

Anti-obesity effect of robusta fermented with *Leuconostoc mesenteroides* in high-fat diet-induced obese mice

SOO-YOUNG CHOI¹, SEUNG-HYUN RYU¹, JIN-IL PARK¹, EUI-SUK JEONG^{1,2},
JONG-HYUNG PARK¹, SEUNG-HOON HAM¹, HEE-YEON JEON¹, JUN-YOUNG KIM¹,
INN-GOO KYEONG³, DONG-GOO KIM³, JI-YOUNG SHIN³ and YANG-KYU CHOI¹

¹Department of Laboratory Animal Medicine, College of Veterinary Medicine, Konkuk University, Seoul 05029; ²Laboratory Animal Center, Daegu-Gyeongbuk Medical Innovation Foundation, Daegu 41061; ³R&D Center, CosisBio Corporation, Chungbuk 27867, Republic of Korea

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Abstract. Robusta beans cultivated with *Monascus ruber* (RMR) were successively fermented with *Leuconostoc mesenteroides* (LM) and the antiobesity effects were examined. To produce an obese mouse model to investigate the hypolipidemic effects, ICR mice were fed the same high-fat diet for 6 weeks. Treatment groups were given 10 or 20% RMR-LM. Body weight changes in the 20% RMR-LM group were lower compared with those in the control group. Visceral adipose tissue weight and adipose size were significantly lower in the 20% RMR-LM group compared with those in the control group. Significant improvement in glucose tolerance was observed in the 10 and 20% RMR-LM groups compared with the control group. The 20% RMR-LM group exhibited a significant reduction in serum glucose concentration. Hepatic mRNA levels of sterol regulatory element-binding protein 1, fas cell surface death receptor, and peroxisome proliferator-activated receptor γ , which are associated with lipid, and fatty acid metabolism, in the 20% RMR-LM group were significantly lower compared with those in the control group. The results of the present study demonstrated that 20% RMR-LM may be used to prevent obesity, and ameliorate diabetes and lipid metabolism imbalances.

Introduction

Obesity is a medical condition characterized by increased adipose mass resulting from a chronic imbalance between energy intake and expenditure (1). In response to the increase

in obesity, it has become increasingly more common to use pharmaceutical treatments for obesity, which are costly and not very effective (2,3). Currently, there is a focus in anti-obesity treatments on alternative therapies, including natural products and herbal medicines. Functional, health-enhancing foods, or nutraceuticals, are food products that influence specific physiological functions in the body (4).

As the interest in natural products has increased, a microbial culture and fermentation products were used to develop food additives and healthy food supplements. Recently, the study of the physiological activities, including antioxidant activity and immune activation, of liquid mycelium cultures has progressed steadily (5). In addition, mycelium fermentation with natural products has the advantage of being able to utilize all of the useful natural components of the mycelium (5).

The natural product robusta have been reported to have various effects and physiological activities. Many herbal-derived polyphenolic compounds are suggested to be able to prevent obesity via hypolipidemia effects and adipose tissue reduction and thus suppress the development of the metabolic, hepatic, and cardiovascular alterations associated with obesity (6). Robusta had been shown to contain melanoidin, which has been shown to have antioxidant activity *in vivo*, and a large amount of chlorogenic acid, which is a functional material component (7). Yamabushitake mushroom (*Hericium erinaceum*) is a well-known edible and medical mushroom used in East Asia. It has been reported that *H. erinaceum* contains many biologically active compounds, which have shown interesting biological activities, such as hypolipidemic, cytotoxic, anti-microbial, and anti-tumor effects (8). Oral administration of dried Yamabushitake mushroom powder can improve mild cognitive impairment in humans, but little is known about the anti-obesity efficacy of *H. erinaceum* (9).

