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Doctoral Thesis

약제 투여에 의한 장내미생물총의 변화와 그에 따른 대사증후군 및 노로바이러스 감염에 대한 영향 가능성: 메트포르민 치료와 비타민 A 보조치료

Changes in Gut Microbiota by Drug Treatments and Possible Intermediate Effects on Metabolic Syndrome and Norovirus Infection: Metformin Treatment and Vitamin A Supplementation

2013년 12월

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A thesis submitted to the faculty of the Seoul National University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Public Health, Graduate School of Public Health.

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ABSTRACT

Changes in Gut Microbiota by Drug Treatments and Possible Intermediate Effects on Metabolic Syndrome and Norovirus Infection: Metformin Treatment and Vitamin A Supplementation

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Gastrointestinal tract, especially the small and large intestine, is a prominent part of the digestion and absorption of nutrients and the immune system. Gut microbiota is the name given to the vast collection of symbiotic microorganisms living in the intestine. It contains tens of trillions of microorganisms, including up to 1,000 bacterial species. Recently, next generation sequencing (NGS) technique-based metagenomic analyses make it possible to reveal the total microbial communities including uncultured bacteria and understand the interaction between host and gut microbiota. Various health problems such as metabolic syndromes, autoimmune disease, and infection disease were implicated by microbial disorder. However, because of the complexity of microbial community, mechanical interactions between gut microbiota and metabolic disorder are not fully understood until now. In this study, we investigated the change of gut microbiota by metformin treatment and vitamin A administration from two different mouse models.

First, the specific bacterial community by metformin treatment, first-line oral antidiabetic agent, was revealed using high-fat diet (HFD)-induced obese mouse model.

The metformin treatment in HFD-induced obese mouse changed gut microbiota to have low diversity and unique microbiota in comparison to untreated obese mice. The composition of phylum *Proteobacteria* (2.1 ± 2.8%) *and Verrucomicrobia* (12.4 ± 5.3%) were significantly increased after metformin treatment during HFD (HF-Met), especially, the genome of *Akkermansia muciniphila* which was suggested as an intestinal mucin-degrading bacterium was highly increased during metformin treatment. In addition, abundance of *Akkermansia muciniphila* was showed in BHI medium supplemented with metformin *in vitro* test. In the analysis of metabolic functions, total 18 KEGG pathways were significantly predicted to be upregulated in HF-Met, among them, sphingolipid and fatty acid metabolism belong to lipid metabolism were most remarkable. These results demonstrated that gut microbiota significantly changed in the process of improvement of metabolic disorders by metformin treatment. Moreover, we could suggested that the change of gut microbiota composition by metformin treatment was strongly linked to improvement of metabolic syndrome.

Second, we investigated the changes in gut microbiota by MNV inoculation during retinoic acid (RA) administration. RA, the metabolite of dietary vitamin A, plays essential roles in innate immune responses for virus infection. Recently, it has been reported that gut microbiota is highly related with both innate and adaptive immunity. But, the relation between RA administration and gut microbiota was not characterized until now. Here, mice were inoculated with murine norovrius (MNV) during RA administration (1 and 10 mg/kg/d), those gut microbiota was compared to MNV- or RA-negative controls. Bacterial diversity was most significantly changed in MNV-inoculated mice during RA administration, especially, *Lactobacillus* was significantly increased by 1 mg/kg/d RA administration. Moreover, abundance of *Lactobacillus* has a positive correlation with RIG-1, MDA-5, and F4/80, and was negatively correlated with MNV positive. Therefore, we could predict that the change of gut microbiota by RA administration is related with innate immunity for virus infection.

In conclusion, gut microbiota was significantly changed by metformin treatment in improvement of metabolic disorders as well as incidence of metabolic syndrome. And,

specific gut microbiota was characterized by MNV inoculation during RA administration. From those results, we could suggest that gut microbiota plays important role in improvement of metabolic syndrome and activation of innate immunity against virus infection, eventually, further studies should be required to verify the beneficial effects of gut microbiota in human health.

Keywords: Gut microbiota, Metformin, Metabolic syndrome, Retinoic acid (RA), Norovirus, Cytokines

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LIST OF ABBREVIATIONS

DNA: deoxyribonucleic acid

h: hour

HDL: high-density lipoprotein

HFD: high-fat diet

KEGG: Kyoto Encyclopedia of Genes and Genomes

MNV: murine norovirus

ND: normal diet

NGS: next-generation sequencing

NoV: norovirus

OGTT: oral glucose tolerance test

OUT: operational taxonomic unit

PCoA: principal coordinates analysis

PCR: polymerase chain reaction

PFU: plaque forming units

PICRUSt: Phylogenetic Investigation of Communities by Reconstruction of Unobserved States

QIIME: quantitative insights into microbial ecology

RA: retinoic acid

RNA: ribonucleic acid

T2D: type 2 diabetes

TG: total cholesterol

CHAPTER Ⅰ.

BACKGROUNDS

Gut microbiota and health

Human intestine contains tens of trillions of microorganisms, including up to 1,000 bacterial species (Fig. 1.1). That is currently considered as a new organ carrying out specific and crucial functions to maintain our optimal health (*1*). But, the large portion of microbial society and those function is still unknown.

Microbial communities in human interact with their energy harvesting form food, metabolic processes, and immune modulation (Fig. 1.2). Various health problems such as metabolic syndromes, inflammatory bowel disease (IBD), asthma, atopy, and cardiovascular disease were implicated by microbial disorder (Fig. 1.2) (*2*). Microbiota is the ecological community of commensal, symbiotic, and pathogenic microorganisms in and on human body, microbiome is those genomes of all the organisms. Gut microbiota in particular have been involved in host metabolism and immune system. However, because of the complexity of microbial community, mechanical interactions between gut microbiota and metabolic disorder are not fully understood until now.

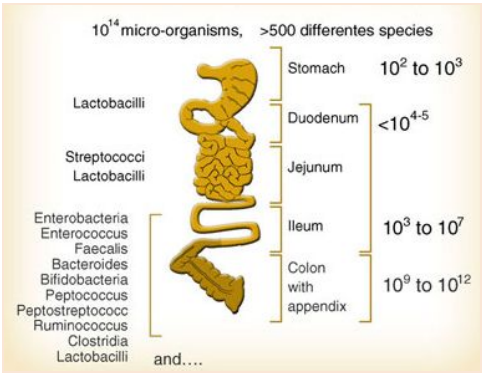


Figure 1.1. The number of gut microbiota comprising human intestine.

Human microbiome research is at the cutting edge of science, recently, Human Microbiome Project (HMP), Metagenomics of the Human Intestinal Tract (MetaHIT), and International Human Microbiome Consortium (IHMC) were established to understand human biology, health and disease.

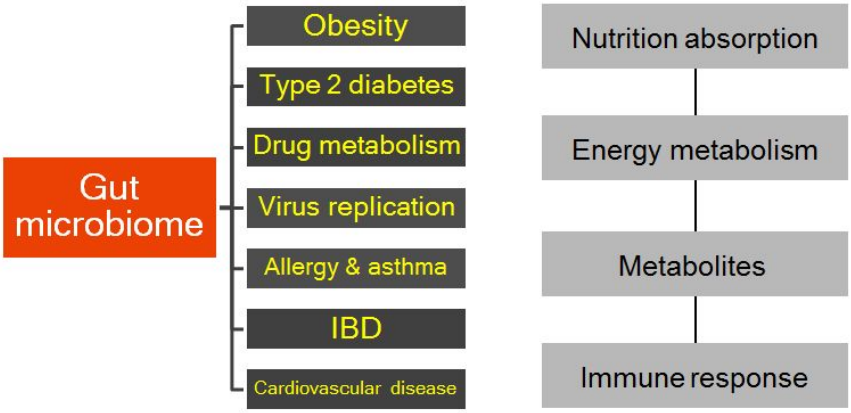


Figure 1.2. Various health problems related with gut microbiota.

Next-generation sequencing (NGS)

NGS technique is currently revolutionizing way to understand the microbial communities exist in environmental and human body. Advances in sequencing technologies coupled with new bioinformatic developments have allowed us to investigate the microbes in human body. Lately NGS technique has been rapidly developed in a few years, now NGS provides a cost-effective method for sequencing targeted genes in various application such as MiSeq, a personal sequencer. The major advantage of NGS comparing former methods is the high throughput microbial taxonomic data from each samples (Fig 1.3). From NGS platform, the various applications of whole genome, transcriptome, epigenome, and metagenome are capable.

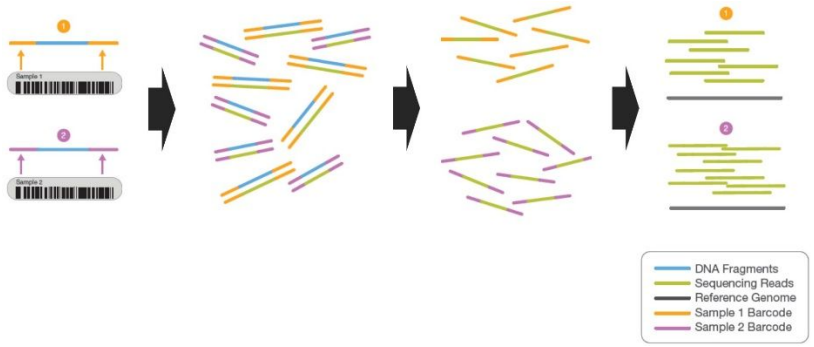


Figure 1.3. Conceptual overview of sample multiplexing in NGS.
(Source: http://res.illumina.com/documents/products/illumina_sequencing_introduction.pdf)

Vitamin A and epithelial immunity

Vitamin A is a group of unsaturated nutritional organic compounds. Retinoic acid (RA), the metabolite of dietary vitamin A, contributes to both innate immune tolerance and the elicitation of adaptive immune responses (*3, 4*). Recent evidence indicates that loss of RA causes to impaired immunity, whereas excess RA can induce inflammatory disorders. In animal studies, impaired T cell responses observed in infection model during retinoid receptor deficiency. Recently, vitamin A supplementation showed the decrease of NoV infection rate and clinical symptoms, and the responses of various intestinal cytokines were modified by vitamin A during NoV infection in epidemiology study (*5*). In recognition and response to viral RNA, retinoic acid-inducible gene 1 (RIG-1) and melanoma differentiation-associated gene 5 (MDA-5) signaling pathway is crucial role in antiviral responses (*6*). And, immunodeficient mice lacking those cytokines associated with immune responses resulted in destruction of bacterial community (*7, 8*).

Norovirus

Norovirus (NoV) is the most frequent etiological viral agent of acute gastroenteritis in all age groups worldwide. Human NoV (HuNoV) causes approximately 90% of

epidemic nonbacterial outbreaks of gastroenteritis around the world, and responsible for 50% of all foodborne outbreaks of gastroenteritis in the United States (Fig. 1.4). NoV is a highly contagious, typically, transmitted person to person, via contaminated food, water, or fomites. Virus infection accompanies diarrhea, vomiting, nausea, abdominal pain, and fever, those last one to three days. Especially, NoV gastroenteritis by is fatal in children and the elderly, cause significant morbidity and mortality among them. But, there is no effective treatment for NoV infection until now, vaccine is suggested promising prevention of NoV infection. Effective inactivation of NoV in environment might be the best way prior to transmit to human so far, but, those are not applicable in clinical patients. In recent population study, vitamin A supplementation significantly prevented the incidence of acute gastroenteritis by norovirus and decreased the clinical symptoms, those were highly associated with cytokines (*5*).

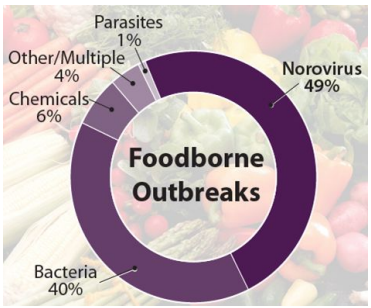


Figure 1.4. Known Causes of Foodborne Illness Outbreaks, U.S., 2006-2010.
(Source: http://www.cdc.gov/norovirus/trends-outbreaks-figure-3.html)

Due to the absence of human NoV culture system, murine norovirus (MNV) which is culturable in mouse macrophage cell line RAW 264.7 has been accepted as a suitable surrogate for human NoV (*9*). Especially, MNV has been used in inactivation studies by chemical (e.g. chlorine or/and ozone) and physical (e.g. UV irradiation). Inactivation of NoV in various environments is important because of the high persistence and infectivity of NoV.

Murine norovirus (MNV) is a common viral infection of mice in many animal facilities.

In immunocompetent strains, MNV infection is variable in length (few days to weeks), and does not induce clinical signs (*10*). But, it was reported that MNV causes histopathological alteration in the small intestine and spleen of immunocompromised mice lacking recombination-activating gene 2 (RAG2), signal transducer and activator of transcription 1 (STAT-1), alpha/beta interferon (IFN-α/β), and IFN-γ receptors (*11*).

Objectives and Hypotheses

The objectives of this study were that to 1) reveal specific bacterial communities in improvement of metabolic disorders by metformin treatment, 2) characterized the gut microbiota between vitamin A administration and MNV inoculation, 3) predict the effect of gut microbiota on metabolism and innate immunity.

Hypothesis 1.
The specific gut microbiota is characterized in improvement of metabolic disorders by metformin treatment.

Hypothesis 2.
Significant changes in gut microbiota are induced by vitamin A supplementation and norovirus inoculation.

Hypothesis 3.
Gut microbiota changed by metformin treatment and vitamin A supplementation shows significant association with various biomarkers representing energy metabolism and innate immunity.

CHAPTER Ⅱ.

CHARACTERISTICS OF GUT MICROBIOTA IN METABOLIC IMPROVEMENT BY METFORMIN TREATMENT

INTRODUCTION

Gut microbiota play a role in energy harvesting from food, metabolic processes, and immune modulation. Different characteristics of gut microbiome were reported in various state of health such as obesity and type 2 diabetes (*12, 13*). Dysbiosis of gut microbiota is known to cause various metabolic and immunological related health problems such as metabolic syndromes (*14*), inflammatory bowel disease (IBD) (*15*), asthma (*16*), atopy (*17*), and cardiovascular disease (*18*). Previous study reported that germ-free mice did not develop obesity by high-fat diet (HFD) (*19*). The disruption of gut microbiota by antibiotic treatment had significant effect on various metabolic disorder or recovery including glucose tolerance, adiposity, and adipose inflammation (*20, 21*). The composition of gut microbiota also has been significantly influenced by the types of food intake, it is suggested that gut microbiota plays a crucial role in weight loss and gain, glucose tolerance, and fat deposition (*22, 23*). Experimental evidences revealed that decrease of total gut microbiota, increase of Gram-negative bacteria, and increase of phylum *Firmicutes/Bacteroidetes* ratio were related with both the change of energy homeostasis in the gut and metabolic syndrome such as obesity and T2D of host (*1, 14*).

The change and reconstruction of gut microbiota has been aware as a therapeutic strategy, especially, fecal microbial transplantation has been applied for the treatment of *Clostridium difficile* infection and IBD in clinical use (*24, 25*). Mechanism action of transplantation is still unclear, the change of metabolic and immunological activities by reconstructed microbial community could be targeted for the treatment of diseases. For example, the treatment of *Akkermansia muciniphila* that was identified from enterotype of gut microbiota showed the improvement of metabolic disorders (*26*), which was associated with inflammation (*27*). Therefore, treatment using gut microbiota is expected for metabolic syndrome including obesity and T2D in future.

Metformin is commonly applied oral antidiabetic agent in the biguanide class, acts to suppress glucose production in liver, increase insulin sensitivity, and enhance peripheral

glucose uptake in hepatic and skeletal muscle (*28*). AMP-activated protein kinase (AMPK) increased by metformin plays an important role in energy balance and glucose metabolism, cellular AMP/ATP ratio caused by increasing ATP consumption and reducing ATP production were related with AMPK activation (*29, 30*). Besides, metformin treatment inhibited phosphoenolpyruvate carboxykinase (*Pepck*), glucose-6-phosphatase (G6Pase), and regulated glucose transporter (GLUT) family in mechanism of action (*31, 32*). However, metformin regulated the hepatic gluconeogenesis and improved hyperglycemia independently of the AMPK pathway (*33*), indicated that the metformin effect on metabolic disorders was related with energy state in body. However, due to the high complexity of gut microbiota, it is difficult to identify the role and underlying mechanisms of gut microbiota while during metformin treatment for metabolic disorder. Therefore, in this study, we investigated the change of composition and their metabolic functions of gut microbiota during the treatment of metabolic disorders by metformin.

MATERIALS AND METHODS

Animal model

Age-matched male and female C57BL/6 mice were purchased from Orientbio Inc. (Seongnam, South Korea) and housed in specific pathogen-free (SPF) facility. Experimental design in this study was showed in Figure 2.1. To induce metabolic disorders such as obesity and type 2 diabetes (T2D), 6-weeks-old mice were fed on HFD (60% lipid, TD.06416, Harlan Laboratories Inc.) for 28 weeks. Metformin (300 mg/kg, 1,1-Dimethylbiguanide hydrochloride, D150959, Sigma-Aldrich, USA) was administered every day during HFD for last 10 weeks (HF-Met). As negative controls, HFD without metformin treatment (HF), diet change group from HFD to normal diet (ND, 5% lipid, Rodent NIH-31 Auto, Zeigler Bros., Inc.) (HF-N), ND without metformin treatment (N), and metformin treatment during ND (N-Met) were included in all procedures. All experimental protocols in this study were approved by the Seoul National University Institutional Animal Care and Use Committee (SNU-111208).

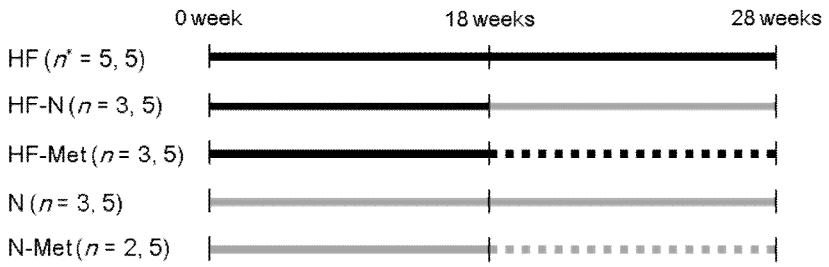


Figure 2.1. Design of high-fat diet-induced obese mouse model for the effect of metformin treatment on gut microbiota. The change of gut microbiota in improvement of metabolic disorders by metformin treatment was performed using high-fat diet-induced obese mouse model. Mice fed on HFD (60% lipid) and ND (5% lipid) for 28 weeks, and metformin was administrated every day for last 10 weeks during high-fat diet. Tissues and stool samples for pyrosequencing was obtained at 28 weeks after high-fat diet or normal diet. *: Number of male and female mice. HF: high-fat diet only

without metformin treatment, HF-N: diet change from high-fat diet to normal diet, HF-Met: metformin treatment during high-fat diet, N: normal diet only without metformin treatment, and N-Met: metformin treatment during normal diet.

Metabolic measurements for diagnosis of metabolic syndrome

Body weight, food intake and glucose level were measured every weeks. Level of serum glucose was measured using Accu-Chek Performa system (Roche, USA) after starvation for 12 h. To estimate glucose tolerance, oral glucose tolerance test (OGTT) was performed. Mice were administrated orally with 2 g glucose/kg body weigh in PBS and glucose levels were measured 30, 60, and 120 min after oral administration. Levels of serum total cholesterol (TG), and high-density lipoprotein (HDL) were measured using EnzyChrom^{TM} HDL and LDL/VLDL Assay Kit (EHDL-100, BioAssay System, USA). Serum insulin was analyzed using flow cytometry (Luminex 100/200, Luminex, USA), and performed at Komabiotech (Seoul, South Korea).

Tissue preparation for histological analysis

Mice were food-deprived overnight before sacrifice and then anaesthetized using Zoletil 50 (30 mg/kg of body weight, Virbac Laboratories) and Rompun (10mg/kg of body weight, Bayer), intraperitoneally. Cardiac puncture was rapidly performed to obtain blood samples. Fat pads, small intestine, liver, and pancreas were dissected, and liver was then weighted. To process histochemistry, tissue samples were fixed with 4% paraformaldehyde, followed by hematoxylin and eosin (H&E) staining. Stained tissues of liver, pancreas, and jejunum were further performed pathology analysis of steatosis and inflammation by pathologist (Reference Biolabs, Seoul, South Korea). In addition, immunochemistry for small intestinal mucosa was performed using anti-MUC5AC antibody (LS-C87775, LSBio, Seoul, South Korea) (Reference Biolabs, Seoul, South Korea).

Metabolic and inflammatory biomarkers for evaluation of metabolic disorders

AMP-activated protein kinase alpha 1 (AMPKα1), peroxisome proliferator-activated receptor alpha (PPARα), glucose transporter 2 (GLUT2), and glucose-6-phosphatase (G6Pase) were analyzed in liver, and adiponectin, leptin, monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor alpha (TNFα), and interleukin-6 (IL-6) was analyzed in fat pad. The expression pattern of mucin 2 and 5 genes (MUC2 and MUC5) was analyzed in ileum of small intestine. Tissues, approximately 100 mg, were totally homogenized using PT-2000 E homogenizer (POLYTRON, Switzerland) prior to RNA extraction. Total RNAs were extracted using easy-spin™ Total RNA Extraction Kit (iNtRON, Seongnam, South Korea), and then cDNA synthesis was followed using High Capacity RNA-to-cDNA Kit (4387406, Applied Biosystems, USA) according to the manufacturer's instructions. To quantify mRNA expression level of metabolic and inflammatory biomarkers, QuantiTect® SYBR® Green PCR Kit (204143, Qiagen, USA) and 7300 Real Time PCR System (Applied Biosystems) was used. Analyzed biomarkers and those oligonucleotide primer sets were described in Table 2.1. The reaction mixture (25 μl) for real-time PCR was composed of 2x QuantiTect SYBR Green PCR Master Mix (12.5 μl), primers (forward and reverse, 50 pmol 0.2 μl, respectively), RNase-free water (11.1 μl), and template DNA (1 μl). GAPDH was used as the internal control.

Table 2.1. Primer sets for biomarkers of metabolic and inflammatory.

