Dietary fermented products using *koji* mold and sweet potato-*shochu* distillery by-product promotes hepatic and serum cholesterol levels and modulates gut microbiota in mice fed a high-cholesterol diet

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**ABSTRACT**

It has been reported that fermented products (FPs) prepared from sweet potato-*shochu* distillery by-product suppressed weight gain and decreased serum cholesterol levels in mice under normal dietary conditions. Furthermore, from the information gained from the above data regarding health benefits of the FPs, the aim of this study was evaluating the effects of dietary FPs on lipid accumulation and gut microbiota in mice with or without cholesterol-load in the diet. C57BL/6N mice were fed normal (CO) diet, CO with 10% FPs (CO + FPs) diet, cholesterol loaded (HC) diet, or HC with 10% FPs (HC + FPs) diet for 8 weeks. The mice were then euthanized, and blood samples, tissue samples, and feces were collected. The adipose tissue weight and liver triglyceride levels in the HC + FPs diet groups were significantly reduced compared to that in the HC diet groups. However, FPs significantly increased the serum non-high-density lipoprotein cholesterol (HDL-C) levels, the ratio of non-HDL-C to HDL-C and hepatic total cholesterol levels in mice fed cholesterol-load diet compared with that of the HC diet group. Since dietary FPs significantly decreased the protein expression levels of cholesterol 7 alpha-hydroxylase 1 in the HC + FPs diet groups, the cholesterol accumulation in FPs group may be explained by insufficient catabolism from cholesterol to bile acid. In addition, the dietary FPs tended to increase *Clostridium* cluster IV and XIVa, which are butyrate-producing bacteria. Related to the result, n-butyrate was significantly increased in the CO + FPs and the HC + FPs diet groups compared to their respective control groups. These findings suggested that dietary FPs modulated the lipid pool and gut microbiota.

**Subjects**  Food Science and Technology, Microbiology, Nutrition

**Keywords**  Sweet potato-*shochu*, Distillery by-product, Dietary fiber, Short chain fatty acids, Gut microbiota, Koji, Cholesterol
INTRODUCTION

Obesity is caused by an imbalance between energy intake and energy consumption (Spiegelman & Flier, 2001), and it is a risk factor for the disruption of lipid metabolism (Furukawa et al., 2017). In Japan, the clinical criteria of metabolic syndrome include abnormal values for more than two of the following parameters: weight, triglycerides (TG), high-density lipoprotein (HDL) cholesterol in serum, and blood pressure (Alberti et al., 2009). Metabolic syndrome related to the disruption of lipid metabolism increases the risk of cardiovascular disease and type 2 diabetes (Grundy, 2016; DeBoer et al., 2016). Metabolic syndrome is a growing social problem, and the efficacy of various functional foods such as green tea (Suzuki et al., 2013), coffee (Watanabe et al., 2017), and soybean (Hashidume et al., 2016) on metabolic syndrome has been evaluated. It is expected that food materials having preventive effects on the disruption of lipid metabolism will be developed in future.

Shochu is a traditional Japanese liquor and is one of the distilled spirits made from sweet potato, barley, rice, buckwheat, and brown sugar (Sameshima, 2004). The sweet potato-shochu distillery by-product (SSDB) discharged from shochu industry is utilized as materials for methane fermentation or animal feed (Kamizono et al., 2010). It is reported that the SSDB contains various nutritional components such as proteins, vitamins and minerals, and functional components such as S-adenosylmethionine and polyamines (Mukai et al., 2017). It has been reported that SSDB has an anti-cancer effect (Sasaki et al., 2005). Among them, by-products of awamori (one of the traditional Japanese spirits) lowered serum total cholesterol (TC) levels (Ishibashi, 2007; Nohara et al., 2010). Moreover, it is used for the preparation of bread (Sho et al., 2008) and vinegar manufacture (Kawano et al., 2008), and also for the production of chitosan (Yokoi et al., 1998) and nisin (Furuta et al., 2008).

Koji is cultured by solid fermentation using koji molds on rice and barley. Because koji mold produces many types of enzymes and secretes them as it grows, it is known that the koji mold is traditionally used for solid fermentation to produce fermented foods like shochu, sake (Japanese rice wine), miso (fermented soybean paste), soy source, and vinegar. Among them, because of the long experience of eating in Japan, koji mold is one of the safest microorganisms. It was indicated that dietary rice koji-fermented product produced by using koji mold reduced weight gain and adipose tissue in mice (Yoshizaki et al., 2014). Yoshimoto et al. (2004) reported that SSDB treated with Bacillus subtilis and cellulase is rich in caffeic acid, one of the many polyphenols that has beneficial physiological functions. In addition, glucosylceramide contained in the koji mold is capable of acting as a prebiotic (Hamajima et al., 2016), and barley-derived β-glucan improves metabolism in mice fed high-fat diet through short chain fatty acids (SCFAs) produced by intestinal bacteria (Miyamoto et al., 2018). It is also observed that Aspergillus species also contain polysaccharides, such as β-glucan (Ishibashi et al., 2004). Furthermore, it has been reported that the intestinal microbiota is altered by a high cholesterol diet (Caesar et al., 2016; Bo et al., 2017) and that SCFA production is increased by dietary fiber intake (Lattimer & Haub, 2010; Slavin, 2013). As described above, FPs are rich in polysaccharides, and we
hypothesized that it would benefit cholesterol metabolism through changes in intestinal microbiota and SCFA production.

The aim of this study was evaluating the effects of dietary FPs on lipid accumulation and gut microbiota in mice with or without cholesterol-load in the diet. To the best of our knowledge, our study is the first to report the effects of FPs on cholesterol loading.

**MATERIALS AND METHODS**

**SSDB production**

We recovered SSDB derived from *shochu* process after atmospheric distillation in Kirishima Shuzo Co., Ltd. (Miyazaki, Japan). SSDB was filtered through a filter press 40D-8 (Yabuta industries Co., Ltd., Hyogo, Japan). The filtrate was preserved at −20 °C and thawed prior to use. Kawachi NK, black koji of NK type, was purchased from Kawachi Gen-ichiro store Co., Ltd. (Kagoshima, Japan).

**Preparation of FPs made from SSDB using koji mold**

To prepare the FPs, we followed a previously reported method (*Kosakai et al., 2019*). Kawachi NK (2 g) was suspended in 20 mL of sterile distilled water by vortexing and ultrasonication. SSDB (4 L) was poured into a 10 L jar fermenter (MDL-10 L; B. E. Marubishi Co., Ltd., Tokyo, Japan) and 3 × 10⁵ conidiospores per mL of medium were inoculated. The culture was incubated at 30 °C at 250 rpm without aeration for 48 h, followed by a rotation speed of 200 rpm at an aeration rate of 0.5 vvm for 48 h. FPs were recovered by suction filtration through a filter paper (No. 2; Toyo Roshi Kaisha, Ltd., Tokyo, Japan), and were freeze-dried and powdered. The experiment procedure from liquid-fermentation to powdering was repeated 18 times, mixed and used for performing animal experiments.

**Nutrient value of FPs**

The nutrient contents, such as moisture, protein, fat, ash, carbohydrates, dietary fiber, and energy of FPs were analyzed by Japan Food Research Laboratories (Tokyo, Japan) in accordance with Cabinet Office Ordinance No. 10 of 2015 in Japan. Moisture and protein were measured by heating-drying method and combustion method, respectively. Fat and ash were measured gravimetrically after the extraction and the incineration, respectively. Carbohydrate was calculated by subtracting the amount of moisture, protein, fat, and ash from 100. The energy values were calculated by means of Atwater’s factors (protein and carbohydrate 4 kcal/g and fat 9 kcal/g, respectively). Chitin–chitosan of FPs was calculated as glucosamine equivalent after acid hydrolysis with hydrochloric acid (HCl). Briefly, 400 µL of 4 N HCl solution was added to freeze-dried and powdered FPs (10 mg) and incubated at 96 °C for 18 h. According to a previous method of *Blix (1948)*, the glucosamine concentration of the supernatant was measured and taken as the amount of chitin-chitosan in FPs. β-glucan of FPs was measured by the mushroom and yeast beta-glucan assay kit (Megazyme international, Wicklow, Ireland).
Mice and diet

