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The Effects of Increasing Dietary Fat on Serum Lipid Profile and Modification of Gut Microbiome in C57BL/6N Mice

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Abstract: Hyperlipidemia is a condition where the blood shows an elevated level of lipid, such as cholesterol and triglyceride. It is considered a risk factor for all coronary artery death globally. Association of microbiome with non-communicable diseases (NCDs) including hyperlipidemia has been reportedly associated. In this study, we hypothesize that the change in microbiome is correlated to the change in serum lipid level, which resulted by increasing dietary fat consumption. The 32 male, 14-week-old, C57BL/6N were divided into 4 groups, each group received control diet, 10%, 20%, and 40% kcal fat diet prepared from purified pork lard, respectively for 28 days. Fasting serum lipids and fecal microbiome were then analyzed. The group of animals assigned to 40% kcal fat showed significantly increased serum cholesterol, LDL, and HDL ($p < 0.05$). Microbiome analysis revealed the abundance of *Muribaculaceae* and *Saccharimonadaceae* were significantly decreased ($p < 0.05$). On the contrary, the abundance of *Clostridia_UCG014*, *Akkermansiaceae*, *Bacteroidaceae*, *Oscillospiraceae*, and *Erysipelotrichaceae* were significantly increased ($p < 0.05$). Spearman correlation indicated that the abundance of *Akkermansiaceae* and *Bacteroidaceae* were positively associated with the increased of serum cholesterol and LDL ($p < 0.05$), while the abundance of *Muribaculaceae*, *Clostridia_UCG-014*, and *Saccharimonadaceae* were negatively associated ($p < 0.05$). These results suggest that dietary fat have ability to manipulated microbiome with relative to elevation of serum lipid profile.

Key words: hyperlipidemia, gut microbiome, mice model

1 Introduction

Cardiovascular disease (CVD) is recognized as one of the worldwide leading causes of death. With the total deaths of 17.9 million in 2016, CVDs account for approximately 31% of all global deaths[1)]. One of the most commonly known CVDs risk factors, hyperlipidemia[2)], is the most intense field of medical research for CVDs prevention and manipulation.

Hyperlipidemia, the most recognized CVDs risk factors, refers to an elevation of either blood total cholesterol (hypercholesterolemia), or blood triglyceride (hypertriglyceridemia). First line of defense against hyperlipidemia is lifestyle changes, including consumption of healthy diet and exercise. When it comes to drug treatment, statin is the most common prescription drug against hypercholesterolemia, which works by inhibition of HMG-CoA reductase, the key enzyme in cholesterol synthesis in the body. Ezetimibe, fibrates, and niacin are also important as prescriptive drugs against hypercholesterolemia[3)]. On the other hand, treatment of hypertriglyceridemia is much more complicate, and untreated hypertriglyceridemia usually progress to the onset of non-alcoholic fatty liver disease (NAFLD)[4)].

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High fat diet (HFD) containing 40% fat calorie have been widely used as hyperlipidemia-induced experimental diet since it can effectively increase serum lipid profile in C57BL/6N[5]. It also suitable in both short term and long term induction of hyperlipidemia[6]. Similar to human diet, Western diet normally contains 30–40% fat calories, higher than other types of diet, for example, Asian diet, which contains 20–30% fat calories[7]. Based on the differences of dietary fat level in different diet regimens, it seems that Asian diet is superior than Western diet in term of health benefit.

Manipulation of gut microbiome have also been intensely studied to discover the potential microbiome associated with improvement of hyperlipidemia in HFD-fed mice. High abundance of *Allobaculum*, *Bifidobacterium*, and *Coriobacteriaceae* was found to be associated with lean phenotype, including reduced cholesterol and triglyceride in HFD-fed mice[8]. Short-chain fatty acids (SCFA) producing bacteria including *Ruminococcaceae* and *Lachnospiraceae* were enhanced in HFD-fed mice with low cholesterol and triglyceride[9].

In this current preclinical study, we aim to investigate the correlations between increasing dietary fat level, serum lipid profiles and changes in microbiome of adult C57BL/6N. These correlations will enable us to better understand the effect of hyperlipidemia on changes of microbiome for further research.

2 Materials and Methods

2.1 Animals and diets

The animal study protocol was approved by National Laboratory Animal Center Animal Care and Use Committee (NLAC-ACUC), National Laboratory Animal Center, Mahidol University, Thailand under the protocol number RA2019-65. Thirty two 14-week-old male C57BL/6N mice were purchased from Nomura Siam International (Bangkok, Thailand). Mice were housed in individual cage with shredded paper strips provided as enrichment. Mice were allowed ad libitum food and water. The room temperature was set at 22.3℃ and 30-70% relative humidity, with a 12 hours light cycle. The animal room is equipped with positive pressure ventilation system.