Target gene	Forward (5' – 3')	Reverse (5' – 3')
AMPKα1	TGTTCCAGCAGATCCTTTCC	ATAATTGGGTGAGCCACAGC
PPARα	TCTTCACGATGCTGTCCTCCT	CTATGTTTAGAAGGCCAGGC
GLUT2	ATTCGCCTGGATGAGTTACG	CAGCAACCATGAACCAAGG
G6Pase	GAGTCTTGTCAGGCATTGCT	GAGTCTTGTCAGGCATTGCT
Adiponectin,	TTGCAAGCTCTCCTGTTCCT	TCTCCAGGAGTGCCATCTCT
Leptin	TGACACCAAAACCCTCATCA	AGCCCAGGAATGAAGTCCA
MCP-1	AGGTCCCTGTCATGCTTCTG	TCTGGACCCATTCCTTCTTG
TNFα	GCCACCACGCTCTTCTGCCT	GGCTGATGGTGTGGGTGAGG
IL-6	AGTTGCCTTCTTGGGACTGA	TCCACGATTTCCCAGAGAAC
MUC2	ACCCGCACTATGTCACCTTC	GGGATCGCAGTGGTAGTTGT
MUC5AC	QuantiTect® Primer Assay (Cat. no.: QT01161104, Qiagen)	
GAPDH	GAAATCCCATCACCATCTTCCAGG	GAGCCCCAGCCTTCTCCATG

Next generation sequencing of gut microbiota and bioinformatics pipeline

For experiment procedures, fresh stool samples frequently collected, and stored in -70°C until analysis. Total DNA was extracted from the frozen feces using QIAamp® DNA Stool Mini Kit (51504, Qiagen, USA) and QIAcube system (Qiagen, USA). For each samples, 16S rRNA genes were amplified using 27F/534R primer set for amplification of V1-V3 region (27F: forward primer, 5'-*CCTATCCCCTGTGTGCCTTGGCAGTCTCAG-* ***AGAGTTTGATCCTGGCTCAG***-3'; 534R: reverse primer containing a unique 10-based barcode for tagging each PCR product designated by *NNNNNNNNN*, *5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG*<u>*NNNNNNNNN*</u>***ATTACCGCGGCTGCTGG***-3') (*34*). Amplified PCR products were purified using QIAquick PCR Purification Kit (Qiagen, USA). Bacterial 16S rRNA pyrosequencing was performed using the GS-FLX system (Roche, USA) at the Macrogen (Seoul, South Korea).

To analyze 16S rRNA sequence, quality filtering steps including sequence length (> 200 bp), end-trimming, number of ambiguous bases, and minimum quality score was preprocessed. After denosing, sequences were assigned to operational taxonomic units

(OTUs, 97% identity), followed by selecting representative sequence using Quantitative Insights Into Microbial Ecology (QIIME 1.5.0). After then, alpha-diversity, beta-diversity, taxonomic composition analysis, and Principal Coordinate Analysis (PCoA) were performed. To estimate taxonomic abundance and characterize the difference between groups, LDA Effect Size (LEfSe) was used (*35*). UniFrac distance between categorized samples was visualized using Cytoscape software (v2.8.3). In addition, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) was performed to predict the functional genes in the sampled microbial community based on KEGG pathway database (*36*). Heatmap of functional gene abundance was generated using MultiExperiment Viewer software (MEV) (v4.8.1).

Cultivation of *Akkermansia muciniphila* in BHI with metformin

Brain-heart infusion (BHI, Difco) supplemented with 0.1 M metformin and 0.1 M phenformin was prepared for evaluating the growth of *Akkermansia muciniphila*. Stool sample from HFD-Met mice group was inoculated in BHI broth, incubated in anaerobic chamber. After 3 and 6 hours post-incubation, total DNA was extracted with easy-spin™ Total DNA Extraction Kit (iNtRON, Seongnam, South Korea). The quantification of *Akkermansia muciniphila* and total bacteria was performed using SYBR qPCR and TaqMan qPCR, respectively (*37, 38*). The percentage of *Akkermansia muciniphila* was calculated comparing total bacteria as 100%.

Statistical analysis

All features were expressed by average and standard deviation of each group. To quantity the level of metabolic and inflammatory biomarkers, qPCR was repeated 3 times. Relative quantification method, $2^{-\Delta\Delta Ct}$ ($\Delta\Delta Ct = (C_{t.Target} - C_{t.GAPDH})_{Group1} - (C_{t.Target} - C_{t.GAPDH})_{Group2}$), was used for analysis of gene expression of biomarker comparing GAPDH internal control. Statistical significance was assessed using one-way ANOVA followed by Duncan's post-hoc test, with P values of < 0.05 considered significance. In relative abundance analysis using LEfSe based on Kruskal-Wallis and Wilcoxon test,

significance was considered P value < 0.05, and threshold on the logarithmic LDA score was 3.0-4.0. To estimate the correlation between metabolic and inflammatory biomarkers and bacterial abundance, Spearman correlation coefficient was performed. Figures of biomarkers and abundance were converted to value of relative quantification and percentage of phylum bacteria, respectively.

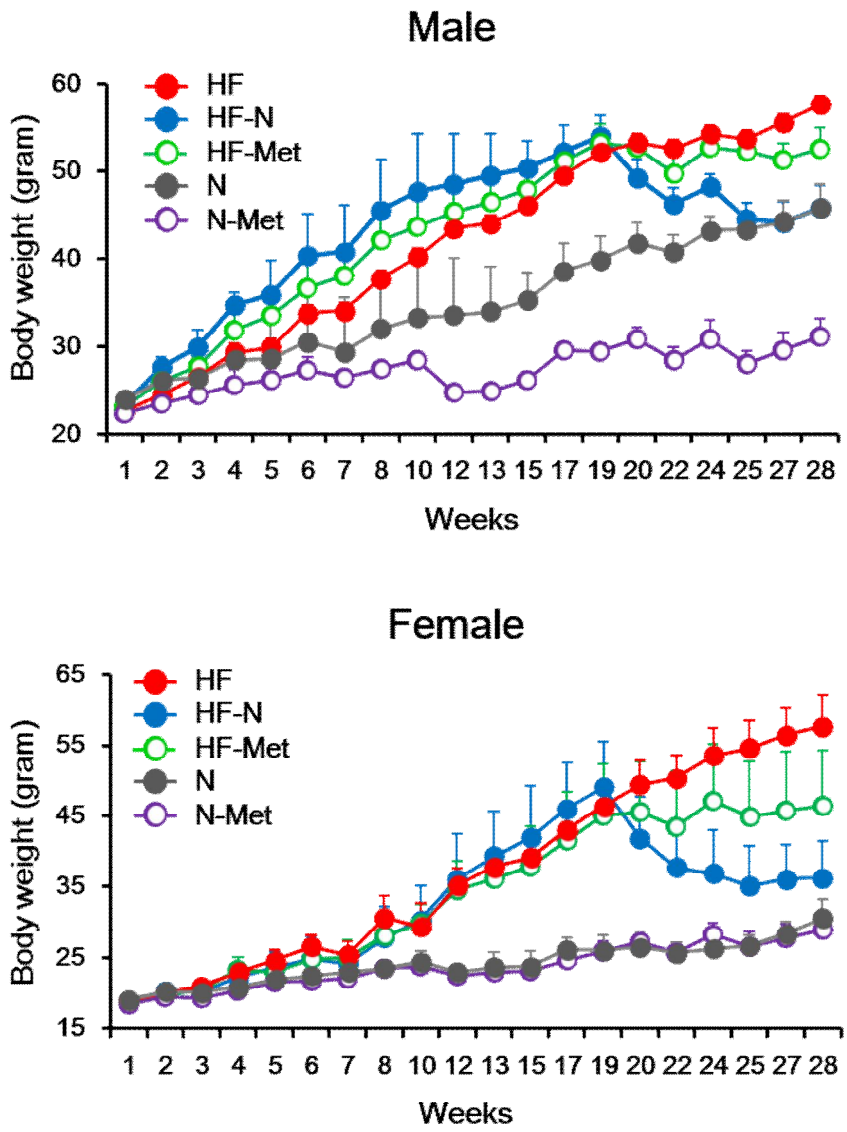
RESULTS

Metabolic disorders and improvement by metformin treatment

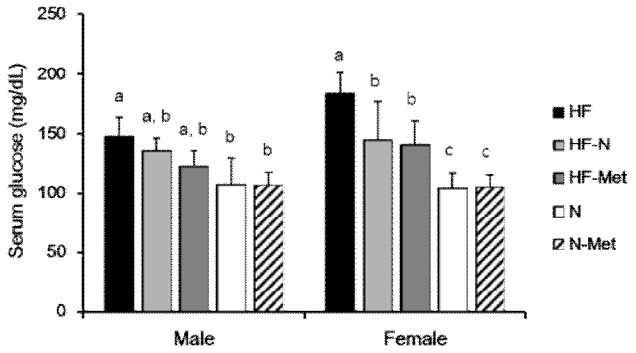
Figure 2.2 showed the change of body weight, glucose level, TG, and HDL as diet change to ND and metformin treatment. Body weight was significantly increased in both male and female mice by HFD than ND. After diet change from HFD to ND, body weight was significantly decreased, and metformin treatment kept body weight to steady state. Glucose level was significantly increased by HFD in male ($P < 0.001$) and female ($P = 0.001$) mice, recovered by ND ($P = 0.032$) and metformin treatment ($P = 0.016$), especially in female mice (Fig. 2.3B). Impaired glucose tolerance, risk factor for T2D, was estimated by OGTT in this study, was significantly recovered after diet change to ND in both male ($P = 0.036$) and female mice ($P = 0.008$), but not after metformin treatment (Fig. 2.3C). In addition, the homeostatic model assessment-insulin resistance (HOMA-IR), estimated with glucose and insulin level, showed the tendency of insulin resistance recovery after ND and metformin treatment, although statistically not significant (Fig. 2.3D). And, the homeostatic model assessment-beta cell function (HOMA-β) showed not significance (Fig. 2.3E). Daily calorie intake was not changed in male and female mice by metformin treatment (Fig. 2.3A).

Both TG and HDL were significantly increased with HFD than ND (Fig. 2.2). TG was significantly decreased with ND than metformin treatment in both male ($P < 0.001$) and female ($P < 0.001$) mice. In metformin treatment, TG was significantly decreased in female ($P = 0.023$), not male ($P = 0.875$) (Fig. 2.2C). HDL was also significantly decreased after ND in both male ($P < 0.001$) and female ($P = 0.001$) mice, and showed the tendency of decrease after metformin treatment in both male ($P = 0.055$) and female ($P = 0.055$) mice, although statistically not significant (Fig. 2.2D). There was no significant effect of metformin treatment on metabolic change of mice with ND only.

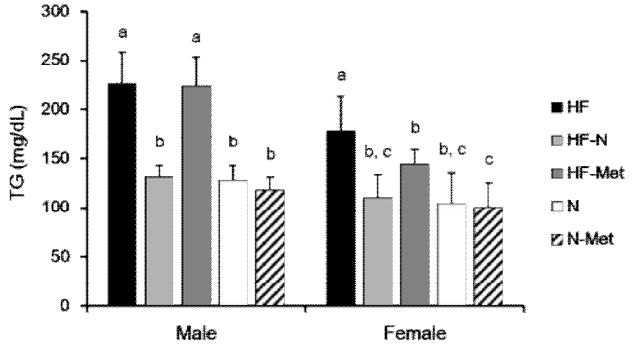
A


Male
HF
HF-N
HF-Met
N
N-Met
Body weight (gram)
60
50
40
30
20
1 2 3 4 5 6 7 8 10 12 13 15 17 19 20 22 24 25 27 28
Weeks
Female
HF
HF-N
HF-Met
N
N-Met
Body weight (gram)
65
55
45
35
25
15
1 2 3 4 5 6 7 8 10 12 13 15 17 19 20 22 24 25 27 28
Weeks

B


Serum glucose (mg/dL)
250
200
150
100
50
0
a
a, b
a, b
b
b
a
b
b
c
c
Male
Female
HF
HF-N
HF-Met
N
N-Met

C


TG (mg/dL)
300
250
200
150
100
50
0
a
b
a
b
b
a
b, c
b
b, c
c
Male
Female
HF
HF-N
HF-Met
N
N-Met

D

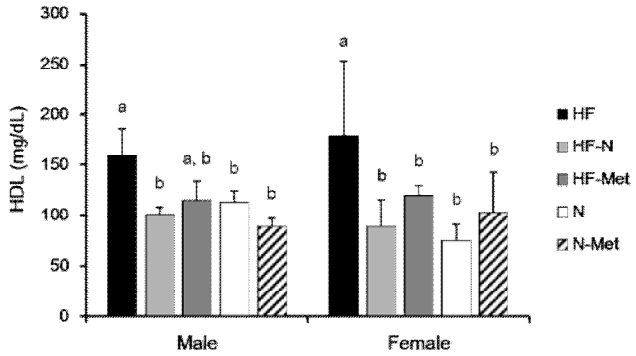
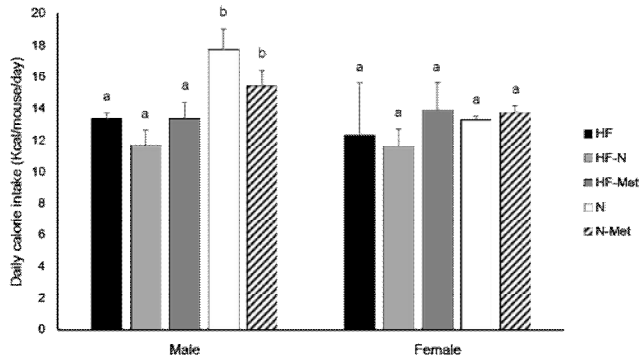
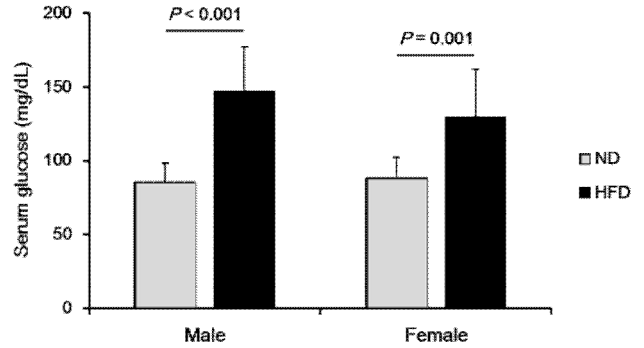

HDL (mg/dL)
300
250
200
150
100
50
0
a
b
a, b
b
b
a
b
b
b
b
Male
Female
HF
HF-N
HF-Met
N
N-Met

Figure 2.2. The effect of metformin treatment and diet on body weight, glucose level, and total cholesterol (TG), and high-density lipoprotein (HDL). Mice were induced metabolic disorders by HFD for 18 weeks, after conducting metformin treatment and diet change to ND for following 10 weeks, the improvement of metabolic disorders were showed in body weight, glucose level, and TG, and HDL (n = 41). (A) Change of body weight of male and female mice. (B) Serum glucose level. Measurement was performed at 21 weeks. (C) Serum total cholesterol (TG). (D) Serum high-density lipoprotein (HDL) level. Both TG and HDL were measured at 10 weeks after diet change to normal diet and metformin treatment.

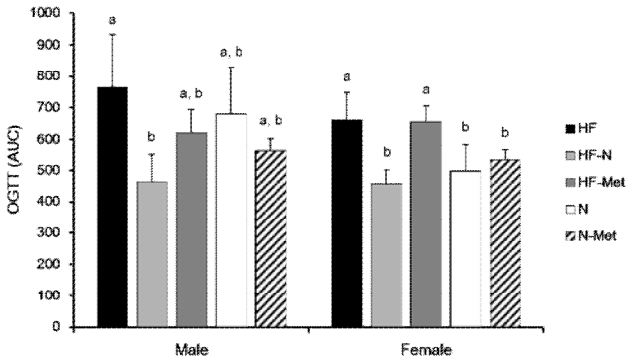
A



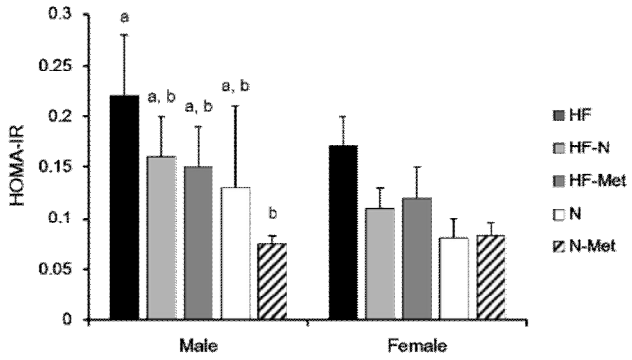
B



C


OGTT (AUC)
1000
900
800
700
600
500
400
300
200
100
0
a
b
a, b
a, b
a, b
a
b
a
b
b
Male
Female
HF
HF-N
HF-Met
N
N-Met

D


HOMA-IR
0.3
0.25
0.2
0.15
0.1
0.05
0
a
a, b
a, b
a, b
b
Male
Female
HF
HF-N
HF-Met
N
N-Met

E

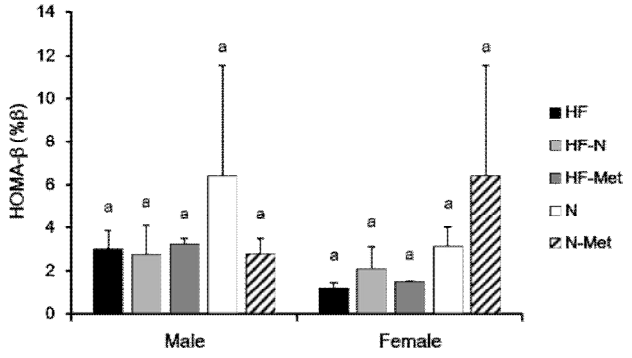
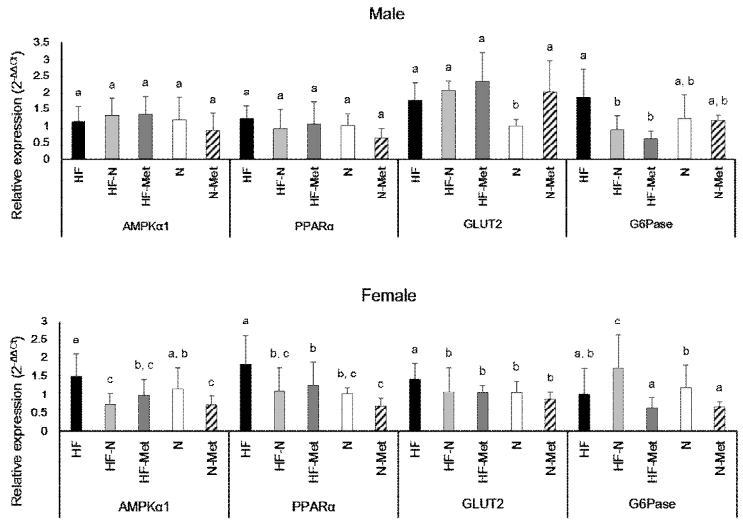

HOMA-β (%β)
14
12
10
8
6
4
2
0
a
a
a
a
a
a
a
a
a
a
Male
Female
HF
HF-N
HF-Met
N
N-Met

Figure 2.3. The effect of metformin treatment and diet on calorie intake and glucose tolerance. Metabolic disorders and improvement by diet and metformin treatment were evaluated by various metabolic indicators. (A) Amount of feed intake of each cages was converted into daily calorie intake. (B) Serum glucose level at 12 weeks after HFD was significantly different comparing to ND. (C) Glucose tolerance was calculated and expressed with the area under the curve (AUC). (D and E) HOMA-IR and HOMA-β was calculated with glucose and insulin level measured at 3 week after metformin treatment.

Expression patterns of metabolic and inflammatory biomarkers

Figure 2.4 described the relative expression level of various metabolic and inflammatory biomarkers from liver and fat pad. AMPKα (P =0.023) and GLUT2 (P = 0.007) were significantly less expressed, and PPARα (P = 0.008) was increased in female HF-Met group. In male, only G6Pase was statistically significant (P = 0.004). In HF-N, AMPKα (P < 0.001), PPARα (P = 0.029), and G6Pase (P = 0.009) was significantly increased. In fat pad, leptin (P = 0.004) and MCP-1 (P = 0.008) were increased in male HF-Met, and only TNFα (P = 0.001) was significantly decreased in female. In HF-N, the expression of adiponectin (P = 0.007), MCP-1 (P < 0.001), TNFα (P < 0.001), and IL-6 (P < 0.001) were statistically significant. Among them, leptin (P = 0.004) and MCP-1 (P = 0.011) in male, and G6Pase (P < 0.001), MCP-1 (P < 0.001), and IL-6 (P < 0.001) in female were significantly different with metformin treatment during HFD.

A



B

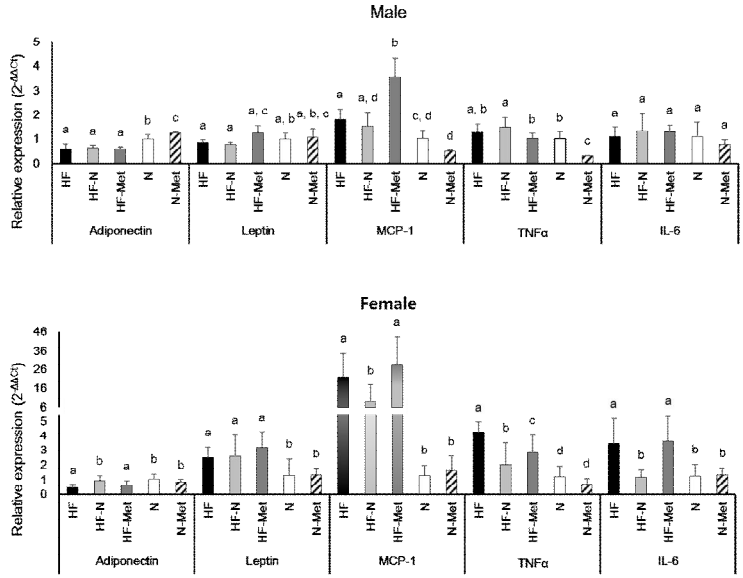


Figure 2.4. Metabolic and inflammatory biomarkers in liver and fat pad. Relative mRNA expression level of metabolic and inflammatory biomarkers was analyzed using qPCR

in (A) liver and (B) fat pad. Relative quantification method, $2^{-\Delta\Delta Ct}$ ($\Delta\Delta Ct = (C_{t.Target} - C_{t.GAPDH})_{Group1} - (C_{t.Target} - C_{t.GAPDH})_{Group2}$), was used for analysis of gene expression of biomarker comparing GAPDH internal control. Statistical significance was assessed by Duncan's post-hoc test.

