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Original Article

Modulation of Pro-inflammatory and Anti-inflammatory Cytokines in the Fat by an Aloe Gel-based Formula, QDMC, Is Correlated with Altered Gut Microbiota

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ABSTRACT

Abnormal inflammatory responses are closely associated with intestinal microbial dysbiosis. Oral administration of Qmatrix-diabetes-mellitus complex (QDMC), an Aloe gel-based formula, has been reported to improve inflammation in type 2 diabetic mice; however, the role of the gut microbiota in ameliorating efficacy of QDMC remains unclear. We investigated the effect of QDMC on the gut microbiota in a type 2 diabetic aged mouse model that was administered a high-fat diet. Proinflammatory (TNF-α and IL-6) and anti-inflammatory (IL-4 and IL-10) cytokine levels in the fat were normalized via oral administration of QDMC, and relative abundances of *Bacteroides*, *Butyricimonas*, *Ruminococcus*, and *Mucispirillum* were simultaneously significantly increased. The abundance of these bacteria was correlated to the expression levels of cytokines. Our findings suggest that the immunomodulatory activity of QDMC is partly mediated by the altered gut microbiota composition.

Keywords: Qmatrix-diabetes-mellitus complex; Gut microbiota; Anti-inflammatory; Proinflammatory; Type 2 diabetes

INTRODUCTION

Increasing evidence has demonstrated that the gut microbiota is closely related to inflammation and metabolic disease. A high-fat diet (HFD), one of the main causes of metabolic diseases, induces dysbiosis of gut microbiota, which leads to an elevated count of certain bacteria containing LPS as well as increased intestinal permeability (1). Moreover, various dietary supplements, including phytochemicals that exhibit anti-inflammatory effects and enhance metabolism, have been reported to overcome the dysbiosis of gut microbiota (2-4).

Recently, the abundance of certain bacteria was closely associated with the mode of action of medication therapy for type 2 diabetes (T2D), which closely related to inflammation, and was considered as a therapeutic target. For example, the administration of *Akkermansia*

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Conflict of Interest
The authors declare no potential conflicts of interest.

Abbreviations
APO, apolipoprotein; HFD, high-fat diet; HFD+QDMC, Qmatrix-diabetes-mellitus complex administration during high-fat diet feeding; IPGTT, intraperitoneal glucose tolerance testing; LDA, linear discriminant analysis; LEfSe, linear discriminant analysis effect size; QDMC, Qmatrix-diabetes-mellitus complex; RD, regular diet; SCFA, short-chain fatty acid; T2D, type 2 diabetes.

Author Contributions
Conceptualization: An J, Lee H, Lee S, Kim J, Song Y, Kong H, Kim K; Data curation: An J, Lee H; Formal analysis: An J, Kim J, Lee H, Park IH; Methodology: An J, Lee H, Lee S, Kim J; Writing - original draft: An J, Lee H; Writing - review & editing: An J, Lee H, Lee S, Kim J, Song Y, Park IH, Kong H, Kim K.

muciniphila, whose abundance increases by treatment with metformin, which is known as first line pharmacotherapy for T2D, reduces inflammation and helps in regulating blood glucose (5,6). Recovery from the imbalance of gut microbiota in T2D patients is related to metabolic inflammation, gut permeability, and short-chain fatty acid (SCFA) production, which are crucial for improving metabolism in the intestinal epithelium of the host (7,8).

Qmatrix-diabetes-mellitus complex (QDMC) is a Aloe gel-based formula, which is supplemented with aloesin and chromium-enriched yeast to process Aloe gel manufactured by the Q Matrix process (9-11). As reported in previous studies, QDMC ameliorates immune dysregulation and insulin resistance (10,12,13).

In the present study, we hypothesized that the inflammatory responses of QDMC may be closely related to changes in the gut microbiota. To demonstrate this, these changes were assessed in HFD-induced type 2 diabetic aged mouse model after oral administration of QDMC. Moreover, we examined the correlation with the metabolic profiles and inflammatory cytokines.

MATERIALS AND METHODS

Preparation of QDMC

QDMC was provided by Univera, Inc. (Seoul, Korea), and comprises chromium, aloesin, and processed Aloe gel (13). The preparation of processed Aloe gel was described in our previous study (11). Briefly, Aloe gel processing involves the incubation of *Aloe vera* gel with cellulase, termination of the reaction by heating, followed by passage through a charcoal column. QDMC was prepared by adding aloesin and chromium-enriched yeast to the processed Aloe gel (13).

