Original Article

Miso, fermented soybean paste, suppresses high-fat/high-sucrose diet-induced muscle atrophy in mice

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(Received 24 May, 2023; Accepted 12 July, 2023; Released online in J-STAGE as advance publication 20 July, 2023)

This study investigated the effects of miso, a traditional fermented soybean food in Japan, on muscle mass atrophy. Eight week old male C57BL/6J mice were fed high fat/high sucrose diet with or without miso for 12 weeks. A miso diet increased soleus muscle weights ($p<0.05$) and reduced intraperitoneal glucose tolerance and insulin tolerance ($p<0.05$). The miso diet down-regulated the Tnfα and Ccl2 expression, related to inflammation, and Trim63 and Fbxo32 expression, related to muscle atrophy, in the soleus muscle ($p<0.05$). The miso diet increased short-chain fatty acids levels, including acetic, propanoic, and butanoic acids, in the feces, serum, and soleus muscle ($p<0.05$). According to the LEfSe analysis, the miso diet increased family Prevotellaceae, family Christensenellaceae, family Dehalobacterium, family Desulfitibacter; family Deferribacteraceae, order Deferribacterales, class Deferribacteres; and family Gemmatimonadaceae, order Gemmatimonadetes, and class Gemmatimonadales, whereas the miso diet decreased family Microbacteriaceae, order Micrococcales, class Actinobacteria, and family Lactobacillaceae. Miso suppressed high fat/high sucrose diet induced impaired glucose tolerance, low muscle strength, and muscle atrophy by improving dysbiosis and increasing short-chain fatty acids production and provides new insights into the preventive effects of fermented foods on sarcopenia.

***Key Words*: sarcopenia, obesity, gut microbiota, fermented food, short-chain fatty acid**

Sarcopenia, defined as the age-related decline in muscle strength, mass, and function[1] is now recognized as an urgent treatment target for older people. Sarcopenia is recognized as the risk of cardiovascular disease[2] and mortality.[3–5] To maintain appropriate muscle mass or induce muscle hypertrophy, sufficient exercise and nutrient intake are required.[6,7] Gut microbiota is closely associated with muscle atrophy.[8–10] Metabolites, represented by short-chain fatty acids and amino acids, are produced from orally intake diet by gut microbiota.[10] Dysbiosis, the alteration of gut microbiota induced by changing the diet, is associated with alterations in gut microbiota-related metabolites.[11] Gut microbiota have been reported to be affected by a variety of factors, including drugs as well as diet.[12] We previously reported that the differences in gut microbiota between Japanese type 2 diabetics and healthy Japanese may be due to acquired factors such as habitual dietary intake, including sucrose intake.[13] High fat/high sucrose diet (HFHSD) is associated with a reduction in short-chain fatty acid (SCFA) production,[14,15] a well-known gut microbiota-related metabolite, and leads to muscle atrophy.[16]

Miso, a traditional fermented soybean food in Japan, contains vegetable proteins, carbohydrates, fat, vitamins, minerals, and microorganisms.[17] Miso effectively protects against hypertension, cancers, and mortality.[17,18] High miso intake is associated with lower insulin resistance.[19,20] Moreover, the glycemic control of diabetic people with habitual miso soup intake were better than that of diabetic people without.[21] In addition, we recently revealed that the prevalence of sarcopenia in diabetic people with habitual miso soup intake was lower than that in diabetic people without.[22] Therefore, there is a possibility that miso, a traditional fermented soybean food in Japan, has a protective effect on muscle atrophy. However, the mechanisms underlying the effect of miso on muscles are yet to be clarified.

We hypothesized that miso alters microbe-associated metabolites, SCFAs, by improving the dysbiosis induced by HFHSD, thereby inhibiting muscle dysfunction and atrophy. The present study investigated the effects of miso on the muscle mass of C57BL/6J mice fed an HFHSD with or without miso and showed that miso suppressed HFHSD-induced muscle atrophy by improving dysbiosis in the intestine, increasing SCFA production, and improving muscle inflammation.

Materials and Methods

Animals. The Committee for Animal Research at the Kyoto Prefectural University of Medicine approved the experimental procedures (M2022-84). In this study, we purchased 12 littermate C57BL/6J male mice with 7 week old from Shimizu Laboratory Supplies (Kyoto, Japan) and mice were housed in a specific pathogen-free, controlled environment. The mice were fed HFHSD (D12327, which included 40% carbohydrate, 20% protein, and 40% fat, coconut oil; Research Diets, Inc., New Brunswick, NJ) with free water or HFHSD with 0.9% barley miso (Fundokin Shoyu Co., Ltd., Oita, Japan) dissolved in water for 12 weeks starting at 8 weeks of age. Body weight was

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doi: 10.3164/jcbn.23-36

measured every week. At 20 weeks of age, after an overnight fast, the mice were killed by administering a combination anesthetic of 4.0 mg/kg midazolam, 0.3 mg/kg medetomidine, and 5.0 mg/kg butorphanol.[23]

Measurement of oral intake. At 20 weeks, mice's oral intake was measured. The mice were housed individually, fed weighed food through the cage, and the remaining amount of food was weighed after 24 h. The oral intake was calculated by subtracting the final food amount from the initial food amount.