The ascomycetous fungus *Monascus ruber* has been used in food, medicine, and industry in Asian countries. The use of fungi for the production of commercially important products has increased rapidly over the past half century, and pigment-producing microorganisms and microalgae are quite common in nature (10). It has been reported that *M. ruber* produces monacolin K, which has antifungal and

Correspondence to: Dr Yang-Kyu Choi, Department of Laboratory Animal Medicine, College of Veterinary Medicine, Konkuk University, 120 Neungdong-ro, Gwangjin-gu, Seoul 05029, Republic of Korea
E-mail: yangkyuc@konkuk.ac.kr

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immunosuppressive effects, and *M. ruber* is an effective treatment for hypercholesterolemia and cholesterol imbalances because it decreases blood cholesterol levels (11). A previous study on red mold rice production by *Monascus* species under monoculture conditions showed that secondary metabolite production is greatly affected by the fermentation medium, cultivation conditions, and types of *Monascus* species used in the fermentation process (12).

There is an increasing interest in the use of health functional materials, especially lactic acid bacteria (13). *Leuconostoc mesenteroides* is an epiphytic bacterium that is widely spread in the natural environment and used in the production of various useful products in the biochemical and pharmaceutical industries. In addition, it has been used to modify a variety of bioactive substances in an effort to improve their functionality.

After the primary fermentation of the mycelium of *M. ruber* with robusta as nutrients, the primary fermentation products were cultivated with *L. mesenteroides* in a secondary fermentation. It was expected that the two-step fermentation (Step-by-step) would enhance the physiological activity of the extracts, enabling their use in developing food additives and health supplements. The goal of this research was to investigate the effect of robusta fermented with *L. mesenteroides* on treating obesity.

Materials and methods

Preparation of first fermentation product. *M. ruber* were obtained from the National Institute of Agricultural Sciences (NAS, Jeollabuk-do, Korea) to ferment robusta. Briefly, the first seed cultivation of *M. ruber* were cultured on potato dextrose agar (PDA) for 10~15 days at 25~30°C and then cultured in potato dextrose broth (PDB) for 4~7 days in a shaking incubator (Jeio Tech Co., DaeJeon, Korea). For robusta fermented with *M. ruber* (RMR) 100 g of Vietnam robusta bean was soaked in 200 ml water for 2 h, sterilized for 120 min at 121°C, and allowed to cool down at room temperature. Robusta and *M. ruber* were mixed in ratio of 10:1, cultured for 7 days at 25°C, and roasted after hot-air drying to produce the first solid fermented product.

Preparation of second fermentation product. Robusta cultivated with *M. ruber* (RMR) were secondarily fermented with *Leuconostoc mesenteroides* (RMR-LM). Briefly, *L. mesenteroides* a pure lactic acid bacteria, was separated from kimchi. Separated *L. mesenteroides* was transferred into Lactobacilli MRS agar and cultivated for 24 h at 30°C in a CO₂ incubator (Sanyo Electric Co., Japan). The first fermentation products, glucose (cornstarch dextrose 100%), *L. mesenteroides*, and water were mixed in ratio of 1:1:1:10 and cultured for 5~7 days at 30°C. When the cultivation was finished, the secondary fermentation products, RMR-LM, were extracted by hot water extraction. The extracts were filtered through 8 µl filter paper and sterilized for 10 min at 100°C for use in experiments. The RMR-LM, the stock solution of each extract was defined as 100% extract. Extracts were diluted with sterilized water to examine the effects of different concentration of RMR-LM.

Animals and experimental designs. Male 5-week-old ICR mice (Hsd:ICR) were purchased from the Koateck (Gyeonggi-do, Korea) company. The mice were acclimatized for 1 week to allow adaptation, during which time they were fed a high-fat diet (60% kcal as fat; D12492; Research Diet, Inc., New Brunswick, NJ, USA) to induce obesity. Each group of mice (n=10), weighing between 28-34 g, was randomly divided and was allowed free access to the liquid extracts and a high-fat diet to maintain obesity for 6 weeks. Control mice had free access to water instead of liquid extracts. The groups were composed of the 10% RMR-LM extract and 20% RMR-LM extract groups. All mice were housed in a pathogen-free room under a constant 12-h light-dark cycle at 22±2°C temperature and 50±10% humidity. Body weight and food intake were recorded once a week at the appointed time for 6 weeks. All of the animal experimental protocols were approved by the Institutional Animal Care and Use Committee of Konkuk University.

