Chain Fatty Acids in Murine Lethal Sepsis

Model

by

David Dang, Master of Science

Utah State University, 2021

Major Professor: Dr. Robert E. Ward

Department: Nutrition, Dietetics, and Food Science

The objective of this study was to determine the effects of dietary short and medium chain fatty acids in on the stress-induced inflammatory response using a murine lipopolysaccharide (LPS) model. It was hypothesized that a diet containing short and medium chain dietary fatty acids would be protective, in terms of survival, against lipopolysaccharide induced inflammation.

Mice were fed a purified AIN-76A diet that either contained corn oil, anhydrous milk fat, a combination of corn oil with milk fat globule membrane or medium chain triglyceride oil, or a milk fat globule membrane and anhydrous milk fat combination (5% by weight). Diets were balanced to contain the approximately the same amount of macro- and micronutrients and only differ in fat source. After being on the diet for 7 weeks, mice were challenged by intraperitoneal injection, with LPS (10 mg/kg) or saline. Primary endpoints for this experiment were: survival, intestinal permeability, production of proinflammatory cytokines, and histopathology of the large intestine.

No significant differences were found with weight gain and body mass measurement. Diet did not affect fasting glucose or glucose sensitivity. Within 30 hours

of LPS injection there was 0% survival of all mice across all diets. LPS challenge caused hyperpermeability of the gut compared to sham mice. Also, LPS challenge increased proinflammatory cytokines. In conclusion, the current data indicates that the addition of dietary fats that are high in short and medium chain fatty acids to a purified diet may not have a protective effect against LPS-induced inflammation.

(50 pages)

PUBLIC ABSTRACT

Effect of Dietary Short and Medium Chain Fatty Acids in Murine Lethal Sepsis

Model

David Dang

Sepsis is defined as the presence of an infection in combination with systemic inflammation in a host. One of the detrimental results of sepsis is multiple organ failure which eventually leads to death. Lipopolysaccharide (LPS), an endotoxin that can be found on the outer surface of gram-negative bacteria, can be used as a model for sepsis. LPS present in the circulatory system of hosts can induce symptoms that are seen in patients during septicemia.

Previous studies have suggested that including milk fat in a diet may protect against a lethal dosage of LPS in rodents. Milk fat has a unique fatty acid composition in which approximately 20-30% is made up of short and medium chain fatty acids. Interestingly, studies have also indicated that medium chain triglyceride (MCT) oil can also have a protective effect against LPS induced inflammation. The objective of this research was to determine if these dietary fatty acids, would have any effect against inflammation that is induced by LPS using an animal model.

ACKNOWLEDGMENTS

First, I would like to express my sincere gratitude to my advisor Professor Robert E. Ward for the continuous support of my Master study and related research, for his patience, motivation, and immense knowledge.

Besides my advisor, I would like to thank the rest of my thesis committee: Dr. Korry Hintze, and Dr. Donald McMahon, for their insightful comments and encouragement.

I thank my fellow lab mates and colleagues at Utah State University for the stimulating discussions, for the sleepless nights we were working together before deadlines, and for all the fun we have had in the last two years.

Last but not the least, I would like to thank my family: my parents and to my brother for supporting me throughout my study. My motivation to succeed is rooted from you all.

In memory of Dr. Robert I. Krieger
1943-2016
My oldest mentor and friend.

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LIST OF ABBREVIATIONS

AIN	American Institute of Nutrition
AMF	Anhydrous Milk Fat
CD14	Cluster of Differentiation 14
CO	Corn Oil
FITC-DEXTRAN	Fluorescein Isothiocyanate-Dextran
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HMGB1	High mobility group box 1
IFN- γ	Interferon gamma
IL-10	Interleukin 10
IL-12P70	Interleukin 12P70
IL-17	Interleukin 17
IL-1A	Interleukin 1A
IL-1B	Interleukin 1B
IL-2	Interleukin 2
IL-3	Interleukin 3
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-6	Interleukin 6
LPS	Lipopolysaccharide
MCP-1	Monocyte chemoattractant protein 1
MCT	Medium Chain Triglyceride
MFGM	Milk Fat Globule membrane
MIP-1A	Macrophage inflammatory protein 1a
MRI	Magnetic resonance imaging
PBS	Phosphate buffer saline
PUFA	Poly Unsaturated Fatty Acid
RANTES	Regulated on Activation Normal T Cell Expressed and Secreted
SCFA	Short chain fatty acid
T2D	Type 2 Diabetes
TNF- α	Tumor necrosis factor alpha

CHAPTER 1

INTRODUCTION

Sepsis is defined as the presence of an infection in combination with systemic inflammation in a host (Nguyen et al., 2006). One of the detrimental results of sepsis is multiple organ failure which eventually leads to death. Indicators of systemic inflammation include irregular body temperature ($>38^{\circ}\text{C}$ or $<36^{\circ}\text{C}$), a high pulse rate ($>90\text{bpm}$), and either a very high or low white blood cell count (Nguyen et al., 2006). During sepsis, the host response begins to activate monocytes, macrophages, and neutrophils that interact with endothelial cells through numerous pathogen recognition receptors (Beutler, 2004). Furthermore, the cellular activation allows for the mobilization of plasma cytokines which include tumor necrosis factor, interleukins, and eicosanoids (Nguyen et al., 2006). The mechanism behind sepsis is unclear and there is currently no known preventative measure or treatment for the illness. However, recent studies have suggested that short and medium chain fatty acids may play a role in protecting against septicemia (Kono et al., 2003, Snow et al., 2011). One source of these types of saturated fatty acids is dairy fat.

In the past, epidemiological data have linked the consumption of saturated fats to certain cardiovascular diseases such as coronary heart disease (Hu et al., 1999). However, in the last few years, scientists have begun to question whether dairy fats should be categorized with other sources of saturated fat, as more recent epidemiological studies suggest that dairy consumption is inversely related to metabolic diseases such as Type 2 diabetes (T2D) (Elwood et al., 2008, Tong et al., 2011, Kalergis et al., 2013, Hirahatake et al., 2014). The idea that dairy fat may protect against the development of metabolic diseases is interesting since certain risk factors that are associated with these diseases, such as insulin resistance, high levels of low-

density lipoprotein cholesterol, elevated blood triglyceride levels, and increases in inflammatory markers, are a concern in the U.S. population (Ervin, 2009, Mozaffarian and Ludwig, 2015). Several different proposed mechanisms and pathways suggest that the protective effect from dairy fat comes from a multitude of sources which includes a calcium dependent pathway (Zemel, 2003a, b, Sun and Zemel, 2006, Sun and Zemel, 2008), its role in preventing mitochondrial dysfunction (Sun and Zemel, 2007, 2009, Zemel and Zhao, 2009, Bruckbauer and Zemel, 2011, Li et al., 2012), and its supply of a bioactive lipid (trans-palmitoleate)(Mozaffarian et al., 2010, Mozaffarian et al., 2013). Unlike most foods, dairy fat contains a relatively high amount of short and medium chain saturated fatty acids.

Fatty acids are typically categorized by their carbon chain length and degree of unsaturation. Medium and short chain fatty acids are saturated fatty acids typically 4 to 14 carbons long. Dairy fat contains this group of fatty acids along with other longer chain fatty acids. The fatty acids in bovine milk come from two sources: *de novo* synthesis or the diet. Medium and short chain fatty acids, which are present in milk fat at up to 10.9% (Månsson, 2008), are metabolized differently than longer chain fatty acids. The metabolism of fats begins in the intestine, where triglycerides are broken down to free fatty acids by pancreatic lipases and bile salts. Longer chain free fatty acids are reesterified into triglycerides and packed in chylomicron vesicles and are transported into the lymphatic and circulatory system. Unlike longer chained fatty acids, medium and short chain fatty acids act as free molecules that can be transported directly to the liver via the portal vein (Figure 1).