Analytical procedures and glucose and insulin tolerance tests. An intraperitoneal glucose tolerance test (iPGTT) (2 g/kg body weight) was performed on 20-week-old mice after 16-h fasts. Then, insulin tolerance test (ITT) (0.5 U/kg body weight) was also performed after 6-h fasts. For both procedures, measurement of blood glucose was performed by collecting a drop of blood using a glucometer at the times indicated (Gultest Neo Alpha; Sanwa Kagaku Kenkyusho, Nagoya, Japan). The area under the curves (AUCs) of the iPGTT results and the ITT results were analyzed.

Evaluation of muscle function and atrophy. A grip strength meter (model DS2-50N, Imada Co., Ltd., Toyohashi, Japan) was used for grip strength measurement in 20-week-old mice. The weight of the soleus muscle of mice was measured as previously reported method.[24]

Gene expression in the soleus muscle. Gene expression of Trim63, Fbxo32, Tnfα, and Ccl2 in the soleus muscle were performed. Methods for total RNA extraction, reverse transcription, and RT-PCR are described in detail in the Supporting information. The relative expression levels of each targeted gene were normalized to Gapdh threshold cycle (CT) values and quantified using the comparative threshold cycle $2^{-\Delta\Delta CT}$ method as described previously.[25] Signals from a C57BL6/J mouse fed an HFHSD were assigned a relative value of 1.0. Six mice from each group were examined, and RT-PCR was performed in triplicates for each sample.

Measurement of short-chain fatty acid concentrations in fecal, serum, and soleus muscle samples. The SCFA compositions of the murine feces of the rectum, serum, soleus muscle and white adipose tissue (eWAT) samples were analyzed using gas chromatography–mass spectrometry (GC/MS) on an Agilent 7890B/7000D System (Agilent Technologies, Santa Clara, CA), same as previously.[26] Methods are described in detail in the Supporting Information.

Fecal microbiota analysis. Fecal samples were collected from the appendix, stored in a cryotube. frozen by liquid nitrogen, and stored in liquid nitrogen until DNA extraction. Three fecal samples were collected individually from the appendix of three mice, excluding one large mouse and one small mouse from each group. Microbial DNA was extracted from the frozen fecal samples using the QIAamp® DNA Feces Mini Kit (Qiagen, Venlo, Netherlands), following the manufacturer's instructions.[22] Whole-genome shotgun sequencing was performed using the HiSeq 2000/2500/4000 system (Illumina, San Diego, CA) at the Bioengineering Lab. Co., Ltd. (Sagamihara, Japan).

Statistical analysis. The data were shown as mean and SD. The differences between the groups were evaluated using the *t* test for parametric continuous data or Mann–Whitney *U* test for non-parametric continuous data. The differences in categorized variables between the groups were evaluated using Pearson's chi-square test. Prism ver. 8.0 software (GraphPad, San Diego, CA) was used. Statistical significance was set at $p<0.05$. Differences between groups were detected by LEfSe analysis. A Kruskal–Wallis sum-rank test was performed to identify significantly different species, and a linear discriminant analysis (LDA) was performed on the identified species to further evaluate the effect size of these differences. Significant differences were set at $p<0.05$ using the Benjamini–Hochberg procedure[27] and a logarithmic LDA score threshold of 2.0.[28]

Results

Miso suppresses HFHSD-induced impaired glucose tolerance, muscle strength, and muscle atrophy. Miso suppresses HFHSD-induced impaired glucose tolerance, muscle strength, and muscle atrophy The body weights of mice fed HFHSD with miso [35.0 (1.9) g vs 37.5 (1.8) g at 20 weeks, $p = 0.007$] (Fig. 1A) and the oral intake at 20 weeks of age [3.34 (0.51) g vs 4.07 (0.72) g, $p = 0.018$] (Fig. 1B) were suppressed than those of mice fed HFHSD. iPGTT and ITT revealed that the glucose and insulin tolerance in mice fed HFHSD with miso was significantly reduced compared to those in mice fed HFHSD [AUC of iPGTT; 20,558 (2,091) mg/dl × min vs 23,685 (1,409) mg/dl × min, $p = 0.001$ and AUC of ITT; 13,723 (1,707) mg/dl × min vs 17,053 (976) mg/dl × min, $p<0.001$] (Fig. 1C–F). The absolute and relative soleus muscle weights in mice fed HFHSD with miso were higher than those in mice fed HFHSD [absolute soleus muscle; 8.0 (0.3) g vs 6.1 (0.8) g, $p<0.001$ and relative soleus muscle; 0.23 (0.01) vs 0.20 (0.01), $p<0.001$] (Fig. 1G and H). Furthermore, grip strength in mice fed HFHSD with miso was higher than that in mice fed HFHSD [grip strength; 35.6 (2.2) g vs 30.2 (6.1) g, $p = 0.017$ and grip strength/BW; 2.62.6 (0.22) vs 2.01 (60.18), $p<0.001$] (Fig. 1I and J).