Experimental animal

Four-week-old male C57BL/6N mice were purchased from Samtako Inc. (Osan, Korea). Mice were housed with libitum access to food and water in a temperature-humidity controlled (22°C±2°C, 55%±5%) animal facility, under a 12 h light–dark cycle. Five-week-old mice were fed an HFD (45% kcal fat; Feed Lab Inc., Trenton, NJ, USA) for 39 weeks to induce obesity and T2D. QDMC (100 mg/kg/day) dissolved in carboxymethyl cellulose solution was orally administered daily for the final 16 weeks of HFD feeding (HFD+QDMC, n=5). Moreover, the animal groups fed with a regular diet (RD) (10% kcal fat; Purina, Seongnam, Korea) (n=5) and an HFD-without QDMC (HFD, n=5) were used as negative controls. Animal groups fed with RD and HFD were treated only with carboxymethyl cellulose solution. The experiments in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of Sahmyook University for the care and use of laboratory animals (SYUIACUC 2015001).

Metabolic measurements

Body weight was measured every week, and the blood glucose level was checked using the Accu-Chek Performa system (Roche Diagnostics, Indianapolis, IN, USA) after fasting for 12 h. Intraperitoneal glucose tolerance testing (IPGTT) was conducted after 16 wk of oral administration of QDMC. A glucose solution dissolved in phosphate-buffered saline was intraperitoneally administered to the mice (2 g/kg of body weight) and their glucose levels were measured at 0, 15, 30, 60, 120, and 240 min after injection. The animals were sacrificed by ether anesthesia, and blood was collected via cardiac puncture and then centrifuged at 10,000 rpm for 5 min to obtain serum. The levels of triglyceride, total cholesterol, high

density lipoprotein, low-density lipoprotein, apolipoprotein (APO) A1, and APO B in serum were measured using a biochemistry analyzer (AU480; Beckman Coulter, Brea, CA, USA).

Gut microbiota analyses

Total DNA from the cecum, including fecal materials was extracted using the PowerSoil DNA Isolation Kit (Mo Bio Laboratories Inc., Solana Beach, CA, USA). Partial sequences of 16S rRNA genes were amplified based on the 16S rRNA amplification protocol of the Earth Microbiome Project (14). The primer set which used to amplifying 16S rRNA was described in previous study (2). Moreover, an index PCR was performed to attach the dual indices and adapter to the amplified PCR products using AmpONE™ α-Pfu DNA polymerase (GeneAll, Seoul, Korea) and a Nextera® XT Index Kit v2 (Illumina, San Diego, CA, USA). After amplification, the PCR products were purified using Expin™ PCR SV (GeneAll). Furthermore, partial bacterial 16S rRNA genes were sequenced using the MiSeq Reagent Kit V3 (600 cycles) and MiSeq platform (Illumina).

Before analyzing the 16S rRNA sequences, the BCL files were converted into raw FASTQ files including read1, index, and read2 sequences using CASAVA-1.8.2. After preprocessing (quality filtering and trimming steps using the FASTX-Toolkit), the sequences were assigned to operational taxonomic units (97% identity); representative sequences were selected using QIIME 1.7.0 software (15). Next, taxonomic composition, alpha diversity, and beta diversity were analyzed. Linear discriminant analysis (LDA) effect size (LEfSe) was used to estimate the taxonomic abundance and characterize differences between groups (16). A heat map of functional gene abundance was generated using the MultiExperiment Viewer (MeV) software (ver. 4.8.1; http://www.tm4.org/).