The long-term goal of this proposed project is to investigate the potential health effects of short and medium chain fatty acids. This research will examine the roles of fats in the diet, specifically, the role of short and medium chain saturated fatty acids

found in dairy fat and medium chain triglyceride oil (MCT) in a model for sepsis. If they are shown to have a protective effect, further studies of these dietary fats would be warranted.

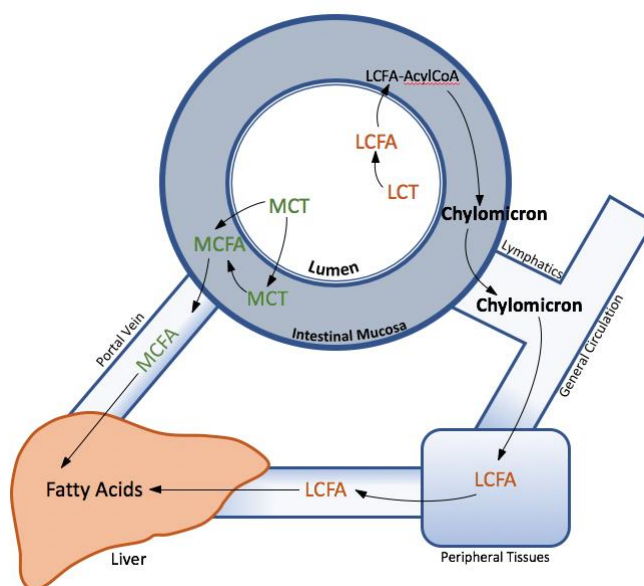


Figure 1. Metabolism of SCFA/MCFA. MCTs are more rapidly absorbed compared to longer chain triglycerides, which require a transport lipoprotein to pass through the lymphatic system and a carnitine-dependent pathway at the hepatic site.

CHAPTER 2

LITERATURE REVIEW

Gut permeability, intestinal microflora, and Gut-Liver Axis

It has been suggested that gut leakiness plays a role in allowing endotoxins to enter the blood and activate cellular macrophages, monocytes, and neutrophils (Rao, 2009). A key component of the gastrointestinal tract is a layer of epithelial cells that separate the body from the external environment. The epithelial layer is one way the body ensures protection from a variety of environmental pathogens entering the blood capillary system and causing an immune response (Arrieta et al., 2006). Specifically, the small intestine epithelial cells regulate the transportation of materials between the lumen and extracellular space. The movement of molecules through the small intestine membrane can come from either transcellular transport, which relies on active transport, or movement through the paracellular space, which occurs solely via passive diffusion. The integrity of tight junctions between adjacent epithelial cells have been shown to regulate the amount of materials that can diffuse across the membrane (Arrieta et al., 2006, Anderson and Van Itallie, 2009). The permeability of the gut is especially important considering the gut microbiota and the number of microorganisms that can leave the lumen and enter the bloodstream.

The gastrointestinal microflora is made up of a diverse community of microorganisms. The roles that the intestinal microflora can play include the production of short-chain fatty acids, synthesis of vitamins, and other metabolic contributions (Bentley and Meganathan, 1982, Canny and McCormick, 2008). The absence of intestinal microflora, shown in germ-free mice, causes the loss of these important metabolic functions (Wostmann, 1981). However, the beneficial effects that come with having a diverse gut microbiome have a limit. Residing on the outer cell membrane of

gram-negative bacteria, which are found naturally in the gastrointestinal tract, is a glycolipid called Lipopolysaccharide (LPS) (Thurman, 1998). LPS, which has been shown to signal for the proinflammatory immune response and the cause of endotoxemia, can be found in the gut lumen (Van Deventer et al., 1988). The concentration of LPS increases in the gut during sepsis (Canny and McCormick, 2008), thus, compromise of the intestinal barrier would allow for the passage of more endotoxin into the bloodstream (Mei et al., 2016). The direct cause of gut leakiness is still unknown. Endotoxemia has been associated with gut leakiness which was linked to certain high-fat diets in obese patients (Rao, 2009, Zhong et al., 2015). Ethanol-induced intestinal permeability in mice showed higher levels of plasma endotoxin, which was further exacerbated through oral administration of LPS (Lambert et al., 2003). One of the targets of LPS is a CD14 LPS receptor that lies on the surface of macrophages in the liver (Kono et al., 2003).

Kupffer cells are one of the resident macrophages that reside in the liver. The main role of these cells is to remove endotoxins in the blood (Wheeler, 2003). In response to the removal of endotoxins, Kupffer cells release proinflammatory cytokines such as tumor necrosis factor-alpha (TNF- α) (Dinarello, 2000). LPS has been shown to be signal dependent on the membrane bound CD14 receptor on Kupffer cells (Kono et al., 1998). Knockout of the receptor has been shown to slow the development of metabolic diseases, which further indicates the involvement of LPS in developing systemic inflammation (Cani et al., 2008). It has been reported that activated Kupffer cells can be inhibited by gadolinium chloride (GdCl₃), a compound toxic to Kupffer Cells, thus preventing liver injury (Adachi et al., 1994). However, gadolinium chloride is not suitable for clinical use due to its toxicity. Therefore, a better mechanism for preventing injury via inhibition of activated Kupffer cells is needed.

Diet and Inflammation

Diet has been shown to play a crucial role in the development and treatment of chronic and acute inflammation in humans (Giugliano et al., 2006, Galland, 2010). Dietary fat is one of the macronutrients that can be directly linked to inflammation. For instance, a selection of important mediators of inflammation such as eicosanoids, prostaglandin E₂, and leukotriene B₄, are all derived from the precursor omega-6 (ω -6) poly-unsaturated fatty acid (PUFA), arachidonic acid (AA) (James et al., 2000). AA is formed from the metabolism of linoleic acid, which is an essential fatty acid, through a series of elongation and desaturation (Nakamura et al., 2004). Existing evidence indicates that a diet with high dietary PUFAs concentration, depending on if it's an omega-3 (ω -3) or ω -6 fatty acid, can either decrease or exacerbate the inflammatory responses. It was shown that biomarkers for inflammation such as proinflammatory cytokines TNF- α are higher in mice that consume an ω -6 rich diet using corn oil, compared to an ω -3 enriched diet during infection (Ghosh et al., 2013). An increase in other proinflammatory cytokines such IL-1, IL-6, IL-2 and IFN- γ are commonly associated with linoleic acid rich diets due to arachidonic acid-derived eicosanoids (Calder, 2001). Furthermore, mice fed a high ω -6 diet had elevated levels of proinflammatory microbes in the gut and also severe intestinal damage during infection (Ghosh et al., 2013). In contrast to the abundance of data that exists for PUFAs and their roles in inflammation, little is known about the dietary fats in dairy and their potential effects on markers of inflammation.

Milk Fat Globule Membrane

One of the unique components of milk is the milk fat globule membranes (MFGM). Inside the mammary epithelial cells, triacylglycerol droplets are secreted

from the rough endoplasmic reticulum, into the cytosol, with a phospholipid/cholesterol monolayer. As the fat droplet migrates through the apical membrane of the cell, it is coated with a triple phospholipid layer membrane, which consist of glycoproteins, cholesterol, and phospholipids, and proteins. Phospholipids such as sphingomyelin, phosphatidylcholine, and phosphatidylethanolamine, which comes from the membrane, make up approximately 30% of the total lipid content of milk fat globules (Black et al., 1998, Cavaletto et al., 2008, Hernell et al., 2016). The phospholipids in MFGM have been shown to play a role in intestinal maturation, in which a decrease in lactase activity and vacuolated enterocytes, which are located along the villi of the intestinal wall, reflects the maturity of intestinal cells (Motouri et al., 2003). The beneficial effect it has on gut integrity and proliferation of epithelial cells is seen most prominently during the neonatal stages in animals and humans (Greenspon et al., 2009, Greenspon et al., 2011). In a past study, a diet that consisted of fat coming from MFGM in combination with milk fat improved gut epithelial integrity and prevented hyper-permeability in mice during a state of acute inflammation induced by LPS (Snow et al., 2011). In this study, mice that had a diet with MFGM as a source of fat had a lower concentration of plasma fluorescence (which is used as an assessment of gut integrity) and survived 48 hours after administration of a lethal dosage of LPS compared to the control group (Snow et al., 2011). It was suggested that the reduction in gut permeability ultimately protected the mice from death after being given a possibly lethal dosage of LPS by disallowing further release of inflammatory toxins that reside in the gut. Interestingly, a study with a similar experimental design in which medium chain triglyceride (MCT) oil was orally given to rats produced similar results in survival and intestinal permeability. An increase in survival was seen as dose response to MCT and a decrease HRP activity, which was used as an indicator for intestinal permeability, was seen in

rats that had supplemented MCT oil (Kono et al., 2003). A dosage of 5g/kg of MCT oil, was shown to be effective in preventing septic shock in this study. The similar result between these two studies brings into consideration the possibility that the protective effects from both diets comes from the similar fatty acid composition of the diets.

