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Effect of Dietary Maillard Reaction Products on Insulin Sensitivity, Metabolic Inflammation and Intestinal Inflammation in Mice Fed the Total Western Diet

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EFFECT OF DIETARY MAILLARD REACTION PRODUCTS ON INSULIN SENSITIVITY, METABOLIC INFLAMMATION AND INTESTINAL IMFLAMMATION IN MICE FED THE TOTAL WESTERN DIET

by

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ABSTRACT

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The effects of dietary Maillard reaction products (MRPs) on weight gain, glucose metabolism and intestinal health in mice were investigated. Three groups of C57BL6/J mice (n=10/gp) were fed the Total Western Diet (TWD) with different levels of MRPs: MRP0, TWD with low level of MRPs; MRP1, TWD with medium level of MRPs, and MRP2, TWD with high level of MRPs. In this study, only the casein (protein) and sugar components of the diet were cooked to generate MRPs, while in previous studies, whole diet pellets, including lipids, were cooked to generate MRPs. Thus, a complementary experiment was conducted to show the difference in the lipid oxidation level of the diet between this study and previous studies.

No significant differences in weight gain were found between three groups during 13 weeks. No significant differences were observed in histology or colitis scores of mice colons after 10 days of oral administration of dextran sodium sulfate (DSS). The results of the oral glucose tolerance test (OGTT) showed that normalized area under the curve (AUC) of blood sugar during 120 minutes of MRP0 was significantly higher than that of MRP2 ($p < 0.05$),

indicating that dietary MRPs led to an improvement in glucose metabolism.

In the gut, significant differences in alpha diversity of fecal microbiome at phylum level ($p < 0.01$) and class level ($p < 0.01$) were observed. Significant differences in beta diversity of the cecal microbiome ($p < 0.004$) and the fecal microbiome ($p < 0.001$) were observed at all taxonomic levels. Different levels of MRPs in the TWD showed significantly different effects on taxonomic abundance of fecal microbiome at all other taxonomic levels except species level ($p < 0.05$), and on taxonomic abundance of cecal microbiome at OTU level, species level and phylum level ($p < 0.05$). As for gut metabolites, valeric acid, propionic acid and butyric acid in the cecal contents, along with propionic acid and valeric acid in the fecal contents were significantly different between the three groups ($p < 0.05$). Baking diets to promote the Maillard reaction, as has typically been done with dietary MRP studies, showed in addition to MRP generation, there were significant effects on lipid oxidation. This finding suggests a possible explanation of the opposite effect of MRPs on glucose metabolism in this study compared to previous studies.

In conclusion, dietary MRPs did not induce obesity in mice. None of the mice showed any symptom of colitis after receiving DSS for 10 days, illustrating that MRPs did not increase susceptibility to DSS-induced colitis. Intake of MRPs decreased the area under the curve for blood glucose during the OGTT, indicating a beneficial effect on glucose metabolism. Alpha diversity of fecal microbiome decreased with the higher MRPs level in diet, and the significance of this observation is unclear. The microbiota composition and microbial fermentation products SCFAs in the hindgut of mice were changed by intake of MRPs. As SCFAs could play a role in the improvement of insulin sensitivity in mice, further studies are needed to investigate if the changes of cecal and fecal SCFAs are related to a better or worse health condition.

(74 pages)

PUBLIC ABSTRACT

Effect of Dietary Maillard Reaction Products on Insulin Sensitivity, Metabolic Inflammation, and Intestinal Inflammation in Mice Fed the Total Western Diet

Siyu Xiao

Maillard reaction products (MRPs) are generated when proteins or amino acids are heated with reducing sugars. In previous studies, baking whole diet pellets at a high temperature has been the most common way to promote MRP formation. However, baking diets also induces other chemical reactions besides MRP production, for example lipid oxidation. In this study, only casein and sugars were cooked to generate MRPs. Thus, a complementary experiment was conducted to determine how baking diet pellets affects lipid oxidation.

Previous rodent studies showed MRPs either induced weight gain and or impaired glucose tolerance. On the other hand, dietary MRPs were shown to alleviate colitis induced by dextran sodium sulfate (DSS). The aim of this study was to elucidate the effects of dietary MRPs generated by a different treatment method in a different diet on weight gain, glucose metabolism and intestinal health in mice. Three groups of C57BL6/J mice (n=10/gp) were fed the Total Western Diet (TWD) with different levels of MRPs: MRP0, TWD with low level of MRPs; MRP1, TWD with medium level of MRPs, and MRP2, TWD with high level of MRPs.

Mice receiving different levels of MRPs in their diet showed no significant difference in weight gain. Mice receiving 10 days of oral administration of dextran sodium sulfate (DSS) showed no significant difference in histology and colitis score of their colons. The results of the oral glucose tolerance test (OGTT) showed that the normalized area under the curve (AUC) of blood sugar during 120 minutes of MRP2 was significantly lower than that of MRP0 ($p < 0.05$),

indicating that increased intake of MRPs either promoted insulin secretion, increased insulin sensitivity in target tissues, or both. Alpha diversity of fecal microbiome showed significant differences at phylum level ($p < 0.01$) and class level ($p < 0.01$). Beta diversity of the cecal microbiome ($p < 0.004$) and the fecal microbiome ($p < 0.001$) showed significant differences at all taxonomic levels (OTU level, species, genus, family, order, class and phylum). Different levels of MRPs in the TWD induced significantly different taxonomic abundance of cecal microbiome at OTU level, species level and phylum level ($p < 0.05$), and significantly different taxonomic abundance of fecal microbiome at all other taxonomic levels except Species level ($p < 0.05$). Short chain fatty acids as microbial fermentation products were measured in the cecal and fecal contents using gas chromatography with flame ionization detection (GC-FID). Valeric acid, propionic acid and butyric acid in the cecal contents, along with propionic acid and valeric acid in the fecal contents were significantly different between the three groups ($p < 0.05$). Baking diets to promote MRP formation was shown to significantly increase lipid oxidation. This suggests that the negative effects of MRPs on glucose metabolism shown in previous studies may be actually caused by ingestion of lipid oxidation products, rather than MRPs.

In conclusion, ingestion of MRPs affected both glucose tolerance and the gut microflora. MRPs did not induce obesity in mice, nor did any of the mice show symptoms of colitis after receiving DSS for 10 days. Intake of MRPs decreased overall incremental blood glucose concentration during OGTT, indicating that it could reduce the risk of high blood glucose, which would be beneficial for health. High alpha diversity of gut microbiota is associated with healthy state according to previous human studies. Alpha diversity of fecal microbiome decreased with higher MRPs level in diet, and the significance of this observation is unclear. The microbiota composition and microbial fermentation products SCFAs in the hindgut of mice were changed

by intake of MRPs. As SCFAs could play a role in the improvement of insulin sensitivity in mice, further studies should investigate possible mechanisms of this beneficial effect.

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LIST OF SYMBOLS, ABBREVIATIONS AND DEFENITIONS

1. Total Western Diet (TWD)
2. Short chain fatty acid (SCFA)
3. Maillard reaction products (MRPs)
4. Advanced glycation end products (AGEs)
5. Oral glucose tolerance test (OGTT)
6. Carboxymethyllysine (CML)
7. Polyunsaturated fatty acids (PUFA)
8. Gas-chromatography (GC)
9. Gas-chromatography Flame ionization detector (GC-FID)
10. Dextran sodium sulfate (DSS)
11. Area under the curve (AUC)
12. Thiobarbituric acid (TBA)
13. Peroxide value (PV)
14. Anisidine value (AnV)

I. INTRODUCTION

During food processing and food storage, there are two major chemical reactions that can influence the quality of food, the Maillard reaction and lipid oxidation. To better understand diet and health, rodent studies have been conducted to investigate the health effects of products of these two reactions. Rodent studies can provide us with useful information on how diet can affect health. Yet in many rodent studies, the background diets and constituents of interest are not provided at physiologically relevant levels, and often the results do not translate well to human studies.

The Maillard reaction occurs during food processing and storage. Reducing sugars react with the amino acids and generate Maillard Reaction Products (MRPs) [1]. They can provide foods with a favorable smell or color. An example of the formation of brown pigmented material, knowns as melanoidins, through the Maillard reaction is shown in Figure 1 [2]. As the Maillard reaction involves the degradation of essential amino acids, it will result in the loss of nutritional value [3].

MRPs are formed by proteins or amino acids being heated with a reducing sugar. Advanced glycation end products (AGEs) are formed in the later stage of the analogous reactions in biological systems, in which aldoses combine with proteins in the plasma [4]. Consequently, AGEs are often used to describe MRPs formed inside the body. Carboxymethyllysine (CML) is one well-known MRP. According to human and rodent studies, CML is associated with the development of diabetes and cardiovascular disease [5] [6]. As around 30% of dietary CML enters the circulation via absorption from the gut [7], dietary CML is considered as an important indicator of MRPs which can increase the risk of diabetes and cardiovascular disease. Researchers also found that consuming a high

MRP diet significantly lowered long chain n-3 fatty acids concentrations in human plasma [8]. However, a rat study indicated that MRPs alleviated colitis induced by rats receiving 5% dextran sodium sulfate (DSS) in drinking water [9]. In addition, in a previous mouse study conducted by Dr. Robert Ward in France, higher dietary CML induced a decrease in total blood glucose during an oral glucose tolerance test and an increase in gut microbes of the genus *Sutterella*. It has been demonstrated in previous studies that *Sutterella* was associated with susceptibility to DSS-induced colitis [10].

In rodent studies conducted to investigate the health effects of Maillard reaction products, chow, the AIN-93G diet and high fat ('diet induced obesity'; DIO) diet have most commonly been used. Chow is formulated with grains and cereals with the addition of vitamins, minerals and vegetable oil to provide adequate nutrition. As the composition of the ingredients in chow are complex and subject to cultivar and seasonal variation, it is difficult to ensure that the specific composition of chows used in different experiments have the same composition. That may make the data from different experiments in one study inconsistent if the rodents are fed with different batches of chow. The AIN-93G diet is a purified diet which was formulated to meet all nutritional requirements of rodents and is formulated with simple, compositionally consistent ingredients which makes the data from different experiments reproducible. DIO formulas are similar to the AIN-93G, in that they are composed of purified ingredients, but their high fat content from soybean oil or lard induces obesity in rodents.

One problem with chow and the purified diets described above is that they do not reflect what Americans actually eat. In order to properly study the effects of MRPs on health, we strove to provide mice with diets that differed in MRPs content on a

macronutrient and micronutrient background of a true ‘Western Diet’. Thus, we chose to use the Total Western Diet (TWD) to feed the mice. The TWD was formulated to mimic actual dietary intake in the US at the 50th-percentile of intake for all macronutrients and micronutrients, according to the National Health and Nutrition Examination Survey (NHANES). By using the allometric scaling, this diet provides high translatability from human intake to mice intake [11]. Comparison of TWD and the commonly used diets are shown in Table 1.

Baking whole diet pellets at a high temperature is the most common way to promote MRP formation. However, baking diets to induce MRP production also likely destroys heat labile vitamins and promotes lipid oxidation. Previous rodent studies have demonstrated that lipid oxidation in diet is associated with insulin resistance [12] [13]. Thus, it is not clear that MRPs in the baked diets fed to mice in previous studies were responsible for the deterioration in insulin sensitivity. In this study, we added the Maillard reaction products which were generated under appropriate condition into the uncooked Total Western Diet instead of cooking the whole diet as the previous studies, so that there was very low possibility for the lipids in TWD to undergo oxidation process. This study design gave us a clear understanding of the effects of MRPs itself in the diets on mice health, without the interference of lipid oxidation products. In addition, a complementary experiment was conducted to test if the cooking method used in previous studies could induce lipid oxidation in the commonly used DIO diet and our TWD. The accuracy of the results from this study can be proved if high level of lipid oxidation was induced by cooking the whole diet pellets in previous studies while lipid oxidation was minimized in this study.