2.2 Diet preparation

Following 1 week quarantine and 1 week adaptation to the environment, mice were randomly divided into 4 dietary groups as follows: mice fed a commercial rodent diet (082 diet, Perfect Companion Group Co., Ltd., Bangkok, Thailand) (Control group), mice fed a 10% fat diet (10% fat), mice fed a 20% fat diet (20% fat), and mice fed a 40% fat diet (40% fat). The dietary fat level of the control diet was 12.5%. The palm oil was used in preparation of the control diet, while pork lard was used in the preparation of all experimental diets. The fatty acid composition was shown (Table 1). All diets were prepared in agar form. Mice were fed by the prepared diets for 4 weeks. Body weight and food intake of all animals were recorded on a daily basis throughout the experiment.

2.3 Collection of blood and feces

Blood samples were collected from mice under isoflurane anesthesia and sacrification immediately by cervical dislocation. Blood was collected by cardiac puncture. The collected blood were incubated at room temperature for 10 min, centrifuged at 2,000× g for 10 min at 4℃, the sera were collected and stored at −80℃ until further analysis. The feces were collected into a clean 1.5 ml centrifuge tube and freeze in liquid nitrogen and then stored at −80℃ until further analysis.

2.4 Serum biological analysis

Cobas C311 biochemistry analyzer (Roche®, Switzerland) was used to measure serum concentration of total cholesterol, triglyceride, low-density lipoprotein cholesterol (LDL), high-density lipoprotein cholesterol (HDL), blood urea nitrogen (BUN), creatinine, alanine aminotransferase (ALT), and aspartate aminotransferase (AST).

2.5 Gut microbiome analysis

The collected fecal samples were subjected to fecal DNA extraction using QIAcube HT and QIAamp 96 DNA QIAcube HT kit (Qiagen, Germany). The extracted DNA samples were subjected to PCR amplification to target the variable V3-V4 region, using KAPA HiFi HS Ready Mix (Kapa Biosystems, Ltd, London, UK). The PCR products were then subjected to sequencing on an Illumina MiSeq platform using Nextera XT v.3 library preparation kit. Once the sequencing was completed, the raw data will be received in fastq format and will be subjected to a quality control and data analysis using Quantitative Insights Into Microbial Ecology version 2 (QIIME2) software suite. Using QIIME2, several tools, such as, sequence demultiplexing and denoising into amplicon sequence variants (ASVs), alpha and beta diversity analysis, alignment, and taxonomic classification can be available for analysis of the sequencing results.

2.6 Statistical analysis

Results were presented as mean ± SEM. One-way ANOVA was applied to analyze the significant difference between sets of data, with Tukey applied as post hoc analysis. Differences were considered statistically significant with the $p<0.05$. Principal component analyses (PCA) was performed using QIIME2View program. The correlation analysis was done using spearman correlation with considered statistically significant of $p<0.05$. GraphPad Prism

Table 1 Fatty acid composition of the oils used in this study.

Fatty acids (mg/100 g of oil)	Palm oil	Pork lard
C18:3 Alpha-linolenic acid (ALA)	243.62	533.02
C18:4 Stearidonic acid	12.90	26.20
C20-3 *Cis*-11,14,17-Eicosatrienoic acid	–	127.79
C20:4 *Cis* 9,11,14,17-Eicosatetraenoic acid	–	13.12
C20:5 *Cis* 5,8,11,14,17-Eicosapentaenoic acid (EPA)	–	–
C22:3 *Cis* 13,16,19-Docosatrienoic acid	–	–
C22:5 *Cis* 7,10,13,16,19-Docosapentaenoic acid (DPA)	–	100.87
C22:6 *Cis* 4,7,10,13,16,19-Docosahexaenoic acid (DHA)	–	26.81
Total omega 3	**256.52**	**827.81**
C18:2 *Cis* 9,12-Octadecadienoic acid	10,915.51	10,934.20
C18:3 Gamma-Linolenic acid methyl ester (GLA)	–	35.80
C20:2 Eicosadienoic acid methyl ester	–	758.96
C20:3 Homogamma-Linolenic acid methyl ester	–	163.01
C20:4 Arachidonic acid methyl ester (ARA)	–	457.44
C22:2 Docosadienoic acid methyl ester	–	14.06
C22:4 Adrenic acid methyl ester	–	270.08
C22:5 Docosapentaenoic acid methyl ester	–	24.88
Total omega 6	**10,915.51**	**12,658.43**
C18:1 *Cis* 9-Oleic acid	43,896.24	40,032.36
C20:1 *Cis* 11-Eicosenoic acid	159.72	1207.10
C20:3 *Cis* 5,8,11-Eicosatrienoic acid	–	50.86
C22:1 Erucic acid	–	18.82
C24:1 Nervonic acid	–	–
Total omega 9	**44,055.96**	**41,309.14**
Saturated fat (g/100 g)	0.04	0.04
Monounsaturated fat (g/100 g)	0.05	0.05
Polyunsaturated fat (g/100 g)	0.01	0.01
Cholesterol (mg/100 g)	0.00	97.00

