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Yoshida, Ryutaro ; Yano, Yoshihiko ; Hoshi, Namiko ; Okamoto, Norihiro ; Sui, Yunlong ; Yamamoto, Atsushi ; Asaji, Naoki ; Shiomi, Yuuki ;…

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Full title

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

Names of authors:
Ryutaro Yoshida, Yoshihiko Yano, Namiko Hoshi, Norihiro Okamoto, Yunlong Sui, Atsushi Yamamoto, Asaji Naoki, Yuuki Shiomi, Eiichiro Yasutomi, Yuri Hatazawa, Hiroki Hayashi Yoshihide Ueda, Yuzo Kodama

Author affiliation:
Division of Gastroenterology, Department of Internal Medicine, Kobe University Graduate School of Medicine

Contact information for corresponding author:
Yoshihiko Yano, Division of Gastroenterology, Department of Internal Medicine, Kobe University Graduate School of Medicine, Chuo-ku, Kobe 650-0017, Japan
Email: yanoyo@med.kobe-u.ac.jp

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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.

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

51 **1. INTRODUCTION**

52 Obesity and related diseases, such as type 2 diabetes, hypertension, cardiovascular
53 disease, and some cancers, have been common problems worldwide because Western-
54 style meals have become more popular and the calorie intake from food is increasing
55 (Bleich et al., 2008). Steatosis is a condition in which fat (triglycerides) is deposited in
56 hepatocytes, and fatty liver disease is a general term for liver damage caused by steatosis.
57 This fatty liver disease is largely classified into alcoholic and non-alcoholic. And non-
58 alcoholic fatty liver disease (NAFLD) is the liver's expression of an obesity-related disease
59 (Marchesini et al., 2003). NAFLD is strongly related to liver cirrhosis and cancer and is
60 becoming the leading cause of liver cancer in Western countries. NAFLD is expected to
61 become the most frequent indication for liver transplantation by 2030 (Byrne & Targher,
62 2015). Therefore, efforts to suppress the development of fatty liver is a major issue
63 worldwide.

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

70 Short-chain fatty acids (SCFAs) such as acetic acid, propionic acid, and butyric acid are
71 produced from dietary fiber decomposed by the gut microflora. Then, SCFAs are
72 absorbed in the small intestine and used as an energy source (Wolever et al., 1989). It
73 has been also reported that obesity and fatty liver caused by HFD can be suppressed by
74 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

99 RS content is increased to about 70%. The degree of polymerization (DP) of this
100 Amylofiber SH® is about 60. The control diet was AIN93G, and the diet of high-fat diet
101 with resistant starch (HFD+RS) was made to contain as much RS as possible. It is usually
102 that fatty liver model mice are administered with 60% lipid content HDF for 8 weeks.
103 However, the HFD was prepared by reducing the amount of β-corn starch in AIN93G and
104 adding lard instead in this study. The lipid content that causes steatosis was set at 45%,
105 because previous report showed the steatosis even after 12 weeks of treatment with
106 HDF containing 45% lipid (Duan et al., 2018). Using this HFD as a base, we further
107 reduced the amount of β-corn starch and replaced it with RS to prepare the HFD+RS. The
108 HFD+RS matched the HDF in terms of the total calories and calories of the three
109 macronutrients, which included RS (21.5%). The composition of each diet is shown in
110 Table 1. The diets were prepared by Oriental Yeast Co. Ltd. (Tokyo, Japan).
111

112 **2.2. Animal experiments**

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

123 cholesterol (TC), low density lipoprotein cholesterol (LDL-C), high density lipoprotein
124 cholesterol (HDL-C), and triglyceride (TG) levels were measured using an ALT, AST, TC,
125 LDL-C, HDL-C, and TG kits, respectively (Wako Pure Chemical, Osaka, Japan). This study
126 was approved by the Institutional Animal Care and Use Committee of Kobe University
127 (approval numbers: P160505 and P210309).
128

129 **2.3. Liver histology**

130 Liver tissues were fixed overnight at 4 °C in 10% formaldehyde and embedded in
131 paraffin wax. The paraffin sections were cut (5 µm thick), mounted on glass slides, and
132 stained with hematoxylin and eosin (HE). Steatosis was evaluated as the approximate
133 percentage of hepatocytes with fatty cells labeled with HE staining in a blinded manner.
134

135 **2.4. Liver RNA extraction and quantitative PCR**

136 Liver tissues were placed in RNAlater (Qiagen, CA, USA) and stored at −80 °C until use.
137 Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, MA, USA), and
138 cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied
139 Biosystems, CA, USA). Real-time PCR was performed using SYBR Green (Applied
140 Biosystems) and ABI 7500 real-time PCR system (Applied Biosystems). The following
141 primers (forward and reverse, respectively) were used: acetyl-CoA carboxylase (*ACC*), 5'-
142 CTCCCGATTCATAATTGGGTCTG-3', and 5'-TCGACCTTGTTTTACTAGGTGC-3'; fatty acid
143 synthase (*FAS*), 5'-GCGGGTTCGTGAAACTGATAA-3', and 5'-GCAAAATGGGCCTCCTTGATA-
144 3'; sterol regulatory element-binding protein 1 (*SREBP1*), 5'-GCAGCCACCATCTAGCCTG-3',
145 and 5'-CAGCAGTGAGTCTGCCTTGAT-3'; peroxisome proliferator-activated receptor
146 gamma (*PPARγ*), 5'-GGAAGACCACTCGCATTCCTT-3', and 5'-