Miso suppresses HFHSD-induced inflammation and muscle atrophy in the soleus muscle. The relative expression of genes related to inflammation, Tnfα ($p<0.001$) and Ccl2 ($p<0.001$), in the soleus muscle of mice fed HFHSD with miso were lower than those of mice fed HFHSD (Fig. 2A and B). In addition, the relative expression of genes related to muscle atrophy, Trim63 ($p<0.001$) and Fbxo32 ($p<0.001$), in the soleus muscle of mice fed HFHSD with miso were lower than those of mice fed HFHSD (Fig. 2C and D).

Miso suppresses the reduction of SCFAs by HFHSD. SCFA levels, including acetic acid, propanoic acid, and butanoic acid, were measured. SCFA levels in the feces [acetic acid; 6.43 (3.05) nmol/μg vs 2.10 (1.45) nmol/μg, $p<0.001$, propanoic acid; 85.3 (4.03) nmol/μg vs 65.4 (2.61) nmol/μg, $p<0.001$, and butanoic acid; 23.6 (10.7) nmol/μg vs 4.07 (7.01) nmol/μg, $p<0.001$], serum [acetic acid; 212.6 (22.4) nmol/μg vs 180.2 (10.7) nmol/μg, $p<0.001$, propanoic acid; 13.1 (6.5) nmol/μg vs 2.81 (2.29) nmol/μg, $p<0.001$, and butanoic acid; 325.9 (16.3) nmol/μg vs 228.3 (23.8) nmol/μg, $p<0.001$], soleus muscle [acetic acid; 2.19 (0.58) nmol/μg vs 0.76 (0.02) nmol/μg, $p<0.001$, propanoic acid; 1.00 (0.25) nmol/μg vs 0.36 (0.02) nmol/μg, $p<0.001$, and butanoic acid; 0.38 (0.10) nmol/μg vs 0.13 (0.01) nmol/μg, $p<0.001$] and eWAT [acetic acid; 0.39 (0.05) nmol/μg vs 0.25 (0.02) nmol/μg, $p<0.001$, propanoic acid; 0.22 (0.04) nmol/μg vs 0.14 (0.02) nmol/μg, $p<0.001$, and butanoic acid; 0.07 (0.01) nmol/μg vs 0.04 (0.01) nmol/μg, $p<0.001$] of mice fed HFHSD with miso were significantly higher than those of mice fed HFHSD (Fig. 3).

Miso alters gut microbiota. The gut microbiota was investigated using shotgun metagenomic sequencing. According to the LEfSe analysis, the families Prevotellaceae, Christensenellaceae, Dehalobacterium, Desulfitibacter; family Deferribacteraceae, order Deferribacterales, class Deferribacteres; and family Gemmatimonadaceae, order Gemmatimonadetes, and class Gemmatimonadales were increased in mice fed HFHSD with miso, and family Microbacteriaceae, order Micrococcales, class Actinobacteria, and family Lactobacillaceae were increased in mice fed HFHSD (Fig. 4A and B).

Discussion

In this study, we showed that miso, the traditional fermented soybean food in Japan, suppressed impaired glucose tolerance, muscle dysfunction, and muscle atrophy, which were induced by HFHSD. Miso suppressed inflammation in skeletal muscle

