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FULL COMMUNICATION

Fermented Green Tea Extract Alleviates Obesity and Related Complications and Alters Gut Microbiota Composition in Diet-Induced Obese Mice

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ABSTRACT Obesity is caused by an imbalance between caloric intake and energy expenditure and accumulation of excess lipids in adipose tissues. Recent studies have demonstrated that green tea and its processed products (*e.g.*, oolong and black tea) are introduced to exert beneficial effects on lipid metabolism. Here, we propose that fermented green tea (FGT) extract, as a novel processed green tea, exhibits antiobesity effects. FGT reduced body weight gain and fat mass without modifying food intake. mRNA expression levels of lipogenic and inflammatory genes were downregulated in white adipose tissue of FGT-administered mice. FGT treatment alleviated glucose intolerance and fatty liver symptoms, common complications of obesity. Notably, FGT restored the changes in gut microbiota composition (*e.g.*, the *Firmicutes*/*Bacteroidetes* and *Bacteroides*/*Prevotella* ratios), which is reported to be closely related with the development of obesity and insulin resistance, induced by high-fat diets. Collectively, FGT improves obesity and its associated symptoms and modulates composition of gut microbiota; thus, it could be used as a novel dietary component to control obesity and related symptoms.

KEY WORDS: • ***fatty liver*** • ***green tea*** • ***health functional food*** • ***insulin resistance*** • ***obesity***

INTRODUCTION

THE BALANCE BETWEEN energy intake and energy expenditure is critical for maintenance of energy homeostasis in humans, and an imbalance in energy metabolism caused either by increased intake or reduced expenditure can cause lipid accumulation in various tissues, predominantly adipose tissue, and lead to obesity.[1] In addition to changes in appearance and related cosmetic concerns, obesity is also a major risk factor for various metabolic diseases, including insulin resistance, type 2 diabetes, hyperglycemia, dyslipidemia, hypertension, hepatic steatosis, cancer, atherosclerosis, and cardiovascular diseases.[1] Thus, obesity is defined as a major metabolic disease closely associated with serious health problems, and it has become an important focus of research worldwide.

The gut microbiota are the resident microorganisms in the digestive tracts of animals and humans, which benefit hosts by aiding nutrient digestion and bioconversion of food chemicals.[2] In addition, abnormal changes in the gut microbiota could have undesirable effects on the health of hosts (*e.g.*, diarrhea and leaky gut syndrome) by inducing severe immune responses.[2] Interestingly, changes in the gut microbiota have recently been reported to be closely associated with the development and treatment of obesity and related metabolic disorders, including type 2 diabetes.[3–7] For instance, the diversity of the gut microbiota reflects the metabolic status of the host.[8] Germ-free mice were reported to be immune to diet-induced obesity,[9,10] whereas transplantation of the gut microbiota from conventional to germ-free mice caused rapid body weight gain in the latter without any changes in food intake.[10] In addition, the ratio of *Firmicutes* to *Bacteroidetes* is closely associated with the progression of obesity.[11–13] Recently, changes in microbiota composition have been reported in the gut of type 2 diabetic patients.[3,6,14] Furthermore, changes in gut microbiota (*e.g.*, *Lactobacillus* spp., *Bifidobacterium* spp., and *Bacteroides-Prevotella* spp.) by antibiotic treatment alleviated obesity and obesity-related complications.[7] Accumulating evidence indicates an interaction between gut microbiota dynamics and metabolic disorders.

Fermentation, the microorganism-mediated conversion of organic molecules, is commonly used in food processing and preservation. Representative fermented food products include soy sauce, *doenjang*, *kimchi*, and yogurt, all of which have improved health benefits, indicating that

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microbial fermentation produces novel organic compounds with beneficial biological activities.[15,16] Similarly, teas can be consumed after processing (*e.g.*, oolong, black, and puer tea). These are reported to exert beneficial effects on metabolism differing from those of conventional green teas, according to animal and human studies.[17,18] However, some processed teas including puer tea are processed by resident microorganisms, and the composition of those teas may vary from each other.[19] To make a standardized tea product, we made fermented green tea (FGT). FGT is a novel fermented product of dried green tea leaves fermented by *Bacillus subtilis*, a microorganism used to produce fermented soybean products (*e.g.*, *doenjang*, *cheonggukjang*, and *ganjang*) in East Asia, especially in Korea. Similar to other processed teas, FGT is expected to exhibit beneficial effects on health management and energy metabolism. In this article, we examined whether FGT is able to modulate energy metabolism in high-fat fed obese mice.