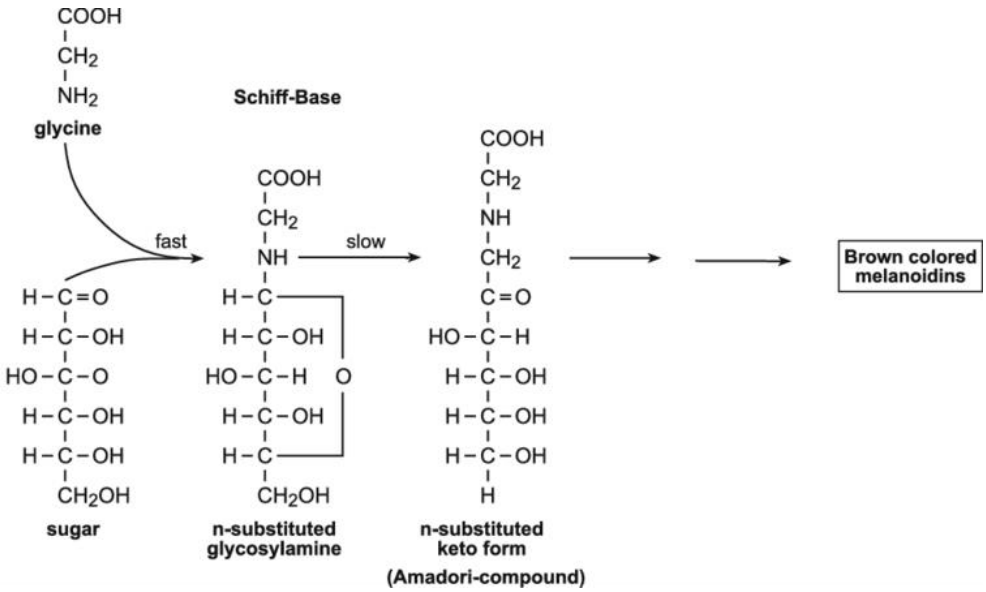

Figure 1. Formation of melanoidin [2]

Table 1. Comparison of TWD and the commonly used diets

	Composition	Macronutrient composition (% by weight)	Reproducibility
Chow	complex, unpurified	unclear	No
AIN-93G diet	simple, purified	~20% protein, ~7% fat, ~60% carbohydrate	Yes
DIO diet	simple, purified	~26% protein, ~35% fat, ~26% carbohydrate	Yes
TWD	simple, purified	19.3% protein, 16.5% fat, 59.1% carbohydrate	Yes

II. LITERATURE REVIEW

1. Total Western Diet

The Total Western Diet (TWD) is a novel rodent diet that was formulated to represent typical American daily nutritional intake in macronutrient composition and micronutrient composition. The TWD was formulated to provide nutrients at the 50th-percentile of intake, according to the National Health and Nutrition Examination Survey (NHANES) [11]. The formula was determined by dividing daily intake values (both sexes) from NHANES tables by 2070 kcal/day. Compared to the commonly used AIN-93 diet, the TWD provides fewer calories from carbohydrates and proteins, and more calories from fat. Lower levels of polyunsaturated fat and complex carbohydrates are also a feature of the TWD. Conversely, the TWD contains more saturated fat, monounsaturated fat and simple sugar.

Table 2. Formulation of the Total Western Diet Based on Energy Density

	NHANES			TWD
	10th	mean	90th	
Macronutrient (mg/kcal)				
Total fat (mg)	24.6	37.8	69.1	37.8
Saturated (mg)	7.7	12.7	16.9	12.7
Monounsaturated (mg)	9.4	13.9	20.3	13.9
Polyunsaturated (mg)	4.9	7.9	10.1	7.9
n6 PUFA (mg)	5.1	7.1	10.4	7.1
n3 PUFA (mg)	0.4	0.7	1.1	0.7
Cholesterol (mg)	106.3	133.3	173.9	133.3
Total carbohydrates (mg)	80.2	123.7	183.1	123.7
Complex carbohydrates (mg)	38.6	58.4	87	58.4
Simple sugars (mg)	38.2	58	87	58
Dietary fiber (mg)	4.4	7.3	11.8	7.3
Total protein (mg)	26.9	37.7	52.2	37.7
Micronutrient (unit/kcal)				
Minerals				
Calcium (μg)	253.6	457	772	457
Phosphorus (μg)	415.9	626.6	909.7	626.6
Potassium (μg)	810.6	1212.1	1730.4	1212.1
Sodium (μg)	1071	1608.7	2327.5	1608.7
Magnesium (μg)	88.9	133.8	195.7	133.8
Iron (μg)	4.5	7.1	11.1	7.1
Zinc (μg)	3.8	5.6	8.2	5.6
Copper (μg)	0.4	0.6	0.9	0.6
Selenium (ng)	33.8	48.3	73.9	48.3
Vitamins				
Niacin (μg)	7.9	11.5	16.8	11.5
Vitamin B_6 (μg)	0.6	0.9	1.4	0.9
Thiamin (μg)	0.6	0.8	1.2	0.8
Riboflavin (μg)	0.7	1	1.6	1
Folate (mg)	0.2	0.3	0.4	0.3
Vitamin K (ng)	22.9	42.9	85	42.9
Vitamin B_{12} (ng)	1.4	2.5	4.5	2.5
Vitamin A (mIU)	501	977.3	1824.2	977.3
Vitamin D (mIU)	36.2	88.9	194.7	88.9
Vitamin E (mIU)	3.5	5.6	8.9	5.6
Choline (μg)	89.9	147.3	235.7	147.3

2. Maillard Reaction and Its Health Effects

2.1 Maillard Reaction Products (MRPs)

Heating is an important method to process foods. The advantages of heat processing include the killing of harmful microbes and the formation of characteristic aromas and colors. The disadvantages of heat processing include the destruction of vitamins, polyunsaturated fatty acids (PUFAs) and phenolic components. During the past forty years, many studies have been conducted to investigate the nutritional effects of consuming heat-processed foods. As food browning is an important part of the heating process, more and more studies are focused on the Maillard reaction. Also known as non-enzymatic browning, the Maillard reaction occurs when proteins or amino acids are heated with a reducing sugar, in which the free carbonyl groups of reducing sugars react with free amino groups of free amino acids or proteins [1]. One of the negative effects of Maillard reaction is a deterioration of protein quality. In many studies, the nutritive value loss is associated with the destruction of lysine. The loss of lysine is attributed to fructoselysine which is not bioavailable [3]. The decrease in protein digestibility and the inhibition of glycolytic and proteolytic enzymes are also the negative consequences of Maillard reaction [14]. Moreover, the production of pathogenic compounds such as mutagenic compounds (methylglyoxal, diacetyl and glyoxal etc.) [15] and toxic compounds (melanoidins and carboxymethyllysine etc.) [16] have drawn researchers' attention.

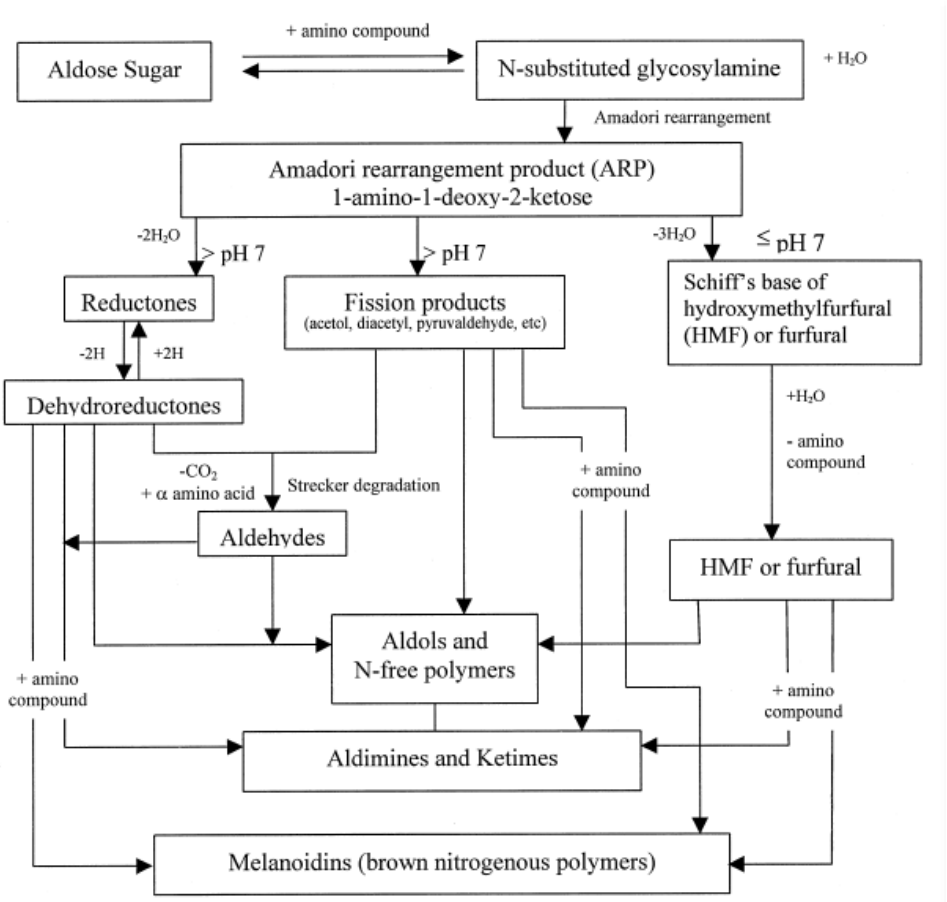

Figure 2. Maillard reaction scheme of Hodge [17]

The pathway for Maillard reaction was modeled by Hodge [17]. The Maillard reaction is initiated with the condensation of carbonyl group from reducing sugar and free amino group from protein or free amino acid. The unstable N-substituted glycosylamine generated from glycation reaction undergoes Amadori rearrangement to form 1-amino-1-deoxy-2-ketose. This Amadori rearrangement product will eventually turn into aldimines and ketimes or melanoidins which are considered as Maillard reaction products [18]. Though melanoidins give the food characteristic color and flavor, negative effects of dietary melanoidins on health, including promoting cardiovascular complications and diabetes mellitus, have been demonstrated [19].

2.2 Advanced Glycation End Products (AGEs)

MRPs are formed by proteins or amino acids being heated with a reducing sugar in foods. Advanced glycation end products (AGEs) are formed in the later stage of the analogous reactions in biological systems [4], so AGEs are synonyms for MRPs in some publications. It has been demonstrated that dietary MRPs (or dietary AGEs) contribute to the development of atherosclerosis by forming crosslinks between the molecules of basement membrane and activating the receptor for advanced glycation end products (RAGE) which is related to oxidative stress and chronic inflammation [19].

2.3 Inhibition of Maillard reaction

One study investigating the influence of cooking methods on the formation of MRPs suggested that the content of MRPs in dry-heated foods are 10- to 100-fold more than in the same food that was not heated. Methods to inhibit the formation of MRPs during cooking include using moist heating or cooking under lower temperature. In addition, manipulation of water activity and temperature, and use of a low pH can also be utilized to limit the Maillard reaction. The amino group is protonated at lower pH, which makes it less nucleophilic and harder to react with carbonyl group. Consequently, adding MRP inhibitory compounds during cooking, such as aminoguanidine, lemon juice and vinegar is another method to reduce the formation of MRPs [20].

2.4 Carboxymethyllysine

Carboxymethyllysine (CML) is one advanced glycation end-product, and MRP. It is an aldimine (Figure 2) [17]. The main pathway for CML formation is the oxidation of

fructosyl-lysine [5]. Cardiovascular disease is correlated to the accumulation of CML in the aortic area of diabetic rats [21]. Around 30% of dietary CML (dCML) enter the circulation via absorption [7]. Because the formation of CML as a glycoxidation product is irreversible, CML is commonly used as a biomarker for oxidative stress in human studies [22].