Intraperitoneal glucose tolerance test. For the glucose tolerance test, the mice were fasted for 6 h on the day before the final treatment, and the basal blood glucose levels (0 min) were determined from the tail vein. Glucose was then intraperitoneally injected (1 g/kg body weight), and additional blood glucose levels were measured at 15, 30, 60, and 120 min using a commercial glucometer (Accu-Chek Active; Roche, Mannheim, Germany). The area under the curve (AUC) for the glucose tolerance test was calculated.

Serum biochemical analysis. After 6 h of fasting, the mice were sacrificed, and blood samples drawn from the caudal vena cava for analysis. Serum glucose (GLU), triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-c), low-density lipoprotein cholesterol (LDL-c), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels were analyzed using a Hitachi 7020 Automatic Analyzer (Hitachi, Tokyo, Japan).

Histopathological analysis. The visceral adipose tissue (epididymal and retroperitoneal pad) was dissected, weighed, and fixed in 10% neutral buffered formalin solution. The sections were stained with hematoxylin and eosin (H&E). The slides were examined under a BX51 light microscope (Olympus Corp., Tokyo, Japan). The adipocyte size in the adipose tissue was determined by dividing the number of the adipocytes by the total area counted.

Quantitative real-time PCR analysis. Total RNA was extracted from frozen liver using TRIzol (Ambion Inc., Austin, TX, USA) according to the manufacturer's instructions. The cDNA as a template was synthesized using M-MLV reverse transcriptase (Invitrogen Life Technologies, Merelbeke, Belgium). Gene expression was quantified using Real-Time PCR with a Bio-Rad CFX96 real-time PCR detection system (Bio-Rad, Mississauga, ON, Canada). The DNA primers for the target genes used in this study are listed in Table I. β-actin was used as an internal control for normalization.

Statistical analysis. All obtained data were analyzed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA,

Table I. The primer sequences for the cDNAs of the housekeeping gene and target genes used in real-time PCR.

Gene	Forward primer	Reverse primer
β -actin	5'-AGCCTTCCTTCTTGGGTATGG-3'	5'-CACTTGCGGTGCACGATGGAG-3'
FAS	5'-AGGGGTGACCTGGTCCTCA-3'	5'-GCCATGCCAGAGGGTGGTT-3'
PPAR γ	5'-CAAGAATACCAAAGTGCATCAA-3'	5'-GAGCTGGGTCTTTTCAGAATAAAG-3'
LPL	5'-CGCTCCATTCATCTTCA-3'	5'-CTTGTTGATCTCATAGCCCA-3'
SREBP-1	5'-GGAGCCATGGATTGCACATT-3'	5'-GGCCCCGGAAGTCACTGT-3'
ACC	5'-GGAGATGTACGCTGACCGAGAA-3'	5'-ACCCGACGCATGGTTTTCA-3'

