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Effects of exercise and microbiota transplant on the memory of obesity-induced mice

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This study attempted to investigate the association between changes in the intestinal environment and the brain using a model that received aerobic exercise and microbiome transplantation. All mice were fed a diet containing 60% fat. For the obesity with nonexercise microbiome transplantation group, feces from donors that did not undergo exercise were administered. For the obesity with exercise microbiome transplantation group, feces from donors who underwent exercise were administered. Treadmill exercise started 16 weeks after the intake of the high fat feeding and continued for 24 weeks. The short-term memory and spatial learning memory were determined by step-down avoidance test and Morris water maze task, immunohistochemistry for glial fibrillary acidic protein, western blot analysis for brain-derived neurotrophic factor and tropomyosin receptor kinase B were performed in the hippocampus. Exercise was the most effective way to reduce obesity, improve memory function, suppress inflammation, and increase brain-derived neurotrophic factor expression. Intestinal microbiota transplantation was the second most effective after exercise. However, there was no significant difference in the fecal microbiota transplant group according to whether or not exercise was performed.

Keywords: Obesity, Microbiome, Microbiota transplant, Exercise, Memory

INTRODUCTION

Obesity is an excessive accumulation of body fat due to excessive caloric intake, lack of balanced energy consumption, hormonal abnormalities, psychological and environmental factors. Obesity increases the production of cytokines such as interleukin-1, interleukin-6 and tumor necrosis factor-α that are involved in inflammation as well as metabolic disorders and various diseases (Weisberg et al., 2003).

There are about 70 trillion microbiomes in the human body that weigh between 1 and 2 kg (Stilling et al., 2014). The microbiome is also primarily influenced by genetic factors, but acquired factors such as dietary habits, antibiotic abuse and lifestyle are more influential (Stilling et al., 2014). The term microbiome combines the terms microbe (microorganism) and biome (a biogeographic unit made up of a biological community) to contain the genetic information of microorganisms in the human body. The first study of a potential link between the microbiome and obesity was by Turnbaugh et al. (2006). Microbiome studies have investigated the role of the microbiome of healthy and young individuals on aging (Smith et al., 2017), obesity (Goodrich et al., 2014), and brain cognitive function (Mayer et al., 2015).

Lactobacillus is a microorganism that suppresses intestinal pathogens, reduces cholesterol, and enhances the immune system of the human body. *Lactobacillus acidophilus*, a type of *Lactobacillus* bacteria, plays a role in improving insulin resistance (Anderson et al., 2012). *Lactobacillus gasseri* acts by reducing adiponectin and subcutaneous fat (Kadooka et al., 2010). *Lactobacillus plantarum* is known to reduce the size and weight of adipocytes (Takemura et al., 2010). *Lactobacillus bacteria* are known to influence obesity

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through a microbiome that alters obesity induction and control in the presence or absence of Christensenellaceae bacteria (Goodrich et al., 2014). In contrast, there are certain microbiota that cause obesity. The best known are Firmicutes and Bactroidetes. The former activates enzymes that store fat while consuming caloric energy, while the latter leads to inefficient consumption of caloric energy (Hjorth et al., 2018). The association between obesity and the microbiome was reported by Rajilić-Stojanović et al. (2015). The microbiome has been shown to increase ghrelin and insulin secretion from the pancreas as short fatty acid chains act on the hypothalamus through the brain blood barrier. This means that obesity control not only involves the gut, but also requires an interaction between the gut and the brain.

Aerobic exercise is recommended for fat loss (Cox et al., 2004). In particular, the most effective exercise recommended for obesity reduction is low-intensity aerobic exercise (Donnelly et al., 2004). Cook et al. (2016) reported that physical activity induces changes in the gut environment to improve gut immune function. Physical activity has been shown to alter the composition and function of the gut microbiota (Allen et al., 2018).

Changes in the intestinal environment and changes in the microbiome have been suggested as causes of obesity. Therefore, this study attempted to investigate the association between changes in the intestinal environment and the brain using a model that received aerobic exercise and microbiota transplantation.

