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The Effect of Dietary Interventions on Intestinal-associated Metabolites and Immune Parameters

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THE EFFECT OF DIETARY INTERVENTIONS ON INTESTINAL-ASSOCIATED METABOLITES AND IMMUNE PARAMETERS

by

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Abstract

The obesity epidemic has driven the search for dietary interventions for weight reduction. In the current study we determined the role of dietary composition and also timing of feeding on weight loss, with a specific focus on changes in intestinal parameters. Mice were divided into different groups with different feeding protocols. Dietary interventions altered weight gain and body composition and intestinal parameters including small intestinal length and cecum weight. There was no difference in splenic or colonic B and T cell populations, but regulatory T cells numbers were altered with the vegan-based diet.

List of abbreviations

ADF: Alternate Day Fasting

BSCFA: Branched Chain Short Chain Fatty Acid

CH: Chow

CR: Caloric Restriction

DF: Daniel Fast

DIO: Diet Induced Obesity

GI: Gastrointestinal

HF: High Fat

IBD: Inflammatory Bowel Disease

IBS: Inflammatory Bowel Syndrome

IC: Ion Chromatography

SCFA: Short Chain Fatty Acid

SEM: Standard Error of the Mean

SW-CH: Switch to Chow

TRF: Time Restricted Feeding

T-reg: T-regulatory cells

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INTRODUCTION

The human intestinal microbiota is complex as it comprises hundreds of bacterial species. To be precise, adults have approximately 250 ml colonic contents, with 10^{11} bacterial cells per ml. The collective genome of these organisms is at least 100 times that of the eukaryotic genome. Modern molecular biology methodologies helped us to understand the colonic microbial composition and hence having a better understanding of colon's dominant phyla (Gijs Den Besten et al. 2013; Byrne et al. 2015; Kasubuchi et al. 2015; Kimura et al. 2013). These microbes help the host by providing protection against pathogens, enhancing the metabolic capabilities, modulating gastrointestinal (GI) development and educating the immune system. Although there are variations in number, type and function of microbes along the length of GI tract, most are concentrated within large bowel where their primary role is fermentation of undigested food components like carbohydrate/fiber. *Bifidobacterium, Lactobacillus, Bacteroides, Clostridium, Escherichia, Streptococcus* and *Ruminococcus* are the most common gut bacteria in adults. Of these, *Bacteroidetes* or *Firmicutes* phyla constitute the 60% of the bacteria found (Manichanh et al. 2006). Comparing the microbiome of subjects who consume diets high in fiber or high in animal fat and protein, it has been found that long term dietary habits influence the intestinal microbiota composition. Currently we do not fully comprehend the impact of the environment and dietary habits of modern humans on the microbial ecology of the human gut (De Filippo et al. 2010).

In the US, many chronic diseases are related to diet, including cardiovascular disease, hypertension, and diabetes mellitus. This diet is typically high in fat and simple carbohydrates (Cordain et al. 2005). Recent lifestyle innovation has definitely raised

concerns particularly regarding the Western diet's role in alteration of genetic composition as well as metabolic activity of our resident gut microbiota (Conlon and Bird 2015)(David et al. 2014b). Growing epidemics of chronic illness in the developed world, including obesity and inflammatory bowel disease, are suspected to be related to changes in gut-associated microbial communities induced by such diets. It is still unclear how diet alters specific bacterial communities. It had been found that the gut microbiome of mice was altered as early as a single day after dietary macronutrients were shifted (Faith et al. 2012)(Turnbaugh et al. 2009). But in contrast, studies of dietary interventions in human cohorts measuring community changes after weeks or months of intervention failed to find significant diet-specific effects, or else have demonstrated responses among a limited number of bacterial taxa (David et al. 2014a).

Interaction of Microbiome with Diet

Commensal bacteria play a key part in the digestion of food. For example, specific species including Bacteroides spp., Eubacterium spp., Bifidobacterium spp., Fusobacterium spp., Peptostreptococcus spp. help to extract nutrients and generate metabolites that are beneficial for the host. These nutrients and metabolites are implicated in homeostasis and the development and functioning of the immune system. The gut microbiome therefore plays an instrumental role in host immunity through the nutrients and metabolite dependent mechanisms (Manuscript 2014) (Artis 2008).

Obesity has become an enormous public health concern due to its role in the development of many chronic diseases. In the US, the prevalence of obesity has increased by more than 75 % over the course of last 25 years. Two thirds of the current US population is

overweight and 1 in 3 adults are found to be clinically obese in the present scenario (Dibaise, Frank, and Mathur 2012). It is now well accepted that obesity and its associated low grade chronic inflammation can contribute to the development of insulin resistance and type 2 diabetes and other detrimental health consequences(Wellen and Hotamisligil 2005) (Jung and Choi 2014). Previous studies focused on adipose tissue as the source of obesity-associated inflammation, but recent evidence support the idea that inflammation associated with diet-induced obesity (DIO) is linked to intestinal bacteria (Ding et al. 2010). The gut microbiome has therefore become important target for dietary interventions and pharmacologic agents. Currently, no direct association of specific microbes with obesity has been observed. It is complicated by the fact that both dietary intake and the host's genetic variation has an influence on gut microbiota's structure as well as phenotype (Clemente, Clemente, and Garc 2017).

Factors affecting gut microbiota composition

Factors such as diet, medication, probiotics, host's genetics and environmental factors influence the composition of gut microbiota. Of these factors, diet is main factor driving the colonic bacteria (Laparra and Sanz 2010)(Xiao et al. 2017). As high as 57 % of the variation in microbiota attributed dietary changes as compared to genetic variation accounting to only 12% (Baothman et al. 2016). People around the world have long been consuming probiotics in their diet as different fermented foods and increasing the number of health-promoting bacterialike *Lactobacilli* and *Bifidobacteria* species positively changes the microbiota balance and increases beneficial metabolites and short chain fatty acids (Arora, Singh, and Sharma 2013).

Production of SCFAs by Gut Microbiota

A major function of the intestinal gut microbiota is fermentation of carbohydrates and proteins that escape digestion in the small intestine. Amongst the wide range of metabolites produced by the microbiota are short chain fatty acids (SCFA)(Scheppach 1994)(Morrison and Preston 2016). These molecules are characterized by containing fewer than six carbons, existing in straight, and branched-chain conformation and are volatile fatty acids. The most abundant (90–95%) of SCFA present in colon are acetic acid (C2), propionic acid (C3), and butyric acid (C4). Although carbohydrates (CHO) are the main substrate for SCFA generation, amino acids such as valine, leucine, and isoleucine can also be converted into branched-chain SCFA (BSCFA) that includes isobutyrate, isovalerate, and 2-methyl butyrate and form a minor (5%) source of SCFA (Ríos-covián et al. 2016) (C. Kim et al. 2014)(Sun et al. 2017).

SCFA have several functions within the host's body including being a source of energy. It has been found that the SCFA provide around 10 percent of the total daily calorific requirement for humans (Gijs Den Besten et al. 2013). It has been also observed that the SCFA can reduce cholesterol levels. (Gijs Den Besten et al. 2013)(Fushimi et al. 2017). Additional functions include influencing colonic mobility, and blood flow, and altering gastrointestinal pH (Tazoe, H., Otomo, Y., Kaji, I., Tanaka, R., Karaki, S. I., & Kuwahara 2008).

Impact of Diet on Gut Microbiota Composition and SCFA Production

Changes in the composition of gut microbiota are driven by changes in diet, the host genetic background and the colonic milieu. The colonic milieu is also in turn affected by microbial activity. This leads to the variation of the microbial population between individuals (Gijs Den Besten et al. 2013)

The composition and activities of gut microbiota are strongly affected by diet and these impact the health of the host (Rajoka et al. 2017) (Graf et al. 2015) (G. D. Wu et al. 2011).The amount and types of fiber consumed have dramatic effects on the composition of the intestinal microbiota and consequently on the type and amount of SCFAs produced (G den Besten et al. 2013). Short-term diet changes have also been shown to alter the human gut microbiome has also been demonstrated (Ríos-covián et al. 2016). A high fiber dietary pattern with subsequent and consistent production of SCFAs with a healthy gut microbiota results in a reduced risk of advanced colorectal adenoma (Chen et al. 2013). It has also been found that prebiotic substrates, that selectively improve the growth of beneficial microbiota like *Bifidobacterium* and *Lactobacillus*, induce changes in SCFA production by the microbiota of healthy individuals by protecting against a range of chronic diseases like CRC, IBD, type 2 diabetes (Majid, Emery, and Whelan 2011)(Conlon and Bird 2015). Besides diets and probiotic substrate, the dairy products fermented with beneficial bacteria also modify the intestinal microbiota toward more butyrate producers in comparison to chemically-acidified milk (Ríos-covián et al. 2016) (Veiga et al. 2014).

In a study of overweight and obese population's dietary intervention, high levels of fecal SCFA related with obesity status(Schwiertz et al. 2010). Although it was reported

initially that high-fat feeding and obesity were associated with lower ratios of *Bacteroidetes* relative to *Firmicutes,* subsequent studies challenged this association (Murphy, Velazquez, and Herbert 2015). Obesity, as indicated by recent studies, is in fact correlated with decreased microbial diversity. As illustrated in gut microbiota profiles in TLR5 knockout mice, TLR2 knockout mice, NOD mice, ob/ob mice, and db/db mice compared with wild-type mice, the composition of the gut microbiota was modulated by genetic background (Harris et al. 2012). Observed changes in the gut microbiota in these mouse models may also be induced by altering the food intake. In some cases, phenotype similarity was found when microbiota was transplanted from knockout mice into wild-type recipient (Montgomery et al. 2013). The strongest drivers for gut microbial composition in humans and mice is thought to be long term habitual dietary intake including fat (Murphy, Velazquez, and Herbert 2015). Because fat also determines the obesity development, it is not understood if the changes in microbiota are due to obese state or from HF feeding based on commonly used models. Most commonly used strains to study genetic and diet-induced obesity are the obesity prone C57BL/6JBomTac (BL6) mouse strain and obesity-resistant mouse strain 129S6/SvEvTac (Sv129) (Xiao et al. 2017)

The inter-relationship between diet, gut microbial ecology and energy balance was investigated using mouse model of obesity, produced by consumption of prototypic Western diet. Blooms in single uncultured clade within Mollicutes class of Firmicutes was produced due to diet-induced obesity (DIO). Subsequent dietary manipulations limiting weight gain diminished the bloom. It was observed that greater fat deposition

occurred when germ-free recipients were transplanted with microbiota from DIO mice rather than from lean donors (Fulton et al. 2008).