MATERIALS AND METHODS

Preparation of FGT

FGT extracts were provided by Mizon Co. (Siheung, South Korea). Briefly, green tea leaves were dried at 150°C for 10 min. The dried leaves were then mixed with 1% sucrose and 5×10^7 colony forming unit *B. subtilis* and fermented at 50°C for 72 h. After fermentation, samples were further incubated at 90°C for 96 h to allow postmaturation. FGT leaves were redried and then extracted using 50% alcohol (20-fold v/w dried green tea leaf) at 70°C for 2 h. FGT was produced from the extracts by freeze drying. The average yield of FGT from dried green tea leaves was 23%. Catechin composition of FGT was analyzed by HPLC (Waters Alliance 2695; Waters, Milford, MA, USA) assay using an ODS (C18) column. A gradient elution was performed with various proportion of solvent A (water–acetic acid with 97:3 w/v) to solvent B (methanol), with a flow rate of 1 mL/min as follows: 0–25 min, 80:20, 26–50 min, 70:30, and 51–55 min, 80:20. The acquisition wavelength was set in the range of 280 nm. The catechin compositions of FGT and green tea are shown in Table 1.

Animal experiments

All animal experiments were performed according to a protocol approved by the Animal Experiment Committee of Korea University (Protocol No. KUIACUC-20090420-4). Six-week-old male C57BL/6 mice were purchased from Central Laboratory Animal, Inc. and fed normal chow *ad libitum* for 1 week to allow adaptation. After adaptation, the mice were divided into three groups (normal diet [ND], high-fat diet [HFD], and HFD-FGT [FGT]; $n = 16$/group). The HFD and HFD-FGT groups were fed a 45% calorie from fat diet (24% w/w of protein, 41% w/w of carbohydrate, and 24% w/w of lipid to a total calorie of 4.73 kcal/g; Research Diet, New Brunswick, NJ, USA), whereas the control group was fed AIN-76A diet (20.3% w/w of protein, 66% w/w of carbohydrate, and 5% w/w of lipid for a total of 3.9 kcal/g; Research Diet). All the diets for *in vivo* study were sterilized before use. During the experiment, FGT (500 mg/kg; dissolved in 0.1% methylcellulose; Sigma, St. Louis, MO, USA) was administered orally and daily. The ND and HFD groups were administered the vehicle (0.1% methylcellulose) instead of FGT. Body weight and food intake were measured biweekly. At the end of FGT administration, fecal samples were prepared.

Half of the mice from each group were starved overnight in preparation for the oral glucose tolerance test (OGTT). OGTT assay was measured as described previously.[20] Briefly, mice were orally injected with 500 μL of glucose (0.1 g/mL). The plasma glucose level was measured 0, 30, 60, and 120 min after glucose administration with Freestyle blood glucose meter (Therasense, Uppsala, Sweden).

The remaining mice were sacrificed, and liver and gonadal white adipose tissues were prepared and weighed. After measurement of the tissue weights, half of the liver tissues were fixed immediately with formalin for further analyses. Following fixation, the liver tissue was paraffin-sectioned and stained with hematoxylin–eosin. Liver triglyceride level was measured as described previously.[21] Briefly, 100 mg of liver tissue was mixed with 350 μL of ethanolic potassium hydroxide solution and incubated for overnight at 55°C. Then, samples were brought up to 1 mL with 50% ethanol and centrifuged at 1500 *g* for 5 min. Supernatants were moved to new microcentrifuge tubes, 50% ethanol was added up to 1.2 mL, and vortexed. Two hundred microliters of mixture was moved to a new tube, 215 μL of 1 M $MgCl_2$ added, and incubated on ice. Samples were centrifuged at 1500 *g* for 5 min and supernatants were measured by Triglyceride Reagent (Sigma). Results were obtained by measuring absorbance at 540 nm using TECAN M200 PRO fluorometric plate reader (Tecan, Männedorf, Switzerland).