MATERIALS AND METHODS

Experiment animals

This experimental protocol was approved by the Kyung Hee University Institutional Animal Care and Use Committee (approval number KHUASP [SE]-20-17). The male BL6 mice (8-month-old) were housed under controlled temperature (24°C ± 2°C) and lighting conditions. For 8 weeks, all mice were fed a diet containing 60% fat. The mice were randomly divided into 4 groups (n = 9 per group): obesity group, obesity with exercise group, obesity with nonexercise microbiome transplantation group, and obesity with exercise microbiome transplantation group. For obesity with nonexercise microbiome transplantation group, the feces of mice that did not exercise were administered. For obesity with exercise microbiome transplantation group, feces of mice that did exercise were administered.

Treadmill exercise protocol

Treadmill exercise started 16 weeks after the intake of the high fat feeding and continued for 24 weeks, as previously described method (Park et al., 2019). The exercise groups started exercising on a treadmill 5 min of warm-up at a 0° inclination at 3 m/min, 40 min of the main exercise at 10 m/min, and 5 min of cool down at 3 m/min for the first 2 weeks. Following this period, animals performed 40 minutes of the main exercise at 10 m/min during weeks 1–2, 40 min of the main exercise at 13 m/min during weeks 3–4, 50 min of the main exercise at 13 m/min during weeks 5–8. The treadmill exercise was performed once a day, 6 days per week for 8 consecutive weeks. The exercise of the exercise microbiome transplantation group was performed in the same way as the exercise group.

Fecal microbiota transplantation

For fecal microbiota transplantation, the method of Jang et al. (2020) was used. For the obesity with nonexercise microbiome transplantation group, feces from donors that did not undergo exercise were administered. For the obesity with exercise microbiome transplantation group, feces from donors who underwent exercise were administered. After anesthetizing the fecal donor mice, 100 mg of fresh feces were received from the fecal donor mice and resuspended in 5-mL physiological saline. After centrifugation of the resuspension for 5 min, 100 μL of feces was administered to recipient mice by oral gavage. Donor feces were administered to the microbiome transplantation groups every 3 days for 8 consecutive weeks.

Glucose tolerance test

Oral glucose tolerance test (OGTT) was performed as previously described (Park et al., 2019). We administered intraperitoneal injection of glucose (1.5 g/kg) after a 12 hr fast, and blood glucose was measured at 0, 15, 30, 45, 60, and 120 min after glucose injection.

Step-down avoidance test

The short-term memory was determined using the step-down avoidance test, according to the previously study (Park et al., 2021). On the 8 weeks after starting exercise, all animals were trained. In the training session, all animals were placed on a platform (7 × 25 cm in width, 2.5 cm in height). The platform faced a 42 × 25 cm grid of parallel, 0.1 cm caliber stainless steel bars spaced 1 cm apart. All animals received electrical foot shock (0.2 mA) for 2 sec immediately upon stepping down. One day after training session, the latency (sec) was determined. Latency was the time interval of all animals stepping down from platform and placing all four

paws on the grid. Over 300 sec in latency was counted as 300 sec.

Morris water maze task

Spatial learning memory was assessed using the Morris water maze task with a previously described method (Park et al., 2019). The Morris water maze task was performed 24 hr after completion of the step-down avoidance test as previously described. This Morris water maze task requires mice to train the spatial positioning of water (30°C±1°C) filled with a hidden platform circular pool (50 cm high, 180 cm diameter). A hidden platform (40 cm high and 15 cm diameter) was placed 2 cm below the water level in the center of the northern quadrant and was disguised as transparent against a black background. The learning test consisted of three trials per day for 5 consecutive days. Probe trials (60 sec) with the platform removed were performed for 24 hr after the end of the 6-day training. The percentage of time spent in the target quadrant was considered an indicator of spatial learning memory performance.

Tissue preparation

The mice were sacrificed immediately after determination of behavior test. To prepare the brain slices, the mice were fully anesthetized Zoletil 50 (10 mg/kg, intraperitoneally; Vibac Laboratories, Carros, France), after which the mice were perfused with 50 mM phosphate-buffered saline (PBS) and then fixed with 4% paraformaldehyde in 100 mM phosphate buffer (pH, 7.4) through heart. Freezing microtome (Leica, Nussloch, Germany) was used to make 40-μm coronal sections.

