Acid-treated high-amylose corn starch suppresses high-fat diet-induced steatosis

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ABSTRACT

Resistant starch (RS) has been reported to improve steatosis as well as obesity. Type 4 resistant starch (RS4), a chemically modified starch, is particularly hard to digest and suggesting higher efficacy. However, because the effects of RS4 on steatosis are not yet fully understood, the effects of RS4 on steatosis were examined using a murine high-fat diet model. Seven-week-old male mice were divided into three groups and fed a normal diet, a high-fat diet (HFD), or a high-fat diet with added RS (HFD+RS). Amylofiber SH® produced from acid-treated corn starch was used as the dietary RS. At 22 weeks old, hepatic steatosis and short chain fatty acid (SCFA) content and gut microbiota in cecum stool samples were analyzed. The ratio of body weight to 7 weeks was significantly suppressed in the HFD+RS group compared to the HFD group (132.2 ± 1.4 % vs. 167.2 ± 3.9 %, p = 0.0076). Macroscopic and microscopic steatosis was also suppressed in the HFD+RS group. Analysis of cecum stool samples revealed elevated SCFA levels in the HFD+RS group compared with the HFD group. Metagenome analysis revealed that *Bifidobacterium* (17.9 ± 1.9 % vs. 3.6 ± 0.7 %, p = 0.0019) and *Lactobacillus* (14.8 ± 3.4 % vs. 0.72 ± 0.23 %, p = 0.0045), which degrade RS to SCFA, were more prevalent in the HFD+RS group than the HFD group. In conclusion, RS4 suppressed steatosis, and increased *Bifidobacterium* and *Lactobacillus*, and SCFAs. RS4 may prevent steatosis by modulating the intestinal environment.

(Word count: 247)

Kew words: RS4, steatosis, SCFA, microbiota, high fat diet

1. INTRODUCTION

Obesity and related diseases, such as type 2 diabetes, hypertension, cardiovascular disease, and some cancers, have been common problems worldwide because Western-style meals have become more popular and the calorie intake from food is increasing (Bleich et al., 2008). Steatosis is a condition in which fat (triglycerides) is deposited in hepatocytes, and fatty liver disease is a general term for liver damage caused by steatosis. This fatty liver disease is largely classified into alcoholic and non-alcoholic. And non-alcoholic fatty liver disease (NAFLD) is the liver's expression of an obesity-related disease (Marchesini et al., 2003). NAFLD is strongly related to liver cirrhosis and cancer and is becoming the leading cause of liver cancer in Western countries. NAFLD is expected to become the most frequent indication for liver transplantation by 2030 (Byrne & Targher, 2015). Therefore, efforts to suppress the development of fatty liver is a major issue worldwide.

It has been reported that the gut microbiota in patients with obesity and NAFLD was changed from that of healthy individuals, and the changes in the gut microbiota can cause various obesity-related diseases and make steatosis worse (Ley et al., 2006, Miele et al., 2009). Low dietary fiber intake is associated with higher rates of obesity, and dietary fiber supplementation has been reported to change the gut microbiota and improve obesity (Mayengbam et al., 2019).

Short-chain fatty acids (SCFAs) such as acetic acid, propionic acid, and butyric acid are produced from dietary fiber decomposed by the gut microflora. Then, SCFAs are absorbed in the small intestine and used as an energy source (Wolever et al., 1989). It has been also reported that obesity and fatty liver caused by HFD can be suppressed by oral intake of SCFA (Lu et al., 2016, Shimizu et al., 2019). Resistant starch (RS) is a type of

75 starch that is not easily digested or absorbed in the small intestine. RS is a starch, but like
76 dietary fiber, it is decomposed by the gut microflora into SCFA (Pelpolage et al., 2020).
77 Amylofiber SH® (J-Oil Mills Inc., Tokyo, Japan) is one of the RS, and is extracted from corn
78 with a high amylose content and is acid-treated to further increase the RS content. It is
79 a type of chemically modified starch subgrouped into type 4 resistant starch (RS4). Many
80 studies have reported inhibitory effect of RS on obesity and steatosis, but most of studies
81 evaluating the effects of RS on suppressing fatty liver are using type 2 resistant starch
82 (RS2). In addition, although limited reports have shown that RS4 is more effective than
83 RS2 in suppressing high-fat diet (HFD)-induced obesity (31.0 ± 0.4g vs. 33.1 ± 1.0g, p
84 <0.05) after 24 weeks of treatment (Shimotoyodome et al., 2010), there are very few
85 studies using RS4, which has a higher resistant starch content and is less digestible than
86 RS2. That is the reason, the inhibitory effects of RS4 on fatty liver are still not fully
87 understood. Therefore, in this study, we examined the inhibitory effects of RS4 on fatty
88 liver using a murine HFD-induced fatty liver model.
89
90 **2. Materials and Methods**
91 **2.1. Diet preparation**
92 Amylofiber SH®, which is extracted from corn with a high amylose content (about 50%–
93 70%) and is acid-treated to increase the RS content, was kindly provided by J-Oil Mills
94 Inc. Amylofiber SH® is an acid-treated high amylose corn starch of the RS4 type
95 (Nagahata et al., 2013). It was prepared by hydrolyzing high amylose corn starch, which
96 is originally RS2 extracted from corn with very high amylose content (the RS content is
97 about 50%), in a hydrochloric acid solution with a concentration of 1.5% for 24 hours at
98 50°C. The yield from performing this hydrolysis is over 100% (about 120-160%), and the

RS content is increased to about 70%. The degree of polymerization (DP) of this Amylofiber SH® is about 60. The control diet was AIN93G, and the diet of high-fat diet with resistant starch (HFD+RS) was made to contain as much RS as possible. It is usually that fatty liver model mice are administered with 60% lipid content HDF for 8 weeks. However, the HFD was prepared by reducing the amount of β-corn starch in AIN93G and adding lard instead in this study. The lipid content that causes steatosis was set at 45%, because previous report showed the steatosis even after 12 weeks of treatment with HDF containing 45% lipid (Duan et al., 2018). Using this HFD as a base, we further reduced the amount of β-corn starch and replaced it with RS to prepare the HFD+RS. The HFD+RS matched the HDF in terms of the total calories and calories of the three macronutrients, which included RS (21.5%). The composition of each diet is shown in Table 1. The diets were prepared by Oriental Yeast Co. Ltd. (Tokyo, Japan).