2.5 The Health Effects of Dietary MRPs

2.5.1 Weight Change

Many studies demonstrated that the intake of a high MRP diet induced significant weight gain compared to the intake of low MRP diet. The results of those studies illustrated that MRPs could promote obesity which can be harmful for health. A study investigated the effect of different MRP intake levels in mice diet on weight gain using db/db mice. Db/db mice are genetically obese mice that are deficient in the leptin receptor, and which are prone to hyperphagia. The AIN-93G diet was chosen as low MRP diet while PicoLab Rodent Diet 20 was chosen as high MRP diet. The MRP content of high MRP diet was 3.4-fold higher than low MRP diet, which was measured by ELISA. Researchers found that the weighs of high MRP diet-fed db/db mice were significantly higher than those of low MRP diet. However, there was a problem with this experimental design. The mice in experimental group and control group did not receive same diet, so besides the different MRP contents in each diet, the different components could also have effect on weight gain [23].

In another study, researchers used a high fat diet (35% fat, PicoLab Rodent Diet D12492) as low-MRP high-fat diet (LMRP-HF), and they obtained high-MRP high-fat

diet (HMRP-HF) by heating LMRP-HF at 120°C for 30 min. The results showed that mice consuming the HMRP-HF diet gained more weight than mice consuming the LMRP-HF diet [24]. A third mouse study measured the effect of different fat contents and MRP contents in diet on weight change. AIN-93G (18.4% protein, 7.2% fat and 58.6% carbohydrate) was used as high fat diet, while another standard rodent diet (24% protein, 4% fat and 46.16% carbohydrate) was used as low fat diet. The high fat diet was heated at 100 ˚C for 20-60 seconds to make low MRP high fat diet (LMRP-HF). LMRP-HF was heated additional 30 minutes at 125 ˚C to create a high MRP high fat diet (HMRP-HF). Low fat diet was heated at 60-80 ˚C for 20-30 seconds and at 65-85 ˚C for 5 seconds to make low MRP low fat diet (LMRP-LF). Then LMRP-LF was heated at 125 ˚C for additional 30 minutes to generate high MRP low fat diet (HMRP-LF). HMRP-HF group had significantly higher weight gain than LMRP-HF, HMRP-LF and LMRP-LF, and there were no significant differences in weight gain among those three groups [25].

However, in other studies dietary MRPs did not affect weight gain. In one study, TestDiet 58G7 (a mouse chow) was used as a low MRP diet, and a high MRP version was produced by heating in an autoclave at 120°C for 15 min. There were no differences in weight gain between the groups [26]. In another study which was focused on the effect of dietary MRPs on experimental fatty liver disease, a methionine and choline- deficient (MCD) diet was used to induce non-alcohol fatty liver disease (NAFLD) in mice. The MCD diet was used as low MRP diet. The MCD diet was baked at 160 ˚ for 1 h to produce a high MRP diet. No significant differences were found for weight loss, liver weight and liver percent body weight between high MRP group and low MRP group [27].

2.5.2 Insulin Sensitivity

Insulin is a hormone that allows glucose in blood to be absorbed into cells. It helps to maintain blood glucose. Insulin sensitivity is a measure of how effective the cells can use glucose with a specific amount of insulin. Lower insulin sensitivity can induce the inefficient utilization of blood glucose, thus cause high blood glucose which may result in diabetes. Mixed results of the effect of MRPs on insulin sensitivity have been reported in both human and animal studies. A human study investigated the effect of mild-steam cooked diets and high-temperature cooked diets with 2.5-fold higher CML content on insulin sensitivity. The result showed that participants consuming the high-temperature cooked diet for one month had significantly lower insulin sensitivity compared to those consuming mild-steam cooked diet [8]. In mice, a study investigated the effect of low-MRP diet (LMRP) and high-MRP diet (HMRP) on diabetes. The fasting serum insulin level of HMRP-fed db/db mice was significantly higher than that of LMRP-fed mice. The glucose tolerance was significantly higher in LMRP-fed db/db mice compared with HMRP-fed db/db mice [23]. Another mouse study demonstrated that significantly impaired glucose tolerance was displayed in the mice consuming high MRP content diet compared to those consuming low MRP content diet [24].

However, in one mouse study, no significant differences were observed in fasting insulin, glucose or HOMA-IR between the regular MRP content diet group and high MRP content diet group [26]. A human study concerning the effect of CML in infant formula on insulin sensitivity of infants was conducted. Because of the heat treatment in the process of making infant formula, its MRP content is around 670-fold higher than breast milk. After comparing the healthy infants fed with MRP-rich infant formula to

other healthy infants fed with breast milk, there was no significant difference shown in their insulin sensitivity [28].

2.5.3 Gut Microbiota

A rat study showed that the cecal contents of rats fed with a glycated fish protein diet were rich in *Allobaculum*, *Akkermansia*, *Turicibacter* and *Lactobacillus animalis*, comparing to the cecal contents of rats fed with fish protein diet, heated fish protein diet and AIN-93G diet [29]. In mice, two groups of mice were individually fed with standard AIN-93G diet with regular MRP content and cooked AIN-93G diet (175 °C for 45 min) with high MRP content. The high MRP diet contained twice the amount of CML compared to the regular MRP diet. The alpha diversity of mice fecal microbiota decreased but the Helicobacter levels of it increased in high MRP group [30]. In an animal study investigating the effect of heating process of food on gut microbiota in two vertebrates, researchers found that receiving thermally treated food (steamed for 15 min; the highest temperature, 100 °C, for 2-3 min) significantly decreased alpha diversity of gut microbiota in both mice and catfish compared to receiving non-thermally treated food [31]. Alpha diversity refers to the species abundance in one microbial ecosystem, which shows how many kinds of species are in one sample. In previous human studies, lower alpha diversity of the microbiome was associated with obesity [32].

2.5.4 Protein Digestion

Maillard reaction products generated from glucose and lysine lowered the dietary protein digestibility in rats [33]. Similar result was obtained in another rat study, in which

protein digestion was reduced in both young and adult rats fed with MRPs [34]. MRPs can lower the digestibility of protein, and thus excess protein will enter the colon for possible fermentation by the microbiome [35]. In young animals, higher amount of *Lactobacilli* and lower amount of *Coliforms* and *Staphylococci* can be induced by additional protein fermentation in colon [36]. Proteins can act as one of energy resources for gut microbiota, but only some of the species survive on fermenting protein. When higher amount of proteins enter the colon, those specific species may grow and multiply faster. Intake of MRPs can induce higher level of protein in colon, thus eventually change the gut microbiota composition.

2.5.5 MRPs Digestion

Among all MRPs, melanoidins which are generated from later stage of Maillard reaction have drawn many researchers' attention [37]. It was concluded by *in vivo* studies that melanoidins resist digestion in the upper gastrointestinal tract and can be metabolized by microbiota in the hindgut [38].

2.5.6 Short Chain Fatty Acids (SCFAs)

Short chain fatty acids usually refer to the fatty acids that contain fewer than six carbons. They are the metabolic end products of the microbial fermentation of carbohydrates and proteins in the intestine [39]. Butyric acid has been shown to protect the colonic mucosa, prevent colon cancer and maintain normal acidity in colon, so increasing formation and transportation of butyrate may have protective effect of the colon [40]. The insulin sensitivity improvement effect of butyric acid was demonstrated

in mice fed with high fat diet [41]. A study using 3T3-L1 cultured adipocytes indicated that both propionic acid and valeric acid can improve insulin responsiveness for glucose uptake [42]. Acetic acid is the primary acid in vinegar, and the most abundant SCFA in the gut. In a recent mouse study, the anti-obesity and anti-inflammation effects of vinegar were demonstrated [43]. Another human study showed that vinegar had an effect of improving insulin-stimulated glucose uptake in muscles [44]. All those studies demonstrated that these short chain fatty acids could play a role in improving insulin sensitivity in mice, human or cells.

Mixed results were obtained on the effects of dietary MRPs on SCFAs contents in previous animal studies. An animal study showed that rats fed with a glycated fish protein diet had significantly higher butyrate concentration in their cecal contents and fecal contents compared with rats fed with fish protein diet, heated fish protein diet and AIN-93G diet [29]. In another rat study, a regular AIN-93G diet was used as the low MRP diet, and the high MRP diet was made by heating AIN-93G diet at 125 °C for 3h. Cecal acetic acid content was significantly reduced in rats fed with the high MRP diet [45]. Mice fed with high heat-treated diet had lower SCFAs concentration in cecal contents than those fed with standard AIN-93G diet [30].

2.5.7 DSS-induced Colitis

Ulcerative colitis is a kind of inflammatory bowel disease whose symptom is inflammation of the colon mucosa [46]. Oral administration with dextran sodium sulfate (DSS) is the most commonly used method to induce experimental colitis in rodents. DSS can erode the intestinal epithelial monolayer so that luminal microbes will have access to

the mucosa and proinflammatory substances will permeate the rest of tissues [47]. In one study, mice from treatment group received tap water with 1.5% DSS, while mice from control group received only tap water. Damage was more evident in the colons of mice given DSS with higher levels of inflammatory cytokines and intestinal microbiota modification [48]. A recent study indicated that MRPs generated from glucose and lysine could alleviate colitis in rats induced by providing 5% DSS in their drinking water [9].

2.5.8 Long Chain n-3 Polyunsaturated Fatty Acids (PUFAs)

It has been suggested that n-6 PUFA-derived eicosanoids are proinflammatory, however n-3 PUFA-derived eicosanoids are anti-inflammatory [49]. When more n-3 PUFAs are in the human inflammatory cells, less eicosanoids will be synthesis from n-6 PUFAs [50]. One human studied showed that significantly lower plasma concentrations of long chain n-3 PUFAs were found in the subjects that consumed high-heat treated diets with high MRP content for one month compared to the subjects that consumed a mild-steam cooked diet [8]. The result suggested that high-heat treated diet lowered the concentration of long chain n-3 PUFAs in plasma, thus may induce more formation of proinflammatory eicosanoids from n-6 PUFAs in inflammatory cells, which might eventually cause more inflammation.

3. Lipid Oxidation and Its Health Effects

3.1 Lipid Oxidation

Lipid oxidation can be catalyzed by many factors, which are called initiators, such as transition metals, temperature, oxygen, ultraviolet light and enzymes [51]. The

initiation step of lipid oxidation is the free radical (R˙) formation due to the existence of initiators. Then in the propagation step, the free radicals react with triplet oxygen and generate peroxy radicals (ROO˙) which continue to react with new unsaturated lipids and form new free radicals and lipid hydroperoxides (ROOH). When the concentrations of free radicals and peroxy radicals become very high, the high probability of collisions make those radicals generate non-radical products, which is called termination step [52].