147 GTAATCAGCAACCATTGGGTCA-3′; and hypoxanthine phosphoribosyltransferase (*HPRT*),
148 5′-GTTGGATACAGGCCAGACTTTGTTG-3′, and 5′-CCAGTTTCACTAATGACACAAACG-3′. And
149 the relative expression to *HPRT*, a housekeeping gene, was calculated.
150

151 **2.5. Immunohistochemistry**

152 Paraffin-embedded liver tissues were deparaffinized with xylene and washed with
153 graded ethanol. The inactivation of endogenous peroxidase was achieved using
154 Peroxidase-Blocking Solution (Dako North America, Inc., CA, USA) for 10 min. These
155 tissue sections were washed with phosphate-buffered saline (PBS) and incubated
156 overnight with anti-Pparγ (1:100; Proteintech, IL, USA) at 4 °C. Following incubation with
157 the secondary antibody, staining with 3,3-diaminobenzidine (DAB; Dako North America,
158 Inc.) was used for visualization and images were captured using a microscope.
159

160 **2.6. Measurement of SCFA**

161 The fecal SCFA levels were measured by a modified method originally reported by
162 García-Villalba et al. (2012). The sample (0.1 g) was placed in a bead tube and 0.9 ml of
163 0.5% phosphoric acid solution was added. The tube was mixed and then heat-treated at
164 85 °C for 15 min. The sample was cooled after grinding, centrifuged (14,000 × *g* for 10
165 min), and the supernatant was transferred to a new tube, mixed with an equal volume
166 of ethyl acetate, and centrifuged again (14,000 × *g*, 10 min). The ethyl acetate layer was
167 transferred to a vial and the internal standard (4-methylvaleric acid) was added to
168 generate the sample solution. After extraction the concentration of SCFA in each sample
169 was measured by gas chromatography using the 7890B Gas Chromatography System
170 (Agilent Technologies Inc., CA, USA). Helium was used as the carrier gas at 1.2 mL/min.

171 The detector temperature was kept at 250°C. The oven temperature program was as
172 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
173 to a final temperature of 250°C, held for 4 min. One microliter of extract was injected in
174 the splitless mode. In this study, butyric acid, acetic acid, and propionic acid were
175 detected as the major cecum SFCAs.
176
177 **2.7. DNA extraction from feces and analysis of microbiota**
178 DNA extraction and amplification of the V3–V4 region of 16*S* rRNA was performed as
179 previously reported (Takahashi et al., 2014, Muyzer et al., 1993, Caporaso et al., 2011,
180 Hisada et al., 2015). The barcoded amplicons were paired-end sequenced using the
181 MiSeq system (Illumina, CA, USA). Chimeric sequences were removed by usearch 6.1.
182 Sequence reads were identified using the RDP Web site (Wang et al., 2007). Taxonomic
183 assignment of sequence reads was performed using Metagenome @ KIN Ver 2.2.1
184 analysis software (World Fusion, Tokyo, Japan).
185
186 **2.8. Statistical analysis**
187 Data are presented as mean ± standard error of the mean (SEM) and were evaluated
188 by one-way analysis of variance followed by Tukey's test to evaluate differences between
189 two groups. Values of $p < 0.05$ were considered statistically significant. Analyses were
190 performed using Prism 8 (GraphPad Software Inc., CA, USA),
191
192 **3. Results**
193 **3.1. Body weight and liver weight**
194 We first tested the effects of the RS on obesity by feeding mice with the HFD, HFD+RS,

195 or control diet, starting at 7 weeks of age. Body weight was recorded weekly. The mean
196 body weights in each groups are plotted in Figure 1A. A difference in body weight was
197 apparent at about 12 weeks of age and became greater over time. At 22 weeks, the
198 percent change in body weight was greater in both the HFD (167.2% ± 3.87%) and
199 HFD+RS (148.8% ± 5.66%) groups than in the control group (132.2% ± 1.43%; both $p <$
200 0.05). Comparing the HDF+RS and HDF groups, the percent change in body weight was
201 lower in the HDF+RS group than that in the HDF group ($p = 0.0076$; Figure 1B). At 22
202 weeks of age, the liver weight of the HFD+RS group (1.37 ± 0.047 g) was lower than that
203 of the HFD group (1.62 ± 0.053 g, $p = 0.0112$; Figure 1C). Furthermore, the weight of the
204 epididymal fat, the main fat-storing white adipose tissue, was also lower in the HFD+RS
205 group than in the HFD group (1.06 ± 0.11 g vs. 1.73 ± 0.14 g, $p = 0.0003$; Figure 1D).
206 Overall, these data suggest that RS could reduce HFD-induced obesity.
207 To exclude the possibility that the effects of the HFD+RS on body weight, liver weight,
208 and adipose tissue weight were due to changes in appetite and increased stool weight
209 due to the addition of RS to the diet, we measured the total food intake and stool weight.
210 We found no differences in either food intake (3.15 ± 0.15 vs. 3.13 ± 0.11 g/mouse/day,
211 $p = 0.996$) or stool weight (0.19 ± 0.021 vs. 0.20 ± 0.02 g/mouse/2hour, $p = 0.983$)
212 between the HFD and HFD+RS groups (Figure 1E,F). However, food intake was
213 significantly different between the control and HFD groups (2.32 ± 0.21 vs. 3.15 ± 0.15
214 g/mouse/day, $p = 0.0029$) and between the control and HFD+RS groups (2.32 ± 0.21
215 g/mouse/day vs. 3.13 ± 0.11 g/mouse/day, $p = 0.0037$). These results suggest that the
216 reduction in body weight and fat accumulation in the HFD+RS group is not simply due to
217 changes in food ingestion or defecation.
218