2.2. Animal experiments

Five-week-old male C57BL/6J mice were purchased from CLEA Japan Inc. (Tokyo, Japan). After 2 weeks of maintenance on a normal diet (CE2), the mice were grouped into three groups with similar weights. Mice in each group (*n* = 10, 2 or 3 mice per cage) were fed the HFD, HFD+RS, or control AIN93G diets from 7 to 22 weeks of age. The mice were weighed weekly. Food intake and stool weight were measured over 3 days at 21 weeks of age. To determine stool weight, stools were collected during the same 2-hour period on 3 days and the weight was measured. At 22 weeks, the mice were killed, and the body weight, white adipose tissue weight, and liver weight were measured. Plasma, liver, and cecum stool samples were collected and stored at −80 °C for further analysis. The plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), total

cholesterol (TC), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C), and triglyceride (TG) levels were measured using an ALT, AST, TC, LDL-C, HDL-C, and TG kits, respectively (Wako Pure Chemical, Osaka, Japan). This study was approved by the Institutional Animal Care and Use Committee of Kobe University (approval numbers: P160505 and P210309).

2.3. Liver histology

Liver tissues were fixed overnight at 4 °C in 10% formaldehyde and embedded in paraffin wax. The paraffin sections were cut (5 μm thick), mounted on glass slides, and stained with hematoxylin and eosin (HE). Steatosis was evaluated as the approximate percentage of hepatocytes with fatty cells labeled with HE staining in a blinded manner.

2.4. Liver RNA extraction and quantitative PCR

Liver tissues were placed in RNAlater (Qiagen, CA, USA) and stored at −80 °C until use. Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, MA, USA), and cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA). Real-time PCR was performed using SYBR Green (Applied Biosystems) and ABI 7500 real-time PCR system (Applied Biosystems). The following primers (forward and reverse, respectively) were used: acetyl-CoA carboxylase (*ACC*), 5'-CTCCCGATTCATAATTGGGTCTG-3', and 5'-TCGACCTTGTTTTACTAGGTGC-3'; fatty acid synthase (*FAS*), 5'-GCGGGTTCGTGAAACTGATAA-3', and 5'-GCAAAATGGGCCTCCTTGATA-3'; sterol regulatory element-binding protein 1 (*SREBP1*), 5'-GCAGCCACCATCTAGCCTG-3', and 5'-CAGCAGTGAGTCTGCCTTGAT-3'; peroxisome proliferator-activated receptor gamma (*PPARγ*), 5'-GGAAGACCACTCGCATTCCTT-3', and 5'-

GTAATCAGCAACCATTGGGTCA-3’; and hypoxanthine phosphoribosyltransferase (*HPRT*), 5’-GTTGGATACAGGCCAGACTTTGTTG-3’, and 5’-CCAGTTTCACTAATGACACAAACG-3’. And the relative expression to *HPRT*, a housekeeping gene, was calculated.

2.5. Immunohistochemistry

Paraffin-embedded liver tissues were deparaffinized with xylene and washed with graded ethanol. The inactivation of endogenous peroxidase was achieved using Peroxidase-Blocking Solution (Dako North America, Inc., CA, USA) for 10 min. These tissue sections were washed with phosphate-buffered saline (PBS) and incubated overnight with anti-Pparγ (1:100; Proteintech, IL, USA) at 4 °C. Following incubation with the secondary antibody, staining with 3,3-diaminobenzidine (DAB; Dako North America, Inc.) was used for visualization and images were captured using a microscope.

2.6. Measurement of SCFA

The fecal SCFA levels were measured by a modified method originally reported by García-Villalba et al. (2012). The sample (0.1 g) was placed in a bead tube and 0.9 ml of 0.5% phosphoric acid solution was added. The tube was mixed and then heat-treated at 85 °C for 15 min. The sample was cooled after grinding, centrifuged (14,000 × *g* for 10 min), and the supernatant was transferred to a new tube, mixed with an equal volume of ethyl acetate, and centrifuged again (14,000 × *g*, 10 min). The ethyl acetate layer was transferred to a vial and the internal standard (4-methylvaleric acid) was added to generate the sample solution. After extraction the concentration of SCFA in each sample was measured by gas chromatography using the 7890B Gas Chromatography System (Agilent Technologies Inc., CA, USA). Helium was used as the carrier gas at 1.2 mL/min.

The detector temperature was kept at 250°C. The oven temperature program was as follows: 50°C; then 10°C/min to 90°C; 15°C /min to 150°C; 5°C /min to 170°C; 20°C/min to a final temperature of 250°C, held for 4 min. One microliter of extract was injected in the splitless mode. In this study, butyric acid, acetic acid, and propionic acid were detected as the major cecum SFCAs.

2.7. DNA extraction from feces and analysis of microbiota

DNA extraction and amplification of the V3–V4 region of 16*S* rRNA was performed as previously reported (Takahashi et al., 2014, Muyzer et al., 1993, Caporaso et al., 2011, Hisada et al., 2015). The barcoded amplicons were paired-end sequenced using the MiSeq system (Illumina, CA, USA). Chimeric sequences were removed by usearch 6.1. Sequence reads were identified using the RDP Web site (Wang et al., 2007). Taxonomic assignment of sequence reads was performed using Metagenome @ KIN Ver 2.2.1 analysis software (World Fusion, Tokyo, Japan).

2.8. Statistical analysis

Data are presented as mean ± standard error of the mean (SEM) and were evaluated by one-way analysis of variance followed by Tukey's test to evaluate differences between two groups. Values of $p < 0.05$ were considered statistically significant. Analyses were performed using Prism 8 (GraphPad Software Inc., CA, USA),

3. Results

3.1. Body weight and liver weight

We first tested the effects of the RS on obesity by feeding mice with the HFD, HFD+RS,

or control diet, starting at 7 weeks of age. Body weight was recorded weekly. The mean body weights in each groups are plotted in Figure 1A. A difference in body weight was apparent at about 12 weeks of age and became greater over time. At 22 weeks, the percent change in body weight was greater in both the HFD (167.2% ± 3.87%) and HFD+RS (148.8% ± 5.66%) groups than in the control group (132.2% ± 1.43%; both $p < 0.05$). Comparing the HDF+RS and HDF groups, the percent change in body weight was lower in the HDF+RS group than that in the HDF group ($p = 0.0076$; Figure 1B). At 22 weeks of age, the liver weight of the HFD+RS group (1.37 ± 0.047 g) was lower than that of the HFD group (1.62 ± 0.053 g, $p =0.0112$; Figure 1C). Furthermore, the weight of the epididymal fat, the main fat-storing white adipose tissue, was also lower in the HFD+RS group than in the HFD group (1.06 ± 0.11 g vs. 1.73 ± 0.14 g, $p = 0.0003$; Figure 1D). Overall, these data suggest that RS could reduce HFD-induced obesity.