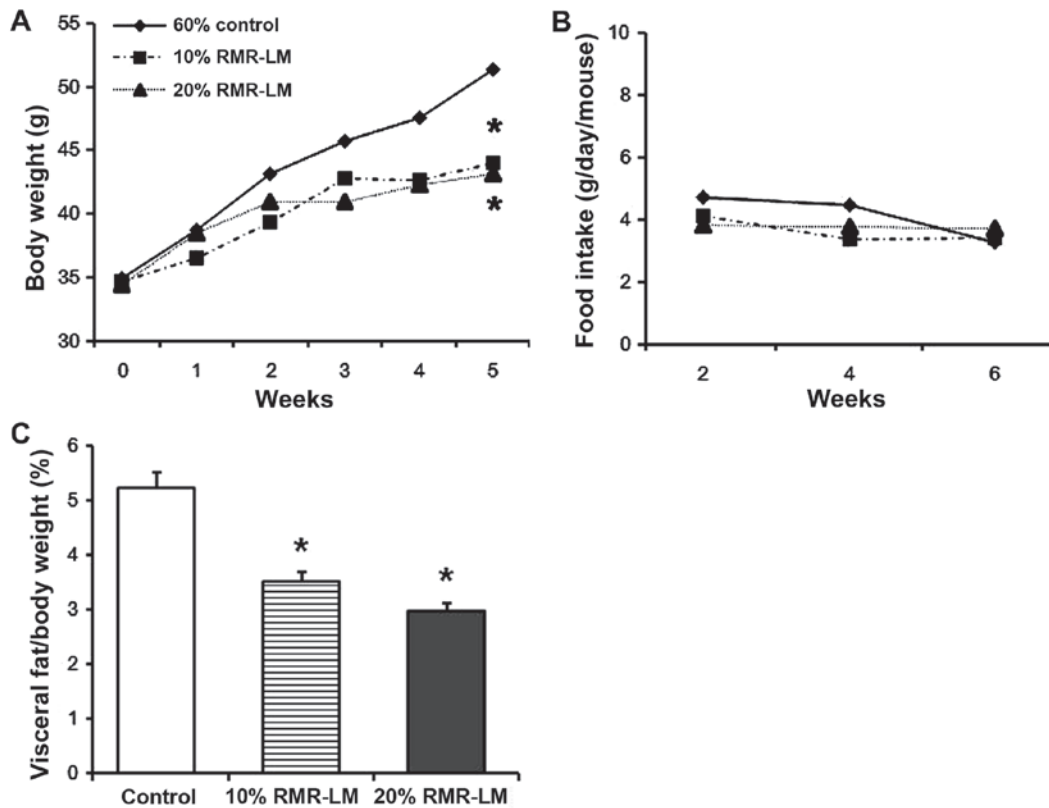


Figure 1. Body weight changes, average food intake levels, and total visceral fat weights. (A) Body weight changes; (B) Average food intake; (C) Total visceral fat/body weight. Average food intake calculated as g/day/mouse. Data are expressed as the mean \pm SD (n=10 per group). *P<0.05 vs. control.

USA) and expressed as the mean \pm standard deviation (*P<0.05). Statistical significance was evaluated by one-way ANOVA with Dunnett's multiple comparison test. P<0.05 was considered to indicate a statistically significant difference.

Results

Body weight changes, food intake levels, and total visceral fat weights. To examine the effects of RMR-LM concentration on anti-obesity, the mice were treated with distilled water (control group), 10% RMR-LM and 20% RMR-LM. The body weight gains of the 10% RMR-LM group and 20% RMR-LM group were significantly (*P<0.05) lower than that of the control group after 6 weeks (Fig. 1A). The food intake levels of all groups were not significantly different, although the body weight gain of the control group was significantly higher than those of the two RMR-LM groups (Fig. 1B). The effects of the extracts on

visceral fat weight are shown in Fig. 1C. The total visceral fat weight decreased with increases in the dose of RMR-LM extract. Both the 10% RMR-LM group and 20% RMR-LM group has a significantly lower total visceral fat weight than the control group.

Adipocyte size in visceral adipose tissue. The number of adipocytes in the 20% RMR-LM group was significantly higher than in the control group (Fig. 2A), and as a result, the adipocyte size in the 20% RMR-LM group was significantly smaller than in the control group (Fig. 2B).

Antidiabetic effects determined by intraperitoneal glucose tolerance tests. To investigate the antidiabetic effects of RMR-LM, glucose tolerance tests were performed after 6 weeks of treatment as seen in Fig. 2. In the control group, the fasting blood glucose level increased following the injection