version 7 (La Jolla, United States) was used for graphic design and IBM SPSS Statistics for Windows, version 25.0 was used for all statistical analysis.

3 Results

3.1 Body weight, diet intake and calorie intake

The control animals showed the lowest body weight, while the 40% fat groups showed highest body weight (Fig. 1). Considering diet intake and calorie intake, the control animals also showed the lowest intake, while the 40% fat groups showed the highest intake (Fig. 2). The body weight seems to be increased as the diet intake and calorie intake increased.

3.2 Serum lipid profiles

The 40% fat group showed the highest serum cholesterol, LDL, and HDL compared to the control group ($p<0.05$)

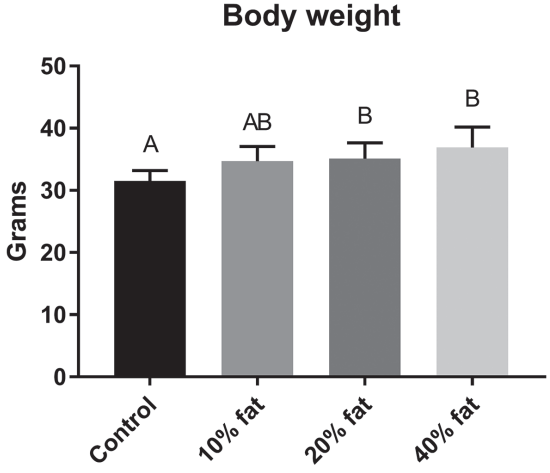

Fig. 1 Body weight. Body weight of all animals at the end of the experiment. Differences in upper case letters indicate significant differences between treatments ($p<0.05$).

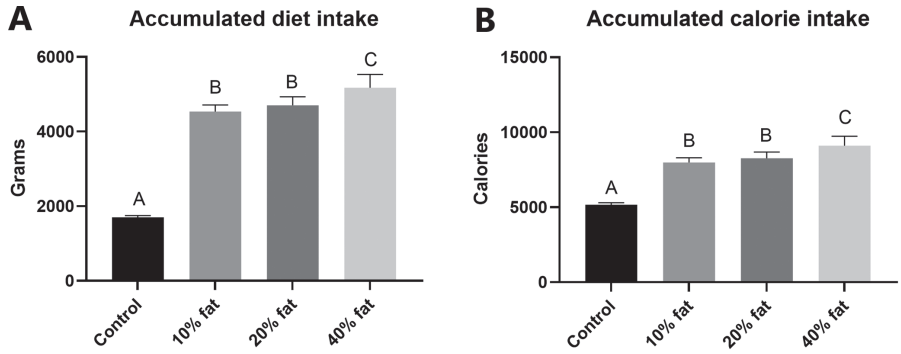

Fig. 2 Accumulated diet intake and accumulated calorie intake. (A) Accumulated diet intake per animal throughout experiment. (B) Accumulated calorie intake per animal throughout experiment. Differences in upper case letters indicate significant differences between treatments ($p<0.05$).