Short Chain Fatty Acids and Treg Cells

There is a close relation between nutrition and immunity. Of all the cells in the body, the immune system has the most energy consuming cells (Wolowczuk et al. 2008)(Çehreli 2018). So, any imbalance in nutrition strongly affects immune system(Çehreli 2018). Intestinal immune system has to evolve along with our gut microbiota in order to maintain intestinal health. SCFAs a bacterial metabolites has been shown to have induced Treg cells (Baothman et al. 2016). Disruption of this homeostasis results in intestinal inflammation and inflammatory diseases such as, IBD, IBS, (Brown et al. 2012). Through the expansion and differentiation of colonic Foxp3+ regulatory T cells (Tregs), the mucosal immune system is regulated by gut commensal microbes (Gijs Den Besten et al. 2013; Byrne et al. 2015; Kasubuchi et al. 2015; Kimura et al. 2013). Amongst the $CD4^+$ T cell population (T helper (Th) cells) CD4 effector T cells control adaptive immunity against pathogens and cancer, while Tregs suppress potentially deleterious activities of effector cells (Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y, Cheng G, Yamasaki S, Saito T, Ohba Y 2011). Using the method of candidate microbe approach, bacterial species and strain specific molecules affecting intestinal immune response have been identified (H. J. Wu and Wu 2012). In a study using mice, it was demonstrated that SCFA control the size and function of colonic Treg pool. This population of cells can protect against colitishrough induction of Tregs there was adaptive immune-microbiota coadaptation and promotion of colonic homeostasis and

health (Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y, Cheng G, Yamasaki S, Saito T, Ohba Y 2011). Colonic Tregs is also induced by *Clostridia* and has been shown to play a vital role in suppressing inflammation as well as allergic responses, but the molecular mechanism by which colonic Tregs are induced remains a mystery. In mice it has been found that butyrate and propionate, microbial fermentation products, are able to induce the differentiation of colonic Tregs. (Smith et al. 2013)

In comparison to other organs, $Foxp3^+$ Tregs are more abundant in the gut lamina propria (LP) of the colon. Intestinal bacteria have been postulated to affect the quantity and function of mucosal Tregs(Geem et al. 2016). Daily treatment with probiotic strains of *bifidobacteria* and *lactobacilli* been found to modify the inflammatory status of mice associated with Tregs (Candelaa, M; Pernab, F; Carnevalic 2008). In addition, an intervention using mice inoculated the human commensal Bacteroides fragilis induced Treg differentiation and increased colonic interleukin-10 (IL-10) production(Nakanishi, Sato, and Ohteki 2015). Although it is clear that the alteration in the indigenous microbial flora correlates with inflammatory diseases, we have to further establish the role in the number and functioning of mucosal Tregs (Murakami et al., n.d.).

$Foxp3^+$ Tregs have a key role in maintaining immune homeostasis as they attenuate immune responses. Many research groups have focused on Tregs cells over the course of last ten years by characterizing different subsets. Helios which is the member of Ikaros family was recently reported to be a marker that differentiate naturally occurring thyimic-derived T-regs from peripherally induced naive $CD4^+$ T cells (Akimova et al. 2011).

Hypothesis

We hypothesize that diet composition and feeding schedule will alter microbiome metabolites and gut-associated Tregs.

Aim 1.

To determine the amount and composition of SCFA generated in the cecum of mice undergoing different diets and feeding protocols.

Aim 2.

To determine if different diets and feeding protocols alter the T cell repertoire, specifically focusing on regulatory T cells associated with the large intestine.

MATERIALS AND METHODS

4-week-old C57BL/6 male mice were purchased from Envigo. Upon their arrival, they were co-housed 2 animals per cage in a USDA approved animal facility on the University of Memphis campus and entrained to an alternating 12-hour dark and 12-hour light schedule (7 pm – 7 am) for two weeks while having *ad libitum* access to standard rodent chow and water. Mice were housed in an area used for circadian rhythm studies and light was therefore well-regulated. After 2 weeks of entrainment, 8 mice remained on the chow diet (remained on this diet for the extend of the study. Chow control), while 48 mice were switched to a high fat diet consisting of 45% fat (mostly lard), 35% carbohydrate and 20% protein and allowed to eat *ad libitum*. This diet induces obesity in the C57BL/6 mouse strain. After 6 weeks of high fat feeding the mice started the intervention and were divided into 6 groups based on diet composition (see Table 1) and feeding times as follows:

HF – continued *ad libitum* access to the 45% high fat diet.

Sw-Chow – *ad libitum* access to the original chow diet.

DF - *ad libitum* access to a purified, fiber-rich, vegan-based diet.

CR - caloric restricted groups were fed the high fat diet at 80% of *ad libitum* of intake as determined during week 6 of high fat feeding prior to intervention.

ADF - alternate day fasting group was fed the high fat diet but followed feeding protocol that allowed *ad libitum* access to food every other day, while fasting for 24h in between feedings.

TRF - time restricted feeding group had access to the high fat diet for 6 hours every day, during the first half of the active (dark) phase (8 am- 2pm)

All groups had *ad libitum* access to water and remained on their respective diets for an additional 8 weeks. Food consumption was measured daily, and mouse weight determined every other day.

Body composition was determined by EchoMRI immediately prior to the dietary intervention and before sacrifice.

Blood was collected via facial vein immediately prior to sacrifice. Mice were sacrificed using isoflurane and cervical dislocation and tissues harvested immediately. Cecal samples and small intestinal contents were collected and immediately frozen in liquid nitrogen

Table 1. Composition of experimental diets.

Macronutrient	DF	HF	CHOW
	kcal%	kcal%	kcal%
Protein	15	20	24
Carbohydrate	59	35	58
Fat	25	45	18
kcal/gm	3.9	4.73	3.1

Ingredient of DF and HF

	DF		HF	
Ingredient	**gm**	**kcal**	**gm**	**kcal**
Casein	0	0	200	800
Soy Protein	170	680	0	0
DL-Methionine	3	12	0	0
Corn Starch	0	0	72.8	291
Corn Starch-Hi Maize 260	533.5	2134	0	0
(70% Amylose and 30% Amylopectin)				
Maltodextrin	150	600	100	400
Sucrose	0	0	172.8	691
L-Cystine	0	0	3	12
Cellulose, BW200	100	0	50	0
Inulin	50	50	0	0
Soybean Oil			25	225
Lard	0	0	177.5	1598
Flaxseed Oil	71	639	0	0
Safflower Oil, High Oleic	59	531	0	0
Ethoxyquin	0.04	0	0	0
DiCalcium Phosphate			13	0
Mineral Mix S10001	35	0	10	40
Calcium Carbonate	4	0	5.5	0
Mineral Mix S10026			10	0
Vitamin Mix V10001	10	40	0	0
Choline Bitartrate	2	0	2	0
Ascorbic Acid Phosphate, 33% active	0.41	0	0	0
Potassium Citrate, 1 H2O	0	0	16.5	0
Cholesterol	0	0	0	0
FD&C Red Dye #40	0.05	0	0.05	0

Table2: Chow Diet

			Amino Acids		
Calcium	%	1	Aspartic Acid	%	1.4
Phosphorous	%	0.7	Glutamic Acid	%	3.4
Non-Phytate Phosphorous	%	0.4	Alanine	%	1.1
Sodium	%	0.2	Glycine	%	0.8
Potassium	%	0.6	Threonine	%	0.7
Chloride	%	0.4	Proline	%	1.6
Magnesium	%	0.3	Serine	%	1.1
Zinc	mg/kg	70	Leucine	%	1.8
Mangenese	mg/kg	100	Isoleucine	%	0.8
Copper	mg/kg	15	Valine	%	0.9
Iodine	mg/kg	6	Phenylalanine	%	1
Iron	mg/kg	200	Tyrosine	%	0.6
Selenium	mg/kg	0.23	Methionine	%	0.4
Vitamins			Cystine	%	0.3
Vitamin A [e,f]	IU/g	15	Lysine	%	0.9
Vitamin D_3 [e,g]	IU/g	1.5	Histidine	%	0.4
Vitamin E	IU/kg	110	Arginine	%	1
Vitamin K_3 (menadione)	mg/kg	50	Tryptophan	%	0.2
Vitamin B_1 (thiamin)	mg/kg	17	**Fatty Acids**		
Vitamin B_2 (riboflavin)	mg/kg	15	C16:0 Palmitic	%	0.7
Niacin (nicotinic acid)	mg/kg	70	C18:0 Stearic	%	0.2
Vitamin B_6 (pyridoxine)	mg/kg	18	C18:1ω9 Oleic	%	1.2
Pantothenic Acid	mg/kg	33	C18:2ω6 Linoleic	%	3.1
Vitamin B_{12} (cyanocobalamin)	mg/kg	0.08	C18:3ω3 Linolenic	%	0.3
Biotin	mg/kg	0.4	Total Saturated	%	0.9
Folate	mg/kg	4	Total Monounsaturated	%	1.3
Choline	mg/kg	1200	Total Polyunsaturated	%	3.4

Immune cell isolation, flow cytometry

The spleen and colon were immediately processed after harvest for immune cell isolation and analysis. Splenocytes were isolated using a sterile 40 μm cell strainer and syringe plunger and rinsing with RPMI 1640 (Roswell Park Memorial Institute medium) solution containing 2% FBS (Fetal bovine serum). Cells were pelleted with centrifugation and media removed. Cell pellet were suspended in ACK (Ammonium Chloride Potassium) lysis buffer (for red blood cell lysis) and incubated at room temperature for 2 minutes and the suspension were diluted with RPMI 1640 containing 2% FBS. Cells were washed with PBS containing 2% FBS and finally resuspended in 2-3ml PBS with 2% FBS. Live cells were quantified using Trypan Blue and a hemocytometer.

The colon were isolated from cecum to rectum and contents gently removed. Individual colons were cut open longitudinally washed in RPMI/10%FBS/1 mM HEPES (N-2-hydroxyethylpiperazine-N -2-ethanesulfonic acid; RPMI-10/HEPES) and cut into 5 mm sections followed by 6 washes in RPMI-10/HEPES. To obtain single-cell suspensions, colon sections were incubated in two serials 1-hour digestions in collagenase-E (95 IU/mL; Sigma-Aldrich, St Louis, MO). After the final incubation, the tissue was sheared using a 30 ml syringe with 16 gauge needle. In order to homogenize, the tissue fragments were aspirated continuously. The homogenate were passed over a 40 μm cell strainer and the cells were pelleted by centrifugation at 1500 rpm at 4°C for 5 minutes. Cells were resuspended in 44% Percoll that is overplayed onto 66% Percoll and spun at 2300 rpm for 20 minutes at room temperature to enrich the mononuclear cells. A band of white blood cell collected and transferred to a 15-ml conical tube and the cells were washed

with PBS with 2% FBS. The cells were resuspended in PBS with 2% FBS and counted as mentioned previously.

Cells isolated from spleen and colon were incubated with Trustain FcX (Biolegend, San Diego)

to block Fc receptors. Cells were then stained with the following fluorescently conjugated antibodies for identification: PeCY7 anti-mouse CD 3 (clone- 145-2C11, Biolegend), APC anti-mouse CD4 (clone GK 1.5), PacBlue anti-mouse CD8α (Clone-53-6.7, Biolegend), PE anti-mouse TCR β-chain (clone H57-597, Biolegend), PacBlue anti-mouse/human Helios (clone-22F6, Biolegend), PE Anti-mouse/Rat FOXP3 (clone FJK-16s, eBioscience). Dead cells were excluded using LIVE/DEAD™ Fixable Aqua (Life Technologies, Eugene, OR). After staining all samples were fixed by incubating with Fixation/Permeabilization solution (eBioscience, San Diego, CA) overnight at 4˚C according to the manufacturer's instructions.

