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ajpendo.00323.2017](https://journals.physiology.org/doi/full/10.1152/ajpendo.00323.2017)

1 **Mechanistically different effects of fat and sugar on insulin resistance,**
2 **hypertension and gut microbiota in rats**

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23 determinations, M.H. and O.J. made the IsoPs determinations; G.D. and I.M. made the
24 determinations of lipid mediators of inflammation; M.R.N. and M.R. evaluated liver
25 function; S.R.-R. and M.H. analyzed the data; S.R.-R. and J.L.T. wrote the paper.

26
27 Running head: Fat, sugar, insulin resistance and microbiota

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35 **ABSTRACT**

36

37 Insulin resistance (IR) and impaired glucose tolerance (IGT) are the first manifestations
38 of diet-induced metabolic alterations leading to type-2 diabetes, while hypertension is
39 the deadliest risk factor of cardiovascular disease. The roles of dietary fat and fructose
40 in the development of IR, IGT and hypertension are controversial.

41 We tested the long-term effects of an excess of fat or sucrose (fructose/glucose) on
42 healthy male Wistar Kyoto (WKY) rats. Fat affects IR and IGT earlier than fructose
43 through low-grade systemic inflammation evidenced by liver inflammatory infiltration,
44 increased levels of plasma interleukin-6, prostaglandin E₂ and reduced levels of
45 protective short-chain fatty acids without triggering hypertension. Increased populations
46 of gut Enterobacteriales and *Escherichia coli* may contribute to systemic inflammation
47 through the generation of lipopolysaccharides. Unlike fat, fructose induces increased
48 levels of diacylglycerols (lipid mediators of IR) in the liver, urine F₂-isoprostanes
49 (markers of systemic oxidative stress) and uric acid, and triggers hypertension. Elevated
50 populations of Enterobacteriales and *E. coli* were only detected in rats given an excess
51 of fructose at the end of the study.

52 Dietary fat and fructose trigger IR and IGT in clearly differentiated ways in WKY rats:
53 early low-grade inflammation and late direct lipid toxicity, respectively; gut microbiota
54 plays a role mainly in fat-induced IR; and hypertension is independent of inflammation-
55 mediated IR. The results provide evidence which suggests that the combination of fat
56 and sugar is potentially more harmful than fat or sugar alone when taken in excess.

57

58 **Keywords:** diabetes, hypertension, microbiota, obesity

59 INTRODUCTION

60 Obesity and type 2 diabetes (T2D) together with hypertension and
61 hypercholesterolemia are the main risk factors of cardiovascular disease (CVD), the
62 leading cause of death worldwide. An unhealthy lifestyle, the combination of a poor diet
63 with physical inactivity, is the single greatest contributor to the appearance of all these
64 warning signs (36). Obesity, insulin resistance (IR, first revealed by high fasting
65 insulinemia) and impaired glucose tolerance (IGT) appear to be the first manifestations
66 of the so-called metabolic syndrome (12), and hypertension is probably the deadliest
67 CVD triggering factor (16).

68 The relationships between fat accumulation, IR and hypertension are still unclear in
69 both humans and animal models. Whereas most diet-induced rodent models of obesity
70 and/or hypertension are insulin resistant, they show significant differences. Rats fed a
71 high-fat diet become overweight but rarely develop hypertension unless their diet is
72 supplemented with salt (1, 39); while those fed a high-fructose or high-sucrose (50%
73 fructose) diet may become hypertensive while remaining normoweight (10, 40, 49).
74 There is wide consensus that ectopic lipid accumulation in key organs such as liver and
75 muscle originates IR, but how particular lipids contribute to systemic IR and the
76 relevance of their location is an active area of research (44). Visceral adipose tissue (AT)
77 has been associated with low-grade inflammation and the metabolic complications of
78 obesity, mainly because it releases free fatty acids and pro-inflammatory adipokines into
79 the portal vein for direct transport to the liver (9, 14). Meanwhile, particular lipids such
80 as diacylglycerols (DAGs) and ceramides may impair insulin signaling in liver and
81 muscle independently of inflammation by altering the phosphorylation pattern of the
82 insulin receptor substrate (IRS): a key protein in the intracellular insulin signaling
83 pathway (13, 44). DAGs may be formed from triacylglycerides (TAGs) by lipolysis or

84 via *de novo* synthesis of TAGs from free fatty acids (13).

85 The relationship between IR and hypertension is also a controversial issue. Whereas
86 it has been proposed that IR is the main upstream event leading to hypertension (49),
87 other evidence suggests that different factors may increase blood pressure more
88 decisively than IR (27).

89 As it is becoming evident that diet-induced changes in populations of gut
90 microbiota play a role in the development of obesity and related disorders (42), the
91 study of the crosstalk between the host organism and its associated microbiota is
92 emerging as an active area of research. Specifically, gut microbiota has been shown to
93 trigger IR through the action of pro-inflammatory products of bacterial metabolism (3).

94 To substantiate preventive dietary strategies against metabolic alterations, it is
95 important to characterize early pre-disease events and to define molecular and
96 physiological markers that are suitable for evaluating nutritional or behavioral
97 interventions. As the molecular mechanisms linking lipid accumulation, IR and
98 hypertension are still largely unknown, we compared two animal models of diet-induced
99 metabolic changes: Wistar Kyoto (WKY) rats fed a high-fat (HF) or high-sucrose (HS)
100 diet. Both models develop incipient IR and/or IGT, while only the HS-fed rats become
101 hypertensive. This paper focuses on the differential mechanisms associated with dietary
102 fat and sugar (fructose) as triggering factors of IR, IGT and hypertension, and it
103 examines the possible role played by gut microbiota in these effects.