Short and Medium Chain Fatty acids

The production of short and medium chain fatty acids in dairy fat (which are products of microbial activity in the rumen) has long been studied (McGuire and Bauman, 2002, Parodi, 2009). The amount of saturated fatty acids make up approximately 70% of the total weight of the fat in milk (Månsson, 2008). Of the saturated fatty acids, about 11% are short chain fatty acids (SCFA) (Månsson, 2008). SCFA are typically esterified at the sn-3 position on a triglyceride (Månsson, 2008). When consumed by humans, milk triglycerides are digested by lipases in the stomach. The preference for hydrolyzing at the sn-3 position of the triglyceride by lipases allows for the release of the SCFA (Månsson, 2008). More specifically, dietary fats, more specifically in the form of long-chain fatty acids, once hydrolyzed, are absorbed in mixed micelles and are transported in chylomicrons (Kono et al., 2003). Unlike long-chain fatty acids, short and medium chains fatty acids can be absorbed and transported directly to the liver via the portal vein, where they are used for energy (Kono et al., 2003). In one study, the metabolism of butyrate was shown to provide a positive effect in an animal model by systemically reducing the expression of high-mobility group box protein 1 (HMGB1) and also significantly increasing the survival rate of the treated group compared to control during septic shock (Zhang et al., 2007). This positive effect could be due to the direct absorption of the SCFA into the liver.

A series of studies have indicated that medium-chain triglyceride diets may have protective effects against inflammation and promote insulin sensitivity. Diets

supplemented with MCTs have been shown to suppress early alcohol-induced liver injury, proinflammatory cytokines in endotoxin-challenged mice, and intestinal inflammation in both animal and cell culture models (Hoshimoto et al., 2002, Mañé et al., 2009, Carlson et al., 2015, Geng et al., 2016). It was reported that dietary MCTs prevented alcoholic liver injury by inhibiting the activation of Kupffer cells (Kono et al., 2003). It has also been shown that diets supplemented with MCTs improve glucose tolerance in rats and diabetic patients, which suggests that MCTs may have anti-diabetic benefits (Han et al., 2007, Terada et al., 2012). Findings from Kono et al. (2003) have suggested that medium chain fatty acids play a direct role in inhibiting the production of proinflammatory cytokines, which protects mice from septic shock and death, through the down-regulation of macrophage surface receptor CD14 found in the liver when given a lethal dosage of an endotoxin.

Overview

Both MCT and MFGM have a protective effect against mediators of sepsis. What is missing in the literature is evidence that links these two entities together. Therefore, this proposed study will evaluate the roles of short and medium chain fatty acids in dairy fat and medium chain triglyceride oil. By using an animal model of sepsis, we can test our hypothesis of SCFA/MCFA having a protective effect.

CHAPTER 3

THE EFFECTS OF DIETARY MILK FAT AND MEDIUM CHAIN TRIGLYCERIDE OIL DURING AN INFLAMMATORY REPOSE

The hypothesis of this proposed research is:

A purified diet with a fat composition coming from short and medium chain fatty acids from dairy fat or medium chain triglyceride will protect against acute inflammation induced by lipopolysaccharide.

The objective of this proposed research is:

- 1) Establish an animal model to determine the effects of dietary fatty acids on the inflammatory response to lipopolysaccharide.

Diet Formulation

For this study, five purified diets varied only in fat composition were formulated (Envigo Teklad Diets, Madison, WI USA). The diets were based on the American Institute of Nutrition -76A (AIN-76A) diet, which has a 5% by weight basis for its dietary fat (Table 2). The macro- and micronutrients in the diets were adjusted to be equivalent (Table 1). Each diet only differed in its type of dietary fat: corn oil (CO), medium chain triglyceride oil + corn oil (MCT+CO), anhydrous milk fat (AMF), milk fat globule membrane + corn oil (MFGM+CO), or milk fat globule membrane + anhydrous milk fat (MFGM+AMF). The milk fat globule membrane isolates diet was formulated previously in the Ward lab (Snow et al., 2011) in which 125 grams of the isolate were used to have approximately 10% of the fat as phospholipid and the other half being anhydrous milk fat (MFGM/AMF) which was shown to have a protective effect against sepsis (Snow et al., 2011).

Table 1. Composition of Diets

	Corn Oil	Medium Chain Triglyceride Oil + Corn Oil	Anhydrous Milk Fat	Milk Fat Globule Membrane + Corn Oil	Milk Fat Globule Membrane +Anhydrous Milk Fat
Ingredients					
Protein (g/kg of diet)					
Casein	183	183	183	103	103
Whey Protein Isolate	17	17	17	-	-
DL-Methionine	3	3	3	3	3
Carbohydrate (g/kg of diet)					
Sucrose	494.9	494.9	494.9	494.9	494.9
Cellulose	50	50	50	50	50
Lactose, monohydrate	5	5	5	5	5
Corn Starch	150	150	150	150	150
Fat					
Corn Oil	50	25	-	25	-
Medium Chain Triglyceride Oil	-	25	-	-	-
Anhydrous Milkfat	-	-	50	-	25
MFGM Powder	-	-	-	125	125
Vitamins and minerals (g/kg of diets)					
Mineral Mix	35	35	35	35	35
Vitamin Mix	10	10	10	10	10
Choline Bitartrate	2	2	2	2	2
Ethoxyquin, antioxidant	0.01	0.01	0.01	0.01	0.01

Table 2.Percentage of Macronutrients in the Diets

	Corn Oil	Medium Chain Triglyceride Oil + Corn Oil	Anhydrous Milk Fat	Milk Fat Globule Membrane + Corn Oil	Milk Fat Globule Membrane +Anhydrous Milk Fat
Protein	17.8	17.8	17.8	17.8	17.8
Carbohydrate	64.9	64.9	64.9	64.9	64.9
Fat	5.2	5.2	5.2	5.1	5.1

Mice Study 1

Animals and Preliminary Measures

BALB/c mice were randomly assigned to each diet (n=12) for 6 weeks. The preliminary measures during this stage of the experiment included monitoring for weight changes in the animal, body composition using Magnetic Resonance Imaging (MRI), and glucose sensitivity by oral glucose tolerance test (OGTT). Weight was measured weekly during the 6-week diet treatment. Body composition was measured twice, at baseline prior to treatment and at terminal, before LPS challenge.

Oral Glucose Tolerance Test

An OGTT was conducted at the end of the 6-week treatment period before the LPS challenge. OGTT was done by using a glucose monitoring system from a local medical supplier. A stock solution of 200 mg/mL was made. Mice were gavaged with a total of 2 g glucose/kg bodyweight. Blood was retrieved from the tail-end of the animal via tail snip. Six mice were randomly selected from each group for this procedure. Glucose was measured in triplicate at baseline, 15, 30, 60, 90, and 120 minutes, post glucose gavage.

Lipopolysaccharide Challenge

After 6 weeks, mice were injected (i.p.) with 10mg LPS/kg body weight to induce septic shock. Six hours after LPS challenge, mice were sacrificed by CO₂ asphyxiation and cardiac puncture. Serum, colon, liver, skeletal muscle, and visceral adipose were collected and flash frozen. The remaining were monitored for 48 hours for survival. A flow diagram of the process is presented in Figure 6.