Blood samples were prepared in serum separation tubes and centrifuged. Separated plasma was moved to new microcentrifuge tubes. Plasma concentration of glucose, cholesterol, triglyceride, alanine aminotransferase (ALT), and asparatate aminotransferase (AST) levels were measured in plasma analyzer.

Table 1. Catechin and Caffeine Compositions of Green Tea and Fermented Green Tea

Component	*Green tea (% w/w)*	*FGT (% w/w)*
Catechin	26.6	7.85
GC	1.52	1.30
EGC	11.7	2.17
C	0.39	0.39
EC	2.16	0.68
EGCG	8.74	1.6
GCG	0.82	1.23
ECG	1.23	0.48
Caffeine	8.13	6.28
Total	34.7	14.1

C, catechin; EC, epicatechin; ECG, epicatechin gallate; EGC, epigallocatechin; EGCG, epigallocatechin gallate; FGT, fermented green tea; GC, gallocatechin; GCG, gallocatechin gallate.

DNA preparation and gut microbiota analysis

Microbial DNA was prepared from mouse fecal samples using the QIAamp DNA Stool Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. Primers targeting the 16S rDNA genes, which are conserved among microbial species, were used to determine the gut microbiota composition, as described previously.[22] Gut microbiota composition was evaluated using real-time polymerase chain reaction (RT-PCR, Rotor-Gene 3000; Qiagen, Venlo, Limburg, The Netherlands). A total of 100 ng of each DNA sample was used as the template. For purposes of validation, a proportion of the DNA samples were also meta-analyzed by Chunlab, Inc. (Seoul, Korea) (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/jmf). The gut microbiota composition (*Firmicutes*/*Bacteroidetes* ratio) RT-PCR and meta-analysis results were identical. Primer sequences are provided in Supplementary Table S1.

RNA isolation, cDNA synthesis, and qRT-PCR

RNA was isolated using TRIzol™ reagent (Invitrogen, Carlsbad, CA, USA), following the manufacturer's protocol. Each RNA sample (2 μg) was subjected to cDNA synthesis using the RevertAid™ First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). Relative mRNA levels were determined by quantitative real-time

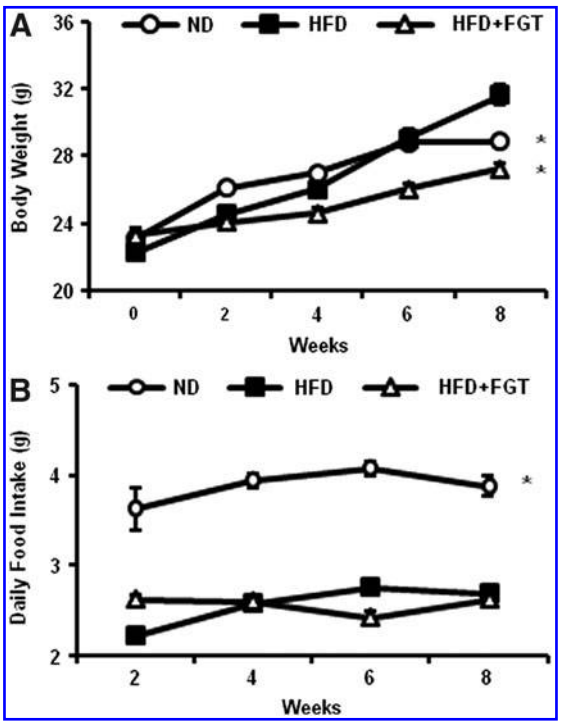

FIG. 1. Fermented green tea (FGT) extract attenuates body weight gain without a change in food intake in high-fat diet (HFD)-fed obese mice. **(A)** Body weight changes and **(B)** cumulative food intake from the animal experiments are presented as line graphs. $^{*}P < .05$ versus HFD.

