



Title	Impact of difructose anhydride III, raffinose, and fructooligosaccharides on energy intake, gut hormones, and cecal fermentation in rats fed a high-fat and high-sucrose diet
Author(s)	Hira, Tohru; Yanagihara, Kurumi; Koga, Toshiki et al.
Citation	Bioscience, Biotechnology and Biochemistry, 81(11), 2186-2194 <a href="https://doi.org/10.1080/09168451.2017.1373591">https://doi.org/10.1080/09168451.2017.1373591</a>
Issue Date	2017
Doc URL	<a href="https://hdl.handle.net/2115/85186">https://hdl.handle.net/2115/85186</a>
Rights	This is a pre-copyedited, author-produced version of an article accepted for publication in Bioscience biotechnology and biochemistry following peer review. The version of record Tohru Hira, Kurumi Yanagihara, Toshiki Koga, Keisuke Takahashi, Taizo Nagura, Hirokatsu Uchino, Hiroshi Hara, Impact of difructose anhydride III, raffinose, and fructooligosaccharides on energy intake, gut hormones, and cecal fermentation in rats fed a high-fat and high-sucrose diet, Bioscience, Biotechnology, and Biochemistry, Volume 81, Issue 11, 2 November 2017, Pages 2186-2194 is available online at: <a href="https://doi.org/10.1080/09168451.2017.1373591">https://doi.org/10.1080/09168451.2017.1373591</a> .
Type	journal article
File Information	Biosci Biotechnol Biochem_81_2186.pdf



1 **TITLE**

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4 diet

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20  
21 **RUNNING TITLE:**

22 Effect of DFA II, raffinose and FOS on diet-induced obesity

23

24 **ABSTRACT**

25 We investigated the effects of dietary supplementation of difructose anhydride III (DFA  
26 III), raffinose (Raf), and fructooligosaccharides (FOS) on diet-induced obesity  
27 development. Male rats were fed normal or high-fat and high-sucrose (HFS) diet, with or  
28 without supplementing (3%) DFA III, Raf, or FOS, for 8 or 5 weeks. Supplementing DFA  
29 III to the HFS diet decreased energy intake compared to the non-supplemented HFS  
30 diet. Accordingly, body weight gain and fat accumulation reduced in DFA III-fed rats.  
31 Cecal acetate production and plasma glucagon-like peptide-1 (GLP-1) and peptide-YY  
32 (PYY) were elevated in DFA III-fed rats, while Raf and FOS partially affected these  
33 parameters. These results demonstrate that DFA III suppressive effect on excessive  
34 energy intake driven by the palatable obesogenic diet, possibly due to combined effects  
35 of increased anorexigenic factors such as cecal acetate production and GLP-1/PYY  
36 secretion.

37

38 **KEY WORDS**

39 Difructose anhydride III, Raffinose, Fructooligosaccharides, Diet-induced obesity, GLP-1

40

41

42

## 43 INTRODUCTION

44 Overeating is one of the critical triggers of obesity that lead to metabolic diseases such  
45 as glucose intolerance, dyslipidemia, hypertension, diabetes, and arterogenesis, not  
46 only in adults but also in children [1-4]. Consuming excess amount of “normal” diet daily,  
47 or frequent ingestion of “energy-dense diets” such as high-fat and high-sugar diet can be  
48 considered as overeating. Because of their high palatability, humans and animals ingest  
49 high-fat and high-sugar diets beyond the energy requirement.

50 Various dietary interventions that protect from diet-induced obesity have been tested.  
51 Fermentable dietary fibers and indigestible oligosaccharides are known to prevent diet-  
52 induced obesity. Anti-obesity effects of resistant maltodextrin and inulin-type fructans  
53 including fructooligosaccharides (FOS or OFS) have been demonstrated in animal and  
54 human studies [5-11]. Fermentable fibers promote production of short chain fatty acids  
55 (SCFAs), and SCFAs stimulate secretion and production of gut hormones such as  
56 glucagon like peptide-1 (GLP-1) and peptide YY (PYY) in the distal gut. These factors  
57 affect glucose, lipid, and energy metabolisms [12]. Therefore, it is possible that other  
58 fermentable oligosaccharides also exert beneficial effects against diet-induced obesity.

59 Raffinose ( $\beta$ -D-fructofuranosyl-O- $\alpha$ -D-galactopyranosyl- (1,6)- $\alpha$ -D-glucopyranoside) is  
60 an indigestible trisaccharide composed of glucose, fructose, and galactose. Previous  
61 studies have demonstrated various effects of dietary raffinose on immunity [13,14],  
62 allergy [15,16], and calcium absorption [17], mainly through enhanced gut fermentation.  
63 However, it remains unclear whether dietary raffinose has any impact on obesity,  
64 glucose, or lipid metabolism.

65 Difuctose anhydride III (di-D-fructofuranosyl 1,2':2, 3' anhydride, DFA III) is an

66 indigestible disaccharide composed of two fructose molecules. DFA III is produced from  
67 inulin [18], and it promotes intestinal mineral absorption [19-21] and shows prebiotic  
68 effects [22,23]. Although DFA III lowers cholesterol [24] in isoflavone-fed rats and  
69 suppresses energy intake in female rats with or without ovariectomy [25], its effect in  
70 diet-induced obesity in animal models has not been demonstrated.

71 In the present study, we first examined the effects of dietary DFA III and raffinose on  
72 glycemia and lipid accumulation in rats fed with an obesogenic high-fat and high-sucrose  
73 (HFS) diet under isocaloric feeding conditions. The experiment showed that DFA III  
74 supplementation resulted in reduced energy intake. Therefore, we further examined the  
75 effect of DFA III, as compared with FOS, on energy intake, glycemia, plasma gut  
76 hormone concentrations, and cecal fermentation in rats fed the HFS diet supplemented  
77 with or without DFA III or FOS, ad libitum.

78

## 79 **MATERIALS AND METHODS**

### 80 **Animals and diets**

81 Male Sprague–Dawley rats (5-week-old) were purchased from Japan SLC, Inc.  
82 (Shizuoka, Japan), and were fed an American Institute of Nutrition (AIN)-93G-based diet  
83 [26] for a one-week acclimation period. Each rat was housed in a separate cage and had  
84 free access to food and water, except for the days preceding the glucose tolerance test  
85 and sacrifice. The experiment was performed in a temperature-controlled room maintained  
86 at  $22 \pm 2$  °C with a 12 h light/12 h dark cycle (08:00–20:00 light period). The study was  
87 approved by the Hokkaido University Animal Committee, and the animals were handled in  
88 accordance with the Hokkaido University guidelines for care and use of laboratory animals.

89 After one week of acclimation, rats were fed individual test-diets, as shown in Table 1. The  
90 HFS diet contained 30% wt/wt fat and 40% wt/wt sucrose [11,27]. All diets contained 65  
91 g/kg of fiber. DFA III, raffinose (Raf), and FOS were supplemented in the diet at doses of  
92 30 g/kg diet (3%) by replacing 15 g of cellulose and 15 g of sucrose. The energy density  
93 of DFA III, FOS, and Raf was equally estimated at 2 kcal/g as the common value for non-  
94 digestible fermentable oligosaccharides, and that of cellulose was estimated at 0 kcal/g,  
95 which have been authorized by the Ministry of Health, Labor, and Welfare of Japan [28].