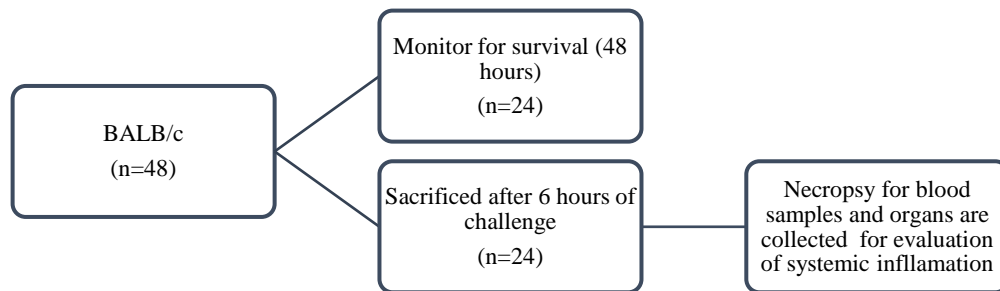


Figure 2. Experimental design for mouse study 1. Mice (n=12) were divided into two groups with an n=6 in each group. Mice that underwent an OGTT were selected as the sacrificed group after LPS was challenged. This selection method was used to ensure that the same treatment was conducted on each mouse within both groups. Six hours after the challenge, samples were collected.

Evaluation of gut barrier integrity in mice and colon inflammation

Mice that were assigned to be sacrificed were gavaged with Fluorescein Isothiocyanate –dextran (FITC-dextran ~4,000 Da) (600mg/kg body weight, 60mg/mL) to assess gut permeability. The oral gavage of FITC-dextran was performed at the same time as the LPS injection, 6 hours prior to sacrifice. FITC-dextran recovered in the blood plasma was analyzed using a fluorescence plate reader at $\lambda(\text{excitation}) = 493 \text{ nm}$ and $\lambda(\text{emission}) = 517 \text{ nm}$. and was used to assess the integrity of the gut in the animals.

Colons were cleaned with 1X PBS, fixed with 70% ethanol and then Swiss-rolled for storage for 24h in 10% formalin. After 24h of storage, 10% formalin was replaced with 70% ethanol for final storage before histological analysis. Colons were evaluated for inflammatory changes using a histologic scoring system described for mouse small intestine (Rees, 1998, Burns et al., 2001). An overall inflammatory score was given to each tissue which indicated the extent of lesions and inflammation.

Statistical Analysis

A completely randomized design was used to evaluate the response of dietary milk fat or medium chain triglyceride oil on mice when challenged with LPS. Shapiro-Wilk normality test was used to determine if data points were normally distributed. A log-transformation was used for data points that were not normally distributed. Tukey's multiple comparisons were used for posthoc analysis. The analysis was performed with GraphPad Software Prism 7 (GraphPad Software Inc., La Jolla, CA USA). Data is displayed as mean \pm SEM.

Results

Body Weight, Mass, and Food Intake

After being on the diet for 6 weeks, there were no significant differences between the four groups. Lean and fat body mass were measured at baseline and at the end. Feed efficiency was calculated as a ratio of the total food intake over kilocalories for 6 weeks. No significant differences were noted in feed efficiency between each diet group.

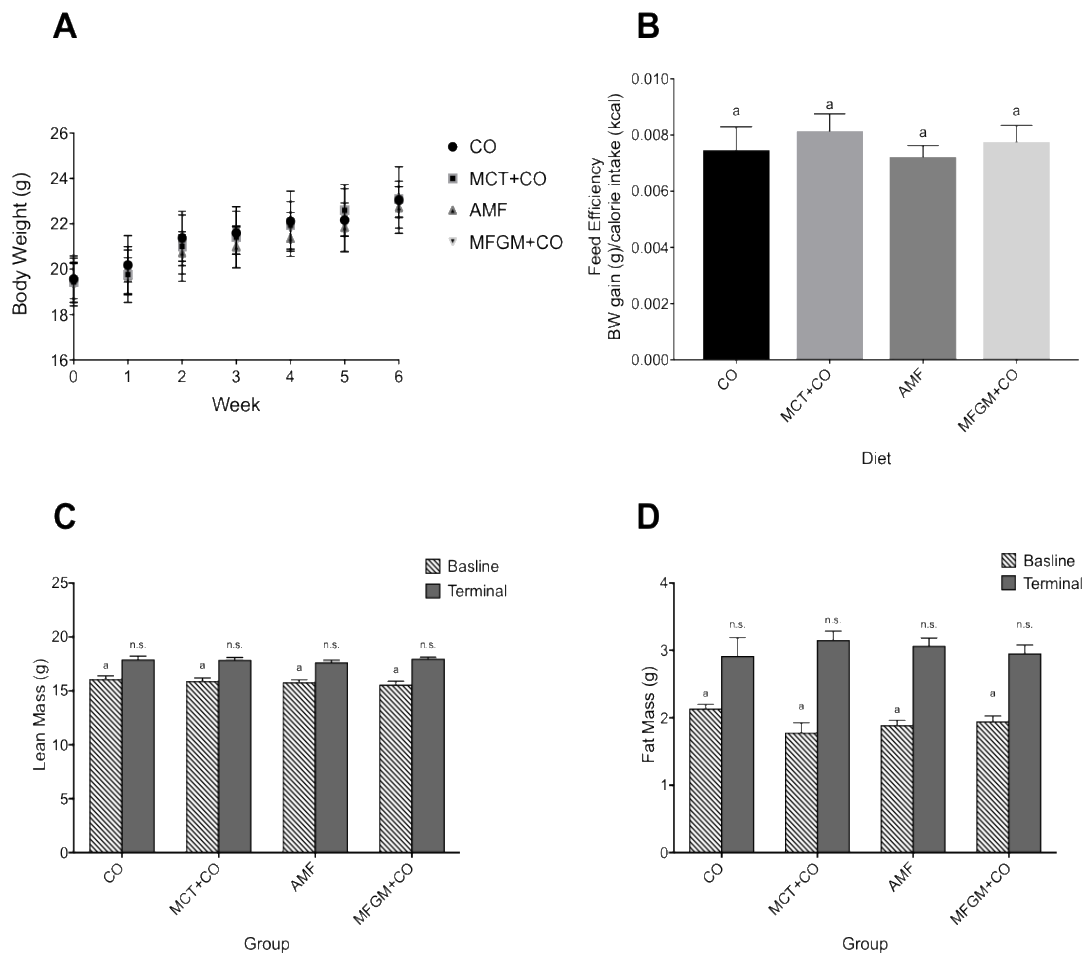


Figure 3. Weight and food intake measurements. An n=12 is assigned per group. A) Change in body weight monitored for 6 weeks. No differences were seen after 6 weeks of diet treatment. B) Feed efficiency was calculated as a ratio of the cumulative food

intake over total kilocalories. C) and D) No significant differences were seen after 6 weeks on the diet.

Oral Glucose Tolerance Test

A measure of fasting glucose and an oral glucose tolerance test was conducted to evaluate glucose sensitivity. The results indicate that a diet consisted of either milk fat or MCT oil does not have any significant effect on glucose sensitivity relative to a corn oil base diet.