All studies were carried out using 5-week-old male mice. C57BL/6N mice purchased from Japan SLC, Inc. (Shizuoka, Japan) and maintained at 22°C in a humidity-controlled room at 55 ± 5% with a 12-h light-dark cycle. All mice were acclimatized for 1 week and randomly assigned to four groups based on the body weights, which were fed control AIN-93G diet (CO, \(n = 8\)), CO + 10% FPs (CO + FPs, \(n = 8\)), high-cholesterol diet including 0.50% cholesterol (HC, \(n = 7\)), and HC + 10% FPs (HC + FPs, \(n = 7\)). Based on the results of nutrient composition of FPs, the increased nutrient components by addition of FPs were, respectively, subtracted as follows: fat, soybean oil; carbohydrates, corn starch protein, casein; dietary fiber, cellulose. Indeed, the weight increased with addition of cholesterol and sodium cholate was subtracted from corn starch. Detailed diet compositions are provided in Table 1. The mice were fed the respective diets and provided with water ad libitum. The body weights and food intake were recorded for 8 weeks every other day and feces were collected as the same frequency as. To evaluate the time-periods during which the effects of FPs are detected, we analyzed the body weights of mice by dividing their feeding periods into middle and final body weight. Food intake and feeding efficiency calculated as follows: food intake (g food intake/day/100 g body weight) and feeding efficiency (g weight gain for 8 weeks/10 g food intake for 8 weeks). The feces were pooled for each individual and preserved every 2 weeks at −80°C. Animal Experiment Committee of Miyazaki University approved this study (2013-024). The animal studies were

| Table 1 Composition of the experimental diet. |
|-----------------|-----|-----|-----|-----|
| Component (g/kg) | CO  | CO + FPs | HC  | HC + FPs |
| Casein           | 200.00 | 159.37 | 200.00 | 159.37 |
| Corn starch      | 397.50 | 385.58 | 390.00 | 378.08 |
| Pregelatinized corn starch | 132.00 | 132.00 | 132.00 | 132.00 |
| Sucrose          | 100.00 | 100.00 | 100.00 | 100.00 |
| Soybean oil      | 70.00  | 65.46  | 70.00  | 65.46  |
| Cellulose        | 50.00  | 7.09   | 50.00  | 7.09   |
| Vitamin mix¹     | 10.00  | 10.00  | 10.00  | 10.00  |
| Mineral mix²     | 35.00  | 35.00  | 35.00  | 35.00  |
| Cystin           | 3.00   | 3.00   | 3.00   | 3.00   |
| Choline bitartrate | 2.50  | 2.50   | 2.50   | 2.50   |
| tertiarly Butylhydroquinone | 0.01 | 0.01 | 0.01 | 0.01 |
| Cholesterol      | 0.00   | 0.00   | 5.00   | 5.00   |
| Sodium cholate   | 0.00   | 0.00   | 2.50   | 2.50   |
| FPs              | 0.00   | 100.00 | 0.00   | 100.00 |
| Total            | 1,000.01 | 1,000.01 | 1,000.01 | 1,000.01 |

Notes:

¹ AIN-93G vitamin mixture.
² AIN-93G mineral mixture.

FPs, fermented products prepared from sweet potato-shochu distillery by-product; CO, control diet; CO + FPs, control diet containing 10% FPs; HC, high-cholesterol diet including 0.50% cholesterol and 0.25% sodium cholate. HC + FPs: high-cholesterol diet containing 10% FPs.
conducting in accordance with the Guide for the Care and Use of Laboratory Animals of the University of Miyazaki and in compliance with the Law Concerning the Protection and Control of Animals (Japan Law No. 105), Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain (Notification no. 88 of the Ministry of the Environment, Japan), and The Guidelines for Animals Experimentation (the Japanese Association for Laboratory Animal Science). After an overnight fast at the end of the feeding period, blood was collected from the heart under a triple anesthesia mix of 0.75 mg/kg medetomidine hydrochloride (Domitor; Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan), 4.0 mg/kg midazolam (Dormicum; Astellas Pharma Inc., Tokyo, Japan), and 5.0 mg/kg butorphanol tartrate (Betorphal; Meiji Seika Pharma, Co., Ltd., Tokyo, Japan) and the mice were euthanized. Plasma was collected after centrifugation (2,000×g for 30 min at 4 °C) and stored at −80 °C until analysis. Epididymal/perirenal white adipose tissues and the livers were harvested, rinsed in saline, weighed, corrected by weight of each animal, frozen in liquid nitrogen, and stored at −80 °C.

**Biochemical analyses of plasma and liver samples**

Plasma TG and TC levels, and aspartate aminotransferase (AST) and alanine transaminase (ALT) activities were measured using the TG E-test, cholesterol E-test and transaminase CII-test according to the manufacturer’s instructions (each from Wako), respectively.

The liver contents of TG and TC were measured after lipid extraction (Fujii et al., 2019). Briefly, liver tissue samples (100 mg) were taken in TM-626S tubes (TOMY SEIKO Co., Ltd., Tokyo, Japan). After adding four tablets of zirconia beads (Φ2.0) and 1 mL of phosphate-buffered saline to the tubes, the samples were homogenized (1,430×g for 3 min at 4 °C) by a beads cell disruptor Micro Smash MS-100R (TOMY SEIKO Co., Ltd., Tokyo, Japan). The homogenized cell suspension samples (800 µL) were transferred to test tubes containing 3.2 mL of chloroform-methanol (2:1) and mixed by vortexing for 2–3 min. After the samples were centrifuged (1,000×g for 1 min), the underlayers were collected into spitz tubes. The collected samples were dried using nitrogen gas in a water bath at 37 °C and dissolved with 2-propanol containing 5% (v/v) Tween-20.

**Western blotting**

The liver tissue samples were homogenized in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 30 mM Na₃P₂O₇, and 2% (v/v) Triton X-100 along with a protease inhibitor cocktail (Nacalai tesque, Inc., Kyoto, Japan). The protein concentration was determined using a BCA protein assay kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Equal amounts of protein (20 µg) were loaded onto 4–12% NuPAGE Bis-Tris gels (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and then transferred to PVDF membrane Power Blotter Select Transfer Stacks (Pore size: 0.2 µm, Thermo Fisher Scientific, Inc., Waltham, MA, USA) by electrophoretic transfer with 3-(N-morpholino) propanesulfonic acid running buffer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Subsequently, nonspecific binding sites were blocked with Blocking One-P (Nacalai tesque, Inc., Kyoto, Japan) for 30 min at 25 °C and the blots were incubated overnight at 4 °C. Next, the membrane was treated with antibodies against sterol.
regulatory element-binding protein-2 (SREBP-2, ab30682, 1:1,000), 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR, ab174830, 1:1,000), liver X receptor α (LXRα, ab176323, 1:2,000), cholesterol 7 alpha-hydroxylase (Cyp7a1, ab65596, 1:1,000), β-actin (ab8226, 1:5,000) (all purchased from Abcam plc (Cambridge, UK)), and microtubule-associated protein 1 light chain 3 (LC3-I/-II, 12,741, 1:1,000) purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). After washing three times, the antigen-antibody complexes were visualized for 1 h at 25 °C with anti-rabbit IgG-HRP (Abcam plc, ab6721, 1:2,000) or anti-mouse IgG-HRP (Abcam plc, ab6789, 1:5,000). Signals were visualized using Pierce ECL western blotting substrate (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Subsequently, bands were quantified using the chemiluminescent imaging system, WSE-6300 LuminoGraph III (ATTO Corporation, Tokyo, Japan). Expression values were obtained as relative expression levels of the target proteins (SREBP-2, HMGR, LC3-I/-II, LXR and Cyp7a1) normalized using a corresponding β-actin as an internal control with image analysis software CS Analyzer 4 (ATTO Corporation, Tokyo, Japan).

Fecal cholesterol analysis
Freeze-dried feces were homogenized with dry ice using a laboratory crusher LAB MILL OML-1 (Osaka Chemical Co., Ltd., Osaka, Japan). Fecal cholesterol levels were analyzed by gas chromatography (GC) according to a previous report (Li et al., 2015). The crushed feces (100 mg) were transferred to 1.5 mL tubes and dissolved with 1 mL of methanol, followed by ultrasonic extraction for 30 min and centrifugation at 100×g for 10 min at 4 °C. The supernatants obtained (200 µL) were collected into spitz tubes and extracted with 1 mL of ethyl acetate after addition of 10 µL of an internal standard (100 µg/mL 5α-cholestane), 10 µL of methanol, and 500 µL of distilled water. Next, the samples were mixed using a vortex mixer for 10–20 min and centrifuged at 1,000×g for 10 min. The supernatants were dried under a nitrogen flow at 40 °C, and the residues were reconstituted with 200 µL of N, O-bis(trimethylsilyl) trifluoroacetamide containing 1% trimethylchlorosilane (Sigma-Aldrich, St. Louis, MO, USA) and were derivatized. One aliquot of each sample was used as a sample for GC analysis. The GC instrument consisted of a GC system (GC-2014; Shimadzu Corporation, Kyoto, Japan) and a capillary column SH-Rxi-5Sil MS (30 m × 0.25 mm i.d., thickness: 0.25 µm, Shimadzu Corporation, Kyoto, Japan). The parameters used were as follows: column temperature, 280 °C; injection temperature, 300 °C; detection temperature, 300 °C; carrier gas, helium (0.9 mL/min); injection mode, split (1:50); injection volume, 3.0 µL.