Transcriptome analysis in the fat

The expression levels of TNF-α (forward primer: 5′-ATGAGCACAGAAAGCATGATCCGC-3′, reverse primer: 5′-CTCAGGGCCCGTCCAGATGAAACC-3′), IL-6 (forward primer: 5′-GGCCTTCCCTACTTCACAAG-3′, reverse primer: 5′-ATTTCCACGATTTCCCAGAG-3′), IL-1β (forward primer: 5′-CAGGATGAGGACATGACACC-3′, reverse primer: 5′-CTCTGCAGACT CAAACTCCAC-3′), IL-4 (forward primer: 5′-ATGGGTCTCAACCCCCAGCTAGT-3′, reverse primer: 5′-CTGAAGGACCTTTCGGATTTCTCG-3′), IL-10 (forward primer: 5′-ATTTGAATTCC CTGGGTGAGAAG-3′, reverse primer: 5′-CACAGGGGAGAAATCGATGACA-3′), GAPDH (forward primer: 5′-CAACTTTGGCATTGTGGAAGG-3′, reverse primer: 5′-ATGGAAATTGTGAGGGAGATGC-3′) (17,18) were measured in the epididymal fat. Total RNA was isolated from the fat tissue using RiboEx (GeneAll), and cDNA synthesis was performed using a HyperScript™ RT premix (GeneAll), according to the manufacturer's instructions. SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) was used to quantify mRNA levels using the StepOnePlus real-time PCR system (Applied Biosystems). GAPDH was used as an internal control.

Statistical analyses

The values are expressed as the mean±SD in each group. Relative abundance analysis was performed using LEfSe based on the Kruskal-Wallis and Wilcoxon tests. The logarithmic LDA score threshold was set as 3.0. Statistical significance was assessed using Mann-Whitney *U* test, and significance was defined as $p<0.05$. Correlation analyses were performed using RStudio (RStudio Inc., Boston, MA, USA) and $p<0.05$ was considered to indicate statistical significance.

RESULTS

Metabolic improvement by QDMC

QDMC significantly improved the blood glucose level and glucose tolerance in mice compared to mice fed with only HFD (45% kcal fat) (**Fig. 1A-C**). APO B significantly decreased in the QDMC group (3.6±0.6 mg/dL) compared to the HFD group (4.6±0.5 mg/dL) (**Fig. 1D**). Body weight and APO A1 remained unchanged by QDMC when compared to the HFD group.

Effect of QDMC on the gut microbiota

In total, 261,242 sequences were generated from 15 samples, with an average of 14,513±6,531 sequences recovered per sample for use in comparative analyses. Differences in microbial diversity were observed in each group (**Fig. 2**). The alpha diversity of the gut microbiota was analyzed using Chao1 richness and Shannon indices and revealed no significant differences among the groups (**Fig. 2A**). Principal coordinate analyses of the weighted and unweighted UniFrac distances revealed separation among the RD, HFD, and QDMC-treated groups (**Fig. 2B**).

Effect of QDMC on the composition of gut microbiota

The ratio of *Firmicutes* to *Bacteroidetes* significantly increased after HFD, and no significant decrease was observed in the QDMC-treated group (**Fig. 2C**). Most abundant bacterial phyla and genera are confirmed in the heat map (**Fig. 2D**). The families *Deferribacteraceae*, *Odoribacteraceae*, and *Bacteroidaceae* revealed significantly greater abundance in the QDMC-treated group than in the other groups (**Fig. 3A**). The abundances of the *Mucispirillum*, *Streptococcus*, *Butyricimonas*, *Anaerotruncus*, *Ruminococcus*, and *Bacteroides* genera were significantly increased in the QDMC-treated group (**Fig. 3A**). Among these, the relative abundance of