104

105 MATERIALS AND METHODS

106

107 *Animals.* A total of twenty-seven male WKY rats (Envigo, Indianapolis, IN, USA),
108 aged 8-9 weeks were used. All animal manipulation was carried out in the morning to
109 minimize the effects of circadian rhythms. All the procedures strictly adhered to the
110 European Union guidelines for the care and management of laboratory animals
111 (directive 2010/63/UE) under license from the regional Catalan authorities (reference
112 no. DAAM7921), and approved by the Spanish CSIC Subcommittee of Bioethical
113 Issues.

114 *Experimental design and sample collection.* The rats were housed (n = 3 per cage)
115 under controlled conditions of humidity (60%), and temperature (22 ± 2 °C) with a 12 h
116 light-12 h dark cycle. They were randomly divided into 3 dietary groups (n = 9 per
117 group): the standard (STD) group, fed a standard diet (2014 Teklad Global 14% Protein
118 Diet from Envigo) and mineral water (Ribes, Girona, Spain); the high-fat (HF) group,
119 fed an HF diet (TD 08811 45% kcal Fat Diet, from Envigo) and mineral water; and the
120 high-sucrose (HS) group, fed the standard diet and 35% (w/vol) sucrose solution in
121 mineral water as the only source of liquid intake. The diets were chosen with the aim of
122 mimicking real nutritional conditions in humans who consume a moderate excess of fat
123 or sugar (glucose + fructose). All the animals were fed *ad libitum* with free access to
124 water or sucrose solution.

125 Fecal samples were collected by abdominal massage at the start of the experiment
126 (time 0) and after weeks 1, 3, 9, 12, 20 and 24. The energy content of the feces from
127 week 20 was determined by differential scanning calorimetry using a TGA/SDTA851e
128 thermobalance (Mettler Toledo; Columbus, OH, USA) with an integrated SDTA signal.
129 After weeks 10 and 16, blood samples were collected from the saphenous vein after

130 overnight fasting. After week 23, the rats were placed in metabolic cages for urine
131 collection. After week 24, they were fasted overnight and anaesthetized
132 intraperitoneally with ketamine (Merial Laboratorios, Barcelona, Spain) and xylazine
133 (Química Farmacéutica, Barcelona, Spain) at doses of 80 and 10 mg/kg body weight,
134 respectively. Blood was collected by cardiac puncture and plasma was immediately
135 obtained by centrifugation. Perigonadal fat, quadriceps muscle and liver samples were
136 collected, washed with 0.9% NaCl solution, weighed and immediately frozen in liquid
137 N₂. All the samples were stored at -80 °C until analysis.

138 *Biometric measurements.* Feed and drink intake as well as body weight were
139 measured weekly. Feed and drink intake per day as a function of body weight were
140 estimated by dividing the total intake per cage by the weight of the animals in that cage
141 and the number of days and then they were averaged over the total number of cages in a
142 group. Fat and fructose intake were calculated from the experimental measurements and
143 the composition of the feed and drink: fat, 0.04 g/g standard feed (groups STD and HS)
144 and 0.23 g/g HF feed (HF group); and fructose, 0.175 g/mL water (HS group) and 0.17
145 g/g HF feed (HF group). Energy intake was calculated as estimates of metabolizable
146 energy based on the Atwater factors, assigning 4 kcal/g protein, 9 kcal/g fat, and 4
147 kcal/g available carbohydrate.

148 Systolic and diastolic blood pressure was measured at time 0 and after weeks 4, 9,
149 15 and 22 by the tail-cuff method, using a non-invasive automatic blood pressure
150 analyzer (Harvard Apparatus, Holliston, MA, USA).

151 *Measurement of uric acid.* Total urine uric acid was determined by a
152 spectrophotometric method using a uricase/peroxidase kit from BioSystems (Barcelona,
153 Spain) by measuring the absorbance at 520 nm on a SpectraMax M5 spectrophotometer
154 (Molecular Devices, Sunnyvale, CA, USA). Creatinine levels in urine were determined

155 by a colorimetric method using a commercial kit (C-cromatest Linear Chemicals,
156 Montgat, Spain) by measuring absorbance at 510 nm.

157 *Glycemic status.* Fasting blood glucose and plasma insulin levels were measured
158 after weeks 10 and 16 on fasted animals. Blood glucose concentration was measured by
159 the enzyme electrode method, using an Ascensia ELITE XL blood glucose meter (Bayer
160 Consumer Care AG, Basel, Switzerland); plasma insulin levels were measured using
161 Milliplex xMAP multiplex technology on a Luminex xMAP instrument (Millipore,
162 Austin, TX, USA). The standard curve was generated for the range 69-50,000 pg/mL,
163 using a five-parameter logistic curve fit. Homeostasis model assessment for insulin
164 resistance (HOMA-IR) was calculated according to the formula $HOMA-IR = \text{fasting}$
165 $\text{blood glucose in mmol/L times fasting plasma insulin in } \mu\text{U/mL divided by } 405$ (31).
166 Insulin units (U) were calculated using the conversion $1U = 0.0347 \text{ mg insulin}$. Oral
167 glucose tolerance tests (OGTT) were performed after weeks 13 and 21 on fasted
168 animals. A solution of glucose (1 g/kg body weight) was administered by oral gavage
169 before the tests and blood glucose concentration measured 15, 30, 45, 60, 90 and 120
170 min after glucose intake.