Fecal bile acid analysis
Fecal bile acid (BA) components were analyzed by TechnoSuruga Laboratory Co. Ltd., (Shizuoka, Japan) using liquid chromatography in combination with hybrid quadrupole time-of-flight mass spectrometry (LC-QTOF-MS) according to a previous report (Kakiyama et al., 2014). The feces (100 mg) were transferred to bead tubes and dissolved with 0.9 mL of 50 mM sodium acetate buffer mixed with ethanol (1:3), followed by homogenization and heat treated for 30 min at 85 °C. After centrifugation (19,000×g for
10 min), the supernatant was diluted four times with water and applied to a Bond Elute C\textsubscript{18} cartridge (Agilent Technologies Inc., Santa Clara, CA, USA). BAs were eluted with ethanol (5 mL). After the solvent was evaporated, the residue was dissolved in 1 mL of 50% ethanol. To an aliquot of this solution, 50% ethanol and internal standard (d\textsubscript{4}-cholate and 23-nordeoxycholate, 1 \mu M in 50% ethanol) was added. Precipitated solids were removed by filtration through an Ultrafree-MC LG hydrophilic PTFE filter (UFC30LG00, 0.2 \mu m; Merck Millipore Ltd, Burlington, MA, USA). One aliquot of each sample was used as a sample for LC-QTOF-MS analysis. The LC-QTOF-MS instrument consisted of a Xevo G2-S QTOF (Waters Corporation, Milford, MA, USA) equipped with an electrospray ionization (ESI) probe and Waters ACQUITY UPLC systems (Waters Corporation, Milford, MA, USA). Injection volume was 4 \mu L. An Acquity UPLC BEH C\textsubscript{18} column (150 \times 2.1 mm id, 1.7 \mu m particle size; Waters Corporation, Milford, MA, USA) was employed at 60 °C. For the gradient analysis, the mobile phase A was water with 0.1% formate and the mobile phase B was acetonitrile with 0.1% formate.

The separation was carried out by linear gradient elution at a flow rate of 0.5 mL/min. The gradient elution program of B was 25–35% for 0.5 min, 35–40% for 6.5 min, 40–50% for 4.0 min, 50–95% for 2.0 min, 95% for 1.0 min, 95–25% for 0.1 min and 25% for 4.9 min. The data acquisition was performed in negative ion ESI mode. The desolvation gas was nitrogen, and the collision gas was argon. The desolvation gas flow rate was 1,000 L/h. The source temperature was 150 °C and the desolvation temperature was 450 °C. The capillary voltage was 0.5 kV, cone voltage was 20.0 V. For mass accuracy, leucine-enkephalin was employed as the lock spray solution. The scan time for each function was set at 0.6 s. The data acquisition range was m/z 50–850. Next, we have used the TargetLynx Application Manager, an option with Waters MassLynx 4.1 software.

**SCFA analysis**

Fecal SCFA levels were analyzed by TechnoSuruga Laboratory using GC according to a previous report (García-Villalba et al., 2012). The feces (100 mg) were transferred to bead tubes and suspended in 0.9 mL of water with 0.5% phosphoric acid, followed by heat treated for 30 min at 85 °C and centrifugation (19,000\times g for 10 min). The supernatants obtained (400 \mu L) were collected into tubes. Each milliliter of the supernatant was extracted with the equal amount of ethyl acetate and centrifuged at 19,000\times g for 10 min. Prior to analysis, a 200 \mu L volume of the ethyl acetate phase was transferred into a tube and an internal standard (1 mM 4-methyl valerate) containing 1% formate equivalent to the ethyl acetate phase added. One aliquot of each sample was used as a sample for GC analysis. The GC instrument consisted of a GC system (7890B; Agilent Technologies Inc., Santa Clara, CA, USA), a capillary column DB-WAXetr (30 m \times 0.25 mm i.d., thickness: 0.25 \mu m, Agilent Technologies Inc., Santa Clara, CA, USA) and a guard column DB-WAXetr (5 m \times 0.25 mm i.d., thickness: 0.25 \mu m, Agilent Technologies Inc., Santa Clara, CA, USA). The parameters used were as follows: column temperature, initially 50 °C, then increased to 90 °C at 10 °C/min, to 150 °C at 15 °C/min, to 170 °C at 5 °C/min, and finally to 250 °C at 20 °C/min and kept at this temperature for 4 min; injection temperature, initially 50 °C and finally to 250 °C at 250 °C/min; detection temperature,
250 °C; carrier gas, helium (1.2 mL/min); injection mode, pulsed splitless; injection volume, 1.0 µL.

Gut microbiota analysis
Gut microbiota were analyzed by TechnoSuruga Laboratory. Fecal samples were collected and DNA extraction was performed according previous report (Takahashi et al., 2014). The variable V3–V4 region of the bacterial 16S rDNA was amplified by PCR (Takahashi et al., 2014). Sequencing was conducted using an Illumina Miseq sequencing system (Illumina, San Diego, CA, USA) with MiSeq Reagent Kit version 3 (600 cycle, Illumina, San Diego, CA, USA), according to the manufacturer’s instructions. Overlapping paired-end reads were merged using the fastq-join program (http://code.google.com/p/ea-utils/). Only reads that had quality value scores of ≥20 for more than 99% of the sequence were extracted for further analysis. In addition, chimeric sequences were filtered out by UCHIME algorithm in USEARCH platform which performs reference based detection (USEARCH v6.1.544) (Edgar et al., 2011). The determined 16S rDNA sequences were subjected to homology searching using Metagenome@KIN software (World Fusion Co., Ltd., Tokyo, Japan) against the Ribosomal Database Project MultiClassifier ver. 2.11 (16S rDNA) (confidence: 0.8) for microorganisms identified at the phylum and the genus level (Wang et al., 2007). Furthermore, the relative abundance of each taxonomic classification was calculated from the classification and the number identified.

Statistical analysis
All data are the means ± SE. All statistical analyses were performed using IBM SPSS Statistics version 24.0 (IBM Corporation, Armonk, NY, USA). Statistical analysis between two groups was performed employing an independent t-test. Analyses between four groups were performed by one-way analysis of variance with Tukey’s HSD post hoc test. A p-value < 0.05 was considered significant.

RESULTS
Nutrient composition of FPs
Nutritive values of FPs per dry weight were investigated. FPs comprised 48.3% carbohydrate (37.8% dietary + 10.5% others), 35.8% proteins, 7.9% ash, 4.0% fat, 4.0% moisture and 297.0 kcal/100 g. Similar to previous reports (Kosakai et al., 2019), FPs were rich in dietary fiber such as 16.7% β-glucan and 12.4% chitin-chitosan.

Effect of FPs on growth parameters in mice
There was no significant difference observed between the middle body weights of the CO group and the CO + FPs group; however, final body weights in the CO + FPs group were significantly lower than those in the CO group (Table 2). On the contrary, both the middle and final body weights of the HC + FPs group were significantly lower than that of the HC group, and the effects on body weights were detected earlier in cholesterol loading conditions (Table 2). In order to evaluate the relationship between food intake and
suppression of weight gain, food intake, and feeding efficiency were confirmed. Food intake of the groups that were fed diets containing FPs (CO + FPs and HC + FPs groups) were not significantly changed compared with those of the respective control groups (CO and HC groups). There was no significant difference in feeding efficiency between the CO and the CO + FPs groups, however, indicating that FPs significantly lowered feeding efficiency in the cholesterol loaded conditions (Table 2).

Regarding tissue weights, the weight of liver was not significantly different between the CO group and the CO + FPs group, and was significantly increased in the HC + FPs group compared with those of the HC groups (Table 2). On the contrary, the weight of perirenal and epididymal adipose was significantly reduced in the HC + FPs group compared with that in the HC groups, and that in the CO + FPs group did not.

**Effect of FPs on biochemical parameters in serum and liver**

We analyzed the mice serum as shown in Table 3. The serum TC levels in the CO + FPs group were significantly lower than those in the CO group; conversely the serum TC levels of the HC + FPs group were significantly higher than those of the HC group (Table 3). Especially, FPs significantly increased the serum non-HDL-C levels, and the ratio of non-HDL to HDL was also higher in mice fed cholesterol-loaded diet compared with that of the HC group (Table 3). Also, in the liver, the TC levels were significantly increased only in the HC + FPs group (Table 3). However, serum TG levels were not significantly different between the CO group and the CO + FPs group, and were significantly reduced in the HC + FPs group compared with those of the HC groups (Table 3). Furthermore, the TG levels in the livers of the CO + FP and the HC + FPs group were significantly decreased than those in the livers of the CO and HC groups, respectively (Table 3). The serum AST and ALT activities were significantly higher in the HC + FPs group than those in the other groups (Table 3). There were no significant differences in serum total BA levels and total protein concentration between all the groups (Table 3).