Foxp3 intracellular staining was perform after fixation/permeabilization.

All samples were analyzed using an Invitrogen "Attune" NxT Flow Cytometer (ThermoFisher Scientific, Waltham, MA). The data obtained were further analyzed using FlowJo software (FlowJo LLC, Ashland, OR).

SCFA Analysis, Ion Chromatography

Ion chromatography (Dionex) is composed of GP50 gradient pump with AG15 guard column, AS15 analytical column and ED50 electrochemical detector**.**

Cecum contents were collected and immediately frozen in liquid nitrogen and store at -80°C until analysis. For analysis the samples were thawed, and weight recorded. 10 ml of dilute H2SO4 (0.18M; 1:100 dilution of H2SO4 stock (36 Normality)was added to cecum sample weighing more than 60mg and 5ml dilute H2SO4 for samples weighing less than 60 mg. The sample were sonicated for one minute, while being kept on ice. The suspension was further diluted to 10 % in a labeled microfuge tube by pipetting 100µL sample and 900µL of the dilute H2SO4 and then centrifuged at 1400 rpm speed for 10 minutes. A disposable transfer pipette was used to place the supernatant in a 3 mL syringe with attached 0.2µL filter. Using the syringe, the contents were transferred into a labeled glass AS50 auto-sampler vials for analysis by ion chromatography was programmed by injecting 200 µl of each sample. For eluent, 100mM eluent (KOH) was used. Acetate, lactate, proprionate, chloride, formate and isobutyrate, butyrate were used as standards.

Statistical Methods

All data are presented as means ± SEM and statistical significance was established at $P < 0.05$. One-way analysis of variance (ANOVA) and post-test Tukey were used to compare group means. For weight data a two-way ANOVA with Tukey's multiple comparison test was performed. All statistical analysis was performed with Graph Pad Prism Software (Version 7, San Diego, CA)

RESULTS

Food consumption, body weight and body mass and composition

Food consumption and body mass of all animals were monitored during the intervention. All high fat groups consumed similar amounts (in grams) of food, while a higher volume of the less energy dense diets were consumed by the Chow and DF groups (CH 3.7g, HF 2.7gm SW-CH 3.5g, DF 3.5g, CR 2.3g, ADF 2.0g, TRF 2.2g Figure 1A demonstrate average daily calorie consumption over the 8 weeks period of the intervention. When calculating the average daily calorie consumption for the study, the HF and DF groups consumed significantly more calories per day than any of the other groups (Fig. 1B, $P < 0.001$)

After the 3rd week of the intervention there was a significance increase in the weight of the HF group (Figure 1C, $P < 0.0001$) with the high fat group having the highest body mass at the end of intervention (Chow 27.71g ±3.13, HF 39.56g ±4.9, Sw-Chow 28.7g ±2.4, DF 28.9g ±1.4, CR 33.85g ±2.8, ADF 28.68g ±1.2, TRF 28.54g ±1.7). Weight change was determined over the 8 week intervention period and the HF group gained significantly more weight during the 8 weeks compared to all other groups (Figure 1D, $P < 0.0001$). Interestingly, the Sw-Chow group lost significantly more weight than the CR group, despite consuming an equal amount of calories ($P= 0.0003$).

To determine if weight loss was due to loss in fat or lean mass, body composition was determined at the beginning and end of intervention. The greatest difference in body fat was induced by the low-fat diets (Figure C, HF +6.8g ± 1.3, SW-CH -6.23g ± 2.4, DF -4.1g ± 1.3; HF vs DF or Sw-Chow, $P<0.0001$). The restricted high fat diet groups also had significantly less body fat, albeit not to the extent of the low-fat diets (Figure 1E, HF

+6.8g ± 1.3, CR 0.01g ±1.26, ADF -2.4g ±1.5, TRF -1.8g ± 1.7, HF vs CR, ADF or TRF, *P*<0.0001). Despite the reduced fat, the low-fat diets maintained nonfat/lean (Figure 1F).

Figure 1

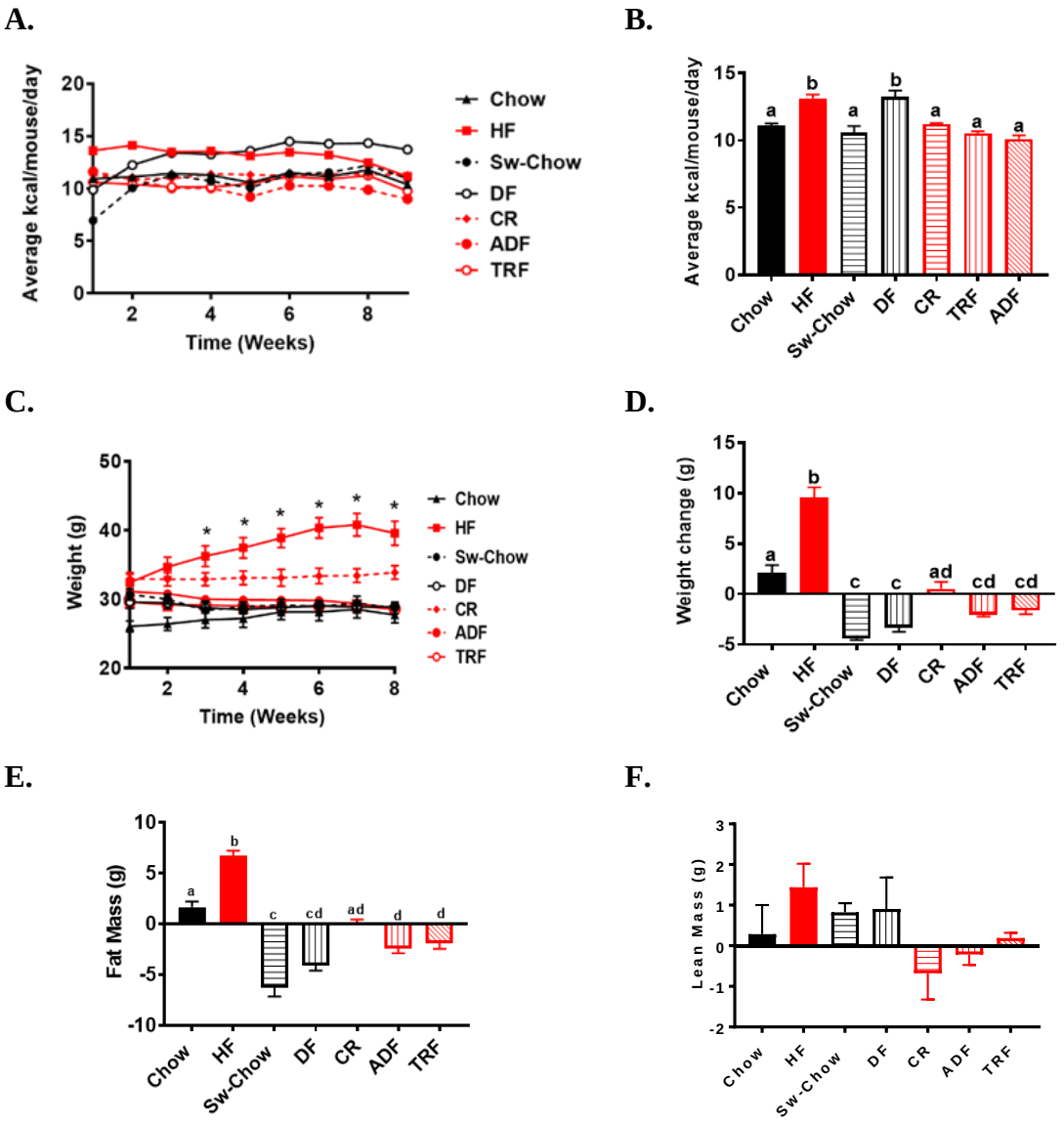


Figure 1. Food consumption (A) and body mass were monitored every day and other day respectively during the 8 week intervention. (B)_Average daily kcalorie consumption was calculated, *P*≤0.002 Bars without a common letter differed sifnificantly (C) After weeks on respective diets the HF group differed from all groups, *P*<0.001 (D) Body weight difference was calculated from pre-and post-intervention weight, *P*<0.0001(E) Fat and (F) Lean mass difference pre- and post-intervention *P* > 0.9 Bars without a common letter differed significantly. All values are mean ±SEM. N=7-9.

Changes in intestinal parameters

After euthanasia intestines were immediately removed from stomach to rectum. The length of the small intestine and colon were measured, and cecum weighed. There was a significant reduction in total intestinal length of all in the groups consuming the high fat diets compared to the Chow control (Chow 48.2cm ±2.051, HF 43.86cm ±2.201, Sw-Chow 46.76cm ±3.78, DF 47.25cm ±1.069, CR 44.53cm ±2.047, ADF 43.69cm ±1.29, and TRF 42.88cm ±1.82; Figure 2A, Chow *vs* HF, CR, ADF or TRF, $P < 0.03$), while no difference was observed in intestinal length of when consuming the low fat diets (Chow *vs* Sw-Chow or DF, $P>0.2$). This trend was consistent for the small intestinal length (Chow 39.4cm ±2.4, HF 35.86cm ±2.5 Sw-Chow 37.14cm ±2.4, DF 38.03cm±1.1, CR 36.09cm ±1.9, ADF 35.28c ± 1.0, TRF 34.64cm ± 1.3, Figure 2B, Chow *vs* HF, CR, ADF or TRF, $P < 0.02$, Chow *vs* Sw-Chow or DF, $P>0.3$). The only significant difference observed in the colon length was between the low-fat diets (DF and Sw-Chow) vs TRF (Figure 2C, $P\leq0.02$).