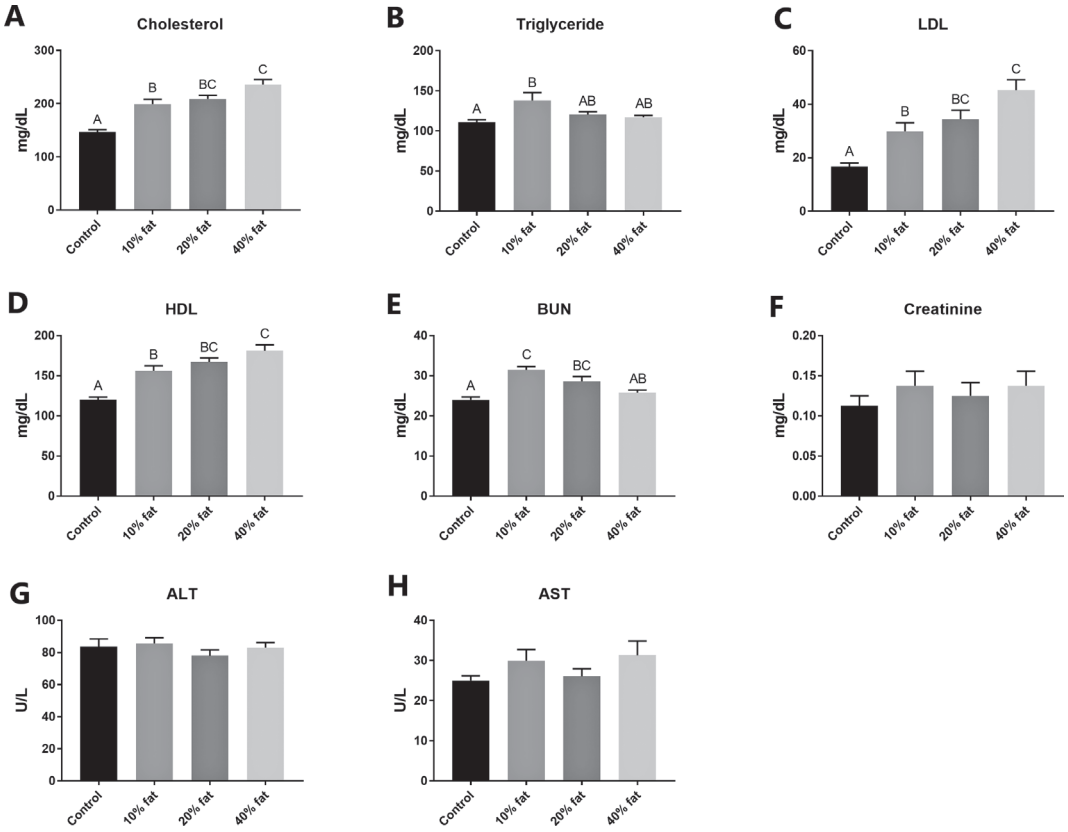

Fig. 3 Serum lipid profile, liver and kidney function enzymes. At the end of the experiment, cholesterol (A), triglyceride (B), LDL (C), HDL (D), BUN (E), creatinine (F), ALT (G), and AST (H) were analyzed. Differences in upper case letters indicate significant differences between treatments ($p<0.05$).

(Fig. 3). Contrary to the cholesterol, the 10% fat group showed the highest serum triglyceride level ($p<0.05$). Similar to the serum triglyceride, the 10% fat group showed the statistically higher serum BUN than the 20% fat and 40% fat group, as well as the control ($p<0.05$). No significant different were observed from serum creatinine, AST, and ALT in all groups.

3.3 Correlation between dietary fat and serum lipid profile

Positive associations between increasing dietary fat level and serum lipid profiles were observed (Fig. 4). As the animals consumed diet with higher dietary fat, they tended to have increasing serum cholesterol, triglyceride and LDL.

3.4 Profile of fecal microbiome

A 3D PCA analysis showed a clear separation of fecal microbiome between experimental and control groups (Fig.

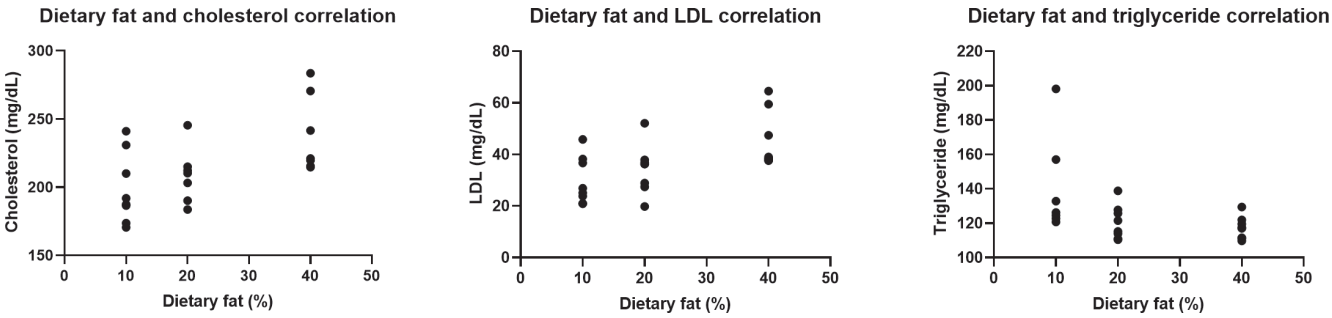

Fig. 4 Correlation between dietary composition and lipid profile. At the end of the experiment, a correlation between dietary fat and cholesterol, dietary fat and LDL, and dietary fat and triglyceride were shown.