### Table 2 Effect of FPs on growth parameters in mice.

|                     | CO    | CO + FPs | HC    | HC + FPs |
|---------------------|-------|----------|-------|----------|
| Body weight (g)     |       |          |       |          |
| Initial             | 20.88 ± 0.32<sup>a</sup> | 20.91 ± 0.31<sup>a</sup> | 20.68 ± 0.26<sup>a</sup> | 20.80 ± 0.24<sup>a</sup> |
| Middle              | 27.12 ± 0.69<sup>a</sup> | 25.44 ± 0.35<sup>b</sup> | 25.46 ± 0.39<sup>a</sup> | 23.40 ± 0.05<sup>b</sup> |
| Final               | 32.70 ± 0.73<sup>a</sup> | 29.62 ± 0.75<sup>b</sup> | 28.81 ± 0.65<sup>b</sup> | 24.31 ± 0.27<sup>c</sup> |
| Food intake (g/day/100 g-body weight) | 11.09 ± 0.46<sup>a</sup> | 10.95 ± 0.31<sup>a</sup> | 13.06 ± 0.44<sup>b</sup> | 12.67 ± 0.25<sup>b</sup> |
| Feeding efficiency (g/10 g) | 0.59 ± 0.05<sup>a</sup> | 0.48 ± 0.04<sup>b</sup> | 0.39 ± 0.03<sup>b</sup> | 0.20 ± 0.02<sup>c</sup> |
| Liver weight (g/100 g-body weight) | 3.51 ± 0.08<sup>a</sup> | 3.61 ± 0.04<sup>b</sup> | 4.09 ± 0.04<sup>b</sup> | 7.22 ± 0.76<sup>c</sup> |
| Perirenal adipose weight (g/100 g-body weight) | 1.95 ± 0.17<sup>a</sup> | 1.39 ± 0.15<sup>b</sup> | 1.04 ± 0.15<sup>b</sup> | 0.28 ± 0.04<sup>d</sup> |
| Epididymal adipose weight (g/100 g-body weight) | 4.26 ± 0.26<sup>a</sup> | 3.27 ± 0.32<sup>b</sup> | 2.92 ± 0.33<sup>b</sup> | 1.01 ± 0.09<sup>c</sup> |
| Fecal wet weight (g/day) | 0.29 ± 0.01<sup>a</sup> | 0.33 ± 0.01<sup>b</sup> | 0.32 ± 0.01<sup>b</sup> | 0.36 ± 0.01<sup>c</sup> |

**Notes:**

Values represent mean ± SE, n = 7–8. Means in a row followed by differing superscript letters (a, b, c) indicate statistically significant difference, *p* < 0.05.

FPs, fermented products prepared from sweet potato-shochu distillery by-product; CO, control diet; CO + FPs, control diet containing 10% FPs; HC, high-cholesterol diet including 0.50% cholesterol and 0.25% sodium cholate; HC + FPs, high-cholesterol diet containing 10% FPs; Initial, body weight on Day0; Middle, body weight on Day28; Final, body weight on Day56. Fecal wet weight was measured using feces pooled from the 1st to 8th week.
Effect of FPs on the expression of cholesterol- and bile acids-related proteins in the liver

Fermented products did not affect mature SREBP-2, HMGR, LC3-I and -II protein expressions (Fig. 1A). The relative expression levels of LXRα in the HC + FPs group were significantly decreased than in the CO + FPs group, however, there were no significant differences with the addition of FPs than without. The expression levels of Cyp7a1 in the CO + FPs group were significantly lower than those in the CO groups, however, there were no significant differences between in the HC and the HC + FPs group (Figs. 1A and 1B).

Effect of FPs on cholesterol and bile acid excretion into feces

As shown in Table 2, fecal wet weight of the groups that were fed diets containing FPs (CO + FPs and HC + FPs groups) were significantly increased compared with those of the respective control groups (CO and HC groups). The results of the feces pooled from the 7th to 8th week of feeding indicated that the fecal TC levels were not significantly different between the CO and the CO + FPs groups; however, these levels significantly increased in the HC + FPs groups compared with those in the HC group (Table 3). When the BA contents in feces pooled on the day before euthanize were analyzed for the two groups loaded with cholesterol (HC and HC + FPs groups), there were no significant differences between these groups (Table 4). Cholate, which is the primary BA, was significantly increased by FPs administration, but chenodeoxycholate and α-muricholate

| Table 3  | Effect of FPs on serum, hepatic and fecal biochemical parameters in mice fed with different experimental diet. |
|----------|----------------------------------------------------------------------------------------------------------------|
| CO       | CO + FPs                                                   | HC                             | HC + FPs                             |
| Serum    |                                                             |                                |                                    |
| TC (mg/dL)| 123.76 ± 4.66a                                            | 102.76 ± 3.89b                 | 99.93 ± 3.98b                       | 159.43 ± 7.95c                      |
| HDL-C (mg/dL)| 77.11 ± 8.80a                          | 68.76 ± 3.15ab                  | 55.34 ± 2.56bc                      | 38.91 ± 0.73c                       |
| non-HDL-C (mg/dL)| 46.65 ± 4.66a       | 33.99 ± 1.65a                    | 45.59 ± 3.12a                       | 120.52 ± 8.02b                      |
| non-HDL-C/HDL-C | 0.69 ± 0.13a                  | 0.50 ± 0.03a                    | 0.82 ± 0.07a                        | 3.11 ± 0.22b                        |
| TG (mg/dL)| 85.21 ± 7.58a                                            | 88.88 ± 3.04a                   | 58.81 ± 4.87b                       | 33.80 ± 1.65c                       |
| total BA (µmol/L)| 7.24 ± 4.24a                   | 7.97 ± 3.13a                    | 4.73 ± 1.86a                        | 11.22 ± 2.97a                       |
| AST (U/L) | 63.62 ± 8.37a                                            | 96.45 ± 16.72ab                 | 164.67 ± 45.57bc                    | 192.09 ± 38.05b                     |
| ALT (U/L) | 27.45 ± 3.99a                                            | 21.73 ± 4.20a                   | 20.99 ± 3.50a                       | 139.23 ± 27.17b                     |
| Total protein (mg/L)| 45.07 ± 0.61a     | 46.81 ± 1.38a                   | 44.33 ± 1.13a                       | 47.45 ± 0.73a                       |
| Liver    |                                                             |                                |                                    |
| TC (mg/g liver) | 5.48 ± 0.36a                                            | 4.13 ± 0.09b                    | 29.51 ± 2.92b                       | 103.59 ± 8.01c                      |
| TG (mg/g liver) | 99.68 ± 13.91a                           | 62.16 ± 4.51bc                  | 84.82 ± 8.29ab                      | 43.23 ± 4.28c                       |
| Feces    |                                                             |                                |                                    |
| TC (mg/g feces)| 17.88 ± 0.89a                                               | 16.05 ± 0.76a                 | 87.02 ± 5.83b                       | 250.87 ± 13.68c                     |

Notes:
Values represent mean ± SE, n = 7–8. Means in a row followed by differing superscript letters (a, b, c) indicate statistically significant difference, p < 0.05.
FPs, fermented products prepared from sweet potato-shochu distillery by-product; CO, control diet; CO + FPs, control diet containing 10% FPs; HC, high-cholesterol diet including 0.50% cholesterol and 0.25% sodium cholate; HC + FPs, high-cholesterol diet containing 10% FPs; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; non-HDL-C, non-high-density lipoprotein cholesterol; TG, triglyceride; BA, bile acids; AST, aspartate transaminase; ALT, alanine transaminase. Fecal TC was measured using feces pooled from the 7th to 8th week.
were significantly decreased; however, there was no significant difference in β-muricholate levels between the two groups (Table 4). In addition, the secondary BAs such as deoxycholate, lithocholate, ursodeoxycholate, and hyodeoxycholate were significantly reduced (Table 4), but there was no significant difference in taurodeoxycholate and ω-muricolate levels between the two groups. Besides, when the results of BAs composition were separately analyzed from primary and secondary BA, no significant difference was found in primary BA the two groups, but secondary BA was significantly reduced by the addition of FPs (Table 4).