96 In experiment 1, rats were divided into four groups (n = 8–10, each group). The control  
97 group was fed the control diet ad libitum and other three groups (HFS, H-DFA, and H-Raf)  
98 were fed the HFS diet with or without DFA III/raffinose supplementation. In the first 2 days,  
99 rats were fed individual test-diets ad libitum. Then, the amount diets were adjusted to have  
100 the same energy intake among HFS, H-DFA, and H-Raf groups, based on the energy  
101 intake of the previous day. The amounts of test-diets provided to these three groups were  
102 adjusted for equal consumption, but not restricted in the lowest-consumption group,  
103 throughout the experimental period. After 2, 4, and 6 weeks of feeding the test-diet, tail  
104 vein blood was collected to measure fasting (overnight fasted) glucose and insulin  
105 concentrations in the plasma. Blood samples were immediately added with heparin (final  
106 concentration 50 IU/mL) and aprotinin (final concentration 500 KIU/mL). Plasma was  
107 separated by centrifugation at  $2,300 \times g$  for 10 min at 4 °C and stored at -80 °C. After 8  
108 weeks (experiment 1) of test-diet feeding and overnight fasting, rats were anesthetized  
109 using sodium pentobarbital injection (50 mg/kg of body weight, Somnopentyl injection;  
110 Kyoritsu Seiyaku Corporation, Tokyo, Japan). Blood samples were collected from the portal  
111 vein using a syringe containing heparin (final concentration 50 IU/mL), aprotinin (final

112 concentration 500 KIU/mL), and DPP-IV inhibitor (final concentration 50  $\mu$ mol/L; DPP4-  
113 010; Merck Millipore). Plasma was collected and stored, as described above. Adipose  
114 tissues (mesenteric, epididymal, retroperitoneal), cecal tissue, and cecal content were  
115 collected after exsanguination. The weights of the contents were calculated by subtracting  
116 tissue weight from total cecal weight. The cecal contents were homogenized with deionized  
117 water (300 mg of cecal content in 1.5 mL water). The pH values of homogenates were  
118 measured using a compact pH meter (B-212, Horiba, Ltd., Kyoto, Japan).

119 In experiment 2, rats were divided into six groups (n = 8, each group), and fed the  
120 respective test-diet (Table 1) ad libitum as follows: control diet, control diet supplemented  
121 with 3% DFA III (C-DFA) or 3% FOS (C-FOS), HFS diet, HFS diet supplemented with 3%  
122 DFA III (H-DFA) or 3% FOS (H-FOS). After 2 and 4 weeks of test-diet feeding, tail vein  
123 blood was collected to measure fasting (overnight fasted) glucose and insulin  
124 concentrations in the plasma. Blood samples were treated as described above. After 5  
125 weeks of test-diet feeding and overnight fasting, rats were anesthetized and blood and  
126 tissue samples were collected as described above.

127

## 128 **Measurement of plasma samples**

129 Glucose and insulin concentrations were measured using a Glucose CII Test Kit (Wako  
130 Pure Chemical Industries, Ltd., Osaka, Japan) and a rat insulin enzyme-linked  
131 immunosorbent assay (ELISA) kit (AKRIN-010T; Shibayagi Co., Ltd., Gunma, Japan; inter-  
132 assay coefficient of variability (CV): < 5%, intra-assay CV: < 5%), respectively. Total GLP-  
133 1, active GLP-1, and total PYY concentrations were measured using a multi-species GLP-  
134 1 total ELISA kit (EZGLP1T-36K; Merck Millipore, Darmstadt, Germany; intra-assay CV: <

135 5%, inter-assay CV: < 12%), a GLP-1 (active) ELISA assay (EGLP-35K; Merck Millipore;  
136 intra-assay CV:  $7.4 \pm 1.1\%$ , inter-assay CV:  $8 \pm 4.8\%$ ), and a MILLIPLEX MAP rat  
137 metabolic hormone magnetic bead panel metabolism multiplex assay (Merck Millipore;  
138 intra-assay CV: 3%, inter-assay CV: 11%), respectively. To estimate the degree of insulin  
139 resistance, the homeostatic model assessment for insulin resistance (HOMA-IR) was  
140 calculated using the following formula [29]:  $\text{HOMA-IR} = \{\text{glucose (mg/dL)} \times \text{insulin}$   
141  $(\mu\text{U/mL})\}/2,430$ , where 1 ng of insulin is equivalent to 26  $\mu$ units.

142

### 143 **Measurement of organic acids in the cecal content**

144 The concentration of organic acids such as succinate, acetate, propionate, and butyrate  
145 in the rat cecal contents were measured using high performance liquid chromatography  
146 (HPLC) [14]. Briefly, the cecal content homogenates were mixed with equal volumes of 0.4  
147 N HCl. After overnight incubation at 4 °C, the supernatant was filtered through a membrane  
148 filter and loaded onto an HPLC column (RSpak KC-811; Showa Denko K.K., Tokyo, Japan),  
149 and we monitored the wavelength at 445 nm using the post-column method with  
150 bromothymol blue as a pH indicator.

151

### 152 **Statistical analyses**

153 Data were expressed as means and standard errors (SE) of the mean. Statistical  
154 analyses were performed using the JMP Pro version 12.0 software (SAS Institute, Inc.,  
155 Cary, NC, USA). Statistical significance was assessed using a one-way (experiment 1) or  
156 two-way (experiment 2) analysis of variance (ANOVA), as appropriate. Significant  
157 differences ( $P < 0.05$ ) between treatments were determined using the Tukey's HSD test,

158 as appropriate.

159

160

## 161 **RESULTS**

### 162 **Experiment 1**

163 Among the three HFS-fed groups, H-DFA group consumed less amount of diet from  
164 the beginning of test-diet feeding. To adjust the energy intake among the three HFS-fed  
165 groups, HFS and H-Raf groups were fed reduced amounts of the diet, while H-DFA group  
166 was fed ad libitum. As shown in Table 2, the total energy intake did not differ among the  
167 three groups. Notably, the total energy intake of HFS-fed group was not significantly  
168 higher than that of the control group which was fed the control diet ad libitum. Final body  
169 weights also did not differ significantly between the treatments. Adipose tissue weights  
170 were slightly higher in three HFS-fed groups, but not significantly different compared to  
171 that of the control group.

172 We monitored fasting glucose and insulin concentrations in the plasma, taken from the  
173 tail vein after 2, 4, and 6 weeks of test-diet feeding. However, no significant differences  
174 were observed in these parameters between the treatments (data not shown).

175 The tissue and content weights of cecum (Table 3) decreased in the HFS group  
176 compared to the control group, but these were maintained or increased by  
177 supplementation of DFA III or raffinose. Acetate concentration ( $\mu\text{mol/g}$  content) and  
178 amount ( $\mu\text{mol/cecum}$ ) were highest in the H-DFA group, and the amount of acetate was  
179 lowest in the HFS group. Similar trends were observed in case of propionate and  
180 butyrate concentrations and amounts. Overall, short chain fatty acids productions were

181 relatively reduced in the HFS group, restored or increased in the H-DFA group, and  
182 partially restored in the H-Raf group, as compared to the control group. In contrast,  
183 succinate concentration was highest in the HFS group.