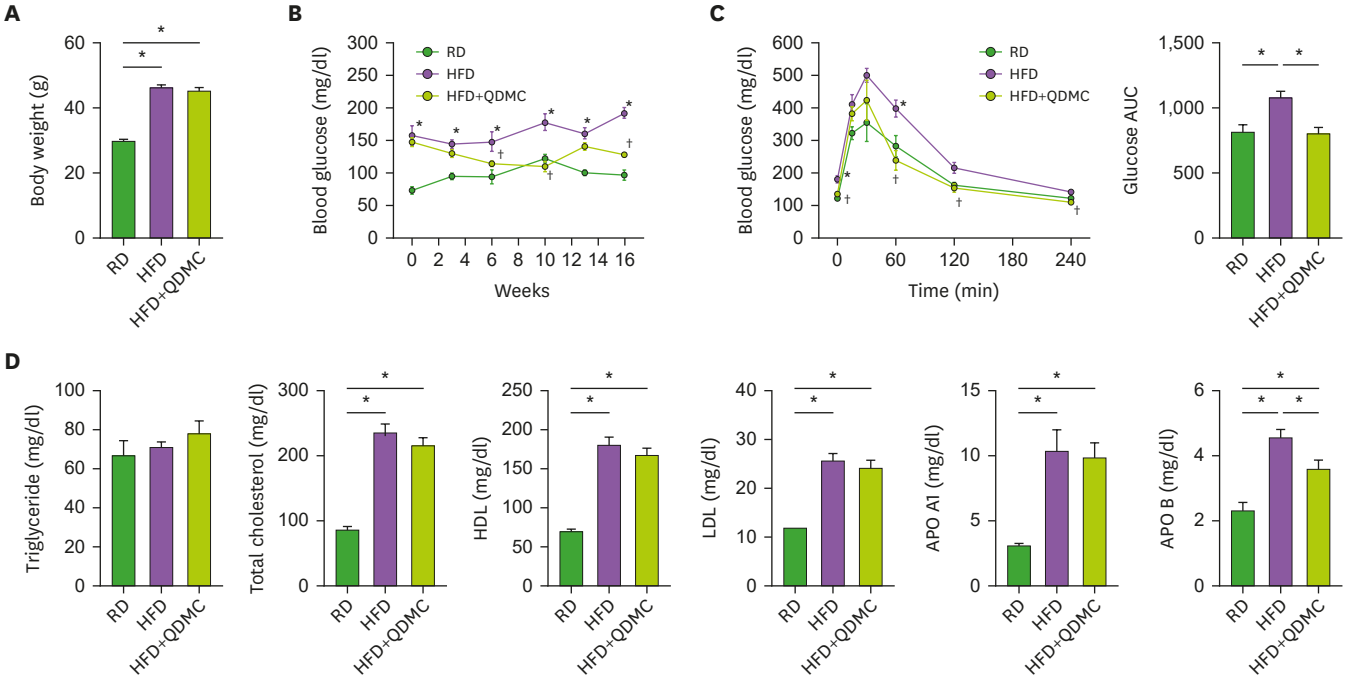

Figure 1. Effect of QDMC on body weight (A), blood glucose level (B), IPGTT (C), total cholesterol, HDL, LDL, APO A1 and APO B (D) results. Six-week-old C57BL/6N mice were fed an HFD (45% lipid) for 23 wk to induce obesity, and then administrated QDMC (100 mg/kg/day) daily for an additional 16 wk. RD: n=5; HFD: n=5; HFD+QDMC: n=5. Statistical analyses were performed using *t*-test and Mann-Whitney test.
HDL, high density lipoprotein; LDL, low density lipoprotein.
*p<0.05, compared with each group, in blood glucose curve; †p<0.05, compared with HFD group.