Cecum weight was increased in all low fat dietary groups (Chow 0.4g ± 0.03, HF 0.1g ± 0.05, Sw-chow 0.4g ± 0.1, DF 0.4g ±0.07,CR 0.1g ±0.03, ADF 0.1g ±0.02, TRF 0.1g ±0.01, Figure 2D, $P<0.0001$) with no difference in cecum weight between groups consuming the high fat diet ($P\geq0.9$)

Mice fed the low-fat diets (chow, switch to chow and DF) had heavier cecal (0.4± 0.04, 0.4 ± 0.1, and 0.4± 0.07). While the High Fat groups (HF, CR, ADF, TRF) showed decreased cecal weight (HF 0.15g ±0.06, CR 0.15g ± 0.03, ADF 0.18 ± 0.02, TRF 0.15g ± 0.02,) [Fig. 2) A, B C and D]

Figure 2

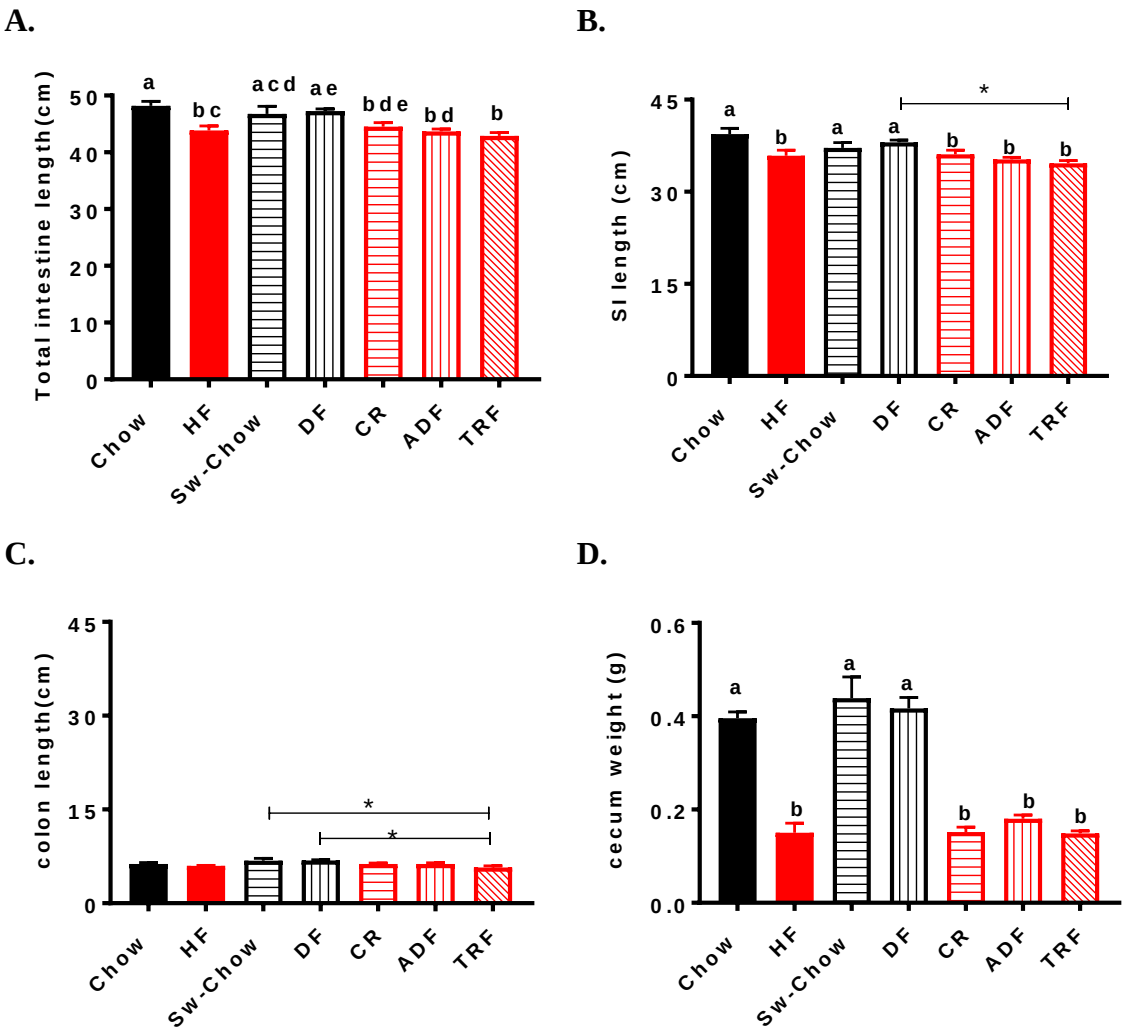


Figure 2. (A) Total intestine length was measured. Bars without a common letter differed $P< 0.03$, B and C) Small intestine and colon length was measured. Bars without a common letter differed $P< 0.02$, * $P\leq 0.01$ D) Cecum weight was determined immediately after euthanasia. Bars without a common letter differed $P\leq 0.02$. All values are mean ±SEM, N=7-9.

Diet components alters Short Chain Fatty Acids (SCFA) amount and composition

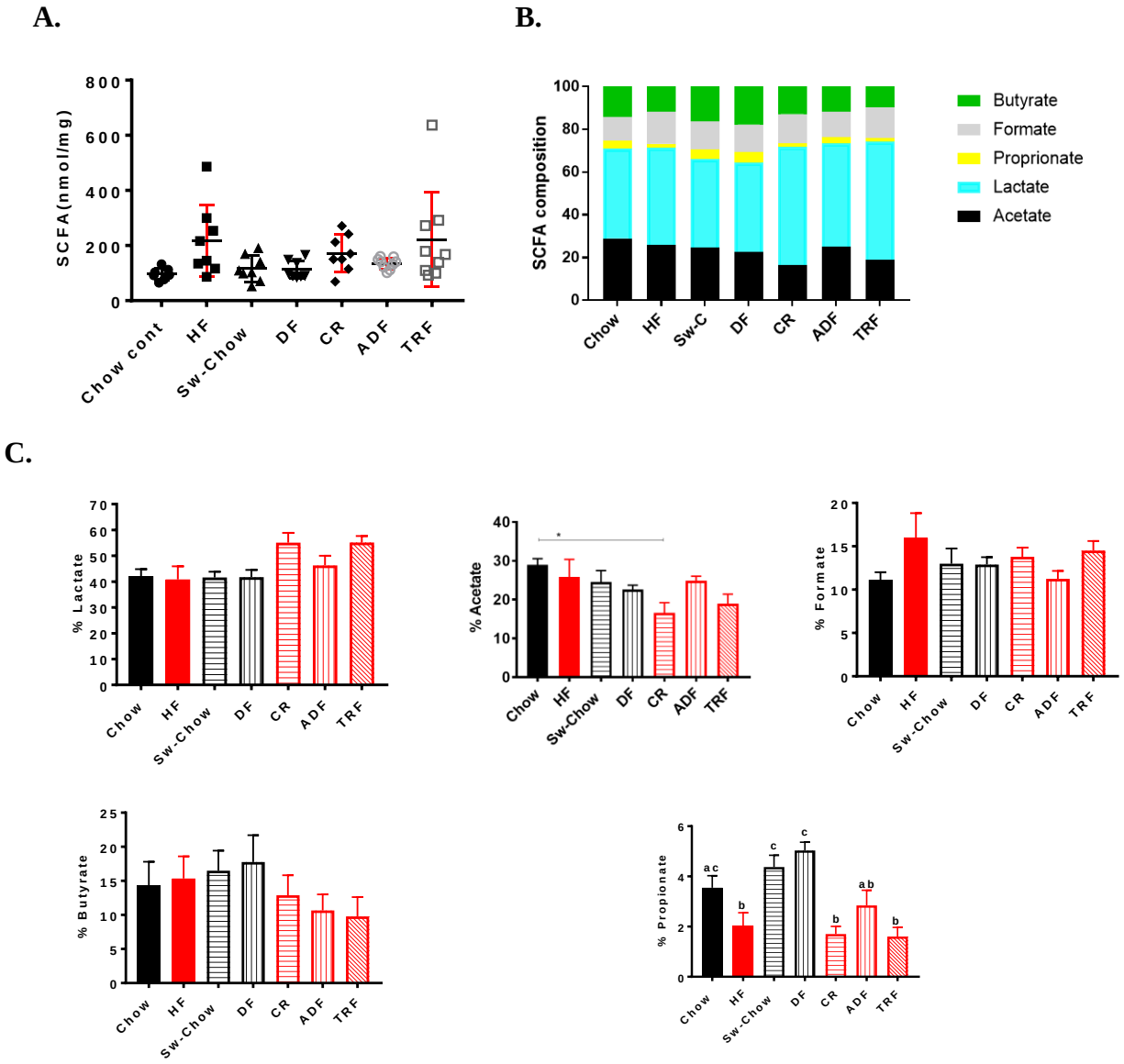
Cecal contents were harvested and microbial metabolites identified using ion chromatography. Short fatty acids (SCFA) measured included acetate, butyrate and propionate. No iso-butyrate was detected. Lactate was also measured. There was a trend towards an increase in the total amount of fatty acids and lactate in all the groups fed the high fat diet (Figure 3A, Chow 165.6nmol/mg ±28.67, HF 380.9 nmol/mg ±225.2, Sw-Chow 195.6nmol/mg ±66.34, DF 190.7 nmol/mg ±30.4, CR 390.7 nmol/mg ±149.1, ADF 262.5 nmol/mg ±74.1, TRF 490.7nmol/mg ±356.4]), but only the TRF group showed a significant increase compared to the lower fat diets (Figure 3A, $P<0.02$).. The concentration for each SCFA and lactate were as follows in table 3:

Table 3:

SCFA	Chow	HF	SW-CH	DF	CR	ADF	TRF
Acetate	48.2±11.5	77.1±19.9	48±17.2	42.6±5.8	65.0±33.0	63.7±13.2	83.4±57.1
Lactate	69.3±142	163.7±109.2	79.2±23.0	77.9±10.4	218.3±100.8	127.2±62.2	269.7±20.0
Butyrate	23.5±15.1	65.6±53.3	35.6±26.7	36.4±27.6	47.4±35.2	25.7±13.02	56.0±57.8
Propionate	6.0±2.7	5.3±2.1	8.2±3.1	9.6±2.6	5.7±2.2	7.0±2.9	5.5±1.8
Formate	18.6±6.4	69.2±75.9	24.62±11.7	24.2±4.5	54.2±24.5	29.5±10.7	76.05±71.8

The percentage of Lactate, Acetate and Butyrate (Figure 3B) did not differ significantly ($P\geq0.9$) although all groups consuming the high fat diet under restricted conditions appear to increase lactate (Figure 3C), with decreased acetate (Figure 3D) and butyrate (Figure 3E) with the ADF groups having the least amount of change. However, propionate was significantly increased in the low-fat diets (Sw-Chow and DF) vs all high fat fed groups (Figure 3F, $P<0.02$)