184 Total GLP-1 concentrations (Fig. 1) in the portal plasma after 8 weeks of test-diet  
185 feeding were measured. The GLP-1 concentration in the HFS group tended to be lower  
186 than that in the control group. In contrast, GLP-1 concentrations in H-DFA and H-Raf  
187 groups did not decrease, as compared to the control group. Moreover, concentration in  
188 the H-Raf group was significantly higher than that in the HFS group.

189 Although supplementation of DFA III or Raf to the HFS diet did not clearly affect  
190 adiposity and glucose tolerance under isocaloric feeding condition, DFA III showed  
191 suppressive effect on energy intake. In experiment 2, we examined effects of  
192 supplementing DFA III and FOS to the HFS diet and to the control diet under ad libitum  
193 feeding condition on energy intake, adiposity, glucose tolerance, cecal fermentation, and  
194 gut hormones involved in satiety induction.

195

## 196 **Experiment 2**

197 Significant effects (increments) of the HFS diet were detected using the 2-way ANOVA  
198 on body weight, weigh gain, and energy intake (Table 4). Significant effects of fiber on  
199 body weight gain and total energy intake were also detected, as rats fed DFA III-  
200 supplemented diets showed lowest values compared with rats fed cellulose- or FOS-  
201 containing diets (both control and HFS diets). Adipose tissue weights also increased by  
202 the HFS diet, but rats fed DFA III-supplemented diets showed lower epididymal fat  
203 weights than rats fed cellulose- or FOS-containing diets. Significant effect of fiber was

204 detected using the 2-way ANOVA.

205 Figure 2 shows daily changes in the difference in cumulative energy intake of test  
206 groups compared with that in the control group fed the control diet containing only  
207 cellulose as dietary fiber. The result clearly demonstrates that rats fed with the HFS diet  
208 had larger energy intake from beginning of the test period, and the cumulative difference  
209 continuously increased throughout the experimental period. H-FOS group showed  
210 incremental changes, similar to the HFS group, while H-DFA group showed only slight  
211 increment until end of the experiment. The C-DFA group had lower energy intake than  
212 the control group throughout the experiment.

213 We monitored fasting glucose and insulin levels to assess glucose tolerance after 2  
214 and 4 weeks of test-diet feeding (Fig. 3). Significant effects were not detected using the  
215 2-way ANOVA, but HFS diet showed a tendency of increased glucose level after 2 weeks  
216 ( $P = 0.053$ ). Plasma GLP-1 levels were significantly affected by replacing the fiber ( $P =$   
217  $0.034$  and  $0.001$ , after 2 and 4 weeks, respectively). After 4 weeks, rats fed DFA-  
218 supplemented diets had highest GLP-1 levels among all treatments. When compared  
219 within the three HFS diet-fed groups, plasma GLP-1 concentrations in the H-DFA rats  
220 were significantly higher than those in the HFS-fed rats (Tukey's test,  $P < 0.05$ ).

221 After 5 weeks of test-diet feeding, we collected portal plasma under anesthesia. Total  
222 GLP-1 and active GLP-1 concentrations in the portal plasma (Fig. 4) were significantly  
223 affected by fiber (2-way ANOVA). Active and total GLP-1 concentrations in C-DFA and C-  
224 FOS groups were significantly higher than those in the control group, and GLP-1  
225 concentrations in the H-DFA and H-FOS groups had higher tendency than those in the  
226 HFS group. The concentration of PYY, another L-cell-derived hormone, in the portal

227 plasma of the C-DFA and C-FOS groups were significantly higher than that in the control  
228 group. Although the HFS diet significantly elevated PYY levels ( $P < 0.0001$ , 2-way  
229 ANOVA), supplementing DFA III further increased plasma PYY concentration as it is  
230 significantly higher than that in HFS group.

231 Cecal content weight (Table 5) increased significantly in the C-DFA group, as  
232 compared to the control group, while the H-DFA group showed insignificant increments,  
233 compared to the HFS group, suggesting suppressive effect of the HFS diet on the DFA  
234 III-enhanced cecal fermentation. This is supported by lower concentrations of acetate  
235 and propionate in the H-DFA group compared to the C-DFA group. However, when  
236 compared within the three HFS-fed groups, acetate and propionate concentrations in the  
237 H-DFA group, but not in the H-FOS group, were significantly higher than those in the  
238 HFS group ( $P < 0.05$ , Tukey's test). Succinate concentration was more than 3-fold higher  
239 in the HFS group compared to that in the control group, while H-DFA and H-FOS had  
240 modestly increased concentrations compared to the control group.

241

242

## 243 **DISCUSSION**

244 The present study aimed to investigate the effects of indigestible oligosaccharides on  
245 development of diet-induced obesity. FOS are known to exert anti-obesity or anti-  
246 hyperglycemic effects, but effects of DFA III and Raf on diet-induced obesity remained  
247 unclear. In the first experiment comparing the effects of DFA III and Raf under isocaloric  
248 feeding conditions, DFA III seemed to prevent excess energy intake. The anorexigenic  
249 effect of DFA III was reproduced in the second experiment under ad libitum feeding

250 conditions. FOS or Raf did not reduce the energy intake at identical doses (3% wt in the  
251 diet). Rats fed DFA III showed highest acetate content in the cecum and increased  
252 plasma GLP-1 and PYY concentrations compared to rats fed the diet containing only  
253 cellulose as fiber. These results suggest anti-obesity effect of dietary DFA III as one of  
254 fermentable oligosaccharides with relatively higher potency than FOS and raffinose.

255 In experiment 1, except for the control group, rats were fed the test-diets (HFS, H-  
256 DFA, H-Raf) under isocaloric conditions to examine the effects of DFA III and raffinose on  
257 adiposity and glucose metabolism, independent of energy intake. The results show no  
258 apparent effects of DFA III and raffinose on parameters related to adiposity and glucose  
259 metabolism. However, energy intake was lower in the H-DFA group than in the HFS and  
260 H-Raf groups, when rats were fed the diet ad libitum at the beginning of experimental  
261 period. The HFS group did not show significant increment in body weight, adipose tissue  
262 weights, and glycemia, compared to the control group because of adjustment of energy  
263 intake among the three HFS-fed groups. These results suggest that DFA III has  
264 preventive effect against diet-induced obesity and glucose intolerance through reduced  
265 consumption of the high-energy diet.