Expression of mucin by metformin treatment

Expression level of mucin was estimated with MUC2 and MUC5 gene in small intestine, those were significantly more expressed after metformin treatment in female mice (Fig. 2.5A; MUC2: $P = 0.003$, MUC5: $P = 0.010$), but not in male (MUC2: $P = 0.110$, MUC5: $P = 0.884$). Figure 2.5B showed immunohistochemical analysis of small intestinal mucous with anti-MUC5AC in female mice with and without metformin treatment during HFD.

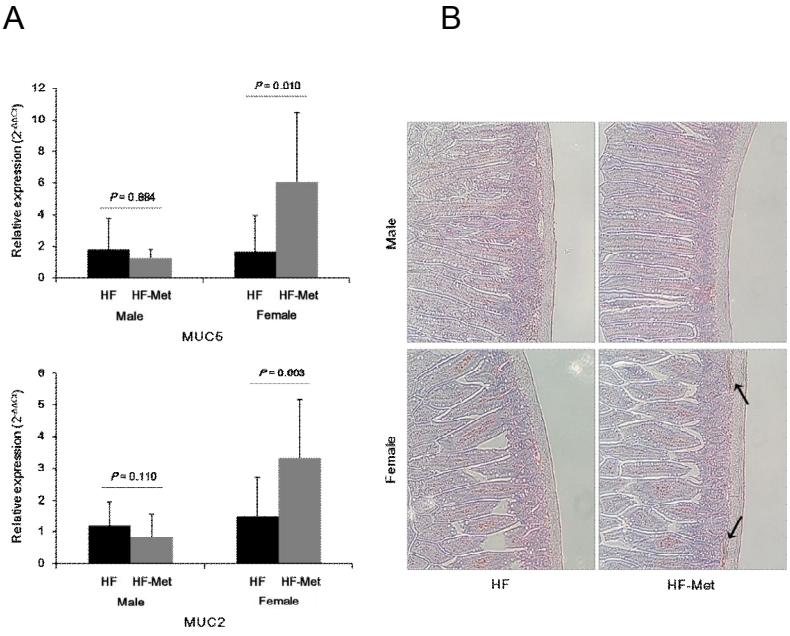


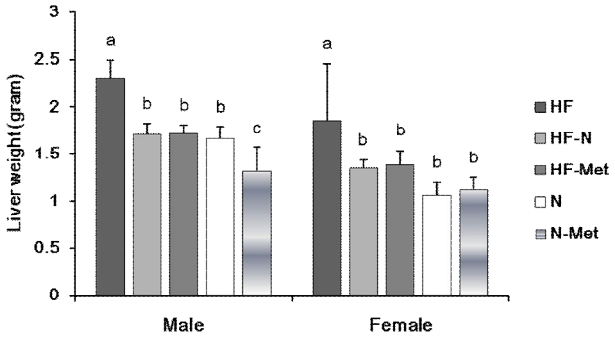
Figure 2.5. Expression level of mucin in small intestine. mRNA expression levels of MUC2 and MUC5 were relatively increased after metformin treatment during HFD in

female mice. Relative quantification method, $2^{-\Delta\Delta Ct}$ ($\Delta\Delta Ct = (C_{t.Target} - C_{t.GAPDH})_{Group1} - (C_{t.Target} - C_{t.GAPDH})_{Group2}$), was used for analysis of gene expression of biomarker comparing GAPDH internal control. Statistical significance was assessed by Mann-Whitney U Test. (B) Thickened intestinal mucosa by metformin treatment was additionally confirmed by immunohistochemistry with anti-MUC5AC. Marked arrows are stained MUC5AC. Magnification 10×.

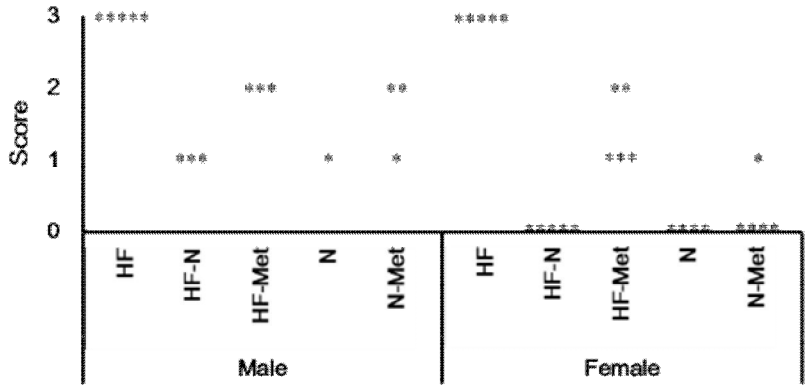
Histological change of liver by metformin treatment

The weights of liver in HF, HF-N, and HF-Met mice groups were measured after dissection. The weights of liver of HF-N and HF-Met were significantly different from those of HF in both male (HF-N: *P* =0.036, HF-Met: *P* =0.036) and female (HF-N: *P* =0.095, HF-Met: *P* =0.095) (Fig. 2.6A). Pathological diagnosis of liver section was further analyzed, pathological diagnosis was evaluated with score as follows: 0-3 for extend of steatosis; and 0 = no involvement; 1 = mild; 2 = moderate; 3 = severe (*39*). Severe steatosis of liver by fed HFD was recovered as change to ND and metformin treatment (Fig. 2.6B-C). There is no difference of inflammation score diagnosed by pathologist in HF-N and HF-Met mice groups.

A



B



C

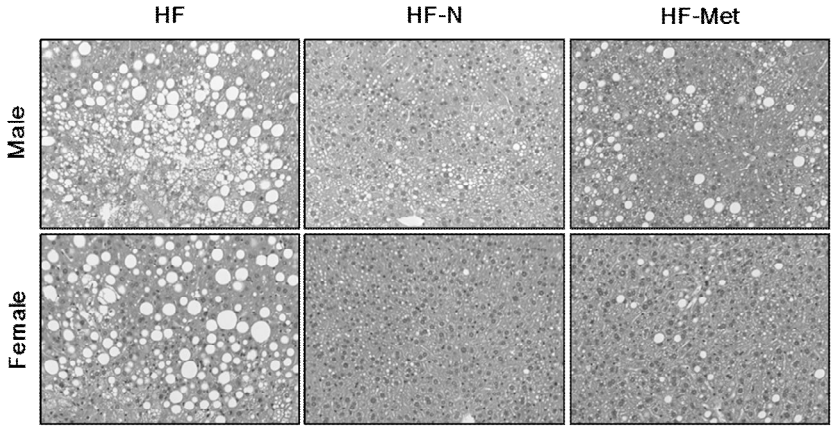


Figure 2.6. Histology of liver. Histological change of liver was observed by diet change to ND and metformin treatment in HF and N mice groups (A) Weight of liver was decreased by ND and metformin treatment. (B) Score 0-3 for extend of steatosis was evaluated by pathologist. 0 = no involvement; 1 = mild; 2 = moderate; 3 = severe. Each dot signify diagnosed each liver samples. (C) Steatosis of liver was observed by optical microscope, magnification 20×. Steatosis was improved by ND and metformin treatment.

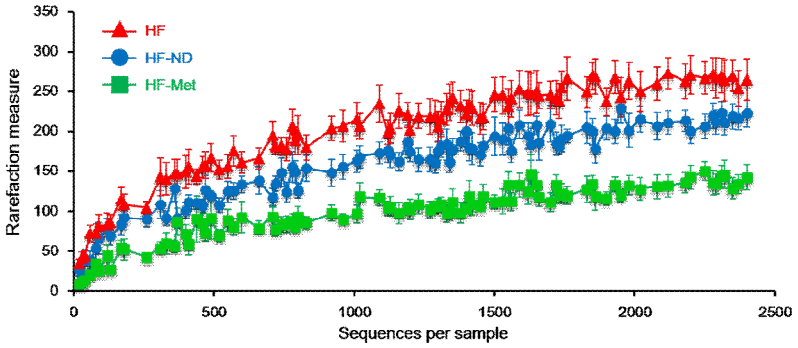
Summary of sequence reads

Total 302,689 sequences were generated from 40 stool samples. Among them, 238,522 sequences were obtained after quality filtering, and those average sequences per sample were 5,963 ± 1,127. Collected sequences of samples were subsequently analyzed and classified to seven phylum, *Actinobacteria*, *Bacteroidetes*, *Deferribacteres*, *Firmicutes*, *Proteobacteria*, *Tenericutes*, and *Verrucomicrobia* (Fig. 2.8A).

Bacterial diversity of gut microbiome

Figure 2.7 described the difference of microbial diversity between mice. Alpha-diversities of gut microbiota of three groups were significantly decreased in the order of HF, HF-N and HF-Met (Fig. 2.7A). But, unlike HF group, there is no significant effect of metformin treatment on alpha diversity of gut microbiota in ND groups (data not shown). PCoA based on phylogenetic analysis clearly indicated clustering of gut mircrobiota within groups. Three groups of HF, HF-Met, and HF-N were clearly separated in PCoA plot (Fig. 2.7B). Furthermore, UniFrac distance between the groups was measured, UniFrac distance by metformin treatment was much higher than that caused by subsequent diet change from HFD to ND (Fig. 2.7C).

A



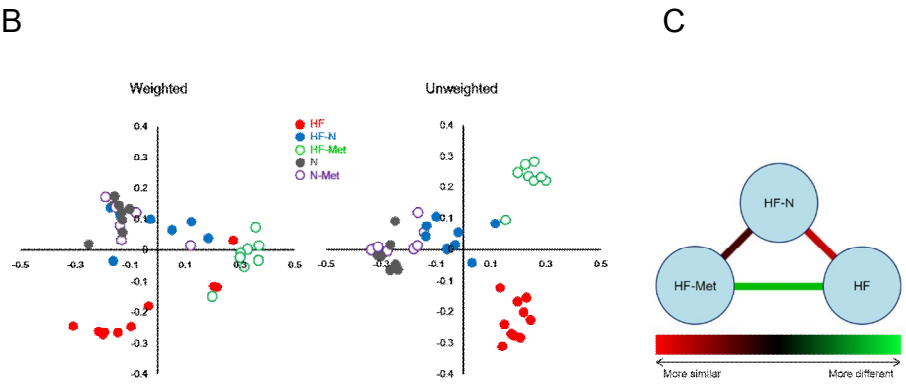


Figure 2.7. Microbial diversity and difference of bacterial community between groups categorized by diet and metformin treatment. Bacterial diversity was significantly decreased by metformin treatment and ND comparing to HFD, those were clearly clustered by groups categorized by diet and metformin treatment. Moreover, bacterial clustering by groups by weighted analysis explained that relative abundance of bacterial community was significantly changed by metformin treatment and diet. Bacterial communities between HF-Met and HF mice groups were more different than those between HF-N and HF. (A) Rarefaction curve of bacterial diversity from 40 mice stool samples (B) Bacterial communities clustered using weighted and unweighted principle coordinate analysis (PCoA) (C) Visualized UniFrac distance between groups.

Relative abundance of gut microbiota by metformin treatment

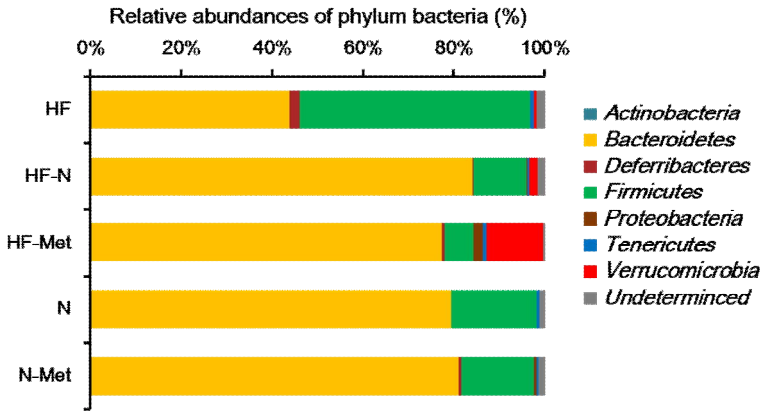
The different bacterial community structure was described in Figure 2.8. The composition of phylum *Bacteroidetes* in HF mice group (43.8 ± 22.4%) was significantly lower than ND (79.4 ± 10.0%) (Fig. 2.8A). When metformin was treated, the composition of phylum *Bacteroidetes* was increased to 77.5 ± 8.7%, which level was becoming close to ND. On the contrary, the composition of phylum *Fimicutes* was significantly higher in HF (Fig. 2.8C-D) (50.7 ± 19.2%). The compositions of phylum *Proteobacteria* (2.1 ± 2.8%) and *Verrucomicrobia* (12.4 ± 5.3%) were significantly increased in HF-Met, but not HF-N. Phylum *Bacteroidetes* was more abundant in female comparing male in HF (Fig. 2.11A). Between N and HF group, phylum *Fimicutes*,

Deferribacteres, and *Verrucomicrobia* were significant abundant in HF (Fig 2.9A). In addition, phylum *Tenericutes* was abundant in male of HF-N, genus *Parabacteroides* was abundant in female of N group (Fig. 2.11C-D).

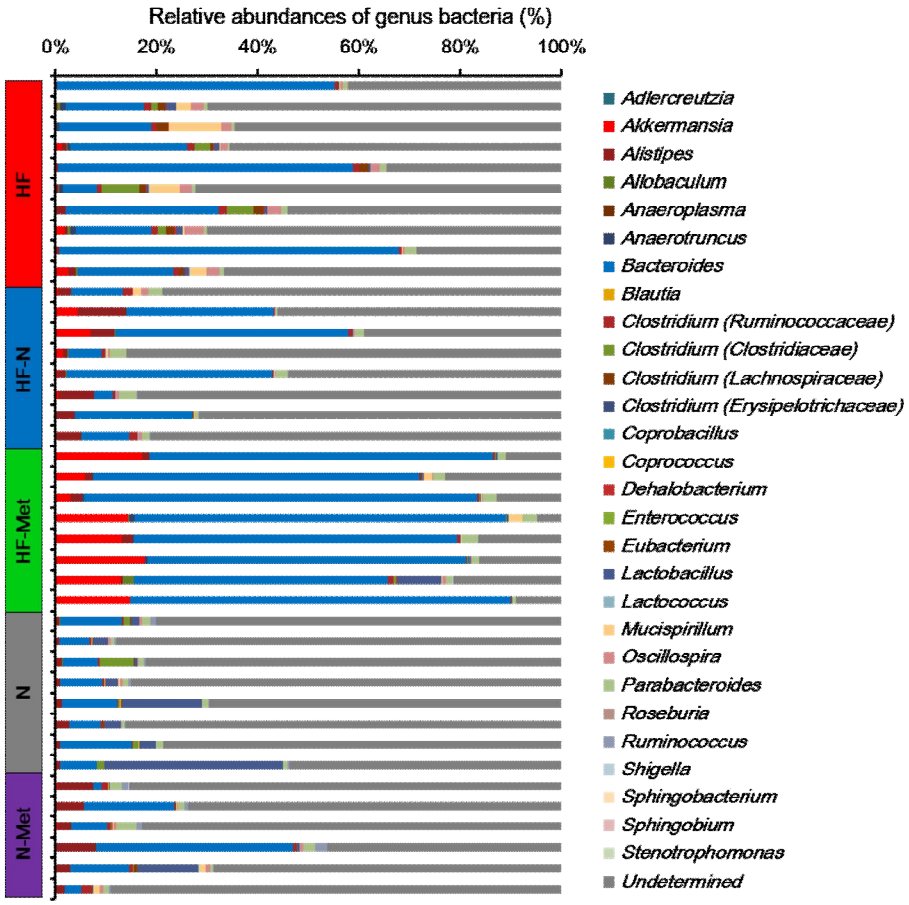
In HF-Met group, family *Bacteroidaceae, Verrucomicrobiaceae*, and *Clostridiales Family XIII. Incertae Sedis*, and genus *Clostridium* and *Akkermansia, and* species *Akkermansia muciniphila* and *Clostridium cocleatum* were remarkably changed comparing other HF and HF-N (Fig. 2.8C-D, Fig. 2.9B). In ND, bacterial abundance was also influenced by metformin treatment. Family *Rikenellaceae, Ruminococcaceae*, and *Verrucomicrobiaceae*, and genus *Alistipes, Akkermansia*, and *Clostridium* were abundant in N-Met comparing N group (Fig. 2.10). In comparison of difference between male and female in HF-Met, genus *Coprobacillus* was abundant in male, genus *Clostridium* and *Bacteroides*, family *Lactobacillaceae*, and class *Bacteroidia* were abundant in female (Fig. 2.11B).

There was no significant difference between male and female in N-Met group.

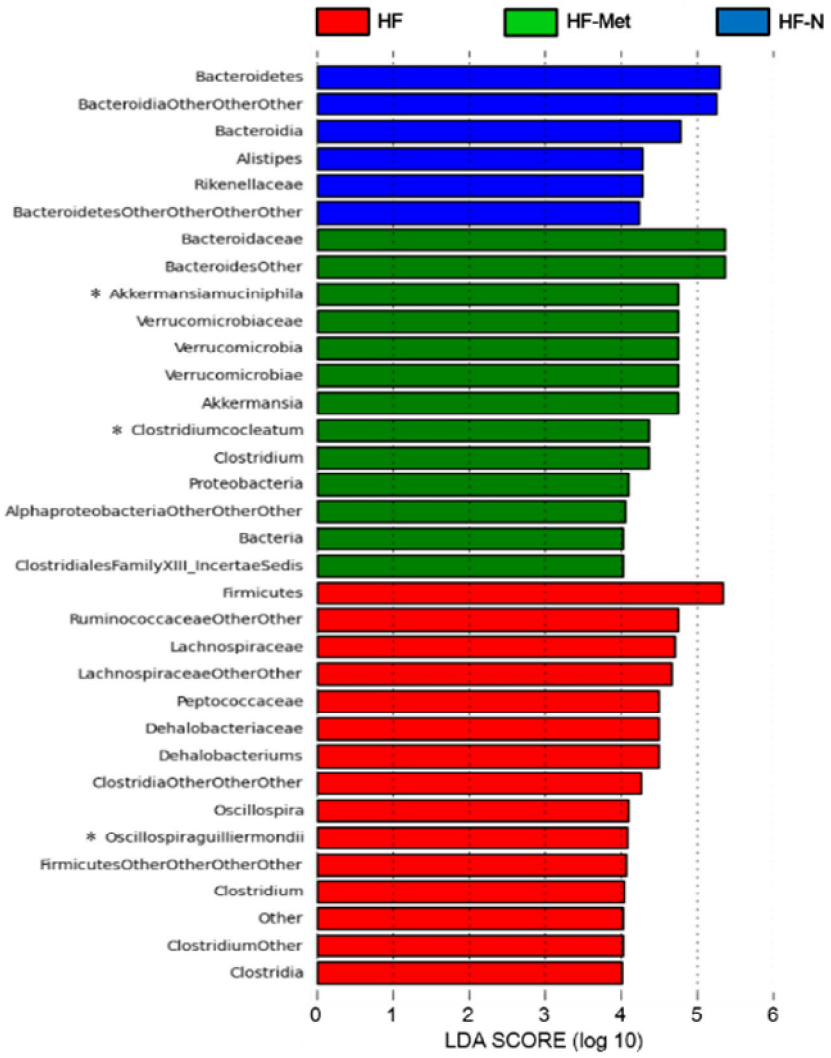
A



B


Relative abundances of genus bacteria (%)
0%
20%
40%
60%
80%
100%
HF
HF-N
HF-Met
N
N-Met
Adlercreutzia
Akkermansia
Alistipes
Allobaculum
Anaeroplasma
Anaerotruncus
Bacteroides
Blautia
Clostridium (Ruminococcaceae)
Clostridium (Clostridiaceae)
Clostridium (Lachnospiraceae)
Clostridium (Erysipelotrichaceae)
Coprobacillus
Coprococcus
Dehalobacterium
Enterococcus
Eubacterium
Lactobacillus
Lactococcus
Mucispirillum
Oscillospira
Parabacteroides
Roseburia
Ruminococcus
Shigella
Sphingobacterium
Sphingobium
Stenotrophomonas
Undetermined

C


HF
HF-Met
HF-N
Bacteroidetes
BacteroidiaOtherOtherOther
Bacteroidia
Alistipes
Rikenellaceae
BacteroidetesOtherOtherOtherOther
Bacteroidaceae
BacteroidesOther
* Akkermansiamuciniphila
Verrucomicrobiaceae
Verrucomicrobia
Verrucomicrobiae
Akkermansia
* Clostridiumcocleatum
Clostridium
Proteobacteria
AlphaproteobacteriaOtherOtherOther
Bacteria
ClostridialesFamilyXIII_IncertaeSedis
Firmicutes
RuminococcaceaeOtherOther
Lachnospiraceae
LachnospiraceaeOtherOther
Peptococcaceae
Dehalobacteriaceae
Dehalobacteriums
ClostridiaOtherOtherOther
Oscillospira
* Oscillospiraguilliermondii
FirmicutesOtherOtherOtherOther
Clostridium
Other
ClostridiumOther
Clostridia
0
1
2
3
4
5
6
LDA SCORE (log 10)