To exclude the possibility that the effects of the HFD+RS on body weight, liver weight, and adipose tissue weight were due to changes in appetite and increased stool weight due to the addition of RS to the diet, we measured the total food intake and stool weight. We found no differences in either food intake (3.15 ± 0.15 vs. 3.13 ± 0.11 g/mouse/day, $p =0.996$) or stool weight (0.19 ± 0.021 vs. 0.20 ± 0.02 g/mouse/2hour, $p =0.983$) between the HFD and HFD+RS groups (Figure 1E,F). However, food intake was significantly different between the control and HFD groups (2.32 ± 0.21 vs. 3.15 ± 0.15 g/mouse/day, $p = 0.0029$) and between the control and HFD+RS groups (2.32 ± 0.21 g/mouse/day vs. 3.13 ± 0.11 g/mouse/day, $p = 0.0037$). These results suggest that the reduction in body weight and fat accumulation in the HFD+RS group is not simply due to changes in food ingestion or defecation.

3.2. Gross and microscopic findings of the liver

To further investigate the effects of RS, we performed histological evaluations of liver tissue. Macroscopically, several specimens in HFD group had whitish areas–suspected steatosis (Figure 2A). However, no such findings were found in the livers of the HFD+RS and control groups. Microscopic observations confirmed the presence of steatosis in the whitish regions of the livers in the HFD group (Figure 2B). When we evaluated the percentage of hepatocytes with fatty deposits by HE staining, moderate fatty liver (defined as 50%–80% of cells containing fatty deposits) was detected in 5 of the 10 liver specimens from the HFD group (Figure 2C). However, fatty liver was not observed in the HFD+RS or control groups. There was a significant difference between the HFD and HFD+RS groups in terms of cells containing fatty deposits (32.5% ± 10.1% vs. 2.5% ± 1.1%, $p = 0.0035$). These results indicate that that RS may suppress the development of fatty liver in mice fed an HFD.

3.3. Blood examinations

Comparing the HFD and HFD+RS groups, we found no significant difference in the AST level (58.3 ± 9.99 vs. 44.8 ± 3.30 IU/L, $p = 0.63$; Figure 3A), but there were significant differences in the ALT (19.1 ± 2.46 vs. 12.0 ± 0.69 IU/L, $p = 0.0115$; Figure 3B). As for cholesterol, the HFD+RS group had significantly lower than the HFD group in the TC level (99. 9 ± 7.88 vs. 165.5 ± 9.45 mg/dL, $p < 0.0001$; Figure 3C), in the LDL-C level (6.5 ± 0.93 vs. 10.2 ± 0.96 mg/dL, $p < 0.0001$; Figure 3D), and in the HDL-C level (58.0 ± 2.81 vs. 76.4 ± 2.15 mg/dL, $p < 0.0001$; Figure 3E). As for TG (Figure 3F), the HFD+RS group was also significantly lower than the HFD group (29.1 ± 5.27 vs. 61.0 ± 8.80 mg/dL, $p = 0.0303$) and even lower than the control group, but not significantly different from the control

group (29.1 ± 5.27 vs. 47.8 ± 9.44 mg/dL, p = 0.4904). Consistent with the histological findings, these blood test data suggest that RS suppresses liver damage associated with steatosis.

3.4. Expression of genes related to fatty acid synthesis and TG accumulation

To investigate the mechanism by which RS reduced obesity and steatosis in mice, we assessed the expression levels of several genes involved in fat accumulation. Real-time PCR was performed using liver tissue samples to examine the gene expression levels of *ACC*, *FAS* and *SREBP1*, which are involved in fatty acid synthesis in the liver (Horton et al., 2002), and *PPARγ*, which is involved in TG accumulation in the fatty liver (Matsusue et al., 2003). Although there were no significant differences in the mRNA expression levels of *ACC* (Figure 4A), *FAS* (Figure 4B) and *SREBP1* (Figure 4C), the relative expression level of *PPARγ* tended to be greater in the HFD group than in the control group (0.99 ± 0.087 vs. 0.66 ± 0.13, p = 0.076; Figure 4D). This suggests that *PPARγ* may play an important role in the development of fatty liver in our experimental setting. Consistent with this role, the gene expression of *PPARγ* tended to be lower in the HFD+RS group than in the HFD group, although the difference was not statistically significant (0.73 ± 0.069 vs. 0.99 ± 0.087, p = 0.1915; Figure 4D).

3.5. Expression of the PPARγ protein

Next, to examine the protein expression of PPARγ among three groups, immunohistochemical analysis was carried out. Nuclear staining of PPARγ was detected in most of hepatocytes with fatty liver as well as normal liver. No significant difference was shown among three groups (Figure 4E).

3.6. Gut microbiota

Because the gut microbiota can influence obesity of the host (Ley et al., 2006, Turnbaugh et al., 2006), we compared the gut microenvironment between the three groups using stools samples. Four samples were randomly selected in each group. First, the components of the gut microbiota were analyzed by 16*S* rRNA sequencing. There were no significant differences in the alpha diversity among the three groups in terms of species richness (Chao1) and evenness (Shannon) (Figure 5A,B). In β-diversity, the distance between the data points for each group is an indicator of the similarity in gut microbiota composition. The data points for each group formed clusters with the data points in roughly equivalent locations, suggesting comparable compositions (Figure 5C). We also analyzed the composition of bacteria at the genus level (Figure 5D). *Bifidobacterium* and *Lactobacillus*, which are known to produce SCFA (LeBlanc et al., 2017), were both present in control group but they were markedly reduced in HFD group but had recovered in the HFD+RS group to a level exceeding that of the control group. The HFD+RS group had significantly greater proportions of *Bifidobacterium* (17.99% ± 1.94% vs. 3.59% ± 0.69%, p = 0.0019) and *Lactobacillus* (14.79% ± 3.44% vs. 0.72% ± 0.23%, p = 0.0045) compared with the HFD group. Moreover, the HFD+RS group tended to have higher proportions of *Bifidobacterium* (17.99% ± 1.94% vs. 14.65% ± 3.30%, p = 0.5613) and *Lactobacillus* (14.79% ± 3.44% vs. 7.54% ± 2.57%, p = 0.1385) compared with the control group, although the differences were not statistically significant (Figure 5E,F). Overall, our data suggest that dietary RS dramatically alters the components of gut microbiota and that RS preferentially increased *Bifidobacterium* and *Lactobacillus*.

3.7. Cecum stool SCFA content

RS is decomposed into SCFAs by gut microbiota (Pelpolage et al., 2020), such as *Bifidobacterium* and *Lactobacillus*, and SCFAs are known to reduce obesity (Shimizu et al., 2019). Six samples were randomly selected in each group. We found that caecum content in the HFD+RS group had significantly greater acetic acid (18.83 ± 1.10 vs. 13.28 ± 1.20 μmol/g, $p = 0.0027$) and propionic acid (3.50 ± 0.45 vs. 1.77 ± 0.16 μmol/g, $p = 0.0159$) levels compared with those in the HFD group (Figure 6A,B). Although butyric acid tended to be greater in the HFD+RS group than in the HFD group (4.00 ± 0.60 vs. 2.98 ± 0.31 μmol/g, $p = 0.5978$), although the difference was not statistically significant (Figure 6C).