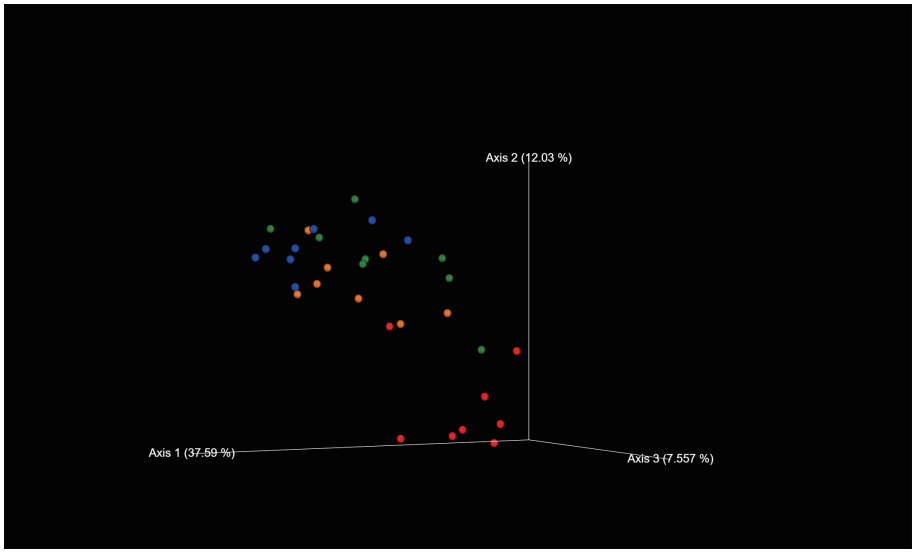

Fig. 5 Principal component analyses (PCA) of fecal microbiome. At the end of the experiment, PCA of fecal microbiome were calculated using Bray-Curtis measurements. Control group is represented by red circles, 10% fat group is represented by orange circles, 20% fat group is represented by green circles, 40% fat group is represented by blue circles.

5). Microbiome of control animals were clearly isolated. In contrast, microbiome of all 3 experimental groups showed some overlapping clusters. Briefly, the 10% fat group showed a slight separation from others, while the 20% fat and 40% fat groups showed an overlap with each other.

3.5 Abundance of microbiome

The fecal microbiome of the control group was dominated by *Muribaculaceae* (52.5%), followed by *Lachnospiraceae* (16.7%), *Lactobacillaceae* (5.9%), *Clostridia_UCG014* (4.7%), *Prevotellaceae* (3.1%), *Ruminococcaceae* (3.0%), *Eggerthellaceae* (2.6%), *Saccharimonadaceae* (2.5%), *Akkremansiaceae* (1.9%), and *Bacteroidaceae* (1.7%) (Fig. 6A).

Comparing to the control, the relative abundance of *Muribaculaceae* and *Saccharimonadaceae* were significantly decreased in other groups (Figs. 6B-6H). On the contrary, *Clostridia_UCG014*, *Akkermansiaceae*, *Bacteroidaceae*, *Oscillospiraceae*, and *Erysipelotrichaceae* were significantly increased compared to the control.

3.6 Serum lipid profiles and microbiome correlation

Spearman correlation indicated positive correlation between serum lipid profiles and the abundance of *Akkermansiaceae* and *Bacteroidaceae*. Negative correlation between serum lipid profiles and the abundance of *Muribaculaceae*, *Clostridia_UCG-014*, and *Saccharimonadaceae* were also indicated (Fig. 7).

4 Discussion

As the animals consumed higher dietary fat diets, their bodyweight increased. This observation might due to the fact that higher dietary fat groups tend to accumulated higher diet intake as well as higher calorie intake. This in-