**SCFAs in feces**

Analysis of fecal SCFAs showed that total SCFAs and propionate levels significantly increased in the HC + FPs group than in the CO group, however, there were no significant differences in the CO + FPs and the HC + FPs diet groups compared to their respective
control groups. (Table 5). No significant differences were observed between each groups in fecal acetate. (Table 5). n-Butyrate was significantly increased in the CO + FPs and the HC + FPs groups compared to their respective control groups; furthermore, the n-butyrate levels in the HC + FPs group were significantly higher than those in the CO + FPs group (Table 5).

Gut microbiota
The effects of FPs on intestinal environment were evaluated by analysis of the intestinal flora using pooled feces in the different groups one day before the mice were euthanized. At the
phylum level, FPs reduced the abundance of *Firmicutes* (Fig. 2A). The abundance of *Bacteroidetes* increased with the addition of FPs only in the normal diet group, but did not increase in the cholesterol-loaded diet group (Fig. 2A). The number of *Verrucomicrobia* increased only in the HC + FPs group compared with those of the other groups (Fig. 2A). Detailed analysis at the genus level showed that the abundance of *Clostridium* cluster IV did not change, while that of *Clostridium* cluster XI was significantly higher only in the HC group (composition ratio CO, CO + FPs, and HC + FPs: 0.0%, HC: 12.8%). *Clostridium* cluster XIVa increased in the CO + FPs and the HC + FPs groups (1.5-fold in the HC + FPs group compared with that of the HC group) (Fig. 2B). In loading FPs, the abundance of *Clostridium* cluster IV and XIVa increased 3.7-fold in the CO + FPs group and increased 1.3-fold in the HC + FPs group compared to their respective control groups, respectively. Besides, *Akkermansia* significantly increased only in the HC + FPs groups compared with that of the other groups (Fig. 2B).

**DISCUSSION**

In this study, we reported that FPs were rich in dietary fiber, such as β-glucan and chitin-chitosan (*Kosakai et al., 2019*). Consequently, CO + FPs and HC + FPs diet
contained 1.7% β-glucan and 1.2% chitin-chitosan. Therefore, it is reasonable to attribute the body-fat lowering effect of FPs to these dietary fibers. Our results are similar to those of Miyazawa et al. (2018) who reported that ICR mice fed a diet supplemented with 1.0% chitosan for 6 weeks showed decreased body weight compared with mice fed high-fat diet. It has been reported that dietary chitosan-suppressed weight gain was apparent from the 5th week onward, wherein chitosan prevented intestinal absorption of lipids (Han et al., 2005; Sumiyoshi & Kimura, 2006). Our data agree with this report, in that 5 weeks were required to exert the inhibitory effects of FPs on weight gain. Therefore, at least in part, it is reasonable to explain the suppression of weight gain, which is caused by inhibiting lipid absorption irrespective of cholesterol intake. We also reported that FPs reduced weight gain in normal diet conditions (Kosakai et al., 2019) and also led to a similar decrease in cholesterol-loaded conditions. Therefore, it can be suggested that dietary FPs lowered weight gain by decreasing the weight of adipose tissues in mice fed not only a normal diet but also high-cholesterol diet.

It was indicated that the effect of dietary cholesterol in this animal strain tended to appear in the liver and to be less responsive to the serum parameter (Sehayek et al., 2000), being reasonable for our study. It is reported that dietary chitosan (Liu et al., 2018), and 1,3- or 1,3-1,6-β-glucan derived from yeast (Vetvicka & Vetvickova, 2009) and fungus (Rop, Mlcek & Jurikova, 2009) prevent elevation of serum TC levels in cholesterol-fed animals. Hence, we hypothesized that FPs, being rich in β-glucan and chitin-chitosan, reduce serum TC levels in cholesterol-loaded conditions. It has become necessary to investigate the effects of FPs using modified mice with cholesterol metabolism similar to humans (Hartvigsen et al., 2007; Chan, 2015). Partially, the increased serum TC levels in our study suggest that there are components that offset the effects of dietary fiber and further raise the cholesterol pool. Besides, the raised serum ALT levels indicated the induction of liver injury. It has been reported that dietary chitosan (Liu et al., 2018) and reishi extract (Meneses et al., 2016) cancelled the raised serum AST and ALT levels; however, these results differ from those observed in our studies. Besides, our studies indicated that dietary FPs decreased the hepatic and serum TG levels in cholesterol-loaded conditions. This observation is convincing for us because reishi extract, which is rich in 1,3-β-glucan (Meneses et al., 2016) and chitosan (Miyazawa et al., 2018; Liu et al., 2018) reduced hepatic and serum TG levels, and both are representative fibers present in the FPs of our study.

Hepatic cholesterol levels are strictly maintained through manipulation of the activity of transcriptional factor, SREBP-2, resulting in the modulation of HMGCR expression which is a rate-limiting enzyme for cholesterol synthesis. It has been reported that the expression of SREBP-2 and LC3-II, an autophagy-related protein, are negatively correlated with each other (Kim & Guan, 2011; Eid et al., 2017). Furthermore, LXRα is a nuclear receptor and regulates the expression of Cyp7a1, which is a rate-limiting enzyme for BA synthesis. Although a previous study indicated that an intake of 5% chitosan increases the activities of hepatic Cyp7a1 in Sprague-Dawley rats (Moon et al., 2007), FPs decreased its protein expression instead of showing an increase. Therefore, cholesterol accumulation in FPs group may be explained by insufficient catabolism from cholesterol to BA.
To interpret the present data, information on the type of mouse strains used may be critical factor. It has been reported that cholesterol- and BA metabolism- associated protein expressions do not change by dietary cholesterol load in some mice strains (Khanuja et al., 1995; Lammert et al., 1999). Among the measured proteins, only Cyp7a1 but not others were decreased under dietary cholesterol-loading as also shown in a previous study (Xu et al., 2004). It is important to accumulate information of the effects of FPs on other strains or models to predict their effects on human cholesterol metabolism. We also focused on the excretion of BA and its composition, because chitosan has been reported to bind with BAs and increase their excretion in feces, which is found to be higher than that with cellulose and glucomannan (Gallagher et al., 2000). However, FPs failed to promote BA excretion. The protein expression levels of Cyp7a1 tended to decrease in the HC + FPs group than in the HC group (Fig. 1). As the result, the BA excretion from the liver may be reduced, and BA absorption from the intestinal tract may have been promoted. It was reported that fecal lipid excretion was increased by dietary chitosan and/or glucomannan than that by cellulose in Wistar rats (Gallagher et al., 2000), and water-soluble chitosan bound to cholesterol rather than cellulose (Jin et al., 2017). Therefore, it is reasonable to consider that dietary fiber contained in FPs inhibits lipid absorption and promotes its excretion into feces. In contrast, cholesterol is excreted into the intestinal tract via ABCG5/ABCG8 (Lu, Lee & Patel, 2001). Further studies are needed to clarify the effect of FPs on intestinal ABC transporter function.

Intriguingly, FPs decreased secondary BA excretion; such BAs have been reported to cause DNA damage through ROS production (Ridlon, Kang & Hylemon, 2006; Payne et al., 2007), cell senescence (Friedman, 2008), and liver cancer (Yoshimoto et al., 2013). Several reports revealed that Bacteroides species (Clostridium cluster XI and XIVa and Bacteroidetes fragilis) (Hirano & Masuda, 1982; Brook, 1989; Ridlon et al., 2014; Fiorucci & Distruuti, 2015) are responsible for the dehydrogenation of primary bile, resulting in secondary BA production. Here, Clostridium cluster XI and XIVa were 0.4 times the cumulative amount in the HC + FPs group compared with those in the HC groups, suggesting that gut microbiota that contributed to secondary BA production were present. Changes of the abundance of Clostridium cluster XI and XIVa at the genus level were also in agreement with the results of laminarin (Nguyen et al., 2016), suggesting the effect of dietary fiber intake of FPs.

Gut microbiota produces SCFAs and provides benefits to the host (Lattimer & Haub, 2010). Among them, propionate has an inhibitory effect on cholesterol synthesis (Ide, Okamatsu & Sugano, 1978; Amaral et al., 1992; Lattimer & Haub, 2010). Although FPs promoted propionate level in the feces, FPs failed to inhibit cholesterol accumulation and the protein expression involved in cholesterol synthesis in mice. Therefore, it is considered that upregulation of propionate is not enough to downregulate cholesterol synthesis as indicated in our study. It has been reported that Clostridium cluster IV and XIVa, which are the main component strains of the genus Firmicutes, produce butyrate and greatly contribute to maintain the intestinal immune homeostasis (Atarashi et al., 2013). In our study, dietary FPs increased the abundance of Clostridium cluster IV and XIVa, suggesting that they partially contributed to increase butyrate production.
Among the genus *Akkermansia*, *Akkermansia muciniphila* was the only intestinal bacterium reported to be present; it has been reported to produce acetate and propionate using mucin (*Derrien et al.*, 2004). It was suggested that intestinal bacteria that convert acetate to butyrate are one of the causes of increasing SCFA levels in feces. Since butyrate suppresses inflammation (*Vinolo et al.*, 2011) and has anti-cancer activity (*Canani et al.*, 2011), FPs might be a putative candidate for the inhibition of intestinal inflammation caused by upregulation of SCFAs with subsequent reduction of secondary BAs.