D

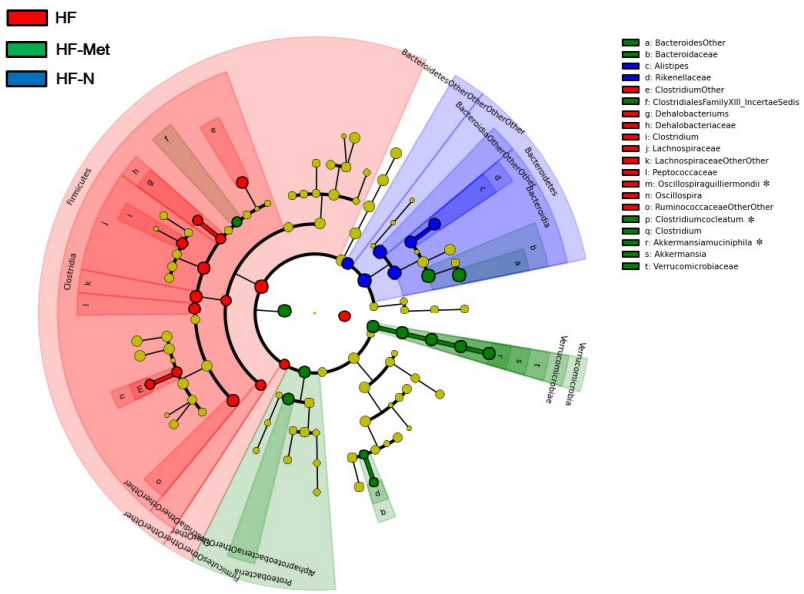
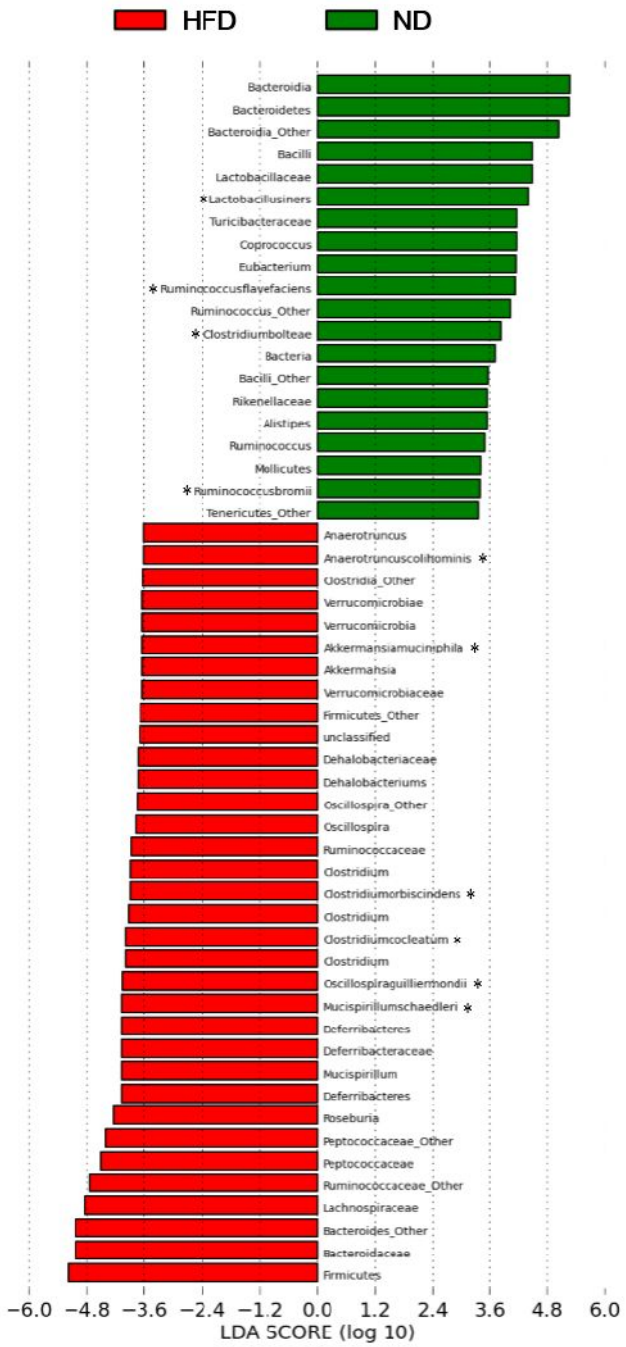


Figure 2.8. Taxonomic comparison from bacterial 16S rRNA genes. Taxonomic comparison was performed using 40 stool samples collected from both male and female mice. Total seven of phylum and 28 of genus were identified, bacterial abundance by metformin treatment and ND was statistically analyzed. (A) Bacterial classification by phylum level. (B) Bacterial classification by genus level. (C-D) LEfSe result and cladogram were produced with *P* value 0.05 in both Kruskal-Wallis among classes and Wilcoxon test between subclasses. Threshold on the logarithmic LDA score was 4.0. *: Bacteria species.

A


HFD
ND
Bacteroidia
Bacteroidetes
Bacteroidia_Other
Bacilli
Lactobacillaceae
*Lactobacillusiners
Turicibacteraceae
Coprococcus
Eubacterium
*Ruminococcusflavefaciens
Ruminococcus_Other
*Clostridiumbolteae
Bacteria
Bacilli_Other
Rikenellaceae
Alistipes
Ruminococcus
Mollicutes
*Ruminococcusbromii
Tenericutes_Other
Anaerotruncus
Anaerotruncuscolihominis *
Clostridia_Other
Verrucomicrobiae
Verrucomicrobia
Akkermansiamuciniphila *
Akkermansia
Verrucomicrobiaceae
Firmicutes_Other
unclassified
Dehalobacteriaceae
Dehalobacteriums
Oscillospira_Other
Oscillospira
Ruminococcaceae
Clostridium
Clostridiumorbiscindens *
Clostridium
Clostridiumcocleatum *
Clostridium
Oscillospiraguilliermondii *
Mucispirillumschaedleri *
Deferribacteres
Deferribacteraceae
Mucispirillum
Deferribacteres
Roseburia
Peptococcaceae_Other
Peptococcaceae
Ruminococcaceae_Other
Lachnospiraceae
Bacteroides_Other
Bacteroidaceae
Firmicutes
−6.0 −4.8 −3.6 −2.4 −1.2 0.0 1.2 2.4 3.6 4.8 6.0
LDA SCORE (log 10)

B

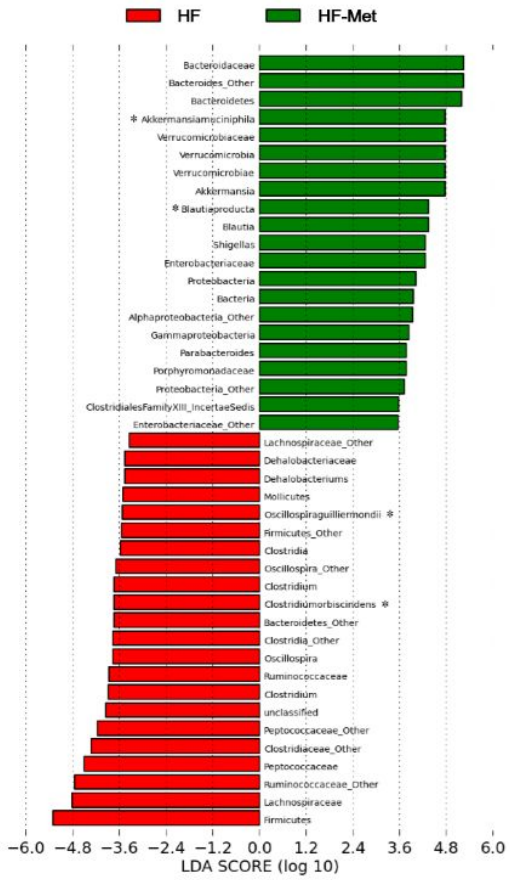
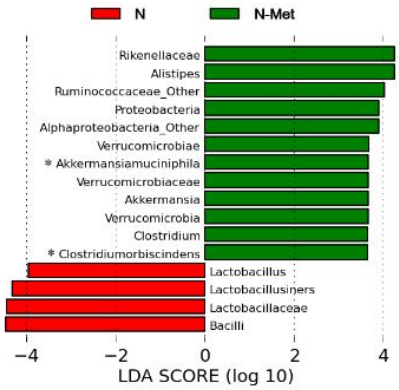


Figure 2.9. Taxonomic comparison by diet and metformin treatment from bacterial 16S rRNA genes. Relative bacterial abundance of HFD mice was changed by diet change to ND and metformin treatment, those were statistically analyzed. Metformin treatment during HFD caused distinguishing bacterial abundance in comparison to diet change to ND. (A) Difference of bacterial abundances by HFD and ND. (B) Specific Bacterial abundances in HF-Met comparing to HF mice group. LEfSe result was produced with *P* value 0.05 in both Kruskal-Wallis among classes and Wilcoxon test between subclasses. Threshold on the logarithmic LDA score was 3.0.

A



B

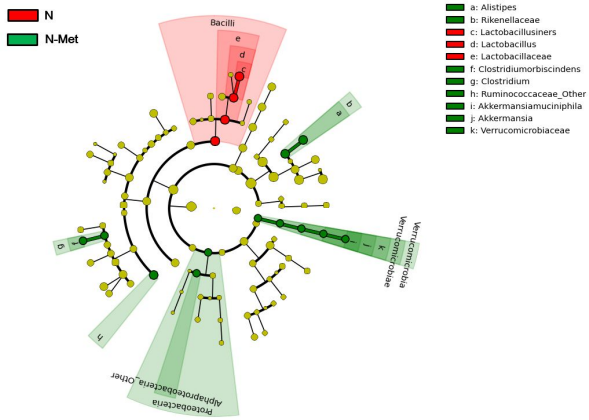
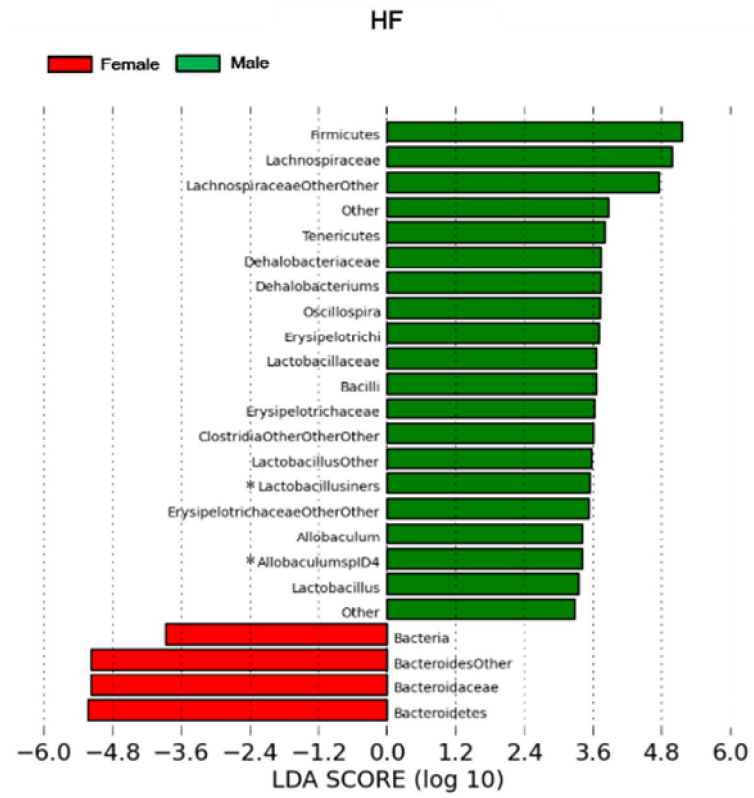
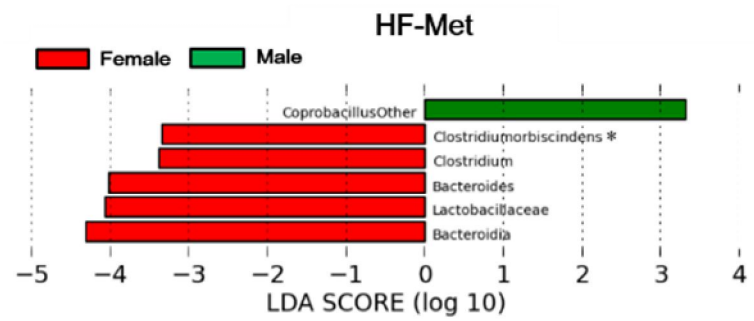


Figure 2.10. Taxonomic comparison by metformin treatment in ND. Metformin administration caused the increase of species *Akkermansia muciniphila* in ND as well as HFD. (A) LDA Score and (B) cladogram were produced with *P* value 0.05 in both Kruskal-Wallis among classes and Wilcoxon test between subclasses. Threshold on the logarithmic LDA score was 3.0. *: Bacteria species.

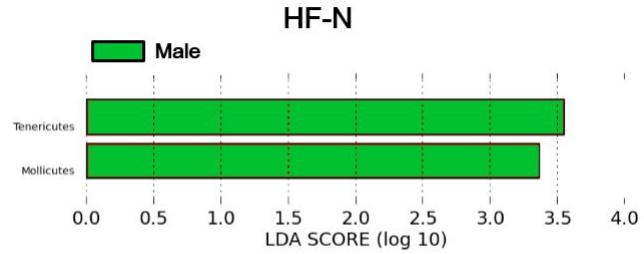
A


HF
Female
Male
Firmicutes
Lachnospiraceae
LachnospiraceaeOtherOther
Other
Tenericutes
Dehalobacteriaceae
Dehalobacteriums
Oscillospira
Erysipelotrichi
Lactobacillaceae
Bacilli
Erysipelotrichaceae
ClostridiaOtherOtherOther
LactobacillusOther
* Lactobacillusiners
ErysipelotrichaceaeOtherOther
Allobaculum
* AllobaculumspID4
Lactobacillus
Other
Bacteria
BacteroidesOther
Bacteroidaceae
Bacteroidetes
−6.0 −4.8 −3.6 −2.4 −1.2 0.0 1.2 2.4 3.6 4.8 6.0
LDA SCORE (log 10)

B


HF-Met
Female
Male
CoprobacillusOther
Clostridiumorbiscindens *
Clostridium
Bacteroides
Lactobacillaceae
Bacteroidia
−5 −4 −3 −2 −1 0 1 2 3 4
LDA SCORE (log 10)

C



D

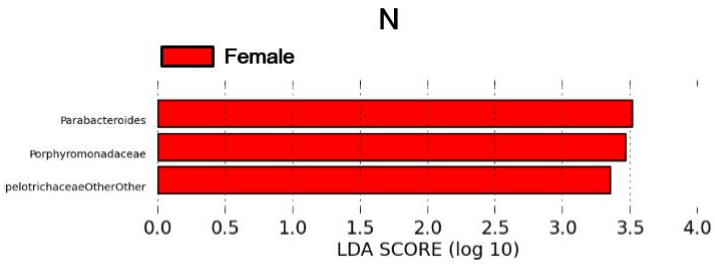


Figure 2.11. Taxonomic comparison between male and female. Relative bacterial abundance was statistically different between male and female mice in each group. Taxonomic comparison was analyzed in HF (A), HF-Met (B), HF-N (C), and N group (D), respectively. LDA Score were produced with *P* value 0.05 in both Kruskal-Wallis among classes and Wilcoxon test between subclasses. There was no significant difference between male and female in N-Met group. Threshold on the logarithmic LDA score was 3.0. *: Bacteria species.

Prediction of KEGG pathways from gut microbiota after metformin treatment

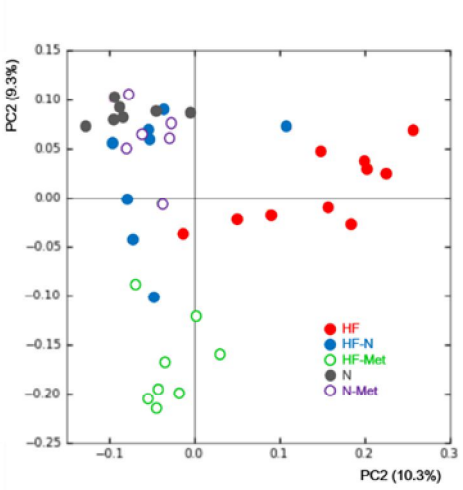
PICRUSt was performed to predict KEGG pathways from gut microbiota which was changed by metformin treatment and diet. After normalizing by abundance, relative abundance of KEGG pathways was estimated (Fig 2.13). A total of 245 KEGG pathways were generated. These were categorized into cellular processes (*n*=10),

environmental information processing (n = 13), genetic information processing (n = 24), human diseases (n = 26), metabolism (n = 130), organismal systems (n = 16), and unclassified (n = 26) (Fig. 2.13). PCoA plot shows the clear clustering of three groups (HF, HF-N, and HF-Met) (Fig. 2.12).

First, total 11 and 30 KEGG pathways were significantly upregulated when diet change from HFD to ND and metformin treatment during HFD comparing HF mice group, respectively. Among them, only two KEGG pathways of chloroalkane and chloroalkene degradation and nitrotoluene degradation in HF-Met group were overlapped with HF-N. On the other hand, 12 KEGG pathways were overlapped with those in N-Met group. Exclusive of overlapped KEGG pathways, total unique 18 KEGG pathways were identified by metformin treatment during HFD, those were described in Table 2.2.

Statistically significant KEGG pathways for each group were additionally identified by LEfSe (Figure 2.12B). In HF group, porphyrin and chlorophyll metabolism, methane metabolism, cysteine and methionine metabolism, purine metabolism, lysine biosynthesis, starch and sucrose metabolism, and one carbon pool by folate were significantly abundant based on LDA score. With change of HFD to ND, metabolic pathways including pyrimidine metabolism, amino acid related enzymes, histidine metabolism, peptidases, and peptidoglycan biosynthesis were significantly enriched. One the other hand, with metformin treatment during HFD, different metabolic pathways such as lipopolysaccharide biosynthesis, sphingolipid metabolism, fructose and mannose metabolism, pentose and glucuronate interconversions, and propanoate metabolism were significantly enriched.

A


PC2 (9.3%)
PC2 (10.3%)
HF
HF-N
HF-Met
N
N-Met

B

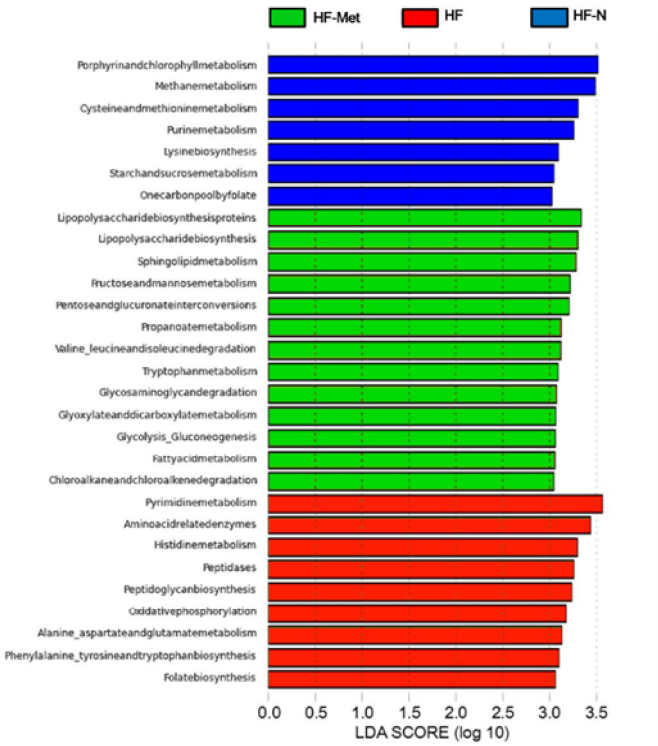

HF-Met
HF
HF-N
Porphyrinandchlorophyllmetabolism
Methanemetabolism
Cysteineandmethioninemetabolism
Purinemetabolism
Lysinebiosynthesis
Starchandsucrosemetabolism
Onecarbonpoolbyfolate
Lipopolysaccharidebiosynthesisproteins
Lipopolysaccharidebiosynthesis
Sphingolipidmetabolism
Fructoseandmannosemetabolism
Pentoseandglucuronateinterconversions
Propanoatemetabolism
Valine_leucineandisoleucinedegradation
Tryptophanmetabolism
Glycosaminoglycandegradation
Glyoxylateanddicarboxylatemetabolism
Glycolysis_Gluconeogenesis
Fattyacidmetabolism
Chloroalkaneandchloroalkenedegradation
Pyrimidinemetabolism
Aminoacidrelatedenzymes
Histidinemetabolism
Peptidases
Peptidoglycanbiosynthesis
Oxidativephosphorylation
Alanine_aspartateandglutamatemetabolism
Phenylalanine_tyrosineandtryptophanbiosynthesis
Folatebiosynthesis
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5
LDA SCORE (log 10)

Figure 2.12. Comparison of KEGG pathways predicted using PICRUSt by diet and metformin treatment. Groups categorized by metformin treatment and diet were clearly clustered by KEGG pathways predicted using PICRUSt as well as bacterial diversity. Total 245 KEGG pathways were generated, significantly increased KEGG pathways by metformin treatment are further analyzed using PCoA and LEfSe. (A) Clustering of five groups by KEGG pathways using PCoA. (B) LEfSe showed the statistically significant abundance of KEGG pathways in HF-N, HF-Met, and HF mice groups. LEfSe result showed sequential significant ranking in $P < 0.05$ in both Kruskal-Wallis among classes and Wilcoxon test between subclasses. Threshold on the logarithmic LDA score was 3.0.

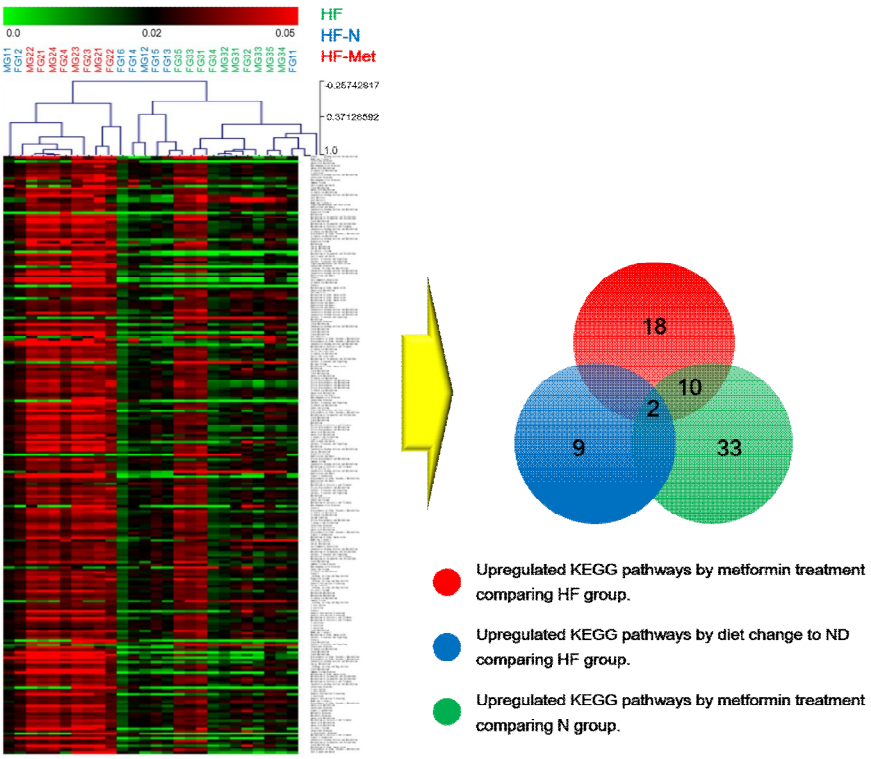


Figure 2.13. Prediction of KEGG pathways by diet and metformin treatment using PICRUSt. Visualized heatmap of relative abundance was generated using 245 KEGG pathways. Stool samples were hierarchically clustered by Pearson correlation. Total 30

KEGG pathways were predicted to be increased by metformin treatment during HFD. Among them, 2 and 12 KEGG pathways were overlapped with those of HF-N and N-Met group, respectively. Finally, 18 unique KEGG pathways were predicted to be increased by metformin treatment during HFD.