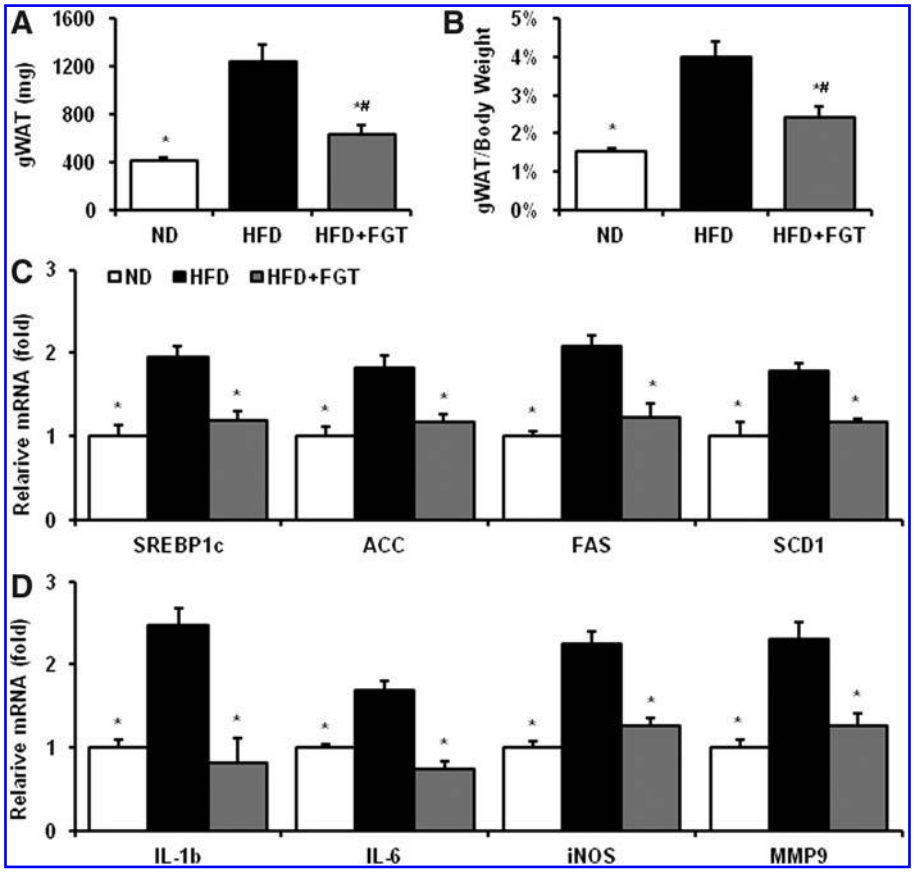

FIG. 2. FGT decreases fat mass and alters gene expression profiles in white adipose tissue. **(A)** The average gonadal white adipose tissue (gWAT) masses and **(B)** average gWAT mass/body weight ratios are shown as bar graphs. The expression levels of **(C)** lipogenic genes (*e.g.*, sterol regulatory element binding protein 1c [SREBP1c], acetyl-CoA carboxylase [ACC], fatty acid synthase [FAS], and stearoyl-CoA desaturase 1 [SCD1]) and **(D)** inflammatory genes (*e.g.*, interleukin-1β [IL-1β], interleukin-6 [IL-6], inducible nitric oxide synthase [iNOS], and matrix metalloproteinase 9 [MMP9]) were determined in the adipose tissue of mice by quantitative real-time PCR (qRT-PCR), normalized by GAPDH, and are shown as bar graphs. $^{*}P < .05$ versus HFD, $^{\#}P < .05$ versus normal diet (ND).

Table 2. Plasma Profile of Fermented Green Tea-Administered Mice

	Glucose (mg/dL)	*Cholesterol (mg/dL)*	*Triglyceride (mg/dL)*	*ALT (mg/dL)*	*AST (mg/dL)*
ND	83.25 ± 3.76	113.71 ± 6.63	67.13 ± 4.28	57.73 ± 8.52	101.87 ± 10.83
HFD	164.43 ± 5.24*	191.03 ± 4.59*	107.25 ± 6.01*	45.35 ± 2.73	105.73 ± 18.29
HFD + FGT	140.14 ± 8.05#	145.69 ± 9.46#	83.00 ± 7.80#	33.96 ± 2.66	110.11 ± 17.71

*$P < .01$ versus ND, #$P < .05$ versus HFD.
ALT, alanine aminotransferase; AST, asparatate aminotransferase; HFD, high-fat diet; ND, normal diet.

PCR (qRT-PCR) using the appropriate primers (Bioneer, Daejeon, Korea). Primer sequences are provided in Supplementary Table S2.

Statistical analyses

The results are representative of the data from three or more independent experiments. All values are the means of triplicate samples. Error bars represent standard error. A *P*-value $< .05$, as calculated by one-way ANOVA followed by Tukey honest significant difference, was interpreted as statistically significant.