Western blotting for brain-derived neurotrophic factor and tropomyosin receptor kinase B

As previously described method (Park et al., 2019), the brain hippocampus was homogenized on ice and lysed in a lysis buffer. Protein content was measured using a Bio-Rad colorimetric protein assay kit (Bio-Rad, Hercules, CA, USA). Protein of 30 μg was separated on sodium dodecyl sulfate-polyacrylamide gels and transferred onto a nitrocellulose membrane, which was incubated with mouse β-actin antibody (1:3,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit brain-derived neurotrophic factor (BDNF) antibody (1:1,000; Santa Cruz Biotechnology), rabbit tropomyosin receptor kinase B (TrkB) antibody (1:1,000; Cell Signaling Technology, Danvers, MS, USA). Horseradish peroxidase-conjugated anti-mouse for β-actin and anti-rabbit for BDNF and TrkB antibodies were used.

Immunohistochemistry for glial fibrillary acidic protein

Immunohistochemistry for glial fibrillary acidic protein (GFAP) in the dentate gyrus was performed previously mentioned method (Park et al., 2021). The sections were incubated in PBS for 10 min, washed 3 times in PBS, and then incubated in 1% H_2O_2 for 20 min. The sections were incubated overnight with rabbit anti-GFAP antibody (1:500; Cell Signaling Technology). They were then incubated with the appropriate biotinylated secondary antibody (1:200; Vector Laboratories, Burlingame, CA, USA) for another 1 hr 30 min, washed, and incubated in ABC complex kit (1:100; Vector Laboratories). Labeling was visualized using 0.03% DAB, and the sections were mounted onto gelatin-coated slides. The slides were air-dried overnight at room temperature, and the coverslips were mounted using Permount (Thermo Fisher Scientific, Waltham, MA, USA).

Collection of fecal samples, sequencing and metagenomic analysis

As previously described method (Jang et al., 2020; Kim et al., 2021), the fecal samples collected from whose gastrointestinal cultures were submitted to the laboratory in accordance with the guidelines in sterile test tubes and stored at -80°C. Total DNA from the feces was extracted from 200 mg of feces per sample using QIAamp Fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Sequencing Library protocols to amplify the V3 and V4 region. The input gDNA 2ng was polymerase chain reaction (PCR) amplified. The cycle condition for 1st PCR was 3 min at 95°C for heat activation, and 25 cycles of 30 sec at 95°C, 30 sec at 55°C and 30 sec at 72°C, followed by a 5-min final extension at 72°C.

The 1st PCR product was purified with AMPure beads (Agencourt Bioscience, Beverly, MA, USA). Following purification, the 2 μL of 1st PCR product was amplified for final library construction. The cycle condition for 2nd PCR was same as the 1st PCR condition except for 10 cycles. The final purified product is then quantified using qPCR according to the qPCR Quantification Protocol Guide (KAPA Library Quantification kits for Illumina Sequencing platforms) and qualified using the TapeStation D1000 ScreenTape (Agilent Technologies, Waldbronn, Germany). And we sequenced using the MiSeq platform (Illumina, San Diego, CA, USA) The barcoded16S rRNA gene amplicons were sequenced using the Illumina MiSeq platform at Macrogen Inc. (Seoul, Korea). For the whole metagenome shotgun sequencing, DNA representing the fecal microbiome communities extracted from the feces were sequenced using paired-end shotgun sequenc-

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ing using the Illumina Hi-Seq 2000 platform at Macrogen Inc. (Seoul).

Data analysis

Western blot results were quantified by setting the control group as 1.00, and detected bands were calculated densitometrically. The numbers of GFAP-positive cells in dentate gyrus were counted hemilaterally under a light microscope (Olympus, Tokyo, Japan). The data were analyzed with one-way analysis of variance and then Tukey *post hoc* tests. All values are expressed as the mean±standard error of the mean, and P value <0.05 was considered significant.