Table 2.2. List of KEGG pathways predicted from the abundance of gut microbiota by diet change to normal diet and metformin treatment.

High-fat diet		Normal diet
Change to normal diet (*n* = 11)	**Metformin treatment (*n* = 30)**	**Metformin treatment (*n* = 45)**
Benzoate degradation	Aminobenzoate degradation	African trypanosomiasis
Biosynthesis of ansamycins	Arachidonic acid metabolism	alpha-Linolenic acid metabolism
Chloroalkane and chloroalkene degradation	**Ascorbate and aldarate metabolism**	Aminoacyl-tRNA biosynthesis
D-Alanine metabolism	**Biosynthesis of unsaturated fatty acids**	Aminobenzoate degradation
Dioxin degradation	**Bisphenol degradation**	Amyotrophic lateral sclerosis (ALS)
Glycerolipid metabolism	Caprolactam degradation	Apoptosis
Nitrotoluene degradation	Carotenoid biosynthesis	Arachidonic acid metabolism
Porphyrin and chlorophyll metabolism	Chlorocyclohexane and chlorobenzene degradation	Atrazine degradation
Protein kinases	Ether lipid metabolism	Bacterial chemotaxis
Tetracycline biosynthesis	**Fatty acid elongation in mitochondria**	Bacterial motility proteins
Xylene degradation	**Fatty acid metabolism**	Bile secretion
	Flavonoid biosynthesis	Biosynthesis and biodegradation of secondary metabolites
	Fluorobenzoate degradation	Biosynthesis of siderophore group nonribosomal peptides
	Geraniol degradation	Caprolactam degradation
	Glycosaminoglycan degradation	Cardiac muscle contraction
	Limonene and pinene degradation	Carotenoid biosynthesis
	Linoleic acid metabolism	Chagas disease (American trypanosomiasis)
	Lipoic acid metabolism	Chlorocyclohexane and chlorobenzene degradation
	Lipopolysaccharide biosynthesis	Colorectal cancer
	Lipopolysaccharide	D-Arginine and

biosynthesis proteins	D-ornithine metabolism
Nitrotoluene degradation	Electron transfer carriers
Sphingolipid metabolism	Ether lipid metabolism
Steroid biosynthesis	Ethylbenzene degradation
Steroid hormone biosynthesis	Flagellar assembly
Stilbenoid, diarylheptanoid and gingerol biosynthesis	Flavone and flavonol biosynthesis
Styrene degradation	Flavonoid biosynthesis
Synthesis and degradation of ketone bodies	Fluorobenzoate degradation
Toluene degradation	Germination
Tryptophan metabolism	Influenza A
Valine, leucine and isoleucine degradation	Inorganic ion transport and metabolism
	Insulin signaling pathway
	Limonene and pinene degradation
	Meiosis – yeast
	Nitrotoluene degradation
	Non-homologous end-joining
	Parkinson's disease
	Polycyclic aromatic hydrocarbon degradation
	Polyketide sugar unit biosynthesis
	Small cell lung cancer
	Sporulation
	Steroid biosynthesis
	Stilbenoid, diarylheptanoid and gingerol biosynthesis
	Toxoplasmosis
	Two-component system
	Viral myocarditis

Lists with bold character were unique 18 KEGG pathways predicted by metformin treatment during HFD.

Correlation of bacterial abundance with metabolic biomarkers

Correlation coefficient and significance in HF-Met group were described in Fig. 7. Body weight and glucose level were negatively correlated with *Clostridium orbiscindens* and *Akkermansia muciniphila*, respectively, *Clostridium orbiscindens* was also positively correlated with TNFα. Both MUC2 and MUC5 showed positive correlation with *Clostridium orbiscindens*. PPAR and GLUT2 showed negative correlation with *Clostridium orbiscindens*, *Blautia product*, and *Allobaculum sp. ID4*. AMPKα1 and TG were positively correlated with *Clostridium cocleatum*.

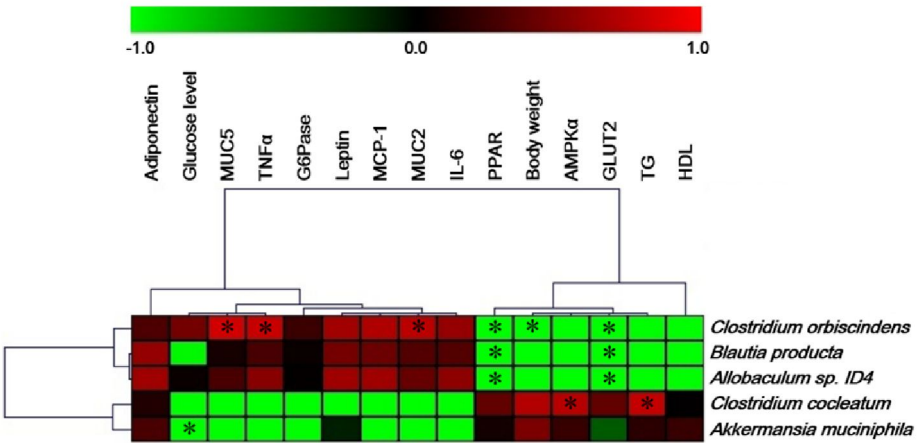


Figure 2.14. Correlation between metabolic biomarkers and bacterial abundance. Correlation was analyzed in HF-Met mice group including both male (n = 4) and female (n = 4) using Spearman correlation. AMPKα1: AMP-activated protein kinase alpha 1, PPARα: Peroxisome proliferator-activated receptor alpha, GLUT2: Glucose transporter 2, G6Pase: Glucose-6-phosphatase, MCP-1: Monocyte chemoattractant protein-1, TNFα: Tumor necrosis factor alpha, IL-6 (Interleukin-6), MUC2: Mucin 2, MUC5: Mucin 5, TG: Total cholesterol, and HDL: High-density lipoprotein. *: Significant in $P < 0.05$.

Correlation of *Akkermansia muciniphila* with other bacteria

Correlation coefficient of *Akkermansia muciniphila* was showed in Fig. 2.15. As increasing of *Akkermansia muciniphila* in metformin-treated mice during HFD, *Lactobacillus iners* and *Mucispirillum schaedleri* were significantly decreased in male and female, respectively. There was no correlation with other bacterial.

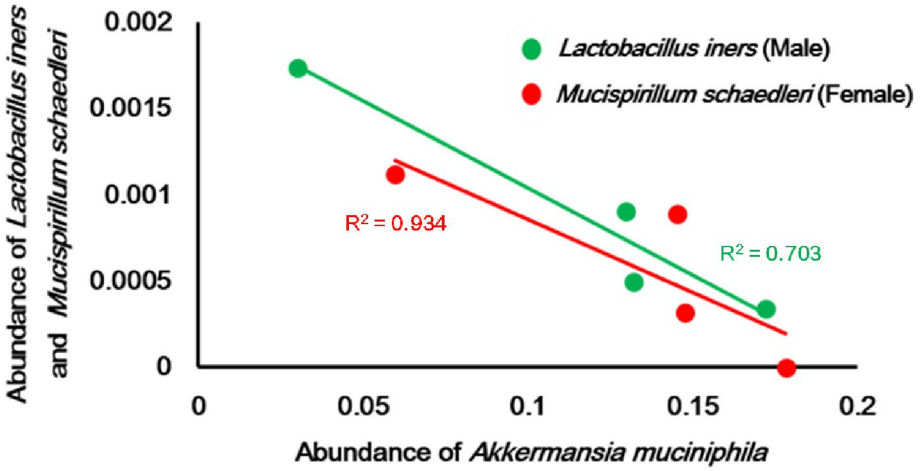


Figure 2.15. Correlation coefficient between abundance of *Akkermansia muciniphila* and other bacteria. Correlation was analyzed in HF-Met mice group including both male (n = 4) and female (n = 4) using Spearman correlation. *Akkermansia muciniphila* showed significant negative correlation with *Lactobacillus iners* and *Mucispirillum schaedleri* in male and female mice, respectively.

Relative enrichment of *Akkermansia muciniphila* in BHI with metformin

Figure 2.16 showed the effect of metformin and phenformin on growth of *Akkermansia muciniphila*. The percentage of *Akkermansia muciniphila* comparing to total bacteria in pooling stool samples of HF-Met mice groups was 14.3 ± 2.6. After 3 and 6 hours post-inoculation, those of BHI broth were 3.0 ± 0.5 and 3.8 ± 1.8, respectively. On the other hand, the percentage of *Akkermansia muciniphila* was significantly increased in BHI broth supplemented with 0.1 M metformin (8.8 ± 3.7 and 6.6 ± 2.8), respectively.

Moreover, BHI supplemented with 0.1 M phenformin, another biguanide class, showed more effective enrichment of *Akkermansia muciniphila* (19.1 ± 4.6 and 14.7 ± 2.1), respectively.

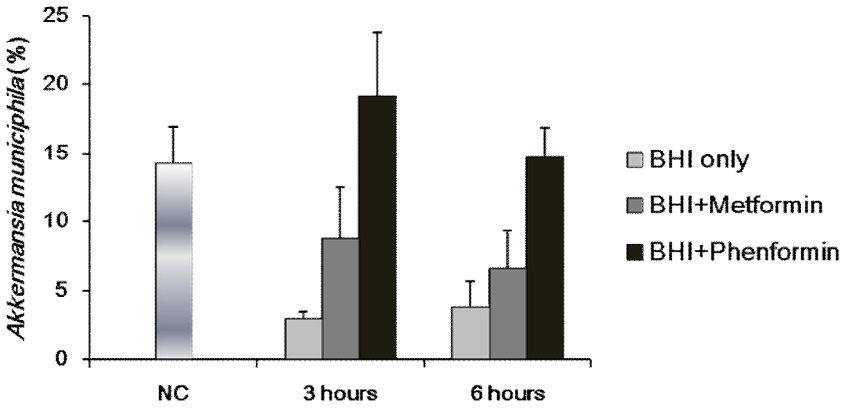


Figure 2.16. Effect of metformin on growth of *Akkermansia muciniphila* comparing to total bacteria in BHI medium. BHI medium supplemented with metformin and phenformin were increased the abundance of *Akkermansia muciniphila* among total bacteria comparing to normal BHI medium at 3 and 6 hours after incubation. Quantification of *Akkermansia muciniphila* and total bacteria was performed using real-time quantitative PCR. Percentage of *Akkermansia muciniphila* was calculated comparing total bacteria as 100%.

DISCUSSION

Increasing microbiome researches describe the association between dysbiosis of gut microbiota and phenotypes of metabolic disorders, those descriptive results have supported that gut microbiota could regulate the development of metabolic disorders including obesity and T2D (*12-14*). In recent trend in microbiome research, large-scale and well-controlled studies have been required to implicate the dysbiosis of gut microbiota in relation to metabolic disorders. Because, the correlation between the relative bacterial abundances was not consistent in same phenotype, experimental significant difference in rodent model did not coincident in humans (*40*). In spite of the limitation of rodent model, various approaches in rodent model systems have been used to establish causal capability between gut microbiota and phenotypes (*41-43*).

Abundance of *Akkermansia muciniphila* was most notable change by metformin treatment. From two recent studies, the association between the abundance of *Akkermansia muciniphila* and obesity and T2D was revealed (*26, 27*). Especially, the abundance of *Akkermansia muciniphila* by metformin treatment was already observed in mouse model (*27*). But, that results were limited to association between gut microbiota and glucose homeostasis for short period, evidences were inadequate to explain the metabolic improvement by metformin treatment via gut microbiota. This study described multilateral analysis in order to establish causal capability of gut microbiota.

First, specific changes in gut microbiota by metformin treatment during HFD were revealed distinguishing to the those in ND. HF-N group was included as a comparative control, because metformin treatment affects food and calorie intake by leptin sensitivity in both human and mouse (*44, 45*), which could be an influent on metabolic recovery. Daily calorie intake was not influenced by metformin treatment and diet change to ND in this study. However, metformin treatment and ND showed the difference in gut microbiota and phenotypes, we expected that the effect of metformin treatment on metabolic recovery was highly related with the change of gut microbiota regardless of energy intake. Moreover, bacterial diversity was significantly decreased by metformin

treatment and diet change to ND, the former was more significant than the later.

Second, pattern of expression of metabolic biomarkers could help us to understand the correlation between gut microbiota and metabolism and to predict mechanism of effect of gut microbiota on metabolic improvement. Especially, unactivated AMPK during metformin treatment for metabolic improvement in this study might disprove the effect of gut microbiota. Activation of AMPK by metformin treatment has been identified main mechanism of action for improving hyperglycemia (*46*), interestingly, metabolic improvement by metformin treatment was observed without the activation of AMPK. Increase of cytosolic AMP by metformin was suggested as a mechanism of AMPK activation (*47*). Nonetheless, mechanism of action of metformin was not fully understood. Metformin effect on hepatic gluconeogenesis recently was observed in AMPK-deficient mouse model (*33*). On the other hand, antagonism of hepatic glucagon by metformin was suggested as another target for T2D improving in recent study (*48*). After metformin treatment during HFD, significant changes in gut microbiota were observed such as relative abundance of *Akkermansia muciniphila*. Therefore, we strongly considered the role of gut microbiota by metformin treatment on metabolic improvement.

Third, different bacterial abundance by metformin treatment was identified, but that did not satisfy us and we further predicted the significant metabolic functions by metformin treatment PICRUSt based on KEGG pathways. Previous study showed different functional gene composition between T2D and normal group (*49*). In this study, significant 18 KEGG pathways by metformin treatment during HFD were predicted. Among them, sphingolipid metabolism, biosynthesis of unsaturated fatty acids, fatty acid elongation in mitochondria, fatty acid metabolism, linoleic acid metabolism, steroid hormone biosynthesis, and synthesis and degradation of ketone bodies belonged to lipid metabolism, in particular, the metabolism of sphingolipid and fatty acid might be involved in process for the improvement of metabolic disorders. Sphingolipid biosynthetic pathway was influenced by saturated fatty acid and ceramide which is an intermediate form of sphingolipid is responsible for fatty acid-mediated attenuation of

insulin-stimulated glucose uptake (*50, 51*). Sphingolipids carry out not only essential structure but also functional roles (*52*), sphingolipids alteration is highly related with metabolic diseases and obesity-associated conditions. Specific sphingolipid signaling pathways influence major biological processes including insulin resistance, lipid metabolism, inflammation, and immune response (*53*). And, lipogenetic gene expression such as fatty acid synthase (FASN) and acetyl-CoA carboxylase 1 (ACC1) was upregulated by *Akkermansia muciniphila* treatment during HFD (*26*), the abundance of *Akkermansia muciniphila* might had an association with lipid metabolism of bacterial community as well as that of host. Those pathways were known to associate with metformin treatment (*54, 55*), therefore, those results explained changed gut microbiota by metformin treatment in HFD could be suggested to play important roles in improvement of metabolic disorders including obesity and T2D by regulation of lipid metabolism. In addition, the abundance of *Akkermansia muciniphila*, gram-negative bacteria, might cause the high prediction of lipopolysaccharide biosynthesis and glycosaminoglycan degradation in glycan biosynthesis and metabolism.

Fourth, causative effect of metformin on abundance of *Akkermansia muciniphila* was proved *in vitro* growth test with BHI medium supplemented with metformin. It was not certain if the abundance of *Akkermansia muciniphila* was directly caused by metformin administration. In this study, both metformin and phenformin in BHI medium relatively increased the growth of *Akkermansia muciniphila* comparing to total bacteria. Interestingly, *Akkermansia muciniphila* was not detected in ND mice at all, slightly increased after HFD. But, after metformin treatment, the abundance of *Akkermansia muciniphila* was significantly increased approximate 18 times (0.68 to 12.4%) than HF group. Therefore, we also guessed that there was the dose-dependent effect of *Akkermansia muciniphila* on metabolic disorder and improvement. In addition, the sequence of *Akkermansia muciniphila* indentified in this study was 100% identical with strain isolated in human intestine and showed therapeutic effect on metabolic improvement (*56*).

At last, bacterial communities and those correlations with metabolic biomarkers were

different by gender in metabolic improvement by metformin treatment. Sex difference in metabolic disorders and metformin treatment has been considered (*57, 58*). In this study, slight difference in gut microbiome was observed between male and female mice, nevertheless, mice and female mice showed the different expression patterns of metabolic biomarkers by metformin treatment. Previous studies explained that hormone in female had protective effect for metabolic disorders (*59, 60*), in clinical, metformin was used for improving the polycystic ovary syndrome in women (*61*). Until now, the effect of gut microbiota on disease by gender did not reported. Genetic factors by gender is highly related with certain diseases such as autoimmune diseases and cancers (*62, 63*), considering the association between gut microbiota and health, we could guess that the gut microbiota differently effect on pathogenesis and treatment by gender.

Approximately, over 300 million people have T2D worldwide (*64*). After metformin was approved in the late 20th century, is most widely prescribed for the treatment of T2D. But, >40% of T2D patients on antihyperglycaemic treatments including metformin remained above the recommended level of HbA1c (*65*). Recent studies reported that drug response such as drug metabolizing and bioavailability was influenced by gut microbiota as well as patient genetic factors (*66-68*). Therefore, specific change of gut microbiota composition could be an indicator to estimate therapeutic efficacy of metformin treatment. In this study, the abundance of *Akkermansia muciniphila* and negative correlation with *Mucispirillum schaedleri Lactobacillus iners* might be a bacterial indicator for metformin efficacy. Moreover, except for *Akkermansia muciniphila*, changes in abundance of various bacteria which were not well characterized so far were observed by metformin treatment during HFD. As Falkow's molecular Koch's postulates was proposed for microbial pathogenicity (*69*), establishment of causative effect of metformin treatment on metabolic improvement via gut microbiota should be promised by novel experimental approaches such as gnotobiotic mouse model in future.

In conclusion, the diversity and composition of gut microbiota were significantly changed by metformin treatment during HFD, those abundance was highly correlated

with various metabolic biomarkers. Furthermore, certain pathways associated with lipid metabolism including sphingolipid and fatty acid metabolism might be a key role of metformin treatment for improvement of metabolic syndrome. We suggest that the specific composition of gut microbiota by metformin treatment could be a favorable therapeutic effect to metabolic improvement including obesity and T2D.

CHAPTER Ⅲ.

CHANGES IN GUT MICROBIOTA BY NOROVIRUS INFECTION DURING VITAMIN A ADMINISTRATION

INTRODUCTION

Retinoic acid (RA), the metabolite of dietary vitamin A, contributes to both innate and adaptive immune responses (*4*). Recent evidence indicates that loss of RA causes to impaired immunity, whereas excess RA can induce inflammatory disorders (*4*). In animal studies, impaired T cell responses observed in infection model during retinoid receptor deficiency. In recognition and response to viral RNA, retinoic acid-inducible gene 1 (RIG-1) and melanoma differentiation-associated gene 5 (MDA-5) signaling pathway is crucial role in antiviral responses by producing type I interferons (IFNs) (*6*). Despite of major advances in the knowledge of essential role of vitamin A, its deficiency is still public health concerns worldwide. An estimated 250 million preschool children are vitamin A deficient (*70*). In previous community-based studies, sufficient vitamin A supplement reduced the mortality from diarrheal diseases by 39%, from respiratory disease by 70% (*71*). In another recent study reported that vitamin A supplementation is related with modification of the various intestinal chemokines and cytokines responding to norovirus (NoV) infection (*5*).

NoV is the most frequent etiological viral agent of acute gastroenteritis in all age groups worldwide. Human NoV (HuNoV) causes approximately 90% of epidemic nonbacterial outbreaks of gastroenteritis around the world, and is responsible for approximately 50% of all foodborne outbreaks of gastroenteritis in the United States (*72, 73*). NoV is highly contagious, typically, transmitted from person to person via direct contact, food, water, or fomites (*74*). Viral infection causes various clinical symptoms including diarrhea, vomiting, nausea, abdominal pain, and fever for 1-3 days. Especially, NoV associated acute gastroenteritis is often fatal in children and the elderly, which causes significant morbidity and mortality among them (*75*). Unfortunately, there is no effective treatment or vaccine for NoV infection until now, recently, vitamin A supplementation could decrease NoV infection rate and clinical symptoms. At the same time, the responses of various intestinal cytokines were modified by vitamin A supplementation during NoV infection (*5*).

Recent studies indicated that the composition of gut microbiota is pivotal role in pathogen infection and mucosal immune responses (*76, 77*). A cross talk between gut microbiota and mucosal immune system was highly associated with inflammation and enteric infection. For example, altered gut microbiota in mice lacking toll-like receptors (TLRs) and myeloid differentiation primary response gene 88 (Myd88) was highly associated with metabolic syndrome, type 1 diabetes (T1D) and host defense against microbial infection (*7, 8, 78, 79*). However, the role of vitamin A in microbial infection via gut microbiota has not been studied yet.

Therefore, the objectives of this study are to 1) determine the gut microbiome by MNV inoculation and vitamin A administration, 2) investigate the correlation between innate immune response and gut microbiota in MNV inoculation during vitamin A administration.