RESULTS

FGT reduces body weight gain without changing food intake

To elucidate the effect of FGT on body weight in mice, we orally administered FGT together with a HFD for 8 weeks. Mice fed a HFD gained significantly more body weight than those fed the ND (Fig. 1A). However, no HFD-induced body weight gain occurred in the HFD-FGT mice. Throughout the experiment, there was no statistically significant difference in food intake between the HFD and HFD-FGT mice (Fig. 1B). This suggests that FGT attenuated body weight gain without a reduction in food intake in mice with diet-induced obesity.

FGT reduces fat mass and alters gene expression profiles in white adipose tissue

To investigate whether the reduction in body weight gain correlates with a loss in fat mass, we measured gonadal fat weight. Similar to the changes in body weight, FGT also reduced gonadal adipose tissue mass significantly (Fig. 2A). The gonadal fat mass-to-body weight ratio was also decreased by FGT treatment as expected (Fig. 2B). These data indicate that the effect of FGT on the retardation of body weight gain might be related to a reduction in fat mass, at least in part.

With HFD, excess lipids from dietary nutrients are primarily stored in fat cells, which alters plasma lipid metabolism. Furthermore, excess free fatty acids trigger proinflammatory responses, leading to insulin resistance and type 2 diabetes.[23–26] Interestingly, the mRNA expression of genes related to lipogenesis was upregulated in adipose tissue of HFD mice; however, FGT treatment alleviated the HFD-mediated induction of lipogenic gene expression (Fig. 2C). Similarly, the induction proinflammatory gene expression by HFD was reversed in the HFD-FGT mice (Fig. 2D). Consequently, FGT decreased plasma glucose and lipid levels without inducing liver toxicity (Table 2). These results demonstrate that FGT reduces lipogenic and proinflammatory gene expression, thereby preventing hyperlipidemia.

FGT alleviates glucose intolerance and fatty liver symptoms in HFD-fed obese mice

We next examined the effects of FGT on glucose tolerance and hepatic steatosis, two obesity-related symptoms, in HFD-fed obese mice. FGT improved glucose tolerance as determined by OGTT assay (Fig. 3A, B). Mice fed the HFD diet exhibited fat accumulation in the liver, causing hepatic steatosis. Large vacuoles containing triglycerides were evident in the livers of HFD mice, but these were diminished in the livers of HFD-FGT mice to a level similar to ND mice (Fig. 4A). Triglyceride levels in the liver were also reduced in HFD-FGT mice (Fig. 4B). Consistent with these results, the expression levels of genes involved in hepatic lipogenesis and inflammation in HFD-FGT mice were significantly reduced in the livers of HFD-FGT mice compared with those of HFD control mice (Fig. 4C, D). Therefore, FGT normalizes glucose tolerance and hepatic steatosis, obesity-related clinical symptoms, in the HFD-fed obese mice.

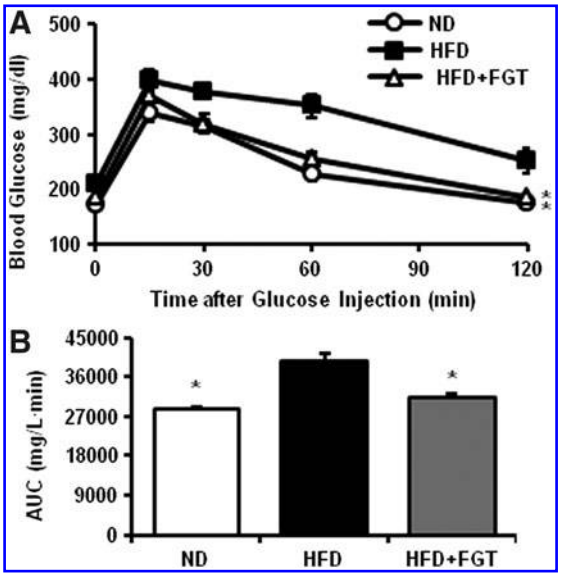

FIG. 3. FGT improves glucose tolerance in HFD-fed obese mice. **(A)** The results of the oral glucose tolerance test and **(B)** the area under the curve (AUC) from the oral glucose tolerance test are shown in **(A)**. *$P < .05$ versus HFD.