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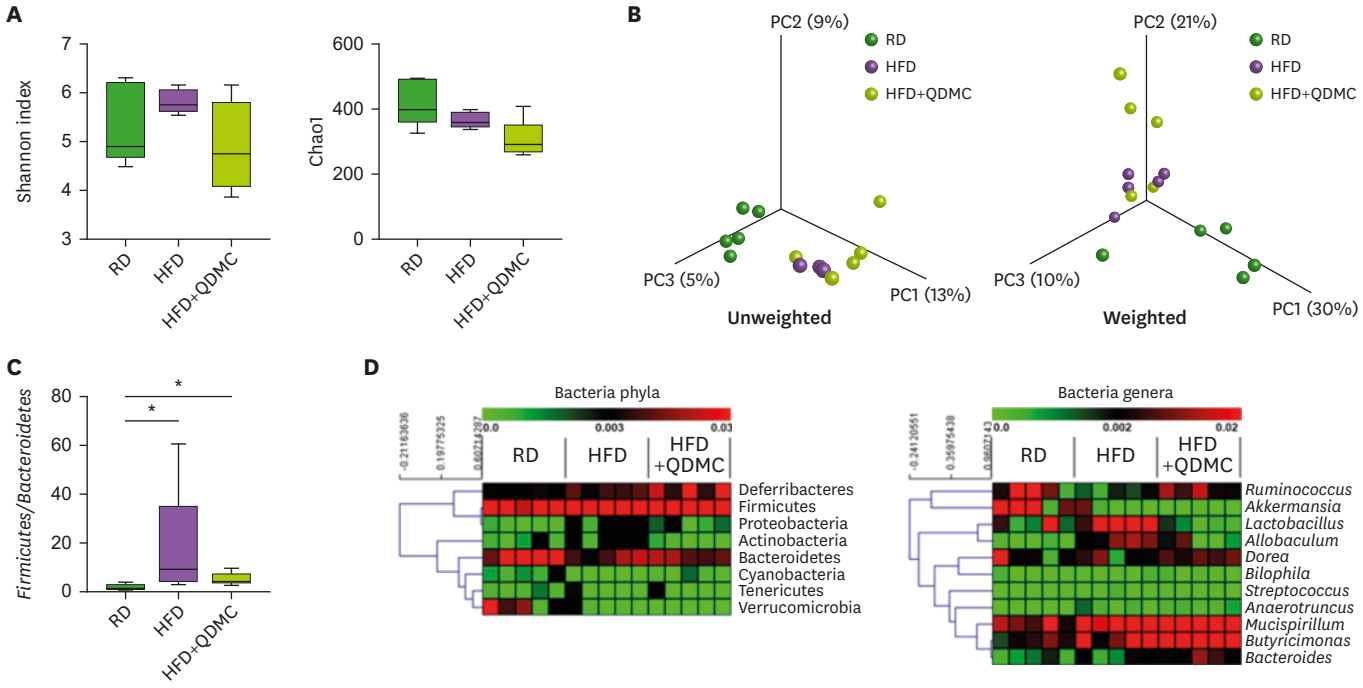

Figure 2. Microbial diversity in bacterial community categorized by diet and QDMC administration. Shannon diversity index and Chao1 richness by diet and QDMC administration (A). Principal coordinate analyses of the weighted and unweighted UniFrac distances of the cecum samples of 15 C57BL/6N mice (B). The ratio of *Firmicutes/Bacteroidetes* according to diet and QDMC (100 mg/kg/day) administration (C). Hierarchical clustering of most abundant bacterial phyla and genera are labeled in the heat map using Spearman's rank correlation (D).
*Statistical significance (*t*-test and Mann-Whitney test), $p<0.05$.

Mucispirillum, *Butyricimonas*, *Ruminococcus*, and *Bacteroides* genera was higher in the QDMC-treated group than that in the HFD group (**Fig. 3B**).

In the QDMC-treated group, body weight was negatively correlated with the relative abundance of *Ruminococcus* and *Bacteroides*, and positively correlated with that of *Anaerotruncus* (**Fig. 3C**). Both blood glucose level and IPGTT were negatively correlated with the abundance of *Butyricimonas*, *Mucispirillum*, and *Ruminococcus*, and positively correlated with the ratio of *Firmicutes/Bacteroidetes*. Correlation analysis revealed that *Butyricimonas*, *Mucispirillum*, and *Ruminococcus* were negatively correlated with glucose profiles (**Fig. 3C**).

Immunological change in the fat

The expression levels of TNF-α and IL-10 were significantly decreased in the QDMC-treated group compared to the HFD-only group. In contrast, IL-6, IL-1β, and IL-4 expression levels were increased in the QDMC-treated group compared to the HFD-only group (**Fig. 4A**).

In the QDMC-treated group, TNF-α expression was negatively correlated with the abundance of *Bacteroides* and the ratio of *Firmicutes/Bacteroidetes*, and positively correlated with the abundance of *Butyricimonas*. Moreover, IL-6 expression was negatively correlated with the abundance of *Butyricimonas* and the ratio of *Firmicutes/Bacteroidetes*, whereas IL-1β expression was negatively correlated with the abundance of *Streptococcus*, *Butyricimonas*, and the ratio of *Firmicutes/Bacteroidetes*, and positively correlated with the abundance of *Mucispirillum*. Furthermore, IL-4 expression was negatively correlated with the abundance of *Anaerotruncus*, and positively correlated with the abundance of *Streptococcus* and the ratio of *Firmicutes/Bacteroidetes*; whereas, IL-10 expression was positively correlated with the abundance of *Butyricimonas* (**Fig. 4B**).

