

Anti-Obesity Effect of a Mixture of Skim Milk, Red Ginseng Extract, and Black Raspberry Extract Fermented with *Lactobacillus acidophilus* on High-Fat Diet-Fed Obese Mice

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Abstract

The aim of this study was to assess the anti-obesity effect of *Lactobacillus acidophilus*-fermented mixture of skim milk, red ginseng extract, and immature fruit of *Rubus coreanus* Miquel (called black raspberry) extract in a mouse model of diet-induced obesity. Male C57BL/6J mice were fed a normal diet (ND), high-fat diet (HFD), high-fat diet supplemented with fermented skim milk (HFD-FSM), or high-fat diet supplemented with the fermented mixture (HFD-FMIX). FSM and FMIX were orally administered (2 g/kg of body weight) on a daily basis. Following a 6-week regimen, the following physical and biochemical measures were assessed: feed intake, body weight, periepididymal fat and perirenal fat mass, size of the epididymal adipocytes, and plasma levels of glucose, insulin, adiponectin, and leptin. No significant differences were observed in all parameters tested in the HFD-fed and HFD-FSM-fed groups, except the plasma leptin level, which was higher in the HFD-FSM-fed group than in the HFD-fed group. In the HFD-FMIX-fed group, feed intake was similar to, but the adiponectin level was higher than that in the HFD-fed group. The HFD-FMIX-fed group showed significantly lower increase in body weight, size of epididymal adipocytes, and plasma levels of glucose and leptin than the HFD-fed group. These results demonstrate that the obesity-related measures can be decreased in obese mice by administration of *L. acidophilus*-fermented mixture of skim milk, red ginseng, and black raspberry fruit extract. These results also suggest that the fermented mixture can possibly be utilized as a functional food material with an anti-obesity effect.

Keywords

Fermentation, *Lactobacillus acidophilus*, Red ginseng, Black raspberry, Anti-obesity

Introduction

Obesity results from energy imbalance caused by excessive intake of high-calorie foods and lack of exercise. It is generally accompanied by metabolic disorders that can cause various diseases such as diabetes, cardiovascular disease, hypertension, hyperlipidemia, and cancers [1]. Thus, several therapeutic strategies, such as behavior modification, exercise, dietary remedies, medications, and surgery, have been developed. Since modification of one's lifestyle helps overcome energy imbalance and subsequent disorders, supplementation with anti-obesity

functional foods has garnered attention for obesity treatment.

Red ginseng is a medicinal herb that has health beneficial effects including anti-obesity effects. Several groups reported that the anti-obesity effect is attributed to ginseng saponins [2-4], which are classified into protopanaxadiol group (ginsenosides: Ra, Rb, Rc, Rd, Rg3, Rh2, and Rs) and protophanxatriol group (ginsenosides: Re, Rf, Rg1, Rg2, and Rh1) depending on their chemical structures. Red ginseng is known to contain large amounts of Rg2, Rg3, Rh1, and Rh2 ginsenosides that are effectively produced during the heating process. Rg3 and Rh2 ginsenosides have anti-obesity effects *in vitro* [3, 4].

Black raspberry (*Rubus coreanus* Miquel; called 'Bokbunja' in Korean) is traditionally used to help restore stamina [5]. Studies have reported that the dried immature fruit is commonly prescribed for various diseases in oriental medicine. In addition, multiple studies have demonstrated the diverse effects of the fruits i.e., the antioxidant effect [6], lipid metabolism enhancing effect [7], and weight control [8].

Lactic acid bacteria (LAB) are representative microorganisms used as probiotics owing to their essential benefits to intestinal health. Since various reports have demonstrated the correlation between intestinal health and obesity development, the anti-obesity effect of LAB or LAB supplementation as a bio-functional material is also extensively studied [9-12].