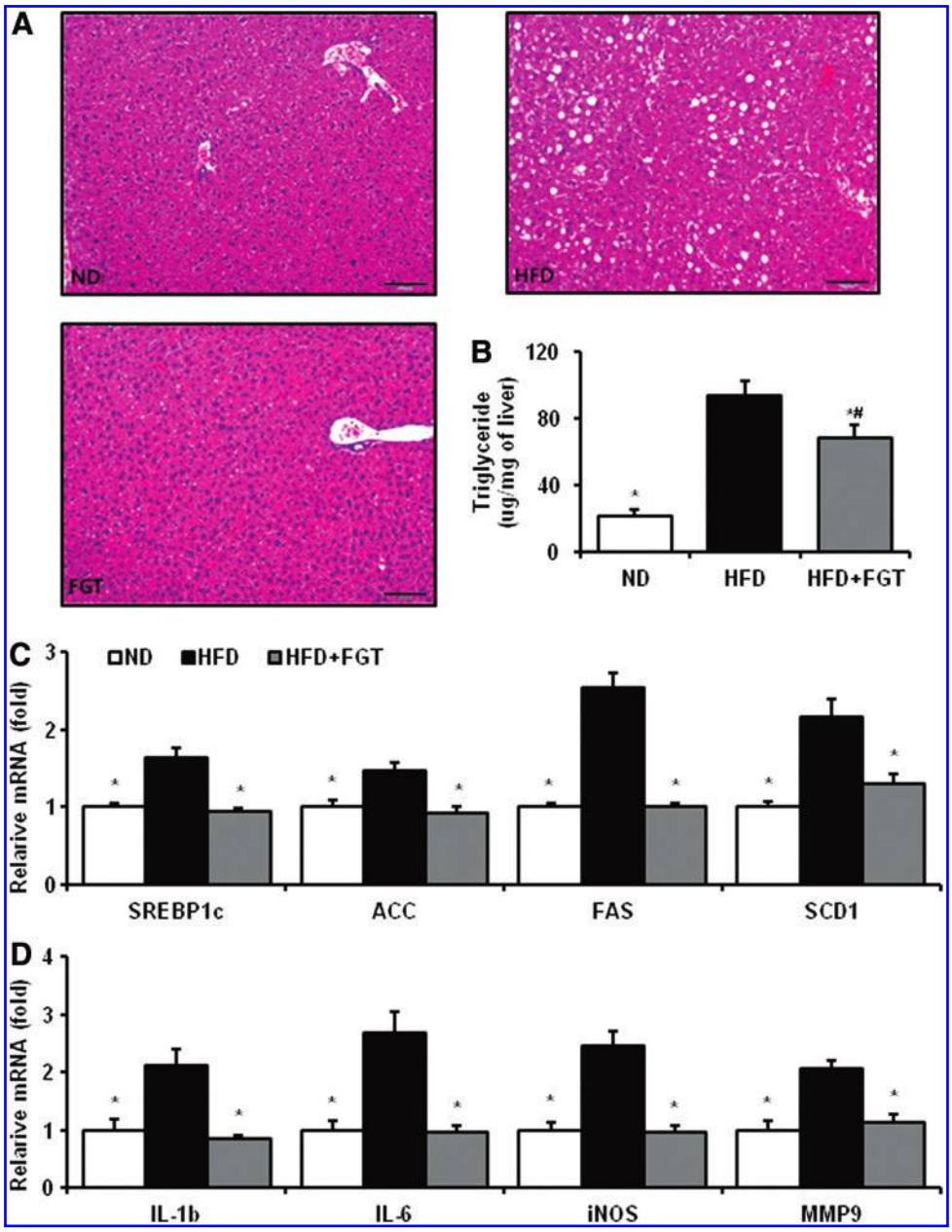

FIG. 4. FGT alleviates fatty liver symptoms. **(A)** Liver histology. Scale bar = 100 μm. Hepatic lipid accumulation shown as small circles was dramatically reduced in mouse livers after FGT feeding. **(B)** Accumulated hepatic triglyceride levels. The mRNA levels of **(C)** lipogenesis-related genes (SREBP1c, ACC, FAS, and SCD1) and **(D)** inflammation-related genes (IL-1β, IL-6, iNOS, and MMP9) were determined in the liver of mice by qRT-PCR, normalized by GAPDH, and presented as bar graphs. $^{*}P < .05$ versus HFD, $^{\#}P < .05$ versus ND. Color images available online at www.liebertpub.com/jmf