Figure 3

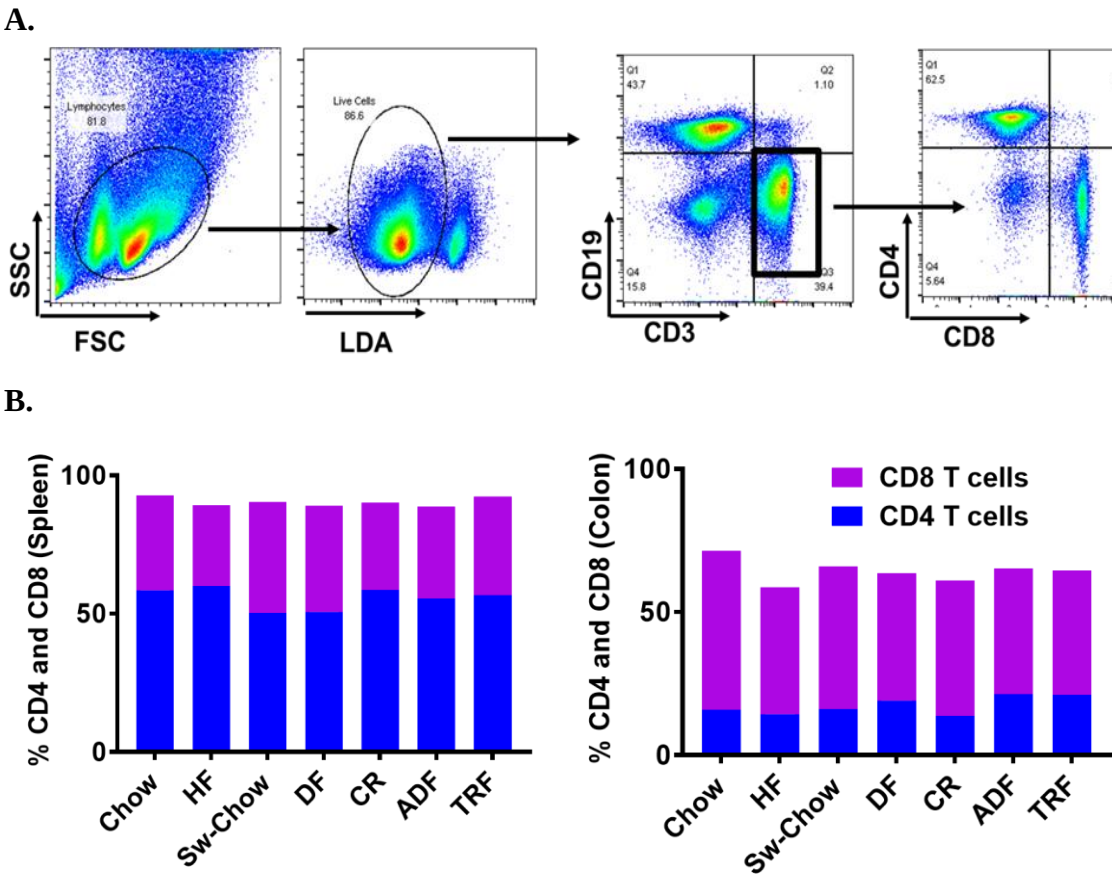


a) Total amount of short fatty acids (SCFA, includes acetate butyrate, formate and propionate. No isobutyrate was detected in any sample) measured in cecal contents. DF and Chow diets differed from TRF group. *$P \leq 0.02$, N=7-9. b) Representation of relative amounts of SCFA and lactate within cecal contents. c) Lactate, Acetate, Butyrate and Propionate percentages of total amount determined. Only propionate showed significant differences between low and high fat diets. Bars without common letter differed, $P<0.02$. All values are mean ±SEM. N=7-9.

Colonic Foxp3+Tregs are increased in the purified diet vs restricted high fat diets

Colonic immune cells were isolated from spleen and colon and lymphocyte population determined. There was no difference in overall CD4+/CD8+ composition in the spleen and colon (Figure 4B) Absolute cell number, and specifically CD4+ T cells were increased in the DF group vs the ADF group ($P<0.05$). Foxp3_ regulatory T cells were also significantly increased vs the ADF and TRF groups ($P<0.05$).

Figure 4:



C.

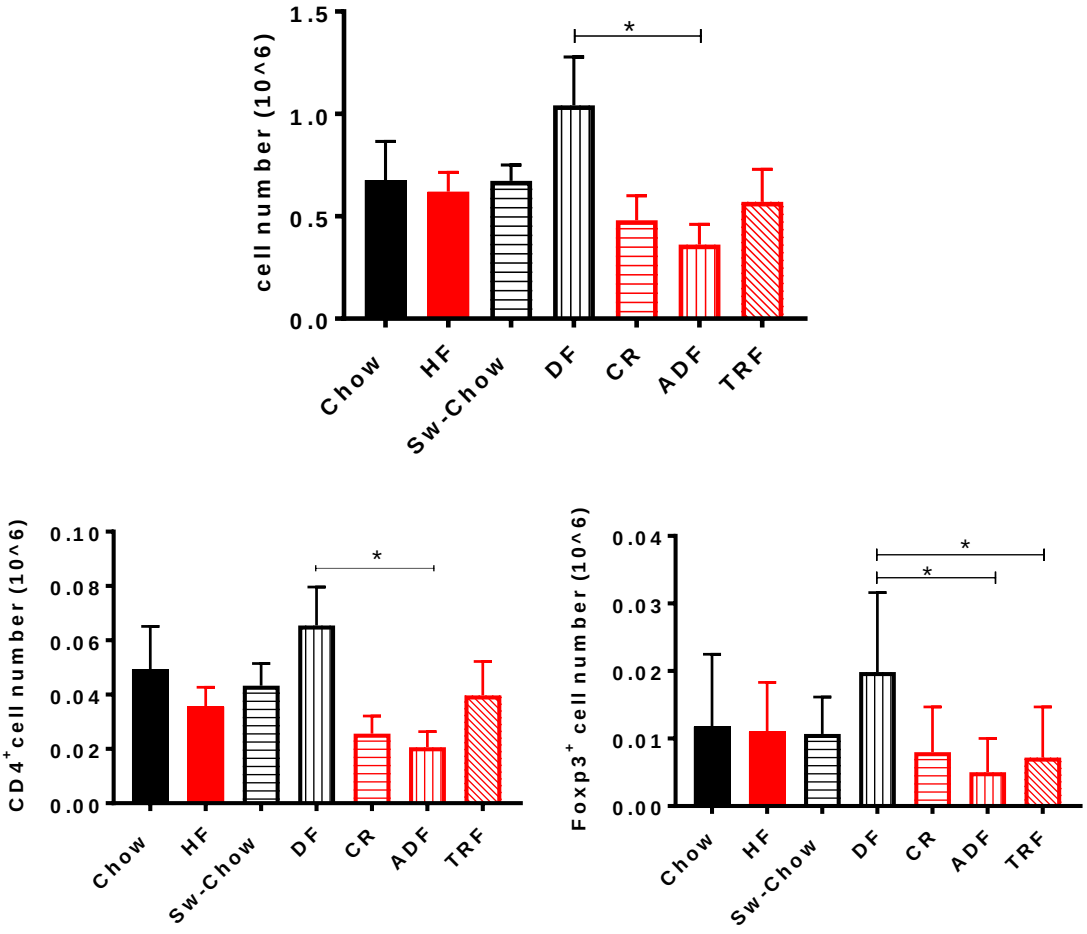


Figure: A) Representative flow cytometry plots to demonstrate gating. (B) Mean CD4+ and CD8+ T cells percentages in spleen (left panel) and colon (right panel). C) Absolute cell number as determined after Collagenase digestion, isolation and counting. CD4+ and Foxp3+ T-reg numbers were calculated. Populations that differed indicated. *P<0.05. All values are mean ±SEM. N=7-9.

DISCUSSION

A recent report indicates that obesity is still increasing. Almost 64.1% and 72.3% of American women and men are categorized as obese or overweight (Huang et al. 2015). Due to this epidemic, many types of dietary interventions are being explored for weight reduction.

In human studies it has been found that the diversity of microbiota is decreased and that the ratio of Bacteroidetes/Firmicutes phyla are altered in obese individuals. This change in the microbiota to an 'obese microbiome' results in increased dietary energy harvest and favor weight gain and fat deposition. (Q. Li et al. 2017). This avenue of research is relatively new and available data are conflicting, but the results provide an exciting perspective. Manipulation of the gut microbiota has now become one viable avenue to combat obesity and improve health. (Koliada et al. 2017).

It is well understood that differences in dietary composition alter the intestinal microbiome as demonstrated by people that have diets high in either protein or carbohydrates (Madsen et al. 2017). The nutrient source allows specific bacteria to flourish. More recent data also suggest that different feeding protocols, such as intermittent fasting has health benefits through alterations in gut microbiota (G. Li et al. 2017). In this current study, we used a murine model to explore the role of various dietary interventions, both alterations in diet composition and restricted feeding protocols, on weight, intestinal microbial metabolites and intestinal immune populations. Mice were fed a high fat diet for six weeks to induce weight gain. At the end of six weeks, the mice were divided into various groups that received either a different diet (lower in fat, higher in carbohydrates) or remained on the same high fat diet, but with restriction in either

amount or time to eat. Time restricted groups followed one of two protocols; an alternate day feeding protocol, or they were only allowed *ad libitum* food access for six hours every day. Food consumption and body weight were followed for 8 weeks at which point the animals were sacrificed and intestinal parameters determined. All mice on intervention diets lost weight during the 8 weeks, while the group that had *ad libitum* access to the high fat diet gained approximately 20% of its starting weight. Interestingly, the groups that were calorie restricted lost less weight than other groups, while consuming a similar number of calories. Of importance also, the vegan-based diet (DF) consumed the same amount of calories as the *ad libitum* fed high fat group but lost the most weight (similar to the group that was switched back to the chow diet). This weight loss was not due to loss of lean mass as the groups consuming the lower fat diet lost the most fat mass while maintaining nonfat/lean mass.

It is known that diets with a higher fiber content result in increased intestinal length (G. D. Wu et al. 2016). Consistent with this result, mice fed the chow or DF diet had longer intestines, with changes both in the small intestine and colon. There was also a significant increase in the cecum weight in the groups fed the chow diet and DF, while all groups consuming the high fat diet (irrespective of restrictions) had the same cecum weight.

Very little is known about the impact of diet on the metabolic potential of gut microbiota even though many studies indicated that dietary changes alter microbial activity. It was reported that a high fat diet causes major alterations in cecal bacterial physiology and metabolite landscape (Daniel et al. 2014). The intestinal immune system has coevolved with the gut microbiota for the maintenance of intestinal health (H. J. Wu and Wu 2012).

The interplay between the immune response and the gut microbiota is complex and is essential for the proper development of the immune system, possibly through the production of SCFA. Disruption of this homeostasis can lead to intestinal inflammation and disease (Kosiewicz et al. 2014). Diet affects the gut microbial composition and activity, and therefore the profile of SCFA synthesized.

To explore the immunoregulatory functions of SCFAs, several studies have been done to analyze SCFA composition in cecal content either between specific-pathogen free (SPF) mice and GF mice or between mice fed with high-fiber diets and low-fiber diets (D. Kim, Zeng, and Núñez 2017). They have reported that acetate, butyrate and propionate are the major bacteria-derived SCFAs that control the mucosal function and also Treg differentiation (Manuscript and Magnitude 2013). There was decrease in SCFA (propionic acid, acetic acid, butyric acid) which was related with a decrease in the number of thymic Treg cells, and treatment with SCFA increased the number of these cells (Çehreli 2018). Acetate is the most abundant SCFA in the colon and it makes up more than half of the total SCFA detected in feces (Louis et al. 2007). In our study, we measured cecum contained short chain fatty acids (SCFA) and we found all high fat dietary groups had increased levels of total SCFA with propionate higher in mice fed the DF and chow diets.

Propionate and butyrate metabolism have received much attention during the last years due to the association between low levels of butyrate and propionate producers and inflammatory diseases, for instance, butyrate producers are normally low in ulcerative colitis (Gijs Den Besten et al. 2013).

Tregs are a subpopulation of CD4+ T cells and can suppress other immune population and the differentiation of colonic Tregs are promoted by specific SCFA. Of three main short chain fatty acids. Butyrate has been found to be the most potent inducer of colonic Tregs (Morrison and Preston 2016) (Luu, Steinhoff, and Visekruna 2017). In a recent study it has been found that SCFAs have a role in colonic regulatory Treg homeostasis. SCFAs did so through Treg-intrinsic expression of GPR43 (Shapiro et al. 2014) . Colonic regulatory T cells expressing the transcription factor Foxp3 are critical for limiting intestinal inflammation and depend on microbiota-derived signals for proper development and function *Bacteroides fragilis* and clostridial species induce responses although gut microbiota affect Tregs responses across mammalian hosts remains still unclear(Corrêa et al. 2016).