RESULTS

The effects of exercise and microbiome transplantation on body weight and blood glucose levels

Compared to the obesity group, all of the obesity with exercise group, the obesity with nonexercise microbiome transplantation group, and the obesity with exercise microbiome transplantation group lost weight. The weight loss was the greatest in the obesity with exercise group (Fig. 1, left).

Compared with the obesity group, blood glucose decreased in all of the obesity with exercise group, obesity with nonexercise microbiome transplantation group, and obesity with exercise microbiome transplantation group. The blood glucose decrease was

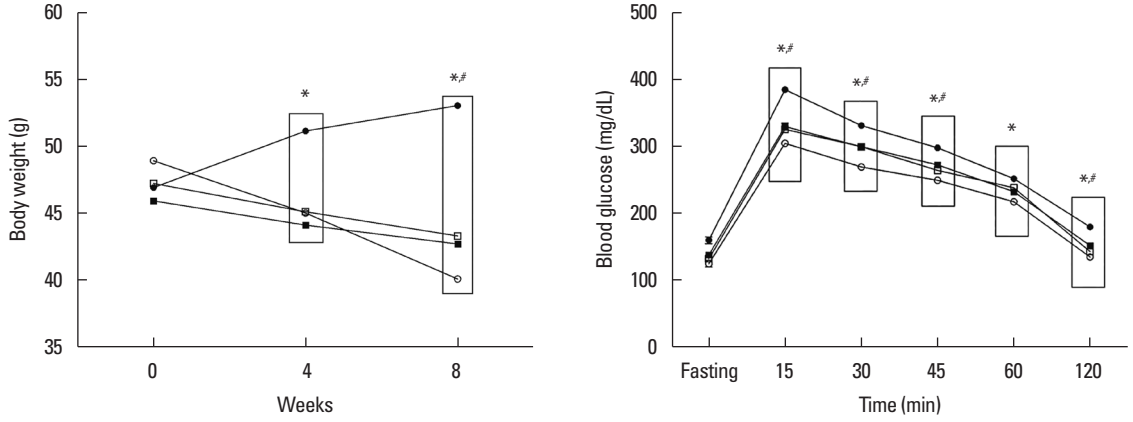

Fig. 1. Effects of exercise and microbiome transplantation on body weight and blood glucose level in obesity mice. Left panel: body weight. Right panel: blood glucose levels. The data are presented as the mean±standard error of the mean. *$P<0.05$ as compared with the obesity group. #$P<0.05$ as compared with the obesity with exercise group. ●, obesity group; ○, obesity with exercise group; ■, obesity with nonexercise microbiome transplantation group; □, obesity with exercise microbiome transplantation group.

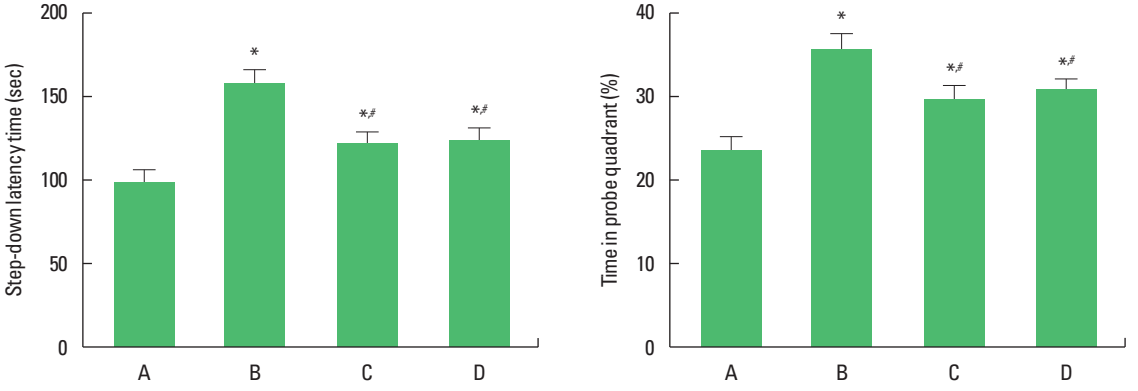

Fig. 2. Effects of exercise and microbiome transplantation on short-term memory and spatial learning memory. Left panel: step-down avoidance test. Right panel: Morris water maze task. The data are presented as the mean±standard error of the mean. *$P<0.05$ as compared with the obesity group. #$P<0.05$ as compared with the obesity with exercise group. A, obesity group; B, obesity with exercise group; C, obesity with nonexercise microbiome transplantation group; D, obesity with exercise microbiome transplantation group.

the greatest in the obesity with exercise group (Fig. 1, right).