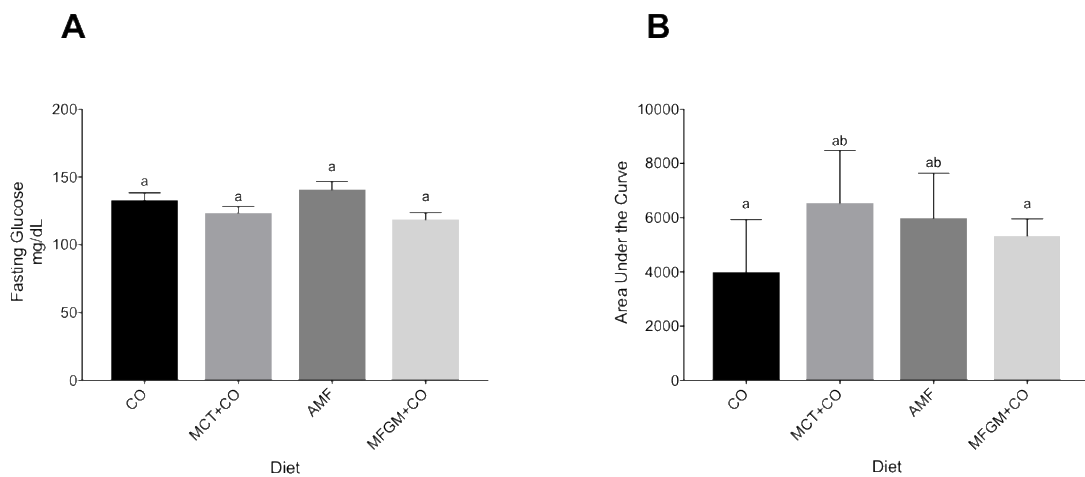


Figure 4. Fasting glucose measurement and OGTT. Diet effect on fasting glucose and glucose tolerance. An n=12 is assigned per group. A) Mean in concentration of milligram per deciliter. Glucose measurements were taken during fasting prior to OGTT. B) Area under the curve was calculated by the sum each area calculated by each time point. (Baseline, 15min, 30min, 60min, 90min, and 120min). Triplicate measurements of glucose concentration were made for each time point.

Survival

After LPS injection, mice were monitored for 48h for survival. Within the first 16h following the injection, mice on the CO, AMF, MCT+CO, and MFGM+CO groups began to die due to the LPS challenge. In less than 24h, 90% of the animals had died. By 24 h, there was 0% survival for the four groups.

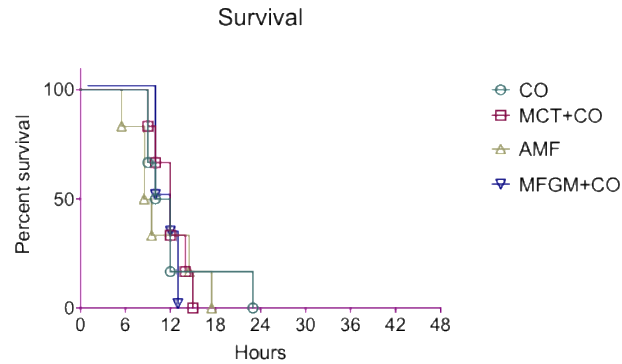


Figure 5. Survival plot. An n=6 was assigned per group. Animals were monitored everything 2-4 hours for 48h.

Intestinal inflammation and epithelial cell permeability

Histological analysis revealed no differences in colon histology scores for mice on CO, AMF, MCT+CO, and MFGM+CO diets. The lesions in these mice consisted of lymphocyte infiltration and aggregates within the submucosa and deep mucosa. The colonic crypt and epithelium were intact. Since the mice in these groups did not survive, it was expected to see a high score. There was no protective effect against intestinal hyperpermeability, which was previously supported in past published literature.

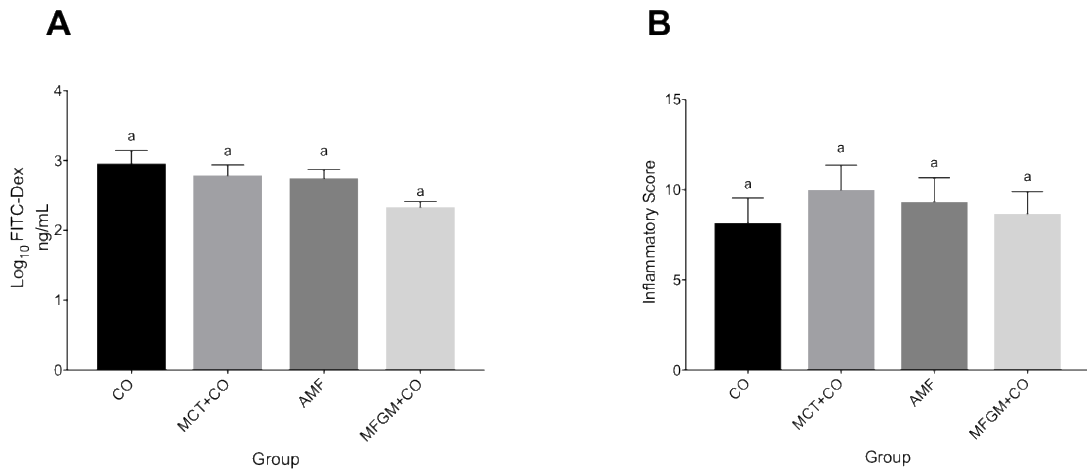


Figure 6. Gut permeability and inflammation measurements. Effect of diets on fluorescence and colon inflammation after LPS challenge. An $n=6$ is assigned per group. A) Data are log₁₀ transformed mean nanogram per milliliter. B) Inflammation parameters were summed together and then multiplied by a factor reflecting the percentage of the colon involved (0–25% (1), 26–50% (2), 51–75% (3), and 76–100% (4), to obtain the overall score (up to 24). Severity and distribution were separately assessed and combined into one score. Colon tissues of mice that were on the MFGM+AMF diet that survived LPS challenge, were also submitted for histology. Interestingly, inflammatory scores for these mice that survived had a delayed effect of inflammation.

Mice Study 2

Changes in dietary medium chain fatty acids in MCT+CO diet

Fatty acid composition analysis using gas chromatography on the diets detected Capric acid (C10:0) in the MCT+CO diet. Previously, it was suggested that the protective effect of medium chain fatty acids was due to Caprylic acid (C8:0) being in the diet (Kono et al., 2003). As a result, for the second mice study, an isolated form of the medium chain triglyceride oil, which only contained C8:0 was used to formulate the MCT+CO diet.

Animals and Preliminary Measures

For this study, BALB/c mice were used (n=90). Mice were randomly assigned to each diet (n=18) but were group housed in triplicates for 7 weeks. The preliminary analyses during this stage of the experiment included monitoring for weight changes in the animal, lean body mass and fat mass using MRI, and glucose sensitivity. Weight was measured weekly during the 7-week treatment. Body composition was measured twice, at baseline prior to treatment and at terminal, before LPS challenge.

Lipopolysaccharide and Sham Challenge

After 7 weeks, mice were injected (i.p.) with 10mg LPS/kg body weight to induce septic shock (n=60). Mice that previously underwent an OGTT (n=30) were given Sham injections. Of the total n=60 LPS challenged mice, n=30 were used for survival analysis. Survival was monitored for 48h. Six hours after the challenge, both Sham (n=30) and the remaining LPS (n=30) challenged mice were sacrificed by CO₂ asphyxiation and cardiac puncture and serum, small and large intestines, and liver was collected and flash frozen.

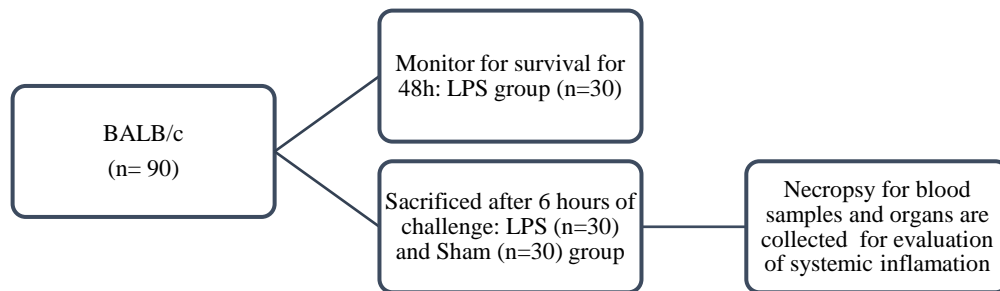


Figure 7. Experimental design for mice study 2. Mice (n=90) will be divided into two groups with survival group having a total of 30 sample size (n=6 per diet group) and a total of 60 samples for the sacrificed group. 6 hours after challenge, sample collection will occur for the sacrificed animal.

