Chemical modification of cornstarch by hydroxypropylation enhances cecal fermentation-mediated lipid metabolism in rats

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Abbreviations: ANOVA, analysis of variance; GLP-1, glucagon-like peptide-1; GPR43, G protein-coupled receptor 43; HACS, high amylose cornstarch; HP, hydroxypropylated; NCS, normal cornstarch; PCoA, principal coordinate analysis; QIIME, Quantitative Insight Into Microbial Ecology; WCS, waxy cornstarch;

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Abstract

Hydroxypropylated (HP)-cornstarch was fed to rats for 4 weeks and the effects on cecal fermentation and lipid metabolism were evaluated. In the cecum of rats fed either HP-normal cornstarch (NCS) or HP-waxy cornstarch (WCS), microbial composition was altered and the relative abundances of the Firmicutes and Bacteroidetes were decreased and increased, respectively, compared to high amylose cornstarch (HACS), NCS and WCS groups. Cecal total-SCFA content in the rats fed HP-NCS and HP-WCS was higher than the rats fed NCS and WCS. In HP-NCS and HP-WCS groups, cecal pH and mesenteric adipocyte area were decreased, and the plasma glucagon-like peptide-1 (GLP-1) level and cecal mucin content were increased compared to HACS, NCS and WCS groups. Plasma GLP-1 level correlated positively with the cecal SCFA content and the serum insulin level, and negatively with the feed intake, while the adipocyte area positively correlated with the serum triglyceride level. Therefore, HP-cornstarch might have possessed beneficial traits that enhanced cecal fermentation and thereby influenced lipid metabolism, equally or greater than that of HACS.

1. Introduction

Starch is one of the main components of food materials and it comprises a mixture of two molecules, amylose and amylopectin. Amylose is an essentially linear chain of α-(1,4)-linked glucose residues, while amylopectin is a branched molecule linked by α-(1, 6) linkages, comprising around 70-80% of starch, making it the major component of starch.[1] High-amylose cornstarch (HACS), one of the major high-amylose starches, resists digestion in the small intestine.[2] Unlike HACS, both normal cornstarch (NCS) and waxy cornstarch (WCS) are reported to be almost completely digested in various *in vivo* and *in vitro* studies.[3,4] The higher digestibility of NCS and WCS can be attributed to the higher peak viscosity and swelling power compared to HACS.[4]

A considerable proportion of HACS escapes small intestinal digestion and enters the large bowel, where it is fermented by microbiota, yielding SCFA, thus lowering the colonic pH and stimulating the proliferation of beneficial bacteria.[5] Short-chain fatty acid in the large bowel was reported to stimulate insulin secretion and suppress feed intake by the stimulation of glucagon-like peptide-1 (GLP-1) secretion,[3,6,7] a hormone secreted from L-cells in the gut. Further, increased SCFA content, mainly acetate and propionate, was reported to activate G protein-coupled receptor 43 (GPR43) expressed in the white adipose tissue,[8,9] where activated GPR43 might lead to the inhibition of fat accumulation.[9] Therefore, regulation of the intestinal microbiota and fermentation is important for host health.

Hydroxypropylated (HP)-starch is a chemically modified starch, widely used as a bulking agent, emulsifier, stabilizer and a thickener in the food industry.[10] Furthermore, HP-starch is reported to be resistant to digestive enzymes *in vivo* and *in vitro*,[3] so it was hypothesized that HP-NCS and HP-WCS would reach the colon with a lower degree of small intestinal digestion, where it would be fermented

by microbiota. It was previously reported that HP-tapioca starch reduced adipocyte size in KKAy mice,[10] so there is a possibility that HP-NCS and HP-WCS might possess beneficial physiological properties related to lipid metabolism also. However, evaluation of the effects of HP-NCS and HP-WCS on physiological properties compared with HACS is less well appreciated. Therefore, effects of HP-NCS and HP-WCS on intestinal fermentation properties and lipid metabolism in rats were evaluated in the present study.

2. Materials and methods

2.1. Materials

High-amylose cornstarch (roughly 70% amylose), NCS (roughly 70% amylopectin), WCS (almost 100% amylopectin), HP-NCS (0.084 degree of substitution) and HP-WCS (0.074 degree of substitution) were supplied by Matsutani Chemical Industry Co., Ltd (Itami, Japan). Indigestible polysaccharide content in HACS was determined by AOAC 2002. 02, and in NCS, WCS, HP-NCS and HP-WCS by AOAC 2001.03.

2.2. Animals and diets

The experimental design was approved by the Animal Experiment Committee of Obihiro University of Agriculture and Veterinary Medicine (approval number 29-94). The animal experiment was conducted as previously described[11] with slight modifications. Thirty male Fischer 344 rats (7-week-

old) weighing 130-160 g were purchased from Charles River Laboratories (Yokohama, Japan). The rats were treated and maintained according to the "Guide for the Care and Use of Laboratory Animals". After a 7-day acclimation period, the rats were randomly assigned to 5 groups and fed one of the experimental diets (Oriental Yeast Co., Ltd., Tokyo, Japan) formulated based on the AIN-93G diet (Supporting Information Table S1) for 4 weeks. Blood samples (1 mL) were collected on the sacrifice day following a 12 hours fasting period and the serum was prepared accordingly[12] to measure the insulin and triglyceride levels. And then, rats were anesthetized[11] between 0900 and 1100 hours and blood samples (1 mL) were collected from the abdominal aorta into syringes containing 0.5 M EDTA-disodium (10 µL), aprotinin (10 µL; 10 mg/mL; Cat. No. 1002646326, 3-8 TIU/mg solid, Sigma-Aldrich Co., Tokyo, Japan) and dipeptidyl peptidase-Ⅳ inhibitor (10 µL; Cat. No. DPP4, Merck Millipore, Billerica, MA, USA). The plasma was separated immediately by centrifugation (1,200 × g at 4℃for 20 min) and was stored at -80℃until GLP-1 and leptin analyses. Liver, cecum, perirenal and epididymal adipose tissues were excised and weighed. Mesenteric adipose tissue was excised and was fixed in freshly prepared 10% neutral buffered-formalin, for staining. The pH of the cecal content suspensions, prepared as previously described[11] was measured immediately[13] and the suspensions were stored at -30℃until further analyses.