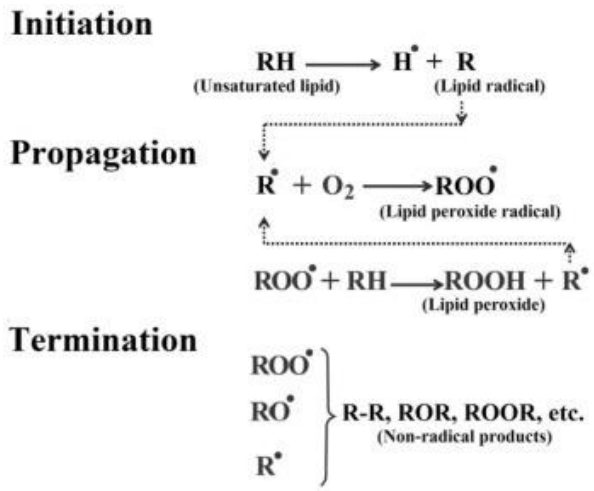

Figure 3. Rationale of lipid oxidation [53]

3.2 Primary Products of Lipid Oxidation

As hydroperoxides are the primary products of lipid oxidation, peroxide value (PV) is an important indicator of the first step of lipid oxidation [54]. Due to the oxidizing ability of hydroperoxides, there are several common methods of measuring PV. In iodometric titration method, saturated iodide ions (I^-) are added to react with peroxides, and the oxidation product molecular iodine are determined by titration [55]. In iron-based spectrophotometric method, Ferrous ions (Fe^{2+}) are oxidized to Ferric ions (Fe^{3+}) by peroxides and the absorption of Ferric thiocyanate is measured at 500-510 nm

[56]. In Fourier Transform Infrared Spectroscopy, typical O-H absorption bands of different compounds generated during lipid oxidation can be measured by infrared spectroscopy, therefore the formation of lipid hydroperoxides can be determined [57]. Basically, fresh oil has a peroxide value of less than 10 mEq/Kg, while oxidized oil with a rancid taste has a peroxide value of 30-40 mEq/Kg.

Besides PV, the absorption peak of conjugated dienes at 230-235 nm [58] and that of conjugated trienes at 268nm [59] can be measured to determine the extent of primary lipid oxidation.

3.3 Secondary Products of Lipid Oxidation

Aldehydes are the main products of decomposition of hydroperoxides. The extent of secondary lipid oxidation can be determined by the content of aldehydes. Malondialdehyde is generated during the degradation of polyunsaturated fatty acids (PUFA). The pink complex formed by malonaldehyde and thiobarbituric acid (TBA) can be detected at 530-535 nm by spectrophotometry, which makes the TBA test one of the most commonly used method of determining the secondary products of lipid oxidation [60]. Another method is to measure p-Anisidine value, in which the yellow products formed by p-anisidine reagent and aldehydes can be detected at 350 nm [61]. TBA test can only measure malonaldehyde content, while p-Anisidine test can measure total aldehyde content which is more accurate.

3.4 Lipid Oxidation in Foods

Lipid oxidation often occurs in the food with high unsaturated fatty acid content.

Methylene bridge (-CH_2-) between two double bonds in polyunsaturated fatty acid (PUFA) has the highly reactive hydrogen atom, while methylene group besides double bond in monounsaturated fatty acid (MUFA) has the hydrogen atom with less reactivity, so it is easier for PUFA to break into free radicals than MUFA. Thus foods with higher PUFA content have higher susceptibility to lipid oxidation. Heat, light, oxygen and metal ions may catalyze lipid oxidation during food storage, as can cooking at high temperature in food preparation. Lipid oxidation gives food a rancid smell and degrades the organoleptic properties of foods. There are several strategies in foods to protect against oxidation, such as adding antioxidants, removing and limiting access to pro-catalysts [62].

3.5 The Health Effects of Dietary Oxidized Lipids

3.5.1 Body Weight

In a mouse study, C57BL/6J mice were divided into two groups, individually fed with a low fat diet with unheated soybean oil or with heated soybean oil. Heated soybean oil which was processed by heating at 190°C for 3 hours contained significantly higher lipid oxidation products than the unheated soybean oil. Researchers found that mice fed with heated soybean oil had significantly lower weight gain than those fed with unheated soybean oil [63]. A similar result was shown in another rodent study, in which oxidized frying oil was prepared by frying wheat flour dough in soybean oil at 205±5 ˚C for 6 hours per day for 4 days. Rats receiving a high fat diet containing oxidized frying oil had significantly lower weight gain than those receiving the high fat diet containing same amount of fresh soybean oil or fish oil [12].

In another mice study, the researchers heated the soybean oil at 190 ˚C for 3 hours, 6 hours and 9 hours under oxygen flow. Then they added those three different oxidation levels of soybean oil into mouse chow to feed mice. No significant difference in weight gain was observed between three groups [13].

3.5.2 Adipose Tissues Weight

In a mouse study, C57BL/6J mice were fed chow with three different oxidation levels of soybean oil. There was no significant difference in fat pad mass between the groups [13]. Another mice study found that mice fed with a diet containing a higher level of oxidized lipid had significantly more fat mass [63]. A rat study showed that oxidized oil had a greater anti-adipogenic effect than fresh oil [12].

3.5.3 Insulin Sensitivity

Results from rodent studies have demonstrated that oxidized oils have negative effect on insulin sensitivity in rats and mice. For example, oxidized frying oil prepared by frying wheat flour dough in soybean oil at 205±5 ˚C for 6 hours per day for 4 days has a negative effect on glucose tolerance, which is a measure of insulin sensitivity [12]. Another mouse study was conducted to test if oxidized soybean oil in the diet can cause hyperglycemia in C57BL/6J mice. The researchers added the soybean oils which were heated at 190 ˚C for 3 hours, 6 hours or 9 hours under oxygen flow and unheated soybean oil into mouse chow to create experimental group diet and control group diet. The mean fasting blood glucose of 6 hours-oxidized oil group was significantly higher than the 3 hours-oxidized oil group. The mean serum insulin of 3 hours-oxidized oil group was

higher than 6 hours and 9 hours-oxidized oil group, while mean serum insulin of 6 hours-oxidized oil group was higher than 9 hours-oxidized oil group, but the differences were not significant. Researchers came to conclusion that higher amount of oxidized oil could either reduce insulin secretion or decrease insulin sensitivity, thus cause the hyperglycemia in mice [13].

4. Gas Chromatography

Gas chromatography (GC) is widely used for the separation of mixed volatile compounds. The retention time is the time for each compound to pass out the column in GC. Different compounds can be distinguished by their own retention time. There are several detectors for GC, one of them is flame ionization detector (FID). In GC-FID, compounds are burned to generate ions, which are measured via a change in detector potential. The electrical signals for those ions can be analyzed for the identification of those compounds [64]. In this study, GC was used to measure long chain n-3 PUFAs concentration in red blood cell and SCFAs concentration in cecal contents and fecal contents.

5. Application of the methods in this study

In this study, the Total Western Diet with different levels of MRPs were used as mice diet. Weight change was measured to see if MRPs can induce obesity. OGTT was conducted to investigate if MRPs can impair glucose tolerance. Gut microbiota and short chain fatty acids in cecal and fecal contents were analyzed to examine if MRPs will lower the diversity and alter the composition of gut microbiota, thus further change their

fermentation products. 1% dextran sodium sulfate was administrated to investigate if MRPs can alleviate DSS-induced colitis. Long chain n-3 PUFA was measured to see if MRPs can cause inflammation. A complementary experiment was conducted to test if the method of cooking whole diet pellets in previous studies could induce lipid oxidation in the commonly used DIO diet and TWD. As lipid oxidation was minimized in TWD in this study, we could confirm that the health effects detected on mice were induced by MRPs instead of lipid oxidation products.

III. HYPOTHESIS AND OBJECTIVES

Hypothesis

Mice fed with diet with higher level of MRPs will have increased weight gain, impaired glucose metabolism and intestinal damage. MRPs will lower the diversity of gut microbiota which is associated with a less healthy state, and alter the composition, thus further change the products from microbial fermentation. MRPs will cause inflammation. Lipid oxidation exists in the cooked diets from previous study, which makes it harder to prove if those health effects were caused by MRPs itself. In this study, we can confirm that the health effects are induced by MRPs.

Objectives

1. Determine the effect of dietary MRPs on the weight gain.
2. Determine the effect of dietary MRPs on the glucose tolerance.
3. Determine if dietary MRPs will alleviate or exacerbate DSS-induced colitis.
4. Determine the effect of dietary MRPs on the diversity, composition and fermentation products of gut microbiota.
5. Determine if dietary MRPs will induce inflammation.
6. Determine if the method of cooking whole diet pellets in previous studies can induce lipid oxidation.

IV. METHODS AND MATERIALS

1. Materials

1.1 Animals

Thirty Male C57BL6/J mice were acquired from Jackson Labs (Bar Harbor, ME). Their cages with clean bedding and water bottles were provided by LARC (Utah State University, Logan, UT).

1.2 Diets

Total Western Diet, casein, sucrose, glucose and fructose were provided by Envigo (Huntingdon, Cambridgeshire, United Kingdom).

1.3 Reagents

Dextran sodium sulfate was acquired from Alfa Aesar (Ward Hill, MA). Mouse insulin ELISA kit was purchased from Thermo Fisher Scientific (Frederic, MD). QIAmp Fast DNA Stool mini Kit was purchased from Qiagen (Germantown, MD). Potassium iodide was purchased from EMD Chemicals Inc. (Jersey City, New Jersey). P-Anisidine was purchased from Sigma-Aldrich Corporation (St. Louis, MO). Butylated hydroxytoluene was purchased from MP Biomedicals Inc. (Solon, OH). Metaphosphoric acid was purchased from Acros Organics (Jersey City, New Jersey). Starch was purchased from Avantor Performance Materials (Center Valley, PA). Potassium hydroxide, sulfuric acid, chloroform, hexanes, methanol, glacial acetic acid were purchased from Fisher Scientific (Hampton, NH).

1.4 Instrument

EchoMRI-700TM was purchased from EchoMRI (Houston, TX). Microwave oven (Model No. JES0738DP1BB; KW: 1.2 M/W) was purchased from General Electric Co. (Louisville, KY). GC2010 was purchased from Shimadzu Scientific Instruments (Columbia, MD).

2. Methods

2.1 Mouse study

In accordance with the formula of the Total Western diet (TWD), the mass ratio of casein, sucrose, glucose and fructose is 42:29:14.5:14.5. To make mice diet for control group (MRP0) and treatment groups (MRP1 and MRP2), part of casein, sucrose, glucose and fructose were isolated and mixed well based on original mass ratio. Casein, glucose and fructose were used to generate MRPs, while sucrose was added because it is the common carbohydrate resource for AIN-93G and DIO diet. Water was added in the mixed powder at 25% of total powder weight to produce a slurry. This mixture was then heated in microwave oven to 125 ˚C at one minute intervals for four minutes in total with stirring after each minute to generate MRPs. This process was followed until the slurry began to turn a shade of light brown according to the previous study in France. The color change of Maillard reaction of casein and reducing sugars is shown in Figure 4. The MRPs obtained from same degree of heat treatment was added back to the uncooked part of TWD at 0%, 20% or 40% of total mass. Based on the previous study in France, loss of lysine happened during Maillard reaction of casein, glucose and fructose. In order to avoid any health effect due to lysine loss, L-lysine was added to the diet which contained

MRPs. The formula of MRP0, MRP1 and MRP2 are shown in Table 3. The experimental part consists of cooked casein and sugars, uncooked casein and sugars, and complementary lysine. The control part consists of the rest uncooked part of the diet, including all the lipids.

After a few days in quarantine, mice were randomly divided into three groups (n=10/gp) that were fed the Total Western Diet (TWD) with different levels of MRPs: MRP0, TWD with 0% MRPs; MRP1, TWD with 20% MRPs, and MRP2, TWD with 40% MRPs.

Weight was recorded weekly, and food intake was recorded twice per week. A Magnetic resonance imaging (MRI) scan was performed to measure the body composition of mice before they received the experimental diets for 10 weeks. A second MRI scan was administered at week 10.

After the second MRI scan, mice were subjected to an Oral Glucose Tolerance Test (OGTT). Mice received 200 mg glucose/ml solution by gavage. The blood glucose concentration at 0 min, 15 min, 30 min, 60 min, 90 min and 120 min were measured. At each time point, triplicate measurements were made.

After one week of recovery from OGTT, mice received 1% dextran sodium sulfate (DSS) solution in the drinking water from week 12 to week 13 for ten days to induce colitis. To investigate if MRPs had an impact on the DSS-induced colitis, a colitis score was determined each day. After 10 days of oral administration of DSS, mice were sacrificed. Plasma, red blood cells, colon, cecal samples and fecal samples were collected and stored at -80 ˚C until further analysis. The process of the animal study is shown in Figure 4.