In our study we have found that there was an increase in absolute number of lymphocytes, with a statistical significance increase in $Foxp3^+$ Tregs in the vegan-based diet compared to the dietary protocols of extended fasting and high fat diet.

Also, some studies report that both microbial as well as dietary factors like probiotics and vitamins influence the induction and suppressor functions of intestinal $Foxp3^+$ Treg cells (Issazadeh-Navikas and Teimer 2012). $Foxp3^+$ Treg cells are a highly activated T cell subset which responds rapidly to environmental and nutritional prompt. Therefore , appropriate nutrient supply is required to fuel the high energetic status of $Foxp3^+$ Treg cells for the regulation of intestinal immunity(Sakaguchi et al. 2008) (Kinoshita and Takeda 2014) .

CONCLUSION

This study demonstrates the reduction in calorie intake irrespective of diet composition will result in with weight loss. We have found that the calories restriction resulted in the smallest weight change, while the plant-based diet caused most weight loss while consuming high amounts of calories. Also, the high fat diet resulted in increased SCFA and lactate.

Propionic acid was increased with low fat diets. Increased levels of Foxp3 regulatory T cells was found with the plant-based diet. The lower fat diets had a more beneficial effect on overall health.

Recommendations for Future Research

The novel findings presented here, highlight the need for further investigation in the effect of nutrition on the microbiome and how it relates to chronic diseases.

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Wu, Hsin Jung, and Eric Wu. *The Role of Gut Microbiota in Immune Homeostasis and Autoimmunity.* Gut Microbes, 2012.

Xiao, Liang, Si Brask Sonne, Qiang Feng, Ning Chen, Zhongkui Xia, Xiaoping Li, Zhiwei Fang, et al. *High-Fat Feeding rather than Obesity Drives Taxonomical and Functional Changes in the Gut Microbiota in Mice*. BioMed Central-Microbiome, 2017.

APPENDIX

IACUC PROTOCOL ACTION FORM

IACUC PROTOCOL ACTION FORM

To:	Richard Bloomer
From	Institutional Animal Care and Use Committee
Subject	Animal Research Protocol
Date	June 21, 2017

The institutional Animal Care and Use Committee (IACUC) has taken the following action concerning your Animal Research Protocol No. **0806** Impact of dietary and caloric restriction models on metabolic health and physical function in male mice

☒ Your protocol is approved for the following period:

From: July 1, 2017 To: June 30, 2020

☐ Your protocol is not approved for the following reasons (see attached memo).

☐ Your protocol is renewed without changes for the following period:

From: To:

☐ Your protocol is renewed with the changes described in your IACUC Animal Research Protocol Update/Amendment Memorandum dated for the following period:

From: To:

☐ Your protocol is not renewed and the animals have been properly disposed of as described in your IACUC Animal Research Protocol Update/Amendment Memorandum dated

Amy L. de Jongh Curry, PhD, Chair of the IACUC

Karyl K Buddington, University Veterinarian and Director of the Animal Care Facilities

IACUC PROTOCOL

FOR USE OF LIVE VERTEBRATES FOR RESEARCH, TEACHING OR DEMONSTRATION

UNIVERSITY OF MEMPHIS

5/3/17

Date submitted to Attending Veterinarian for pre-review:

IACUC Protocol # 0806 Date Submitted to IACUC 5/30/17

Dates Protocol will be in effect: from 7/1/17 to 6/30/20

(not to exceed three years including two yearly renewals)

Is this protocol related to an external grant or contract application? Yes ☐

No ☒

If yes, complete the following:

Agency: _____ Date Submitted _____

Grant # _____

University account for Animal Care Facility per diem charge: _____

If the protocol is not related to an external grant or contract application, complete the following:

University account for Animal Care Facility per diem charge: 211700

Project Title: (If project relates to a grant or contract application, give that title; if multiple protocols relate to one grant, give unique titles for each protocol; if the project is related to a class, give the course name and number):

Impact of dietary and caloric restriction models on metabolic health and physical function in male mice

I. Personnel

Investigator/Instructor: Marie van der Merwe and Richard Bloomer

Department: Health Studies

Academic Rank: Assistant Professor and Professor

Campus phone**:** 678 3476 and 678 5638 Emergency phone: 901 406 7458 / 901 267 3514

Attending Veterinarian: Karyl Buddington

Phone: 901-678-2359 Emergency phone: 901-258-1232

List all individuals that will handle animals using this protocol, their affiliation, and their level of expertise (e.g. relevant qualifications). If the protocol applies to a class then so specify.

Marie van der Merwe, PhD (Molecular Pharmacology), Postdoctoral Fellowship (Bone Marrow Transplantation): More than 10 years of experience using mice as a research model.

Richard Bloomer, PhD: 2 years of prior experience using rodents in research.

Melissa Puppa, PhD: 8+ years of experience working with mice including breeding, exercise training/testing, injections, surgery, dietary interventions, gavage, GTT, electroporation, blood collection, and dissection/necropsy.

Matt Butawan: 2 years of rodent handling experience.

Harold Lee: 1 year of rodent handling experience, including exercise testing (treadmill running).

Sunita Sharma: 6 months animal handling experience; has been to Laboratory Animal Training.

Nick Smith: No experience with animals, but has been to Laboratory Animal Training; training will be provided during experiment.

Jade Caldwell: No experience with animals, but has been to Laboratory Animal Training; training will be provided during experiment.

Kyle Truska: No experience with animals and will attend Laboratory Animal Training; training will be provided during experiment

If additional personnel become involved in handling animals used in this protocol, it is the responsibility of the principal investigator to notify the Animal Care Facility in writing before they start.

Has the investigator/instructor and all personnel listed above received the appropriate vaccinations (tetanus, rabies)? Yes ☒ No ☐ Not Applicable ☐

Will be done by the time the study is initiated.

Is it necessary for personnel listed on this protocol to be tested for TB?

Yes ☐ No ☒

If you have questions about the kind of vaccination or about TB, call the Animal Care Facility at 678 2359.

All U of M personnel involved in this protocol must complete the animal care and use training program before animals can be procured or before experiments/teaching or demonstration. In submitting this protocol, I, as Principal Investigator/Instructor accept the responsibility for compliance with this requirement.

<u>In addition, the Principal Investigator/Instructor must be willing to provide appropriate supervision for all persons working on this protocol.</u> In the case of a class, the Instructor must be responsible for training any students in classes involved prior to using animals.

II. Project Description

A. Summary **(Enter a brief description below of your project, using lay terminology)**:

Obesity has become an epidemic in the Western world, largely due to poor dietary habits. Multiple dietary programs have been studied in recent years, showing promise with regards to weight loss and improvement in multiple health related outcomes, including glucose control, inflammation, and oxidative stress. More recently, a great deal of attention has been placed on the microbiome and the influence of dietary intake on this very important component of overall health. What is unknown is the degree of improvement noted when following each of the popular dietary regimens as compared to simple caloric restriction. That is, are certain protocols more beneficial than others and if so, in regards to which specific outcome(s)? Much debate centers on these questions and no study has sought to make direct comparisons between the well-investigated protocols.

In fact, most dietary protocols have simply been compared to a typical high fat Western diet. Very few comparison studies have been conducted inclusive of the four most common dietary approaches: Caloric Restriction (CR), Dietary Restriction (DR), Time Restricted Feeding (TRF), and Alternate Day Fasting (ADF). The goal of the present study is to directly compare the above four dietary plans to a Western Diet, with regards to body mass/body fat, physical performance, insulin sensitivity, inflammation, oxidative stress, and the microbiome. A diet of standard rodent chow will be used as a control. Outcome measures will be determined after 8 weeks of

assignment to the dietary programs, with a total of 56 animals assigned to one of 7 diet groups (n=8 per group).

4 week old C57BL/6 male mice will be entrained under a 12h light: 12h dark schedule for two weeks with standard rodent chow available ad libitum. During the entrainment period, mice will begin the reverse light-dark schedule, with lights off between the hours of 7am-7pm. This will be done so that the feeding time will be during the active phase ("light off" phase) of the mice. Mice will be housed in Life Sciences in an area that is currently used for studies of the circadian rhythm and therefore the light is well-regulated. After two weeks of entrainment, all but 8 mice will be switched to a Western diet for four weeks, consisting of 45% fat with lard as the fat source. This additional four week period of ad libitum feeding should allow for significant weight gain. Eight mice will continue following a standard chow diet during this 4-week period and serve as a low-fat control diet group. They will maintain this same diet throughout the entire study period. Following the 4-week period, the mice fed the Western diet will be divided into 6 additional groups: Western, CR, DR, TRF, ADF, and chow.

A MRI for the determination of body mass/fat and treadmill run to exhaustion will occur at baseline (prior to starting the specific diet plan) and following eight weeks on the specific diet assignment. Before and after the 4-week period of Western diet feeding and at the end of the intervention period, a glucose tolerance test will be performed. Mice will be fasted for 7 hours and glucose levels measured in 10 ul of blood collected via the tail vein. Glucose will be administered intraperitoneally and blood glucose measured every 30 minutes for 90 minutes from 10 ul of blood collected

from the tail vein. At the end of the dietary intervention, prior to sacrifice, blood will be collected from the facial vein for the determination of lipids, glucose, insulin, oxidative stress markers, cytokines, immune cell populations, and other variables. All mice will be sacrificed by CO2 inhalation. Tissues will be harvested immediately. This form of euthanasia does not affect the outcome measures as determined by our previous studies. Organs to be harvested are liver, spleen, intestine, lymph nodes, adipose tissue, skeletal muscle, and heart.

B. Describe IN DETAIL the procedures you will follow. Include accompanying documentation and reference to previously published work in the box below. Provide a complete bibliographic citation and describe any variations from the published technique. The bibliography may be included in the box below or appended to this protocol.

Mice: 56, 4 week old C57BL/6 male mice will be purchased from Envigo or another vendor. After arrival, mice will be co-housed (as done in reference 1) at the animal facility on the University of Memphis campus.

After arrival at the University of Memphis, mice will be entrained under a 12h light: 12h dark schedule for two weeks with standard rodent chow available ad libitum. During the entrainment period, the light-dark cycle will be reversed with lights off between the hours of 7am-7pm. This will be done so that the feeding time will be during the active phase ("light off" phase) of the mice. Mice will be housed in Life Sciences in an area that is currently used for studies of the circadian rhythm and therefore the light is well-regulated. After two weeks of entrainment, all but 8 mice will be switched to a Western diet, consisting of 45% lard and 41% carbohydrate (20%

sucrose, 9% corn starch, and 12% Maltodextrin). This additional four week period of ad libitum feeding should allow for significant weight gain. Following this period, mice will be divided into 6 additional groups and will consume their respective diets for an additional 8 weeks.