2.3. Measurement of the mesenteric adipocyte size

Mesenteric adipose tissues fixed in 10% neutral buffered-formalin were embedded in paraffin to measure the adipocyte size. Four-micrometer thick tissue sections fixed in paraffin were cut and stained with hematoxylin-eosin. Adipocyte area (μm^2) was measured using the Image J software (National

Institutes of Health, Bethesda, MD, USA), and the mean area was calculated by averaging adipocyte areas of three randomly acquired images (magnification 10×) from each sample.

2.4. Plasma GLP-1, leptin and serum insulin analysis

The plasma GLP-1 and leptin levels were determined using a GLP-1, Active form (High sensitivity) Assay Kit (Cat. No. 27700, Immuno-Biological Laboratories Co., Ltd., Gunma, Japan) and a Mouse/Rat Leptin ELISA Kit (Cat. No. M1305, Morinaga Institute of Biological Science Inc., Yokohama, Japan), respectively, according to the manufacturers' instructions.

The serum insulin levels were determined using a Rat Insulin ELISA Kit (U-E type; Cat. No. AKRIN-130, Shibayagi, Gunma, Japan) according to the manufacturer's instructions.

2.5. Serum lipid profile analysis

The serum triglyceride levels were measured using Toshiba TBA-120FR autoanalyzer (Toshiba Medical Systems Corp., Tochigi, Japan).

2.6. Hepatic and fecal lipid analysis

Total hepatic lipid was extracted as previously described.[14] Hepatic triglyceride levels in the extracted total lipid fraction, dissolved in isopropyl alcohol, were measured using commercially available kits (Cat. No. 290-63701, Wako Pure Chemical Industry, Ltd., Osaka, Japan).

2.7. Cecal microbial analysis

2.7.1. DNA extraction and sequencing

Cecal genomic DNA extracted by RBB+C method[15] was purified using QIAamp DNA stool mini kit (Cat. No. 51504, Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The variable regions, V3 to V4 of the 16S rRNA gene were amplified using Illumina primer overhang adapters and bacterial universal primers as mentioned below: the forward overhang adapter and primer 341F (5′- TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG -3′) and the reverse overhang adapter and primer 805R (5′- GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G GAT TAC HVG GGT ATC TAA TCC -3′). Dual-index barcodes were added to the amplicon targets using the Nextera XT Index kit (Cat. No. FC-131-1002, Illumina, San Diego, CA, USA). The concentrations of the PCR products were measured (QuantiFluor dsDNA system, Cat. No. E2670, Promega, Madison, WI, USA), and were pooled in one tube in equal volumes. Paired-end sequencing was performed using Illumina MiSeq platform (Illumina).[16]

2.7.2. Analysis of 16S rRNA gene sequences

The raw 16S rRNA gene sequence data were analyzed by Quantitative Insight Into Microbial Ecology (QIIME) version 1.9.1 as reported by Warren et al.[16] The biome table was normalized using an equal subsampling size of 5,251 sequences. Distances between bacterial communities in different samples

were calculated by the unweighted Unifrac distance metric[17] in QIIME. Calypso[18] version 8.56 was used for quantitative visualization of the microbial community composition at phylum, genus and species levels.

2.8. Cecal SCFA analysis

The cecal SCFA contents in the rats were determined using HPLC (LC-10AD, Shimadzu, Kyoto, Japan). Samples for HPLC were prepared as previously described.[13] The analytical conditions were as follows: column, RSpak KC-811 (8.0 mm × 300 mm, Shodex, Tokyo, Japan); eluent and flow rate, 2 mM perchloric acid at 1.0 mL/min; column temperature, 47℃; reaction reagent and flow rate, ST3-R (Cat. No. F56120000, 10× diluted, Shodex) at 0.5 mL/min; UV-VIS detector wavelength, 430 nm.

2.9. Cecal mucin analysis

The mucin fractions were isolated from the rat cecal content suspensions as previously described[19] and mucin levels were determined by the fluorometric assay reported by Crowther and Wetmore.[20]

2.10. Cecal IgA analysis

The IgA content in the rat cecal content suspensions was determined using a rat IgA ELISA quantitation kit (Cat. No. E110-102, Bethyl Laboratories, Montgomery, TX, USA) according to the manufacturer's instructions.

2.11. Statistical analysis

Data, except microbial community data, are presented as mean ± SE (n = 6). Significant differences amongst the 5 groups were determined by analysis of variance (ANOVA) paired with Tukey's test (SPSS version 17, IBM Corporation, Armonk, NY, USA). Correlations between the parameters were assessed using Pearson's correlation analysis. For microbial community data, statistical significance of Shannon index was determined by Dunn's Multiple Comparison test-post hoc after Kruskal-Wallis H test (Prism version 7.0a, GraphPad Software, La Jolla, CA, USA), and the relative abundance of phyla, genera and species amongst the groups was determined by Kruskal-Wallis H test using Calypso version 8.56. A p value less than 0.05 was considered as statistically significant.

3. Results and Discussion

3.1. Effect of HP-cornstarch on feed intake, body weight gain, fasting plasma and serum hormone levels and adipose tissue parameters in rats

Feed intake and body weight gain were significantly lower ($p < 0.05$) in the rats fed HP-NCS and HP-WCS diets than the rats fed NCS and WCS diets (Table 1). Similarly, a previous study reported that the feed intake in mice fed HP-tapioca starch diets were lower than that of the mice fed unmodified tapioca starch diet.[10]

The fasting plasma GLP-1 levels in the rats fed HP-WCS diet were significantly higher ($p < 0.05$)

than the rats fed NCS and WCS diets (Table 1). The fasting serum insulin levels in the rats fed HP-NCS and HP-WCS diets were significantly higher ($p < 0.05$) than that of the NCS and WCS groups (Table 1). Pearson's correlation analysis also revealed a positive correlation with the serum insulin levels ($r = 0.378$; $p < 0.05$) and a negative correlation with the feed intake ($r = -0.650$; $p < 0.01$) for the plasma GLP-1 levels. In previous studies also, GLP-1 was reported to stimulate insulin secretion[7] and reduced feed intake.[3] Therefore, increased plasma GLP-1 levels in HP-NCS and HP-WCS groups might have caused the increment in the serum insulin levels and the observed reduction in feed intake in this study.