219 **3.2. Gross and microscopic findings of the liver**

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

232

233 **3.3. Blood examinations**

234 Comparing the HFD and HFD+RS groups, we found no significant difference in the AST
235 level (58.3 ± 9.99 vs. 44.8 ± 3.30 IU/L, p = 0.63; Figure 3A), but there were significant
236 differences in the ALT (19.1 ± 2.46 vs. 12.0 ± 0.69 IU/L, p = 0.0115; Figure 3B). As for
237 cholesterol, the HFD+RS group had significantly lower than the HFD group in the TC level
238 (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
239 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
240 ± 2.15 mg/dL, p < 0.0001; Figure 3E). As for TG (Figure 3F), the HFD+RS group was also
241 significantly lower than the HFD group (29.1 ± 5.27 vs. 61.0 ± 8.80 mg/dL, p = 0.0303)
242 and even lower than the control group, but not significantly different from the control

243 group (29.1 ± 5.27 vs. 47.8 ± 9.44 mg/dL, p = 0.4904). Consistent with the histological
244 findings, these blood test data suggest that RS suppresses liver damage associated with
245 steatosis.
246
247 **3.4. Expression of genes related to fatty acid synthesis and TG accumulation**
248 To investigate the mechanism by which RS reduced obesity and steatosis in mice, we
249 assessed the expression levels of several genes involved in fat accumulation. Real-time
250 PCR was performed using liver tissue samples to examine the gene expression levels of
251 *ACC*, *FAS* and *SREBP1*, which are involved in fatty acid synthesis in the liver (Horton et al.,
252 2002), and *PPARγ*, which is involved in TG accumulation in the fatty liver (Matsusue et
253 al., 2003). Although there were no significant differences in the mRNA expression levels
254 of *ACC* (Figure 4A), *FAS* (Figure 4B) and *SREBP1* (Figure 4C), the relative expression level
255 of *PPARγ* tended to be greater in the HFD group than in the control group (0.99 ± 0.087
256 vs. 0.66 ± 0.13, p = 0.076; Figure 4D). This suggests that *PPARγ* may play an important
257 role in the development of fatty liver in our experimental setting. Consistent with this
258 role, the gene expression of *PPARγ* tended to be lower in the HFD+RS group than in the
259 HFD group, although the difference was not statistically significant (0.73 ± 0.069 vs. 0.99
260 ± 0.087, p = 0.1915; Figure 4D).
261
262 **3.5. Expression of the PPARγ protein**
263 Next, to examine the protein expression of PPARγ among three groups,
264 immunohistochemical analysis was carried out. Nuclear staining of PPARγ was detected
265 in most of hepatocytes with fatty liver as well as normal liver. No significant difference
266 was shown among three groups (Figure 4E).

267

268 **3.6. Gut microbiota**

269 Because the gut microbiota can influence obesity of the host (Ley et al., 2006,
270 Turnbaugh et al., 2006), we compared the gut microenvironment between the three
271 groups using stools samples. Four samples were randomly selected in each group. First,
272 the components of the gut microbiota were analyzed by 16*S* rRNA sequencing. There
273 were no significant differences in the alpha diversity among the three groups in terms of
274 species richness (Chao1) and evenness (Shannon) (Figure 5A,B). In β-diversity, the
275 distance between the data points for each group is an indicator of the similarity in gut
276 microbiota composition. The data points for each group formed clusters with the data
277 points in roughly equivalent locations, suggesting comparable compositions (Figure 5C).
278 We also analyzed the composition of bacteria at the genus level (Figure 5D).
279 *Bifidobacterium* and *Lactobacillus*, which are known to produce SCFA (LeBlanc et al.,
280 2017), were both present in control group but they were markedly reduced in HFD group
281 but had recovered in the HFD+RS group to a level exceeding that of the control group.
282 The HFD+RS group had significantly greater proportions of *Bifidobacterium* (17.99% ±
283 1.94% vs. 3.59% ± 0.69%, p = 0.0019) and *Lactobacillus* (14.79% ± 3.44% vs. 0.72% ±
284 0.23%, p = 0.0045) compared with the HFD group. Moreover, the HFD+RS group tended
285 to have higher proportions of *Bifidobacterium* (17.99% ± 1.94% vs. 14.65% ± 3.30%, p =
286 0.5613) and *Lactobacillus* (14.79% ± 3.44% vs. 7.54% ± 2.57%, p = 0.1385) compared with
287 the control group, although the differences were not statistically significant (Figure 5E,F).
288 Overall, our data suggest that dietary RS dramatically alters the components of gut
289 microbiota and that RS preferentially increased *Bifidobacterium* and *Lactobacillus*.