266 Experiment 2 was conducted to examine the effects of DFA III and FOS on energy  
267 intake and on the development of diet-induced obesity under ad libitum feeding  
268 conditions. Supplementation of DFA III reduced the energy intake when it was added  
269 either in the control diet or the HFS diet, while FOS had no effects on the energy intake.  
270 Accordingly, body weight gain and epididymal fat weight were also reduced by feeding  
271 DFA III-supplemented diets (C-DFA and H-DFA). As a result, the H-DFA group showed  
272 almost similar values of body weight, adipose tissue weight, and fasting glucose

273 concentrations as those of the control group. FOS and inulin-type fructans have been  
274 reported to increase SCFA production, increase GLP-1 production, and decrease energy  
275 intake in rodent models, on adding these oligosaccharides in the diet at around 5–10%  
276 (wt/wt) [5-9,30-32]. The dose of FOS at 3% in the present study seems insufficient to  
277 exert an anorexic effect in the diet-induced obesity model. In contrast, DFA III at 3% in  
278 the diet is effective in preventing excess energy intake. Although DFA III reduced energy  
279 intake when it was added to the control diet, the growth of rats was not impaired in the  
280 present study. The relatively higher efficacy of DFA III than FOS and raffinose on appetite  
281 suppression against the palatable obesogenic diet indicates the translational potential of  
282 DFA III in preventing excess energy intake in humans at a low dose and with low risk of  
283 unfavorable effects such as hyperosmotic diarrhea. In a human study, repeated ingestion  
284 of DFA III at 5 g/day for 12 days did not show any harmful side effects [33].

285 Cecal SCFA (acetate, propionate, butyrate) contents ( $\mu\text{mol}/\text{cecum}$ ) decreased after  
286 continuous feeding with HFS diet in both of the experiments. SCFA contents were  
287 restored overall by supplementing with DFA III, raffinose, and FOS, although these  
288 oligosaccharides had different impacts on individual SCFA levels. Acetate contents were  
289 increased by DFA III in both experiments, while raffinose and FOS had relatively smaller  
290 impact on the acetate production. These results indicate that oligosaccharides are  
291 utilized to produce SCFAs in the cecum, even if they are supplemented in the HFS diet  
292 that suppresses gut fermentation.

293 SCFAs are known to promote secretion and production of GLP-1 in the large intestine  
294 [34,35]. Elevated plasma GLP-1 levels on supplementation of DFA III, raffinose, and FOS  
295 were demonstrated in the present study. Although changes in SCFA production and

296 plasma GLP-1 levels are not simply parallel among all the diet-treatments, increased  
297 SCFAs probably enhanced GLP-1 release in the large intestine. GLP-1 has an anorexic  
298 effect, along with an incretin effect [36]. Moreover, acetate also shows an anorexic effect  
299 [37,38]. Acetate production was largely increased by DFA III in the present study. A  
300 previous study showed similar effects of DFA III, FOS and raffinose on cecal SCFA  
301 production in rats [39], while several following studies demonstrated higher impacts of  
302 DFA III in rats on cecal SCFA production than FOS, especially on acetate content [40-42].  
303 Although experimental conditions vary among studies, the latter studies support the  
304 finding that DFA III has potent effect on gut fermentation compared to FOS. Thus, the  
305 suppressive effect of DFA III on energy intake could be attributed to combined effects of  
306 increased acetate and GLP-1 levels. Plasma GLP-1 concentrations increased rapidly  
307 (within 2 weeks) by DFA III and FOS, and the effect sustained by end of the experimental  
308 period, suggesting that cecal fermentation was already modified after 2 weeks of feeding  
309 these oligosaccharides. Another anorexic gut hormone, PYY [43], was significantly  
310 elevated by DFA III, but not by FOS, when added to the HFS diet. These results suggest  
311 that additive or synergistic effects of increased plasma GLP-1, PYY, and acetate are  
312 involved in suppressive effects of DFA III on excess energy intake. There may be other  
313 mechanisms also involved. Although DFA III failed to stimulate GLP-1 secretion from a  
314 murine GLP-1-producing enteroendocrine cell line (GLUTag) in our preliminary study  
315 (data not shown), postprandial GLP-1/PYY secretions might be transiently potentiated by  
316 the supplementation of DFA III to the diet, through direct/indirect action of DFA III on  
317 enteroendocrine L cells in vivo. Further studies would be need in the future to assess the  
318 hypothesis.

319        Interestingly, increased production of succinate by the HFS diet was attenuated by  
320        supplementation of DFA-III, raffinose, and FOS. Luminal succinate has been reported to  
321        have unfavorable effects such as inhibition of colonic epithelial proliferation, inflammatory  
322        and hypoxic effects [44-46]. Therefore, these oligosaccharides may protect the host from  
323        any harmful effects by reducing succinate production. Clarifying the underlying  
324        mechanism will be an attractive issue for future studies.

325        In summary, rats were fed the HFS diet with or without supplementation of DFA III,  
326        raffinose, and FOS for 5 or 8 weeks. Rats fed DFA III-containing diet showed lower  
327        energy intake compared to rats fed with the HFS diet, resulting in reduced fat  
328        accumulation in DFA III-fed rats. Cecal SCFA productions were increased by these  
329        oligosaccharides, but acetate production was largely increased by DFA III. Plasma GLP-1  
330        concentrations were elevated in DFA III and FOS-fed groups after 2 weeks of the diet  
331        intervention. In addition, plasma PYY concentration was increased in the DFA III fed  
332        group. These results demonstrate that DFA III has suppressive effect on energy intake  
333        against palatable obesogenic diet, possibly through combined effects of increased  
334        anorexigenic factors, including cecal acetate, plasma GLP-1, and PYY concentrations.

335

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- 456

457 **TABLES**

458

459 **Table 1. Test diet composition.**

Test diet	Control	Control +DFA III (C-DFA)	Control +FOS (C-FOS)	HFS	HFS +DFA III (H-DFA)	HFS +FOS (H-FOS)	HFS +Raffinos e (H-Raf)
Ingredient	g/kg of diet						
Cornstarch	397.486	397.486	397.486	-	-	-	-
Casein <sup>1)</sup>	200	200	200	200	200	200	200
Dextrinized cornstarch <sup>2)</sup>	132	132	132	-	-	-	-
Sucrose	85	70	70	384.486	369.486	369.486	369.486
Soybean oil	70	70	70	70	70	70	70
Lard oil	-	-	-	230	230	230	230
Fiber							
Cellulose <sup>3)</sup>	65	50	50	65	50	50	50
DFA III <sup>4)</sup>	-	30	-	-	30	-	-
FOS <sup>5)</sup>	-	-	30	-	-	30	-
Raffinose <sup>6)</sup>	-	-	-	-	-	-	35.3
Mineral mixture <sup>7)</sup>	35	35	35	35	35	35	35
Vitamin mixture <sup>7)</sup>	10	10	10	10	10	10	10
L-Cystine	3	3	3	3	3	3	3
Choline	2.5	2.5	2.5	2.5	2.5	2.5	2.5
<i>tert</i> -Butyl- hydroquinone	0.014	0.014	0.014	0.014	0.014	0.014	0.014
Energy density (kcal/g)	3.90	3.90	3.90	5.05	5.05	5.05	5.03

460 1) Acid Casein (Fonterra, Ltd., Auckland, New Zealand);

461 2) TK-16 (Matsutani Chemical Industry Co., Ltd., Hyogo, Japan);

462 3) Avicel PH102 (Asahi Kasei Chemicals Corporation, Tokyo, Japan);

463 4) Difuctose anhydrate III (Nippon Beet Sugar Manufacturing Co., Ltd., Tokyo, Japan);

464 5) Fructooligosaccharides (Meiologo-P, Meiji Co., Ltd., Tokyo, Japan);

465 6) Raffinose (Nippon Beet Sugar Manufacturing Co., Ltd., Tokyo, Japan); contains 15%  
466 of water as hydrate.