Evaluation of gastrointestinal and hepatic inflammation

Intestinal tissue was cleaned with 1X PBS, fixed with 70% ethanol and then Swiss-rolled for storage for 24h in 10% formalin. After 24h of storage, 10% formalin was replaced with 70% ethanol for final storage before histological analysis. Sections of ileum, jejunum, colon, and liver were evaluated for inflammatory changes using a histologic scoring system described for mouse small intestine (Rees, 1998, Burns et al., 2001). An overall inflammatory score was given to each tissue which indicated the extent of lesions and inflammation.

Analysis of proinflammatory cytokines in blood serum

Pro-inflammatory cytokines such as TNF- α , interleukin 1 (α & β), 12, and 18 are released during an immune response (Netea et al., 2003). Previously, both MCT (Kono et al., 2003) and MFGM (Snow et al., 2011) treatment resulted in a log reduction in the concentration of these pro-inflammatory cytokines; thus, it is important to measure

cytokine production for this study as well in order to see observe any similar findings. Sixteen different cytokines were measured in serum using a Quansys-multiplex plate reader: IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL5, IL6, IL-10, IL-12p70, IL-17, MCP-1, IFN- γ , TNF- α , MIP-1 α , GM-CSF, and RANTES. It has been shown that LPS has a negative effect on gut integrity and increases the concentration of proinflammatory cytokines (TNF- α , IL-1, IL-6, and IL-12) in blood plasma.

Statistical Analysis

A 2x2 factorial design was used to evaluate the response of dietary milk fat or medium chain triglyceride oil on mice when challenged with LPS or Sham. Shapiro-Wilk normality test was used to determine if data points were normally distributed. A log-transformation was used for data points that were not normally distributed. Tukey's multiple comparisons was used for posthoc analysis. The analysis was performed with GraphPad Software Prism 7 (GraphPad Software Inc., La Jolla, CA USA). Data is displayed as mean \pm SEM.

Results

Body Weight, Mass, and Food Intake

Mice were fed the treatment diets for 7 weeks. Results indicated no significant differences ($P > 0.05$) between diet groups in terms of body weight, feed efficiency, and body mass.

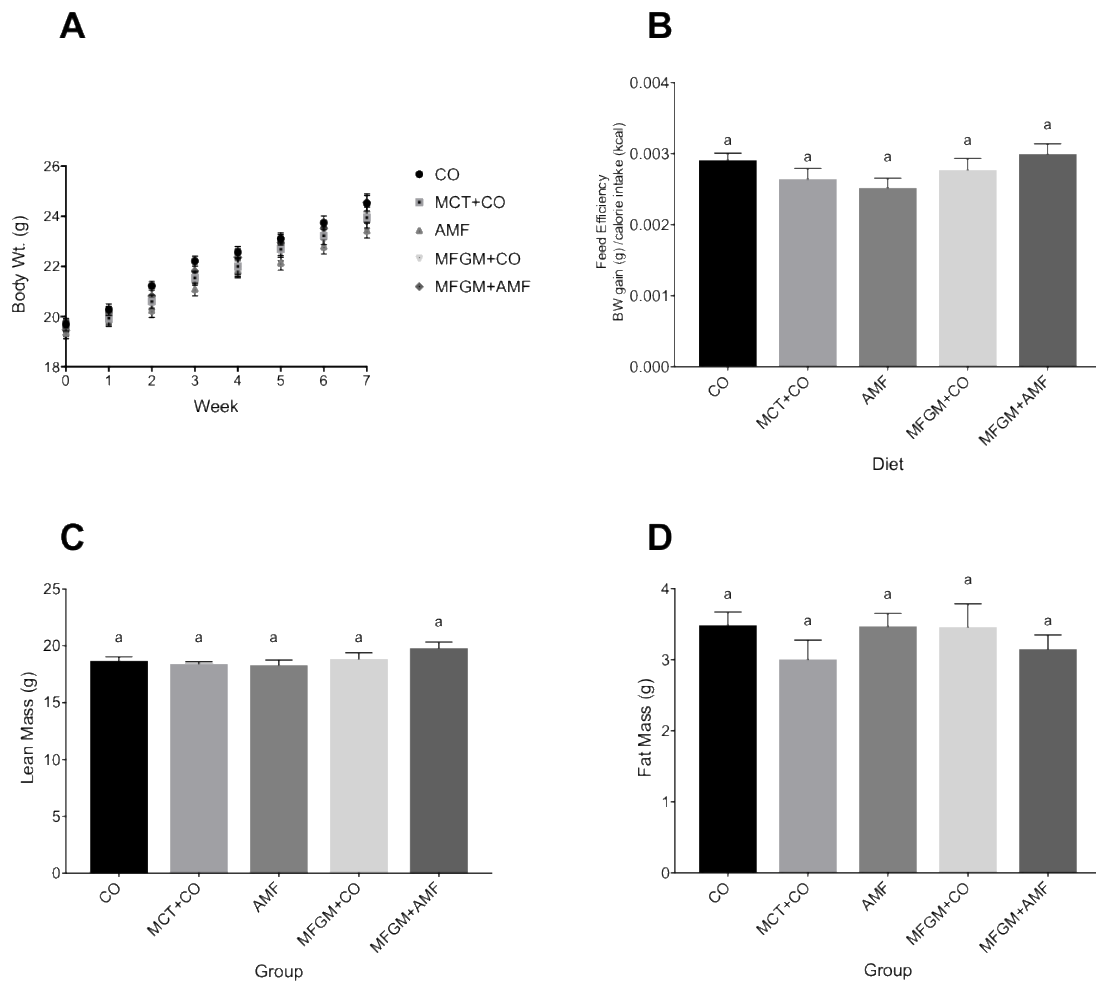


Figure 8. Weight and food intake measurements. An $n=6$ is assigned per group. A) Change in body weight monitored for 7 weeks. No significant differences were seen after 7 weeks of diet treatment. B) Feed efficiency was calculated as a ratio of the cumulative food intake over total kilocalories. C) and D) No significant differences were seen after 7 weeks.

Oral Glucose Tolerance Test

Contrary to what was seen previously in the preliminary study, blood glucose concentration during fasting was not significantly different for mice on the MFGM+AMF diet compared to other diets. Similarly, there were no significant differences seen in any diet groups in terms of glucose tolerance.

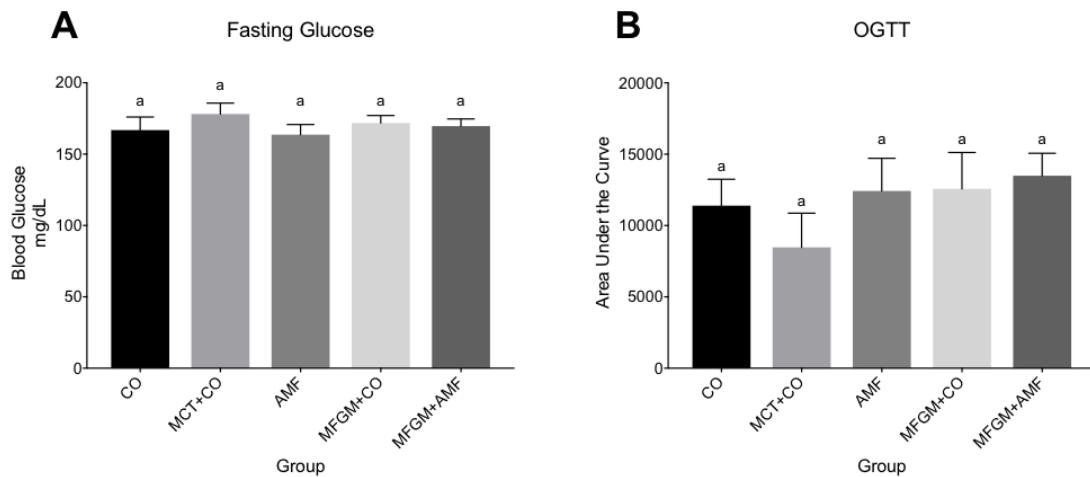


Figure 9. Fasting glucose measurements and OGTT. Diet effect on fasting glucose and glucose tolerance. A) Mean in concentration of milligram per deciliter. B) Area under the curve was calculated by the sum each area calculated by each time point. (Baseline, 15min, 30min, 60min, 90min, and 120min). Triplicate measurements of glucose concentration were made for each time point.