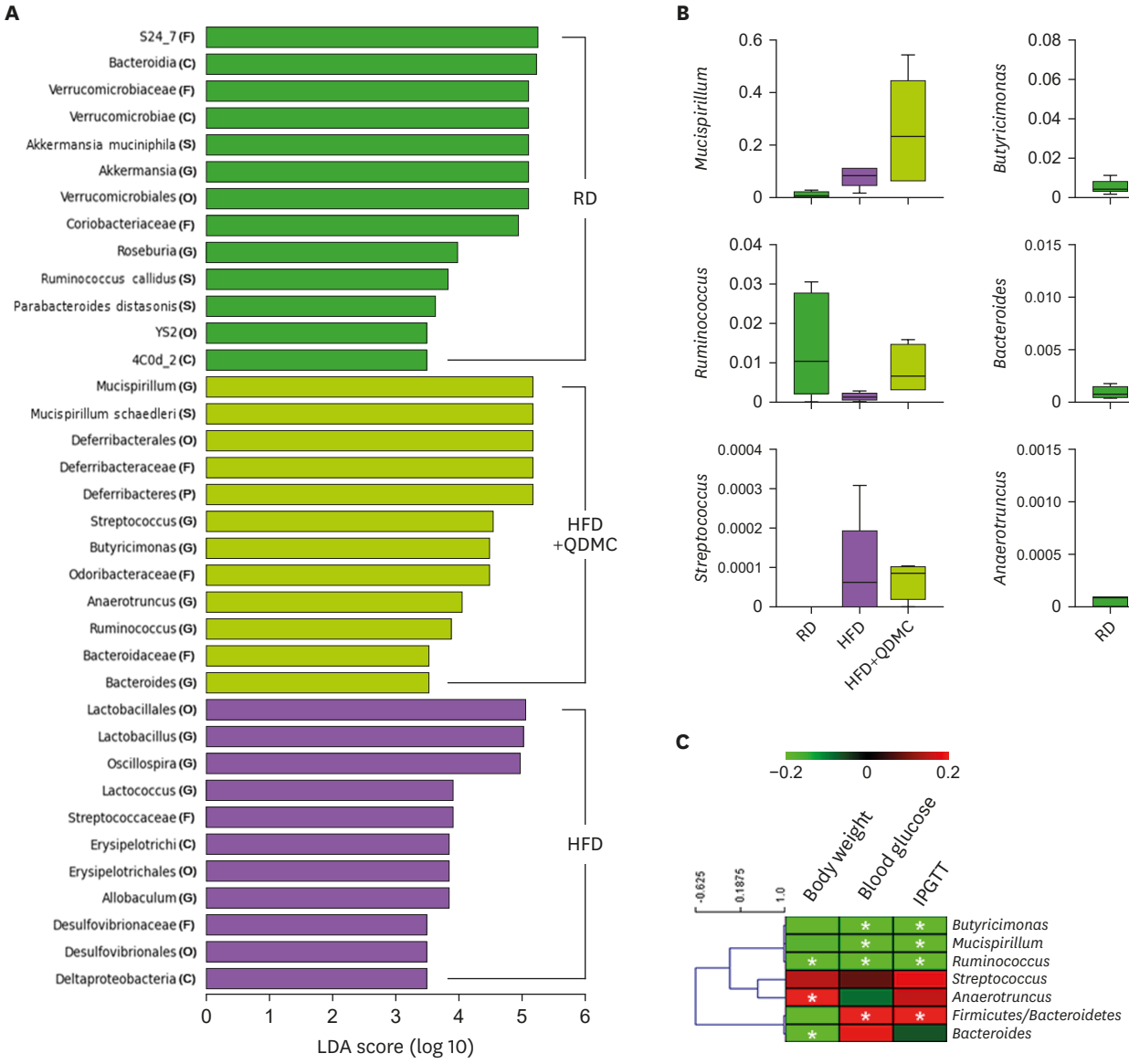

Figure 3. Relative bacterial abundance according to diet and QDMC administration. Significant differences were identified by LEfSe analyses as $p<0.05$, using both the Kruskal-Wallis test (among classes) and Wilcoxon test (between subclasses) (A). The abundance of *Butyricimonas*, *Mucispirillum*, *Streptococcus*, *Anaerotruncus*, *Ruminococcus and Bacteroides* (B). Correlations between metabolic biomarkers (body weight, blood glucose and IPGTT) and bacterial abundance in the QDMC (100 mg/kg/day) administered group (C). *Butrycimonas*, *Mucispirillum and Ruminococcus* were similar as showing negative correlation with glucose profiles.
*Statistical significance (Spearman's correlation coefficient), $p<0.05$.