Leptin, secreted from the white adipose tissue, is a hormone responsible for reducing the feed intake.[21] However, in the current study, the fasting plasma leptin levels in the HACS, HP-NCS and HP-WCS groups were significantly lower ($p < 0.05$) than that of the WCS group (Table 1), and its levels in HACS and HP-NCS groups were also significantly lower ($p < 0.05$) than the rats fed NCS diet. In the current study, perirenal and epididymal fat tissue weights in the rats fed HP-NCS and HP-WCS diets were significantly lower ($p < 0.05$) than the rats fed NCS and WCS diets (Table 1). Mean mesenteric adipocyte area in rats fed HP-NCS and HP-WCS diets was significantly lower ($p < 0.05$) than rats fed HACS, NCS and WCS diets (Table 1 and Supporting Information Figure S1). It was reported that the leptin levels are positively correlated with adipose tissue weight or adipocyte size in mice,[22] and similarly, a positive correlation between the mesenteric adipocyte area and the plasma leptin levels was observed ($r = 0.419$; $p < 0.05$). A previous study also reported that the consumption of HP-tapioca starch decreased the epididymal adipocyte size in mice due to a reduction in energy intake.[10] The low energy densities of the two HP-cornstarch diets, reflected by the higher indigestible polysaccharide content (discussed in the section 3.4) might be also responsible for fat loss and

212 adipocyte size. Therefore, the lower plasma leptin levels might reflect the reduction of the fat mass and
213 the mesenteric adipocyte size in HP-NCS and HP-WCS groups.
214
215 **3.2. Effect of HP-cornstarch on serum lipid levels, liver parameters and fecal parameters in rats**
216
217 The fasting serum triglyceride levels in the rats fed HP-NCS and HP-WCS diets were significantly
218 lower ($p < 0.05$) than the rats fed NCS and WCS diets (Table 2). This study revealed positive
219 correlations for the mesenteric adipocyte area with fasting serum triglyceride levels ($r = 0.596$; $p <$
220 0.01). Therefore, the similar adipocyte size in the rats fed HP-NCS and HP-WCS diets might not only
221 be due to lower feed intake but also to lower serum lipid levels. The adipocyte size was reported to be
222 decreased in the rats with lower serum triglyceride levels previously.[13]
223 The liver weight in the rats fed WCS, HP-NCS and HP-WCS diets was significantly higher ($p < 0.05$)
224 than the rats fed NCS diet (Table 2). Hepatic triglyceride levels amongst the groups were not
225 statistically different (Table 2).
226
227 **3.3. Effect of HP-cornstarch on cecal microbial diversity and abundance in rats**
228
229 There are few reports about the effects of consumption of HP-starch on gut microbiota to date. Alpha-
230 diversity measured by Shannon index in the HP-NCS and HP-WCS groups showed lower values than
231 the other groups, and in the HP-NCS group, it was significantly lower ($p < 0.05$) than the HACS group
232 (Figure 1a). Beta-diversity presented in the principal coordinate analysis (PCoA) plot showed distinct
233 clustering amongst HACS, cornstarch and HP-cornstarch groups (Figure 1b).

The relative abundance of phylum Firmicutes in rats fed HP-NCS and HP-WCS diets was significantly lower ($p < 0.05$) than the rats fed NCS and WCS diets (Figure 2a). And the relative abundance of phylum Bacteroidetes in rats fed HP-NCS and HP-WCS diets was significantly higher ($p < 0.05$) than the rats fed HACS, NCS and WCS diets (Figure 2b), as a result, ratio of Firmicutes:Bacteroidetes in the HP-NCS and HP-WCS groups was significantly lower ($p < 0.05$) than the NCS and WCS groups (Figure 2c). The ratio of Firmicutes:Bacteroidetes is known to be negatively associated with obesity and positively associated with weight loss.[23]

The relative abundances of the genera, *Parabacteroides* and unclassified *Lachnospiraceae* in the rats fed HP-NCS and HP-WCS diets were significantly higher ($p < 0.01$) than the rats fed HACS, NCS and WCS diets (Figure 3a,c). Bacteria belonging to family *Lachnospiraceae* utilize starch and produce SCFA,[24] and genus *Parabacteroides* also has several enzymes which can degrade saccharides.[25] Relative abundances of genera *Bacteroides* and *Ruminococcus* in the rats fed HACS, HP-NCS and HP-WCS diets were significantly higher ($p < 0.05$) than that of the rats fed NCS and WCS diets (Figure 3b,d). Genus *Bacteroides* includes a variety of species expressing a wide range of carbohydrate active enzymes.[26] Within the genus *Ruminococcus*, *R. bromii* is well-known for its ability to degrade RS.[27] Furthermore, the abundance of *Akkermansia muciniphila* in the rats fed HP-WCS diet was significantly higher ($p < 0.05$) than the rats fed HACS and WCS diets (Figure 3e), and its abundance in the rats fed HP-NCS diet was significantly higher ($p < 0.05$) than that of the rats fed NCS diet. It has been previously reported that the depleted cecal *A. muciniphila* abundance in obese and type 2 diabetic mice was normalized upon feeding of prebiotics.[28] As reported elsewhere, a very high abundance of mucus-degrading bacteria, including *A. muciniphila*, in mice cecum degraded the mucus layer and weakened the barrier function.[29] The disproportionate proliferation of mucus-

256 degrading bacteria was observed in mice fed fiber-free diet, due to the lack of energy source for the
257 other bacteria to grow and proliferate.[29] Therefore, the higher abundance of mucus-degrading
258 members in the colonic microbiota of dietary fiber deprived subjects expressed a degraded mucus
259 lining.[29] In contrast, in this study, the diets contained 16-25% indigestible polysaccharide content
260 (discussed in details in section 3.4), thus microbiota might not have been deprived of the energy source.
261 Further, the relative abundance of *A. muciniphila* in all the groups was well within the reference limits
262 of a normal microbiota, according to the previous reports.[30]
263 According to these results, increased abundance of several specific bacterial groups might have led
264 to a lower α-diversity and different microbial community structure in HP-NCS and HP-WCS groups
265 compared to the other types of starch used in this study, similar to the previous reports.[3,31] The lower
266 α-diversity is often reported to accompany with a greater fermentation,[30] and the two HP-cornstarch
267 groups in this study exhibited a similar trend as the previous report. Although the cecal SCFA
268 production in the HACS group was higher than that of the NCS and WCS groups (discussed in the
269 section 3.4), α-diversity among the HACS, NCS and WCS groups was similar. Though the exact reason
270 is unclear, the relative abundance of bacterial genera, except genus *Ruminococcus*, in the HACS group
271 was similar to the NCS and WCS groups, therefore the α-diversity among the HACS, NCS and WCS
272 groups might not be different.
273
274 **3.4. Effect of HP-cornstarch on cecal parameters in rats**
275
276 The cecal content weight in the two HP-cornstarch groups was significantly higher ($p < 0.05$) than the
277 HACS, NCS and WCS groups (Table 3). The cecal pH in the rats fed HP-WCS diet was significantly