171 *Histology of the liver.* Fixed livers were dehydrated in alcohol and embedded in
172 paraffin, then cut into 3 μm thick slices, using an HM 355S Rotary Microtome (Thermo
173 Fisher Scientific, Waltham, MA, USA). Sections were stained with hematoxylin
174 (hematoxylin solution modified acc to Gill III for microscopy; Merck KGaA,
175 Darmstadt, Germany) mixed with eosin (Pharmacy Service of Puerta del Mar Hospital,
176 Cádiz, Spain). The tissue sections were viewed under a NIKON Eclipse 80i light
177 microscope (NIKON Corporation, Minato, Japan). Three variables were graded
178 following the method described by *Taltavull et al.* (48): steatosis, 0 to 3; steatosis
179 localization, 0 to 3; lobular inflammation with lymphoplasmacytic inflammatory

180 infiltration, 0 to 3; and the presence of microgranulomas.

181 *Measurement of diacylglycerols.* Frozen samples of liver, muscle and adipose tissue
182 (AT) were weighed and sonicated (SFX150 Sonifier; Emerson Industrial Automation,
183 St. Louis, MO, USA) until total homogenization. Diacylglycerol (DAG) extracts were
184 prepared in the presence of BHT (butylated hydroxytoluene, 0.01%) and analyzed using
185 a reported method (46) with some modifications. The mixtures were fortified with an
186 internal standard (1,3-17:0 D5 DG, Avanti Polar Lipids Inc., Alabaster, AL, USA; 200
187 pmol) and incubated overnight at 48 °C. After solvent evaporation, the samples were
188 suspended in methanol, centrifuged (9390 g, 3 min) and the supernatants loaded into an
189 Acquity UPLC system connected to an LCT Premier orthogonal accelerated time-of-
190 flight mass spectrometer (Waters, Milford, MA, USA), operated in positive ESI mode.
191 Full-scan spectra from 50 to 1,500 Da were acquired, and individual spectra were
192 summed to produce data points of 0.2 s each. Mass accuracy and precision were
193 maintained by using an independent reference spray (leucine enkephalin) via the
194 LockSpray interference. A C8 Acquity UPLC-bridged ethylene hybrid 100 x 2.1 mm
195 inner diameter, 1.7 µm column (Waters) was used in the separation step. The samples (8
196 µL) were eluted with a binary system consisting of 0.2% (v/v) formic acid, 2 mM
197 ammonium formate in water [A] and in methanol [B] under linear gradient conditions:
198 0.0 min, 80% B; 3 min, 90% B; 6 min, 90% B; 15 min, 99% B; 18 min, 99% B; 20 min,
199 80% B; and 22 min, 80% B, at 30 °C. The flow rate was 0.3 ml/min. Quantification was
200 carried out using the extracted ion chromatogram of each compound, using 50 mDa
201 windows.

202 *Subpopulations of gut microbiota.* The levels of total bacteria and Bacteroidetes,
203 Firmicutes, Enterobacteriales, and *Escherichia coli* were estimated from fecal DNA by
204 quantitative real-time polymerase chain reaction (qRT-PCR). DNA was extracted from

205 feces using QIAamp® DNA Stool Mini Kit from QIAGEN (Hilden, Germany) and
206 quantified using a Nanodrop 8000 Spectrophotometer (ThermoScientific, Waltham,
207 MA, USA). DNA samples were diluted to 20 ng/μL and qRT-PCR was carried out on a
208 LightCycler® 480 II (Roche, Basel, Switzerland) in triplicate. The samples contained
209 DNA (2 μL) and a master mix (18 μL) made of 2X SYBR (10 μL), the corresponding
210 forward and reverse primer (1 μL each), and water (6 μL). All reactions were paralleled
211 by analysis of a non-template control (water) and a positive control. The primers and
212 annealing temperatures are detailed in Table 1. Total bacteria were normalized as 16S
213 rRNA gene copies per mg of wet feces (copies/mg).

214 *Measurement of short-chain fatty acids.* Short-chain fatty acids (SCFAs) in feces
215 were analyzed after week 12 by gas chromatography (GC) using a reported method (45)
216 with some modifications. SCFAs were extracted from freeze-dried feces (~50 mg) with
217 a mixture consisting of acetonitrile/water 3:7 (1 mL), and the internal standard 2-
218 ethylbutiric acid (0.1 mL, 100 mg/L) and 0.1 M oxalic acid (0.5 mL) both in the same
219 solvent, for 10 min using a horizontal shaker. Finally, the suspension was centrifuged
220 (12880 g, 5 min) in a 5810R centrifuge (Eppendorf, Hamburg, Germany) and the
221 supernatant passed through a 0.45 μm nylon filter. Aliquots (0.7 mL) were diluted to 1
222 mL with acetonitrile/water 3:7 and the SCFAs were analyzed using a Trace2000 gas
223 chromatograph (ThermoFinnigan, Waltham, MA, USA) coupled to a flame ionization
224 detector equipped with an Innowax 30 m × 530 μm × 1 μm capillary column (Agilent,
225 Sta. Clara, CA, USA). The method showed good selectivity for acetic acid, propionic
226 acid, butyric acid, isobutyric acid valeric acid and isovaleric acid; sensitivity; linearity;
227 and accuracy (trueness and precision). To check the method trueness and precision, a
228 recovery study at three concentrations was performed on three different days. Precision
229 (RSD < 15%) and recovery (> 70%) were adequate; as was intra-day reproducible.

230 *Biomarkers and lipid mediators of inflammation.* Plasma lipopolysaccharide (LPS)
231 concentration was estimated by reaction with Limulus amoebocyte extract: LAL kit
232 endpoint-QCL1000 (Cambrex BioScience, Walkersville, MD, USA). Plasma samples
233 collected at the end of the study under sterile conditions were diluted 70-fold and heated
234 for 20 cycles of 10 min at 68 °C and 10 min at 4 °C each. An internal control for LPS
235 recovery was included.