In this study, a mixture of skim milk powder, ginseng extract, and immature black raspberry extract was fermented by *Lactobacillus acidophilus* isolated from kimchi. The fermented extract was then administered to high-fat diet-induced obese mice. Following a 6-week regimen, various physical and biochemical measures, such as food intake, body weight, periepididymal fat and perirenal fat mass, size of epididymal adipocytes, and plasma levels of glucose, insulin, adiponectin, and leptin were assessed.

Materials and Methods

Samples and preparation

L. acidophilus of LAB and black raspberry (dried immature fruit) were obtained from Imsil Research Institute of Cheese Science (Seongsu-myeon, Imsil-gun, Korea) and Berry and Biofood Research Institute (Buan-myeon, Gochang-gun, Korea), respectively. Nonfat dry milk and red ginseng extract were purchased from Seoul Dairy Cooperative (Seoul, Korea) and Kunbo Co., Ltd. (Jinan-gun, Korea), respectively. Black raspberry (200 g) was extracted in water (6 L) for 9 h at 100 °C, and then the extracts were freeze-dried.

Analysis of free amino acids

The sample (1 g) was extracted in 70% ethanol (50 mL) for 15 min at 80 °C. After duplicating the extraction, the extracts were filtered and ethanol was evaporated with a rotary vacuum evaporator. The resulting water solution was mixed with ether and then passed through a separating funnel to remove the ether layer. The extracts obtained were dried in a rotary evaporator. After the extract powder was solubilized in

sample dilution buffer (10 mL), the amino acid contents were determined using an amino acid analyzer (Sykam S433, Sykam GmbH, Eresing, Germany), according to the manufacturer's instructions.

Experimental animals and feed

Twenty-four C57BL/6J mice (25 g, male) were purchased from Damul-Science (Daejeon, Korea). After 1-week adaptation under a 12-h light/12-h dark cycle (temperature, 20–24 °C; humidity, 45–55%), 8-week old mice were used in this study. The mice were divided into 4 groups (6 mice per group) using a randomized block design. For 6 weeks, each group was fed different diets; (1) normal diet (ND), (2) high fat diet (HFD), (3) HFD with fermented 10% skim milk diet (HFD-FSM, fermented by *L. acidophilus* at 37 °C for 72 h) and (4) HFD with fermented mixture (HFD-FMIX, fermented by *L. acidophilus* at 37 °C for 72 h) (Table 1). The mixture consisted of 10% w/w skim milk and 2% w/w mixture (1% w/w red ginseng extract and 1% w/w black raspberry extract). Before administration to the mice, the skim milk powder and mixture powder were freshly dissolved in drinking water.

Table 1: Composition of experimental diets.

Ingredients	Unit: gm%			
	ND ^{a)}	HFD ^{b)}	HFD-FSM ^{c)}	HFD-FMIX ^{d)}
Casein	200.00	233.07	224.14	224.14
Sucrose	100.00	201.38	195.73	175.73
Dextrose	132.00	116.54	116.54	116.54
Corn Starch	397.50	84.84	0.00	0.00
Cellulose	50.00	58.27	58.27	58.27
Soybean Oil	70.00	29.13	29.13	29.13
Fermented Skim milk	0.00	0.00	100.00	100.00
Red Ginseng Extract	0.00	0.00	0.00	10.00
Black Raspberry Extract	0.00	0.00	0.00	10.00
Lard	0.00	206.85	206.26	206.26
Mineral (Ca.P free)	35.00	52.44	52.44	52.44
Vitamin Mix	10.00	11.65	11.65	11.65
L-Cystine	3.00	3.50	3.50	3.50
Choline Bitartrate	2.50	2.33	2.33	2.33

^{a)}ND: normal diet group (AIN-93G)

^{b)}HFD: high fat-diet 45 Cal% group

^{c)}HFD-FSM: HFD with fermented freeze-dried 10% skim milk powder (FSM) diet

^{d)}HFD-FMIX: HFD with fermented freeze-dried mixture powder (10% skim milk, 1% red ginseng extract, and 1% black raspberry extract: FMIX) diet group

The HFD-FSM and HFD-FMIX groups were fed an HFD, and orally administered FSM and FMIX in water at 2 g/kg body weight (BW) on a daily basis, respectively. The same volume of drinking water was orally administered to the mice in the ND and HFD groups.