290

291 **3.7. Cecum stool SCFA content**

292 RS is decomposed into SCFAs by gut microbiota (Pelpolage et al., 2020), such as
293 *Bifidobacterium* and *Lactobacillus*, and SCFAs are known to reduce obesity (Shimizu et
294 al., 2019). Six samples were randomly selected in each group. We found that caecum
295 content in the HFD+RS group had significantly greater acetic acid (18.83 ± 1.10 vs. 13.28
296 ± 1.20 µmol/g, p = 0.0027) and propionic acid (3.50 ± 0.45 vs. 1.77 ± 0.16 µmol/g, p =
297 0.0159) levels compared with those in the HFD group (Figure 6A,B). Although butyric
298 acid tended to be greater in the HFD+RS group than in the HFD group (4.00 ± 0.60 vs.
299 2.98 ± 0.31 µmol/g, p = 0.5978), although the difference was not statistically significant
300 (Figure 6C).

301

302 **4. Discussion**

303 RS is a general term for starch that is not easily digested or absorbed in the small
304 intestine. It was reported that RS improves blood glucose levels by reducing postprandial
305 hyperglycemia and improving insulin responsiveness and promotes weight loss in obese
306 individuals (Hasjim et al., 2010, Kendall et al., 2010). In a recent meta-analysis, RS
307 improved fasting glucose, fasting insulin, insulin resistance, insulin sensitivity, and type
308 2 diabetes associated with obesity (Wang et al., 2019). Regarding steatosis, RS derived
309 from potato and banana (RS2) decreased hepatic fat accumulation and improved fatty
310 liver (Klingbeil et al., 2019, Rosado et al., 2020).

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

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

329 There are several possible mechanisms for the suppression of fatty liver by RS. Si et al.
330 (2017) performed real-time PCR of liver tissue and reported that RS4 significantly
331 suppressed the expression of genes associated with fatty acid synthesis (*ACC*, *Fads1*, and
332 *SREBP1*) and genes associated with fat accumulation (*PPARγ*), and cholesterol synthesis-
333 related genes (*HMGCR*) compared with RS2. In our study, the expression of *PPARγ* tended
334 to be greater in the HFD group than in the HFD+RS and control groups, but the expression
335 levels of *ACC*, *FAS*, and *SREBP1* did not differ among the three groups. PPARγ, which is
336 expressed mainly in adipocytes, is required for the differentiation of pre-adipocytes to
337 mature adipocytes and is involved in adipocyte fat accumulation (Matsusue et al., 2008).
338 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

363 these mechanisms.

364 NAFLD has become a major cause of chronic liver diseases worldwide, including
365 cirrhosis and liver cancer. Liver disease is also strongly related to the intestine. Recent
366 studies have shown that the microbiota is closely related to fatty liver (Safari & Gérard,
367 2019). It is clear that adequate diet and exercise are necessary to improve NAFLD. Our
368 study has revealed that dietary Amylofiber SH® is effective for the suppression of fatty
369 liver, at least in a mouse model.

370 There are several limitations of this study. First, we did not investigate whether
371 physical activity or metabolic rate were altered by RS ingestion due to the limitations of
372 the facility. Considering that the amount of food intake and stool weights were not
373 significantly different between the HFD and HFD+RS groups, it is possible that energy
374 expenditure through exercise and metabolism was greater in the HFD+RS group. Second,
375 we were not able to examine steatosis by the amount of RS. In this study, the diet was
376 21.5% RS, which is the maximum amount that can be included in HFD-RS, but the effect
377 of whether steatosis is improved by a smaller content such as 10% or 15% needs to be
378 examined in the future. Third, we cannot exclude the possibility that different effects of
379 RS4 may be observed in different facilities, perhaps by use of a different base diet or
380 caused by host factors. We observed dramatic increases in *Bifidobacterium* and
381 *Lactobacillus*, but hosts lacking *Bifidobacterium* and *Lactobacillus* should not show
382 expansion of those bacteria. We tried to reduce gut microflora by treatment with
383 antibiotics in HFD-fed mice, but this was unsuccessful because antibiotics suppressed
384 the development of obesity in these mice. Antibiotics resulted in an enlargement of the
385 cecum, which occupied the abdominal cavity, and we assume that the mice showed a
386 loss in appetite, which prevented the development of obesity. It would be interesting to

387 investigate whether the suppression of fatty liver by RS4 can be recapitulated using
388 gnotobiotic mice lacking *Bifidobacterium* and *Lactobacillus*. Fourth, the long-term course
389 of RS4 feeding is unknown. Additionally, TG in the liver is not considered to be a major
390 factor in lipotoxity; instead, free fatty acids such as palmitic acid, cholesterol,
391 lysophosphatidylcholine, and ceramides are thought to be important mediators of
392 chronic hepatitis caused by fatty liver (Marra & Svegliati-Baroni, 2018). However, we
393 have not tested these factors. Further studies are needed to elucidate the effects of RS4
394 and answer these questions.
395

396 **5. Conclusion**

397 RS4, a type of RS, reduced fat accumulation and prevented steatosis in HFD-fed mice.
398 Our results suggest this was mediated by increases in *Bifidobacterium* and *Lactobacillus*,
399 and consequently SCFA, in the intestinal environment. Further investigations are
400 warranted to confirm these findings.
401

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405

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408 thank TechnoSuruga Laboratory Co., Ltd. for help with measuring short chain fatty acids
409 and for DNA extraction from feces, and for the microbiota analyses.
410

411 **Conflicts of Interest**

412 The authors have no conflicts of interest to declare.

413

414 **Availability of data and materials**

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

416

417 **List of abbreviations**

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

428

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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