The effects of exercise and microbiome transplantation on short-term memory and spatial learning memory

The latency of the step-down avoidance test was greatest in the obesity with exercise group, and short-term memory was the best. Next, the obesity with nonexercise microbiome transplantation group and the obesity with exercise microbiome transplantation group showed large numbers, and short-term memory was the next best (Fig. 2, left).

The probe quadrant time of the Morris water maze task was the largest in the obesity with exercise group, and the spatial learning memory was the best. Next, the obesity with nonexercise microbiome transplantation group and the obesity with exercise microbiome transplantation group were found to be large, and the spatial learning memory was the next best (Fig. 2, right).

The effects of exercise and microbiome transplantation on BDNF and TrkB expression in the hippocampus

The expression of BDNF was highest in the obesity with exercise group, followed by the obesity with nonexercise microbiome transplantation group and the obesity with exercise microbiome transplantation group (Fig. 3, upper and middle).

The expression of TrkB was highest in the obesity with exercise group, followed by the obesity with nonexercise microbiome

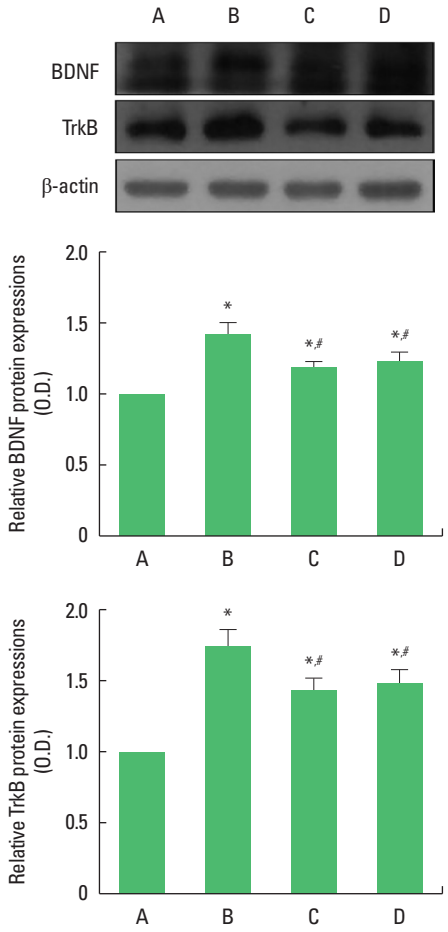

Fig. 3. Effects of exercise and microbiome transplantation on the expressions brain-derived neurotrophic factor (BDNF) and tropomyosin receptor kinase B (TrkB) in the hippocampus. Upper panel: representative expression of BDNF and TrkB. Middle panel: BDNF expression. Lower panel: TrkB expression. The data are presented as the mean ± standard error of the mean. *$P<0.05$ as compared with the obesity group. #$P<0.05$ as compared with the obesity with exercise group. A, obesity group; B, obesity with exercise group; C, obesity with nonexercise microbiome transplantation group; D, obesity with exercise microbiome transplantation group.