lower ($p < 0.05$) than the rats fed HACS, NCS and WCS diets, while it was significantly lower ($p < 0.05$) in HP-NCS group compared to the HACS and NCS groups (Table 3). Cecal acetate, propionate and total-SCFA contents in the rats fed HACS and HP-WCS diets were significantly higher ($p < 0.05$) than the rats fed NCS and WCS diets (Table 3). They were significantly higher ($p < 0.05$) in HP-NCS fed rats than that of the rats fed HACS, NCS, WCS and HP-WCS. *n*-Butyrate content in the rats fed HP-NCS diet were also significantly higher ($p < 0.05$) than the rats fed NCS, WCS and HP-WCS diets. In the current study, indigestible polysaccharide content in the products of HACS, NCS, WCS, HP-NCS and HP-WCS were 16.7%, 1.32%, 1.62%, 22.0% and 24.6%, respectively. Therefore, a large amount of non-digestible materials in HP-NCS and HP-WCS diets might enter the cecum increasing the cecal digesta volume and facilitating microbial growth and function.[3] A similar previous study also reported a lower cecal pH in rats fed HP-potato starch diets compared to the rats fed normal potato starch diet.[32] However, the consumption of HP-tapioca starch did not affect the cecal pH and the SCFA production in mice.[10] Thus, it appears that the effects of HP-starch on microbial fermentation might vary according to the starch source.

It has been reported that the activation of GPR43 in the white adipose tissue is related to the adipose tissue metabolism, which is identified as a SCFA receptor mainly activated by acetate and propionate.[8] Short-chain fatty acid-mediated activation of GPR43, suppressed adipose-specific insulin signaling, improved systemic insulin sensitivity and enhanced energy expenditure, thus leading to the inhibition of fat accumulation.[9] Similarly in the current study, Pearson's correlation test showed a negative correlation between the mesenteric adipocyte area and the serum insulin levels ($r = -0.474$; $p < 0.01$). Further, the activated GPR43 expressed in the intestinal endocrine L-cells, influenced the GLP-1 secretion.[6] In the current study also, the plasma GLP-1 levels were positively correlated with

300 the cecal acetate (r = 0.614; p < 0.01), propionate (r = 0.632; p < 0.01), *n*-butyrate (r = 0.461; p < 0.02)
301 and total-SCFA (r = 0.628; p < 0.01) contents. Thus, GPR43 expressed in the white adipose tissue and
302 intestine might have been activated by SCFA (acetate and propionate), which subsequently suppressed
303 fat accumulation and stimulated the GLP-1 secretion in HP-NCS and HP-WCS groups.
304 Cecal mucin content in the rats fed HP-NCS and HP-WCS diets was significantly higher (p < 0.05)
305 than that of the rats fed HACS, NCS and WCS diets (Table 3). The cecal IgA expression was not
306 statistically significant among the diet groups (Table 3), yet in HP-NCS and HP-WCS groups, it tended
307 to be higher than the NCS group (p = 0.074 and 0.060, respectively), while the HP-WCS group tended
308 to have a higher IgA content than that of the WCS group (p = 0.091). Mucin is a key component of the
309 intestinal barrier that prevents potential pathogens and antigens from entering the underlying
310 epithelium,[33] and IgA is known to block luminal bacterial attachment to epithelial cells.[34] It was
311 reported that cecal SCFA stimulates the secretion of mucin[11] and IgA.[3] Pearson's correlation test also
312 showed positive correlations for the cecal mucin and IgA contents with the cecal acetate (mucin, r =
313 0.769; IgA, r = 0.666; p < 0.01), propionate (mucin, r = 0.725; IgA, r = 0.603; p < 0.01), *n*-butyrate
314 (mucin, r = 0.411; IgA, r = 0.408; p < 0.05) and total-SCFA (mucin, r = 0.760; IgA, r = 0.661; p <
315 0.01) contents. Further, the negative correlation between cecal IgA expression and cecal pH (r = -0.780
316 < 0.01) was also comparable with previous reports.[3] Therefore, the secretion of cecal mucin and IgA
317 in HP-NCS and HP-WCS groups might have been stimulated by the increased cecal SCFA and lowered
318 cecal pH. Further, it was reported that the oral administration of *A. muciniphila* led to increased mucin
319 layer thickness[28] in mice, which is indicative of improved gut integrity. Therefore, an increase in the
320 relative abundance of the *A. muciniphila* and the cecal mucin content in the two HP-cornstarch groups
321 might have been correlated with each other.

4. Conclusions

In conclusion, our observations suggested that the consumption of HP-NCS and HP-WCS could alter the cecal microbial composition and exacerbate cecal fermentation, including secretion of hormones, due to their increased resistance to digestive enzymes. In addition, reduction in adipose tissue mass and adipocyte size could be attributed to the effects of cecal fermentation. These effects in the HP-NCS and HP-WCS were equal to or greater than that of the HACS. Therefore, HP-cornstarch might possess beneficial traits to infer beneficial physiological properties on intestinal fermentation and lipid metabolism.

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Conflict of interest

Authors declare no financial/commercial conflict of interests.

References

[1] S. Jobling, *Curr. Opin. Plant Biol.* **2004**, *7*, 210.

[2] R.M. Sandstedt, D. Strahan, S. Ueda, R.C. Abbot, *Cereal Chem.* **1962**, *39*, 123.

[3] K. Ebihara, M. Tachibe, N. Kaneko, T. Kishida, *J. Nutr. Sci.* **2013**, *2*, 1.

[4] H. Liu, L. Ramsden, H. Corke, *Carbohydr. Polym.* **1999**, *40*, 175.

[5] D.L. Topping, P.M. Clifton, *Physiol. Rev.* **2001**, *81*, 1031.

[6] G. Tolhurst, H. Heffron, Y.S. Lam, H.E. Parker, A.M. Habib, E. Diakogiannaki, J. Cameron, J. Grosse, F. Reimann, F.M. Gribble, *Diabetes* **2012**, *61*, 364.