FGT significantly alters the composition of the gut microbiota

The composition of the gut microbiota has been reported to be an independent risk factor for obesity and metabolic disorders,[2,11–13] and increased *Firmicutes*/*Bacteroidetes* and *Bacteroides*/*Prevotella* ratios have been suggested as signature biomarkers for obesity and type 2 diabetes. We assessed the effect of FGT on the composition of the gut microbiota. Consistent with a previous report,[11] the *Firmicutes*/*Bacteroidetes* ratio was increased in HFD mice. However, FGT administration for 8 weeks reduced this ratio dramatically (Fig. 5A), which was similar in the FGT and normal-chow-diet groups. In addition, FGT restored the HFD-induced changes in the *Bacteroides*/*Prevotella* ratio (Fig. 5B), indicating that the enterotype of FGT-administered mice resembles that of lean mice, despite being fed the HFD. Collectively, these results demonstrate that FGT functions to restore the gut microbiota composition altered by a HFD, which may be a key mechanism underlying the anti-obesogenic activity of FGT in obese mice.

DISCUSSION

Trillions of microorganisms live in human gut.[27,28] They benefit the host by breaking down nondigestible nutrients, regulating the immune system, and bioconverting useful materials.[2] However, abnormal growth of, or compositional changes in, the gut microbiota can cause gut disorders and increase cancer risk.[2] The importance of gut microbiota as a

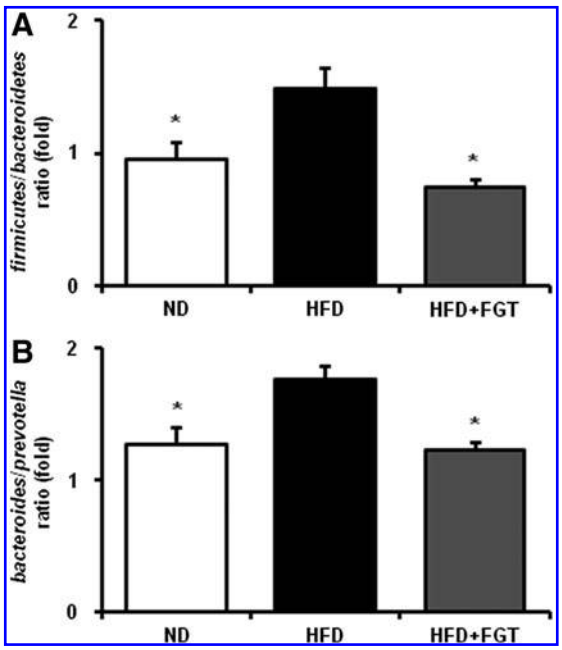

FIG. 5. FGT alters the composition of the gut microbiota. **(A)** The *Firmicutes*/*Bacteroidetes* and (**B**) *Bacteroides*/*Prevotella* ratios are shown. $*P < .05$ versus HFD.

causal factor of obesity has been emphasized recently. Several studies have revealed that the composition of the gut microbiota is dependent on nutrient intake, and changes in the gut microbiota are closely related to body weight. For example, the *Prevotella* enterotype is associated with carbohydrate- or sugar-rich diets, whereas the *Bacteroides* enterotype is related to proteins and saturated fats.[29] Additionally, it has been reported that the gut microbiota of leptin-deficient obese ($ob/ob^{-/-}$) mice contains 50% more *Firmicutes* and 50% less *Bacteroidetes.*[11] In the guts of obese humans, the phylum *Firmicutes* predominates, and weight loss results in a reduction in *Firmicutes* and elevation in *Bacteroidetes* levels.[11–13] Therefore, controlling the composition of the gut microbiota might be crucial for maintaining optimal body weight.

FGT altered the gut microbiota composition to be similar to that of lean mice (Fig. 5A). As Turnbaugh *et al.* suggested, $ob/ob^{-/-}$ mice release more calories during digestion of food.[12] Since the phylum *Firmicutes* predominates in the gut microbiota of obese mice,[11] the host likely receives more calories with increasing *Firmicutes* levels in the gut. Therefore, it might be feasible that in the absence of a change in food intake, FGT-induced alteration of the composition of the gut microbiota (reduced *Firmicutes*) contributed to the reduction in energy intake, thereby attenuating body weight gain and fat mass increase, at least in part.