2.2 Complementary experiment of lipid oxidation

A complementary experiment was conducted to demonstrate the effect of the cooking method of baking whole diet pellets used in previous studies on the lipids in TWD and DIO diet. Eight samples for each diet were prepared, in which half of them were used as control group (uncooked) and another half of them were used as experimental group (cooked). In this experiment, the whole diet instead of sugars and casein themselves were heated at 120℃ for 30 min in oven to induce Maillard Reaction. After the heat treatment, the Peroxide value and p-Anisidine value were measured on uncooked DIO diet / TWD and cooked DIO diet / TWD.

Table 3. Formula of MRP0, MRP1 and MRP2

g/kg	MRP0	MRP1	MRP2
Experimental part			
Casein	190	101.3	12.7
Sucrose	131	71.95	13
Dextrose, anhydrous	65.5	36.025	6.55
Fructose	65.5	36.025	6.55
L-Lysine HCL	0	3.2	6.3
Browned mix of Casein and Sugars	0	203.4	406.8
Experimental part total	452	452	452
Control part			
L-Cystine	2.172	2.172	2.172
Corn starch	230	230	230
Maltodextrin	70	70	70
Cellulose	30	30	30
Olive oil	28	28	28
Soybean oil	31.4	31.4	31.4
Corn oil	16.5	16.5	16.5
Lard	28	28	28
Beef tallow	24.8	24.8	24.8
Anhydrous milkfat	36.3	36.3	36.3
Cholesterol	0.4	0.4	0.4
Mineral mix, nTWD (110422)	35	35	35
Sodium chloride	4	4	4
Vitamin mix, nTWD (110423)	10	10	10
Choline bitartrate	1.4	1.4	1.4
TBHQ, antioxidant	0.028	0.028	0.028
Control part total	548	548	548

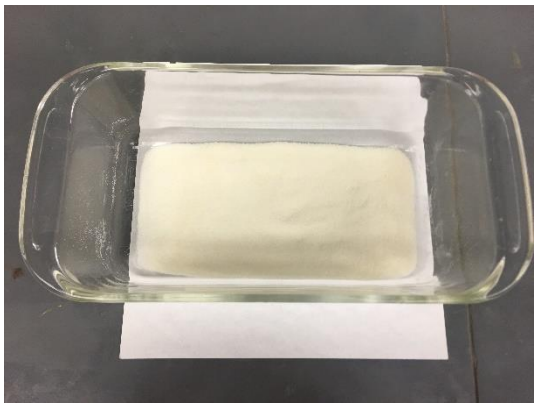

Figure 4A. Mixed powder of casein, reducing sugars and sucrose

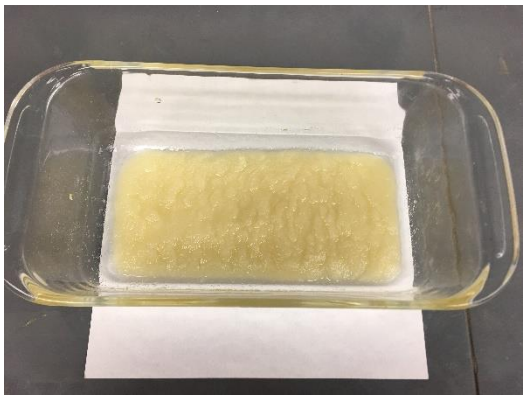

Figure 4B. Slurry of casein, reducing sugars and sucrose

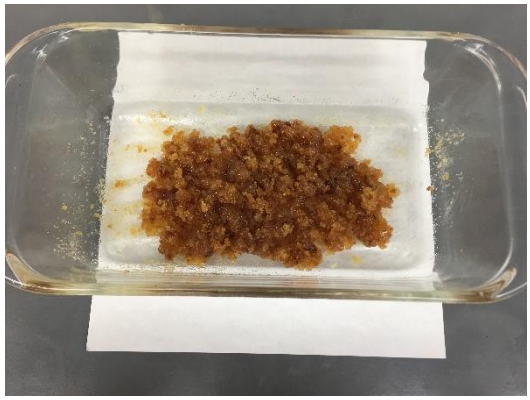

Figure 4C. Mixture of Maillard reaction products after heat treatment

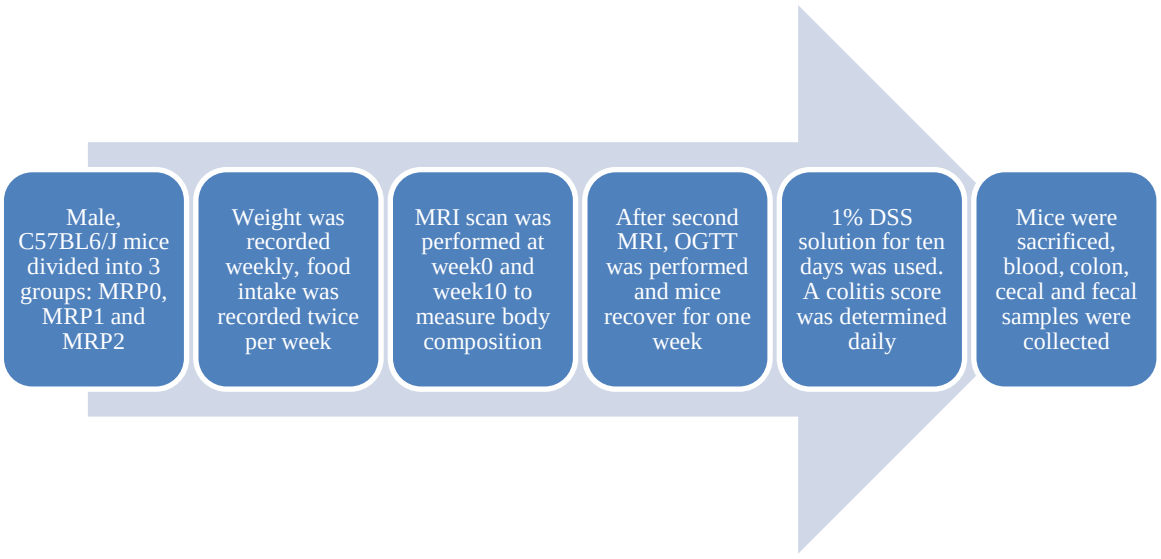

Figure 5. Process of mouse study

2.3 Chemistry Analysis

2.3.1 Body composition

The fat mass and lean mass of all the mice from three groups were measured by EchoMRI system. Their body composition data from the baseline (week 0) and the terminal (week 10) were collected.

2.3.2 Red blood cell concentrations of long chain n-3 PUFAs

The method for measuring long chain n-3 PUFAs in red blood cell was a modification of that of O'Fallon et al [65]. Approximately 50 mg of red blood cell sample were weighed for analysis. The samples were mixed with 350 µL of 10 N KOH and 3.15 mL methanol, and the solution were heated at 55˚C for 1.5 hours. Then the samples were cooled at room temperature. 290 µL of 24 N H_2SO_4 were added to each sample. After another 1.5 hours heat at 55 ˚C, the samples were mixed with 1 mL hexane and vortexed for 30 seconds. The samples were then centrifuged at 2000g for 5 minutes. The top layer was added to GC vial and dried under nitrogen. Then the samples were dissolved in 200

μL of hexane with BHT and injected to GC for analysis of long chain n-3 PUFAs. GC2010 (Shimadzu Scientific Instruments, Columbia, MD) was used for GC analysis.

2.3.3 Short chain fatty acids (SCFAs) concentration in cecal contents and fecal contents

Measuring SCFAs concentration in cecal and fecal contents was based on the method of Ward et al [66]. Approximately 25 mg of cecal samples or fecal samples were mixed with 1 mL of deionized water. 200 μL of metaphosphoric acid/ethyl butyric acid were added to the samples. The samples were centrifuged at 13k for 20 minutes, and the supernatant were injected to gas chromatography with flame ionization detection (GC-FID) for analysis of short chain fatty acids, including acetic, butyric, propionic, isovaleric, caproic, valeric, and isobutyric acid. GC2010 (Shimadzu Scientific Instruments, Columbia, MD) was used for GC analysis.

2.3.4 Gut microbiome of fecal contents and cecal contents

Bacterial DNA in approximately 100 mg fecal sample or cecal sample was extracted using QIAmp Fast DNA Stool mini Kit. Next, normalization and amplification of DNA samples were performed by polymerase chain reaction (PCR). QIIME version 1.9 was used to analyze microbiota sequences. After integrity check, filtering and normalization of data, alpha and beta diversity analysis and classical univariate analysis were performed on all taxonomic levels. Alpha diversity shows the species abundance in one microbial ecosystem, Chao 1 was used for diversity measurement. It takes unobserved species based on low-abundance OTUs into consideration besides observed

OTUs, so that it provides the richness of OTUs within one sample. Beta diversity is used to define how many distinct compositional units are in the site, the dissimilarity was indicated by Bray-Curtis index. Based on the calculation of Bray-Curtis index, the result lies between 0 and 1, higher number means higher dissimilarity. Principal coordinates analysis (PCoA) graph was used for showing the dissimilarities between three groups. Classical univariate analysis provides the information of significant differences on all taxonomic levels.

2.3.5 Histology of colon samples

Colon samples were examined and rated by a histologist at the Utah Veterinary Diagnostics Lab. The scores for colon samples were based on four parameters: inflammation, depth of inflammation, epithelial regeneration and crypt damage. Inflammation and depth of inflammation represent severity and distribution of inflammation respectively. A higher score means a more severe or more extensive inflammation. For the overall score of colon inflammation, inflammation parameters were added up and multiplied by the percentage of the colon involved. Crypt damage shows the extent of destroyed tissues, the score goes higher with more damage. Epithelial regeneration shows if the damaged tissue is repaired or not, the score goes higher with less repair. For the overall score of epithelium assessment, these two parameters were added up and multiplied by the percentage of the colon involved.

2.3.6 Plasma insulin concentration

Mouse insulin ELISA (enzyme-linked immunosorbent assay) kit was used to

measure the plasma insulin concentration of mice. Insulin acted as the target in this test. Plasma samples were added to bind with the primary antibody for insulin on the plate. Then the secondary antibody linked with an enzyme was added to form an enzyme-antibody-target complex. At last the substrate of the enzyme was added to reacts with the complex to produce a detectable color change which can be used to measure the concentration of plasma insulin.

2.3.7 Peroxide value, p-Anisidine value and Totox value

To determine the lipid oxidation level of uncooked or cooked DIO diet and TWD, peroxide value (PV) and p-Anisidine value (AnV) were measured. The Totox value reveals the overall lipid oxidation level. It can be calculated by the following equation:

$$\text{Totox value} = 2PV + AnV$$

Peroxide value of the lipid was examined based on the AOCS Official Methods and Recommended Practices [67]. A 3.5 g of lipid sample was added into a 250 mL Erlenmeyer flask with the glass stopper. Thirty mL of the acetic acid – chloroform solution (volume ratio 3:2) was added to completely dissolve the lipid sample. After 0.5 mL of saturated potassium iodide solution was added, the flask was stoppered and shaken for exactly 1 min. Then after 30 mL of distilled water was added, the flask was stoppered and shaken again to liberate the iodine from the chloroform layer. 1 mL of 1% starch solution was added as indicator. The solution was titrated with 0.1 N sodium thiosulfate until the blue color just disappeared. The peroxide value was calculated by the volume of sodium thiosulfate used and the weight of lipid sample. The rationale of this test can be explained by the following equation:

$$2\ I^- + H_2O + HOOH \Rightarrow HOH + 2\ OH^- + I_2$$

$$2\ S_2O_3^{2-} + I_2 \Rightarrow S_4O_6^{2-} + 2\ I^-$$

P-Anisidine value of the lipid was tested based on the AOCS Official Methods and Recommended Practices [68]. A 0.5 g of lipid sample was added to a 25 mL volumetric flask. Iso-octane was added to dissolve the sample and make up to volume. The absorbance of the lipid solution against pure iso-octane (Ea) was measured at 350 nm. 5 mL of lipid solution and pure iso-octane were removed to test tube A and B respectively. 1 mL of Anisidine reagent (0.25% p-Anisidine in acetic acid) was added into test tube A and B. After being stoppered and shaken, the tubes were left in dark for exactly 10 min. The absorbance of the content of test tube A against test tube B (Eb) was measured at 350 nm. The p-Anisidine value was calculated by Ea, Eb and the weight of lipid sample (W) based on the following equation:

$$AnV = 25 \times (1.2 \times Eb - Ea) / W$$

2.4 Statistical analysis

One-way ANOVA, student's t test and repeated measures ANOVA were used for the analysis of differences of means. A P value<0.05 was used for the definition of statistically significant difference. Data are expressed as mean ± standard error (SE) of the mean. Significant differences between groups are indicated by different superscripts ($p<0.05$). Triplicate measurements were made in OGTT, duplicate measurements were made in complementary experiments of lipid oxidation. Single measurements were made in other experiments.