*See online. https://doi.org/10.3164/jcbn.23-36

doi: 10.3164/jcbn.23-36

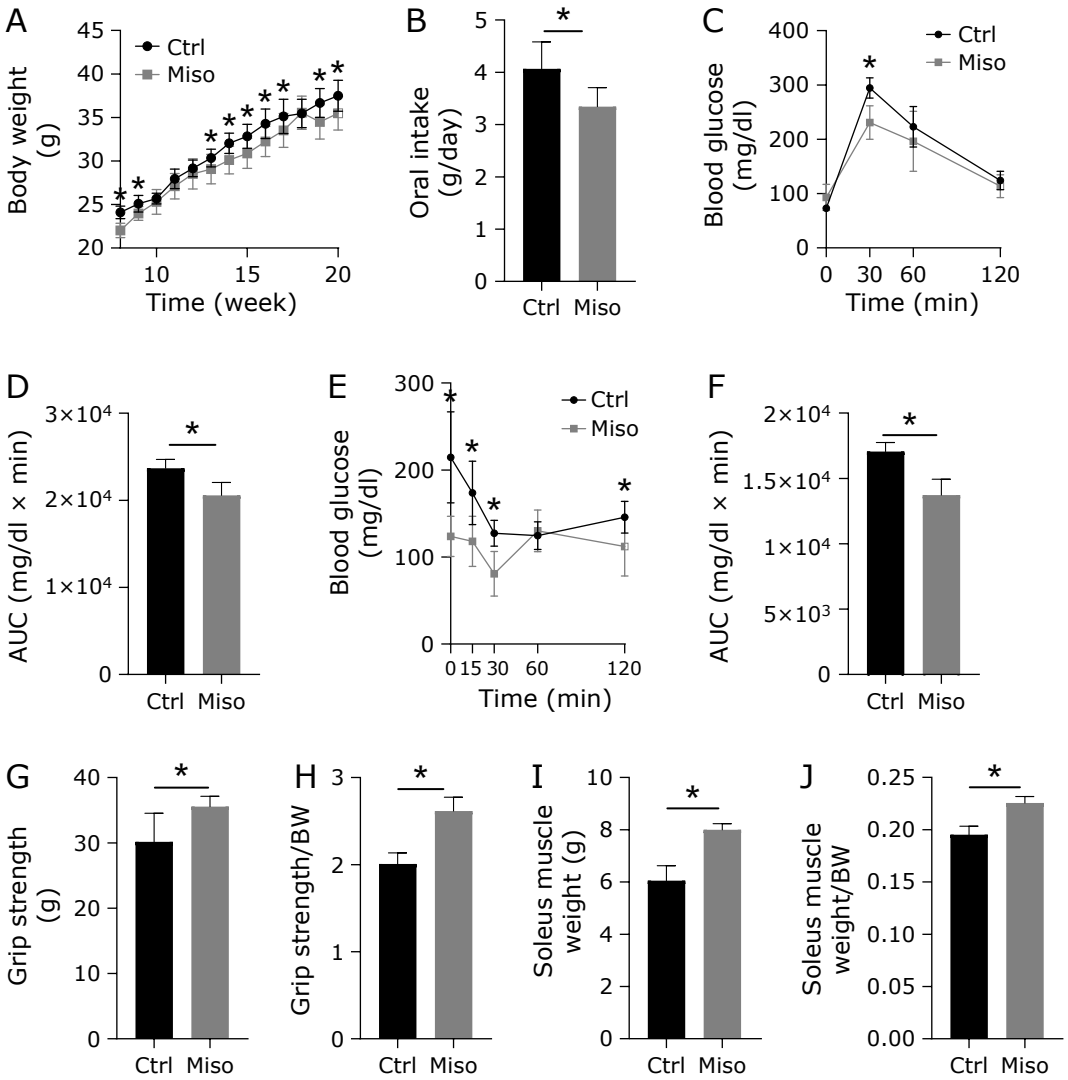

Fig. 1. Miso suppresses HFHSD-induced impaired glucose tolerance, muscle dysfunction, and muscle atrophy. (A) Body weight changes ($n = 6$). (B) Oral intake measured at 20 weeks of age ($n = 6$). (C, D) When the mice reached 20 weeks of age, an intraperitoneal glucose tolerance test (iPGTT) (2 g/kg body weight) was performed, and the area under the curve (AUC) was analyzed ($n = 6$). (E, F) When the mice reached 20 weeks of age, insulin tolerance test (ITT) (0.5 U/kg of body weight) was performed, and the AUC was analyzed ($n = 6$). (G, H) Grip strength of mice was measured at 20 weeks of age. (I, J) The soleus muscle weight of mice ($n = 6$). Soleus muscle weight/BW was evaluated as soleus muscle weight divided by body wight (BW) at sacrifice. The differences between the groups were evaluated using the *t* test for parametric continuous data or Mann–Whitney *U* test for non-parametric continuous data and $*p<0.05$.