236 Levels of plasma interleukin-6 (IL-6) were measured using Milliplex xMAP multiplex
237 technology. Liver function was ascertained by measuring the activities of alanine
238 transaminase (AST) and aspartate transaminase (ALT) in plasma by a spectrophometric
239 method using kits from Spinreact (Sant Esteve de Bas, Spain) and it is expressed as the
240 AST/ALT ratio.

241 Lipid mediators from the metabolism of arachidonic acid (ARA), eicosapentaenoic
242 acid (EPA) and docosahexaenoic acid (DHA) were determined in plasma by liquid
243 chromatography coupled to tandem mass spectrometry (LC-MS/MS) using a method
244 modified from Dasilva *et al.* (6). Erythrocyte-free plasma samples (90 µL) were thawed,
245 diluted in the presence of BHT and spiked with the internal standard 12HETE-d8
246 (Cayman Chemicals, Ann Arbor, MI, USA). Then, the samples were centrifuged (800 g,
247 10 min) and the lipids in the supernatants were purified by SPE. The LC-MS/MS
248 analyzer consisted of an Agilent 1260 Series chromatograph (Agilent) coupled to a
249 dual-pressure linear ion trap mass spectrometer LTQ Velos Pro (Thermo Fisher,
250 Rockford, IL, USA) operated in negative ESI mode. A C18-Symmetry 150 × 2.1 mm
251 inner diameter, 3.5 µm column (Waters) with a C18 4 × 2 mm guard cartridge
252 (Phenomenex, Torrance, CA, USA) were used in the separation step. Samples (10 µL)
253 were eluted with a binary system consisting of [A] 0.02% aqueous formic acid and [B]
254 0.02% formic acid in methanol under gradient conditions of: time 0, 60% B; 2 min,

255 60% B; 12 min, 80% B; 13 min, 80% B; 23 min, 100% B; 25 min, 100% B; and 30 min,
256 60% B, at a flow rate of 0.2 mL/min.

257 *Measurement of isoprostanes.* F₂-isoprostanes (F₂-IsoPs) were determined in urine
258 samples by LC/ESI-MS/MS following a previously reported procedure (35) with
259 modifications. Urine samples (500 µL) were acidified, β-glucuronidase (90 U/mL)
260 (Sigma, Saint Louis, MI, USA) was added and the mixtures were incubated for 2 h at 37
261 °C. After addition of the internal standard [²H₄]15-F_{2t}-IsoP (Cayman, Ann Arbor, MI,
262 USA) (100 µL, 10 µg/L), F₂-IsoPs were purified by SPE. F₂-IsoPs were analyzed using
263 an Agilent 1260 chromatograph fitted with a Mediterranea Sea 18 column (10 cm x 2.1
264 mm i.d., 2.2 µm particle size) (Teknokroma, Barcelona, Spain) coupled to a 4000
265 QTRAP mass spectrometer (Applied Biosystems, Foster City, CA, USA). The
266 instrument was operated in the negative-ion mode with a Turbo V source to obtain
267 MS/MS data. Separation was achieved with a binary system consisting of [A] 0.1%
268 aqueous formic acid and [B] 0.1% formic acid in acetonitrile, at 40° C, with an
269 increasing linear gradient (v/v) of [B]: time 0, 10% B; 7 min, 50% B; 7.1 min, 100% B;
270 8 min, 100% B; 8.1 min, 10% B; and 10 min, 10% B, at a flow rate of 700 µL/min. F₂-
271 IsoPs were detected by MS/MS multiple reaction monitoring (MRM). Calibration
272 curves were prepared using seven matrix-matched standards covering the working
273 concentration range. The LOQ was 0.4 µg/L for 15-F_{2t}-IsoP and 2 µg/L for 5-F_{2t}-IsoP.
274 The results were expressed as ng/mg creatinine, to correct for urine dilution.

275 *Statistical analysis.* All data manipulation and statistical analysis was performed
276 using Graph Pad Prism 5 (Graph Pad Software, Inc., San Diego, CA, USA). The results
277 are expressed as mean values with their standard errors (SEM). Normal distribution and
278 heterogeneity of data were evaluated and statistical significance was determined by two-
279 way ANOVA for repeated measurements or one-way ANOVA, and Tukey's multiple

280 comparisons test was used for mean comparison. Differences were considered
281 significant when $P < 0.05$.

282

283 **RESULTS**

284 *Feed and drink intakes, and energy balance.* Feed intake was lower in animals fed
285 the HF diet than in those in the STD group and even lower in animals given the HS diet
286 (Table 2). Drink intake was higher in animals in the HS group than in those from the
287 other two groups. Energy intake was higher in both the HF and HS groups than in the
288 STD group. Residual energy in feces was similar in the STD and HF groups, and lower
289 in the HS group ($P < 0.05$ vs HF). The HF animals consumed significantly ($P < 0.001$)
290 more fat than the other two groups throughout the experiment (Figure 1A); while HS
291 rats consumed significantly ($P < 0.001$) more fructose (Figure 1B) per 100 g of body
292 weight than rats in the other groups. As the only source of carbohydrate in the HF diet
293 was sucrose (equimolar fructose and glucose), the rats in this group consumed as much
294 glucose as fructose.

295 *Weight gain and lipid accumulation.* Body weight was similar in all the groups at
296 the beginning (235.9 g, SEM 3.6) and no differences were observed between the STD
297 and HS groups during the whole experiment (Figure 1C). After 6 weeks, the HF group
298 had significantly ($P < 0.001$) increased body weight (352.0 g, SEM 10.8) compared to
299 the STD (305.9 g, SEM 7.3) and HS (292.4 g, 9.1) groups; and the differences in weight
300 gain increased until the end of the study (STD: 416.4 g, SEM 12.9; HF: 544.3 g, SEM
301 15.5, after week 24). Perigonadal AT weight was significantly ($P < 0.001$) higher in the
302 HF group than in the other two groups (Figure 1E).