Amount of dietary intake and feed efficiency and body weight

Dietary intake of mice was regularly measured once a

week for 6 weeks. Feed efficiency was calculated by dividing the weight gain by the dietary intake. The body weight was daily monitored at 10:00 am for a week from the first experiment day. The change in body weight was calculated by the following equation:

$$\text{Feed efficiency ratio} = \text{Weight gain (g)} / \text{Dietary intake (g)}$$

Fat gain and change in the fat cell size

At the end of the experiment, the periepididymal fat and perirenal fat were obtained from each group and weighed to check the amount of fat gain. Some epididymal adipocytes were fixed with Bouin's solution and soaked in 20% sucrose overnight, and then 20- μm frozen sections were obtained for Oil Red O staining using the free floating method to observe the changes in the adipocyte size.

Change in the blood glucose and levels of obesity-related hormones in the serum

After the end of the experiment, mice from each group were sacrificed and their blood was collected to measure the blood glucose level using the blood analysis kit (Asan Pharm, Seoul, Republic of Korea), and the anti-obesity effect was observed physiologically.

ELISA analysis was conducted to observe the physiological anti-obesity effect of adiponectin, which includes adipocyte differentiation-related hormone in the serum (using mouse/rat adiponectin ELISA kit, Shibayagi, Japan), leptin (using mouse leptin ELISA kit, KOMA Biotech, Korea), and insulin (using mouse insulin ELISA kit, Shibayagi, Japan). The concentration of adiponectin in the blood was measured by Enavall and Perlmann method [13] using mouse/rat adiponectin ELISA kit (Shibayagi Co., Ltd, Japan). All the analyses were conducted following the manufacturer's instructions. The absorbance was measured at 450 nm using ELISA reader.

Statistical analysis

All results were expressed as the average and standard deviation using SPSS 12.0 for Windows (SPSS Inc., USA), and one-way analysis of variance (ANOVA) was applied. In order to verify the differences between each group, non-paired *t*-test was performed. The significance was assessed at $p < 0.05$.

Results

Change in body weight, dietary intake, and feed efficiency

After 6 weeks, the body weights of the mice were measured and compared among the groups (Figure 1). The average body weight per mouse in the ND and HFD groups was 5.42 ± 1.43 g (22.6% increase compared to that before the experiment) and 8.94 ± 3.01 g (37.9% increase compared to that before the experiment), respectively. The body weight of HFD-induced obese mice significantly increased by 64.9% compared to that reported for the ND group mice (#, $p < 0.01$). The body weights in the HFD-FSM and HFD-FMIX groups were 8.09 ± 1.88 g (33.4% increase compared to that before the experiment) and 5.42 ± 1.02 g (22.5% increase compared to that before the experiment), respectively. The weight of mice in the HFD-FMIX group significantly decreased by 39.4% compared to

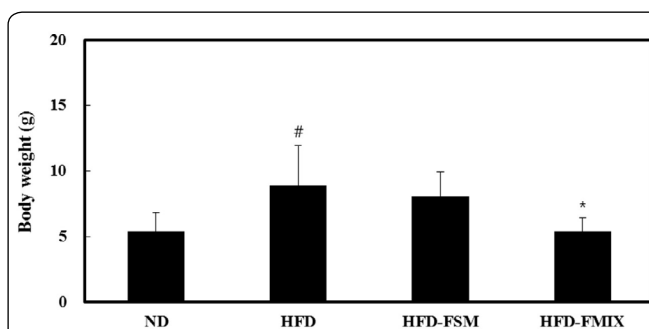


Figure 1: Total body weight of mice fed experimental diets for 6 weeks. ND, normal diet group; HFD, high-fat diet group; HFD-FSM, high-fat diet containing fermented skim milk (*L. acidophilus* and 10% skim milk); HFD-FMIX, high-fat diet containing the fermented mixture (*L. acidophilus*, 10% skim milk, 2% red ginseng concentrate, and *Rubus coreanus* Miquel extract). Values are significantly different from the normal group (ND) (#) and control group (HFD) (*); #, $p < 0.01$; *, $p < 0.01$.