It has also been reported that some intestinal bacteria control host energy metabolism (*Backhed et al.*, 2004; *Velagapudi et al.*, 2010; *Den Besten et al.*, 2013). More *Firmicutes* and less *Bacteroidetes* (*Turnbaugh et al.*, 2006) are reported to be present in obese mice, and *Bacteroidetes* increase and *Firmicutes* decrease in mice fed pectin (*Trompette et al.*, 2014) and laminarin (β-1, 3-glucan) (*Nguyen et al.*, 2016). In our study, the consistency between the suppression of weight gain and modulation of gut microbiota at the phylum level may be suggested to be due to dietary fiber. *Akkermansia* has been shown to be associated with high-fat diets (*Jakobsdottir et al.*, 2013; *Zhong et al.*, 2015). In contrast, there are reports related to anti-obesity (*Everard et al.*, 2013) activities of *Akkermansia*, attracting attention as a useful next-generation microorganism (*Cani & De Vos*, 2017; *Naito, Uchiyama & Takagi*, 2018). Further analysis is necessary to investigate the significance of the increased abundance of *Akkermansia* in this study.

In our study, it is demonstrated that the FPs ingestion significantly reduced fat accumulation but increased the liver and serum cholesterol levels in high-cholesterol diet groups. Furthermore, it is suggested that this action is due to components other than dietary fiber in the diet. Since the effects of FPs change depending on the food composition, we would like to explore conditions that exhibit maximum effects in the future. In addition, it is also suggested that dietary FPs decreased secondary BA and increased SCFA by modulating gut microbiota.

**CONCLUSIONS**

Dietary FPs decreased hepatic and serum TG levels in cholesterol-loaded conditions, while contrary to our hypothesis increasing hepatic and serum TC levels in cholesterol loaded mice, thus suggesting that there are components which offset the effects of dietary fibers in FPs. Moreover, alterations of the gut microbiota accompanied with reduction of secondary BAs and increase of SCFAs were observed in mice fed dietary FPs, which indicated that dietary fiber present in FPs might be responsible for this effect. Further studies are needed to reveal the mechanisms to increase the cholesterol pool by FPs for its utilization as a beneficial food source with anti-obesity effects.

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Toshiki Kosakai, Cho Sho, Kuniaki Kawano, Ken-ichi Iwai and Yoshikazu Takase are salaried employees of Kirishima Shuzo Co. Ltd., and worked on the by-product, SSDB, used in this study. Hirotaka Kato, Kenjiro Ogawa, Kazuo Nishiyama and Masao Yamasaki declare no conflict of interest.

**Author Contributions**
- Toshiki Kosakai conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, authored or reviewed drafts of the paper, wrote the paper.
- Hirotaka Kato performed the experiments, analyzed the data, prepared figures and/or tables.
- Cho Sho conceived and designed the experiments, contributed reagents/materials/analysis tools, approved the final draft.
- Kuniaki Kawano conceived and designed the experiments, contributed reagents/materials/analysis tools, approved the final draft.
- Ken-ichi Iwai conceived and designed the experiments, contributed reagents/materials/analysis tools, approved the final draft.
- Yoshikazu Takase conceived and designed the experiments, contributed reagents/materials/analysis tools, approved the final draft.
- Kenjiro Ogawa contributed reagents/materials/analysis tools, approved the final draft.
- Kazuo Nishiyama contributed reagents/materials/analysis tools, approved the final draft.
- Masao Yamasaki conceived and designed the experiments, contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft.

**Animal Ethics**
The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):
The Animal Experiment Committee of Miyazaki University approved this study (2013-024).
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The following information was supplied regarding data availability:

The raw measurements are available in the Supplemental File.

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REFERENCES
Alberti K, Eckel RH, Grundy SM, Zimmet PZ, Cleeman JI, Donato KA, Fruchart J-C, James WPT, Loria CM, Smith SC Jr. 2009. Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. *Circulation* **120**(16):1640–1645 DOI 10.1161/CIRCULATIONAHA.109.192644.

Amaral L, Morgan D, Stephen A, Whiting S. 1992. Effect of propionate on lipid-metabolism in healthy-human subjects. *FASEB Journal* **6**(5):A1655.

Atarashi K, Tanoue T, Oshima K, Suda W, Nagano Y, Nishikawa H, Fukuda S, Saito T, Narushima S, Hase K. 2013. Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. *Nature* **500**(7461):232–236 DOI 10.1038/nature12331.

Backhed F, Ding H, Wang T, Hooper LV, Koh GY, Nagy A, Semenkovich CF, Gordon JI. 2004. The gut microbiota as an environmental factor that regulates fat storage. *Proceedings of the National Academy of Sciences of the United States of America* **101**(44):15718–15723 DOI 10.1073/pnas.0407076101.

Blix G. 1948. The determination of hexosamines according to Elson and Morgan. *Acta Chemica Scandinavica* **2**:467–473 DOI 10.3891/acta.chem.scand.02-0467.

Bo T, Shao S, Wu D, Niu S, Zhao J, Gao L. 2017. Relative variations of gut microbiota in disordered cholesterol metabolism caused by high-cholesterol diet and host genetics. *Microbiologyopen* **6**(4):e00491 DOI 10.1002/mbo3.491.

Brook I. 1989. Pathogenicity of the *Bacteroides fragilis* group. *Annals of Clinical & Laboratory Science* **19**(5):360–376.

Caesar R, Nygren H, Orešič M, Bäckhed F. 2016. Interaction between dietary lipids and gut microbiota regulates hepatic cholesterol metabolism. *Journal of Lipid Research* **57**(3):474–481 DOI 10.1194/jlr.M065847.

Canani RB, Di Costanzo M, Leone L, Pedata M, Meli R, Calignano A. 2011. Potential beneficial effects of butyrate in intestinal and extraintestinal diseases. *World Journal of Gastroenterology* **17**(12):1519 DOI 10.3748/wjg.v17.i12.1519.

Cani PD, De Vos WM. 2017. Next-generation beneficial microbes: the case of *Akkermansia muciniphila*. *Frontiers in Microbiology* **8**:1765 DOI 10.3389/fmicb.2017.01765.

Chan J. 2015. Animal models of diet-induced *Hypercholesterolemia*. In: Karere GM, Cox LA, Vandelberg JL, eds. *Hypercholesterolemia*. Rijeka: IntechOpen.

DeBoer MD, Gurka MJ, Morrison JA, Woo JG. 2016. Inter-relationships between the severity of metabolic syndrome, insulin and adiponectin and their relationship to future type 2 diabetes and cardiovascular disease. *International Journal of Obesity* **40**(9):1353–1359 DOI 10.1038/ijo.2016.81.
Den Besten G, Van Eunen K, Groen AK, Venema K, Reijngoud D-J, Bakker BM. 2013. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *Journal of Lipid Research* 54(9):2325–2340 DOI 10.1194/jlr.R036012.

Derrien M, Vaughan EE, Pluge CM, De Vos WM. 2004. *Akkermansia muciniphila* gen. nov., sp. nov., a human intestinal mucin-degrading bacterium. *International Journal of Systematic and Evolutionary Microbiology* 54(5):1469–1476 DOI 10.1099/ijs.0.02873-0.

Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. 2011. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 27(16):2194–2200 DOI 10.1093/bioinformatics/btr381.

Eid W, Dauner K, Courtney KC, Gagnon A, Parks RJ, Sorisky A, Zha X. 2017. mTORC1 activates SREBP-2 by suppressing cholesterol trafficking to lysosomes in mammalian cells. *Proceedings of the National Academy of Sciences of the United States of America* 114(30):7999–8004 DOI 10.1073/pnas.1705304114.

Everard A, Belzer C, Geurts L, Ouwerkerk JP, Druart C, Bindels LB, Guiot Y, Derrien M, Muccioli GG, Delzenne NM, de Vos WM, Cani PD. 2013. Cross-talk between *Akkermansia muciniphila* and intestinal epithelium controls diet-induced obesity. *Proceedings of the National Academy of Sciences of the United States of America* 110(22):9066–9071 DOI 10.1073/pnas.1219451110.

Fiorucci S, Distrutti E. 2015. Bile acid-activated receptors, intestinal microbiota, and the treatment of metabolic disorders. *Trends in Molecular Medicine* 21(11):702–714 DOI 10.1016/j.molmed.2015.09.001.