MATERIALS AND METHODS

Animal model

12-weeks-old ICR male mice were purchased from KOATECH. (Pyungtaek, South Korea) and housed in animal biosafety level 2 (ABL-2) facility in Seoul National University College of Medicine. Retinoic acid (RA) (R-2625, Sigma-Aldrich, USA) was suspended in corn oil, and mice were orally administered at the dosage of 1 and 10 mg/kg/day for 8 days. At the 7th day, mice were infected by the peroral route with 5×10^6 PFU of MNV-1 suspended in PBS using oral gavage. All experimental protocols in this study were approved by the Seoul National University Institutional Animal Care and Use Committee (SNU-111208-4).

16S rRNA gene sequencing of gut microbiome using MiSeq platform

Cecum samples were totally homogenized using PT-2000 E homogenizer (POLYTRON, Switzerland) and bead beating with PowerBead Tube (MO BIO Laboratories, Inc., USA) prior to DNA extraction. Total DNA was extracted using QIAamp® DNA Stool Mini Kit (51504, Qiagen, USA) and QIAcube system (Qiagen, USA). Amplification of 16S rRNA was followed by 16S rRNA Amplification Protocol from Earth Microbiome Project (*80*). For each samples, 16S rRNA genes were amplified using 515F/806R primer set for amplification of V4 region (27F: forward primer, 5' - *AATGATACGGCGACCACCGAGATCTACAC TATGGTAATT GT* ***GTGCCAGCMGCCGCGGTAA*** - 3'; 806R: reverse primer containing a unique 12-based barcode for tagging each PCR product designated by *5' – CAAGCAGAAGACGGCATACGAGAT-*<u>*NNNNNNNNNNNN*</u>*-AGTCAGTCAG CC* ***GGACTACHVGGGTWTCTAAT*** - 3'). Amplified PCR products were purified using UltraClean® PCR Clean-Up Kit (MO BIO Laboratory, Inc., USA). Bacterial 16S rRNA pyrosequencing was performed using MiSeq Reagent Kit V3 (600 cycles) by MiSeq platform (Illumina, USA).

Prior to analyze 16S rRNA sequence, BCL files were extracted to raw fastq files

including read1, index, and read2 sequences using CASAVA-1.8.2. After preprocessing of quality filter and trimming steps using FASTX-Toolkit, sequences were assigned to operational taxonomic units (OTUs, 97% identity), followed by selecting representative sequence using Quantitative Insights Into Microbial Ecology (QIIME 1.7.0). After then, taxonomic composition analysis, alpha-diversity, and beta-diversity were performed. To estimate taxonomic abundance and characterize the difference between groups, LDA Effect Size (LEfSe) was used (*35*). In addition, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) was performed to predict the functional genes in the sampled microbial community based on KEGG pathway database (*36*). Heatmap of functional gene abundance was generated using MultiExperiment Viewer (MEV) software (v4.8.1).

Tissue collection for MNV quantification and gene expression

Tissue were collected at 72 h after MNV infection. After anaesthetizing using Zoletil 50 (30 mg/kg of body weight, Virbac Laboratories) and Rompun (10 mg/kg of body weight, Bayer), blood was collected by cardiac puncture and serum was separated using centrifuge. To analyze the MNV quantification and the expression of cytokines, cecum, spleen, liver, and ileum were removed. Cecum and spleen were homogenized and centrifuged (10,000 × g for 30 min), supernatant was used for MNV detection, and the rest solid was stored in -70℃ until RNA extraction. Approximately 1 cm terminal ileum with Payer's patch was stored in RNAlater® Solution (AM7021, Ambion, USA) until RNA extraction.

RNA extraction and gene expression level using quantitative PCR

Prior to total RNA extraction, ileum sample was totally homogenized using PT-2000 E homogenizer (POLYTRON, Switzerland). Total RNA was extracted using easy-spin™ Total RNA Extraction Kit (iNtRON, Seongnam, South Korea) then cDNA synthesis was followed using High Capacity RNA-to-cDNA Kit (4387406, Applied Biosystems, USA) according to the manufacturer's instructions. To estimate the expression level of mRNA

associated innate immunity, QuantiTect® SYBR® Green PCR Kit (204143, Qiagen, USA) and 7300 Real Time PCR System (Applied Biosystems) was used. Analyzed cytokines and those oligonucleotide primer sets were described in table 3.1. The reaction mixture (25 μl) for real-time PCR was composed of 2x QuantiTect SYBR Green PCR Master Mix (12.5 μl), primers (forward and reverse, 50 pmol 0.2 μl, respectively), RNase-free water (11.1 μl), and template DNA (1 μl). GAPDH was used as the internal control.

Plaque assay for MNV quantification

Homogenized spleen samples were centrifuged with 10,000 g for 30 min. Tenfold serial dilutions of supernatant were inoculated into 6-well plate confluent with RAW 264.7 cells. After incubation for 1 hour at 37℃ in 5% CO_2, the inocula was aspirated and replaced with a mixture 3 ml of 1.5% seaplaque agarose and 2X MEM media. Plaques was visible in 36 to 48 hours.

Table 3.1. Primer sets for immunological biomarkers.

Target gene	Forward (5' – 3')	Reverse (5' – 3')
RIG-1	CAAAAACCACCCATACAATCAG	CAAATGTGATGTGTACAGGAAG
IFNα4	TGTGTGATGCAGGAACCTCCT	GGTACACAGTGATCCTGTGG
MDA-5	CTTCCTGGATGTTCTGCGCCAA	CCGTGGGGAGGCAGATAATAAT
F4/80	CTTTGGCTATGGGCTTCCAGTC	GCAAGGAGGACAGAGTTTATCGTG
TLR-3	CGAAAGTTGGACTTGTCATCAA	AGTTGGGCGTTGTTCAAGAG
TNFα	GCCACCACGCTCTTCTGCCT	GGCTGATGGTGTGGGTGAGG
GAPDH	GAAATCCCATCACCATCTTCCAGG	GAGCCCCAGCCTTCTCCATG

Statistical analysis

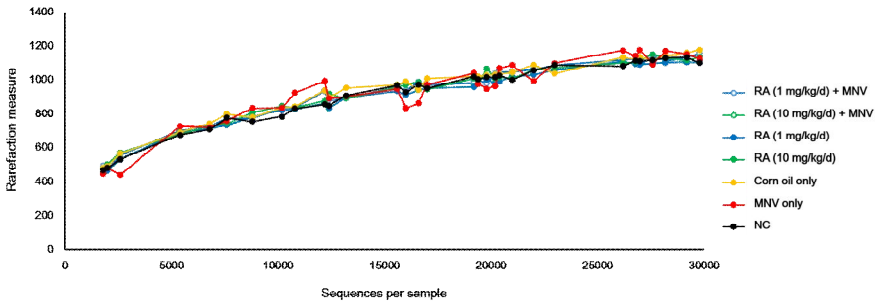
All features were expressed by average and standard deviation of each group. To quantity the level of mRNA *in vivo*, relative quantification method, $2^{-\Delta\Delta Ct}$ ($\Delta\Delta Ct = (C_{t.Target} - C_{t.GAPDH})_{Group1} - (C_{t.Target} - C_{t.GAPDH})_{Group2}$) was used comparing to GAPDH internal control. Statistical significance was assessed using one-way ANOVA followed by Duncan's post-hoc test, with *P* values of < 0.05 considered significance. Significant difference of bacterial similarity between groups was analyzed using Mann-Whitney U test. In relative abundance analysis using LEfSe based on Kruskal-Wallis and Wilcoxon test, significance was considered *P* value < 0.05, and threshold on the logarithmic LDA score was 2.0 - 3.0. To estimate the correlation between cytokines level, MNV positive and bacterial abundance, Spearman correlation coefficient was performed. All statistical analyses were performed using SPSS software 12.0. Both *P* value < 0.05 and 0.01 were used for statistical significance.

RESULTS

Summary of sequencing output

Total 4,066,474 sequences were generated after quality filtering from cecum samples from 45 mice. Minimum and maximum of sequence were 30,756 and 134,726, respectively, average sequences per sample were 90,366 ± 21,960. Collected sequences were subsequently analyzed and classified to 13 phylum, those were *Actinobacteria, Bacteroidetes, Cyanobacteria, Deferribacteres, Euryarchaeota, Firmicutes, Fusobacteria, Lentisphaerae, Proteobacteria, SR1, Tenericutes, TM7,* and *Verrucomicrobia*. Figure 3.1B shows the relative abundance of gut microbiome in family level. Alpha diversity of gut microbiota was not significantly different between groups categorized by RA administration and MNV inoculation (Fig. 3.1A).

A



B

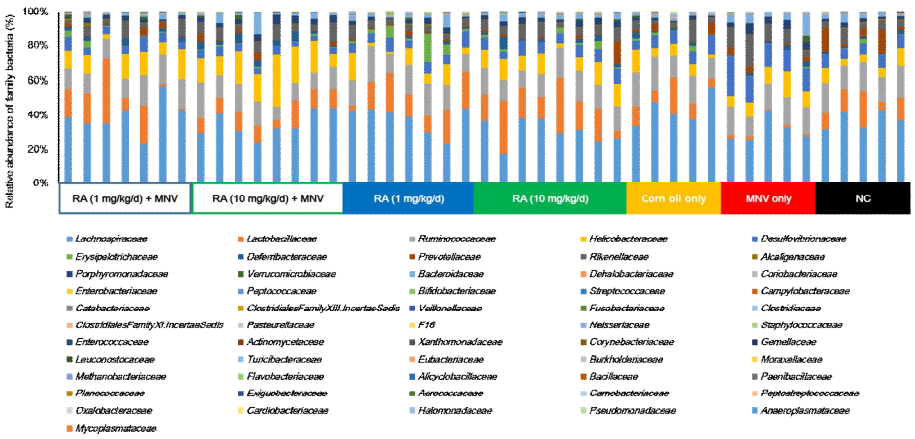
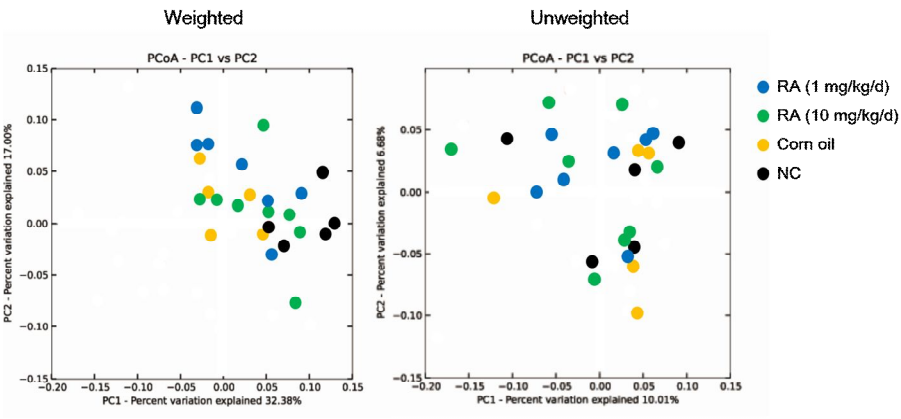


Figure 3.1. Microbial diversity and taxonomic comparison from 16S rRNA genes. Seven groups from 45 mice cecum samples categorized by RA administration and MNV inoculation were measured alpha diversity, there was no significant difference between groups. (A) Rarefaction curve of bacterial diversity. (B) Relative bacterial abundance was classified by family level.

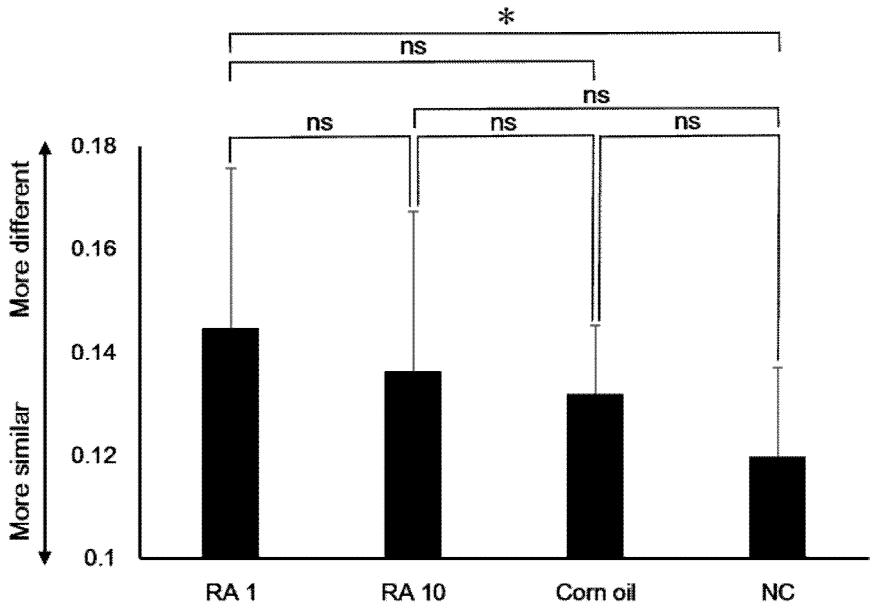
Characteristics in gut microbiota by RA administration

In both weighted and unweighted PCoA plots based on phylogenetic analysis, 25 samples were not clearly clustered by groups. Similarity of bacterial community within mice administered with 1 mg/kg/d of RA was significantly different to those of NC. Genus *Allobaculum, Ruminococcus, and Bifidobacterium* were significantly increased in mice administered with 1 mg/kg/d of RA comparing to NC. And, in high-dose of 10 mg/kg/d, genus *Haemophilus* belong to order *Pasteurellales* was significantly increased. Moreover, KEGG pathways predicted by PICRUSt did not show clear clustering by RA administration as well as the results of bacterial beta-diversity.

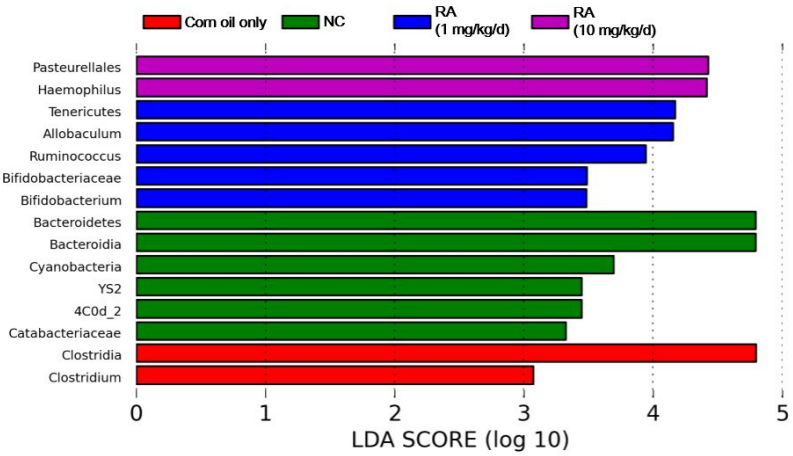
A


Weighted
Unweighted
PCoA - PC1 vs PC2
PCoA - PC1 vs PC2
PC2 - Percent variation explained 17.00%
PC1 - Percent variation explained 32.38%
PC2 - Percent variation explained 6.68%
PC1 - Percent variation explained 10.01%
RA (1 mg/kg/d)
RA (10 mg/kg/d)
Corn oil
NC

B


*
ns
ns
ns
ns
ns
More different
More similar
0.18
0.16
0.14
0.12
0.1
RA 1
RA 10
Corn oil
NC

C



D

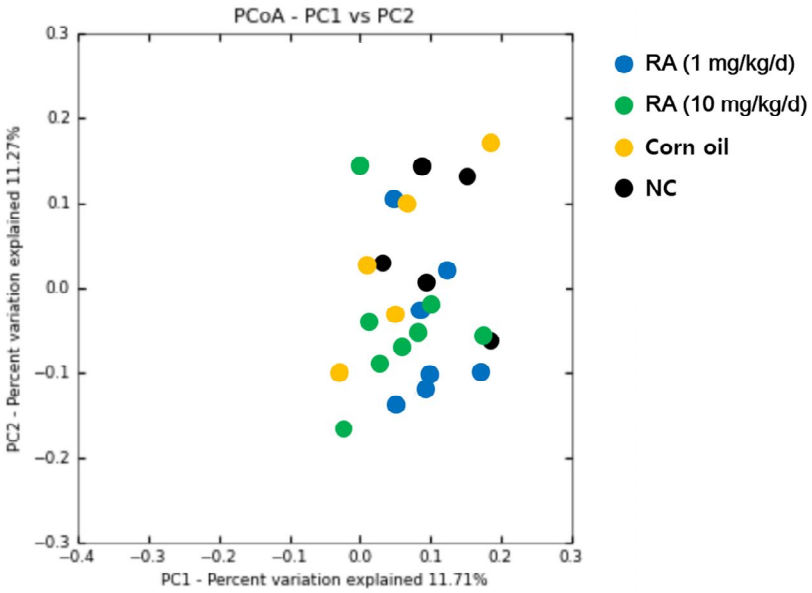


Figure 3.2. Difference of bacterial diversity and abundance by RA administration. 25

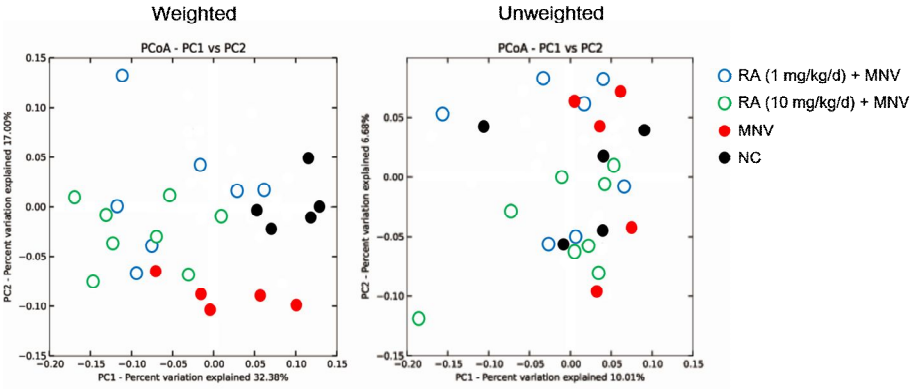
cecum samples were not clearly clustered in both weighted and unweighted principle coordinate analysis (PCoA). Besides, specific bacterial abundance was increase by RA administration comparing to RA negative controls. (A) Weighted and unweighted principle coordinate analysis (PCoA). (B) UniFrac distance between groups categorized by RA administration. Statistically differences between groups were analyzed using Mann-Whitney U test. ***:** Significant in $P < 0.05$. (C) Statistically significant bacterial abundance by RA administration. Significant differences were identified by LEfSe analysis with *P* value 0.05 in both Kruskal-Wallis among classes and Wilcoxon test between subclasses. Threshold on the logarithmic LDA score was 3.0. NC: negative control. RA was suspended in corn oil, and orally administered. (D) PCoA of KEGG pathways predicted by PICRUSt. Results did not show clear clustering.

Characteristics in gut microbiota by MNV inoculation

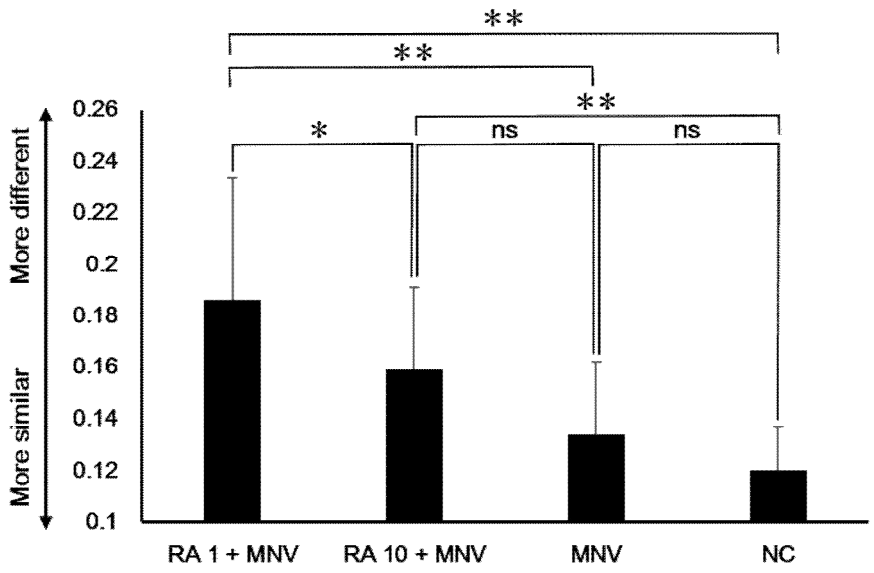
Figure 3.3A showed the clustering of samples by MNV inoculation in weighted PCoA. MNV-inoculated samples were clearly clustered with those of NC. RA-administrated samples were respectively clustered by means of MNV inoculation. Unweighted PCoA did not show the clear clustering. UniFrac distance was no significant difference between MNV-inoculated mice and NC. But, MNV-inoculated mice group during RA administration was significantly different (Fig. 3.3B). Figure 3.3C showed the relative bacterial abundance by MNV inoculation during RA administration. After MNV inoculation, genus *Bilophila, Desulfovibrio*, and LE30 belong to family *Desulfovibrionaceae* were most significantly increased. Besides, genus *Alistipes, Bacteroides, Parabacteroides, Yaniella, Jeotgalicoccus, Facklamia,* and *Oligella* were significantly increased. On the other hand, genus *Alcaligenaceae, Dialister,* and *Lactobacillus* were significantly decreased after MNV inoculation comparing to NC groups. *Lactobacillus* was significantly increased when MNV was inoculated during 1 mg/kg/d of RA administration comparing to only MNV inoculation group and NC. And, in MNV inoculation during high-dose of RA (10 mg/kg/d), *Helicobacter, Acinetobacter, Flexispira,* and *Lachnobacterium* were significantly increased.

Moreover, PCoA showed the clear clustering of predicted KEGG pathways by MNV inoculation during RA administration (Fig. 3.3D). In addition, among predicted KEGG pathways, counts of Glycolysis / Gluconeogenesis (Carbohydrate Metabolism), Benzoate degradation (Xenobiotics Biodegradation and Metabolism), ABC transporters (Membrane Transport), Phosphotransferase system (PTS) (Membrane Transport), and Signal transduction mechanisms (Cellular Processes and Signaling) were significantly changed by MNV inoculation during 1 mg/kg/d RA administration comparing to only MNV-inoculated mice group. As KEGG pathways belong to immune system, only antigen processing and presentation and RIG-1-like receptor signaling pathway were generated, those counts were not significantly different by MNV inoculation and RA administration (data not shown).