4. Discussion

RS is a general term for starch that is not easily digested or absorbed in the small intestine. It was reported that RS improves blood glucose levels by reducing postprandial hyperglycemia and improving insulin responsiveness and promotes weight loss in obese individuals (Hasjim et al., 2010, Kendall et al., 2010). In a recent meta-analysis, RS improved fasting glucose, fasting insulin, insulin resistance, insulin sensitivity, and type 2 diabetes associated with obesity (Wang et al., 2019). Regarding steatosis, RS derived from potato and banana (RS2) decreased hepatic fat accumulation and improved fatty liver (Klingbeil et al., 2019, Rosado et al., 2020).

Amylofiber SH® used in this study is an acid-treated high amylose corn starch of the RS4 type (Nagahata et al., 2013). RS is classified into four categories (RS1–4) based on its chemical properties. Type 1 resistant starch (RS1) is starch that is difficult for digestive enzymes to act on due to the starchy material being encased in an outer skin or shell;

RS2 is starch particles that are themselves resistant to digestion, such as high-amylose corn starch with high amylose content; type 3 resistant starch (RS3) is aged starch that has recrystallized after cooking, such as mashed potatoes; RS4 is a processed starch produced by several different methods, including the addition of ester cross-links between starch molecules using chemical reagents, the addition of chemical constituent groups, and acid hydrolysis and heat treatment (Sajilata et al, 2006). To date, most of studies evaluating the effects of RS on suppressing fatty liver are using type 2 resistant starch (RS2), there are many references showing inhibitory effects of RS2 on obesity and fatty liver. RS4, which is less digestible than RS2, was also shown to elicit greater inhibitory effect on HFD-induced obesity than RS2 (31.0 ± 0.4g vs. 33.1 ± 1.0g, p <0.05) after 24 weeks of treatment (Shimotoyodome et al. 2010). Thus, we investigated the inhibitory effect of RS4 on fatty liver. This study revealed that the fatty liver was less frequent in the HFD+RS group than the HFD group, which is indicative of an inhibitory effect of RS4 on fatty liver.

There are several possible mechanisms for the suppression of fatty liver by RS. Si et al. (2017) performed real-time PCR of liver tissue and reported that RS4 significantly suppressed the expression of genes associated with fatty acid synthesis (*ACC*, *Fads1*, and *SREBP1*) and genes associated with fat accumulation (*PPARγ*), and cholesterol synthesis-related genes (*HMGCR*) compared with RS2. In our study, the expression of *PPARγ* tended to be greater in the HFD group than in the HFD+RS and control groups, but the expression levels of *ACC*, *FAS*, and *SREBP1* did not differ among the three groups. PPARγ, which is expressed mainly in adipocytes, is required for the differentiation of pre-adipocytes to mature adipocytes and is involved in adipocyte fat accumulation (Matsusue et al., 2008). In addition, the gene expression of *PPARγ* is increased in fatty liver, and it is faintly

339 expressed in the normal liver (Matsusue et al., 2003). In our study, PPARγ expression was
340 histologically confirmed in normal liver, and the expression was not significantly different
341 in fatty liver treated with HFD or in liver treated with HFD+RS. These results suggested
342 that RS4 did not directly affect the genes related to fatty acid synthesis and TG
343 accumulation.

344 The improvement in gut dysbiosis is an important mechanism by which RS inhibits
345 fatty liver. In fact, RS4 was previously reported to improve gut dysbiosis (Martínez et al.,
346 2010). Interestingly, in our study, the composition of bacteria in the stool showed an
347 increase in *Bifidobacterium* from an average of 3.59% in the HFD group to 17.99% in the
348 HFD+RS group, and an increase in *Lactobacillus* from 0.72% to 14.79%. As a result, SCFA
349 which was produced by gut bacteria such as *Bifidobacterium* and *Lactobacillus* was
350 increased from an average of 13.28% to 18.83% for acetic acid, from 1.77% to 3.5% for
351 propionic acid, and from 2.98% to 4.00% for butyric acid, respectively. In general, SCFA
352 receptors, such as GPR41 and GPR43, are involved in the mechanisms of SCFA. It was
353 reported that SCFAs increased energy expenditure via GPR41 in sympathetic ganglia
354 (Kimura et al., 2011). Furthermore, SCFA produced by gut bacteria inhibited fat
355 accumulation in white adipocytes via GPR43 (Kimura et al., 2013). Butyric acid, an SCFA,
356 was reported to promote hepatic glycogen metabolism via GPR43 (Zhang et al., 2019).
357 In a fasting glycogen deficient state, the body uses more fat instead of glycogen (Izumida
358 et al., 2013). Based on these findings, it seems that SCFAs might suppress weight gain
359 and fatty liver via GPR43 in white adipocytes, GPR41 in sympathetic ganglia, and GPR43
360 in the liver. Taken together, the evidence suggests that dietary RS4 alters the gut
361 microbiota, favoring the production of SCFAs in the intestine, which helped suppress the
362 development of steatosis in HFD-fed mice. Further studies will be required to confirm

these mechanisms.

NAFLD has become a major cause of chronic liver diseases worldwide, including cirrhosis and liver cancer. Liver disease is also strongly related to the intestine. Recent studies have shown that the microbiota is closely related to fatty liver (Safari & Gérard, 2019). It is clear that adequate diet and exercise are necessary to improve NAFLD. Our study has revealed that dietary Amylofiber SH® is effective for the suppression of fatty liver, at least in a mouse model.

There are several limitations of this study. First, we did not investigate whether physical activity or metabolic rate were altered by RS ingestion due to the limitations of the facility. Considering that the amount of food intake and stool weights were not significantly different between the HFD and HFD+RS groups, it is possible that energy expenditure through exercise and metabolism was greater in the HFD+RS group. Second, we were not able to examine steatosis by the amount of RS. In this study, the diet was 21.5% RS, which is the maximum amount that can be included in HFD-RS, but the effect of whether steatosis is improved by a smaller content such as 10% or 15% needs to be examined in the future. Third, we cannot exclude the possibility that different effects of RS4 may be observed in different facilities, perhaps by use of a different base diet or caused by host factors. We observed dramatic increases in *Bifidobacterium* and *Lactobacillus*, but hosts lacking *Bifidobacterium* and *Lactobacillus* should not show expansion of those bacteria. We tried to reduce gut microflora by treatment with antibiotics in HFD-fed mice, but this was unsuccessful because antibiotics suppressed the development of obesity in these mice. Antibiotics resulted in an enlargement of the cecum, which occupied the abdominal cavity, and we assume that the mice showed a loss in appetite, which prevented the development of obesity. It would be interesting to

investigate whether the suppression of fatty liver by RS4 can be recapitulated using gnotobiotic mice lacking *Bifidobacterium* and *Lactobacillus*. Fourth, the long-term course of RS4 feeding is unknown. Additionally, TG in the liver is not considered to be a major factor in lipotoxity; instead, free fatty acids such as palmitic acid, cholesterol, lysophosphatidylcholine, and ceramides are thought to be important mediators of chronic hepatitis caused by fatty liver (Marra & Svegliati-Baroni, 2018). However, we have not tested these factors. Further studies are needed to elucidate the effects of RS4 and answer these questions.