[7] J.J. Holst, *Am. J. Physiol. Endocrinol. Metab.* **2004**, *287*, E199.

[8] E. Le Poul, C. Loison, S. Struyf, J.Y. Springael, V. Lannoy, M.E. Decobecq, S. Brezillon, V. Dupriez, G. Vassart, J. Van Damme, M. Parmentier, M. Detheux, *J. Biol. Chem.* **2003**, *278*, 25481.

[9] I. Kimura, K. Ozawa, D. Inoue, T. Imamura, K. Kimura, T. Maeda, K. Terasawa, D. Kashihara, K. Hirano, T. Tani, T. Takahashi, S. Miyauchi, G. Shioi, H. Inoue, G. Tsujimoto, *Nat. Commun.* **2013**, *4*, 1.

[10] R. Kato, M. Tachibe, S. Sugano, T. Kishida, K. Ebihara, *J. Food Sci.* **2009**, *74*, H89.

[11] R. Nagata, M. Echizen, Y. Yamaguchi, K.H. Han, K. Shimada, K. Ohba, T. Kitano-Okada, T. Nagura, H. Uchino, M. Fukushima, *Biosci., Biotechnol., Biochem.* **2018**, *82*, 489.

[12] K.H. Han, H. Tsuchihira, Y. Nakamura, K. Shimada, K. Ohba, T. Aritsuka, H. Uchino, H. Kikuchi, M. Fukushima, *Dig. Dis. Sci.* **2013**, *58*, 2177.

[13] K.H. Han, C.H. Lee, M. Kinoshita, C.H. Oh, K. Shimada, M. Fukushima, *Food Funct.* **2016**,

7, 1814.

[14] E.G. Bligh, W.J. Dyer, *Can. J. Biochem. Physiol.* **1959**, *37*, 911.

[15] Z. Yu, M. Morrison, *BioTechniques* **2004**, *36*, 808.

[16] F.J. Warren, N.M. Fukuma, D. Mikkelsen, B.M. Flanagan, B.A. Williams, A.T. Lisle, P. Ó Cuív, M. Morrison, M.J. Gidley, *mSphere* **2018**, *3*, 1.

[17] C. Lozupone, R. Knight, *Appl. Environ. Microbiol.* **2005**, *71*, 8228.

[18] M. Zakrzewski, C. Proietti, J.J. Ellis, S. Hasan, M.J. Brion, B. Berger, L. Krause, *Bioinformatics* **2017**, *33*, 782.

[19] I.M.J. Bovee-Oudenhoven, M.L. Termont, P.J. Heidt, R. Van Der Meer, *Gut* **1997**, *40*, 497.

[20] R.S. Crowther, R.F. Wetmore, *Anal. Biochem.* **1987**, *163*, 170.

[21] J.L. Halaas, K.S. Gajiwala, M. Maffei, S.L. Cohen, B.T. Chait, D. Rabinowitz, R.L. Lallone, S.K. Burley, J.M. Friedman, *Science* **1995**, *269*, 543.

[22] K.Y. Guo, P. Halo, R.L. Leibel, Y. Zhang, *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **2004**, *287*, R112.

[23] P.J. Turnbaugh, R.E. Ley, M.A. Mahowald, V. Magrini, E.R. Mardis, J.I. Gordon, *Nature* **2006**, *444*, 1027.

[24] H. Zeng, C. Huang, S. Lin, M. Zheng, C. Chen, B. Zheng, Y. Zhang, *J. Agric. Food Chem.* **2017**, *65*, 9217.

[25] M. Sakamoto, Y. Benno, *Int. J. Syst. Evol. Microbiol.* **2006**, *56*, 1599.

[26] A.A. Salyers, J.R. Vercellotti, S.E.H. West, T.D. Wilkins, *Appl. Environ. Microbiol.* **1977**, *34*, 529.

[27] X. Ze, S.H. Duncan, P. Louis, H.J. Flint, *ISME J.* **2012**, *6*, 1535.

[28] A. Everard, C. Belzer, L. Geurts, J.P. Ouwerkerk, C. Druart, L.B. Bindels, Y. Guiot, *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 9066.

[29] M.S. Desai, A.M. Seekatz, N.M. Koropatkin, N. Kamada, C.A. Hickey, M. Wolter, N.A. Pudlo, S. Kitamoto, N. Terrapon, A. Muller, V.B. Young, B. Henrissat, P. Wilmes, T.S. Stappenbeck, G. Núñez, E.C. Martens, *Cell* **2016**, *167*, 1339.

[30] T. Genda, T. Kondo, S. Sugiura, S. Hino, S. Shimamoto, T. Nakamura, S. Ukita, T. Morita, *J. Agric. Food Chem.* **2018**, *66*, 11909.

[31] Y. Zhong, M. Nyman, F. Fåk, *Mol. Nutr. Food Res.* **2015**, *59*, 2066.

[32] K. Ebihara, R. Shiraishi, K. Okuma, *J. Nutr.* **1998**, *128*, 848.

[33] S. Hino, N. Takemura, K. Sonoyama, A. Morita, H. Kawagishi, S. Aoe, T. Morita, *J. Nutr.* **2012**, *142*, 1429.

[34] N.K. Childers, M.G. Bruce, J.R. McGhee, *Annu. Rev. Microbiol.* **1989**, *43*, 503.

Figure legends

Figure 1. (a) Alpha-diversity measured by Shannon index and (b) β-diversity presented in the PCoA plot in cecal microbiota of rats fed HACS, NCS, WCS, HP-NCS and HP-WCS diets for 4 weeks (n = 6). Statistical significance of the α-diversity amongst the groups were determined by Dunn's Multiple Comparison test-post hoc after Kruskal-Wallis H test using Prism version 7.0a ($^{**}p < 0.01$).

Figure 2. Relative abundance of the phyla (a) Firmicutes and (b) Bacteroidetes, and (c) Firmicutes:Bacteroidetes (F:B) ratio in the cecum of rats fed HACS, NCS, WCS, HP-NCS and HP-WCS diets for 4 weeks (n = 6). Statistical significance amongst the groups were determined by Kruskal-Wallis H test using Calypso version 8.56 ($^{*}p < 0.05$ and $^{**}p < 0.01$).