Group 1 will have access to a standard rodent chow ad libitum, 24 hours per day.

Group 2 will have access to a purified, high-fiber, vegan-based diet ad libitum, 24 hours per day. We have used this same customized diet in past studies and it results in normal weight/muscle mass gain, while minimizing fat accumulation. This will be the DR arm of the trial.

Group 3 will have access to the Western diet ad libitum, 24 hours per day.

Group 4 will have ad libitum access to the Western diet for 6 hours at the beginning of their active phase (7am-1pm). This will be the TRF arm of the trial. We have used this exact feeding protocol in a recent investigation of mice. No negative health consequences were observed using this protocol.

Group 5 will be on the alternate day fasting (ADF) protocol and have ad libitum access to the Western diet every other day. That is, on day 1 they will have unrestricted access to food during the entire 24 hour period. On day 2 they will receive no food. On day 3, they will have unrestricted access to food, and so on. This same protocol has been used in mice in several studies without incident. Please see references 2-10. It should be noted that if the ADF protocol leads to significant weight loss beyond what would be expected for mice following such a plan (≥20% body mass as compared to their body mass at the start of the intervention period [after 4 weeks of following the ad libitum Wester diet]) and/or mice show signs of stress or impaired health (e.g., scruffy

coat, hunched posture, excessively aggressive behavior), the attending veterinarian will be notified and a rescue protocol will be set in place and will consist of the following: instead of receiving no food on the fasting days, mice will receive 20% of the daily calories in the form of the Western diet during a one hour period at the mid-point of the animals' active phase (12-1pm).

It should be noted that animals in all groups will be monitored daily for signs of stress and impaired health, with particular attention given to animals in the ADF group. Monitoring body weight three times weekly should be more than adequate and should not cause undue stress to the animals due to frequency of handling.

Group 6 will receive 80% of ad libitum intake of the Western diet as determined during the prior 4 week period. This will be the CR arm of the trial. Multiple rodent studies have reduced caloric intake by 20-40% without incident. See references 5 and 11-17.

Al mice will be monitored daily for sign of malnutrition and stress.

The diets will be purchased from Research Diets, which has experience in producing the Western diet and purified vegan diets for rodent studies. The mice will remain on their particular diets for eight weeks and then post-testing will begin. Mice will continue on their diets until all testing is completed (~ end of week 9). Water will be provided ad libitum throughout the study period. The amount of food consumed will be measured daily and the weights of the mice will be taken three times per week at the same time of day. There will be a total of 56 mice assigned in this study.

There will be two mice housed per cage. From our previous studies we know that genetically similar mice eat basically a constant volume of food. We can therefore pair-house the mice and determine an average amount of food consumed. Previous studies

have used a similar set up where 3-5 mice were co-housed (Hatori et al.). If there are any signs of fighting or it appears that one the mice is consuming the majority of the food, those specific mice will be separated into individual cages. Food will be weighed daily. Mice will be weighed 3 times per week at the same time of day. A glucose tolerance test (GTT) will be performed at the beginning of the experiment (when mice are put on their respective diets) and following 8 weeks after the start of the dietary intervention. The final GTT will be performed 48h prior to euthanasia. For the glucose tolerance test, mice will be fasted for a minimum of 7 hours and blood glucose levels determined by blood from tail vein. Mice will be given a 1g glucose/kg body weight intraperitoneally and blood (10ul) collected every 30 minutes for 90 minutes to measure glucose levels. For blood collection, mice will be placed on a flat surface and restraint by gently holding onto the tail without pulling. The tip of the tail will be snipped – 1mm region. This part of the tail has little nerve innervation and does not cause the animal any distress. By "milking" the tail, blood can be collected at multiple time points without having to cut again. Isoflurane cannot be used as it increases blood glucose levels independent of treatment. We have used this exact procedure in a recent study.

In addition to the GTT, prior to commencing the specific diet plans (after the initial 4 week period on the Western diet), animals will undergo a MRI for determination of body mass/body fat. Body composition will be determined using an EchoMRI™ 1100. The MRI is housed in room 115 in Life Sciences, the same location that our mice are housed in. For scanning, animal are placed with cylindrical tube holders and movement restricted to the bottom 7.5 cm as stated in the instrument manual and the study by Jones et al. (Validation of quantitative resonance for the determination of body composition of mice. *Int. J Body Compos Res*. 2009; 7(2):68-72). The animals are scanned without anesthesia, sedation or restraint and are free to move within the holder. The smallest possible holder is used to limit the movement of the mouse (without constraining them) in order to reduce measurement errors induced by motion. Scanning time is approximately 40 seconds. There is no prior training required for the animal. We have prior experience using this system in mice.

Finally, animals will undergo a treadmill run test to exhaustion using a motorized treadmill without incline. Specifically, mice will be acclimated to the treadmill prior to testing. Run to fatigue test will be performed twice in the mice; once prior to starting the 8 week intervention and at the end of the 8 week intervention. Animals will run using a 5% grade at 20m/min for 30 min and 25m/min for the remaining time until they reach exhaustion. A warm up phase will be provided for 15min (5min at 5m/min, 5min at 10m/min, 5min at 15m/min). Fatigue will be defined as the time at which mice are no longer able or willing to keep up with the speed of the treadmill despite gentle hand

prodding for a period of 30 seconds. Very mild electric shock will only be used if mice do not respond well to gentle hand prodding. Our past and current work using running protocols demonstrates that mild shocking is preferable to obtain the best running performance. The frequency and amplitude of shock will be as low as possible to motivate the animals to remain on the treadmill belt, without causing unnecessary distress. We have used small electric shock in prior studies and this is well-accepted in rodent running studies. Equipment will be cleaned upon the completion of testing with ethanol solution. All urine and feces will be cleaned off of the device and the surrounding area.

After 8-9 weeks of dietary intervention, mice will be euthanized (CO_2 inhalation) and with cervical dislocation. Tissues will be harvested immediately. This form of euthanasia does not affect the outcome measures as determined by our previous studies. Organs to be harvested are liver, spleen, intestine, lymph nodes, adipose tissue, skeletal muscle, and heart. Immediately prior to euthanasia, blood will be collected via the facial vein to measure lipids, glucose, insulin, oxidative stress markers, cytokines, immune cell populations, and other variables as needed. Cecum and intestinal contents will be collected for microbiome analysis.

1. Hatori M, Vollmers C, Zarrinpar A, et al. Time-restricted feeding without reducing caloric intake prevents metabolic diseases in mice fed a high-fat diet. Cell Metab. 2012.

2. R. Michael Anson, Zhihong Guo, Rafael de Cabo, et al. Intermittent fasting dissociates beneficial effects of dietary restriction on glucose metabolism and neuronal resistance to injury from calorie intake. Proceedings of the National Academy of Sciences of the United States of America. 2003.

3. Krista A. Varady, D. J. Roohk, & Marc K. Hellerstein. Dose effects of modified alternate-day fasting regimens on in vivo cell proliferation and plasma insulin-like growth factor-1 in mice. Journal of Applied Physiology. 2007.

4. Varady, K. A., Roohk, D. J., Loe, et al. Effects of modified alternate-day fasting regimens on adipocyte size, triglyceride metabolism, and plasma adiponectin levels in mice. Journal of Lipid Research. 2007.

5. Varady, K., Allister, C., Roohk, d., & Hellerstein, M. Improvements in body fat distribution and circulating adioponectin by alternate-day fasting versus calorie restriction. The Journal of Nutritional Biochemistry. 2010.

6. Lu, J., E, L., Wang, W., Frontera, et al. Alternate day fasting impacts the brain insulin-signaling pathway of young adult male C57BL/6 mice. Journal of Neurochemistry. 2011.

7. Dorighello, G. G., Rovani, J. C., Luhman, C. J. F., Paim, B. A., Raposo, H. F., Vercesi, A. E., & Oliveira, H. C. F. Food restriction by intermittent fasting induces diabetes and obesity and aggravates spontaneous atherosclerosis development in hypercholesterolaemic mice. The British Journal of Nutrition. 2014.

8. Beigy, M., Vakili, S., Berijani, S., et al. Alternate-day fasting diet improves fructose-induced insulin resistance in mice. Journal of Animal Physiology and Animal Nutrition. 2013.

9. Joslin, P. M. N., Bell, R. K., & Swoap, S. J. Obese mice on a high-fat alternate-day fasting regimen lose weight and improve glucose tolerance. Journal of Animal Physiology and Animal Nutrition. 2016.

10. Descamps O, Riondel J, Ducros V, Roussel A. Mitochondrial production of reactive oxygen species and incidence of age-associated lymphoma in OF1 mice: effect of alternate-day fasting. Mech Ageing Dev. 2005.

11. Mager, D. E., Wan, R., Brown, et al. Caloric restriction and intermittent fasting alter spectral measures of heart rate and blood pressure variability in rats. FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology. 2006.

12. Lusseau, D., Mitchell, S. E., Barros, et al. The effects of graded levels of calorie restriction: IV. non-linear change in behavioural phenotype of mice in response to short-term calorie restriction. Scientific Reports. 2015.

13. Sharma, N., Wang, H., Arias, E. B., et al. Mechanisms for independent and combined effects of calorie restriction and acute exercise on insulin-stimulated glucose uptake by skeletal muscle of old rats. American Journal of Physiology. Endocrinology and Metabolism. 2015.

14. Ingram, D. K., Weindruch, R., Spangler, E. L., et al. Dietary restriction benefits learning and motor performance of aged mice. Journal of Gerontology. 1987.

15. Means, L. W., Higgins, J. L., & Fernandez, T. J. Mid-life onset of dietary restriction extends life and prolongs cognitive functioning. Physiology & Behavior. 1993.

16. Yu, B. P., Masoro, E. J., Murata, et al. Life span study of SPF fischer 344 male rats fed AdLibitum or restricted diets: Longevity, growth, lean body mass and disease. Journal of Gerontology. 1982.

Sohal, R. S., Agarwal, S., Candas, M., et al. Effect of age and caloric restriction on DNA oxidative damage in different tissues of C57BL/6 mice. Mechanisms of Ageing and Development. 1994.

C. Rationale for Involving Animals and the Appropriateness of Species and Number Used.

Indicate (**here**) briefly the short and/or long-term benefits (to humans and/or other animals) of this use of animals for research, teaching or demonstration. Provide rational for and the number of animals to be used. In addition, state briefly why living animals are required for this study, rather than some alternative model.

The goal of this experiment is to study common feeding patterns in a controlled environment over a moderate period of time to determine the cardio-metabolic health effects of these dietary plans. Results will provide evidence for or against certain models that can then be used by human subjects in an attempt to combat obesity and related co-morbidities. We know from our prior work in rodents that the TRF and DR models are favorable as compared to a WD. However, we are uncertain as to how CR and ADF plans compare. Moreover, we are unaware of studies focused on comparing these plans with regards to the microbiome or physical performance. These are important areas of interest to scientists and humans who are focused on which dietary plan may be "best." As obesity is becoming more of a problem in the Western world, determining which dietary approaches may be best to combat this disease is of great importance.