V. RESULTS

Weight gain and Food intake

Mouse weight gain and food intake were analyzed. No significant differences in weight gain or their food intake were found between three groups from Week 0 to Week 13, which means MRPs did not induce obesity.

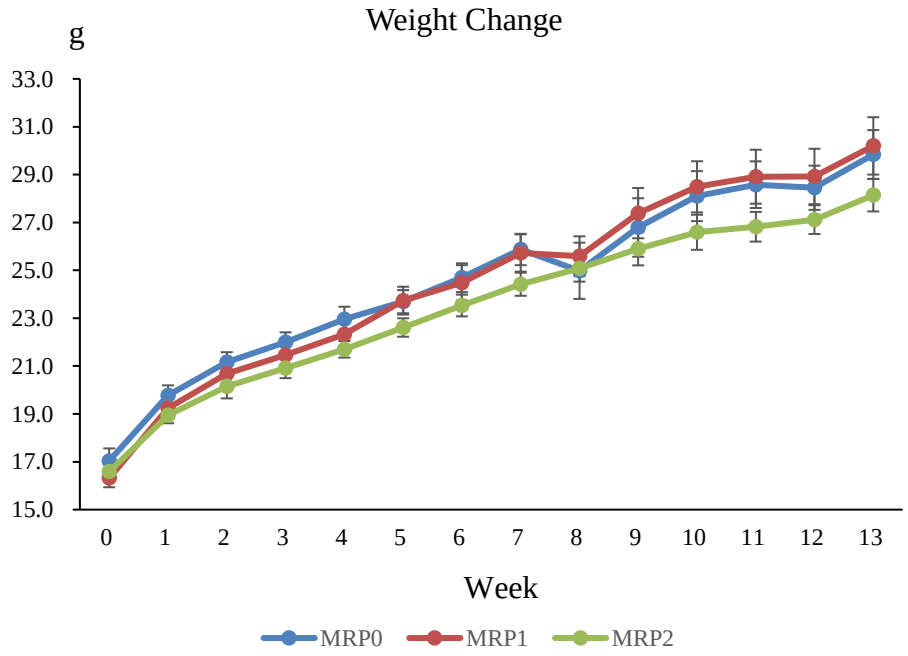

Figure 6. Weight change of mice from week 0 to week 13, error bar represents the standard error of mean.

Table 4. The p values for food intake in 13 weeks between three groups, a p value less than 0.05 is considered as significantly different.

Week	1	2	3	4	5	6	7
P value	0.47	0.32	0.18	0.49	0.66	0.79	0.85
Week	8	9	10	11	12	13	
P value	0.44	0.28	0.91	0.61	0.52	0.31	

Body composition

Based on the results from first MRI scan at week 1 (baseline) and second MRI scan at week 10 (terminal), no significant differences were detected between three groups in fat mass, lean mass or fat / lean ratio, which means intake of MRPs did not make mice gain more fat mass or less lean mass. However, when comparing fat mass and lean mass at baseline with these indexes at terminal within the group, significant differences were found in all three groups, which was due to growth. Mice fed the MRP1 diet has a significantly higher fat/lean mass ratio.

Table 5. Mice fat mass, lean mass or fat / lean ratio at baseline and terminal, data are expressed as mean ± SE, significant differences between groups are indicated by different superscripts ($p<0.05$).

	Baseline fat mass (g)	Terminal fat mass (g)	p value
MRP0	1.91 ± 0.32^{a}	5.76 ± 0.82^{b}	0.0003
MRP1	2.71 ± 0.51^{a}	6.12 ± 0.86^{b}	0.0035
MRP2	2.13 ± 0.06^{a}	4.96 ± 0.63^{b}	0.0003

	Baseline lean mass (g)	Terminal lean mass (g)	p value
MRP0	15.68 ± 0.52^{a}	19.13 ± 0.51^{b}	0.0002
MRP1	14.25 ± 0.89^{a}	19.55 ± 0.26^{b}	<0.0001
MRP2	15.02 ± 0.43^{a}	19.29 ± 0.22^{b}	<0.0001

	Baseline fat / lean	Terminal fat / lean	p value
MRP0	0.126 ± 0.019^{a}	0.297 ± 0.039^{b}	0.001
MRP1	0.227 ± 0.081^{a}	0.311 ± 0.041^{a}	0.368
MRP2	0.143 ± 0.005^{a}	0.258 ± 0.033^{b}	0.003

Oral glucose tolerance test (OGTT)

Blood glucose concentration from 6 time points were collected during 120 min after mice were gavaged with glucose. Normalized area under the curve (AUC) represents the area between the baseline (y = value of starting point) and the curve. Normalized AUC of blood glucose indicates the overall change in blood glucose concentration during study period. It can be calculated by summing up the areas of triangles and trapezoids between every two adjacent time points. Figure 7 shows the curves of blood glucose concentration change for three groups. Normalized AUC is shown in Table 6, it decreased with the increasing MRPs level in mice diet. In addition, normalized AUC was significantly higher in MRP0 group than in MRP2 group ($p < 0.05$), which means the mice in MRP0 group had significantly higher overall incremental blood glucose concentration during 120 minutes than the mice in MRP2 group. The result showed that intake of MRPs can improve glucose metabolism.

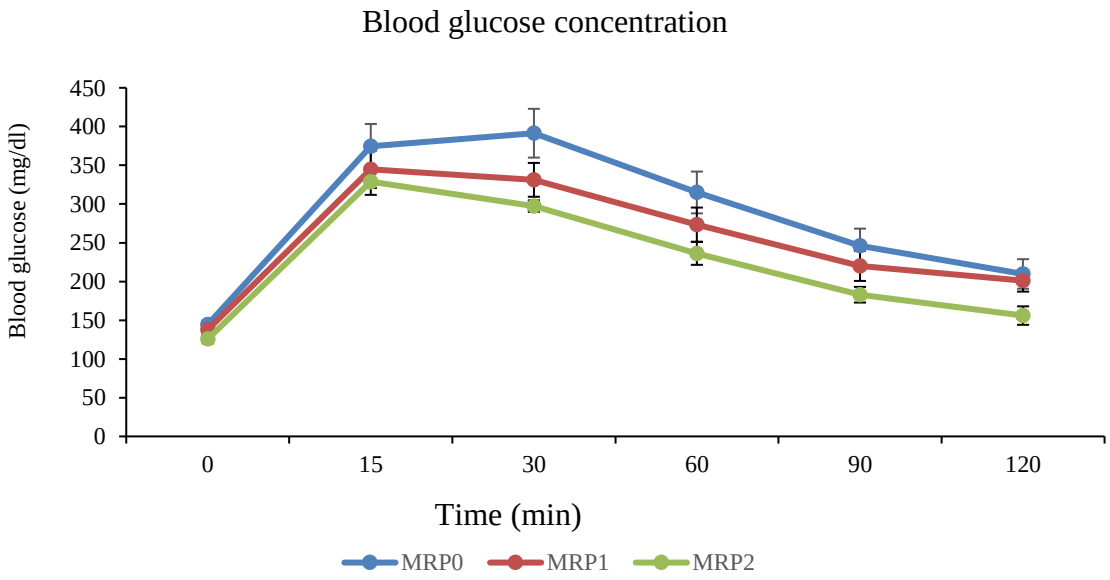

Figure 7. Trend chart of mice blood glucose during 120 min after OGTT

Table 6. Normalized AUC of blood sugar, data are expressed as mean ± SE, significant differences between groups are indicated by different superscripts ($p<0.05$).

	MRP0	MRP1	MRP2
Normalized AUC	18138 ± 2089^{a}	14946 ± 1802^{ab}	12385 ± 1120^{b}

Red blood cell concentrations of long chain n-3 PUFAs and plasma insulin

There was no significant difference in EPA and DHA in red blood cells among three groups, which demonstrated that dietary MRPs did not cause inflammation. No significant difference of plasma insulin among three groups was found, which illustrated that intake of MRPs did not change the insulin secretion.

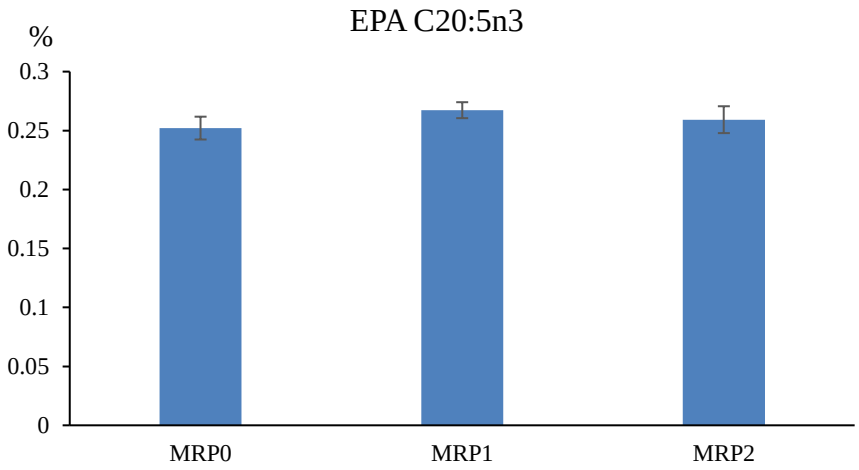

Figure 8A. Percentage of EPA concentration for three groups

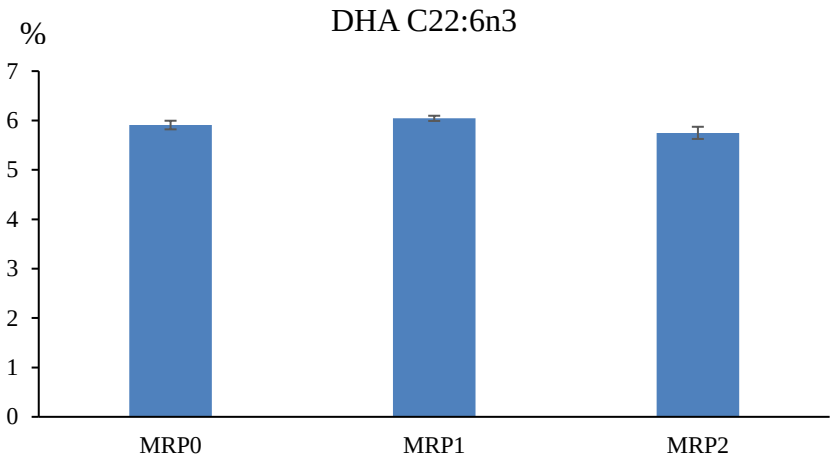

Figure 8B. Percentage of DHA concentration for three groups

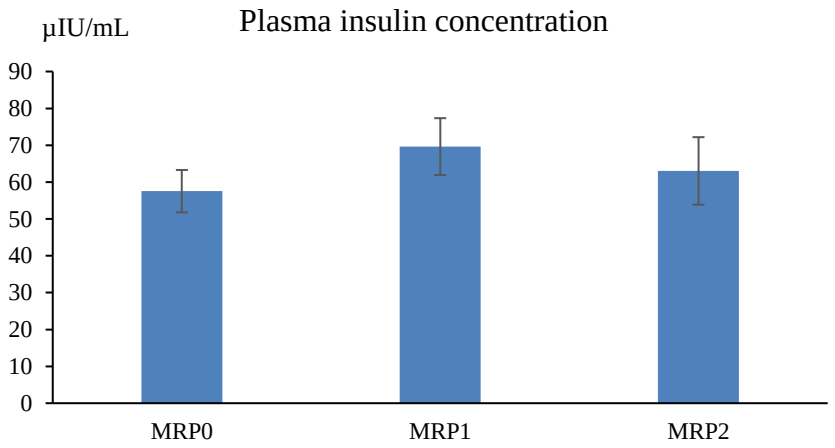

Figure 9. Plasma insulin concentration for three groups

Histology and colitis score of mice colon

No significant difference was found in overall score of colon inflammation or overall score of epithelium assessment among three groups. During the ten days administration of DSS in water, no significant differences of colitis score were shown in three groups. The result revealed that dietary MRPs did not increase susceptibility to DSS-induced colitis.