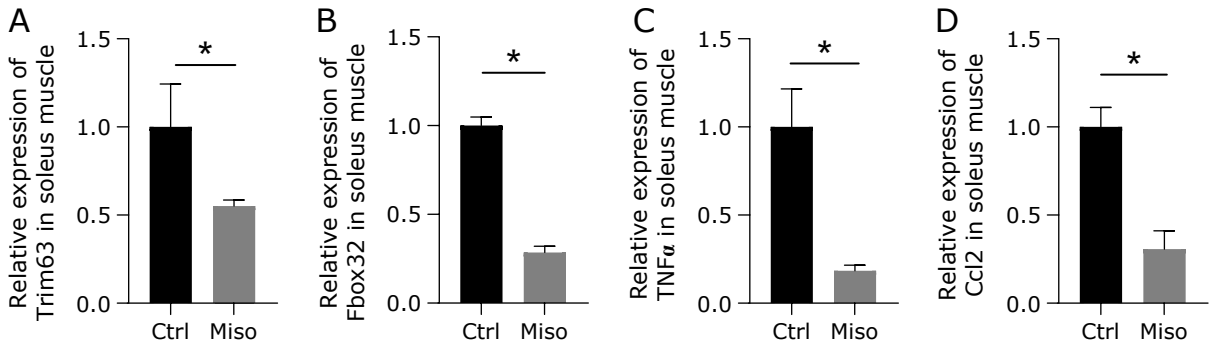

Fig. 2. Inflammation and muscle atrophy-related gene expression in mice fed HFHSD with miso was significantly decreased compared to those fed HFHSD. Gene expression in the soleus muscle ($n = 6$). (A) Tnfa, (B) Ccl2, (C) Trim63, and (D) Fboxo32. The differences between the groups were evaluated using the *t* test and $*p<0.05$.

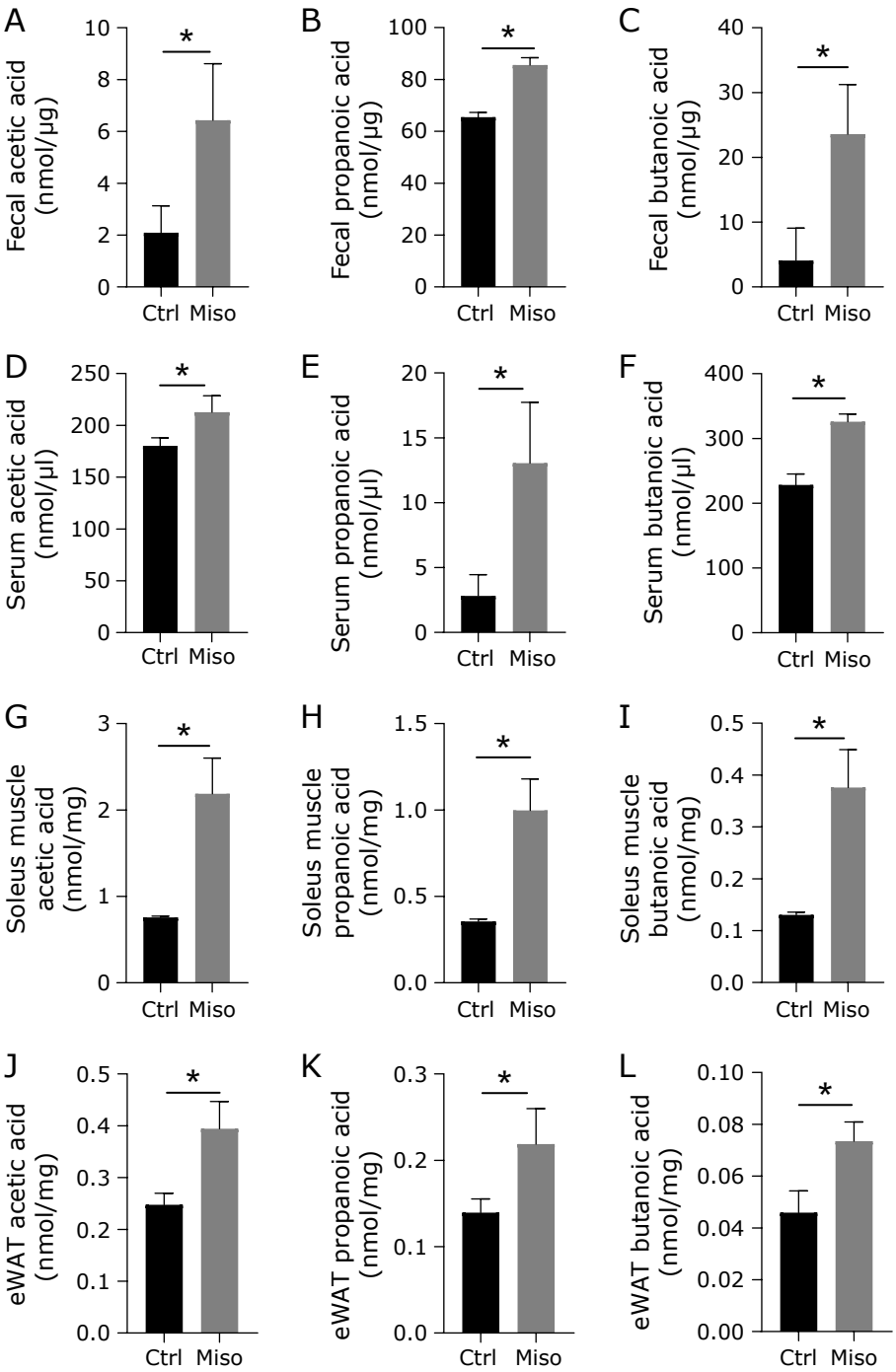

Fig. 3. Levels of short-chain fatty acids (SCFAs) in mice fed HFHSD with miso was significantly increased compared to those fed HFHSD. (A–C) The concentrations of acetic acid, propanoic acid, and butanoic acid in the feces ($n = 6$). (D–F) The concentrations of acetic acid, propanoic acid, and butanoic acid in the serum ($n = 6$). (G–I) The concentrations of acetic acid, propanoic acid, and butanoic acid in the soleus muscle ($n = 6$). (J–L) The concentrations of acetic acid, propanoic acid, and butanoic acid in white adipose tissue (eWAT) ($n = 6$). The differences between the groups were evaluated using the *t* test and $*p<0.05$.