Friedman SL. 2008. Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver. *Physiological Reviews* 88(1):125–172 DOI 10.1152/physrev.00013.2007.

Fujii K, Ota Y, Nishiyaama K, Kunitake H, Yamasaki Y, Tari H, Araki K, Arakawa T, Yamasaki M. 2019. Blueberry leaf polyphenols prevent body fat accumulation in mice fed high-fat, high-sucrose diet. *Journal of Oleo Science* 68(5):471–479 DOI 10.5650/jos.ess18226.

Furuta Y, Maruoka N, Nakamura A, Omori T, Sonomoto K. 2008. Utilization of fermented barley extract obtained from a by-product of barley *shochu* for nisin production. *Journal of Bioscience and Bioengineering* 106(4):393–397 DOI 10.1263/jbb.106.393.

Gallaher CM, Munion J, Hesslink R Jr, Wise J, Gallaher DD. 2000. Cholesterol reduction by glucomannan and chitosan is mediated by changes in cholesterol absorption and bile acid and fat excretion in rats. *Journal of Nutrition* 130(11):2753–2759 DOI 10.1093/jn/130.11.2753.

García-Villalba R, Giménez-Bastida JA, García-Conesa MT, Tomás-Barberán FA, Carlos Espín J, Larrosa M. 2012. Alternative method for gas chromatography-mass spectrometry analysis of short-chain fatty acids in faecal samples. *Journal of Separation Science* 35(15):1906–1913 DOI 10.1002/jssc.201101121.

Grundy SM. 2016. Metabolic syndrome update. *Trends in Cardiovascular Medicine* 26(4):364–373 DOI 10.1016/j.tcm.2015.10.004.

Hamajima H, Matsunaga H, Fujikawa A, Sato T, Mitsutake S, Yanagita T, Nagao K, Nakayama J, Kitagaki H. 2016. Japanese traditional dietary fungus *Aspergillus oryzae* functions as a prebiotic for *Blautia coccoides* through glycosylceramide: Japanese dietary fungus koji is a new prebiotic. *SpringerPlus* 5(1):1321 DOI 10.1186/s40064-016-2950-6.
Han L-K, Zheng Y-N, Yoshikawa M, Okuda H, Kimura Y. 2005. Anti-obesity effects of chikusetsusaponins isolated from Panax japonicus rhizomes. BMC Complementary and Alternative Medicine 5(1):9 DOI 10.1186/1472-6882-5-9.

Hartvigsen K, Binder CJ, Hansen LF, Rafia Å, Juliano J, Hörkkö S, Steinberg D, Palinski W, Witzum JL, Li AC. 2007. A diet-induced hypercholesterolemic murine model to study Atherogenesis without obesity and metabolic syndrome. Arteriosclerosis, Thrombosis, and Vascular Biology 27(4):878–885 DOI 10.1161/01.ATV.0000258790.35810.02.

Hashidume T, Kato A, Tanaka T, Miyoshi S, Itoh N, Nakata R, Inoue H, Oikawa A, Nakai Y, Shimizu M, Inoue J, Sato R. 2016. Single ingestion of soy β-conglycinin induces increased postprandial circulating FGF21 levels exerting beneficial health effects. Scientific Reports 6(1):28183 DOI 10.1038/srep28183.

Hirano S, Masuda N. 1982. Enhancement of the 7 alpha-dehydroxylase activity of a gram-positive intestinal anaerobe by Bacteroides and its significance in the 7-dehydroxylation of ursodeoxycholic acid. Journal of Lipid Research 23(8):1152–1158.

Ide T, Okamatsu H, Sugano M. 1978. Regulation by dietary fats of 3-hydroxy-3-methylglutaryl-coenzyme a reductase in rat liver. Journal of Nutrition 108(4):601–612 DOI 10.1093/jn/108.4.601.

Ishibashi G. 2007. Effects of the kokuto (brown sugar)-shochu lees on the cholesterol metabolism in rat. Journal for the Integrated Study of Dietary Habits 18(3):277–282 DOI 10.2740/jisdh.18.277.

Ishibashi K, Miura NN, Adachi Y, Tamura H, Tanaka S, Ohno N. 2004. The solubilization and biological activities of Aspergillus β-(1→3)-D-glucan. FEMS Immunology & Medical Microbiology 42(2):155–166 DOI 10.1016/j.femsim.2004.04.004.

Jakobsdottir G, Xu J, Molin G, Ahrné S, Nyman M. 2013. High-fat diet reduces the formation of butyrate, but increases succinate, inflammation, liver fat and cholesterol in rats, while dietary fibre counteracts these effects. PLOS ONE 8(11):e80476 DOI 10.1371/journal.pone.0080476.

Jin Q, Yu H, Wang X, Li K, Li P. 2017. Effect of the molecular weight of water-soluble chitosan on its fat-/cholesterol-binding capacities and inhibitory activities to pancreatic lipase. PeerJ 5(143):e3279 DOI 10.7717/peerj.3279.

Kakiyama G, Muto A, Takei H, Nittono H, Murai T, Kurosawa T, Hofmann AF, Pandak WM, Bajaj JS. 2014. A simple and accurate HPLC method for fecal bile acid profile in healthy and cirrhotic subjects: validation by GC-MS and LC-MS. Journal of Lipid Research 55(5):978–990 DOI 10.1194/jlr.D047506.

Kamizono T, Nakashima K, Ohtsuka A, Hayashi K. 2010. Effects of feeding hexane-extracts of a shochu distillery by-product on skeletal muscle protein degradation in broiler chicken. Bioscience, Biotechnology, and Biochemistry 74(1):92–95 DOI 10.1271/bbb.90587.

Kawano K, Morimura S, Fujiwara T, Okuno H, Takase Y, Kida K. 2008. Production of vinegar from sweetpotato cv. murasakimasari shochu distillery wastewater and rice koji. Journal of the Brewing Society of Japan 103(4):301–307 DOI 10.6013/jbrewsocjapan1988.103.301.

Khanuja B, Cheah YC, Hunt M, Nishina PM, Wang DQ, Chen HW, Billheimer JT, Carey MC, Paigen B. 1995. Lith1, a major gene affecting cholesterol gallstone formation among inbred strains of mice. Proceedings of the National Academy of Sciences of the United States of America 92(17):7729–7733 DOI 10.1073/pnas.92.17.7729.

Kim J, Guan K-L. 2011. Regulation of the autophagy initiating kinase ULK1 by nutrients: roles of mTORC1 and AMPK. Cell Cycle 10(9):1337–1338 DOI 10.4161/cc.10.9.15291.
Kosakai T, Nobetsu R, Sho C, Kawano K, Iwai K, Takase Y, Nishiyama K, Yamasaki M. 2019. Novel fermented products made from sweet potato-shochu distillery by-products reduces body fat and serum cholesterol in mice. *Journal of the Brewing Society of Japan* 114(5):294–301.

Lammert F, Wang DQ, Paigen B, Carey MC. 1999. Phenotypic characterization of Lith genes that determine susceptibility to cholesterol cholelithiasis in inbred mice: integrated activities of hepatic lipid regulatory enzymes. *Journal of Lipid Research* 40(11):2080–2090.

Lattimer JM, Haub MD. 2010. Effects of dietary fiber and its components on metabolic health. *Nutrients* 2(12):1266–1289 DOI 10.3390/nu2121266.

Li X-Y, Zhao Z-X, Huang M, Feng R, He C-Y, Ma C, Luo S-H, Fu J, Wen B-Y, Ren L, Shou J-W, Guo F, Chen Y, Gao X, Wang Y, Jiang J-D. 2015. Effect of Berberine on promoting the excretion of cholesterol in high-fat diet-induced hyperlipidemic hamsters. *Journal of Translational Medicine* 13(1):278 DOI 10.1186/s12967-015-0629-3.

Liu S-H, Chiu C-Y, Shi C-M, Chiang M-T. 2018. Functional comparison of high and low molecular weight chitosan on lipid metabolism and signals in high-fat diet-fed rats. *Marine Drugs* 16(8):251 DOI 10.3390/md16080251.

Lu K, Lee M-H, Patel SB. 2001. Dietary cholesterol absorption; more than just bile. *Trends in Endocrinology & Metabolism* 12(7):314–320 DOI 10.1016/S1043-2760(01)00433-7.

Meneses ME, Martínez-Carrera D, Torres N, Sánchez-Tapia M, Aguilar-López M, Morales P, Sobal M, Bernabé T, Escudero H, Granados-Portillo O, Tovar AR. 2016. Hypocholesterolemic Properties and Prebiotic Effects of Mexican *Ganoderma lucidum* in C57BL/6 Mice. *PLOS ONE* 11(7):e0159631 DOI 10.1371/journal.pone.0159631.