583

584

585

586

587 **Figure legends**

588

589 Figure 1. (A) Percent changes in body weight plotted in each group from 7 to 22 weeks
590 of age. (B) Percent changes in body weight, (C) liver weights, and (D) white adipose tissue
591 weights at 22 weeks of age. (E) Food intake. The amount of food ingested per day was
592 measured at 21 weeks of age and repeated three times. Because 2–3 mice were housed
593 in each cage, the data are calculated as g/mouse/day. Total plots of three repeated
594 measurements are shown. (F) Stool weights. Individual mice were placed in a cage for 2
595 hours and the stools were collected and weighed. This was repeated three times over 3
596 days at 21 weeks of age, and the total weights for three repeated measurements are
597 shown. Values are shown as the mean ± standard error of the mean. Control, mice fed
598 with the normal chow; HFD, mice fed with the high-fat diet; HFD+RS, mice fed with high-
599 fat diet supplemented with resistant starch. Asterisks indicate statistical significance ($p <$
600 0.05).

601

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

608

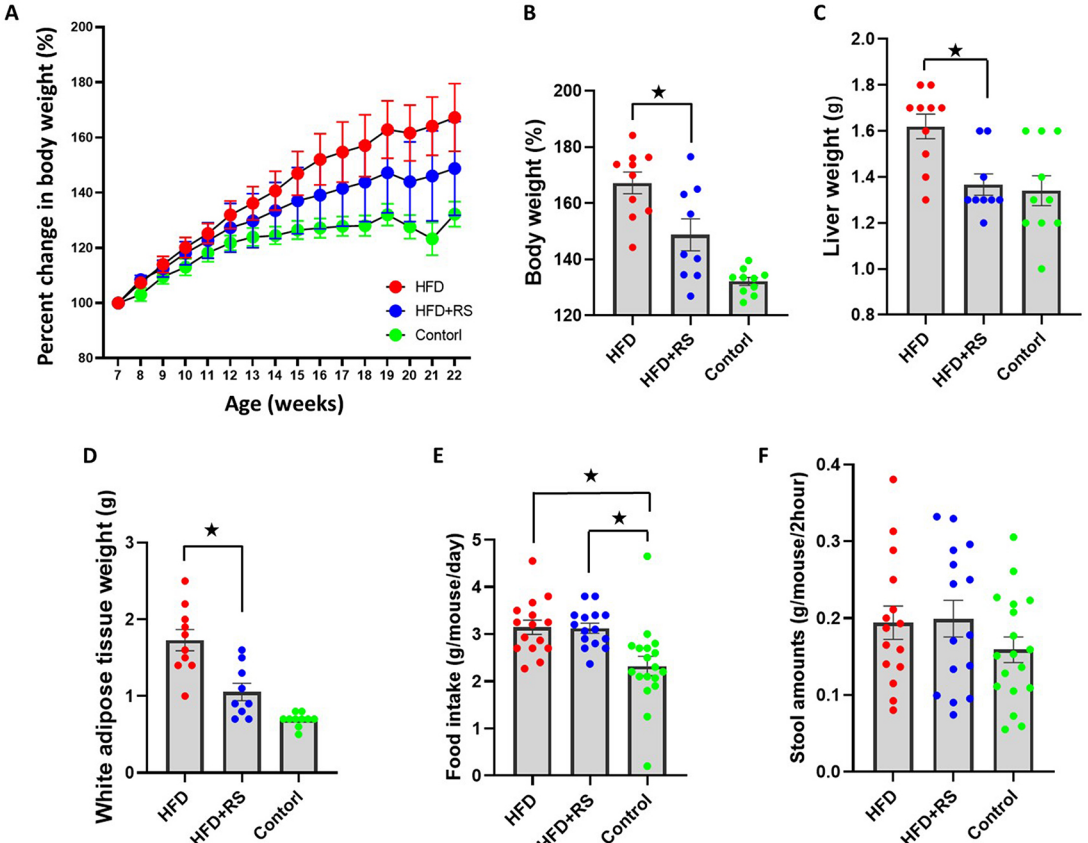
609 Figure 3. Blood examinations. (A) No significant difference among groups was shown in
610 AST levels. (B) ALT, (C) TC, (D) LDL-C, and (E) HDL-C levels in HFD+RS and control groups