Table 2: The effect of HFD-FSM and HFD-FMIX on daily body weight and feed intake, and feed efficiency ratio (FER) in high fat diet-fed mice.

Groups	Increase in the daily body weight (g)	Daily feed intake (g)	Feed efficiency ratio
ND	$0.13 \pm 0.04^{1)}$	3.06 ± 0.16	0.042
HFD	$0.21 \pm 0.04^{2)}$	$2.72 \pm 0.14^{\#}$	0.077 [#]
HFD-FSM	0.19 ± 0.03	2.77 ± 0.10	0.068
HFD-FMIX	$0.13 \pm 0.06^{3)}$	2.74 ± 0.19	0.047 [*]

¹⁾Values represent the mean \pm s.d. (n = 6/group) of mice fed experimental diets for 6 weeks

²⁾Values are significantly different from the normal group (ND) (#, $p < 0.01$)

³⁾Values are significantly different from the control group (HFD) (*, $p < 0.05$)

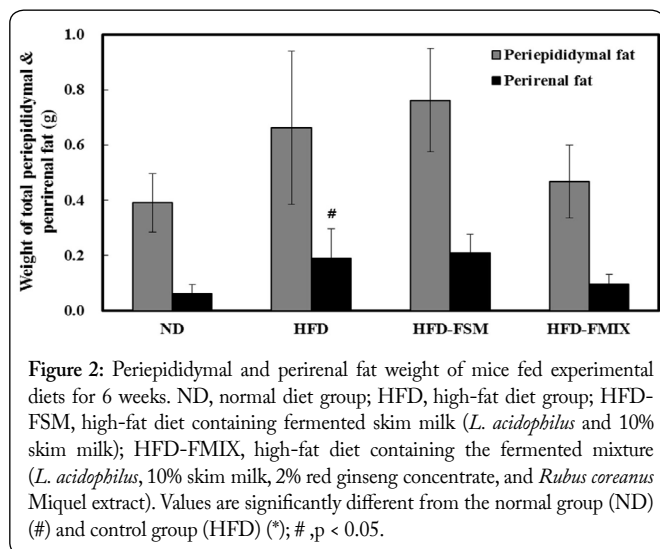
that reported for the HFD group mice (*, $p < 0.01$).

Dietary intake and feed efficiency are shown in Table 2. The daily feed intake in the ND and HFD groups was 3.06 ± 0.16 g and 2.72 ± 0.14 g per mouse, respectively, showing significant reduction in the HFD group compared to the ND group (#, $p < 0.01$). The feed intake in the HFD-FSM group and HFD-FMIX group was 2.77 ± 0.10 g and 2.74 ± 0.19 g, respectively.

The feed efficiencies of the ND and HFD groups were 0.042 and 0.077, respectively. The feed efficiency of the HFD group was significantly higher than that of the ND group (#, $p < 0.01$). The feed efficiencies of the HFD-FSM and HFD-FMIX groups were 0.068 and 0.047, respectively. The feed efficiency of the HFD-FMIX group was significantly lower than that of the HFD group (*, $p < 0.01$).