303 *Blood pressure and urine uric acid.* Systolic and diastolic blood pressures (Figure
304 1D) were similar in the STD and HF groups throughout the experiment. After 23 weeks

305 of intervention, systolic blood pressure was significantly higher ($P < 0.001$) in animals
306 given the HS diet than in those given the STD or HF diet. Diastolic blood pressure was
307 higher ($P < 0.05$ vs STD) in HS-fed animals from week 9 to week 23. Animals fed HS
308 presented a significantly ($P < 0.001$) higher concentration of uric acid in urine at week
309 23 than those of the STD and HF groups (Figure 1F).

310 *Glycemic status.* Fasting blood glucose and plasma insulin concentration were measured
311 after weeks 10 and 16 of the intervention (Table 3). HOMA-IR is used as an indicator of
312 IR. At both times, both fasting glucose and insulin as well as HOMA-IR were
313 significantly higher in the HF group than in the STD group. After 16 weeks of
314 intervention, the plasma insulin concentration and HOMA-IR in the HS group were not
315 different from the values in either the STD or the HF group (Table 3). The OGTT was
316 performed twice during the study, after weeks 13 and 21 (Figure 2). The increase of the
317 area under the curve (AUC) is as an indicator of IGT. After 13 weeks, the levels of
318 postprandial glucose in the HF group were higher than those in the other groups (STD
319 and HS), 30 and 60 min after administration (Figure 2). The AUC corresponding to the
320 HF group was significantly higher ($P < 0.001$) than that in the STD group. By the end of
321 the study (week 21), the plasma glucose levels in groups HF and HS were similar and
322 significantly higher than those of the STD group at all the time points (Figure 2).

323 *Liver histology, total liver triacylglycerols and biochemical measurement of liver*
324 *function.* An excess of dietary fat triggered lobular inflammation and microgranulomas,
325 while an excess of fructose did not (Figure 3A). The livers of animals fed the HF diet
326 showed lobular inflammation with lymphoplasmacytic inflammatory infiltration around
327 the blood vessels (e.g. Figure 3C). Conversely, an excess of fructose induced significant
328 and highly localized steatosis (Figure 3A, D) while an excess of dietary fat did not
329 (Figure 3A, C). The levels of total triacylglycerols ($\mu\text{mol/g liver}$) were STD: 46.8, SEM

330 20.1; HF: 80.4, SEM 19.7; HS: 168.1, SEM 92.6, with no significant differences
331 between the groups. No liver functional damage resulted from any of the diets as
332 revealed by the similar AST/ALT ratio in the three groups (STD: 6.7, SEM 0.6; HF: 6.3,
333 SEM 0.8; HS: 5.7, SEM 1.2)

334 *Diacylglycerols in tissues.* The levels of saturated DAGs 38:0 and 40:0, and
335 unsaturated DAGs 34:2, 34:3, 34:4, 36:5, and 40:5, were lower in liver in the HF group
336 than in the STD and/or HS groups (Table 4). The levels of unsaturated DAGs 32:1,
337 32:2, 34:1, and 36:2 were higher in animals that consumed an excess of fructose than in
338 animals in the other two groups; whereas the levels of DAG 42:12 were lower. The
339 levels of DAGs 36:4, 36:6, 38:1, and 38:6, were lower in both HS and HF groups than
340 in the STD group.

341 In muscle (Table 5), the levels of DAGs 34:3, 38:3, 38:4, 38:5, and 40:5 were lower
342 in the HF group than in the STD group; whereas the levels of DAG 36:1 were higher.
343 The levels of unsaturated DAG 32:1 were higher in the HS group than in the other two
344 groups (STD and HF). The levels of DAG 34:1 were higher and those of 36:4 were
345 lower in both HS and HF groups than in the STD group. In perigonadal AT (Table 6),
346 only the levels of DAG 42:1 were higher in the HF group than in the other two groups
347 (STD and HS). The levels of unsaturated DAGs 32:3, 36:1, 36:2, 38:2, and 40:3 were
348 lower in animals that consumed an excess of fructose than in animals in the STD or HF
349 groups. The levels of DAGs 34:1, 34:2, 34:3, 34:4, 36:3, 36:4, 36:5, 36:6, 38:3, 38:4,
350 38:5, 38:6, and 40:5 were lower in both HS and HF groups than in the STD group.

351 *Subpopulations of gut microbiota and microbial products.* The
352 Bacteroidetes:Firmicutes ratio (Figure 4A) was reduced in HF animals and increased in
353 HS animals compared to STD animals. The proportion of Enterobacteriales and *E. coli*
354 in animals given the HF diet were already significantly increased after weeks 1 and 3,

355 and tended to decrease gradually afterwards (Figure 4B, C). An excess of fructose only
356 increased the populations of Enterobacteriales and *E. coli* at the end of the study (Figure
357 4B, C).

358 The levels of acetic, propionic and isobutyric acids, and total SCFAs were lower in
359 the feces of HF animals than in those fed the STD or HS diet (Table 7).

360 The HF animals showed a non-significant tendency ($P = 0.1$ vs STD group) towards
361 increased plasma concentration of LPS at the end of the study (Figure 5A). The HS
362 animals did not show any increase in plasma LPS.