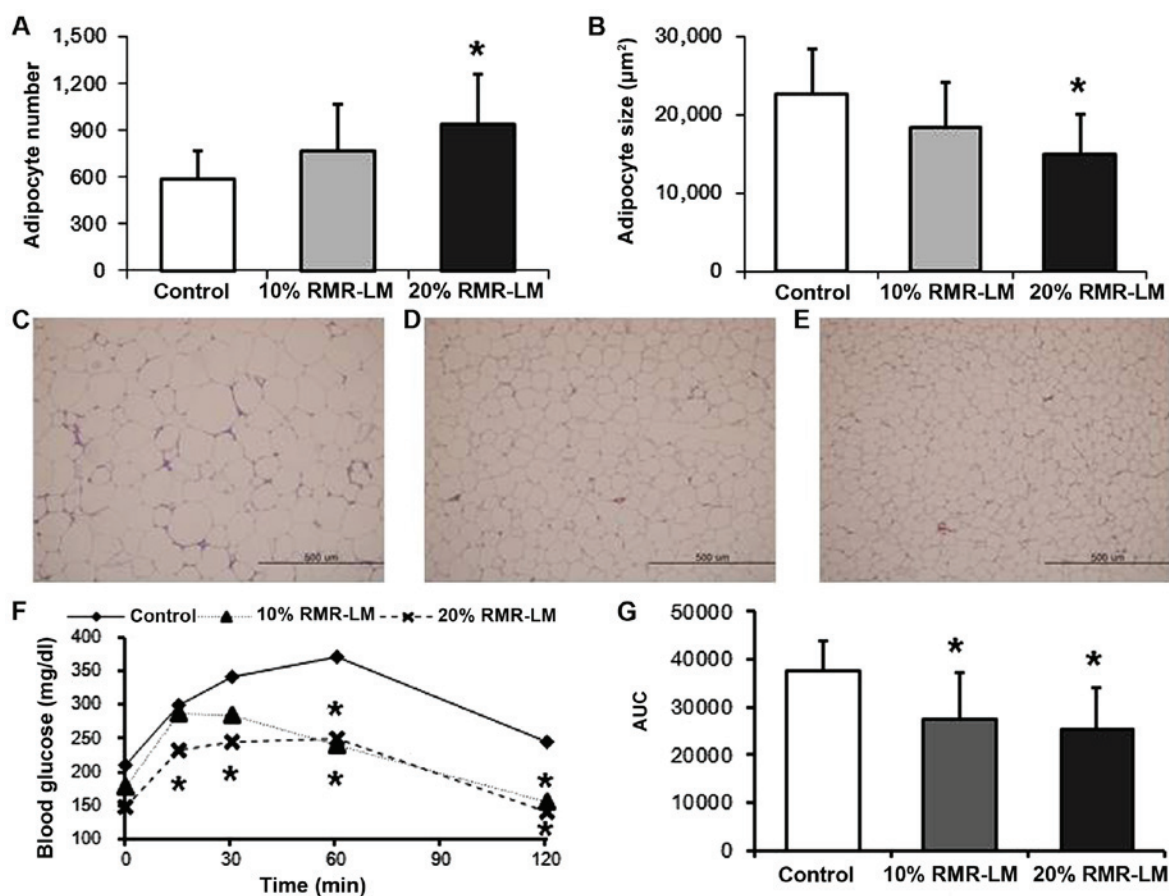


Figure 2. Adipocyte size in visceral adipose tissue intraperitoneal glucose tolerance tests. (A) Adipocyte number; (B) Adipocyte size; (C) Control of visceral adipose tissue, hematoxylin and eosin (H&E) staining; (D) 10% RMR-LM of visceral adipose tissue, H&E staining; (E) 20% RMR-LM of visceral adipose tissue, H&E staining; (F) Intraperitoneal glucose tolerance test; (G) Area under the curve (AUC) of glucose tolerance test. Adipocyte size was calculated by dividing the number of adipocytes by the area counted. H&E staining. Data are shown as the mean \pm SD (n=10 per group). *P<0.05 vs. control.

of glucose. The 20% RMR-LM group showed significantly ($P<0.05$) improved glucose tolerance compared with the control group, indicating that the 20% RMR-LM treatment may improve glucose tolerance in obese mice. The blood glucose concentrations of the 10% RMR-LM group at the 60- and 120-minute intervals were significantly lower than those of the control group. The AUC values for the glucose tolerance test of the 10% RMR-LM group and 20% RMR-LM group were significantly lower than that of the control group.