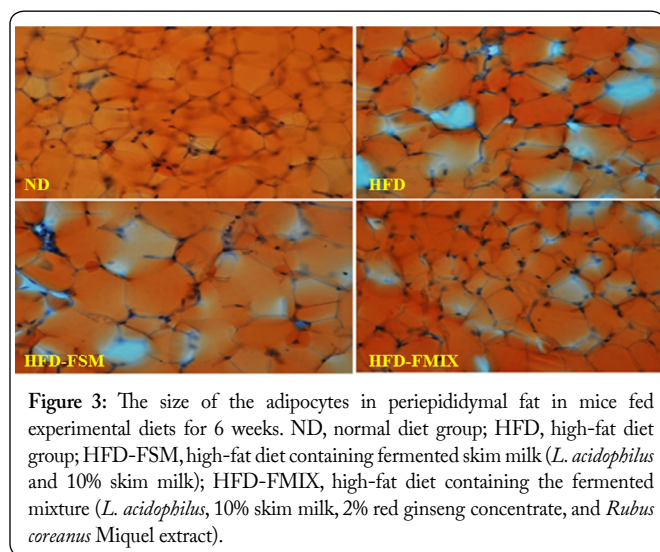
Fat gain and change in the fat cell size

The weights of periepididymal fat and perirenal fat after the 6-week experimental period are shown in Figure 2. Periepididymal fat weight was 0.39 ± 0.105 g in the ND group and 0.66 ± 0.278 g in the HFD group, showing 69.2% weight gain in the HFD group relative to the ND group. The weight of periepididymal fat in the HFD-FMIX group was 29.4% lower than that in the HFD group. The weights of perirenal fat in the ND group and HFD group were 0.06 ± 0.032 g



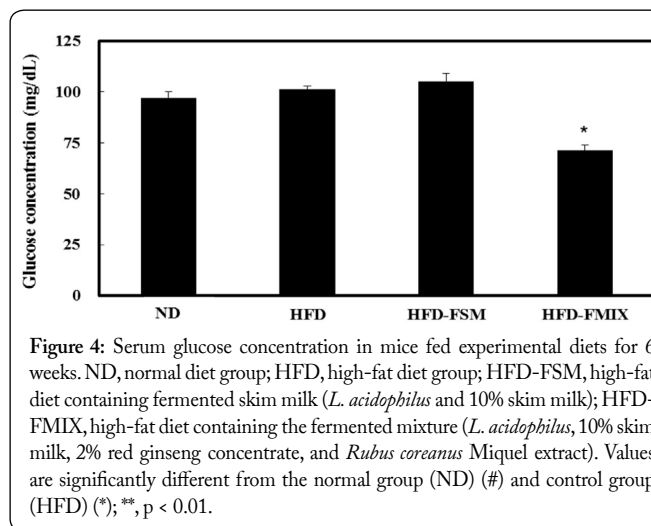
and 0.19 ± 0.108 g, respectively. The perirenal fat weight of the HFD group significantly increased by 67% compared to that observed for the ND group (#, $p < 0.05$). The perirenal fat weight of the HFD-FMIX group (0.09 ± 0.036 g) decreased by 50% compared to that reported for the HFD group; however, it was not significantly different. In conclusion, the total weight gain of periepididymal fat and perirenal fat in the ND group and HFD group was 0.45 ± 0.138 g and 0.85 ± 0.385 g per mouse, respectively. The total fat weight of the HFD group increased by 88.0% compared to that reported for the ND group. In the HFD-FSM group and HFD-FMIX group, the total weights were 0.97 ± 0.249 g and 0.56 ± 0.167 g per mouse, respectively. The total fat weight of the HFD-MIX group decreased by 34.1% compared to that reported for the HFD group.

The change in the adipocyte size is shown in Figure 3. The adipocyte size in the HFD group increased compared to that in the ND group. In the HFD-FMIX group, the size of the adipocytes decreased compared to the HFD group or HFD-FSM group.



Change in blood glucose level

At the end of the 6-week study, the serum glucose



concentration was measured and is shown in Figure 4. The concentration of serum glucose was 97.2 ± 2.8 mg/dL in the ND group and 101.3 ± 1.7 mg/dL in the HFD group. There was no significant difference in the serum glucose level between the ND and HFD groups. The serum glucose level in the HFD-FSM group and HFD-FMIX group was 105.3 ± 3.8 mg/dL and 71.3 ± 2.7 mg/dL, respectively, whereas that in the HFD-FMIX group was significantly lower than the levels in the HFD group (*, $p < 0.01$).