467 7) Mineral and vitamin mixtures were prepared according to the AIN-93G formulation.

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470 **Table 2. Body weight, energy intake, and visceral adipose tissue weights of rats fed**  
 471 **with test-diets for 8 weeks (Experiment 1).**

	Control	HFS	H-DFA	H-Raf	ANOVA <i>P</i> value
Body Weight (g)	444 ± 9	459 ± 5	469 ± 6	460 ± 6	0.096
Total Energy Intake (kcal)	4481 ± 61	4655 ± 28	4656 ± 86	4632 ± 65	0.126
Mesenteric fat (g)	10.06 ± 0.74	11.34 ± 0.61	11.22 ± 0.67	9.57 ± 0.78	0.249
Mesenteric fat (g/100 g BW)	2.25 ± 0.14	2.47 ± 0.13	2.39 ± 0.13	2.07 ± 0.15	0.230
Epididymal fat (g)	10.92 ± 1.05	13.92 ± 0.70	13.87 ± 0.85	11.67 ± 0.74	0.037
Epididymal fat (g/100 g BW)	2.44 ± 0.20	3.03 ± 0.15	2.95 ± 0.16	2.53 ± 0.13	0.039
Retroperitoneal fat (g)	15.52 ± 1.05	17.52 ± 0.98	17.72 ± 1.58	15.06 ± 1.13	0.306
Retroperitoneal fat (g/100 g BW)	3.47 ± 0.18	3.81 ± 0.20	3.76 ± 0.29	3.27 ± 0.23	0.335
Visceral fat <sup>#</sup> (g)	36.5 ± 2.7	42.8 ± 2.0	42.8 ± 2.6	36.3 ± 2.2	0.096
Visceral fat (g/100 g BW)	8.16 ± 0.48	9.31 ± 0.40	9.10 ± 0.45	7.87 ± 0.41	0.088

472 <sup>#</sup> Visceral fat weight is the sum of mesenteric, retroperitoneal, epididymal fat weights

473 Values shown are the means ± SE (*n* = 8–10). Significant differences among the  
 474 treatments were not detected by post-hoc analysis (*P* < 0.05, Tukey–Kramer test).

475

476

477 **Table 3. Cecal tissue, content weights, pH, and short chain fatty acid**  
 478 **concentrations of rats fed test-diets for 8 weeks (Experiment 1).**

	Control	HFS	H-DFA	H-Raf	ANOVA <i>P</i> value
Cecal tissue (g)	1.26 ± 0.06 <sup>b</sup>	0.97 ± 0.05 <sup>c</sup>	1.66 ± 0.07 <sup>a</sup>	1.47 ± 0.10 <sup>ab</sup>	<.0001
Cecal content (g)	2.55 ± 0.19 <sup>b</sup>	1.54 ± 0.17 <sup>c</sup>	3.79 ± 0.43 <sup>a</sup>	2.88 ± 0.36 <sup>ab</sup>	<.0001
Cecal pH	6.8 ± 0.1	6.6 ± 0.1	6.6 ± 0.1	6.5 ± 0.1	0.138
Acetate (μmol/g content)	20.7 ± 1.7 <sup>b</sup>	19.0 ± 1.6 <sup>b</sup>	32.8 ± 3.3 <sup>a</sup>	23.9 ± 1.9 <sup>ab</sup>	0.001
Acetate (μmol/cecum)	52.1 ± 5.3 <sup>bc</sup>	28.6 ± 3.3 <sup>c</sup>	118.8 ± 16.3 <sup>a</sup>	70.1 ± 10.4 <sup>b</sup>	<.0001
Propionate (μmol/g content)	4.61 ± 0.35	3.57 ± 0.24	4.70 ± 0.44	4.42 ± 0.29	0.087
Propionate (μmol/cecum)	11.26 ± 0.44 <sup>bc</sup>	5.66 ± 0.88 <sup>c</sup>	18.51 ± 3.18 <sup>a</sup>	13.20 ± 2.52 <sup>ab</sup>	0.000
Butyrate (μmol/g content)	2.03 ± 0.19 <sup>ab</sup>	1.75 ± 0.30 <sup>b</sup>	3.19 ± 0.50 <sup>a</sup>	2.20 ± 0.27 <sup>ab</sup>	0.025
Butyrate (μmol/cecum)	5.08 ± 0.56 <sup>bc</sup>	2.68 ± 0.50 <sup>c</sup>	11.87 ± 1.76 <sup>a</sup>	6.07 ± 0.80 <sup>b</sup>	<.0001
Succinate (μmol/g content)	20.7 ± 3.1 <sup>b</sup>	37.1 ± 1.8 <sup>a</sup>	21.5 ± 1.5 <sup>b</sup>	22.2 ± 2.7 <sup>b</sup>	<.0001
Succinate (μmol/cecum)	50.5 ± 7.3	58.0 ± 8.5	84.9 ± 14.9	64.0 ± 10.1	0.127

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480 Values shown are the means ± SE (*n* = 8–10). Values that do not share the same  
 481 letter differ significantly between the treatments (*P* < 0.05, Tukey–Kramer test).

482

483 **Table 4. Body weight, energy intake, and adipose tissue weights of rats fed the**  
 484 **control or the HFS diet supplemented with DFA III or FOS for 5 weeks (Experiment**  
 485 **2).**