Figure 3. Relative abundance of the genera (a) *Parabacteroides*, (b) *Bacteroides*, (c) Unclassified. *Lachnospiraceae* and (d) *Ruminococcus*, and (e) *Akkermansia muciniphila* in the cecum of rats fed HACS, NCS, WCS, HP-NCS and HP-WCS diets for 4 weeks (n = 6). Statistical significance amongst the groups were determined by Kruskal-Wallis H test using Calypso version 8.56 ($^{*}p < 0.05$ and $^{**}p < 0.01$).

Figure S1. Hematoxylin-eosin stained mesenteric adipose tissue in rats fed HACS, NCS, WCS, HP-NCS and HP-WCS diets for 4 weeks.

Table 1. Feed intake, body weight gain, fasting plasma and serum hormone levels and adipose tissue parameters in rats fed specific diets for 4 weeks.

	HACS	NCS	WCS	HP-NCS	HP-WCS
Feed intake (g/4 weeks)	338 ± 5^{ab}	352 ± 8^{a}	360 ± 3^{a}	317 ± 5^{b}	322 ± 7^{b}
Body weight gain (g/4 weeks)	53.7 ± 2.0^{b}	56.6 ± 0.1^{b}	64.9 ± 0.4^{a}	42.5 ± 0.6^{c}	45.7 ± 2.3^{c}
Plasma GLP-1 (pmol/L)	5.70 ± 1.01^{ab}	4.43 ± 0.74^{b}	3.68 ± 0.39^{b}	9.91 ± 1.55^{ab}	11.6 ± 2.9^{a}
Plasma leptin (ng/mL)	1.61 ± 0.21^{c}	2.63 ± 0.16^{ab}	3.46 ± 0.26^{a}	1.48 ± 0.19^{c}	2.05 ± 0.30^{bc}
Serum insulin (pg/mL)	119 ± 3^{ab}	107 ± 2^{b}	108 ± 3^{b}	125 ± 5^{a}	131 ± 5^{a}
Perirenal + epididymal fat (g/100 g body weight)	3.51 ± 0.26^{ab}	3.72 ± 0.30^{a}	4.33 ± 0.23^{a}	2.25 ± 0.20^{c}	2.57 ± 0.15^{bc}
Mean mesenteric adipocyte area (μm^2)	2291 ± 109^{a}	2415 ± 196^{a}	2484 ± 146^{a}	1410 ± 108^{b}	1569 ± 175^{b}

Data are expressed as mean ± SE (n = 6). $^{a\text{-}c}$Mean values within a row with unlike superscript letters are significantly different ($p < 0.05$), as determined by ANOVA paired with Tukey's test.

Table 2. Serum triglyceride levels and liver parameters in rats fed specific diets for 4 weeks.

	HACS	NCS	WCS	HP-NCS	HP-WCS
Serum triglyceride (mmol/L)	0.75 ± 0.07^{bc}	0.92 ± 0.06^{b}	1.34 ± 0.09^{a}	0.59 ± 0.09^{c}	0.52 ± 0.05^{c}
Liver					
Weight (g/100 g body weight)	2.42 ± 0.03^{ab}	2.33 ± 0.04^{b}	2.54 ± 0.06^{a}	2.53 ± 0.04^{a}	2.54 ± 0.03^{a}
Triglyceride (μmol/g liver)	12.9 ± 1.0	11.1 ± 1.0	12.3 ± 1.2	10.8 ± 1.3	12.1 ± 0.8

Data are expressed as mean ± SE (n = 6). [a-c]Mean values within a row with unlike superscript letters are significantly different ($p < 0.05$), as determined by ANOVA paired with Tukey's test.

Table 3. Cecal parameters in rats fed specific diets for 4 weeks.

	HACS	NCS	WCS	HP-NCS	HP-WCS
Cecal content					
Weight (g)	2.58 ± 0.15^{b}	1.49 ± 0.19^{b}	1.32 ± 0.06^{b}	7.02 ± 1.12^{a}	7.11 ± 1.45^{a}
pH	7.63 ± 0.06^{a}	7.64 ± 0.03^{a}	7.56 ± 0.06^{ab}	7.25 ± 0.12^{bc}	7.05 ± 0.11^{c}
SCFA (μmol/content)					
Acetate	232 ± 19^{b}	123 ± 13^{c}	107 ± 16^{c}	320 ± 25^{a}	211 ± 25^{b}
Propionate	35.4 ± 2.7^{b}	20.1 ± 2.5^{c}	16.8 ± 2.5^{c}	51.4 ± 1.8^{a}	35.1 ± 5.1^{b}
n-Butyrate	13.8 ± 2.6^{ab}	3.95 ± 0.46^{b}	4.98 ± 0.60^{b}	25.7 ± 9.3^{a}	7.02 ± 0.93^{b}
Total-SCFA	282 ± 22^{b}	147 ± 16^{c}	128 ± 18^{c}	397 ± 30^{a}	253 ± 30^{b}
Mucin (mg/content)	12.0 ± 1.0^{b}	7.11 ± 1.15^{b}	5.04 ± 0.13^{b}	34.9 ± 6.7^{a}	32.7 ± 6.8^{a}
IgA (mg/content)	1.11 ± 0.07	0.57 ± 0.04	0.73 ± 0.05	2.74 ± 0.71	2.82 ± 1.03

Data are expressed as mean ± SE (n = 6). $^{a\text{-}c}$Mean values within a row with unlike superscript letters are significantly different ($p < 0.05$), as determined by ANOVA paired with Tukey's test.