363 *Markers and lipid mediators of inflammation.* The animals fed the HF diet showed
364 increased plasma concentrations of IL-6 and prostaglandin E₂ (PGE₂, EPA metabolite)
365 compared to the STD group at the end of the study (Figure 5B, C). The concentrations
366 of ARA, EPA and DHA were higher in the HS group than in the STD and HF groups
367 (Figure 5D, E, F). No differences were detected in the concentration of LTB₄
368 (leukotriene B₄) or 12HEPE (12-hydroxyeicosapentaenoic acid), ARA and EPA
369 metabolites, respectively (Figure 5G, H). The concentration of the DHA metabolite
370 17HDoHE (17-hydroxy docosahexaenoic acid) was lower in animals fed the HS diet
371 than in those fed the HF diet (Figure 5I).

372 *Isoprostanes as markers of oxidative stress.* The animals fed HS showed increased
373 concentrations of 5-F_{2t}-IsoP and 15-F_{2t}-IsoP ($P < 0.05$) compared to the STD and HF
374 groups after 23 weeks of intervention (Figure 6A, B).

375 **DISCUSSION**

376 The present study explores some molecular factors behind the differential action of
377 an excess of dietary fat or of fructose on normal rats and examines the role that gut
378 microbiota may play in these processes. A prediabetic state was induced to WKY rats by

379 HF or HS¹ diets over a period of 24 weeks. Despite all the information available on the
380 induction of IR and IGT by fat and fructose in rat models, the molecular mechanisms
381 behind this action are still largely unknown. In our models, fat induced a prediabetic
382 state faster than fructose, as evidenced by the results of fasting blood glucose, plasma
383 insulin concentration, HOMA-IR (Table 3) and the OGTT (Figure 2). Only the animals
384 in the HF group presented both IR (HOMA-IR) and IGT (OGTT). The rats that
385 consumed an excess of sugar showed a tendency towards elevated plasma insulin and
386 significant IGT only at the end of the study (Table 3 and Figure 2). The differences in
387 timing between the metabolic response to fat and fructose prompted us to explore the
388 changes induced in some molecular factors known to be mechanistically related to the
389 development of IR and IGT. As low-grade inflammation may trigger IR, we measured
390 inflammation markers in the two models. Unlike the rats in the HS group, the livers of
391 HF-fed animals clearly presented inflammatory infiltrations within the portal space
392 (Figure 3). This is a sign of systemic inflammation that was confirmed by the plasma
393 levels of IL-6 and PGE₂ (Figure 5B, C). IL-6 and other inflammatory markers are
394 elevated in obesity-induced low-grade inflammation-related IR (11, 25); ARA-derived
395 cyclooxygenase (COX)-mediated pro-inflammatory factor PGE₂ is the predominant
396 prostaglandin in white AT, where it regulates adipose functions (26). PGE₂ also inhibits
397 pancreatic beta cell function and insulin secretion (43) which is an effect characteristic
398 of intermediate stages of diabetes, beyond the initial increase in insulin secretion (IR,
399 compensation stage) (50). Conversely, potentially anti-inflammatory EPA and DHA
400 were elevated in animals given an excess of fructose (Figure 5E, F). EPA and DHA are
401 considered to protect against inflammation mainly because they compete with ARA for

¹ In our HS model, IR and probably hypertension are more likely to be triggered by the excess of fructose than by glucose, as glucose can be metabolized and/or stored as glycogen in different organs (e.g. brain, liver and muscle) and it is carefully controlled by insulin everywhere, while fructose is almost entirely processed, mainly in the liver, and escapes metabolic control by insulin (18).

402 the same metabolizing enzymes, and also because they generate protectins and resolvins
403 as metabolites (7, 30). So, the HF animals presented systemic inflammation; while the
404 HS diet not only did not trigger early systemic inflammation, but also may have favored
405 anti-inflammatory pathways.

406 The observation of a non-significant trend ($P = 0.1$) toward elevated plasma LPS
407 concentrations (Figure 5A) suggested that microbial-derived endotoxemia might
408 contribute to the low-grade inflammation in HF-fed animals. LPS is a component of the
409 wall of Gram-negative bacteria present in Bacteroidetes and Enterobacteriales but not in
410 Firmicutes. As the population of Bacteroidetes clearly decreased in HF animals with
411 respect to STD rats (Figure 4A), the increase in LPS and pro-inflammatory mediators
412 detected in the plasma of HF animals (Figure 5A, B, C) might originate from
413 Enterobacteriales (Figure 4B), particularly its major member *E. coli* (Figure 4C). This
414 agrees with observations by other authors (8, 15). Additional research should confirm or
415 refute this suggestion. In addition to LPS, other mediators proposed as the link between
416 gut bacteria and metabolic alterations are bile acids, angiopoietin-like protein 4 and
417 SCFAs: products of microbial fermentation of dietary fiber (23). SCFAs acetate and
418 butyrate generated by Firmicutes may contribute to body fat gain through *de novo*
419 lipogenesis (2). Together with propionate, these SCFAs exhibit a protective effect
420 against inflammation and IR (2). In apparent contradiction, the animals that presented
421 the lowest Bacteroidetes:Firmicutes ratio and gained most weight (HF group) generated
422 the lowest amounts of total SCFAs (Table 7). This may be due to the composition of the
423 diet. Whereas the standard diet (fed to the STD and HS groups) contains wheat
424 middlings, ground wheat and ground corn, the only source of fiber in the HF diet is
425 cellulose (50 g/kg). The lower diversity in the source of fiber may be the cause of the
426 reduced production of protective SCFAs.