by improving dysbiosis and increasing SCFA production and absorption.

Diet habits play an important role in maintaining health, including muscle mass. A previous study showed that theaflavins, a type of phytochemical, have been reported to act protectively against muscle atrophy.[29] Furthermore, dysbiosis, often occurring with dietary changes, alters the production of gut microbiota-related metabolites.[11] Gut microbiota is closely associated with muscle atrophy through SCFAs.[8–10] HFHSD reduces SCFA production[14,15] and upregulates inflammation related gene expression, such as Tnfα and Ccl2, in muscles,[30,31] leading to muscle atrophy.[16] In our study, SCFA levels in the feces, serum, muscle, and eWAT of mice fed HFHSD decreased, and Tnfα and Ccl2 levels in the muscle of mice fed HFHSD increased. Therefore, miso intakes improved the production of SCFAs, one of the important gut microbiota-related metabolites,

doi: 10.3164/jcbn.23-36

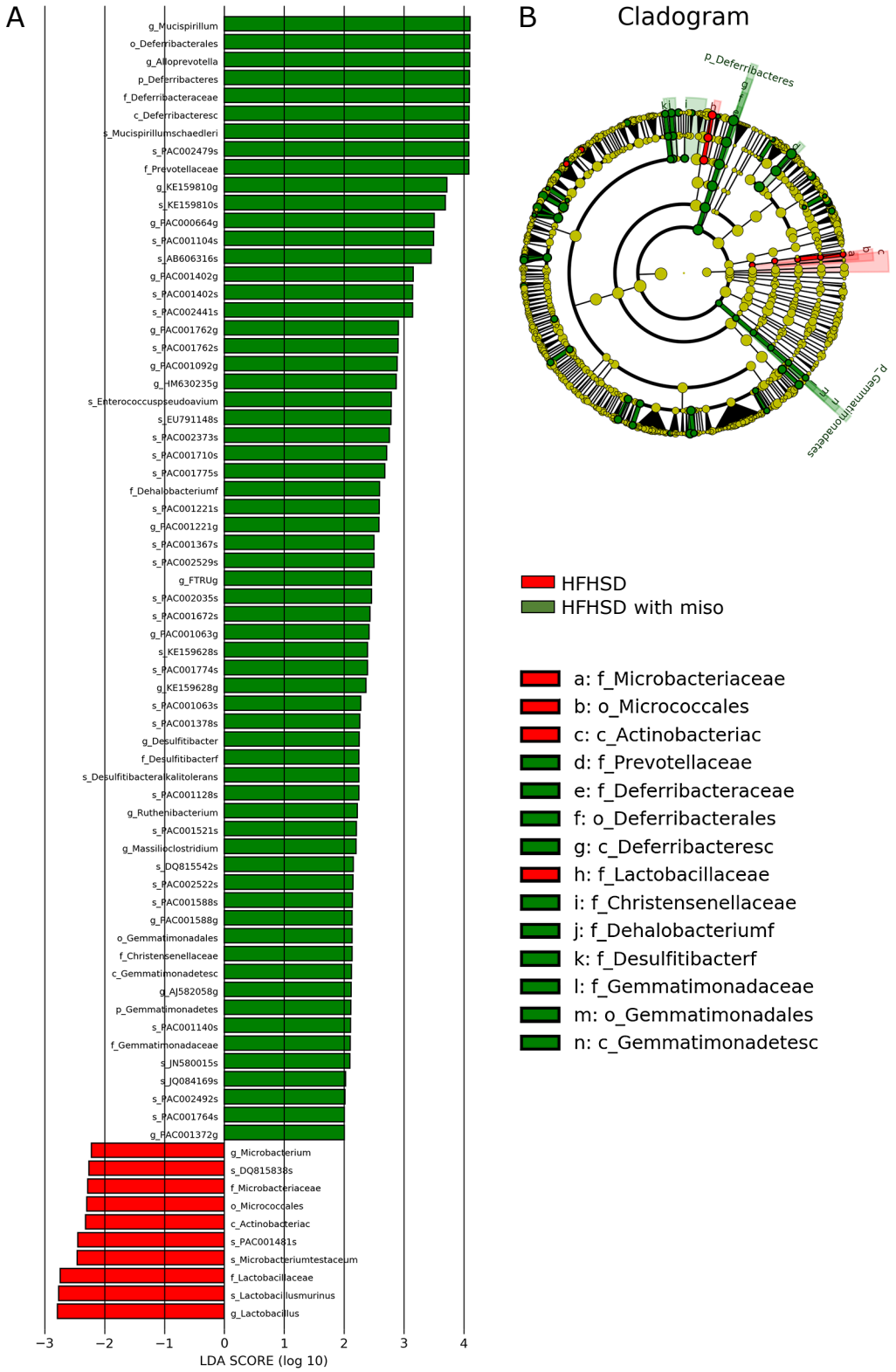

Fig. 4. Changes in the gut microbiota observed using shotgun metagenomic sequencing. (A) Linear discriminant analysis (LDA) scores of gut microbiota of the control (red) and miso (green) groups. (B) LEfSe was used to identify the species with the greatest differences in the abundance between the gut microbiota of control (red) and miso (green) groups ($n = 6$). The brightness of each dot is proportional to the effect size. Only species with a significant LDA threshold value >2 are demonstrated. See color figure in the on-line version.

in the gut, which had been suppressed by the HFHSD and suppressed the inflammation in muscle via an increase in SCFAs.

It has been reported that high-fat high-sucrose diets increases the threshold for self-stimulation in the lateral hypothalamus, which constitutes the reward system, making it difficult to feel brain reward from food intake.[32] Although the detailed mecha-

nism is not known, it is possible that miso consumption may have had an inhibitory effect on raising the threshold for self-stimulation in the lateral hypothalamus.

Fermented foods are produced through controlled microbial growth and conversion of food components through enzymatic action.[33] Various fermented foods, including cheese, alcoholic beverages, pickles, soy sauce, and yogurt, are highly enriched in SCFAs.[34,35] Japan is known to have many kinds of fermented foods due to its mild and humid climate, which provides a favourable environment for fermented microorganisms to grow and an abundance of foodstuffs from which to ferment. Miso, a traditional fermented soybean food in Japan, has a protective effect against sarcopenia[22] and is negatively correlated with insulin resistance in humans.