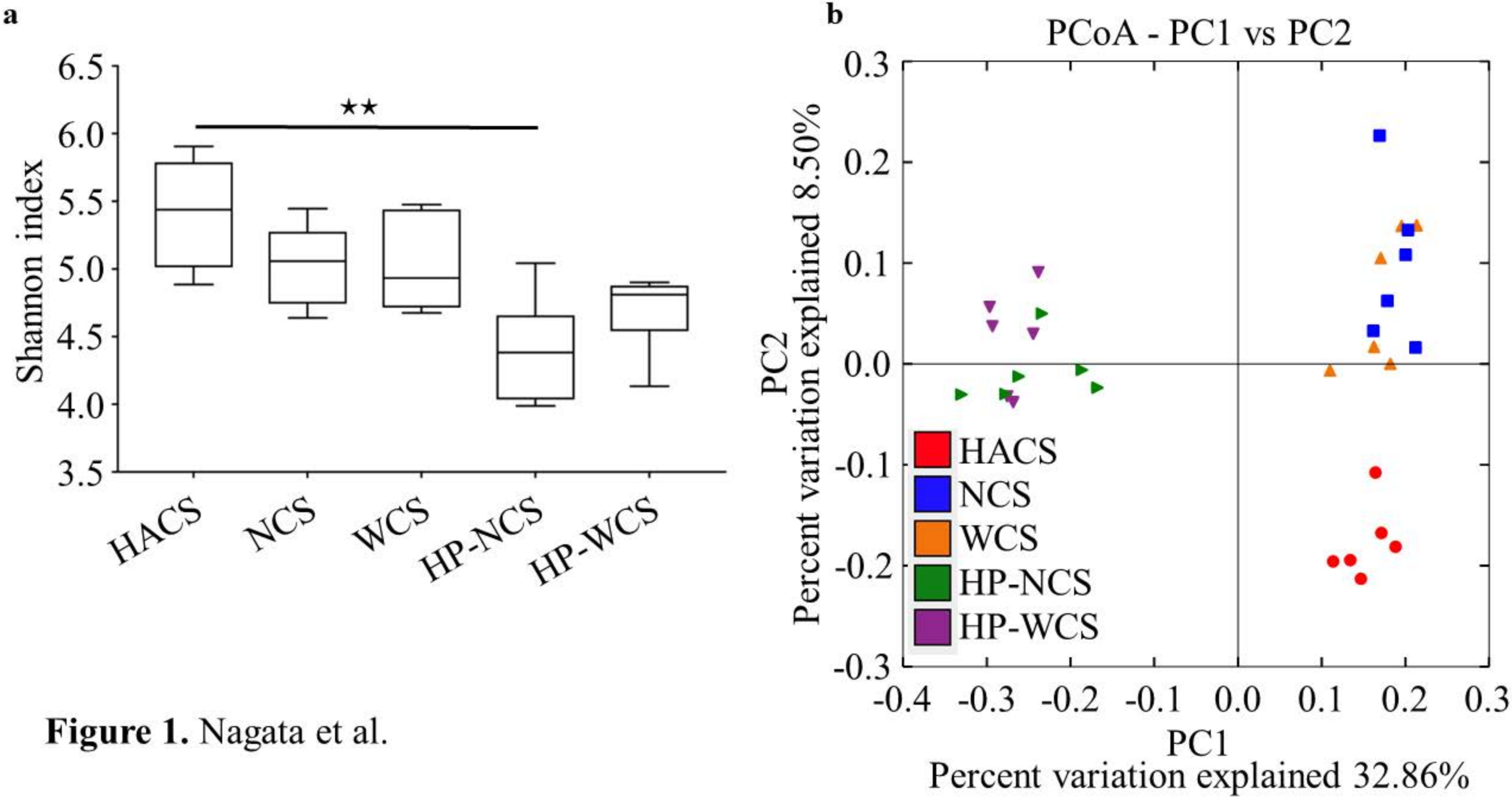
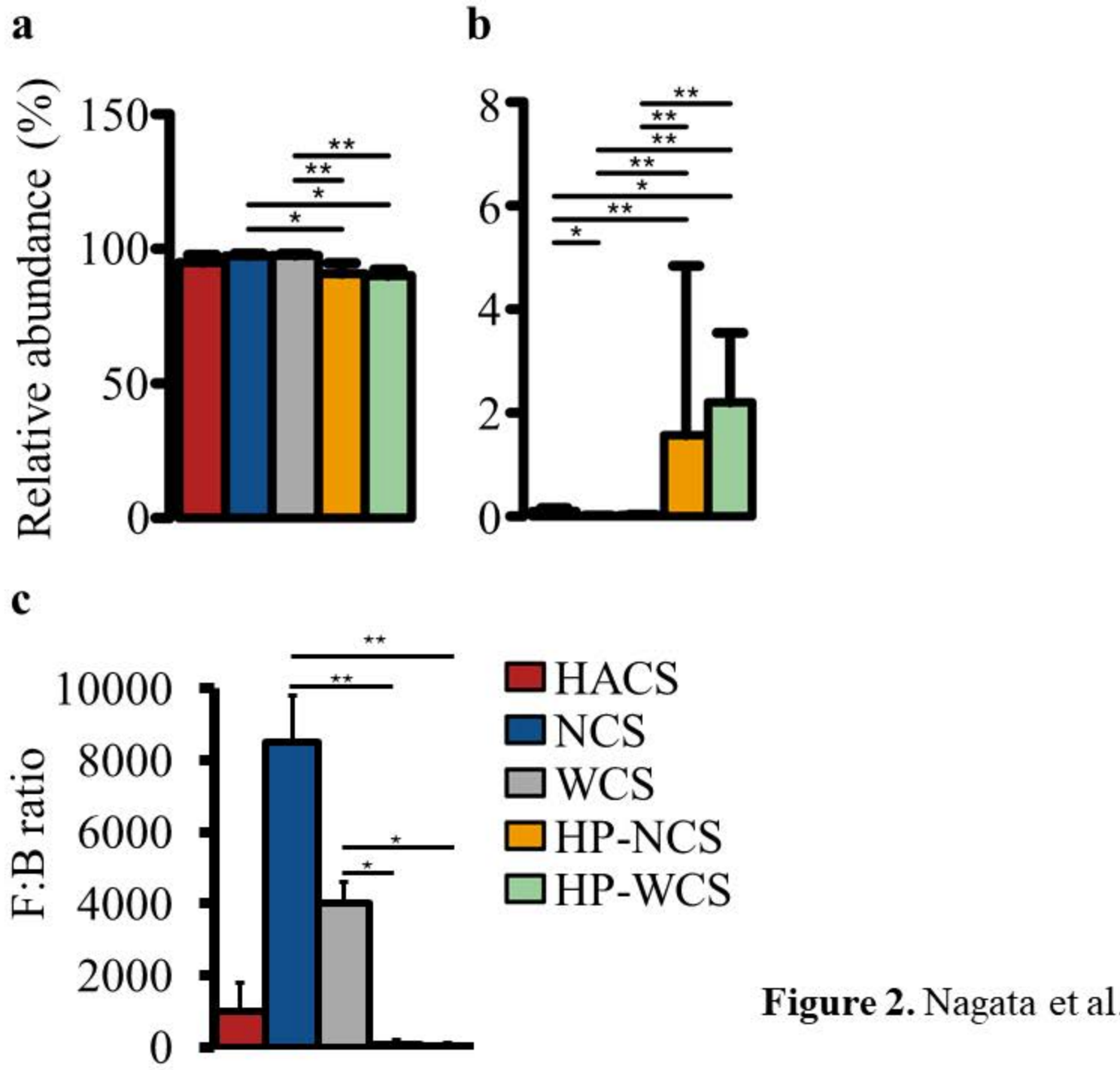


Figure 1. Nagata et al.