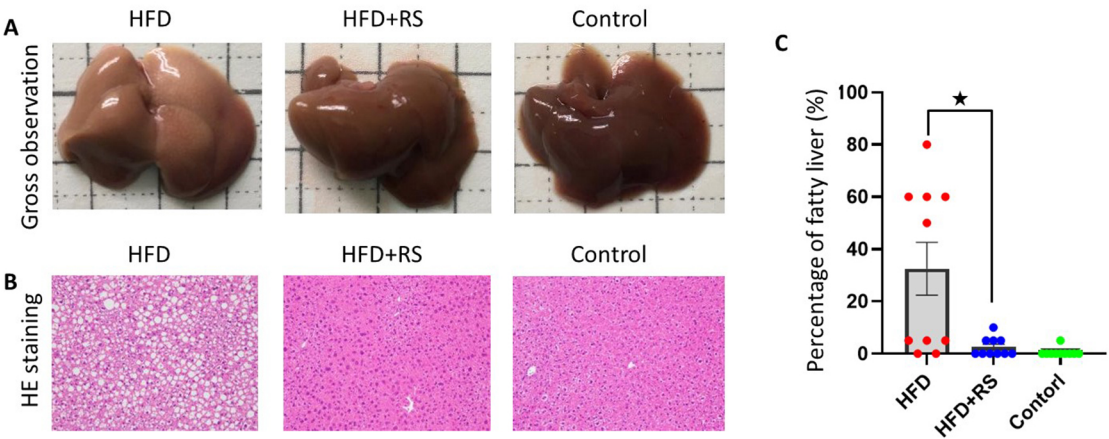
611 were significantly lower than that of HFD group. (F) TG level in HFD+RS was also
612 significantly lower than that of HFD group. Values are shown as the mean ± standard
613 error of the mean. Control, mice fed with the normal chow; HFD, mice fed with the high-
614 fat diet; HFD+RS, mice fed with high-fat diet supplemented with resistant starch.
615 Asterisks indicate statistical significance ($p < 0.05$).
616
617 Figure 4. Real-time PCR analysis of the hepatic expression levels of genes related to fatty
618 acid synthesis (*ACC* [A], *FAS* [B], and *SREBP1* [C]) and triglyceride accumulation in fatty
619 liver (*PPARγ* [D]). Values are shown as the mean ± standard error of the mean. Control,
620 mice fed with the normal chow; HFD, mice fed with the high-fat diet; HFD+RS, mice fed
621 with high-fat diet supplemented with resistant starch. (E) Immunostaining of PPARγ.
622 Cleary positive staining in the nucleus of hepatocytes was detected in all groups.
623
624 Figure 5. Analysis of intestinal microbiota by 16*S* rRNA sequencing. (A) Chao1, (B)
625 Shannon, and (C) β-diversity. (D) Microbiota composition at the genus level. The names
626 of major bacteria were indicated under the graph. (E,F) Percentages of *Bifidobacterium*
627 (E) and *Lactobacillus* (F). Values in A, B, E and F are shown as the mean ± standard error
628 of the mean. Control, mice fed with the normal chow; HFD, mice fed with the high-fat
629 diet; HFD+RS, mice fed with high-fat diet supplemented with resistant starch. Asterisks
630 indicate statistical significance ($p < 0.05$).
631
632 Figure 6. Short-chain fatty acid levels in cecum stool samples. Values are shown as the
633 mean ± standard error of the mean. Control, mice fed with the normal chow; HFD, mice

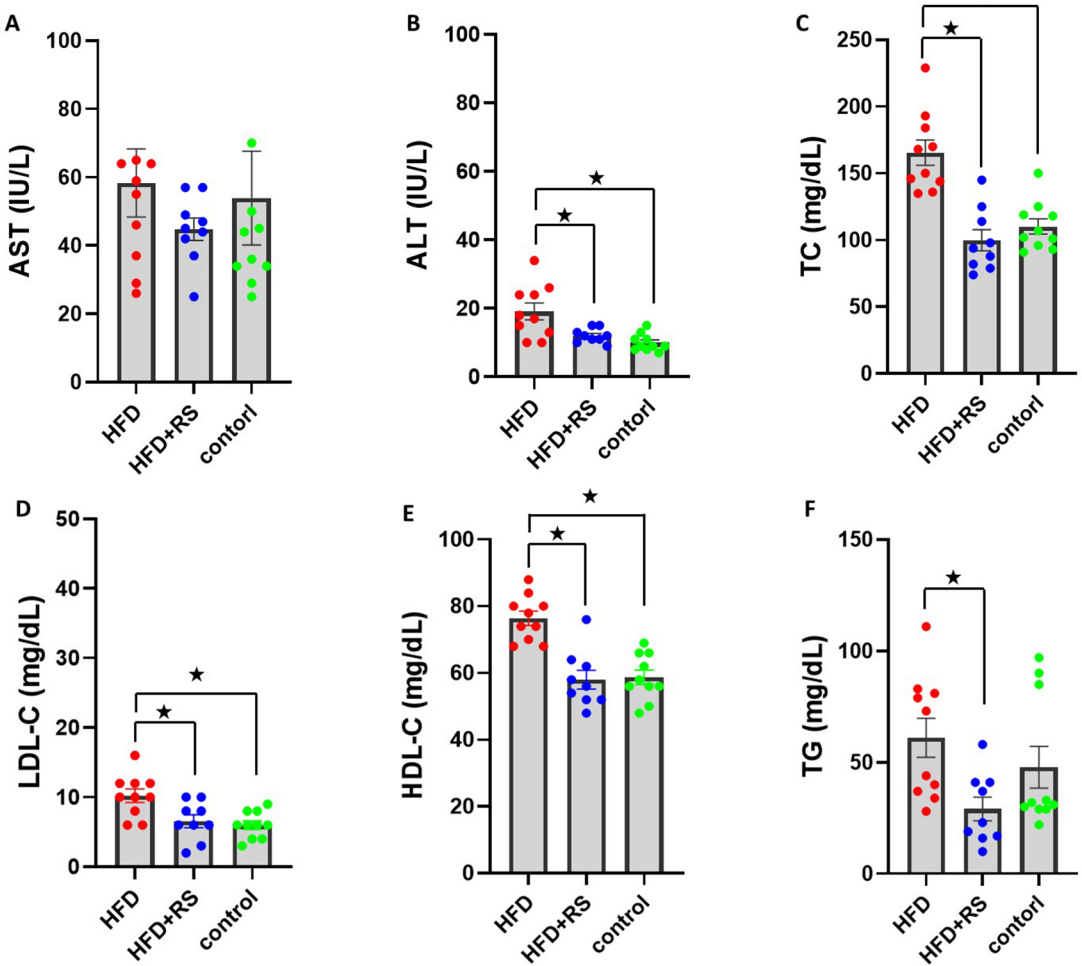
634 fed with the high-fat diet; HFD+RS, mice fed with high-fat diet supplemented with