Interestingly, the *Bacteroides*/*Prevotella* ratio was also recovered by FGT treatment. As mentioned above, *Bacteroides* abundance is increased by a Western diet (*e.g.*, high protein and high fat), whereas *Prevotella* consume carbohydrates and fibers.[29] Feeding HFD resulted in increased *Bacteroides* and reduced *Prevotella*, which was reversed by FGT treatment (Fig. 5B). The change in the *Bacteroides*/*Prevotella* ratio reflects the preference of HFD-FGT mice for carbohydrates and dietary fibers; however, it is also possible that lipids were the main source of dietary nutrients in HFD. By increasing the abundance of carbohydrate-consuming members of the gut microbiota (*Prevotella*), FGT might help to prevent HFD-induced body weight gain. Further studies will reveal the active ingredient and working mechanism of FGT responsible for *Prevotella* growth.

A HFD results in elevated expression of lipogenic genes to allow storage of excess lipids in adipose tissue.[30] These excess lipids, such as free fatty acids, trigger proinflammatory responses, which are causal factors of insulin resistance.[31] According to previous results, control of lipid metabolism is likely important for reversing obesity-mediated metabolic disorders (such as insulin resistance and type 2 diabetes) through the modulation of inflammatory responses. We showed that FGT administration reversed the expression of lipogenic genes and proinflammatory genes in white adipose tissue (Fig. 2C, D), accompanied by increased mRNA expression of β-oxidation-related genes and mitochondrial biogenesis-related genes (Supplementary Fig. S2), thereby reducing plasma lipid levels (Table 2). These data suggest that FGT can alleviate obesity and chronic inflammation, and thus it may be useful for treating metabolic disorders. Indeed, FGT improved glucose tolerance and alleviated fatty liver symptoms (Figs. 3 and 4), indicating that FGT is capable of ameliorating obesity-related complications, including glucose intolerance and hepatic steatosis, similar to other antiobesity agents.[32,33]

Green tea has also been proposed to exert antiobesity effects[34–41] and to induce compositional changes in gut microbiota.[42,43] Since the phylum *Bacteroidetes* is more capable of cleaving the glycosidic linkages of polyphenols compared with *Firmicutes*,[44] it might be feasible that polyphenol supplementation contributes tothe reduction of body weight through the modulation of the gut environment in favor of *Bacteroidetes*, at least in part. However, the concentration of catechins in FGT is much lower than that of green tea (Table 1). Therefore, other—perhaps unknown—molecules, rather than catechins may mediate the antiobesity and gut microbiota-modulating effect of FGT. Interestingly, it has been proposed that polymerized polyphenols, which exert beneficial effect on lipid metabolism, are synthesized during oolong tea production.[45,46] As a processed green tea product, we speculate that FGT might also contain modified polyphenols derived from catechins, and we are now identifying those compounds. Future studies are needed to elucidate the detailed mechanisms and active components involved in FGT-mediated weight control.

In conclusion, we demonstrated that FGT induced changes in the composition of the gut microbiota. These changes may be the mechanism responsible for the FGT-induced reduction in body weight gain and fat mass and improvement of glucose intolerance and fatty liver symptoms in HFD-fed obese mice. Collectively, our data suggest that FGT improved obesity and its associated symptoms and

modulated the composition of gut microbiota, and thus represents a novel dietary component that could be used to control the symptoms of obesity.

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AUTHOR DISCLOSURE STATEMENT

All of the authors have read the journal's policy and disclose the following conflicts. Authors Hyun Woo Jeong, Donghyun Cho, Bum Jin Lee, Ji Hae Lee, Jae Young Choi, and Il-Hong Bae are employees of Amorepacific Corporation. A patent application on the improvement of obesity and glucose intolerance by modulation of gut microbiota was submitted on 21 March 2014 and is currently undergoing revision (submission number 2014-0033262, Republic of Korea). There is one related product on the market in South Korea (S'Lite Slimmer DX). However, this did not alter our adherence to the policies of the *Journal of Medicinal Food* on data and material sharing.

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