427 As DAGs may induce IR independently of inflammation, we measured their levels
428 in liver, muscle and AT. We did not detect any significant increase in the DAG profile in
429 the livers of rats fed HF (Table 4), in agreement with the absence of steatosis (Figure
430 3C). The only DAGs with elevated levels in the HF group were the monounsaturated
431 34:1 (muscle), 36:1 (muscle) and 42:1 (AT). As lipid-induced IR occurs earlier in liver
432 than in muscle (28), we concluded that direct impairment of insulin signaling by lipid
433 metabolites would not explain the observed early fat-induced systemic IGT. In contrast,
434 the animals given an excess of fructose presented steatosis around the blood vessels
435 (Figure 3D) and clearly elevated DAGs in the liver (Table 4) without gaining more
436 weight or accumulating more perigonadal AT than those given the STD diet (Figure 1C,
437 E). The levels of total triglycerides were also elevated in the HS group although the
438 differences were not statistically significant probably due to the fact that the steatosis
439 was highly localized. These observations are consistent with hepatic *de novo*
440 lipogenesis from fructose (20). It is becoming evident that different DAGs have a very
441 different impact on cellular signaling (13). While there is no information so far as to
442 what particular DAG species might impair insulin signaling, it has been reported that
443 cellular signaling proteins and receptors such as the human transfer receptor potential
444 C3 (TRPC3) are differentially activated by different DAGs and that DAG 36:2 (1-
445 stearoyl-2-linoleoyl-*sn*-glycerol) is one of the active species (21, 38). The amounts of
446 DAGs 36:2 and 34:1 (putatively 1-palmitoyl-2-oleoyl-*sn*-glycerol) were dramatically
447 increased (3- to 4-fold) in the livers of HS-fed rats (Table 4), which suggests that DAG
448 36:2 may play a role in late fructose-induced tendency to IR. DAG 34:1, which does not
449 activate the TRCP3 calcium channel (38), may or may not play a role in our model. In
450 muscle, DAG content is much lower than in liver and only the levels of DAG 32:1 were
451 slightly elevated in the muscular tissue of animals given the HS diet; and those of DAG

452 34:1 in both the HF and HS groups (Table 5). As expected, perigonadal AT presented
453 the highest levels of DAGs in all groups (Table 6). None of the significant differences
454 detected corresponded to any increase in DAG levels in the HF or HS group. The
455 overall examination of DAG variations in liver, muscle and AT showed that the only
456 likely contribution of these lipid metabolites to IR or IGT takes place in the liver of HS-
457 fed rats and that DAGs may not play a significant role in the development of IR or IGT
458 in rats fed an HF diet.

459 The levels of urine F₂-IsoPs, which are products of free radical-mediated *in vivo*
460 oxidation of ARA (33) different from COX-derived oxidized prostaglandins such as
461 PGE₂, were only elevated in animals given an excess of fructose at the end of the study
462 (Figure 6), consistently with increased levels of ARA (Figure 5D). This implies that an
463 excess of dietary fat did not trigger significant low-grade inflammation-mediated
464 systemic oxidative stress (OS), which may be attributed to the action of the excess of
465 fructose. In agreement with this observation, increased generation of superoxide radical
466 has been detected in the kidneys of high fructose-fed rats (40). The results also indicate
467 that IR *per se* does not trigger systemic OS, as the animals given an excess of fat
468 developed IR and IGT for the duration of the entire experiment (Table 3, Figure 2)
469 while showing IsoP levels similar to those in the STD group (Figure 6). In contrast with
470 our results, other authors have linked fructose-induced OS to inflammation and
471 hyperinsulinemia (4, 40). Those experiments were performed on Sprague–Dawley rats
472 using shorter intervention times (3-12 weeks). In our experiment (WKY rats), if the
473 inflammatory response was associated with OS in an early compensatory stage
474 (stimulation of insulin secretion) (50), it was no longer evident after 24 weeks of
475 intervention.

476 In the present study, only the HS diet induced hypertension which was statistically
477 significant after 9 weeks (diastolic pressure) and 22 weeks (systolic pressure) (Figure
478 1D). As rats in this group consumed significantly more sugar and water than those given
479 the STD or HF diets, the hypertensive effect may be explained, at least in part, by water
480 movement from tissues into the intravascular space caused by elevated levels of blood
481 glucose (41) together with hyperhydration. The high amounts of fructose available in
482 the liver of rats given the HS diet may also have caused elevated blood pressure via the
483 formation of uric acid (32) which is biosynthesized through the degradation of AMP to
484 inosine monophosphate following a fall in intracellular phosphate that originates in the
485 rapid and uncontrolled phosphorylation of fructose (24). This explanation is supported
486 by our observation that the HS group shows significantly higher levels of urine uric acid
487 than the STD and HF groups (Figure 1F). Late mild IR (Table 3) and IGT (Figure 2)
488 may also contribute to the elevated blood pressure in the HS group, as described in
489 previous studies (49). It should be noted that only hepatic IR (HS group) would in any
490 case bring about hypertension as the animals showing early IR (HF group, Table 3) were
491 normotensive (Figure 1D). The observation that the populations of Enterobacteriales
492 and particularly *E. coli* only increased in the feces of animals in the HS group (Figure
493 4B, C) when IGT became significantly high (week 21, Figure 2) suggests a relationship
494 between gut bacteria and late fructose-related metabolic alterations. In this case though,
495 the diet is less likely to induce late changes in the intestine, therefore the increase in
496 bacterial populations may be a consequence of the systemic action of the diet rather than
497 a cause of it. It has been reported that intestinal uric acid in end-stage renal disease
498 (ESRD) patients increases the populations of bacteria that are able to catabolize uric
499 acid into urea and eventually ammonia (51). As part of the uric acid is excreted through
500 the gut in both humans and rats (19, 47), we hypothesize that uric acid generated by

501 high fructose-fed rats (Figure 1F) may contribute to the increase in the populations of
502 gut Enterobacteriales (Figure 4B), which are microorganisms known to use urease to
503 metabolize urea (5, 34). To test this hypothesis it may be worth monitoring changes in
504 the gut populations of microorganisms known to metabolize uric acid into urea. The late
505 increase in Enterobacteriales might trigger further inflammation.