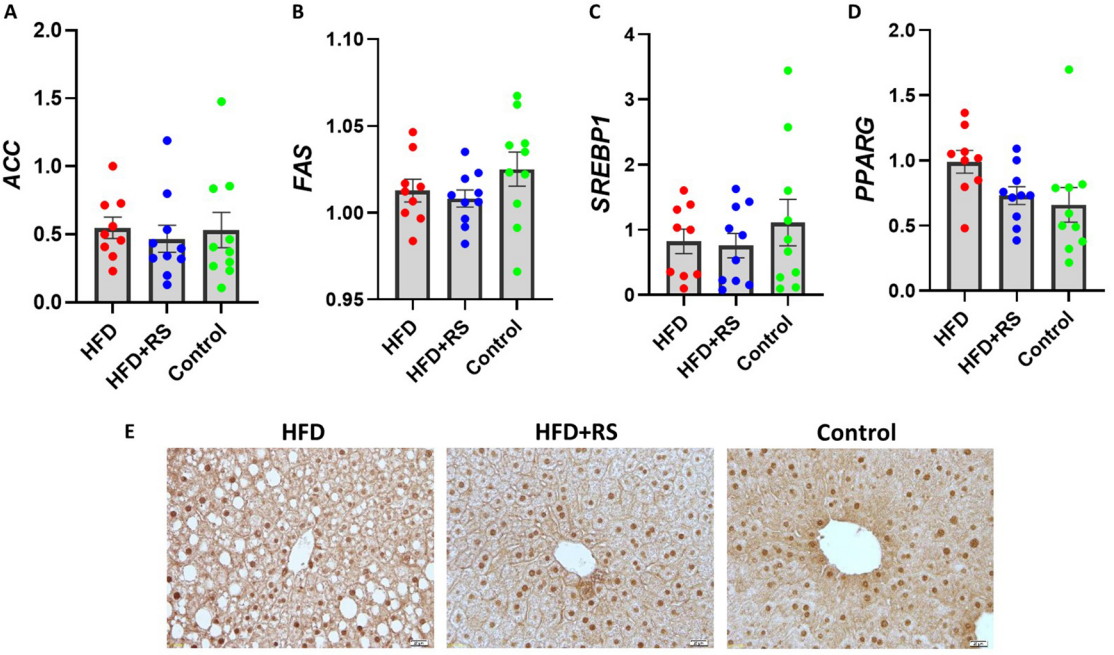
635 resistant starch. Asterisks indicate statistical significance ($p < 0.05$).