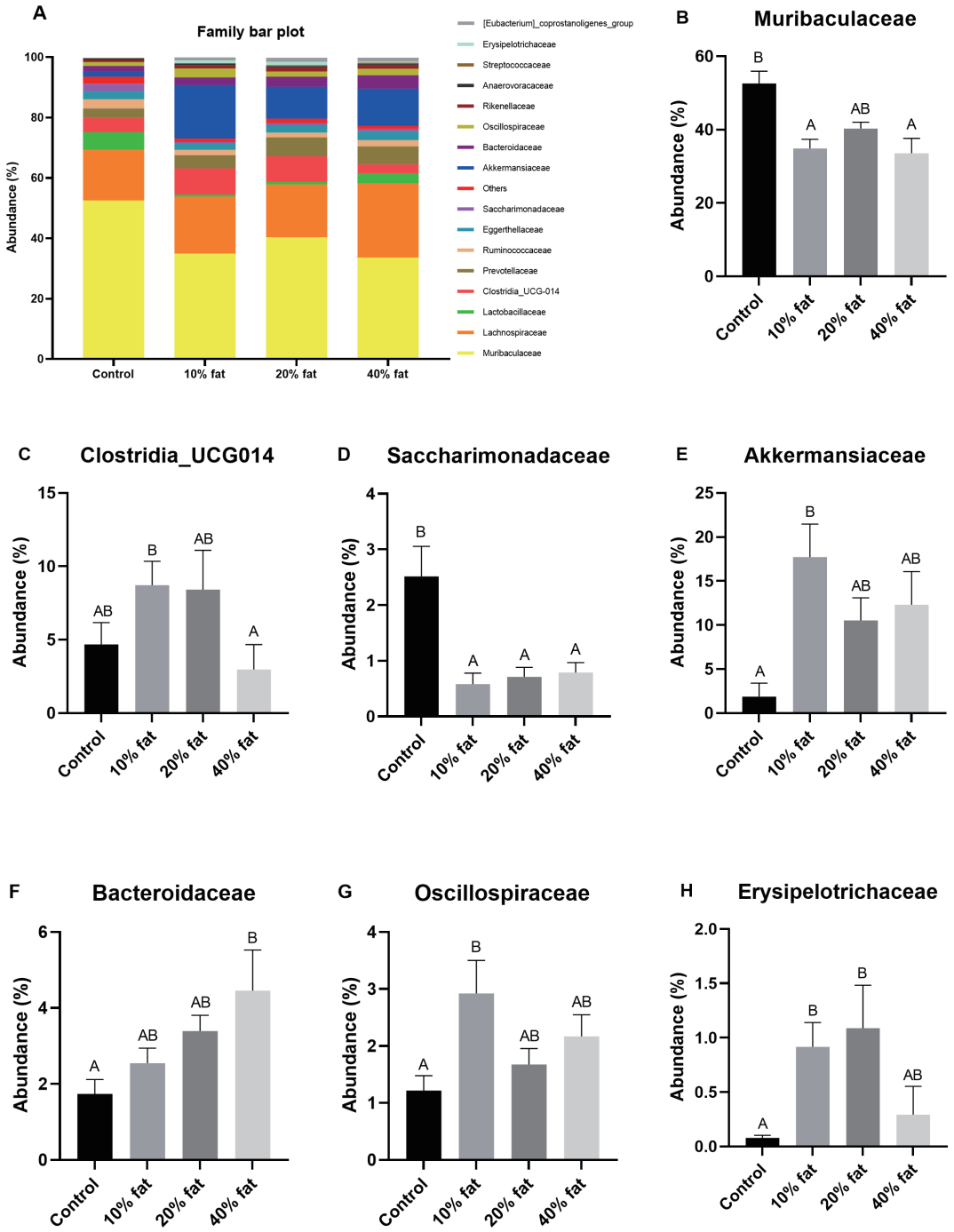

Fig. 6 Comparison of relative abundance of fecal microbiome. Relative abundance of fecal microbiome at family level of each treatment (A), comparison of the abundance of fecal microbiome at family level between treatments are shown for *Muribaculaceae* (B), *Clostridia_UCG014* (C), *Saccharimonadaceae* (D), *Akkermansiaceae* (E), *Bacteroidaceae* (F), *Oscillospiraceae* (G), and *Erysipelotrichaceae* (H). Differences in upper case letters indicate significant differences between treatments ($p<0.05$).

creasing of bodyweight was consistency with many studies. A significant increase of bodyweight in C57BL/6J mice consumed HFD (45% fat), with a significant increase reported after 6 weeks was also reported[10]. Another study reported C57BL/6J mice gained 40% increase of bodyweight after 9 weeks on a 60% fat diet[11]. Similarly, another study reported a 94% increase of bodyweight after a group of C57BL/6J consumed a lard-based HFD for 12 weeks[12].