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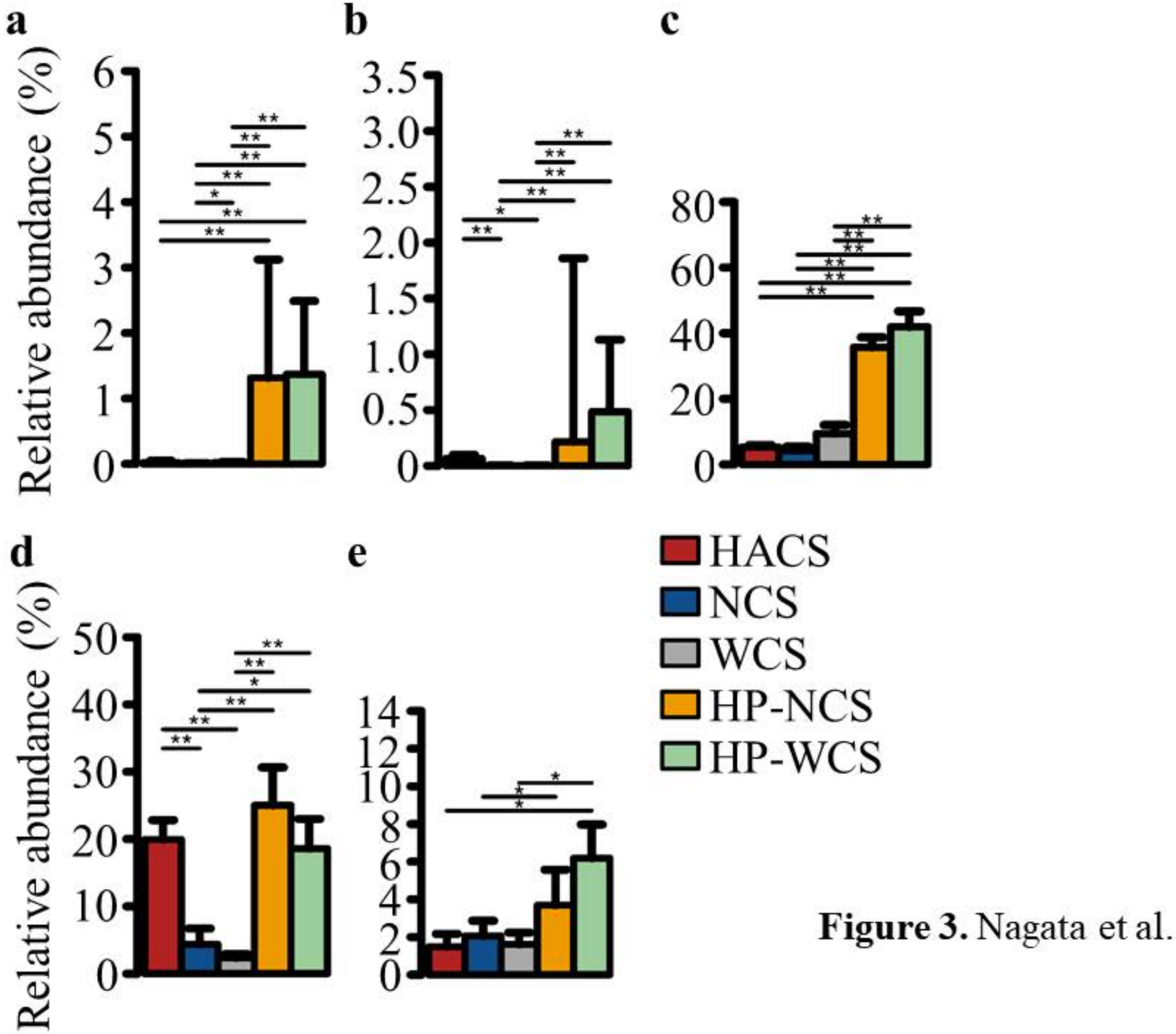

Figure 3. Nagata et al.

Supporting Information

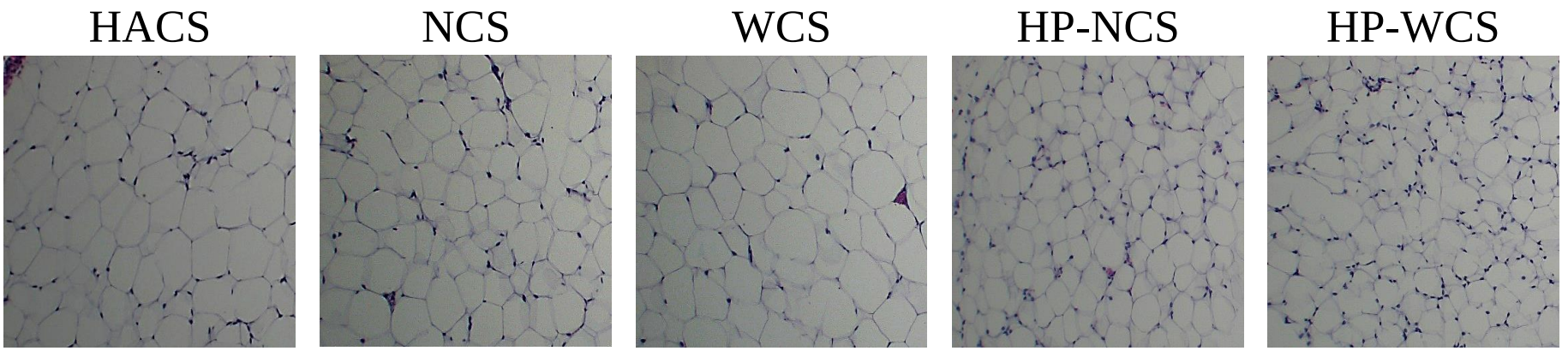

Figure S1. Hematoxylin-eosin stained mesenteric adipose in rats fed HACS, NCS, WCS, HP-NCS and HP-WCS diets for 4 weeks.