[20,22] The main ingredient of miso is soybeans, which contain soy protein and isoflavones as well as various vitamins and minerals. Miso also contains other contents, such as pyroglutamyl leucine, polyamines, melanoidin, trypsin inhibitor, saponin, lecithin, choline, and dietary fiber. Fermented foods have the potential to provide a higher absorption rate of nutrients than when consumed as original foods. Miso exhibits anti-inflammatory effects.[30] A previous study reported soy isoflavones prevent muscle atrophy through down regulation of inflammation markers, including Tnfα,[36] which was identical to the results of this study. Miso soup intake is associated with an increase in SCFAs in the human feces.[31] In this study, the production and absorption of SCFAs in mice fed HFHSD with miso were higher than those in mice fed HFHSD. Gut microbiota, such as family Prevotellaceae, including genus Alloprevotella, which was increased in mice fed HFHD with miso, was associated with SCFA production.[8] The proportions of Prevotella is high in Japanese individuals[37] but was reduced in the patients with type 2 diabetes, whose dietary habits are westernized, compared to healthy people.[13] The family Christensenellaceae is also associated with SCFA production.[38,39] The increase in the Lactobacillaceae family is associated with lower SCFA levels.[38] SCFAs enhance skeletal muscle metabolism by increasing insulin sensitivity and decreasing inflammation. In our study, SCFA levels were higher and inflammation was lower in the muscle of mice fed HFHSD with miso than those in mice fed HFHSD.

Limitations of this study should be mentioned. First, to assess sarcopenia, the mice at least at 12 months of age is desirable. Further study will be performed to clarify the usage of miso on the sarcopenia using the older mice. Second, since soleus muscle is an oxidative muscle, using only soleus muscle could be limiting and results might be influenced by fiber type and metabolism. To investigate not only soleus, an oxidative muscle, but also a glycolytic muscle (extensor digitorum longus or plantaris) and a mixed fiber type muscle (tibialis, quadriceps or gastrocnemius) is desirable. Third, assessment of muscle synthesis pathways and muscle cross-sectional area is also important. Unfortunately, however, we did not evaluate these points. Our results demonstrate important points. First, miso, the traditional fermented soybean paste, improves glucose tolerance and suppresses muscle atrophy, even though it has no effect on whole body weight. Second, miso intake increased SCFA production through improving dysbiosis. This study provides new insight into the significant benefit of taking fermented food.

In summary, this study revealed that miso, the traditional fermented soybean food in Japan, suppressed HFHSD induced impaired glucose tolerance, muscle strength, and muscle atrophy by improving dysbiosis and increasing SCFA production and absorption.