Change in serum levels of adipocyte-related proteins and hormones

After the 6-week experiment, the serum concentration of leptin, adiponectin, and insulin was evaluated and is shown in Figure 5. The concentrations of leptin in the ND, HFD, HFD-FSM, and HFD-FMIX groups were 480.0 ± 8.5 pg/mL, 607.5 ± 12.5 pg/mL, 896.6 ± 29.4 pg/mL, and 243.0 ± 2.5 pg/mL, respectively. The leptin levels in the HFD group significantly increased compared to that in the ND group (##, $p < 0.01$). In contrast, the leptin levels in the HFD-FMIX group significantly decreased by 60.0% compared to that in HFD group (***, $p < 0.001$).

The serum adiponectin concentrations in the ND group and HFD groups were 27.47 ± 0.56 μ g/mL and 10.43 ± 0.53 μ g/mL, respectively. The adiponectin concentration in the HFD group was significantly lower than that in the ND group (###, $p < 0.001$). The adiponectin concentrations in the HFD-FSM group and HFD-FMIX group were 9.23 ± 1.36 μ g/mL and 14.18 ± 1.43 μ g/mL, respectively. The HFD-FMIX group exhibited 36.0% increase in adiponectin concentration compared to the HFD group (**, $p < 0.01$). The serum insulin concentrations in the ND, HFD, HFD-FSM, and HFD-FMIX groups were 10.5 ± 0.1 μ IU/mL, 52.4 ± 0.9 μ IU/mL, 53.4 ± 1.3 μ IU/mL, and 48.2 ± 0.2 μ IU/mL, respectively. The insulin level in the HFD group significantly increased to 399.0% compared to that in the ND group (#, $p < 0.05$). The HFD-FMIX group exhibited significant reduction (8.0%) in insulin level compared to the HFD group (*, $p < 0.05$).

Free amino acid content in the FSM and FMIX

Amino acids in FSM and FMIX are the major fermentation products with anti-obesity effects in this study.