Miyamoto J, Watanabe K, Taira S, Kasubuchi M, Li X, Irie J, Itoh H, Kimura I. 2018. Barley β-glucan improves metabolic condition via short-chain fatty acids produced by gut microbial fermentation in high fat diet fed mice. *PLOS ONE* 13(4):e0196579 DOI 10.1371/journal.pone.0196579.

Miyazawa N, Yoshimoto H, Kurihara S, Hamaya T, Eguchi F. 2018. Improvement of diet-induced obesity by ingestion of mushroom chitosan prepared from *Flammulina velutipes*. *Journal of Oleo Science* 67(2):245–254 DOI 10.5650/jos.ess17159.

Moon M-S, Lee M-S, Kim C-T, Kim Y. 2007. Dietary chitosan enhances hepatic CYP7A1 activity and reduces plasma and liver cholesterol concentrations in diet-induced hypercholesterolemia in rats. *Nutrition Research and Practice* 1(3):175–179 DOI 10.4162/nrp.2007.1.3.175.

Mukai N, Morimoto T, Fuke N, Yoshida S, Kumazaki T, Teramoto T, Uesako D, Sato R, Nishibori N, Kanai M, Yamada O, Fujii T. 2017. Investigation of the content of nutrients and functional ingredients in shochu distillation residue. *Journal of the Brewing Society of Japan* 112(10):695–706.

Naito Y, Uchiyama K, Takagi T. 2018. A next-generation beneficial microbe: *Akkermansia muciniphila*. *Journal of Clinical Biochemistry and Nutrition* 63(1):33–35 DOI 10.3164/jcbn.18.57.

Nguyen SG, Kim J, Guevarra RB, Lee J-H, Kim E, Kim S, Unno T. 2016. Laminarin favorably modulates gut microbiota in mice fed a high-fat diet. *Food & Function* 7(10):4193–4201 DOI 10.1039/C6FO00929H.

Nohara T, Uechi S, Ogura G, Hayashi K, Sunagawa K. 2010. Effect of Awamori lees on growth, serum and liver lipid concentrations in rats fed a high cholesterol diet. *Nippon Shokuhin Kagaku Kaishi* 57(3):93–99 DOI 10.3136/nskkk.57.93.

Payne CM, Weber C, Crowley-Skillicorn C, Dvorak K, Bernstein H, Bernstein C, Holubec H, Dvorakova B, Garewal H. 2007. Deoxycholate induces mitochondrial oxidative stress and
activates NF-κB through multiple mechanisms in HCT-116 colon epithelial cells. *Carcinogenesis* 28(1):215–222 DOI 10.1093/carcin/bgl139.

Ridlon JM, Kang DJ, Hylemon PB. 2006. Bile salt biotransformations by human intestinal bacteria. *Journal of Lipid Research* 47(2):241–259 DOI 10.1194/jlr.R500013-JLR200.

Ridlon JM, Kang DJ, Hylemon PB, Bajaj JS. 2014. Bile acids and the gut microbiome. *Current Opinion in Gastroenterology* 30(3):332–338 DOI 10.1097/MOG.000000000000057.

Rop O, Mlcek J, Jurikova T. 2009. Beta-glucans in higher fungi and their health effects. *Nutrition Reviews* 67(11):624–631 DOI 10.1111/j.1753-4887.2009.00230.x

Sameshima Y. 2004. Technical changes of shochu, and the subject in the 21th century. *Journal of the Brewing Society of Japan* 99(7):495–500 DOI 10.6013/jbrewsocjapan1988.99.495.

Sasaki T, Abe M, Nakayama S, Moriyama K, Tahara H, Takita T. 2005. Novel application of shochu distillery by-products to prophylaxis against mammary carcinogenesis induced by 7,12-dimethylbenz[a]anthracene in rats. *Bioscience, Biotechnology, and Biochemistry* 69(10):1898–1904 DOI 10.1271/bbb.69.1898.

Sehayek E, Shefer S, Nguyen LB, Ono JG, Merkel M, Breslow JL. 2000. Apolipoprotein E regulates dietary cholesterol absorption and biliary cholesterol excretion: studies in C57BL/6 apolipoprotein E knockout mice. *Proceedings of the National Academy of Sciences of the United States of America* 97(7):3433–3437 DOI 10.1073/pnas.97.7.3433.

Sho C, Kurata R, Okuno H, Takase Y, Yoshimoto M. 2008. Characteristics of breads made with sweet potato-derived shochu distillery by-product supernatants. *Nippon Shokuhin Kagaku Kagaku Kaishi* 55(6):287–292 DOI 10.3136/nskkk.55.287.

Slavin J. 2013. Fiber and prebiotics: mechanisms and health benefits. *Nutrients* 5(4):1417–1435 DOI 10.3390/nu5041417.

Spiegelman BM, Flier JS. 2001. Obesity and the regulation of energy balance. *Cell* 104(4):531–543 DOI 10.1016/S0092-8674(01)00240-9.

Takahashi S, Tomita J, Nishioka K, Hisada T, Nishijima M. 2013. Development of a prokaryotic universal primer for simultaneous analysis of bacteria and archaea using next-generation sequencing. *PLOS ONE* 9(8):e105592 DOI 10.1371/journal.pone.0105592.

Trompette A, Gollwitzer ES, Yadava K, Sichelstiel AK, Sprenger N, Ngom-Bru C, Blanchard C, Junt T, Nicod LP, Harris NL. 2014. Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis. *Nature Medicine* 20(2):159–166 DOI 10.1038/nm.3444.

Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JJ. 2006. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 444(7122):1027–1031 DOI 10.1038/nature05414.

Velagapudi VR, Hezaveh R, Reigstad CS, Gopalacharyulu P, Yetukuri L, Islam S, Felin J, Perkins R, Borén J, Orešič M, Bäckhed F. 2010. The gut microbiota modulates host energy and lipid metabolism in mice. *Journal of Lipid Research* 51(5):1101–1112 DOI 10.1194/jlr.M002774.
Vetvicka V, Vetvickova J. 2009. Effects of yeast-derived β-glucans on blood cholesterol and macrophage functionality. *Journal of Immunotoxicology* 6(1):30–35 DOI 10.1080/15476910802604317.

Vinolo MA, Rodrigues HG, Nachbar RT, Curi R. 2011. Regulation of inflammation by short chain fatty acids. *Nutrients* 3(10):858–876 DOI 10.3390/nu3100858.

Wang Q, Garrity GM, Tiedje JM, Cole JR. 2007. Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology* 73(16):5261–5267 DOI 10.1128/AEM.00062-07.

Watanabe S, Takahashi T, Ogawa H, Uehara H, Tsunematsu T, Baba H, Morimoto Y, Tsumeyama K. 2017. Daily coffee intake inhibits pancreatic beta cell damage and nonalcoholic steatohepatitis in a mouse model of spontaneous metabolic syndrome, tsunuma-suzuki obese diabetic mice. *Metabolic Syndrome and Related Disorders* 15(4):170–177 DOI 10.1089/met.2016.0114.

Xu G, Müller O, Stange EF, Fuchs M. 2004. Impaired regulation of sterol regulatory element binding protein 2 in cholesterol gallstone-susceptible mice. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease* 1688(3):274–279 DOI 10.1016/j.bbadis.2004.01.001.

Yokoi H, Aratake T, Nishio S, Hirose J, Hayashi S, Takasaki Y. 1998. Chitosan production from shochu distillery wastewater by funguses. *Journal of Fermentation and Bioengineering* 85(2):246–249 DOI 10.1016/S0922-338X(97)86777-3.

Yoshimoto M, Kurata-Azuma R, Fujii M, Hou D-X, Ikeda K, Yoshidome T, Osako M. 2004. Phenolic composition and radical scavenging activity of sweetpotato-derived shochu distillery by-products treated with koji. *Bioscience, Biotechnology, and Biochemistry* 68(12):2477–2483 DOI 10.1271/bbb.68.2477.

Yoshimoto S, Loo TM, Atarashi K, Kanda H, Sato S, Oyadomari S, Iwakura Y, Oshima K, Morita H, Hattori M, Honda K, Ishikawa Y, Hara E, Ohtani N. 2013. Obesity-induced gut microbial metabolite promotes liver cancer through senescence secretome. *Nature* 499(7456):97–101 DOI 10.1038/nature12347.

Zhong Y, Marungruang N, Fåk F, Nyman M. 2015. Effects of two whole-grain barley varieties on caecal SCFA, gut microbiota and plasma inflammatory markers in rats consuming low- and high-fat diets. *British Journal of Nutrition* 113(10):1558–1570 DOI 10.1017/S0007114515000793.