Survival

After LPS challenge, the survival of the groups was monitored for 48h. Within 18h, mice from the MCT+CO and AMF diets began to succumb to death from the LPS challenge. At 24h, none of the mice fed with MCT+CO, AMF, MFGM+CO, and MFGM+AMF survived. After 30h, there was 0% survival across all diet groups.

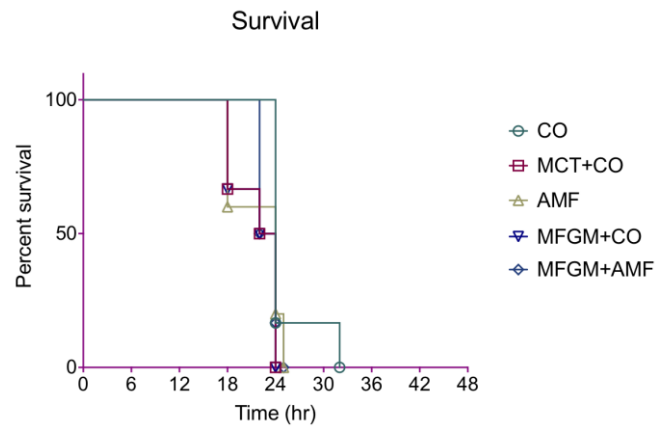


Figure 10. Survival plot. An n=6 is assigned per group. Animals were monitored everything 2-4 hours for 48h.

Intestinal inflammation and epithelial cell permeability

Although data are not presented here, histology conducted on the small intestine, colon, and liver indicated that there were no significant differences seen in the overall inflammation in these tissues. Surprisingly, there were no differences between Sham and LPS treated mice. No significant differences in FITC-Dextran concentration were seen between diet groups challenged with LPS. A lower concentration of FITC-Dextran was seen in Sham mice compared to LPS mice.

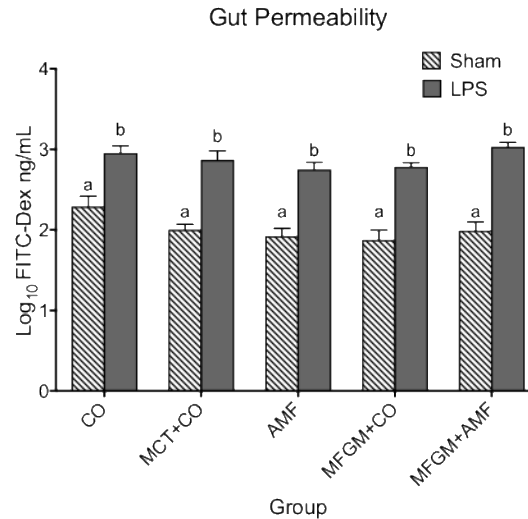
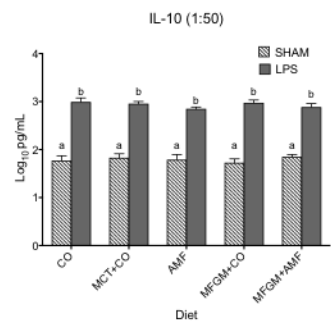
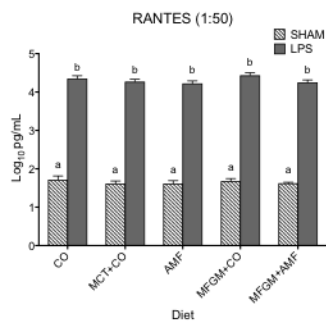
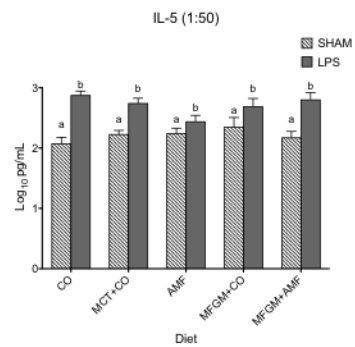
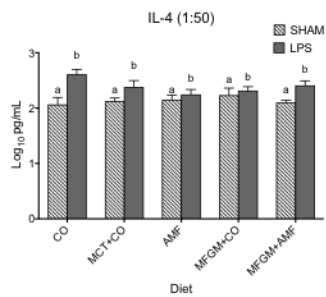
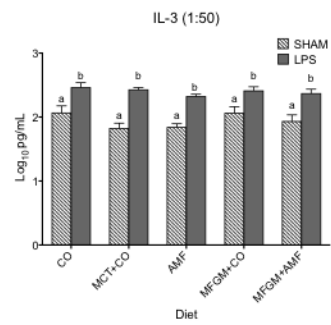
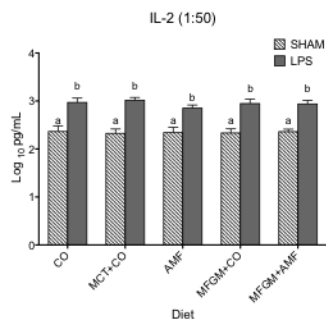
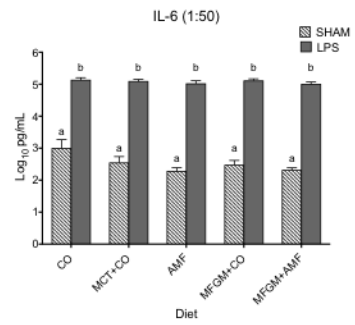
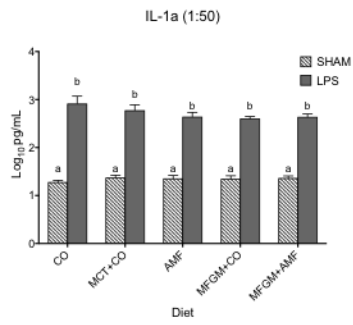


Figure 11. Gut permeability measurements. Effect of diets on fluorescence levels after Sham or LPS challenge. An n=6 is assigned per group. Data are log₁₀ transformed mean nanogram per milliliter. No significant differences in fluorescence levels were seen between any diets for LPS challenged mice.

Serum Cytokines Analysis

Sixteen different cytokines were analyzed which included: IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL5, IL6, IL-10, IL-12p70, IL-17, MCP-1, IFN- γ , TNF- α , MIP-1 α , GM-CSF, and RANTES. Overall, LPS-challenged mice had a higher concentration of plasma cytokines amongst all diet groups compared to Sham mice. Diets that were predicted to reduce the production of pro-inflammatory cytokines (IL-1, IL-6, and TNF- α) did not have any significant effects on the mice.