5. Conclusion

RS4, a type of RS, reduced fat accumulation and prevented steatosis in HFD-fed mice. Our results suggest this was mediated by increases in *Bifidobacterium* and *Lactobacillus*, and consequently SCFA, in the intestinal environment. Further investigations are warranted to confirm these findings.

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Conflicts of Interest

The authors have no conflicts of interest to declare.

Availability of data and materials

Full data sets are available from the corresponding author: yanoyo@med.kobe-u.ac.jp

List of abbreviations

ACC: acetyl-CoA carboxylase; AST: aspartate aminotransferase; ALT: alanine aminotransferase; DP: degree of polymerization; *FAS*: fatty acid synthase; HE: hematoxylin and eosin; HFD: high-fat diet; HFD+RS: high-fat diet with added resistant starch; HDL-C: high density lipoprotein cholesterol; *HPRT*: hypoxanthine phosphoribosyltransferase; LDL-C: low density lipoprotein cholesterol; NAFLD: nonalcoholic fatty liver disease; *PPARγ*: peroxisome proliferator-activated receptor gamma; RS: resistant starch; RS1: type 1 resistant starch; RS2: type 2 resistant starch; RS3: type 3 resistant starch; RS4: type 4 resistant starch; SCFA: short chain fatty acid; SEM: standard error of the mean; *SREBP1*: sterol regulatory element-binding protein 1; TC: total cholesterol; TG: triglyceride

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582 **Table 1. Composition of the experimental diets**

	unit	Control	HFD	HFD+RS
Casein	g/100 g	20	14	14
Cystine	g/100 g	0.3	0.21	0.21
β-corn starch	g/100 g	39.7	10	3.5
α-corn starch	g/100 g	13.2	26	26
Sucrose	g/100 g	10	10	10
Cellulose	g/100 g	5	15	0
Resistant starch	g/100 g	0	0	21.5
Soybean oil	g/100 g	7	2	2
Lard	g/100 g	0	18	18
Mineral mix	g/100 g	3.5	3.5	3.5
Vitamin mix	g/100 g	1	1	1
Total calorie	kcal/100 g	368	391.3	390.7
Protein	kcal/100 g	19.6	12.5	12.5
Lipid	kcal/100 g	17.8	44.4	44
Carbohydrate	kcal/100 g	62.6	43.1	43.6

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Figure legends

Figure 1. (A) Percent changes in body weight plotted in each group from 7 to 22 weeks of age. (B) Percent changes in body weight, (C) liver weights, and (D) white adipose tissue weights at 22 weeks of age. (E) Food intake. The amount of food ingested per day was measured at 21 weeks of age and repeated three times. Because 2–3 mice were housed in each cage, the data are calculated as g/mouse/day. Total plots of three repeated measurements are shown. (F) Stool weights. Individual mice were placed in a cage for 2 hours and the stools were collected and weighed. This was repeated three times over 3 days at 21 weeks of age, and the total weights for three repeated measurements are shown. Values are shown as the mean ± standard error of the mean. Control, mice fed with the normal chow; HFD, mice fed with the high-fat diet; HFD+RS, mice fed with high-fat diet supplemented with resistant starch. Asterisks indicate statistical significance ($p < 0.05$).

Figure 2. (A) Macroscopic finding of the liver. (B) Microscopic findings with hematoxylin and eosin (HE) staining. (C) Approximate percentages of hepatocytes containing fatty deposits. HE-stained sections from each sample were evaluated in a blinded manner. Values are shown as the mean ± standard error of the mean. Control, mice fed with the normal chow; HFD, mice fed with the high-fat diet; HFD+RS, mice fed with high-fat diet supplemented with resistant starch. Asterisks indicate statistical significance ($p < 0.05$).

Figure 3. Blood examinations. (A) No significant difference among groups was shown in AST levels. (B) ALT, (C) TC, (D) LDL-C, and (E) HDL-C levels in HFD+RS and control groups

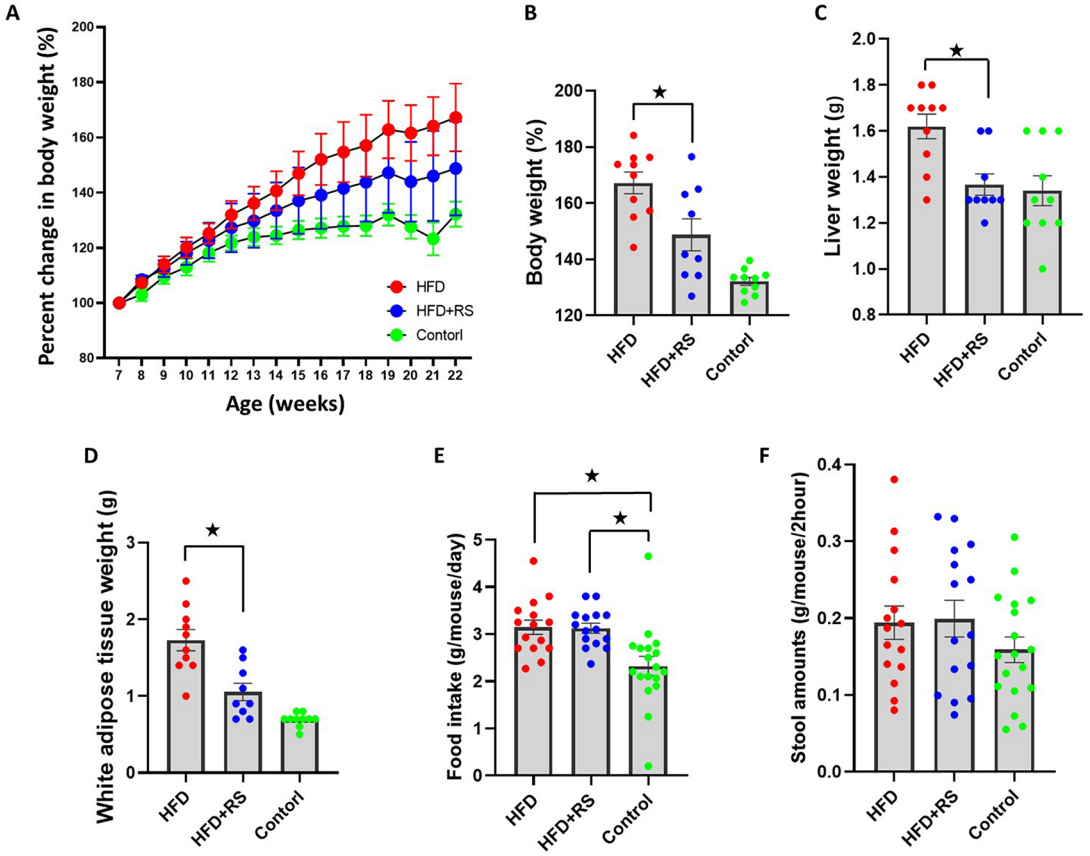
were significantly lower than that of HFD group. (F) TG level in HFD+RS was also significantly lower than that of HFD group. Values are shown as the mean ± standard error of the mean. Control, mice fed with the normal chow; HFD, mice fed with the high-fat diet; HFD+RS, mice fed with high-fat diet supplemented with resistant starch. Asterisks indicate statistical significance ($p < 0.05$).