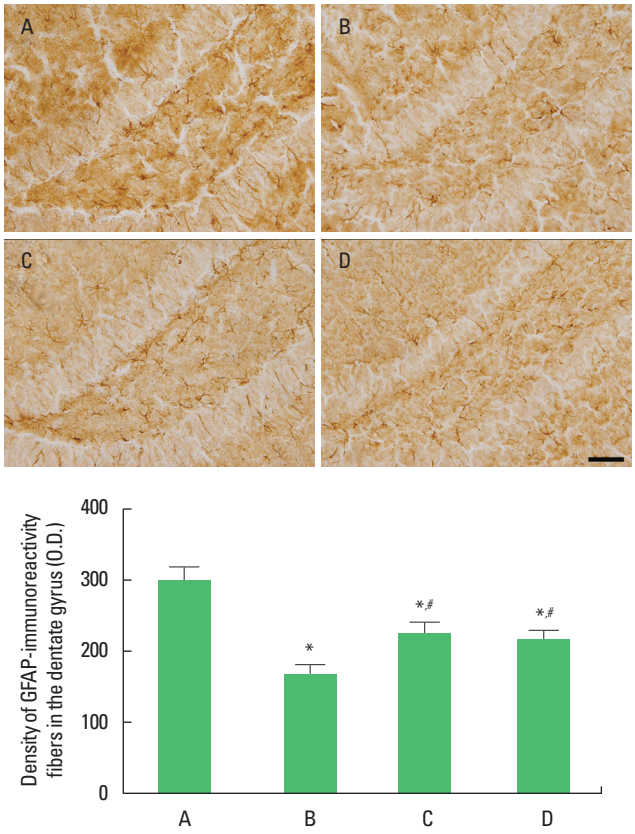

Fig. 4. Effects of exercise and microbiome transplantation on the density of glial fibrillary acidic protein (GFAP)-immunoreactivity fibers in the dentate gyrus. Upper panel: photomicrography of GFAP-positive fibers. Lower panel: density of GFAP-positive fibers. The scale bar represents 200 μm. The data are presented as the mean ± standard error of the mean. *$P<0.05$ as compared with the obesity group. #$P<0.05$ as compared with the obesity with exercise group. A, obesity group; B, obesity with exercise group; C, obesity with nonexercise microbiome transplantation group; D, obesity with exercise microbiome transplantation group.

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transplantation group and the obesity with exercise microbiome transplantation group (Fig. 3, upper and lower).

The effects of exercise and microbiome transplantation on GFAP-positive cells in the hippocampal dentate gyrus

The immunoreactivity of GFAP was lowest in the obesity with exercise group, followed by the obesity with nonexercise microbiome transplantation group and the obesity with exercise microbiome transplantation group (Fig. 4).

The effects of exercise and microbiome transplantation in the gut microbiome

The numbers of *L. acidophilus*, *L. gasseri*, Christensenellaceae, Bactroidetes, and Oscillibacter in feces were highest in the obesity with exercise group, followed by the obesity with nonexercise microbiome transplantation group and the obesity with exercise mi-