Author Contributions

Conceptualization, YH, AM, and MF; methodology, YH, TO, and MH; software, HT and RS; investigation, YH, TO, RB, YY, CM, AK, AM, SM, TS, EU, HT, RS, and NN; data curation, TO; writing—original draft preparation, YH and TO; writing—review and editing, RB, YY, CM, AK, AM, SM, TS, EU, HT, RS, NN, MH, and MF; visualization, TO; supervision, MH and MF; project administration, YH; funding acquisition, YH and MF. All authors have read and agreed to the published version of the manuscript.

Acknowledgments

Barley miso (Fundokin Shoyu Co., Ltd., Oita, Japan) was provided by Fundokin Shoyu Co., Ltd.

Funding

This work was supported by KAKENHI, Grant-in-Aid for Young Scientists (19K20187), the Food Science Institute Foundation and the Tojuro Iijima Foundation for Food Science and Technology.

Conflict of Interest

YH has received personal fees from Sanofi K.K., Mitsubishi Tanabe Pharma Co., Daiichi Sankyo Co., Ltd., and Novo Nordisk Pharma Ltd., outside the submitted work. TS has received personal fees from MSD K.K., Astellas Pharma Inc., Ono Pharma Co., Ltd., Kissei Pharma Co., Ltd., Mitsubishi Tanabe Pharma Co., Kowa Pharma Co., Ltd., Taisho Toyama Pharma Co., Ltd., Kyowa Hakko Kirin Co., Ltd., Takeda Pharma Co., Ltd., Sanofi K.K., Novo Nordisk Pharma Ltd., and Eli Lilly Japan K.K., outside the submitted work. EU has received grants from the Japanese Study Group for Physiology and Management of Blood Pressure (Grant number: 4024). Donated Fund Laboratory of Diabetes therapeutics is an endowment department, supported with an unrestricted grant from Ono Pharmaceutical Co., Ltd., and received personal fees from MSD K.K., Takeda Pharmaceutical Co., Ltd., Astellas Pharma Inc., Kowa Pharmaceutical Co., Ltd., Mitsubishi Tanabe Pharma Co., Nippon Boehringer Ingelheim Co., Ltd., AstraZeneca plc, Novo Nordisk Pharma Ltd., Taisho Toyama Pharmaceutical Co., Ltd., Kyowa Hakko Kirin Company Ltd., Daiichi Sankyo Co., Ltd., and Sumitomo Dainippon Pharma Co., Ltd., outside the submitted work. MH has received grants from Mitsubishi Tanabe Pharma Co., Sumitomo Dainippon Pharma Co., Ltd., Asahi Kasei Pharma, Sanofi K.K., Takeda Pharmaceutical Co., Ltd., Astellas Pharma Inc., Daiichi Sankyo Co., Ltd., Eli Lilly Japan K.K., Novo Nordisk Pharma Ltd., Nippon Boehringer Ingelheim Co., Ltd., and Kyowa Kirin Co., Ltd., outside the submitted work. MF has received grants from Taisho Pharma Co., Ltd., Ono Pharma Co., Ltd., Daiichi Sankyo Co., Ltd., Sanwa Kagaku Kenkyusho Co., Ltd., Nippon Boehringer Ingelheim Co., Ltd., Astellas Pharma Inc., Kissei Pharma Co., Ltd., Sanofi K.K., MSD K.K., Takeda Pharma Co., Ltd., Teijin Pharma Ltd., Eli Lilly Japan K.K., Novo Nordisk Pharma Ltd., Sumitomo Dainippon Pharma Co., Ltd., Abbott Japan Co., Ltd., Mitsubishi Tanabe Pharma Co., Kyowa Hakko Kirin Co., Ltd., Johnson & Johnson K.K. Medical Co., Terumo Co., Nippon Chemiphar Co., Ltd., and Kowa Pharmaceutical Co., Ltd.; and received personal fees from Taisho Pharma Co., Ltd., Sumitomo Dainippon Pharma Co., Ltd., Eli Lilly Japan K.K., Mitsubishi Tanabe Pharma Co., AstraZeneca plc, Nippon Boehringer Ingelheim Co., Ltd., Sanwa Kagaku Kenkyusho Co., Ltd., Astellas Pharma Inc., Kissei Pharma Co., Ltd., Daiichi Sankyo Co., Ltd., Bayer Yakuhin, Ltd., Kyowa Kirin Co., Ltd., Kowa Pharma Co., Ltd., Novo Nordisk Pharma Ltd., Medtronic Japan Co., Ltd., Ono Pharma Co., Ltd., Mochida Pharma Co., Ltd., Takeda Pharma Co., Ltd., Abbott Japan Co., Ltd., Arkley Inc., MSD K.K., Teijin Pharma Ltd., Sanofi K.K., and Nipro Co., outside the submitted work.

doi: 10.3164/jcbn.23-36

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