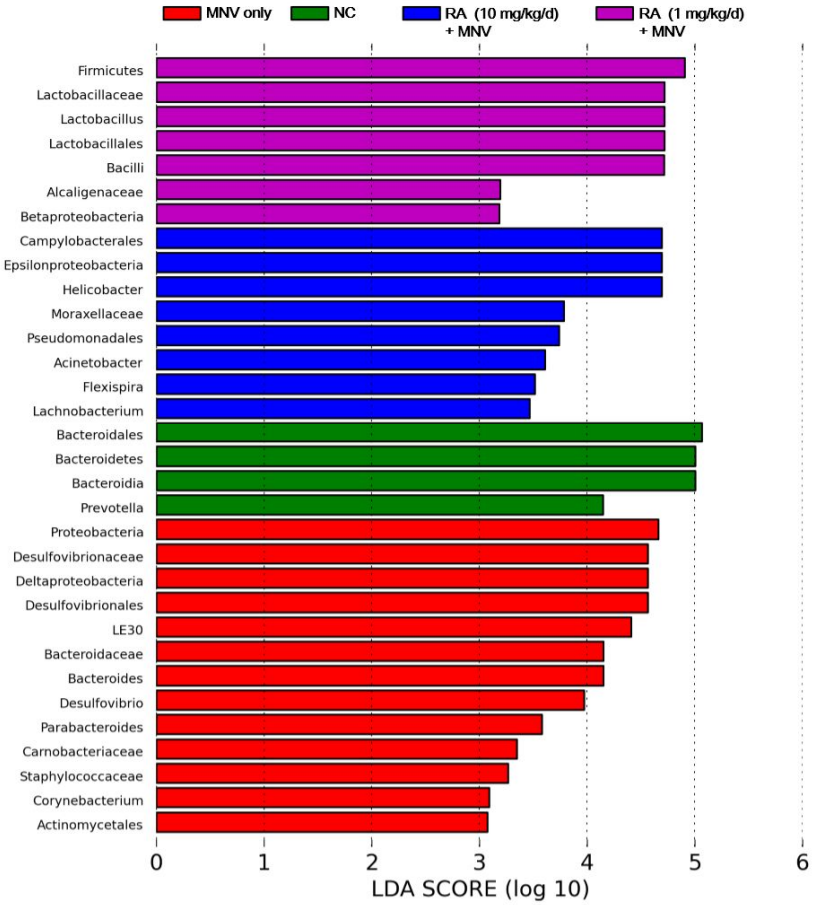
A


Weighted
Unweighted
PCoA - PC1 vs PC2
PC2 - Percent variation explained 17.00%
PC1 - Percent variation explained 32.38%
PC2 - Percent variation explained 6.68%
PC1 - Percent variation explained 10.01%
RA (1 mg/kg/d) + MNV
RA (10 mg/kg/d) + MNV
MNV
NC

B


**
**
**
*
ns
ns
More different
More similar
0.26
0.24
0.22
0.2
0.18
0.16
0.14
0.12
0.1
RA 1 + MNV
RA 10 + MNV
MNV
NC

C


MNV only
NC
RA (10 mg/kg/d) + MNV
RA (1 mg/kg/d) + MNV
Firmicutes
Lactobacillaceae
Lactobacillus
Lactobacillales
Bacilli
Alcaligenaceae
Betaproteobacteria
Campylobacterales
Epsilonproteobacteria
Helicobacter
Moraxellaceae
Pseudomonadales
Acinetobacter
Flexispira
Lachnobacterium
Bacteroidales
Bacteroidetes
Bacteroidia
Prevotella
Proteobacteria
Desulfovibrionaceae
Deltaproteobacteria
Desulfovibrionales
LE30
Bacteroidaceae
Bacteroides
Desulfovibrio
Parabacteroides
Carnobacteriaceae
Staphylococcaceae
Corynebacterium
Actinomycetales
0
1
2
3
4
5
6
LDA SCORE (log 10)

D

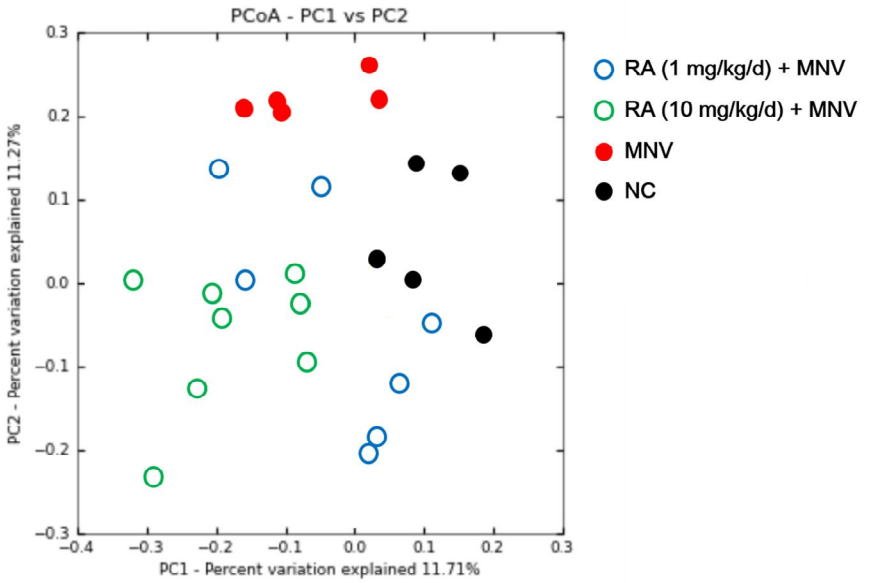


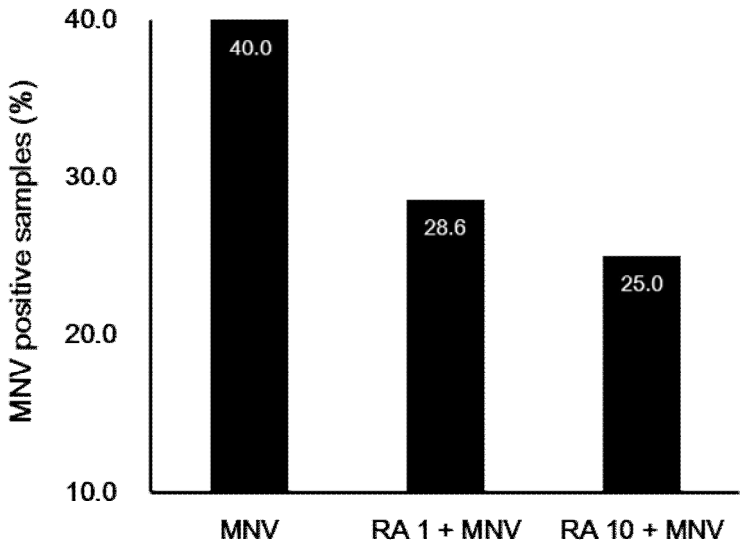
Figure 3.3. Microbial diversity and taxonomic comparison from 16S rRNA genes by MNV inoculation. (A) Bacterial communities clustered by MNV inoculation using weighted and unweighted principle coordinate analysis (PCoA). (B) UniFrac distance between groups categorized by MNV inoculation. Statistically differences between groups were analyzed using Mann-Whitney U test. *: Significant in $P < 0.05$. **: $P < 0.01$. (C) Statistically significant bacterial abundance by RA administration. Significant differences were identified by LEfSe analysis with P value 0.05 in both Kruskal-Wallis among classes and Wilcoxon test between subclasses. Threshold on the logarithmic LDA score was 3.0. NC: negative control. RA was suspended in corn oil, and orally administered. (D) PCoA of KEGG pathways predicted by PICRUSt. Four groups categorized by MNV inoculation and RA administration were clearly clustered. In LEfSe analysis, Glycolysis / Gluconeogenesis, Benzoate degradation, ABC transporters, Phosphotransferase system (PTS), and Signal transduction mechanisms were significantly changed by MNV inoculation during 1 mg/kg/d RA administration

comparing to only MNV-inoculated mice.

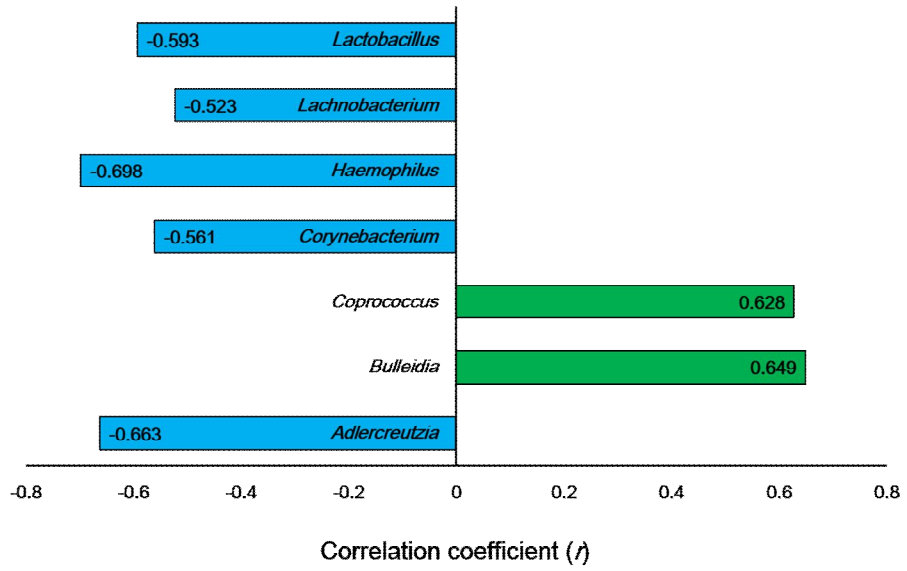
MNV clearance by RA administration and correlation with cytokines and bacterial abundance

MNV positive rate was 40% after 72h post-MNV inoculation, those were decreased to 28.6 and 25.0% by RA administration with 1 and 10 mg/kg/d, respectively (Fig. 3.4A). Figure 3.4B showed the correlation between detection of MNV and bacterial abundance in RA-administrated 15 mice. The detection of MNV had a positive correlation with *Coprococcus* and *Bulleidia*, and negative correlation with *Lactobacillus, Lachnobacterium, Haemophillus, Corynebacterium,* and *Adlercreutzia* (Fig. 3.4B). In addition, only TNFα expression level of RA-administrated mice was significantly decreased after MNV inoculation (Fig. 3.5C). There was no significant expression changes of cytokines by MNV inoculation in RA-untreated mice.

A


40.0
30.0
20.0
10.0
MNV positive samples (%)
40.0
28.6
25.0
MNV
RA 1 + MNV
RA 10 + MNV

B


-0.593 Lactobacillus
-0.523 Lachnobacterium
-0.698 Haemophilus
-0.561 Corynebacterium
Coprococcus 0.628
Bulleidia 0.649
-0.663 Adlercreutzia
-0.8
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
0.8
Correlation coefficient (r)

C

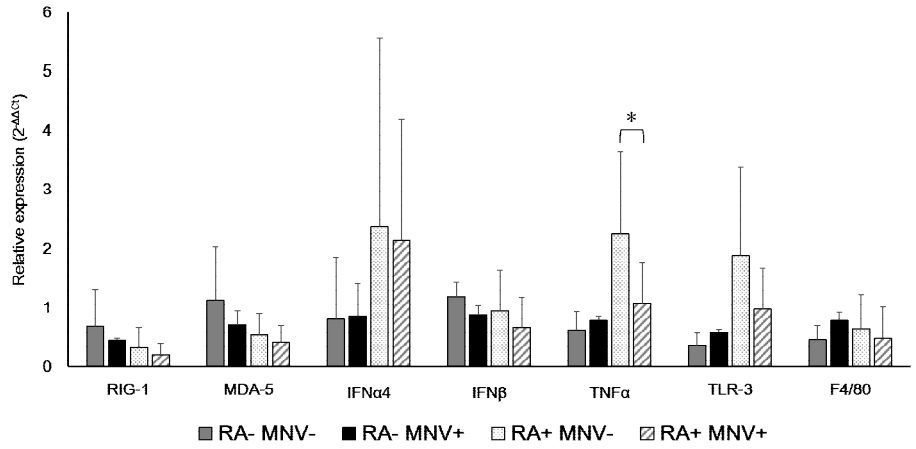
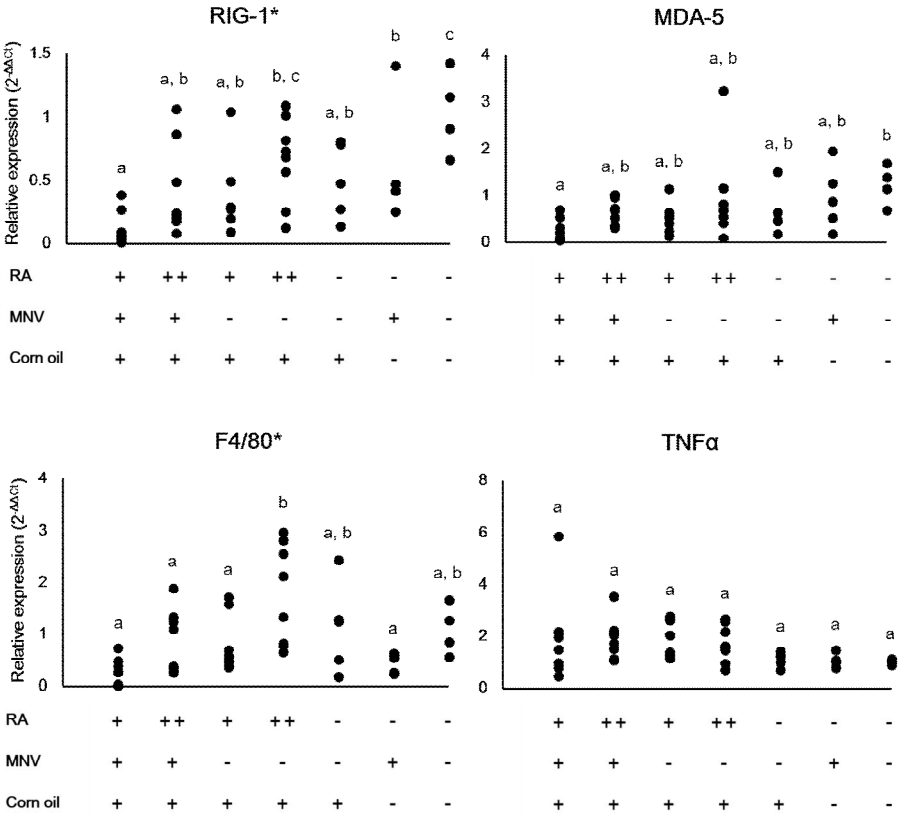


Figure 3.4. Effect of RA administration on MNV clearance and correlation with bacterial abundance and mRNA expression level of cytokines. (A) After 72 h post-MNV inoculation in RA-untreated mice, the percentage of MNV positive was 40% (2/5). Administrating RA with 1 and 10 mg/kg/d, MNV positive rates were decreased to 28.6 (2/7) and 25.0% (2/8), respectively. MNV detection was performed in spleen. (B) Correlation between the detection of MNV and bacterial abundance was analyzed by Spearman correlation coefficient in RA-administrated 15 mice. Total five and two bacteria genus had a negative and positive correlation with MNV defined as positive and negative parameter in significance with $P < 0.05$. (C) Significant difference of cytokine expression level by MNV inoculation during RA administration was analyzed using Spearman correlation coefficient. *: Significant in $P < 0.05$. In RA-untreated mice (n = 10), there was no significant difference of cytokine expression level. Besides, TNFα was significantly decreased by MNV inoculation in RA-treated mice (n = 15).

mRNA expression level of cytokines in ileum

Figure 3.5 described the relative mRNA expression level of cytokines in ileum. The expression level of RIG-1 was decreased in MNV-inoculated mice during 1 mg/kg RA administration comparing to both only MNV inoculation group and NC, MDA-5 was only significantly different to NC. F4/80 was significantly increased when 10 mg/kg of RA was administered comparing to MNV inoculation group. But, the expression level of TLR-3, IFNα4, IFNβ and TNFα were not significantly different between groups. In addition, those correlation between cytokines was analyzed. RIG-1 and MDA-5 has a significant positive correlation ($P < 0.001$). MDA-5 was also positively correlated with F4/80 ($P < 0.001$) and TLR-3 ($P = 0.002$). IFNα4 and IFNβ has a significant positive correlation ($P < 0.001$), and TNFα and TLR-3 has a positive correlation as well ($P < 0.001$).


RIG-1*
MDA-5
F4/80*
TNFα
Relative expression (2-ΔΔCt)
RA
MNV
Corn oil

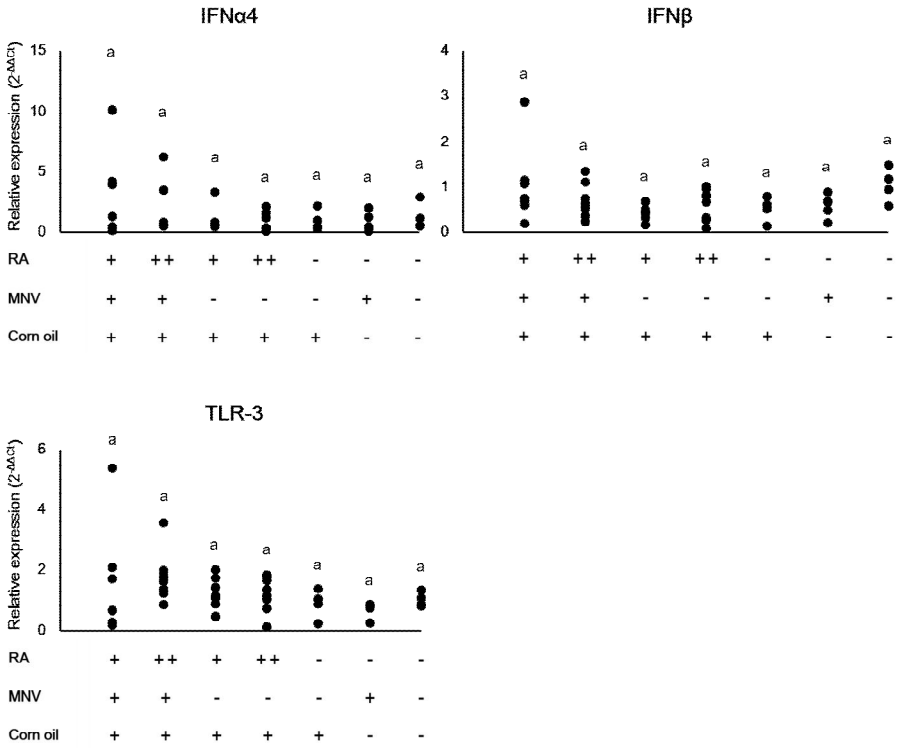
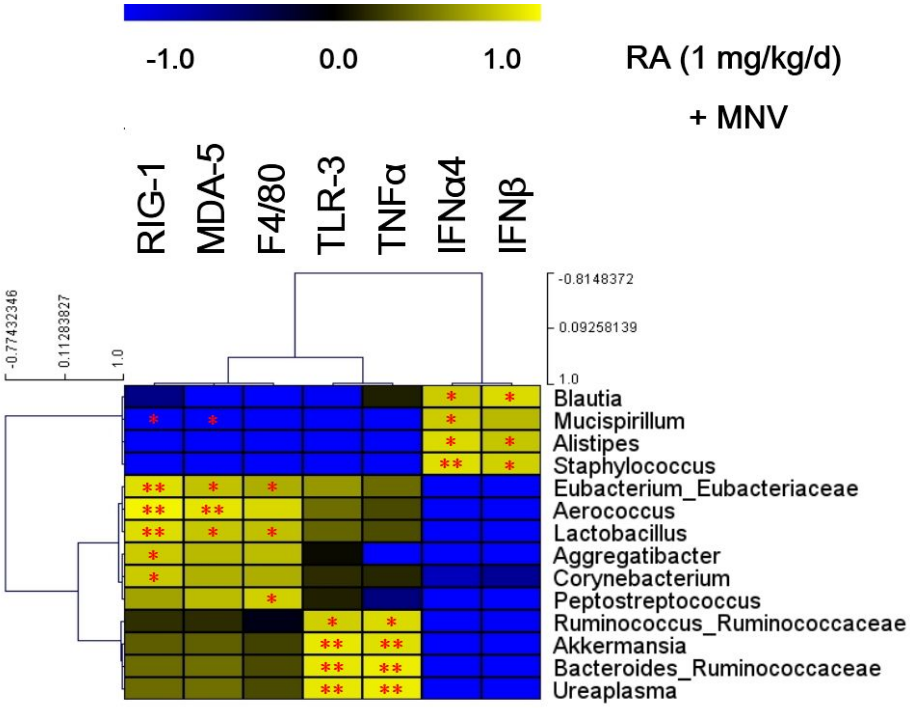


Figure 3.5. mRNA expression level of cytokines associated with innate immune response in ileum tissue. Relative quantification method, $2^{-\Delta\Delta Ct}$ ($\Delta\Delta Ct = (C_{t.Target} - C_{t.GAPDH})_{Group1} - (C_{t.Target} - C_{t.GAPDH})_{Group2}$), was used for analysis of gene expression of biomarker comparing GAPDH internal control. Statistical significance was assessed by Duncan's post-hoc test. *: Cytokines significance with $P < 0.05$ was observed.