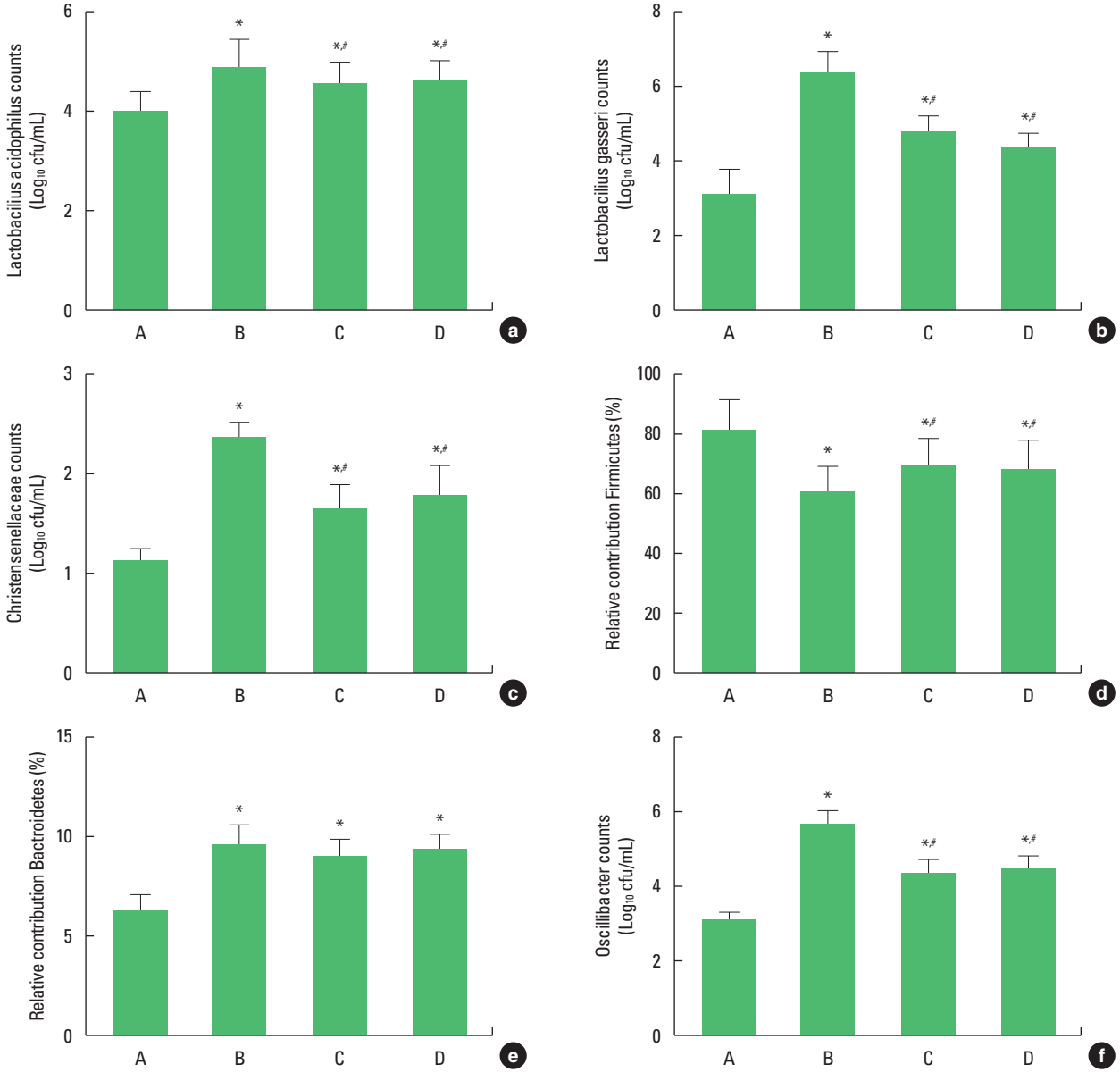

Fig. 5. Effects of exercise and microbiome transplantation on the intestinal microbiota. (a) counts of *Lactobacillus acidophilus*. (b) counts of *Lactobacillus gasseri*. (c) counts of Christensenellaceae. (d) relative contribution Firmicutes. (e) relative contribution Bactroidetes. (f) counts of Oscillibacter. The data are presented as the mean±standard error of the mean (SEM). *$P<0.05$ as compared with the obesity group. #$P<0.05$ as compared with the obesity with exercise group. A, obesity group; B, obesity with exercise group; C, obesity with nonexercise microbiome transplantation group; D, obesity with exercise microbiome transplantation group.

crobiome transplantation group (Fig. 5a–c, f).

The percentage of fecal Firmicutes was highest in the obesity with nonexercise microbiome transplantation group and the obesity with exercise microbiome transplantation group, followed by the obesity with exercise group (Fig. 5d).

The percentage of Bactroidetes was higher in all of the obesity with exercise group, obesity with nonexercise microbiome transplantation group, and obesity with exercise microbiome transplantation group than in the obesity group (Fig. 5e).

DISCUSSION

In this experiment, it was found that exercise had the greatest effect on obesity. Transplantation of the fecal microbiome from exercised mice and fecal microbiome from non-exercised mice was the second most effective in obesity after exercise performance.