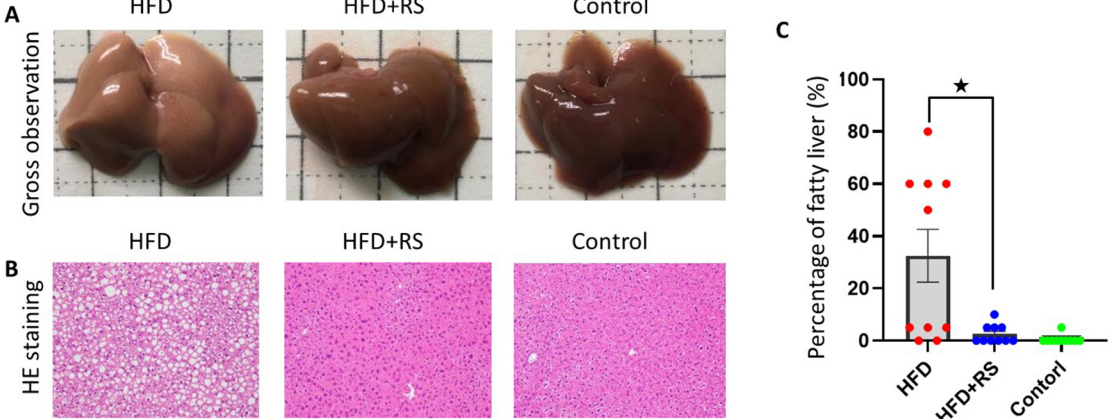
Figure 4. Real-time PCR analysis of the hepatic expression levels of genes related to fatty acid synthesis (*ACC* [A], *FAS* [B], and *SREBP1* [C]) and triglyceride accumulation in fatty liver (*PPARγ* [D]). Values are shown as the mean ± standard error of the mean. Control, mice fed with the normal chow; HFD, mice fed with the high-fat diet; HFD+RS, mice fed with high-fat diet supplemented with resistant starch. (E) Immunostaining of PPARγ. Cleary positive staining in the nucleus of hepatocytes was detected in all groups.

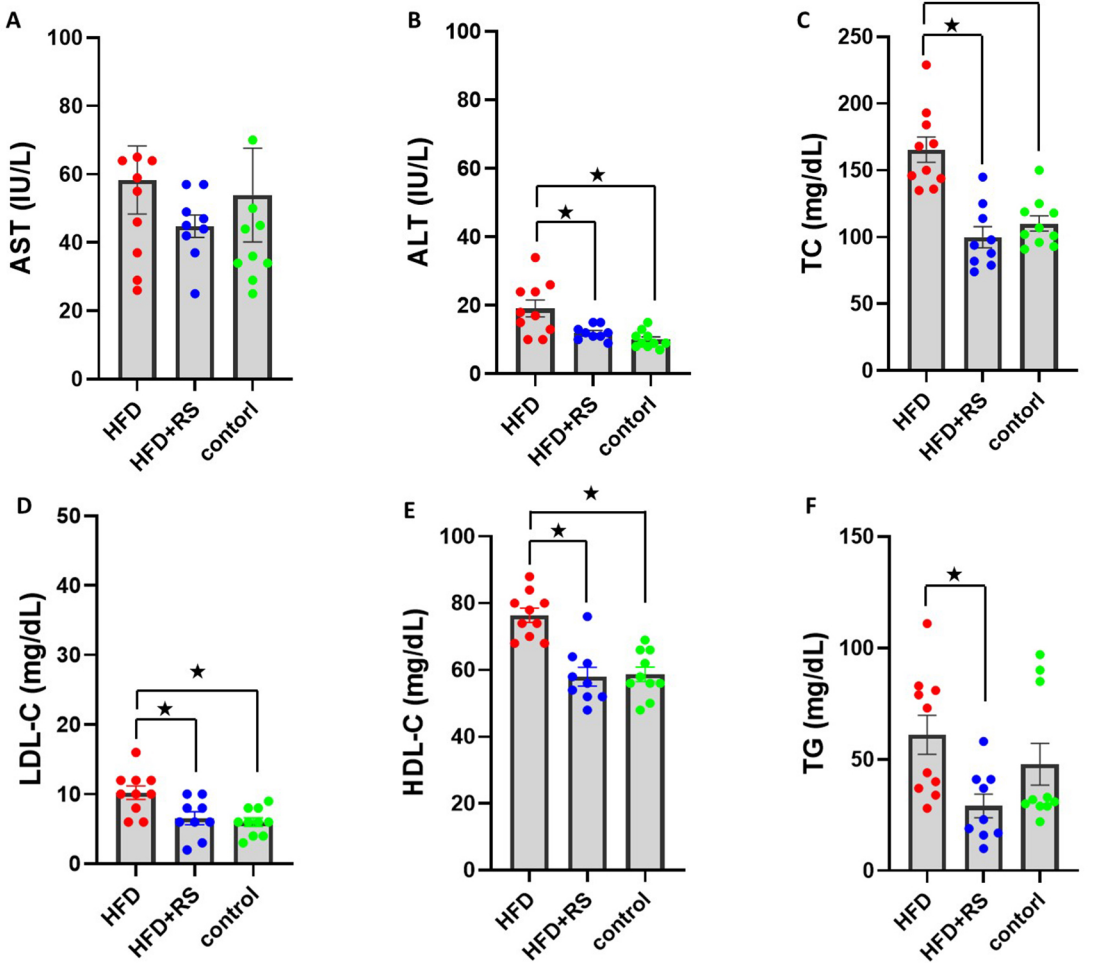
Figure 5. Analysis of intestinal microbiota by 16*S* rRNA sequencing. (A) Chao1, (B) Shannon, and (C) β-diversity. (D) Microbiota composition at the genus level. The names of major bacteria were indicated under the graph. (E,F) Percentages of *Bifidobacterium* (E) and *Lactobacillus* (F). Values in A, B, E and F are shown as the mean ± standard error of the mean. Control, mice fed with the normal chow; HFD, mice fed with the high-fat diet; HFD+RS, mice fed with high-fat diet supplemented with resistant starch. Asterisks indicate statistical significance ($p < 0.05$).