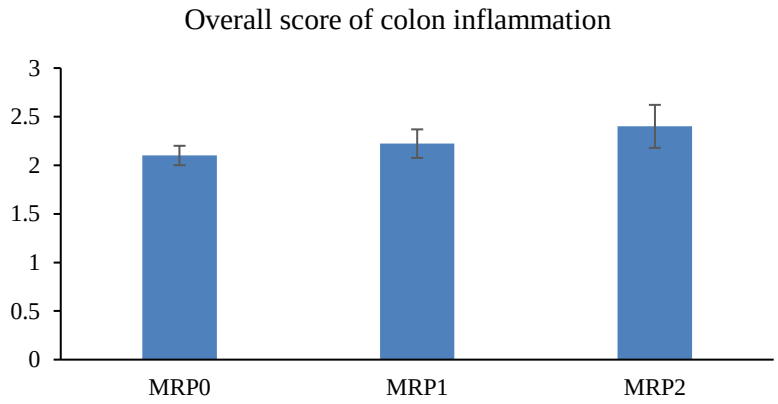

Figure 10A. Overall score of colon inflammation for three groups

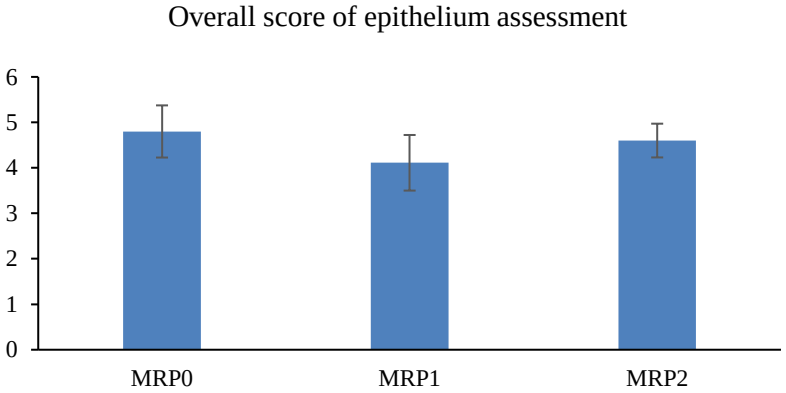

Figure 10B. Overall score of epithelium assessment for three groups

Table 7. The p values for colitis score in 10 days between three groups, significant differences between groups are indicated by different superscripts ($p<0.05$).

Day	1	2	3	4	5
P value	0.18	0.68	0.19	0.26	0.31
Day	6	7	8	9	10
P value	0.22	0.41	0.52	0.45	0.49

Gut microbiome

No significant difference in alpha diversity of cecal microbiome at any taxonomic level was observed. Significant differences in alpha diversity of fecal microbiome at Class level ($p < 0.01$) and Phylum level ($p < 0.01$) were observed. At both levels, MRP0 had significantly higher alpha diversity of fecal microbiome than MRP1 and MRP2. The result demonstrated that dietary MRPs lowered the alpha diversity of fecal microbiome, which is associated with a less healthy condition.

Table 8. P value for Alpha diversity of cecal and fecal microbiome at all taxonomic levels

	OTU	Species	Genus	Family	Order	Class	Phylum
Cecal	0.802	0.402	0.485	0.417	0.316	0.443	0.443
Fecal	0.286	0.065	0.054	0.128	0.209	0.008	0.008

Significant effects of overall treatment on beta diversity of fecal microbiome ($p < 0.001$) and cecal microbiome ($p < 0.004$) were observed at all taxonomic levels (OTU level, Species, Genus, Family, Order, Class and Phylum). In Figure 11, dots in different colors representing different groups showed the beta diversity of each group had distinct distribution, which further indicated that significant differences of beta diversity were found between three groups. The result showed that dietary MRPs changed the gut microbiota composition.

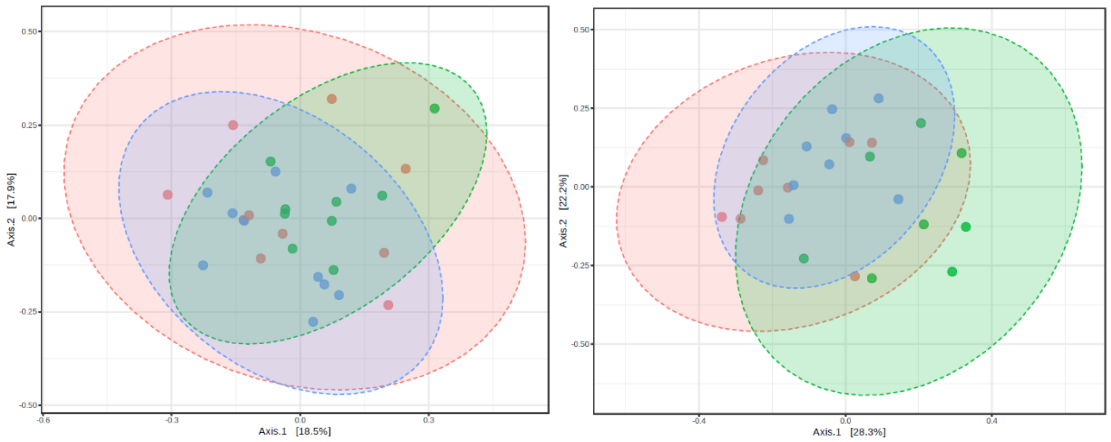

Figure 11. 2D plot for Beta diversity of cecal microbiome (left) and fecal microbiome (right) at OTU level, blue dots represent MRP0, green dots represent MRP1, red dots represent MRP2.

Different levels of MRPs in the TWD showed significantly different effects on taxonomic abundance of cecal microbiome at OTU level, Species level and Phylum level, and on taxonomic abundance of fecal microbiome at all other taxonomic levels except Species level. Data in Table 9 and 10 shows the specific changes of cecal and fecal microbiome composition.

In cecal contents, significant differences at Phylum and Species level were observed between three groups. *Verrucomicrobia* abundance was significantly higher in MRP0 than MRP1 and MRP2. *Gnavus* abundance was significantly lower in MRP0 than MRP1 and MRP2, while *Muciniphila* abundance was significantly higher in MRP0 than MRP1 and MRP2.

In fecal contents, significant differences at Phylum, Class, Order, Family and Genus level were observed between three groups. Abundance of *Bacilli*, *Lactobacillales*, *Streptococcaceae* and *Lactococcus* significantly decreased with the increasing level of MRPs in diet.

Table 9. Taxonomic abundance of cecal microbiome

Taxa	p value (FDR corrected)
Phylum	
p__*Verrucomicrobia*	0.0399
Species	
s__*gnavus*	0.016
s__*muciniphila*	0.016

Table 10. Taxonomic abundance of fecal microbiome

Taxa	p value (FDR corrected)
Phylum	
p__*Tenericutes*	0.0499
Class	
c__*Bacilli*	0.0013
c__*Mollicutes*	0.0374
Order	
o__*Lactobacillales*	0.0009
o__*Turicibacterales*	0.0162
o__*Anaeroplasmatales*	0.0305
Family	
f__*Anaeroplasmataceae*	0.0436
f__*Clostridiaceae*	0.0395
f__*Streptococcaceae*	0.0023
f__*Turicibacteraceae*	0.031
Genus	
g__*Anaeroplasma*	0.0364
g__*Coprobacillus*	0.0327
g__*Lactococcus*	0.0023
g__*Oscillospira*	0.0364
g__*Turicibacter*	0.031

Short chain fatty acids (SCFAs) in cecal and fecal contents

In cecal samples, valeric acid of MRP0 and MRP2 were significantly higher than that of MRP1, propionic acid of MRP2 was significantly higher than that of MRP0 and MRP1, and butyric acid of MRP0 and MRP2 were significantly higher than that of MRP1. In fecal contents, valeric acid of MRP0 was significantly lower than that of MRP1 and MRP2, and propionic acid of MRP0 was significantly lower than that of MRP1. The result illustrated that the microbial fermentation products were changed by dietary MRPs, which may due to lower alpha diversity and altered gut microbiota composition.

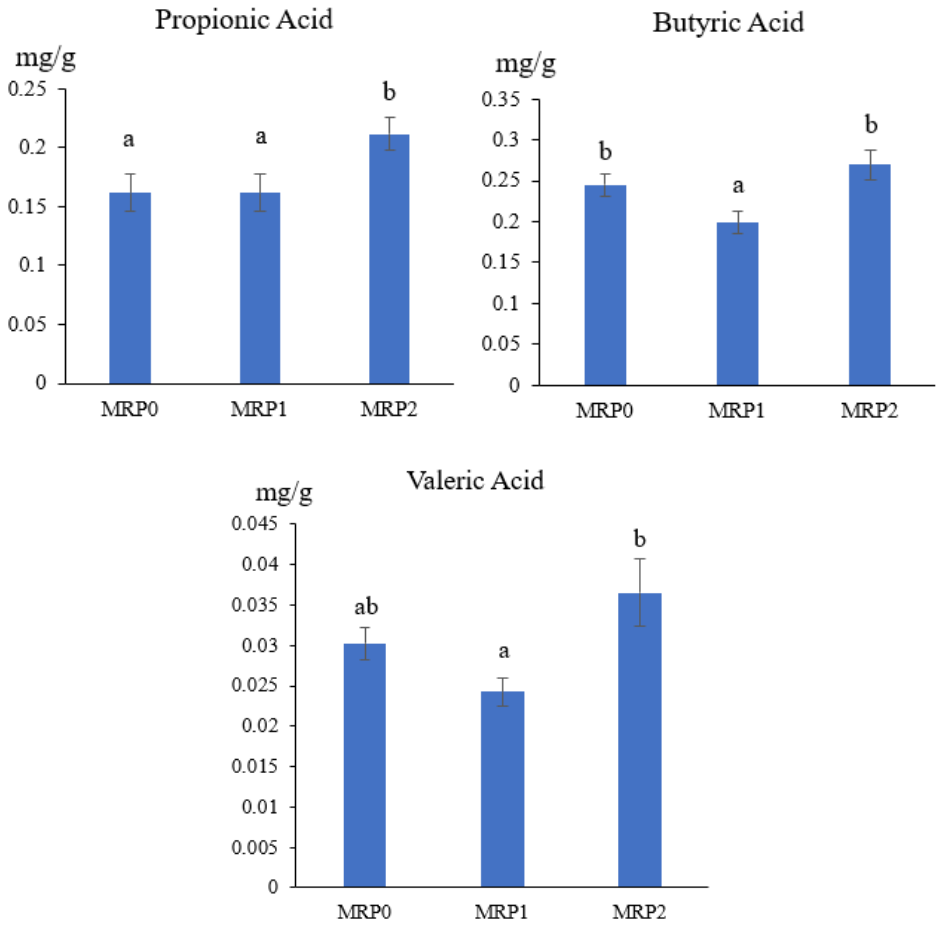

Figure 12A. Propionic acid, butyric acid and valeric acid in cecal contents, significant differences between groups are indicated by different superscripts ($p<0.05$).