		Fiber in the diet			ANOVA <i>P</i> value	2-way ANOVA					
		Cellulose	DFA III	FOS		HFS	Fiber	HFS x Fiber			
Body Weight (g)	Control	370 ± 12	<sup>ab</sup>	355 ± 7	<sup>b</sup>	377 ± 8	<sup>ab</sup>	0.003	0.000	0.050	0.967
	HFS	395 ± 11	<sup>a</sup>	384 ± 6	<sup>ab</sup>	404 ± 4	<sup>a</sup>				
Body Weight Gain (g)	Control	175 ± 9	<sup>bc</sup>	160 ± 5	<sup>c</sup>	182 ± 7	<sup>abc</sup>	0.000	<.0001	0.015	0.971
	HFS	200 ± 11	<sup>ab</sup>	189 ± 6	<sup>abc</sup>	209 ± 3	<sup>a</sup>				
Total Energy Intake (kcal)	Control	2769 ± 66	<sup>ab</sup>	2584 ± 38	<sup>b</sup>	2750 ± 74	<sup>ab</sup>	0.000	<.0001	0.012	0.833
	HFS	2942 ± 92	<sup>a</sup>	2815 ± 38	<sup>ab</sup>	2992 ± 42	<sup>a</sup>				
Mesenteric fat (g)	Control	6.01 ± 0.62	<sup>ab</sup>	5.56 ± 0.48	<sup>b</sup>	6.03 ± 0.46	<sup>ab</sup>	0.006	0.001	0.139	0.625
	HFS	7.50 ± 0.66	<sup>ab</sup>	6.58 ± 0.29	<sup>ab</sup>	7.98 ± 0.30	<sup>a</sup>				
Mesenteric fat (g/100 g BW)	Control	1.62 ± 0.15		1.56 ± 0.11		1.59 ± 0.10		0.047	0.004	0.357	0.566
	HFS	1.89 ± 0.12		1.71 ± 0.07		1.98 ± 0.08	<sup>NS</sup>				
Epididymal fat (g)	Control	8.16 ± 0.65	<sup>ab</sup>	6.58 ± 0.66	<sup>b</sup>	8.26 ± 0.70	<sup>ab</sup>	0.001	0.000	0.031	0.994
	HFS	10.35 ± 0.93	<sup>a</sup>	8.87 ± 0.25	<sup>ab</sup>	10.42 ± 0.68	<sup>a</sup>				
Epididymal fat (g/100 g BW)	Control	2.20 ± 0.16	<sup>ab</sup>	1.85 ± 0.17	<sup>b</sup>	2.18 ± 0.17	<sup>ab</sup>	0.012	0.002	0.073	0.975
	HFS	2.61 ± 0.18	<sup>a</sup>	2.31 ± 0.05	<sup>ab</sup>	2.58 ± 0.17	<sup>a</sup>				
Retroperitoneal fat (g)	Control	9.21 ± 0.90	<sup>bc</sup>	8.38 ± 0.63	<sup>c</sup>	9.42 ± 0.71	<sup>abc</sup>	0.002	<.0001	0.362	0.917
	HFS	11.76 ± 1.37	<sup>ab</sup>	11.42 ± 0.34	<sup>abc</sup>	12.59 ± 0.46	<sup>a</sup>				
Retroperitoneal fat (g/100 g BW)	Control	2.47 ± 0.18	<sup>ab</sup>	2.35 ± 0.15	<sup>b</sup>	2.49 ± 0.18	<sup>ab</sup>	0.006	0.000	0.698	0.876
	HFS	2.95 ± 0.26	<sup>ab</sup>	2.98 ± 0.07	<sup>ab</sup>	3.11 ± 0.10	<sup>a</sup>				
Visceral fat <sup>#</sup> (g)	Control	23.4 ± 2.0	<sup>bc</sup>	20.5 ± 1.7	<sup>c</sup>	23.7 ± 1.8	<sup>bc</sup>	0.001	<.0001	0.102	0.9483
	HFS	29.6 ± 2.9	<sup>ab</sup>	26.9 ± 0.7	<sup>abc</sup>	31.0 ± 1.0	<sup>a</sup>				
Visceral fat (g/100 g BW)	Control	6.29 ± 0.44	<sup>ab</sup>	5.76 ± 0.41	<sup>b</sup>	6.26 ± 0.41	<sup>ab</sup>	0.005	2E-04	0.259	0.9429
	HFS	7.44 ± 0.53	<sup>a</sup>	7.00 ± 0.14	<sup>ab</sup>	7.68 ± 0.26	<sup>a</sup>				

486 <sup>#</sup>Visceral fat weight is the sum of mesenteric, retroperitoneal, epididymal fat weights

487 Values shown are the means ± SE (*n* = 8). Values that do not share the same letter  
 488 differ significantly between the treatments (*P* < 0.05, Tukey–Kramer test).

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492 **Table 5. Cecal tissue, content weights, pH, and short chain fatty acid**  
 493 **concentrations of rats fed the control or the HFS diet supplemented with DFA III or**  
 494 **FOS for 5 weeks (Experiment 2).**

		Fiber in the diet			ANOVA <i>P</i> value	2-way ANOVA		
		Cellulose	DFA III	FOS		HFS	Fiber	HFS x Fiber
Cecal tissue (g)	Control	1.01 ± 0.03 <sup>c</sup>	1.49 ± 0.06 <sup>ab</sup>	1.49 ± 0.06 <sup>ab</sup>	<0.001	<0.002	<0.003	<0.004
	HFS	1.71 ± 0.09 <sup>a</sup>	1.32 ± 0.04 <sup>bc</sup>	1.78 ± 0.15 <sup>a</sup>				
Cecal content (g)	Control	2.27 ± 0.17 <sup>b</sup>	4.22 ± 0.64 <sup>a</sup>	2.03 ± 0.19 <sup>b</sup>	0.000	0.052	<.0001	0.076
	HFS	2.03 ± 0.18 <sup>b</sup>	2.79 ± 0.31 <sup>b</sup>	2.06 ± 0.22 <sup>b</sup>				
Cecal pH	Control	7.71 ± 0.07 <sup>a</sup>	7.48 ± 0.06 <sup>ab</sup>	7.29 ± 0.14 <sup>ab</sup>	0.000	<.0001	0.003	0.065
	HFS	7.07 ± 0.14 <sup>bc</sup>	7.35 ± 0.11 <sup>ab</sup>	6.84 ± 0.11 <sup>c</sup>				
Acetate (μmol/g content)	Control	22.6 ± 1.4	26.6 ± 2.2	25.3 ± 1.8	0.194	0.126	0.075	0.961
	HFS	19.9 ± 1.5	24.1 ± 1.9	23.6 ± 2.0				
Acetate (μmol/cecum)	Control	52.1 ± 6.4 <sup>b</sup>	112.2 ± 20.3 <sup>a</sup>	51.2 ± 5.6 <sup>b</sup>	0.000	0.013	<.0001	0.085
	HFS	39.8 ± 3.8 <sup>b</sup>	65.6 ± 7.2 <sup>b</sup>	46.9 ± 4.0 <sup>b</sup>				
Propionate (μmol/g content)	Control	8.57 ± 0.63 <sup>ab</sup>	10.10 ± 0.62 <sup>a</sup>	9.92 ± 0.68 <sup>a</sup>	0.001	0.000	0.024	0.556
	HFS	5.69 ± 0.47 <sup>b</sup>	7.22 ± 0.56 <sup>ab</sup>	8.43 ± 1.19 <sup>ab</sup>				
Propionate (μmol/cecum)	Control	19.5 ± 2.0 <sup>b</sup>	42.5 ± 6.6 <sup>a</sup>	20.6 ± 2.9 <sup>b</sup>	0.000	0.000	0.000	0.018
	HFS	11.7 ± 1.5 <sup>b</sup>	19.7 ± 2.1 <sup>b</sup>	17.4 ± 2.6 <sup>b</sup>				
Butyrate (μmol/g content)	Control	3.71 ± 0.26 <sup>a</sup>	2.79 ± 0.22 <sup>ab</sup>	3.27 ± 0.41 <sup>ab</sup>	0.022	0.002	0.292	0.507
	HFS	2.43 ± 0.36 <sup>ab</sup>	2.30 ± 0.44 <sup>b</sup>	2.38 ± 0.22 <sup>ab</sup>				
Butyrate (μmol/cecum)	Control	8.39 ± 0.85 <sup>ab</sup>	11.49 ± 1.63 <sup>a</sup>	6.53 ± 0.90 <sup>b</sup>	0.000	0.000	0.000	0.000
	HFS	5.01 ± 0.87 <sup>b</sup>	6.05 ± 0.95 <sup>b</sup>	4.62 ± 0.28 <sup>b</sup>				
Succinate (μmol/g content)	Control	8.0 ± 1.2 <sup>b</sup>	3.3 ± 1.0 <sup>b</sup>	7.5 ± 1.6 <sup>b</sup>	0.000	<.0001	<.0001	0.0026
	HFS	30.1 ± 2.7 <sup>a</sup>	13.0 ± 3.4 <sup>b</sup>	12.8 ± 3.0 <sup>b</sup>				
Succinate (μmol/cecum)	Control	18.1 ± 3.2 <sup>b</sup>	11.1 ± 3.1 <sup>b</sup>	16.0 ± 3.7 <sup>b</sup>	0.000	<.0001	0.008	0.0336
	HFS	61.6 ± 8.4 <sup>a</sup>	33.8 ± 9.7 <sup>b</sup>	25.0 ± 7.0 <sup>b</sup>				

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 497 Values are the means ± SE (*n* = 8). Values that do not share the same letter differ  
 498 significantly between the treatments (*P* < 0.05, Tukey–Kramer test).