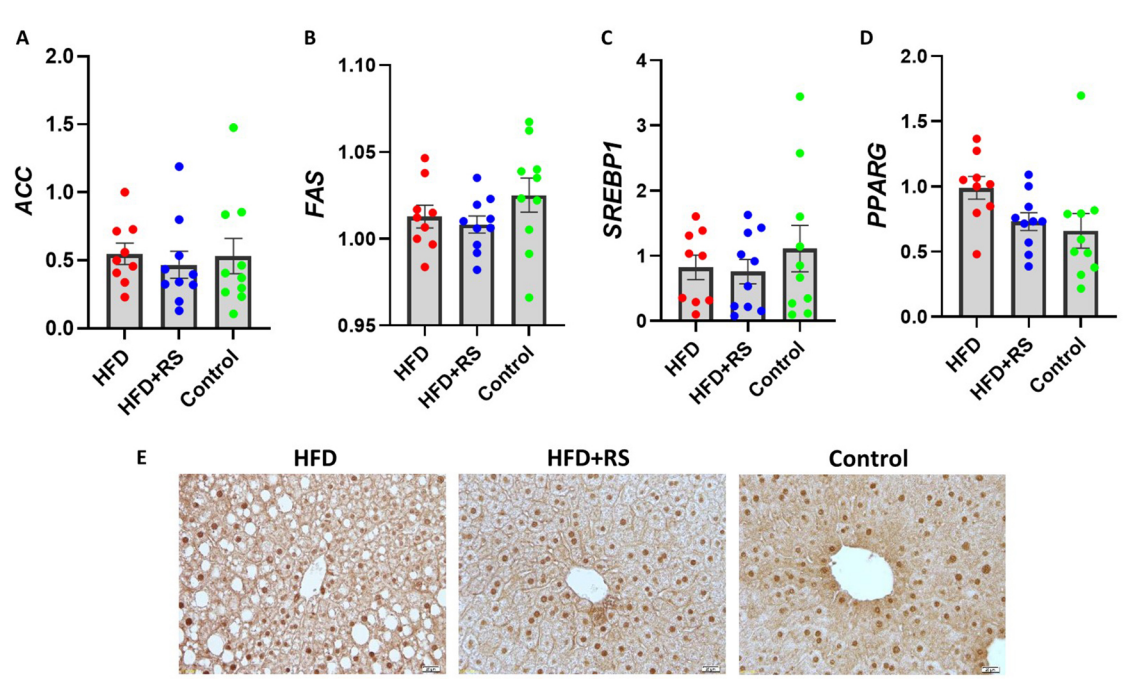
Figure 6. Short-chain fatty acid levels in cecum stool samples. Values are shown as the mean ± standard error of the mean. Control, mice fed with the normal chow; HFD, mice

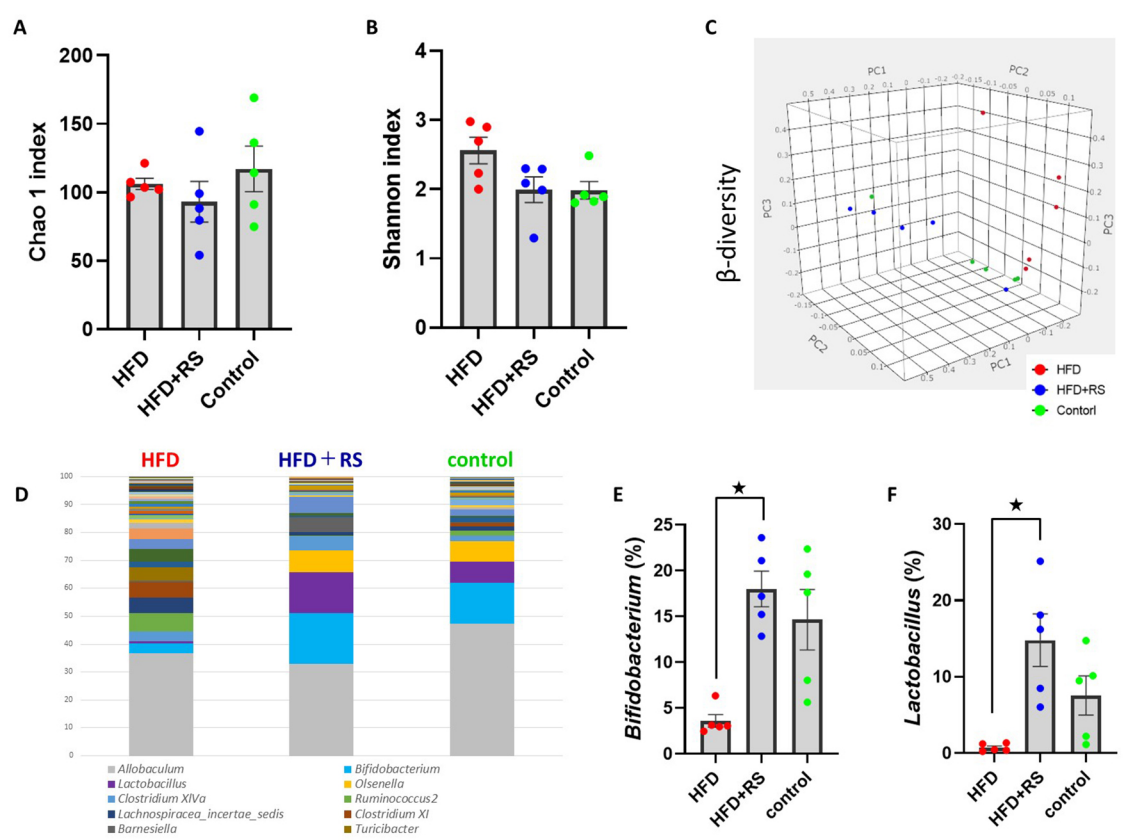
fed with the high-fat diet; HFD+RS, mice fed with high-fat diet supplemented with resistant starch. Asterisks indicate statistical significance ($p < 0.05$).