The C57BL/6 diet induced obesity mouse model has been used previously to study the effect of excess weight on various organ systems. As we are interested in the interaction between the immune system, oxidative stress, physical performance, and other organs, we cannot use isolated cell lines or model organisms such as yeast. Additionally, many reagents have been developed for the use of mouse tissues, especially antibodies that will be used to identify certain outcome measures. As we are

focusing on the effect of the different dietary programs on multiple organ systems, it is only feasible in an animal study.

There will be 8 mice per group for a total of 56 mice. This number is the norm for similar studies of dietary-induced changes in our health-specific parameters. This number should be sufficient to determine statistical significance for the tests planned during this study.

D. Do the procedures described in B above, have the potential to inflict more than momentary pain or distress (this does not include pain caused by injections or other minor procedures)? Yes ☐

No ☒

I have considered alternatives to procedures that might cause more than momentary or slight pain/distress, and I have not found such alternatives. As such, I have used one or more of the following methods and sources to search for such alternatives: **(check below each method used)**

☐ Agricola Data Base ☐ Medline Data Base ☐ CAB Abstracts

☐ TOXLINE ☐ BIOSIS ☐ Lab. Animal Sci. Journal

☐ Lab. Animals Journal ☐ Lab Animal ☐ Animal Welfare Info Center

☐ ATLA (Alternatives to Laboratory Animal Journal) ☐ Quick Biblio. Series

☐ Lab Animal Welfare Bibliography (QL55L27311988) ☐ "Benchmarks"

☐ "Alternatives to Animal Use in Research, Testing and Education"

☐ Current Contents

☐ CARL

☐ Direct contact with colleagues **(if selected, you MUST document this below)**

List search words for the literature search:

Daniel Fast, dietary restriction, vegan fasting, fasting, time restricted feeding, intermittent fasting, caloric restriction, alternate day fasting, chow, obesity, inflammation, oxidative stress, microbiome, treadmill test, EchoMRI, insulin resistance, insulin sensitivity, fatty acids, body composition, glucose, insulin, blood sugar (words used in isolated and in combination in PubMed and Google Scholar).

What is the length of time that the literature search covers?

1960-2017

III. Animal Use

A. List all animal species to be used (example below).

Species Number[1] Age[2] Sex[2] Weight[2] Where Housed (Bldg./Rm#)

e.g. Hooded Wistar rats	45	2 months	male	250-350 gm	Psychology Bld./422I
C57Bl/6 mice	56	4 weeks	Male	15-20 gm	Life Sciences/115

[1]Individuals using ectotherms need to only approximate numbers.

[2]Individuals using fish or other ectotherms need not answer this question.

Is any species threatened or endangered? Yes ☐

No ☒

B. Source of animals

☒ Commercial vendor (Source___ Envigo

Labs_____)

☐ Bred at The University of Memphis

☐ Captured from wild. Identify method of capture: _____

☐ Transferred from another study (IACUC Protocol Number _____

☐ Donated (Source _____)

☐ Tennessee Wildlife Resources Agency

Is the supplier a USDA approved source? Yes ☒

No ☐

If not, explain why:

☐ Animals are already in residence at U of M

C. Will surgery be conducted on animals? Yes ☐

No ☒

If yes, complete this section:

☐ Non Recovery Surgery ☐ Recovery Surgery

☐ Multiple Survival Surgery (**if the latter is checked, complete section F)**

Surgeon(s) (Name/Job/Title/Academic Rank) Location of Surgery (Bldg. & Room #)

D. Will Anesthetic(s), Analgesic(s), or

Tranquilizing agents be administered? Yes

No x☐

If yes, complete this section (example below).

Species & Sex Agent Dose Route Performed by

(Name/Title/Academic

Rank)

e.g. male Hooded Wistar rats	sodium pentobarbitol	50 mg/kg	i.p.	Mr. Smith/Research Technician/B.S.

E. Will euthanasia be carried out? Yes ☒ No ☐

If yes, complete this section (example below).

Species & Sex Agent Dose Route Performed by (Name/Title/Academic Rank)

e.g. male Hooded Wistar rats	sodium pentobarbitol	150 mg/kg	i.p.	Mr. Smith/Research Technician/B.S.
C57BL/6 mice	CO_2	3L/min	Inhalation	Marie van der Merwe/ Assistant Professor Matt Butawan/Research Associate Sunita Sharma/Master's Student (Will be trained by Dr. Karyl Buddington)

If no, describe disposition of animal(s) at conclusion of this study in box below.

F. Will special housing, conditioning, diets or other conditions be required? Yes ☒

No ☐

If yes, please explain in box below.

Mice will be on high fat, chow, or purified diets. Some mice will have restricted access to food, either 7am-1pm or every other day. Some mice will have access to only 80% of their daily ad libitum intake.

G. Will animals be removed from the U of M campus at any time? Yes ☐

No ☒

If yes, please indicate to where and for how long in box below.

H. If they are to be housed for more than 24 hours outside approved facilities at U of M, provide a scientific justification in box below.

IV. Toxic and Hazardous Substances

A. Check off any of the following below that will be used in these experiments?

☐ Infectious agents (Fill out a, b)

☐ Radioisotopes (Fill out a, b, e)

☐ Toxic chemicals or carcinogens (Fill out a, b)

☐ Recombinant DNA (Fill out a)

☐ Experimental drugs (Fill out a)

☐ Malignant cells or hybridomas (Fill out a, c)

☐ Adjuvants (Fill out a)

☐ Controlled substances (Fill out a, d, e)

For each checked off category, answer the questions indicated below:

a. Identify the substance(s) and completely describe their use, including how will be injected or given to the animal(s):

b. Describe all procedures necessary for personnel and animal safety including biohazardous waste, carcass disposal and cage decontamination:

c. If transplantable tumors or hybridoma cells are to be injected into the animals, have the tissues/cells been tested for inadvertent contamination by viruses or mycoplasma? Yes ☐

No ☐

If yes, what was the result (indicate in box below).

d. In the box below, provide a complete list of these substances, and if their use is not explicitly explained in the materials already provided, explain their use and role in the research.

Provide DEA license # covering the use of these substances:

To whom (or what entity) is the license issued?

e. Provide Radioisotope License Number: _____

To whom is the license issued? _____

V. Categories of Animal Experimentation Based Upon Level of Manipulation and Pain: (check off each category that is applicable to this application)

☐ A. Animals will be involved in teaching, research, experiments or tests involving no pain, distress, or use of pain-relieving drugs.

☐ B. Animals will be subject to mild stress only (e.g., food or water deprivation of less than 24 hours for use in behavioral studies such as operant conditioning; physical restraint for less than 30 minutes), and will not be subject to surgery, painful stimuli, or any of the other conditions described below. Procedures described in this protocol have the potential to inflict no more than momentary or slight pain or distress on the animal(s)----that is, no pain in excess of that caused by injections or other minor procedures such as blood sampling.

☐ C. Animals will have minor procedures performed, blood sampling, etc. while anesthetized.

☐ D. Live animals will be humanely killed without any treatments, manipulations, etc. but will be used to obtain tissue, cells, sera, etc.

☐ E. Live animals will have significant manipulations, surgery, etc. performed while anesthetized. The animals will be humanely killed at experiment termination without regaining consciousness.

☒ F. Live animals will receive a painful stimulus of short duration without anesthesia (behavior experiments with flight or avoidance reactions--e.g., shock/reward) resulting in a short-term traumatic response. Other examples in this category are, blood sampling, injections of adjuvants, or drugs, etc.

Injection for glucose tolerance test; possible low grade shock while on treadmill.

☐ G. Live animals will have significant manipulations performed, such as surgery, while anesthetized and allowed to recover. Such procedures cause post-anesthetic pain/discomfort resulting from the experiment protocol (e.g., chronic catheters. surgical wounds, implants) which cause a minimum of pain and/or distress. Also included are mild toxic drugs or chemicals, tumor implants (including hybridomas). tethered animals, short-termed physically restrained animals (up to 1 hour), mother/infant separations.

☐ H. Live animals will have significant manipulations or severe discomfort, etc. without benefit of anesthesia, analgesics or tranquilizers. Examples to be included in this category are: toxicity testing, radiation sickness, irritants, burns, trauma, biologic toxins, virulence challenge, prolonged: restrictions of food or water intake, cold exposure, physical restraint or drug addiction. All use of paralytic agents (curare-like drugs) must be included in this category. Describe any abnormal environmental conditions that may be imposed. Describe and justify the use of any physical restrain devices employed longer then 1 hour.

VI. Justifications for Category G Studies and Deviations from Standard Techniques

Describe in the box below any steps to be taken to monitor potential or overt pain and/or distress during the course of this study and how such pain or distress will be alleviated. Be as detailed as necessary to justify your procedure.

VII. Certifications

(By submitting this protocol, I am acknowledging that I comply with the certifications included in Section VII.) *(check one)*

☒ Animal Use for Research. I certify that the above statements are true and the protocol stands as the original or is essentially the same as found in the grant application or program/project. The IACUC will be notified of any changes in the proposed project, or personnel, relative to this application, prior to proceeding with any animal experimentation. I will not purchase animals nor proceed with animal experimentation until approval by the IACUC is granted.

☐ Animal Use for Teaching/Demonstration. I certify that the information in this application is essentially the same as contained in the course outline and a copy of the laboratory exercises using animals is on file in the IACUC office. The IACUC will be notified of any changes in the proposed project, or personnel, relative to this application, prior to proceeding with any animal experimentation. I will not proceed with animal experimentation until approval by the IACUC is granted.

Estimate the cost of maintaining animals used in this protocol based on current per diem charge at University of Memphis.

Please specify cost per unit of time: $7.20/day ($0.24/cage/day)

Specify anticipated total costs for project duration: $756 (15 weeks)

As supervisor of this project it is required that you inform your department chair concerning any animal per diem costs related to this project that are to be paid by the department.

By submitting this protocol, the Principal Investigator/Course Director indicates that the following have been considered:

1. Alternatives to use of animals.
2. Reduction of pain and stress in animals to the lowest level possible.
3. The proper needs of the animals with respect to housing and care.
4. The lowest number of animals used that will give the appropriate experimental results.
5. Use of the most primitive species that will give the appropriate experimental results.
6. Proper training of all personnel in the care and handling of the species used and in the procedures called for in this protocol before beginning the experiment/teaching or demonstration.

7. That this protocol is not an unnecessary repeat of results already in the literature or in the case of teaching/demonstrations, results that can be demonstrated using models or video material.

Marie van der Merwe/Richard Bloomer

Principal Investigator/Course Director (Type Name)

mvndrmrw@memphis.edu / rbloomer@memphis.edu

e-mail address

5/3/2017

Date

Federal Law requires that members of the IACUC be given adequate time to read and review protocols including any changes or revisions in them.

Pre-review of protocols by the Attending Veterinarian is required before submission to the IACUC. New protocols or modifications or renewals to protocols must be submitted to the IACUC Chair by the 1st business day of the month to be considered for review during that month. Incomplete protocols will be returned to the principal investigator.

E-mail the completed protocol to the IACUC Chair, Dr. Amy de Jongh Curry, adejongh@memphis.edu

February, 2015