Although increasing dietary fat seems to have no impact on kidney, as showed in 10% fat, 20% fat, and 40% fat

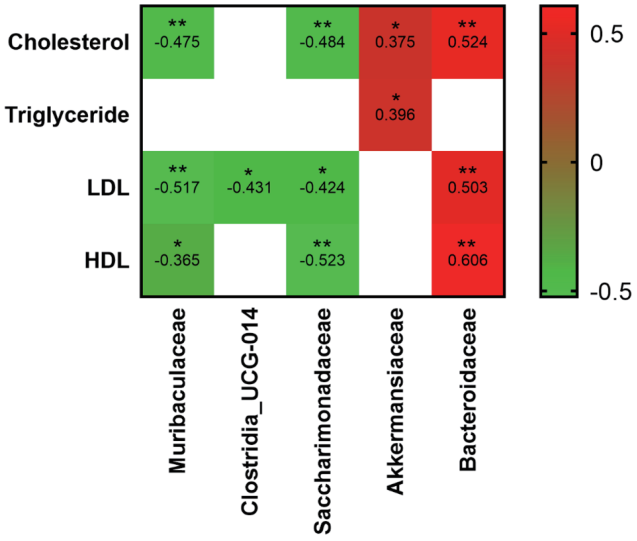

Fig. 7 Correlation between fecal microbiome and serum lipid profile. Spearman correlations of fecal microbiome and serum lipid profile are shown. * indicates $p<0.05$, ** indicates $p<0.01$.

groups indicating high fat diet content was not toxic to kidney. Another study seems to have the similar results with short term HFD in rat[13]. Similarly, another study reported no significant different in urea and creatinine, but their detailed finding showed a significant decrease of Na/K ATPase activity in HFD group, which transferred to deteriorated of renal function[14]. In contrary, some study has revealed that HFD had resulted in decrease glomerular filtration rate (GFR), increase kidney lipid deposition and increase pro-inflammatory cytokines, such as IL-6 and IL-1b[15]. There is a possibility that if the detailed renal functions were to be evaluated, some deteriorated renal function might be revealed, although the BUN and creatinine showed no significant different compared to the control.

According to the results, AST and ALT showed no significant different across all groups. As reported earlier, a similar experiment with twice as long the experimental period showed severely liver damage through AST and ALT[16].

Increasing dietary fat increased serum cholesterol and LDL. This finding is similar to other studies, they showed the increasing of body weight, fat mass, plasma cholesterol, and LDL in C57BL/6N mice fed with HFD for 2, 4, 6, 12 weeks, with the clear elevation after 4 weeks[17]. Another study also showed a significant increase of total cholesterol after only 7 and 14 days of HFD regimen in C57BL/6N[18]. Although hypercholesterolemia was not observed in this trial, a significant increase was observed and sufficient for comparison. Other studies also support the finding, which indicated that plasma cholesterol was increased after 6 and 10 weeks of HFD in their animal subjects[19,20]. Although the dietary fat level in the control diet was 12.5%, similar to that of the 10% fat diet, the diet intake of the 10% fat group was much higher than that of the control group. The accumulated diet intake and calorie intake of the 10% fat was higher than that of the control group, which should responsible for the differences of serum lipid between these two groups.

Microbiome analysis indicated a similarity with another. The PCA analysis using Bray-Curtis calculation showed a clear separation between the microbiome of control group and experimental groups. Since the main fat source of the control diet was palm oil, while that of the experimental diet was pork lard, the microbiome seems to be separated according to the different fat source. This observation is similar to another study in which a clear separation between palm oil and pork lard was observed[21]. Therefore, the changes in microbiome is believed to be caused by different fatty acid composition between the oils, especially the content of cholesterol.

Microbiome reported here shared some similarities with other studies. The decreasing of the abundance of *Muribaculaceae* and increasing of *Lachnospiraceae* in the HFD groups were also observed in another study[22]. Moreover, a similar report showed the increase of the abundance of *Erysipelotrichaceae* after 7 weeks of 60% calories fat diet[23]. A long term 30% calories fat diet at 12 and 28 weeks also increase the abundance of *Erysipelotrichaceae*, compared to the control[24]. Another 16 weeks long term lard-based HFD also showed a decrease in the abundance of *Lactobacillaceae* and increase in the abundance of *Bacteroidaceae*[25], similar to our results.

On contrary to our finding, the increase in the abundance of *Akkermansiaceae* was opposite to what others have reported. An increase in the abundance of *Akkermansiaceae* in lean phenotypic mice was observed instead of high cholesterol[26]. Another study reported a decrease of the abundance of *Akkermansiaceae* in the HFD group[27], which again opposite to our finding.

The correlation between increasing of the abundance of *Bacteroidaceae* and increasing of serum cholesterol and LDL were also similar to other studies. Abundance of *Bacteroidaceae* tends to increase in mice after 4 weeks of HFD[28]. A numerical positive association between *Bacteroides* and serum cholesterol and LDL in HFD fed preclinical trial involved rats and hamsters were also reported[29,30]. Lastly, a clinical trial also showed an approximately 5% increase of the abundance of *Bacteroidaceae* in hypercholesterolemia patients[31]. Beside the *Bacteroidaceae* and serum cholesterol and LDL association, we also have found an increasing abundance of *Bacteroidaceae* as animals consumed diets with higher fat content (Fig. 6F). This has implied the positive association between increasing of the abundance of *Bacteroidaceae* and serum cholesterol and LDL in HFD fed mice.