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 500

501 **FIGURE LEGENDS**

502

503 **Fig. 1. GLP-1 concentration in the portal plasma after 8 weeks of test-diet feeding**  
504 **(Experiment 1).**

505 Blood samples were collected from the portal vein of rats fasted overnight after 8  
506 weeks of test-diet feeding, and the concentrations of total GLP-1 in the plasma were  
507 measured. Values are the means  $\pm$  SE (n = 8–10). Bars that do not share the same letter  
508 differ significantly between treatments ( $P < 0.05$ , Tukey–Kramer test).

509

510 **Fig. 2. Differences in cumulative energy intake compared to energy intake of the**  
511 **control group (Experiment 2).**

512 Cumulative energy intake was calculated from the amount of consumed diet in each  
513 group, and the differences between cumulative energy intakes of each group and the  
514 control group are presented as means  $\pm$  SE (n = 8). Two-way ANOVA p values for  
515 treatment, day, and treatment x day were  $<.0001$ , 0.947, and 0.023, respectively.

516

517

518 **Fig. 3. Plasma glucose, insulin, GLP-1 concentrations, and HOMA-IR under fasting**  
519 **condition 2 and 4 weeks after test-diet feeding (Experiment 2).**

520 Blood samples were collected from the tail vein of rats fasted overnight after 2 and 4  
521 weeks of test-diet feeding. Glucose (A, E), insulin (B, F), and total GLP-1 (D, H) levels  
522 were measured in the plasma. HOMA-IR (C, G) was calculated from glucose and insulin  
523 concentrations. Values are the means  $\pm$  SE (n = 8).  $P$  values of 2-way ANOVA are  
524 presented below each panel. Bars that do not share the same letter differ significantly  
525 between treatments ( $P < 0.05$ , Tukey–Kramer test).

526

527

528 **Fig. 4. GLP-1 and PYY concentrations in the portal plasma of rats fed the control or**  
529 **the HFS diet supplemented with DFA III or FOS for 5 weeks (Experiment 2).**

530 Blood samples were collected from the portal vein of rats fasted overnight after 5  
531 weeks of test-diet feeding, and concentrations of total GLP-1, active GLP-1, and PYY  
532 were measured in the plasma. Values are the means  $\pm$  SE (n = 8).  $P$  values of 2-way  
533 ANOVA were presented below each panel. Bars that do not share the same letter differ  
534 significantly between treatments ( $P < 0.05$ , Tukey–Kramer test).

535

Fig. 1.

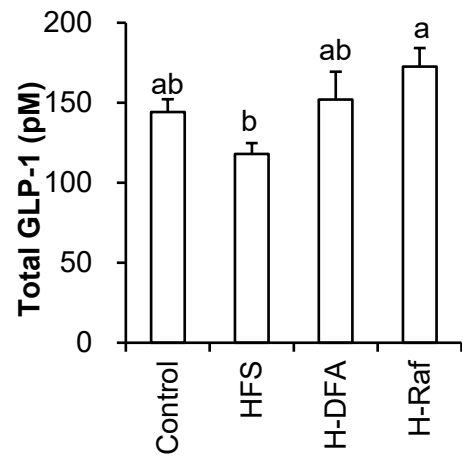
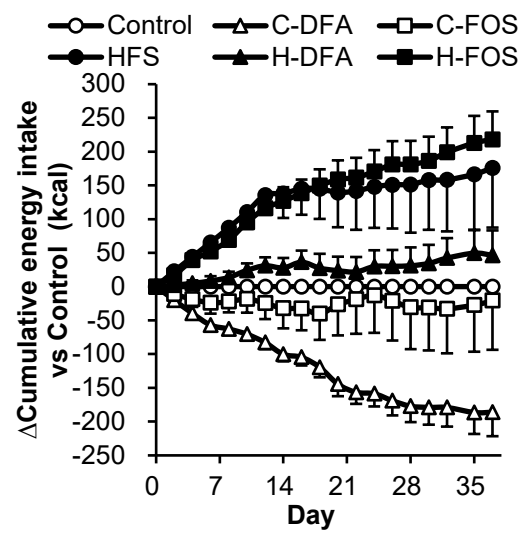
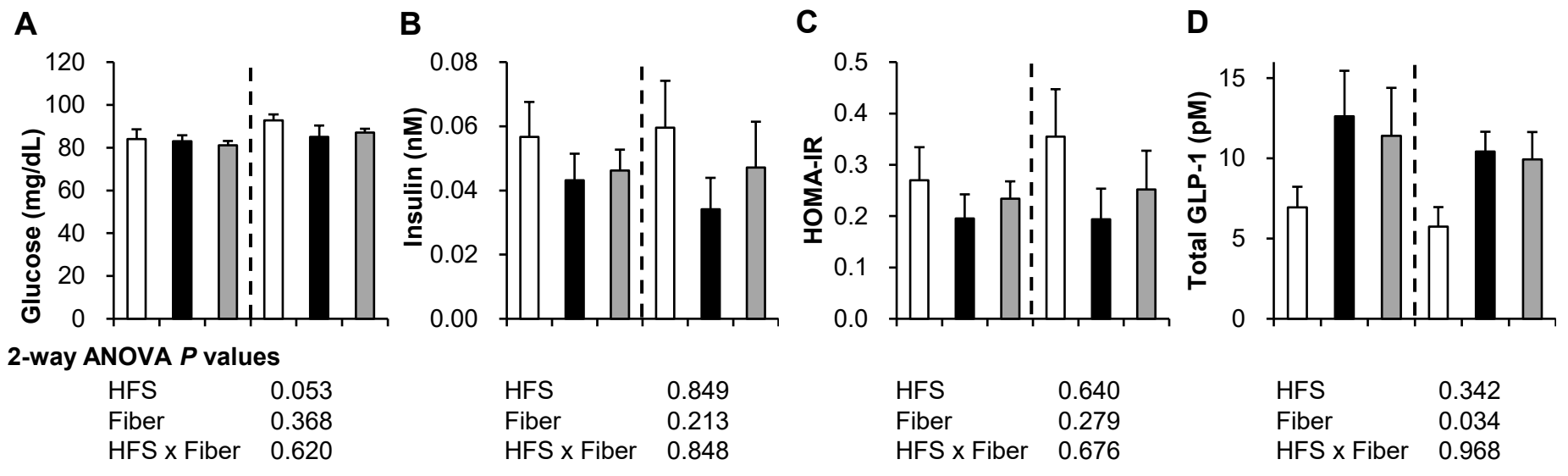


Fig. 2.