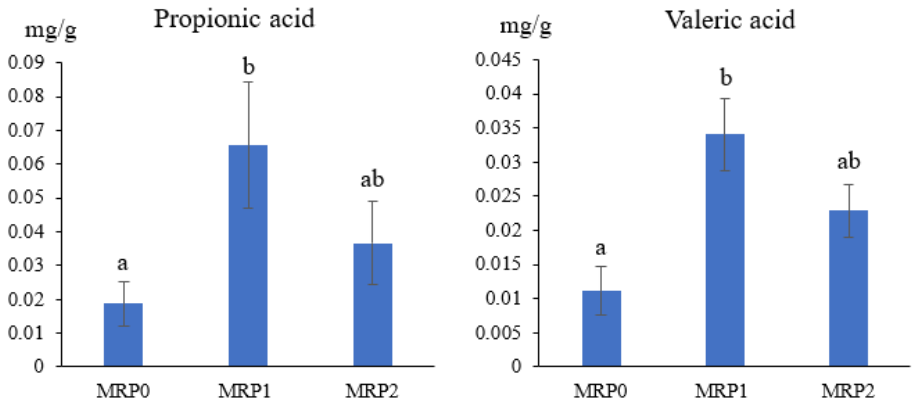

Figure 12B. Propionic acid and valeric acid in fecal contents, significant differences between groups are indicated by different superscripts ($p<0.05$).

Lipid oxidation in DIO and TWD

Peroxide value, p-Anisidine value and Totox value of uncooked or cooked TWD or DIO diet were shown in figure 13. According to table 11, Totox value of cooked TWD and DIO diet were significantly higher than uncooked TWD and DIO diet respectively. The results demonstrated that the method of cooking the whole diets at high temperature for a long time in previous studies could induce high level of lipid oxidation in rodent diets.

Table 11. P value of Peroxide value, p-Anisidine value and Totox value of uncooked / cooked TWD and uncooked / cooked DIO

	PV	AnV	Totox value
TWD	<0.0001	<0.0001	<0.0001
DIO	<0.0001	<0.0001	<0.0001

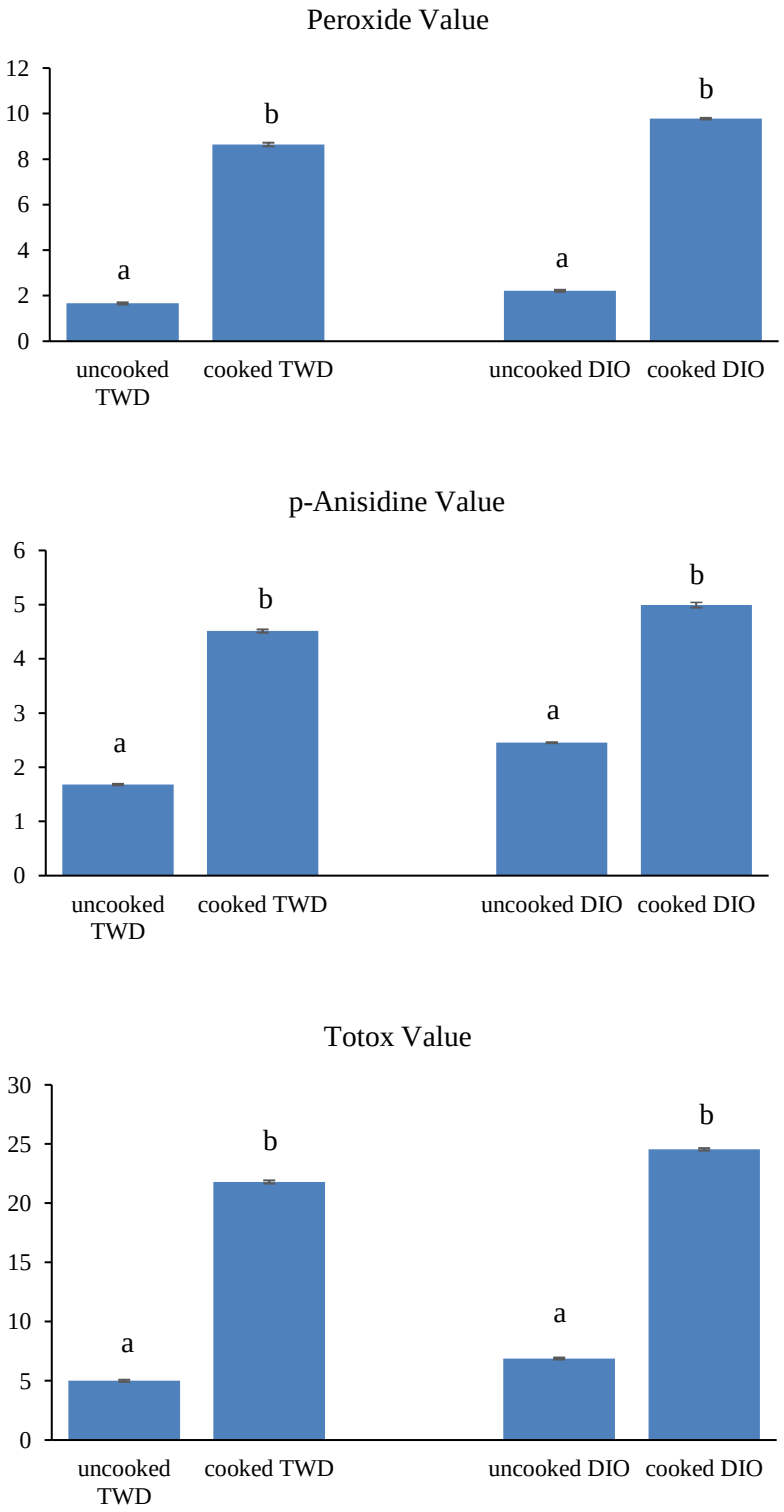

Figure 13. Peroxide, p-Anisidine and Totox value of uncooked or cooked diets

VI. DISCUSSION

In this study, the effects of MRPs on weight gain, glucose metabolism and intestinal health were investigated.

Many studies demonstrated that the intake of high MRP content diet significantly increased weight gain [23] [24] [25]. However, some other studies concluded that no significant difference was found in weight gain in mice fed diets with different levels of MRPs [26] [27], which is in agreement with the results of this study.

The mice had significantly higher fat mass and lean mass after 10 weeks of the experiment, which can be explained by general growth. No significant differences were detected between three groups in fat mass, lean mass or fat / lean ratio, which means intake of MRPs did not differentially affect accretion of fat or lean mass.

The results of OGTT indicate that the dietary MRPs significantly lowered total blood glucose during 120 minutes after oral administration. This result indicates that increasing intake of MRPs increases either insulin secretion, insulin sensitivity in target tissues, or both.

Most human studies and rodent studies have consistently found that dietary MRPs has the opposite effect, in that they appear to decrease insulin sensitivity [8] [23] [24]. However, the results of one rodent study [26] and one human study [28] showed that dietary MRPs have no effect on insulin sensitivity.

In a conclusion, the results of this study showed that MRPs may have beneficial effect on insulin sensitivity in mice. This is different with and even opposite to what we have learned in the previous studies [8] [23] [24] [26] [28]. To demonstrate the effect of the cooking method of baking whole diet pellets used in previous studies on the lipids in

TWD and DIO diet, a complementary experiment was conducted. A significantly higher level of lipid oxidation was observed in both cooked DIO diet and TWD comparing to uncooked DIO diet and TWD. When it comes to lipid oxidation, evidences of the negative effect of lipid oxidation products on insulin sensitivity can be found in previous animal studies. As in previous work, researchers usually cooked the whole diet to generate MRPs, thus they may ignore the occurrence of lipid oxidation in those cooked diets. Moreover, as DIO diet was widely used in the previous studies related to MRPs, the level of lipid oxidation may be much higher due to the high fat content. In this study, lipid oxidation in mice diet was minimized by only cooking sugars and casein. Whether lipid oxidation products abundant in the diets or not may be the reason of the opposite results of this study and the previous studies. Because this study was focused on the effect of MRPs which is mainly generated from the Maillard Reaction, the newly developed diet treatment method can give us a clear understanding on how MRPs itself influence insulin sensitivity. Another possible reason for the positive effect on insulin sensitivity in this study may be the antioxidant activity of MRPs revealed by some *in vitro* studies, including investigating how MRPs reacted to lipid oxidation in oil-in-water [68] and water-in-oil [69] emulsion system. Those studies demonstrated that one of the rationales of MRPs preventing lipid oxidation was to inhibit free radicals. In human bodies, aerobic respiration can cause the generation of reactive oxygen species (ROS) [70], which is one kind of free radicals [71]. ROS can impair insulin sensitivity by restraining the insulin response [72]. If MRPs can inhibit ROS, then it may contribute to the improvement of insulin sensitivity.

No obvious symptom of colitis was shown in any group after 10 days of oral

administration of 1% DSS solution. No obvious colon inflammation or epithelium damage was shown in any group either. MRPs did not increase susceptibility to DSS-induced colitis, which means MRPs did not have negative effect on mucosal immune system.

According to the result of cecal and fecal microbiome, at class level and phylum level, alpha diversity of fecal microbiome significantly decreased with increasing amount of dietary MRPs, which means species richness was lowered by more dietary MRPs. As lower alpha diversity of microbiome is associated with obesity in previous human studies [32], MRPs may have negative effect on mice health by causing lower alpha diversity. There was also a significant difference in beta diversity. The result demonstrated that MRPs induced the gut microbiome composition variation among the groups. Significant differences in taxonomic abundance of cecal and fecal microbiome at different taxonomic levels revealed dietary MRPs could change the amount of some gut microbes. One possible reason is that MRPs decrease protein digestion and more proteins enter the colon. Thus, the specific species who survive on fermenting protein can grow and multiply faster. Another possible reason is that MRPs itself can be metabolized by some species that provide those species with more resources to grow and multiply. The original balance between all species may be broken by those fast-growing species, so that a new balance needs to be achieved. Thus, dietary MRPs may eventually change the composition of gut microbiota.

As to the short chain fatty acids, their concentrations in cecal and fecal contents were significantly different according to different levels of MRPs in diet. Previous studies demonstrated that these short chain fatty acids could play a role in improving

insulin sensitivity in mice, human or cells [40] [41] [42] [43] [44]. In this study, different levels of MRPs in diet induced different concentrations of short chain fatty acids in mice cecal and fecal contents. However, the changes of different SCFAs concentration had different trends, which makes it hard to come to conclusion whether MRPs have a negative or positive effect on mice insulin sensitivity.

In addition, as SCFAs are the products of microbial fermentation, altered gut microbiome species interacting with changed amounts of substrates can possibly form different amount of SCFAs, which can explain the change in SCFAs concentration.

VII. CONCLUSION

In conclusion, MRPs have positive effect on glucose metabolism. It did not induce obesity or increased susceptibility to DSS-induced colitis. It may have negative effect on mice health due to lower alpha diversity. As beta diversity of microbiota and SCFAs in fecal and cecal contents changed, MRPs may interact with gut microbiota to change the microbiota species and fermentation products.

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