The composition of the microbiome in the body has been proposed to determine the degree of obesity induction (Hjorth et al., 2018). In general, energy intake leads to blood sugar control by insulin, and in the case of obesity, an abnormality in the insulin control system accelerates the accumulation of excess energy. Insulin resistance is directly affected by type 2 diabetes and obesity. As the level of obesity increases, so does the level (Glynn et al., 2015). In a study comparing the obesity group and the direct exercise group after obesity induction, and the microbiome group, the latter group showed improvement in insulin resistance in OGTT, which had a positive effect on glucose control. Microbiota transplantation has been shown to induce changes in the recipient's gut environment and improve insulin sensitivity (Vrieze et al., 2012). Direct or indirect effects on obesity induction or inhibition. Although the mechanisms of regulation may differ from exercise, microbiota transplantation has been shown to affect insulin sensitivity and regulation as well as exercise. Simon et al. (2015) reported that insulin regulation was affected by *Lactobacillus* strains, particularly *Lactobacillus reuteri*, and this study also showed that it was involved in increasing beneficial bacteria and improving insulin sensitivity. This suggested that with exercise, the microbiome should be enhanced for maximum effect in controlling obesity.

BDNF has been reported to be associated with obesity and cognitive function (Prickett et al., 2015). BDNF is a neurotrophic factor and a type of neuronutrient that plays an important role in the growth and development of nerve cells and cognitive function (Tsai et al., 2014). TrkB is a BDNF receptor, and dysfunction of TrkB is known to cause diseases such as depression, neurodegenerative disorders, obesity, and eating disorders (Gray et al., 2006). In this study, BDNF and TrkB levels were increased as a result of comparing the exercise group and the microbiome transplant group. The increase has been shown to be more effectively mediated by direct locomotion than by microbiota transplantation. The increase in BDNF due to microbial transplantation causes changes in the Bifidobacterium community, which plays an important role in central nervous system development and neuronal growth (Diaz Heijtz et al., 2011; Sudo et al., 2004).

Microbiome analysis was performed to determine the effects of exercise and microbiota transplantation. Specifically, *L. acidophilus*, *L. gasseri*, Christensenellaceae, Firmicutes, Bacteroidetes, and Oscillibacter were set as variables for microbiome analysis. Goodrich et al. (2014) reported that obesity control is influenced by *Christensenella bacteria*, whereas Bacteroidetes acts to induce inefficient calorie consumption in the body (Hjorth et al., 2018). In this study, it was found that the level of harmful Firmicutes bacteria caused by obesity was significantly lower in the group transplanted with intestinal microbes. *L. acidophilus*, *L. gasseri*, and Christensenellaceae, which are beneficial bacteria, control obesity, promote lipid metabolism, and are also involved in anti-inflammatory action (Million et al., 2012; Rouxinol-Dias et al., 2016). *L. gasseri* has an antiobesity effect, and the obesity-reducing effect is thought to be due to the increase through microbiome transplantation and exercise. Kadooka et al. (2010), *L. gasseri* has been reported to play a role in suppressing obesity in both animals and humans. The Christensenellaceae are abundant in healthy weight groups and their levels increase after weight loss through diet control, indicating their role in obesity control (Alemán et al., 2018; Goodrich et al., 2014). Both the exercise group and the intestinal microbial transplant group showed an increase in the rhododendron family compared to the obesity control group, suggesting that both treatments were effective in strengthening the intestinal microflora to reduce obesity. Similarly, in this study, direct exercise and microbiota transplantation were found to be effective in increasing the levels of obesity-related beneficial bacteria and antiobesity bacteria. Osilybacter is known to affect brain cognitive function, which is reduced in levels through obesity (Olsthoorn et al., 2021).

Obesity induction increases inflammation levels and intestinal permeability and activates the immune system (Muscogiuri et al., 2019), whereas inflammation is thought to affect brain cognitive function. Increased inflammatory gene expression in the prefrontal cortex in fecal transplantation in obese individuals (Arnoriaga-Rodríguez et al., 2020). Similarly, changes in the microbiome in this study affect cognitive function, suggesting a link between

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the gut and the brain. In other words, the gut environment is presumed to be a factor influencing brain function. The results of this study showed that exercise is the most effective way to reduce obesity, improve memory, and suppress inflammation. Intestinal microbiota transplantation was the second most effective after exercise. Microbiota transplantation in young mice has contributed to obesity suppression and may act as an indirect method of obesity reduction. However, there was no significant difference in the fecal microbiome transplant group according to whether or not exercise was performed.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

ACKNOWLEDGMENTS

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