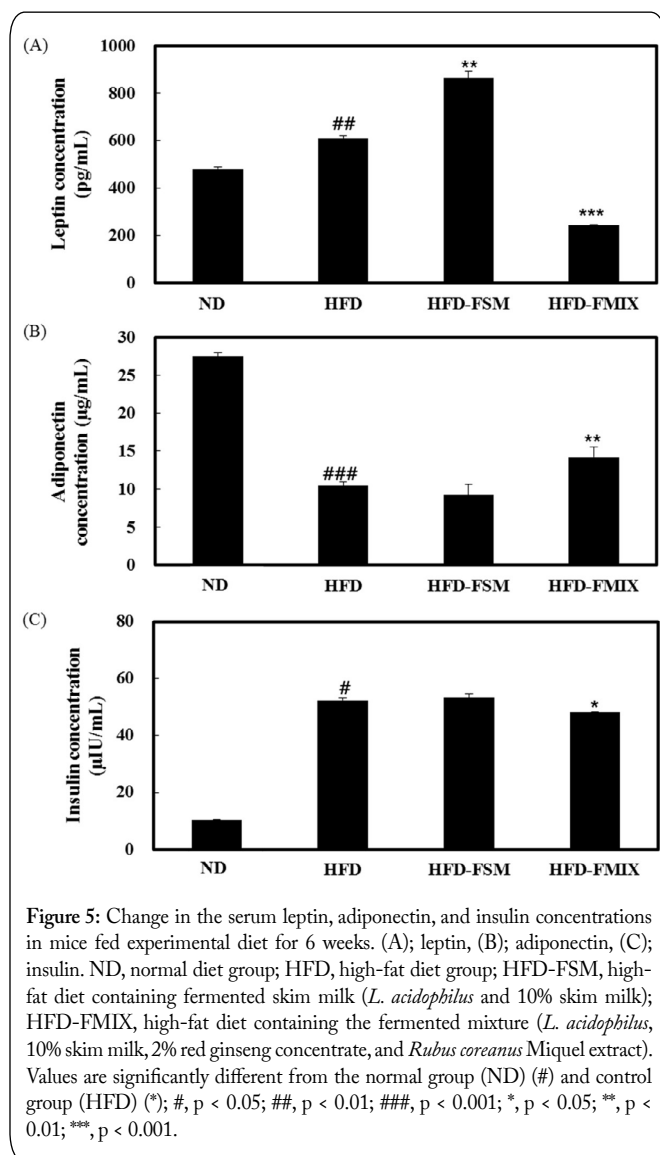


Figure 5: Change in the serum leptin, adiponectin, and insulin concentrations in mice fed experimental diet for 6 weeks. (A); leptin, (B); adiponectin, (C); insulin. ND, normal diet group; HFD, high-fat diet group; HFD-FSM, high-fat diet containing fermented skim milk (*L. acidophilus* and 10% skim milk); HFD-FMIX, high-fat diet containing the fermented mixture (*L. acidophilus*, 10% skim milk, 2% red ginseng concentrate, and *Rubus coreanus* Miquel extract). Values are significantly different from the normal group (ND) (#) and control group (HFD) (*); #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

The content of total free amino acids in FSM and FMIX was 4844.18 mg/L and 6341.28 mg/L, respectively (Table 3). Among the eighteen different free amino acids, the content of seven amino acids (aspartic acid, threonine, serine, asparagine, GABA, ornithine, and arginine) in FMIX was significantly different from that in FSM. Out of the seven free amino acids in FMIX, arginine was detected at a higher level, 836.28 mg/L.

Discussion

In this study, the anti-obesity effect of the mixture of skim milk, red ginseng, and raspberry extract fermented by Kimchi-derived *L. acidophilus* was evaluated *in vivo*. Adult mice were administered the fermented mixture for 6 weeks and various obesity-related physical and biochemical measures were tested. The results demonstrated that (1) supplementation with the fermented mixture reduced body weight, total fat weight, and adipocyte size in obesity-induced mice, and (2) administration of the fermented mixture to obese mice increased the level of plasma adiponectin and decreased the levels of plasma leptin and insulin.

Leptin is positively correlated with the adipocyte size [14,

15], but plasma adiponectin is negatively correlated with leptin, fasting insulin concentration, body mass index (BMI), and fat mass [16-18]. Adiponectin, an adipocytokine produced by adipocytes, activates energy metabolism by increasing insulin sensitivity and promotes fat oxidation of skeletal muscle [19-21]. Thus, the elevated plasma adiponectin level in the HFD-FMIX group may be correlated with reduced body weight and fat mass.

Table 3: Free amino acid contents of the FSM and FMIX.

Amino acid	SM-MIX ¹⁾	FSM ²⁾	FMIX ³⁾
Aspartic Acid	240.10 ± 4.80 _{b3)}	ND ⁴⁾	460.54 ± 21.31 ^a
Threonine	48.23 ± 6.13 ^b	169.79 ± 9.03 ^c	219.06 ± 43.67 ^a
Serine	119.94 ± 0.44 ^b	65.85 ± 3.85 ^c	167.74 ± 34.62 ^a
Asparagine	1261.64 ± 45.64 ^a	ND	1143.82 ± 18.18 ^b
Glutamic Acid	236.61 ± 21.88 ^c	1264.84 ± 51.52 ^a	942.88 ± 30.71 ^b
Glycine	42.48 ± 3.13 ^b	131.29 ± 4.81 ^a	145.99 ± 27.20 ^a
Alanine	119.62 ± 7.53 ^c	669.39 ± 26.97 ^a	476.19 ± 87.46 ^b
Valine	73.08 ± 23.32 ^c	378.14 ± 13.49 ^a	294.63 ± 74.60 ^b
Methionine	ND	54.61 ± 6.73	ND
Isoleucine	37.78 ± 2.52 ^c	164.51 ± 5.66 ^a	132.20 ± 29.56 ^b
Leucine	64.45 ± 3.81 ^c	750.88 ± 25.91 ^a	587.34 ± 45.27 ^b
Tyrosine	48.13 ± 1.18 ^b	125.33 ± 6.47 ^a	107.95 ± 25.49 ^a
Phenylalanine	33.83 ± 2.28 ^b	283.41 ± 11.07 ^a	261.30 ± 51.65 ^a
GABA	170.90 ± 4.47 ^b	ND	309.72 ± 19.99 ^a
Histidine	ND	145.81 ± 4.96 ^a	67.14 ± 12.25 ^b
Ornithine	8.55 ± 1.16 ^b	8.03 ± 0.89 ^b	20.44 ± 4.72 ^a
Lysine	29.35 ± 1.00 ^b	317.97 ± 11.04 ^a	168.06 ± 31.99 ^b
Arginine	329.90 ± 6.97 ^b	314.33 ± 11.99 ^b	836.28 ± 24.04 ^a
Total	2864.59	4844.18	6341.28