Serum biochemistry analysis of the lipid. The fasting serum TG and glucose levels were analyzed (Fig. 3A and B). The level of TG was not significantly different between the control group and the RMR-LM extract groups. The glucose level in the 20% RMR-LM group was significantly lower than that in the control group. The glucose level in the 10% RMR-LM group was also lower than that of the control group, but this difference was not significant. The serum AST level of the 10% RMR-LM group was lower than that of the control group, but these differences were not significant. The serum ALT levels of the 10% RMR-LM and 20% RMR-LM groups were lower than that of the control group, but no significant difference was observed (Fig. 3C and D).

Gene expression analysis in the liver. The hepatic mRNA expression levels of genes related to lipid and fatty acid

metabolism, including sSREBP-1, ACC, FAS, LPL, and PPAR γ , were analyzed by real-time PCR (Fig. 4). The mRNA expression levels of SREBP-1 in liver tissue in both the 10% RMR-LM group and 20% RMR-LM group were significantly ($P<0.05$) down-regulated compared with the control group, especially in the 20% RMR-LM group. The gene expression levels of FAS and ACC in the 20% RMR-LM group were significantly decreased compared with the control group. The PPAR γ is a nuclear receptor that regulates adipocyte differentiation and liver lipid storage. The mRNA expression levels of PPAR γ in liver tissue in both the 10% RMR-LM group and 20% RMR-LM group were significantly ($P<0.05$) down-regulated compared with the control group.

Discussion

In this study, the anti-obesity effects of fermented extracts on the development of obesity in ICR mice were examined. We selected successive two-step fermentation to enhance biological activity based on our data (5,7). Robusta were fermented by successive two-step fermentation of *M. ruber* and *L. mesenteroides* (RMR-LM). The present results clearly show that the 20% RMR-LM treatment is able to suppress diet-induced obesity. The effectiveness of the doses of RMR-LM (10, 20%) used in this study was validated by the significant body

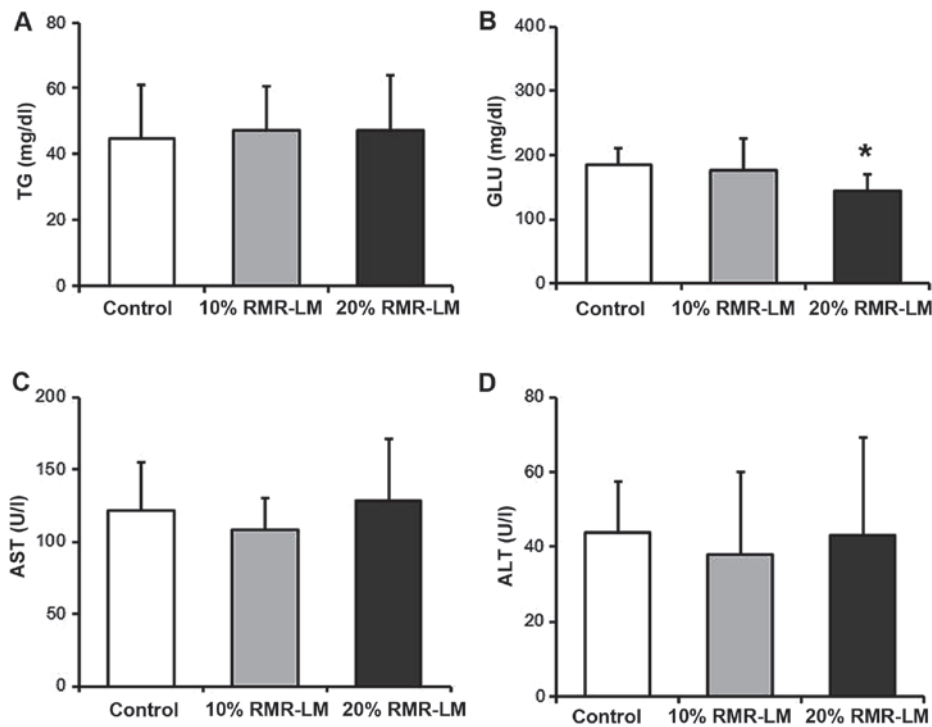


Figure 3. Effects on serum lipid and hepatic profiles by biochemistry analysis. (A) TG level; (B) GLU level; (C) AST level; (D) ALT level. Values are expressed as the mean \pm standard deviation (n=10 per group). *P<0.05 vs. control. TG, triglyceride; GLU, glucose; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