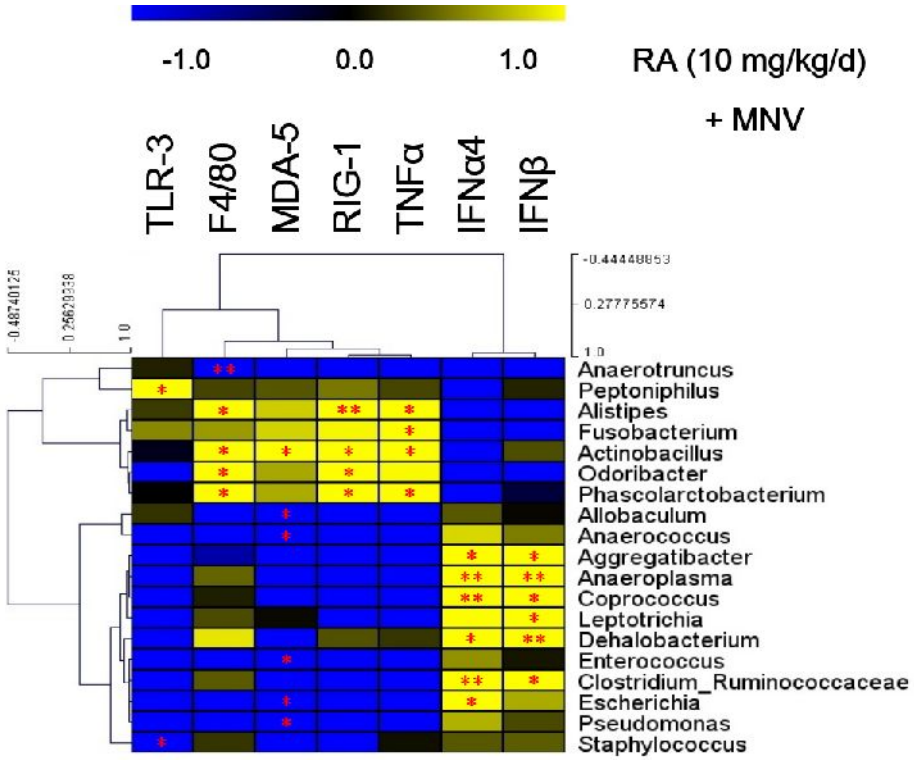
Correlation of bacterial abundance with cytokine level

Figure 3.6 showed the correlation between cytokine levels and bacterial abundance by administered RA dosage. In MNV-inoculated seven mice during RA administration with 1 mg/kg/d, *Lactobacillus*, *Aerococcus*, and *Eubacterium* (family *Eubacteriaceae*) were positively correlated with both RIG-1 and MDA-5. *Ruminococcus* (family *Ruminococcaceae*), *Akkermansia*, *Bacteroides* (family *Ruminococcaceae*) were positively correlated with TLR-3 and TNFα. And, *Blautia*, *Alistipes*, and *Staphylococcus* were positively correlated with IFNα4 and IFNβ. In mice with 10 mg/kg/d RA, RIG-1, TNFα, and F4/80 were positively correlated with *Alistipes*, *Actinobacillus*, and *Phascolarctobacterium*. Additionally, RIG-1 was positively correlated with *Odoribacter*, TNFα was positively correlated with *Fusobacterium*, and F4/80 was positively and negatively correlated with *Odoribacter* and *Anaerotruncus*, resepectively. MDA-5 was positively correlated with *Actinobacillus*, negatively correlated with *Allobaculum*, *Anaerococcus*, *Enterococcus*, *Escherichia*, and *Pseudomonas*. TLR-3 showed positive and negative correlation with *Peptoniphilus* and *Styphylococcus*, respectively. And, both IFNα4 and IFNβ showed positive correlation with *Aggregatibacter*, *Anaeroplasma*, *Coprococcus*, *Dehalobacterium*, and *Clostridium* (family *Ruminococcaceae*).

A


-1.0
0.0
1.0
RA (1 mg/kg/d)
+ MNV
RIG-1
MDA-5
F4/80
TLR-3
TNFα
IFNα4
IFNβ
-0.77432346
0.11283827
1.0
-0.8148372
0.09258139
1.0
Blautia
Mucispirillum
Alistipes
Staphylococcus
Eubacterium_Eubacteriaceae
Aerococcus
Lactobacillus
Aggregatibacter
Corynebacterium
Peptostreptococcus
Ruminococcus_Ruminococcaceae
Akkermansia
Bacteroides_Ruminococcaceae
Ureaplasma

B



C

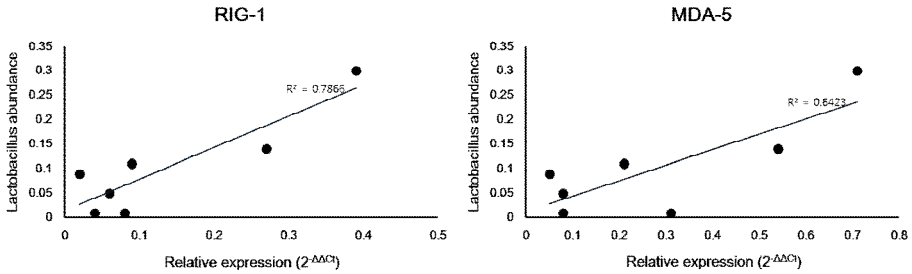


Figure 3.6. Correlation between bacterial abundance and cytokine expression levels. Total 14 and 19 bacteria genus had at least one significance with cytokines were analyzed using Spearman correlation coefficient. (A-B) Heatmap of correlation coefficient was generated in MNV-inoculated mice during RA administration with

different dosage (n = 1 mg/kg/d: 7, 10 mg/kg/d: 8). *: Significant in $P < 0.05$. **: $P < 0.01$. (C) *Lactobacillus*, significantly increased by MNV inoculation during RA administration with 1 mg/kg/d, was positively correlated with the mRNA expression level of both RIG-1 and MDA-5.

DISCUSSION

Vitamin A deficiency impairs systemic immunity and increases the incidence and mortality by infection during childhood. Despite of major advances in the knowledge of essential role of vitamin A, its deficiency is still public health worldwide. An estimated 250 million preschool children are vitamin A deficient (*70*). In previous community-based studies, sufficient vitamin A supplement reduced the mortality from diarrheal disease by 39%, from respiratory disease by 70% (*71*). And, in recent study reported that vitamin A supplementation is related with modification of the various intestinal chemokine and cytokine responses to norovirus infection (*5*). That which immune system against bacterial and viral infection was highly associated with vitamin A supplementation, gut microbiota was definitely considered to be associated with vitamin A. Because, gut microbiota plays important role in maintenance of intestinal homeostasis (*81, 82*). This study was first investigation of gut microbiota by vitamin A, elucidated the characteristics of gut microbiota by RA administration and MNV inoculation.

Gut microbiota was differently changed by dosage of RA. When mice were administered with 1 mg/kg/d of RA, *Allobaculum, Ruminococcus, and Bifidobacterium* were significantly increased, besides, *Haemophilus* belong to order *Pasteurellales* was significantly increased in high-dose of 10 mg/kg/d. *Bifidobacterium*, one of probiotics such as *Lactobacillus*, was known to promote innate and adaptive immune responses to bacterial and viral infection (*83, 84*), we could guess that the significant increase of *Bifidobacterium* was caused by RA administration. Besides, *Allobaculum*, and *Ruminococcus* were not characterized until now. Although there was no correlation with cytokines analyzed in this study, those might be associated with immune system by RA administration. High-dose of RA might rather produce of immune suppression and could promote inflammatory disorders (*4, 85*). *Haemophilus* was relatively increased comparing to other groups, that might be caused by high-dose of RA. Because, genus

Haemophilus includes commensal bacteria along with opportunistic pathogenic species including *Haemophilus influenzae* causing meningitis and bacteremia particularly in young children (*86*).

In recent study, MNV infection does not cause major change of intestinal microbiota in mouse model (*87*). In another human study, increase in *Proteobacteria* was only showed in patients whose gut microbiota was disrupted by HuNoV infection (*88*). On the contrary, this study showed that different bacterial diversity and abundance was characterized by MNV inoculation. In common with human study, increase in *Proteobacteria* was most statistically significant in this mouse model with MNV inoculation. Interestingly in this study, although gut microbiota was not disrupted by MNV inoculation, various bacterial abundances which have not well characterized until now were significantly increased. Decrease in *Lactobacillus* was another major significant change in gut microbiota by MNV inoculation. Although any inflammatory disorders were not founded in MNV-inoculated mice, continuous lack of *Lactobacillus* may cause inflammation and vulnerability to pathogens because of protective effect of various probiotic *Lactobacillus* strains (*83, 89*). In previous experimental inoculation studies with MNV showed that the duration of infection varied depending on the mouse strain (*10, 11*). Moreover, considering existence of different strains of MNV, previous study with only a few MNV isolates has a limitation to identify the MNV pathogenesis and relation with gut microbiota.

Interestingly, the abundance of specific bacteria community unchanged by only RA administration was significantly increased by MNV inoculation during RA administration. In particular, it was notable that the abundance of *Lactobacillus* decreased by MNV inoculation was significantly recovered by 1 mg/kg/d of RA administration. Numerous previous studies reported that probiotics including *Lactobacillus* and *Bifidobacterium* strains have various beneficial effects on human health through their immunomudulatory activity (*90, 91*). In this study, the abundance of *Lactobacillus* was negatively correlated with MNV positive, and was positively correlated with RIG-1, MDA-5, and F4/80 in MNV-inoculated mice during RA

administration with 1 mg/kg/d. Therefore, we considered the possibility of protection effect of *Lactobacillus* against NoV infection. Because, RIG-1 and MDA-5 play essential roles to production of proinflammatory cytokines against virus infection (*6*), especially, MDA-5 was highly associated with MNV recognition (*92*). In addition, interferon induced with helicase C domain 1 (*IFIH1*) gene which encoded MDA-5 in human protected against T1D in genome-wide association study (*93*). Until now, pathology associated with TID was not reported by MNV infection in wild type mice, further studies should be required for identification of both short and long-term effect of MNV infection with different isolates.

From this microbiota study, the changes of various bacterial abundance by MNV inoculation during RA administration, which have not been well characterized so far, were associated with the expression level of cytokines. For examples, *Akkermansia* highly associated with metabolic disorders was positively correlated with both TLR-3 and TNFα (*26*), *Peptostreptococcus* occasionally becomes pathogenic under immunosuppressed condition was negatively correlated with IFNs (*94*). Moreover, among the revealed bacteria showing significant correlation with cytokines in this study, rarely partial results were reported. For examples, *Allobaculum* was negatively correlated with *Bifidobacterium* in HLA transgenic mice model (*95*), and *Mucispirillum* isolated in mucus might interacted with host in gastrointestinal track (*96*). Therefore, as microbiome studies revealed the various bacteria, following studies for characterizing those veiled bacteria should be required.

RA administration both with 1 and 10 mg/kg/d was not significantly changed the expression level of RIG-1 and MDA-5. But, interestingly, after MNV inoculation during RA administration with 1 mg/kg/d RIG-1 and MDA-5 was significantly decreased comparing to only MNV-inoculated mice group. Besides, there was no significance in high-dose of RA (10 mg/kg/d). It is well known that excess retinoic acid promote inflammatory disorders as well as liver toxicity, dry skin, and teratological effect (*4*). In further studies, recommended daily intake of RA for activation of innate immunity against virus infection should be suggested. In addition, although TLR-3 was not

significantly changed by MNV inoculation during RA administration, was positively correlated with MDA-5 and TNFα. In previous studies, MNV titer was increased TLR-3 deficient mice, and TLR-3 played an important role in recognition of double stranded RNA (*92, 97*). In addition, MDA-5 and TLR-3 were required for inducing IFN responses against Rhinovirus which is a single stranded RNA picornavirus as well as MNV (*98*). Therefore, although TLR-3 was not associated with RA in this study, might have minor roles in proinflammatory signaling pathways.

In this study, we first reveal the significant changes in gut microbiota by MNV inoculation during RA administration. Bacterial community in RA-administered mice was significantly changed by MNV inoculation. Especially, *Lactobacillus* was decreased in MNV-inoculated mice without RA administration, on the other hand, as decreasing MNV positive rate by RA administration, *Lactobacillus* abundance was significantly increased. Moreover, there were various significant correlations between *Lactobacillus* abundance and the expression level of cytokines such as RIG-1 and MDA-5 in MNV-inoculated mice during RA administration. Therefore, we could consider the effect of RA administration on MNV infection via the change of gut microbiota, further research should be required for RA effects on gut microbiota related to activation of innate immunity. In addition, various bacterial abundances which were not well characterized until now were changed by MNV inoculation during RA administration, those were correlated with the expression level of cytokines. Therefore, we could also expect the roles of those bacteria on innate immunity in further researches.

CHAPTER Ⅳ.

DISCUSSION AND CONCLUSIONS

Summary and conclusions

Gut microbiome research using NGS is a new frontier of human biology. Gut microbiota is influenced by various medical and dietary interventions, only small part of that has been characterized until now. After the human genome project (HGP), HMP has been aim to identify and characterize the microbiota which is found in associated with human health. As part of microbiome research performed worldwide, we characterized specific gut microbiota by metformim treatment and first showed the changes in gut microbiota by MNV inoculation during vitamin A administration.

Characteristics of gut microbiota by metformin treatment

Metformin is most widely prescribed for the treatment of T2D. In metformin treatment for T2D and obesity, various mechanisms of action are related with antihyperglycemic effect and weight loss including AMPK. Recent studies reported that drug response such as drug metabolizing and bioavailability was influenced by gut microbiota as well as patient genetic factors. Therefore, we formed hypothesis that specific change of gut microbiota could be associated with the improvement of metabolic disorders by metformin treatment because it has been reported that change of gut microbiota was highly related with the development of metabolic disorders.

In this study, bacterial diversity was significantly decreased by metformin treatment in HFD comparing to only HFD group, those compositions were significantly different. Therefore, we considered that specific bacterial community changes by metformin treatment were associated with improvement of metabolic disorders. In particular, Species *Akkermansia muciniphila*, which is known as a mucin-degrading bacterium, highly increased approximate 18 times (0.68 to 12.4%) in HF-Met than HFD. Interestingly, *Akkermansia muciniphila* was not detected in N mice group at all, slightly increased after HFD. In recent study, the consistent inoculation of *Akkermansia muciniphila* improved metabolic disorders (*26*). The sequence of *Akkermansia muciniphila* detected using PCR with AM1/AM2 primer set in this study was 100%

identical with those strain which was isolated in human intestine (*56*). Moreover, metformin and phenformin in BHI medium relatively increased the growth of *Akkermansia muciniphila* comparing total bacteria. Therefore, the abundance of *Akkermansia muciniphila* by metformin treatment indicated the causative capability on metabolic improvement. Moreover, KEGG pathways corresponding to lipid metabolism including fatty acid metabolism, linoleic acid metabolism, sphingolipid metabolism were predicted to be upregulated by metformin treatment in HFD. In recent study, lipogenetic gene expression such as FASN and ACC1 was upregulated by *Akkermansia muciniphila* treatment in HFD. Therefore, we suggested that regulation of lipid metabolism by gut microbiota was highly associated with mechanism of metformin treatment in metabolic improvement.

Changes in gut microbiota by MNV inoculation during vitamin A administration

Gut microbiota was not disrupted by MNV inoculation, various bacterial abundances which have not well characterized until now were significantly changed. In particular, decrease in *Lactobacillus* was a major significant change in gut microbiota by MNV inoculation. In recent study, MNV infection does not cause major change of intestinal microbiota in mouse model (*87*). In another human study, increase in *Proteobacteria* was only showed in patients whose gut microbiota was disrupted by HuNoV infection (*88*). In previous experimental inoculation studies with MNV showed that the duration of infection varied depending on the mouse strain (*10, 11*). Moreover, considering existence of different strains of MNV, previous study with only a few MNV isolates has a limitation to identify the MNV pathogenesis and relation with gut microbiota.

Gut microbiota was differently changed by dosage of RA. When mice were administered with 1 mg/kg/d of RA, *Allobaculum, Ruminococcus, and Bifidobacterium* were significantly increased, besides, *Haemophilus* belong to order *Pasteurellales* was significantly increased in high-dose of 10 mg/kg/d. Except in *Bifidobacterium*, the others were not characterized until now. Although there was no correlation with cytokines analyzed in this study, those might be associated with immune system by RA

administration.

Interestingly, the abundance of specific bacteria community unchanged by only RA administration was significantly increased by MNV inoculation during RA administration. In particular, it was notable that the abundance of *Lactobacillus* decreased by only MNV inoculation was significantly recovered by 1 mg/kg/d of RA administration. Moreover, abundance of *Lactobacillus* was negatively correlated with MNV positive, and was positively correlated with RIG-1, MDA-5, and F4/80 in MNV-inoculated mice during RA administration with 1 mg/kg/d. Therefore, we strongly considered the possibility of protection effect of RA on NoV infection via gut microbiota.

Suggestions for Further Research

We revealed the characteristics in gut microbiota by metformin treatment, MNV inoculation, and RA using NGS, and showed the significant association with biomarkers representing metabolisms and innate immunity. From those results, we needed to look to the effect of gut microbiota on metabolic improvement and activation of innate immunity, and suggested that changed gut microbiota by metformin treatment and RA played important role in energy metabolism and innate immunity. But, causalities between gut microbiota and metabolic improvement, and gut microbiota and activation of innate immunity were not identified in this study. Therefore, to identify the causative effect of gut microbiota, first, novel or candidate bacteria should be isolated from sample showing the phenotypes, and second, gnotobiotic mouse model with fecal transplantation also should be performed in further researches.

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국문 초록 (ABSTRACT IN KOREAN)

약제 투여에 의한 장내미생물총의 변화와 그에 따른 대사증후군 및 노로바이러스 감염에 대한 영향 가능성: 메트포르민 치료와 비타민 A 보조치료

위장관, 특히 대장과 소장은 영양분의 흡수 및 소화와 면역 체계에 중요한 역할을 담당하는 기관이다. 장내 마이크로바이오타는 장내에 공생하고 있는 미생물 군집을 일컫는데, 총 수십 조의 미생물로 이루어졌으며, 약 1,000 종의 박테리아로 구성된다. 이는 최근 차세대 시퀀싱 (NGS) 기술을 기반으로 한 메타지노믹 연구를 통해 밝혀졌는데, 배양이 안 되는 미생물을 포함한 모든 미생물의 염기서열을 밝힐 수 있었고, 장내 미생물과 숙주와의 상호 작용을 보다 폭넓게 이해 할 수 있었다. 최근 연구를 통해, 대사 증후군, 자가 면역 질환, 감염 질환 등 다양한 건강 문제들이 장내 미생물과 밀접한 연관이 있음을 확인되었다. 하지만, 미생물 군집의 복잡성으로 인해, 장내 미생물 및 대사 장애 사이의 메커니즘은 아직까지 완전히 밝혀지지 않고 있다. 이 연구에서는 메트포르민에 의한 대사장애 치료와 비타민 A 투여에 따른 장내 미생물 군집 변화를 마우스 모델을 이용하여 확인하고자 하였다.

첫 번째로, 고지방 식이로 유도된 비만 마우스 모델을 이용하여, 제 2형 당뇨의 1차 치료제인 메트포르민에 의한 특징적인 장내 미생물 군집을 확인하였다. 고지방 식이 중 메트포르민 치료한 마우스에서는 고지방 식이만 실시한 마우스에 비해 장내 미생물 다양성이 낮았고, 고유한 미생물 군집을 나타냈다.

고지방 식이 중 메트로프민 치료가 실시된 마우스에서는 *Proteobacteria*와 *Verrucomicrobia* 문이 각각 2.1±2.8와 12.4±5.3%로 유의적으로 증가하였으며, 특히, 뮤신 분해 미생물로 알려진 *Akkermansia muciniphila*가 가장 유의하게 증가하였다. 또한, 총 18개의 특징적인 KEGG 경로가 고지방 식이 중 메트포르민 치료로 인해 유의하게 증가되었음을 장내 미생물 유전 정보를 바탕으로 예측하였다. 그 중에는 지질 대사에 속하는 sphingolipid와 지방산 대사가 가장 통계적으로 유의하였다. 이러한 결과는 메트포르민 치료로 인해 대사 장애가 개선되는 과정에서 장내 미생물이 유의하게 변화하였음을 설명하며, 더 나아가 특징적인 장내 미생물 군집이 대사 증후군 개선과 매우 밀접한 관련이 있음을 예측할 수 있다.

두 번째로, 레티노산 (Retinoic acid, RA) 섭취 중 뮤린 노로바이러스 감염으로 인한 장내 미생물 변화를 조사하였다. 비타민 A의 대차체인 레티노산은 바이러스 감염에 대한 선천성 면역 반응에서 매우 중요한 역할을 수행한다. 최근 연구를 통해, 장내 마이크로바이오타와 선천성 면역 및 후천성 면역과의 매우 밀접한 연관성이 보고되었다. 하지만, 레티노산 섭취와 장내 미생물 군집과의 연관성은 아직까지 밝혀지지 않았다. 이번 연구에서는 레티노산을 1 mg/kg 과 10 mg/kg으로 매일 경구 투여하면서 뮤린 노로바이러스로 감염시켰을 때 장내 미생물 군집의 변화를 확인하였다. 박테리아 군집의 다양성은 뮤린 노로바이러스 및 레티노산를 각각 투여했을 때보다 레티노산을 섭취하면서 뮤린 노로바이러스에 감염되었을 때 가장 유의하게 변화하였다. 특히, *Lactobacillus*는 뮤린 노로바이러스 감염 시 유의하게 감소하였으나, 1 mg/kg의 레티노산을 매일 섭취 중 감염되었을 때에는 오히려 그 양이 유의하게 증가하였다. 또한, *Lactobacillus*의 양은 RIG−1, MDA −5, F4/80의 발현 양과 양의 상관관계를 나타냈다. 이 연구를 통해, 뮤린 노로바이러스와 레티노산 섭취로 인한 특징적인 장내 미생물 군집을 확인하였고, 이는 선천

성 면역과 밀접한 관련이 있는 다양한 싸이토카인들과 통계적으로 유의한 상관관계를 보여줬다. 그러므로, 레티노산 섭취로 인한 장내 마이크로바이오타의 변화가 바이러스 감염에 대한 선천성 면역 기능에 매우 밀접한 연관이 있다고 예측하였다.

결론적으로, 장내 마이크로바이오타가 메트포르민을 통한 대사 증후군 개선 과정에서도 유의하게 변화하였음을 확인하였다. 또한, 레티노산 섭취 중 뮤린 노로바이러스 감염 시 특징적인 장내 미생물 군집을 확인하였다. 이러한 결과로부터, 장내 마이크로바이오타가 대사 증후군의 개선 및 바이러스 감염에 따른 선천성 면역 기능에 매우 중요한 역할을 한다고 제한할 수 있었고, 궁극적으로 인간 건강에 미치는 장내 미생물의 이로운 효과가 추가적인 연구를 통해 밝혀져야 할 것이다.

주요어: 장내 마이크로바이오타, 메트포르민, 대사 증후군, 레티노산, 노로바이러스, 싸이토카인

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