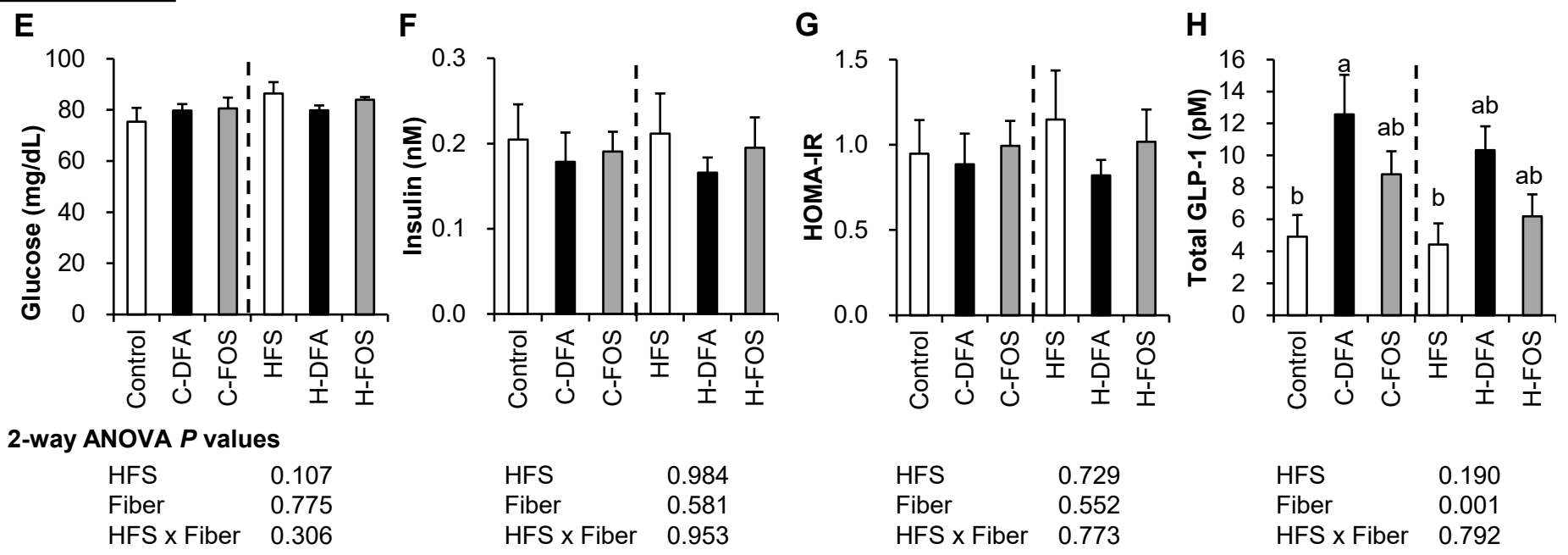


**Fig. 3.**

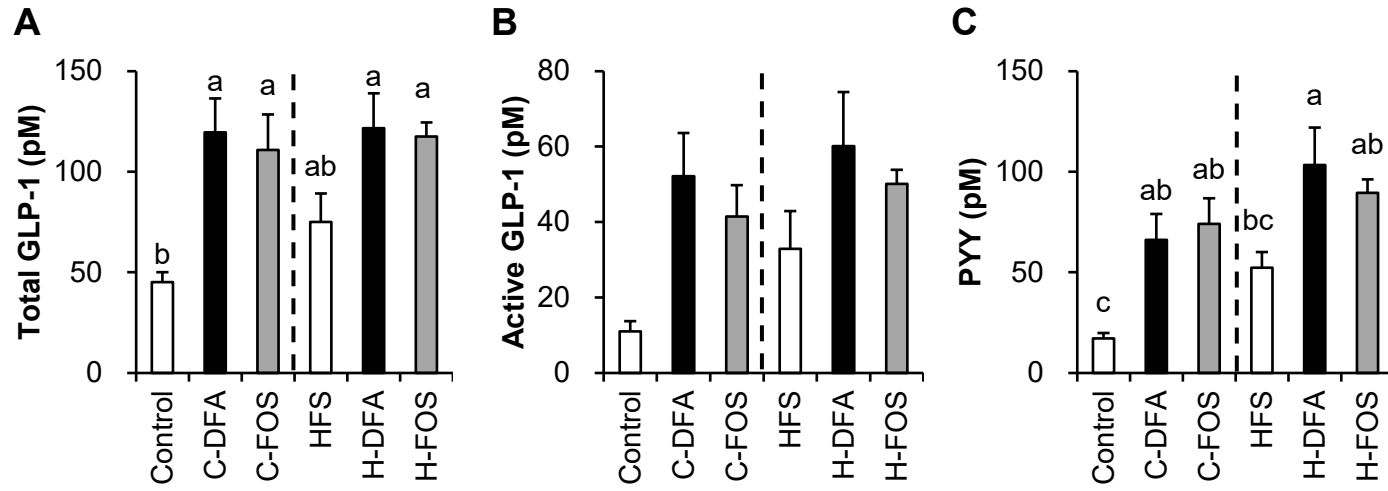
**After 2 weeks**



**After 4 weeks**



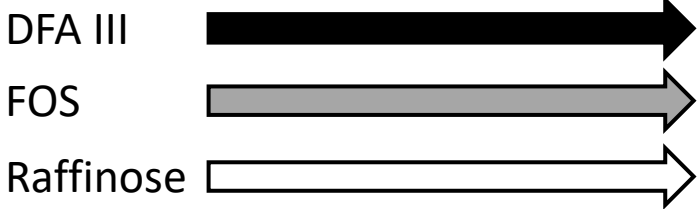
**Fig. 4.**



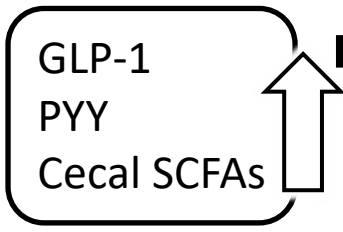
**2-way ANOVA P values**

HFS	0.265	HFS	0.151	HFS	<.0001
Fiber	0.000	Fiber	0.014	Fiber	0.003
HFS x Fiber	0.576	HFS x Fiber	0.796	HFS x Fiber	0.065

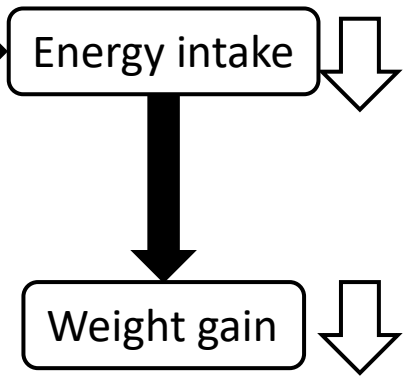
Oligosaccharides



Gut



Output



added at 3%  
in a high-fat/high-sucrose diet  
for 5 weeks

Supplementation of 3% DFA III attenuated energy intake  
of rats fed a obesogenic a high-fat/high-sucrose diet