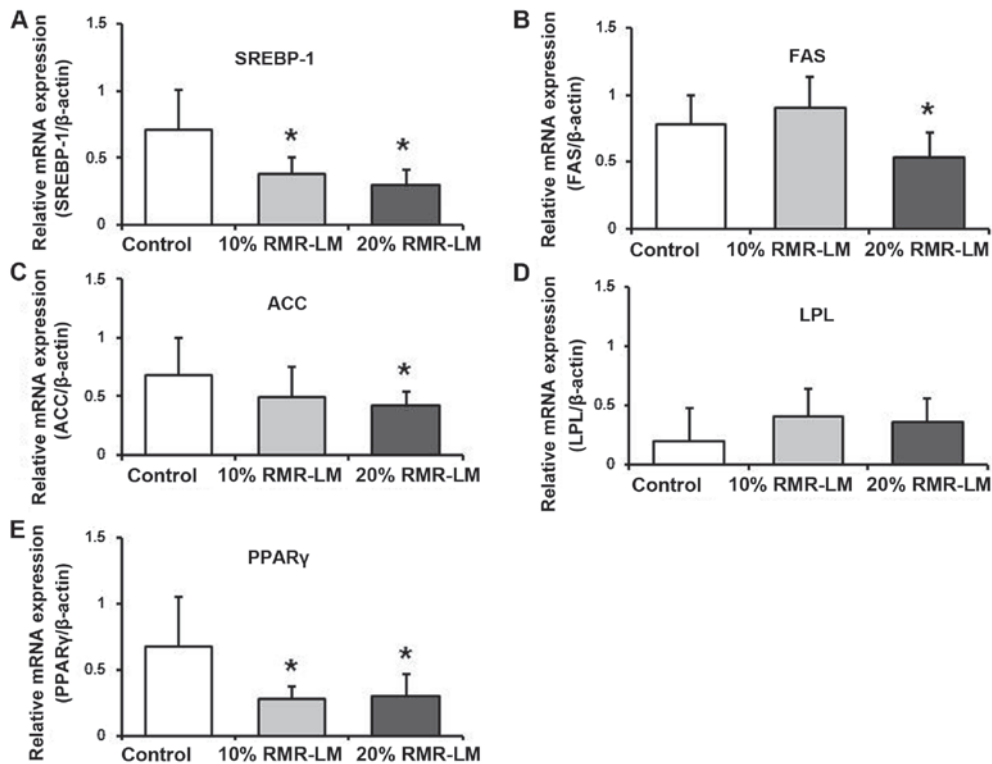


Figure 4. Effects of RMR-LM on relative mRNA expression in the liver. (A) sterol regulation element-binding protein (SREBP)-1; (B) fatty acid synthase (FAS); (C) acetyl-CoA carboxylase (ACC); (D) lipoprotein lipase (LPL); (E) peroxisome proliferator-activated receptor γ (PPAR γ). β -actin was used as an internal control. Data are expressed as the mean \pm standard deviation (n=10 per group). *P<0.05 vs. control.

weight changes, reduction in total visceral fat weights, blood glucose level effects, and intraperitoneal glucose tolerance test results (14).