The outcomes of this study have indicated that there is a positive association between the abundance of *Bacteroidaceae* and serum cholesterol and LDL in C57BL/6N mice. Several researchers have indicated similar associations between high abundance of *Bacteroidaceae* and Western diet, comprises of high protein and animal fat[32, 33]. Moreover, long term and short term consumption of high animal protein, low fiber diet also induce their abundance in clinical studies[34, 35]. These might imply that *Bacteroidaceae* have ability to digest fat and protein, hence increasing their population in HFD environment. Since the experimental diets used in our study comprised lard as fat source, the increasing of the abundance of *Bacteroidaceae* was similar to other reports, along with its association with serum cholesterol and LDL.

The changes in microbiome, as observed in this study, might resulted from the consumption of HFD and lipid metabolism. The consumption of HFD changes microbiome. As showed in Fig. 6F, increasing dietary fat level increased Bacteroidaceae abundance significantly. This might due to the fact that consumption of HFD resulted in the release of more bile acid needed for dietary fat emulsification, leading to increase lipid metabolism[36, 37]. Since bile acid has antimicrobial activity, the abundance of certain groups of microbiome are expected to be decreased due to the release of bile acid for emulsification process[38, 39]. On the other hand, certain groups of microbiome, including some species of Bacteroidaceae, are bile acid tolerant[40, 41], hence, their abundances are expected to increase during the HFD regimen. Therefore, lipid metabolism resulted by HFD consumption affected the abundances of certain groups of microbiome.

Beside abundances of certain groups, metabolites of those who affected also play significant role in host's metabolic diseases. Several studies have reported increasing abundance of Bacteroidaceae in CVD patients. It seems that there is a correlation between HFD consumption, increasing of Bacteroidaceae, and CVD patients[42, 43]. *p*-Cresyl sulfate, one of microbial metabolites, including Bacteroidaceae, have been found to be related to cardiovascular event in elderly[44, 45]. Hence, a possibility that HFD resulted in accumulation of *p*-cresyl sulfate through increasing Bacteroidaceae abundance, and in turn, increasing the risk of cardiovascular event[46, 47]. Therefore, avoiding HFD seems to be a solution to delay the progression of metabolic syndrome, including cardiovascular event, possibly due to lowering Bacteroidaceae abundance, and as a results, lowering *p*-cresyl sulfate accumulation.

This study also has indirectly indicated the advantages of Asian diet regimen over Western diet regimen. Western diet contains approximately 30–40% fat calories[48], which can be represented by the 40% fat group in this study. American diet regimen contains approximately 30–33% fat calories, compared to Japanese diet regimen, which contains approximately 21–29% fat calories[7]. Interestingly, Japanese diet regimen contains approximately 27% fat calories[49], much lower than that of Western diet. Other Asian diet, such as Chinese diet regimen also contains much lesser fat calorie than Western diet, which reported an approximately 20–30% fat calories[50]. Another Asian diet, Thai diet regimen also contains much less fat calorie, at approximately 22% and 29% fat calories[51, 52]. Most Asian diet regimens contain approximately 20–30% fat calories, which can be represented by the 20% fat group in this study, which showed a potential benefits not only in bodyweight but also in lipid profile. Moreover, Western diet seems to increase the risk of hyperlipidemia and obesity through its high fat contents[53, 54]. This could potentially translated to health benefits of Asian diet regimens over Western diet regimen. Based on our finding of increasing *Bacteroidaceae* abundance in elevated serum lipid profile animals, we could expect that consumption of Western diet may increase *Bacteroidaceae* abundance as well increase risk of hyperlipidemia and obesity than consumption of Asian diet.

In conclusion, these findings are believed to contribute to testing of functional ingredients beneficial not only to serum lipid profiles, but also the gut microbiome in both clinical and preclinical settings.

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Author Contributions

Conceived and designed the experiments: AK, VT, SP, KP, PS. Performed the experiments: AK, MW, NN, PJ. Analyzed the data: AK, MW, NN, PJ, VT, SP, SR. Contributed reagents/materials/analysis tools: MW, NN, PJ, PL, PS. Wrote the manuscript: AK, VT, SP, SR, KS, KP. Collected specimens: AK. Commentation: VT, SP, SR, KS, KP.

Supporting Information

This material is available free of charge via the Internet at doi: 10.5650/jos.ess22009

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