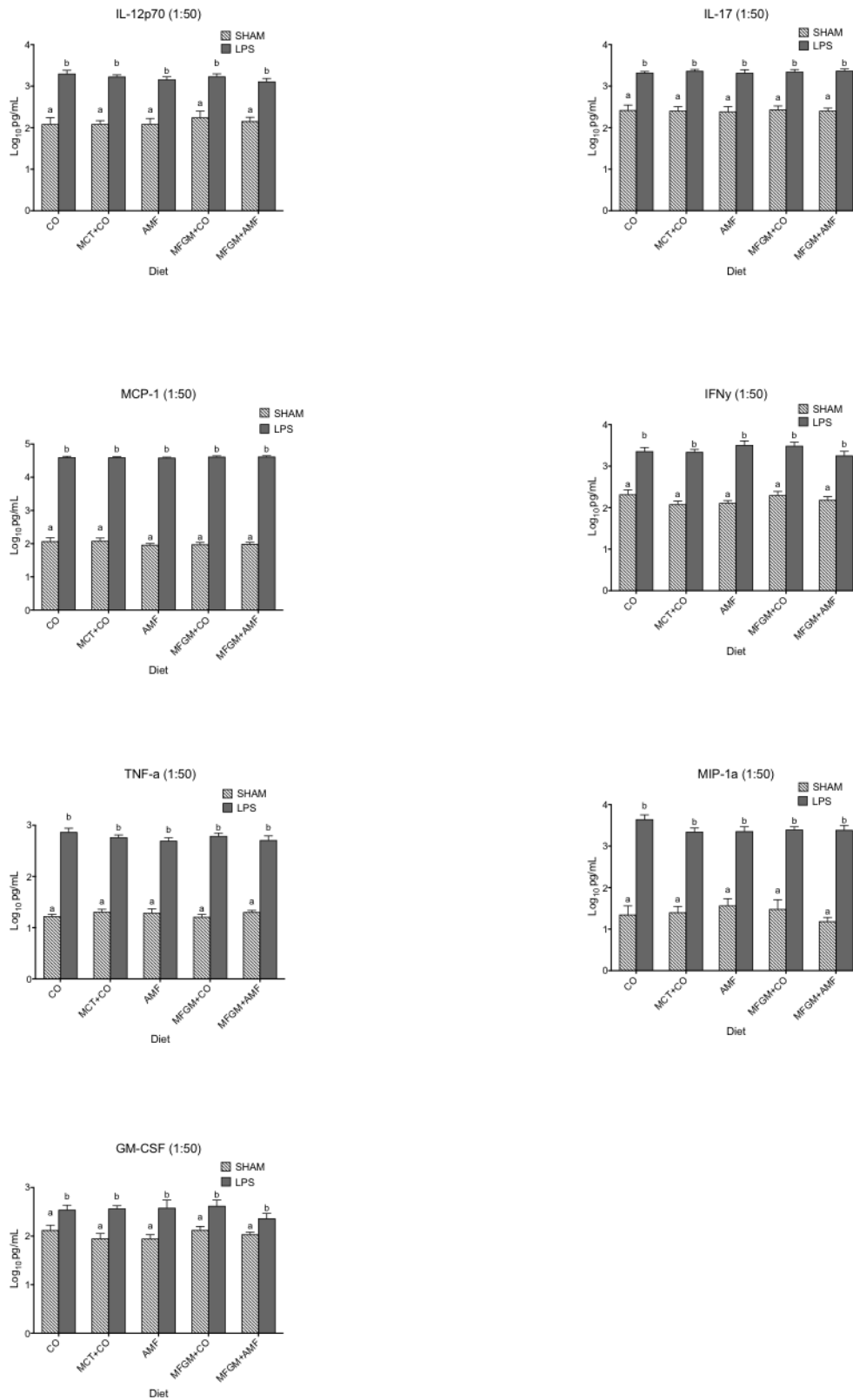


Figure 12. Measurements of pro-inflammatory cytokines. Effect of dietary treatments on serum cytokine concentration for Sham and LPS challenged mice. An n=6 is

assigned per group. No significant differences were seen for all 17 serum cytokines in LPS challenged mice.

Discussion

Intraperitoneal injection of LPS in a murine model is a method commonly used for inducing gastrointestinal leakiness and systemic inflammation through the production of proinflammatory cytokines. It has been previously shown that certain dietary fats, milk fat and medium chain triglyceride oil, can protect against acute inflammation caused by LPS (Kono et al., 2003, Snow et al., 2011). Although these dietary fats came from two completely different sources, they both contain either one specific medium chain fatty acid (Caprylic C8:0) or a variety of short and medium chain fatty acids (C4-C14). For this study it was hypothesized that short and medium chain fatty acids in a purified diet will have a systemic protective effect against LPS. However, our results indicate that these dietary fatty acids had no protective effect against LPS in terms of survival, gut leakiness, or production of pro-inflammatory cytokines. The experiment was designed to clarify an unanswered question that arose in a previous study that suggested milk fat has a protective effect against gastrointestinal leakiness and resilience to acute inflammation/septic shock. As mentioned previously, it was unknown whether the milk fat globule membrane was the source of that protection or if the anhydrous milk fat. Thus, a separate anhydrous milk fat group was included in this study. At the same time, a diet that contained medium chain triglyceride oil was also included in this design to further explore the possibility that medium chain fatty acids could potentially be the reason to why these animals were resilient to LPS.

Initially it was planned that a single study that involved the incorporation of dietary fats coming from corn oil, MCT oil, and milk fat would be conducted. However, after not seeing any treatment effect from mouse study 1, the study was reevaluated at it

was discovered that there was a formulation error in the milk fat diet. The milk fat diet was formulated to have a combination of MFGM and AMF, but CO was used instead. Therefore, mice study 1 did not evaluate the effects of MFGM+AMF but instead it looked at the effects of MFGM+CO during an inflammatory response. A separate single trial run with one experimental group was then conducted, with the same parameter as mice study 1, that had the correct milk fat formulation following mice study 1 (data not shown). Mice that were in this trial survived an LPS challenge. Along with the improvement in survival, the mice also had a lower concentration of serum FITC-dextran and colon inflammatory score when compared to the experimental groups in mice study 1. The trial run gave merit for repeating the entire study with the correct diets.

In mouse study 2, a few alterations to the original design were made. A fatty acid analysis of the previous MCT+CO diet indicated that both Caprylic (C8:0) and Capric (C10:0) were present in the diet. Previously, another study with an MCT diet containing only caprylic acid showed protective effects against LPS-induced shock. This protection involved the down regulation of the CD14 LPS binding receptor located on the surface of Kupffer cell macrophages in the liver and alterations to intestinal permeability. Thus, an MCT+CO diet without Capric acid was used in the second study. After the finding from the colon histopathology from the trial experiment, we decided to evaluate the jejunum and ileum to see if similar effects would be seen in proximal region of the gastrointestinal tract. LPS also seemed to cause pathological changes in the liver in which necrotic and hemorrhagic changes can occur (Kono et al., 2003), thus segments of the liver were also taken for histopathological evaluation. In addition, a sham challenged group was included in the design, as a negative control, to observe any measurable differences for each test within diet groups.

In the repeated study, LPS challenged mice did not survive regardless of diet treatment (Figure 10). Mice were given purified diets for approximately the same timeframe as the preliminary study. Weight gain, feed efficiency, and body mass measurements indicated no significant differences between diet groups (Figure 8). Fasting glucose concentration along with calculated area for OGTT were not statistically different between diet group as well (Figure 9). LPS did influence gut barrier dysfunction in which hyper-permeability was seen in LPS challenged mice compared to the Sham (Figure 11). As expected, LPS challenged mice also had an increase of inflammatory cytokines when compared Sham mice (Figure 12). However, diets had no effect in preventing these negative effects from LPS.

The discrepancy in results from past results and published literature could have stemmed from multiple factors. For instance, in the referenced study in which MCT was shown to be effective in LPS challenged rats, the animals received capric acid through oral gavage at dosage of 5mg/kg once a day. The mechanism of delivery by oral gavage ensures that the test animals receive the required amount of dietary fats. For this study, diets contained approximately 5% fat by weight and by allowing the diets to be consumed ad libitum, mice were consuming less fat compared to the Kono study. Thus, the lower intake in MCT could explain the lethality of LPS at a dosage of 10mg/kg. In the preliminary study, mice that were on the MFGM+AMF diet had a significantly higher feed efficiency relative to the other diet groups for both preliminary and repeated study, which indicates that these mice consumed more of the diet compared to the other groups. Mice in the secondary study were also group housed due to the large sample size, which can limit the amount of diet being consumed by each individual mouse, and thus could have influence the experimental results.

Conclusion

Ultimately, the results from this research indicate that short and medium chain fatty acids in a purified diet do not protect against acute inflammation induced by a high dose of LPS. The fatty acids did not attenuate the hyper-permeability of gut and release of the pro-inflammatory cytokines in LPS-challenged mice. Additionally, LPS did not have any histopathological effect on the small intestine, large intestine, and liver.

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