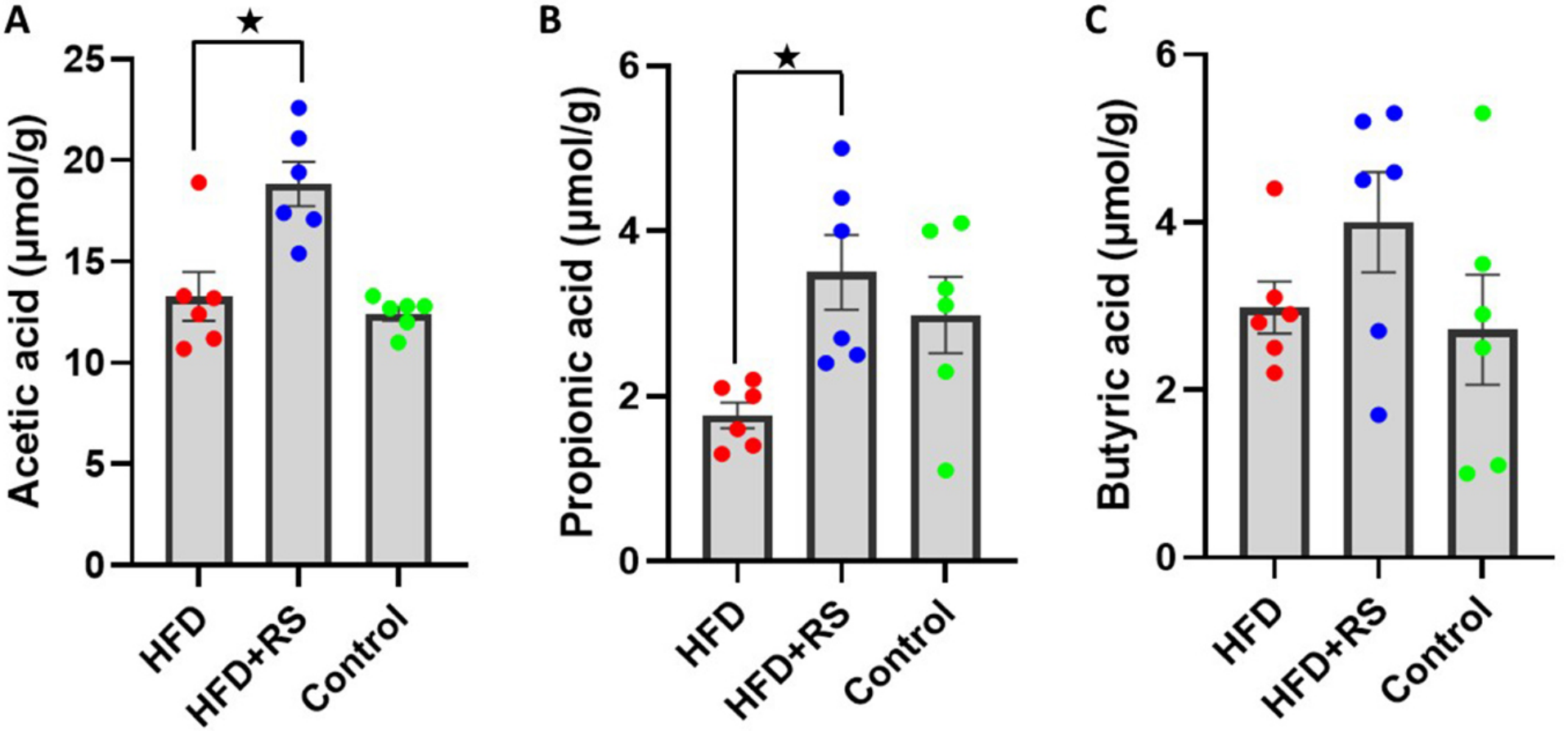

A
Percent change in body weight (%)
Age (weeks)
HFD
HFD+RS
Contorl
B
Body weight (%)
HFD
HFD+RS
Contorl
C
Liver weight (g)
HFD
HFD+RS
Contorl
D
White adipose tissue weight (g)
HFD
HFD+RS
Contorl
E
Food intake (g/mouse/day)
HFD
HFD+RS
Control
F
Stool amounts (g/mouse/2hour)
HFD
HFD+RS
Control


A
HFD
HFD+RS
Control
Gross observation
B
HFD
HFD+RS
Control
HE staining
C
Percentage of fatty liver (%)
100
80
60
40
20
0
★
HFD
HFD+RS
Contorl


A
AST (IU/L)
100
80
60
40
20
0
HFD
HFD+RS
contorl
B
ALT (IU/L)
100
80
60
40
20
0
★
★
HFD
HFD+RS
contorl
C
TC (mg/dL)
250
200
150
100
50
0
★
★
HFD
HFD+RS
contorl
D
LDL-C (mg/dL)
50
40
30
20
10
0
★
★
HFD
HFD+RS
control
E
HDL-C (mg/dL)
100
80
60
40
20
0
★
★
HFD
HFD+RS
control
F
TG (mg/dL)
150
100
50
0
★
HFD
HFD+RS
contorl


A
ACC
2.0
1.5
1.0
0.5
0.0
HFD
HFD+RS
Control
B
FAS
1.10
1.05
1.00
0.95
HFD
HFD+RS
Control
C
SREBP1
4
3
2
1
0
HFD
HFD+RS
Control
D
PPARG
2.0
1.5
1.0
0.5
0.0
HFD
HFD+RS
Control
E
HFD
HFD+RS
Control


A
Chao 1 index
200
150
100
50
0
HFD
HFD+RS
Control
B
Shannon index
4
3
2
1
0
HFD
HFD+RS
Control
C
β-diversity
PC1
PC2
PC3
HFD
HFD+RS
Contorl
D
HFD
HFD＋RS
control
100
90
80
70
60
50
40
30
20
10
0
Allobaculum
Bifidobacterium
Lactobacillus
Olsenella
Clostridium XlVa
Ruminococcus2
Lachnospiracea_incertae_sedis
Clostridium XI
Barnesiella
Turicibacter
E
Bifidobacterium (%)
25
20
15
10
5
0
★
HFD
HFD+RS
control
F
Lactobacillus (%)
30
20
10
0
★
HFD
HFD+RS
control


A
Acetic acid (μmol/g)
25
20
15
10
5
0
★
HFD
HFD+RS
Control
B
Propionic acid (μmol/g)
6
4
2
0
★
HFD
HFD+RS
Control
C
Butyric acid (μmol/g)
6
4
2
0
HFD
HFD+RS
Control