Obesity is associated with an increased risk of morbidity and mortality. It is also recognized as a major risk factor for metabolic diseases, lipid disorders, type-2 diabetes, and

hypertension. The accumulation of fat in the body is a major characteristic of obesity (15,16). To test whether the body weight reduction was caused by a decrease in adiposity, fat pads were dissected and weighed (17). The total visceral fat weight in the 10% RMR-LM and 20% RMR-LM groups was significantly ($^*P<0.05$) lower than that in the control group. The amount of fat mass is increased when the size of adipocytes are multiplied by proliferation and differentiation (17). The adipocyte size was calculated by dividing the number of adipocytes by the area counted. The adipocyte size in the 20% RMR-LM group was significantly smaller than in the control group. An increased adipocyte number led to a decreased adipocyte size that the 20% RMR-LM treatment had an anti-obesity effect.

While the LDL cholesterol are mainly attached to the arterial vessel wall causing arteriosclerosis, or the higher the plasma concentration as a factor that is the cause of heart disease, the risk is increased, HDL cholesterol is to go to the liver to remove the low molecular cholesterol that causes arteriosclerosis in the blood is known as a good cholesterol (7). Although the 20% RMR-LM group exhibited reductions in body weight, and total visceral fat weight, the serum TG level was not significantly different among the groups. The serum AST level and ALT level in the 10% RMR-LM group or 20% RMR-LM group were lower than those in the control group, indicating that the RMR-LM treatments did not cause hepatic toxicity. And, the serum TC level in the 10% RMR-LM group and 20% RMR-LM group was lower than in the control group, but this difference was not significant (data not shown). These findings provided direct evidence that the RMR-LM extract had beneficial lipolytic effects on high-fat diet-induced obesity.

In our experiments, the significant decreases in body weight in the 10% RMR-LM group and 20% RMR-LM group suggested that the RMR-LM extracts may reduce the visceral fat weight. The adipocyte size in the 20% RMR-LM group was significantly smaller than in the control group, indicating that the 20% RMR-LM treatment up-regulated lipolysis in adipocytes. Significant improvements in glucose tolerance were observed in the 10% RMR-LM and 20% RMR-LM groups compared with the control group. These results suggested that the 10% RMR-LM and 20% RMR-LM treatments had a hypoglycemia effect.

To clarify the mechanism on anti-obesity effect of the RMR-LM extracts, expression levels of genes related to lipid and fatty acid metabolism in the liver were investigated by real-time PCR. PPAR γ is a key nuclear receptor transcription factor in adipogenesis and lipogenesis (18) and plays an important role in liver lipid storage and the differentiation of fat cells (19,20). The findings in the present study indicated that both the 10% RMR-LM group and 20% RMR-LM group had significant ($^*P<0.05$) down-regulation of PPAR γ mRNA expression in the liver. The expression of several lipogenic genes is regulated by SREBP-1 at the transcriptional level (21). Our evidence suggests that the 20% RMR-LM extract may decrease lipogenesis partly by regulating PPAR γ signaling. SREBP-1 is a major transcription factor involved in the activation of lipogenic genes such as FAS and ACC (22,23). As the hepatic SREBP-1 mRNA level was significantly lower in the 10% RMR-LM group and 20% RMR-LM group than in the control group, this lower SREBP-1 level could have contributed to the reduction in the FAS and ACC mRNA

levels in the liver. The liver expression levels of FAS and ACC were significantly ($^*P<0.05$) suppressed in the mice in the 20% RMR-LM group. The 20% RMR-LM extract was shown to be beneficial by lowering the body weight and visceral fat weight and preventing hepatic lipid accumulation. The reduction in the hepatic mRNA level of genes related to lipid anabolism such as SREBP-1 and PPAR γ contributes to the anti-obesity effect of 20% RMR-LM.

In conclusion, robusta was cultivated first with *M. ruber* and then fermented with *L. mesenteroides* (RMR-LM). Significant improvements in glucose tolerance were observed in the 10% RMR-LM and 20% RMR-LM groups compared with the control group. The serum glucose level was significantly lower in the 20% RMR-LM group than in the groups. These results suggest that the 20% extract of RMR-LM may be a promising dietary supplement and food additive for inhibition of obesity and amelioration of diabetes and lipid metabolism imbalances.

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