¹⁾SM-MIX: 10% skim milk media with 1% Bokbunja and 1% red ginseng extract before fermentation

²⁾FSM: 10% skim milk media fermented by *L. acidophilus*

³⁾FMIX: 10% skim milk media with 1% Bokbunja and 1% red ginseng extract fermented by *L. acidophilus*

³⁾Data are presented as a mean ± standard deviation. Means with the same alphabet in each column are not significantly different at $p < 0.05$ using one-way ANOVA

⁴⁾ND = Not detected.

Insulin regulates blood glucose concentrations [22, 23]. Consumption of carbohydrates causes an immediate increase in blood glucose levels, followed by the release of insulin from pancreatic β cells. Insulin binds cell surface receptors in the liver, skeletal muscle, and adipose tissues and reduces the blood glucose levels. In addition, insulin can influence plasma leptin concentration; these two hormones act together in the brain to affect the homeostatic systems [24]. In this study, the HFD-FMIX group showed reduced plasma insulin levels compared to the HFD group. It is still unclear whether supplementation with the fermented mixture had a direct effect in decreasing

insulin.

However, multiple studies reported that treatment with red ginseng extract or its functional constituents, saponins, led to a reduction in the mRNA levels of angiogenic factors (e.g., VEGF-A and FGF-2) and matrix metalloproteinases (MMPs) (e.g., MMP-2 and MMP-9), and the expression of hypothalamic neuropeptide Y (NPY) *in vivo* [2-4, 25]. In addition, immature black raspberry contains large amounts of ellagic acid, which can regulate the protein levels of Nrf2, NF- κ B and CPT1 in an HFD-induced rat model of metabolic syndrome [26], and has anti-obesity effects [27]. Therefore, it is speculated that the anti-obesity effect in the HFD-FMIX group might be correlated with the presence of various biologically active saponins in the red ginseng extract and phenolic compounds in the black raspberry extract.

L. acidophilus is associated with cholesterol absorbance and regulation of intestinal microflora [28, 29]. However, in our experiments, *L. acidophilus* did not appear to exert an anti-obesity effect. No significant differences were observed in any obesity-related measurements between the HFD-FSM group and the HFD group.

In terms of free amino acid contents, significant differences were observed between the FSM and FMIX. Arginine exerts an anti-obesity effect, reduces fat content, and inhibits the growth of white adipose tissue [30]. The anti-obesity effect of HFD-FMIX may be attributed to amino acids such as arginine. Therefore, the anti-obesity effect of HFD-FMIX is likely to be attributed to red ginseng saponins, phenolic acids in immature black raspberry, and high content of free amino acids in the FMIX fermented by *L. acidophilus*.

In conclusion, supplementation with a fermented mixture of skim milk, red ginseng, and immature black raspberry extract had an anti-obesity effect in a mouse model of obesity. This suggests that fermented materials combined with functional edible plant and probiotics could be applied for the development of anti-obesity functional foods.

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