DISCUSSION

Aloe species have been widely used for enhancing glucose metabolism and inflammatory conditions (19); however, the role of gut microbiota in ameliorating these effects remains unclear. Several *in vitro* studies have reported that *Aloe vera* increases the SCFA-producing bacteria and lactic acid bacteria such as *Lactobacilli* (8,20,21). This is the first study to assess the composition of gut microbiota after oral QDMC administration in an obese mouse model and to identify the relationship between altered gut microbiota, inflammatory responses, and metabolic profiles.

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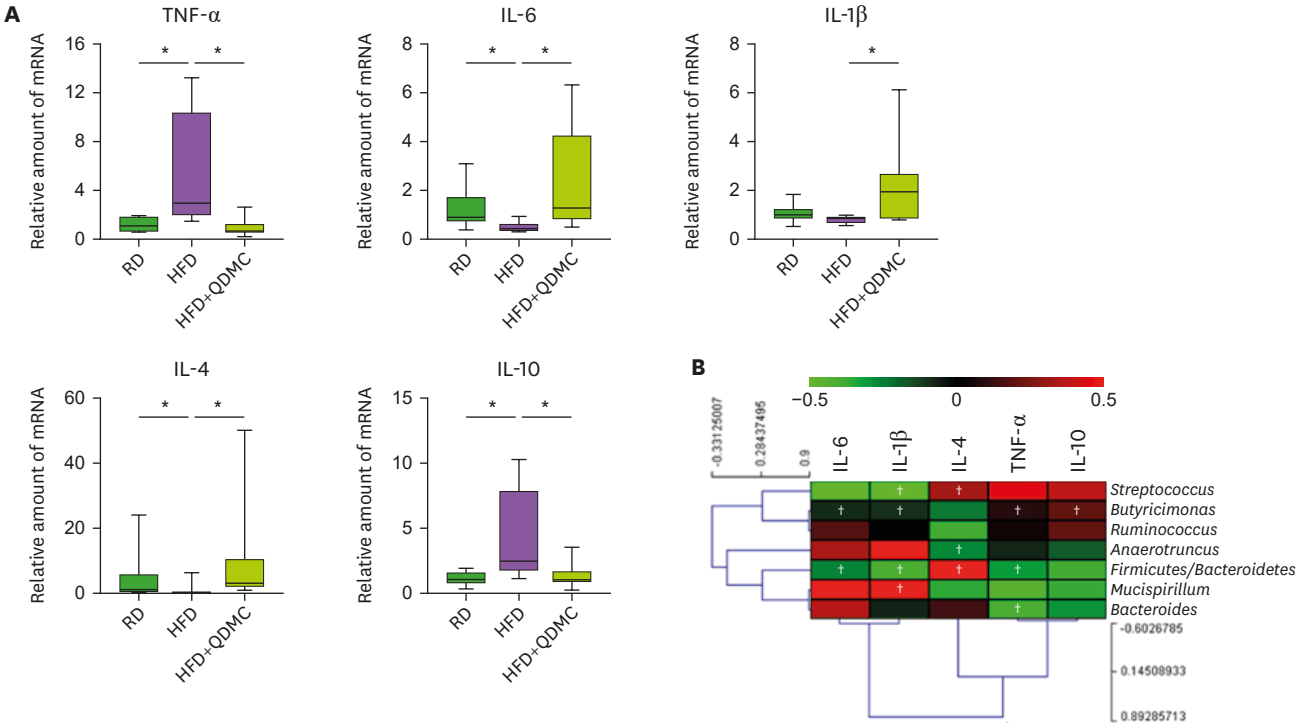

Figure 4. Immunological biomarkers in the epididymal fat. Relative expression level of mRNA measured by qPCR. The level of TNF-α, IL-6, IL-1β, IL-4, and IL-10 were compared by *t*-test and Mann-Whitney test (A). Correlations between immunological biomarkers and bacterial abundance in the QDMC (100 mg/kg/day) administrated group (B).
*p<0.05 compared with HFD group; †Statistical significance (Spearman's correlation coefficient), p<0.05.