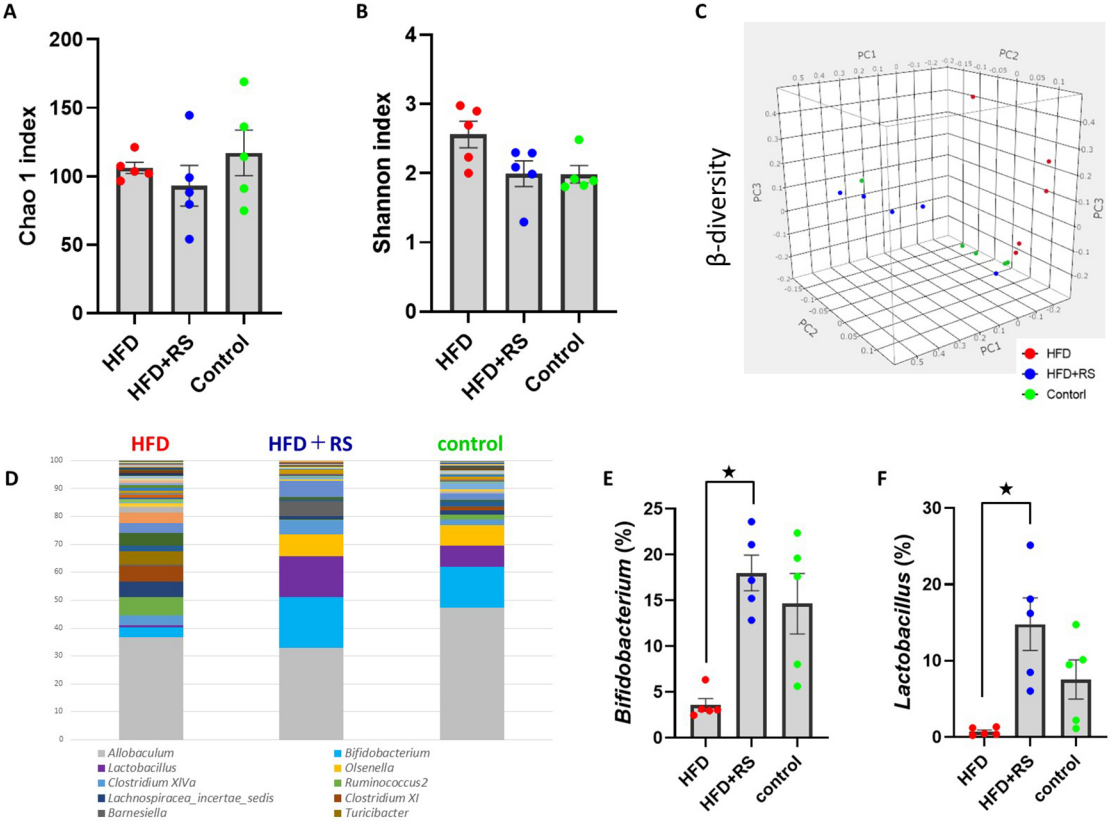
636

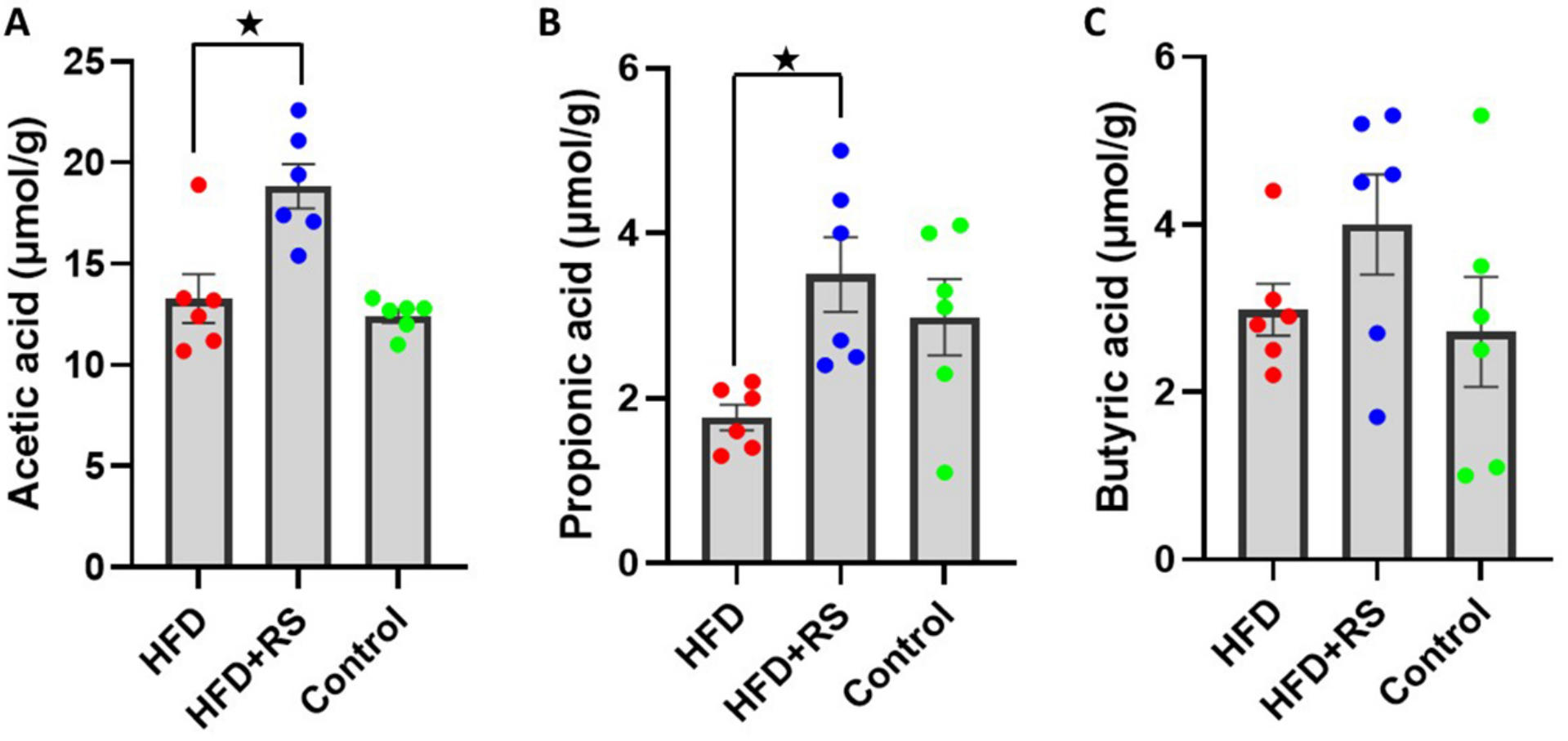

A
Percent change in body weight (%)
Age (weeks)
HFD
HFD+RS
Contorl
B
Body weight (%)
HFD
HFD+RS
Contorl
C
Liver weight (g)
HFD
HFD+RS
Contorl
D
White adipose tissue weight (g)
HFD
HFD+RS
Contorl
E
Food intake (g/mouse/day)
HFD
HFD+RS
Control
F
Stool amounts (g/mouse/2hour)
HFD
HFD+RS
Control


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
Allobaculum
Lactobacillus
Clostridium XIVa
Lachnospiracea_incertae_sedis
Barnesiella
Bifidobacterium
Olsenella
Ruminococcus2
Clostridium XI
Turicibacter
E
Bifidobacterium (%)
HFD
HFD+RS
control
F
Lactobacillus (%)
HFD
HFD+RS
control


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