Altered bacterial community indicated that QDMC treatment had an impact on the gut microbiota. Among these microorganisms, the relative abundance of *Ruminococcus*, *Mucispirillum*, and *Butyricimonas* was remarkably increased by QDMC treatment, which may be closely associated with the glucose regulation effect. *Ruminococcus*, known to digest plant fiber, is normally observed in individuals on a low-fat diet (22,23). *Ruminococcus* abundance varied with pharmacotherapies for T2D as well; among these, metformin, an AMPK inhibitor, was reported to increase *Ruminococcus* abundance (24). *Butyricimonas* is a bacterium that produces butyric acid, which is less abundant in obese and pre-diabetic populations than in normal and healthy populations (25). In recent study reporting the effect of atorvastatin and rosuvastatin on metabolic disorders, an increase in the relative abundance of *Butyricimonas* was observed and was significantly related to glucose regulation (26). Moreover, supplementation with black raspberry ameliorated the blood glucose level, and increased abundance of *Butyricimonas* played a pivotal role in glucose regulation (2). *Mucispirillum* lives in the intestinal mucus layer of rodents and human (27); however, few studies have discussed the correlation of *Mucispirillum* with intestinal inflammation, and hence further research is required (28).

Secretion of inflammatory cytokines is affected by various factors such as metabolic disorders and the composition of gut microbiota; therefore, the expression pattern of pro-inflammatory (TNF-α, IL-6, and IL-1β) and anti-inflammatory (IL-4 and IL-10) cytokines may provide a better understanding of the association between gut microbiota and metabolic improvement by QDMC. Insulin resistance is a key feature of metabolic disorders including hyperglycemia, and the dysfunction of TNF-α in adipose tissue plays a pivotal role in insulin

resistance (29,30). *Ruminococcus* species has been reported to inhibit TNF-α secretion in peripheral blood mononuclear cells sensitized with LPS, and *Ruminococcus* genera in Crohn's disease patients lower than in healthy control (31,32). From these reports provide insightful clues into the correlation between blood glucose improvement, changes in the gut microbiota, and changes in cytokines due to treatment of QDMC. In the present study, the decreased expression level of TNF-α in presence of QDMC may be related to the effect of glycemic regulation of QDMC. IL-6 is a pro-inflammatory cytokine associated with insulin resistance (33). Whether IL-6 induces insulin resistance remains debatable; however, it enhances the transport of insulin-stimulated glucose in adipocytes (34) and enhances AMPK, which further increases glucose uptake in skeletal muscle and adipocytes (35). These results suggest that increased expression of IL-6 by QDMC may play a pivotal role in glucose regulation. The levels of anti-inflammatory cytokine IL-4 are decreased after HFD (36). As expected, IL-4 level was increased in the QDMC-treated group, whereas the IL-10, which is an anti-inflammatory cytokine, was decreased in the QDMC-treated group. According to an interesting recent report, loss of IL-10 increased energy consumption and suppressed obesity (37). Moreover, feeding HFD to IL-10-deficient mice prevented hepatic steatosis and reduced fat and insulin resistance (37,38). These reports explain the effect of QDMC in reducing IL-10 and blood glucose. Based on the results of cytokine expression patterns, the regulation of TNF-α, IL-4, IL-6, and IL-10 in the fat may be crucial for the effect of glycemic regulation of QDMC. Furthermore, the composition of gut microbiota revealed a significant correlation with the metabolic profiles and the expression level of pro- or anti-inflammatory cytokines in this study. In particular, *Butyricimonas* abundance, which was significantly negatively correlated with IL-6 and IL-1β and positively correlated with IL-10 and TNF-α, was considered to highly impact the relationship between these cytokine activities.

In conclusion, relative abundance of *Butyricimonas*, *Ruminococcus*, and *Mucispirillum* was remarkably increased by QDMC treatment. QDMC ameliorated the glucose profiles and expression levels of anti- or pro-inflammatory cytokines in the fat, which were significantly correlated with bacterial abundance. These results suggest that alteration of gut microbiota by treatment of QDMC may be associated with the effect of glycemic regulation and immunomodulatory activity of QDMC.

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