506

507 **CONCLUDING REMARKS**

508 This paper examines two rat models of prediabetes with clearly differentiated
509 mechanisms than can be used to test the effects of drugs and food ingredients. As the
510 effects induced are mild, the models are particularly suited for testing functional food
511 ingredients. A high-fat diet induced obesity and fast IR and IGT via low-grade
512 inflammation in WKY rats. In contrast, a high-sucrose (fructose + glucose) diet induced
513 IGT later than the HF diet, by processes triggered by *de novo* liposynthesis from
514 fructose, probably mediated by active DAGs in the liver. Neither low-grade
515 inflammation (HF group) nor localized steatosis (HS group) severely affected liver
516 function as assessed by measuring the AST/ALT ratio. Hypertension in HS-fed rats may
517 be due to causes other than systemic IR that include an excess of water/glucose intake
518 and the generation of uric acid from fructose. The development of IR coincides with
519 increased populations of *E. coli* in the intestinal tract that may contribute to low-grade
520 inflammation in HF-fed animals or may be a consequence of the metabolic alterations
521 of an excess of fructose in HS-fed animals. The results show that fat and sugar trigger
522 metabolic alterations by largely independent mechanisms and underscore the potentially
523 harmful effect of the excessive intake of both these nutrients together.

524

525

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527

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530

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677

678 **FIGURE CAPTIONS**

679

680

681 **Figure 1.** Fat intake (A), fructose intake (B), body weight (C), blood pressure (D),
682 adipose tissue weight (E) and uric acid in urine (F) of rats (n = 9 per group) fed the
683 standard (STD, □), high-fat (HF, ●) or high-sucrose (HS, ▼) diet for 24 weeks.

684 Data are presented as means with their standard errors. Comparisons were conducted
685 using two-way ANOVA (A, B, C, D) or one-way ANOVA (E, F) and Tukey's multiple
686 comparisons test. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs STD group; δδδ $P <$
687 0.001 vs HF group.

688

689

690 **Figure 2.** Glycemic response in rats (n = 9 per group) fed the standard (STD), high-fat
691 (HF) or high-sucrose (HS) diet. Curves of OGTT after ingestion of a single dose of
692 glucose (1 g/kg body weight) after weeks 13 and 21 of intervention, and the
693 corresponding areas under the curve (AUC).

694 Values are presented as means with their standard errors. Comparisons were conducted
695 using two-way ANOVA (OGTT curves) or one-way ANOVA (AUCs) and Tukey's
696 multiple comparisons test. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs STD group; δ P
697 < 0.05 and δδ $P < 0.01$ vs HF group.

698

699

700 **Figure 3.** Liver histology. Estimation of variables (A) and cuts stained with
701 hematoxilin-eosin: rats fed the STD diet present normal liver (B, 20x), rats fed the HF
702 diet present inflammatory infiltration within the portal triad without steatosis (C, 20x),

703 and rats fed the HS diet present steatosis without inflammatory infiltration (D, 20x).
704 Scores are presented as means with their standard errors. Comparisons were conducted
705 using one-way ANOVA and Tukey's test. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs
706 STD group; $\delta P < 0.05$, $\delta\delta P < 0.01$ and $\delta\delta\delta P < 0.001$ vs HF group.

707

708

709 **Figure 4.** Excreted intestinal bacteria measured by qRT-PCR and expressed as
710 percentages of total bacteria in fecal samples from rats (n = 9 per group) fed a standard
711 diet (STD), high-fat diet (HF) or high-sucrose diet (HS) for 24 weeks of nutritional
712 intervention. A, Bacteroidetes/Firmicutes ratio; B, Enterobacteriales; and C, *E. coli*.

713 Results are presented as means with their standard errors. Comparisons were conducted
714 using one-way ANOVA and Tukey's multiple comparisons test. * $P < 0.05$, ** $P < 0.01$
715 and *** $P < 0.001$ vs STD group; $\delta P < 0.05$, $\delta\delta P < 0.01$ and $\delta\delta\delta P < 0.001$ vs HF
716 group.

717

718

719 **Figure 5.** Plasma biomarkers of inflammation from rats (n = 9 per group) fed a standard
720 diet (STD), high-fat diet (HF) or high-sucrose diet (HS) for 24 weeks of nutritional
721 intervention. A, Lipopolysaccharide; B, IL-6 (determined after 10 weeks of
722 intervention); C, PGE₂; D, arachidonic acid (ARA); E, eicosapentaenoic acid (EPA); F
723 docosahexaenoic acid (DHA); G, LTB₄; H, 12HEPE; and I 17HDoHE.

724 Results are presented as means with their standard errors. Comparisons were conducted
725 using one-way ANOVA and Tukey's multiple comparisons test. * $P < 0.05$ and ** $P <$
726 0.01 vs STD group; $\delta P < 0.05$ vs HF group.

727

728

729 **Figure 6.** Isoprostanes in urine from rats ($n = 9$ per group) fed a standard diet (STD),
730 high-fat diet (HF), and high-sucrose diet (HS) for 24 weeks of nutritional intervention.

731 A, 5F2t; B, 15F2t.

732 Results are presented as means with their standard errors. Comparisons were conducted

733 using one-way ANOVA and Tukey's multiple comparisons test. * $P < 0.05$ vs STD

734 group; $\delta P < 0.05$ vs HF group.

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Table 1. *qRT-PCR primers and conditions*

Target bacteria	Positive control	Annealing temperature (°C)	Sequence (5'-3')	Reference
Total Bacteria	#	65	F: ACT CCT ACG GGA GGC AGC AGT R: ATT ACC GCG GCT GCT GGC	(18)
Bacteroidetes	<i>Bacteroides fragilis</i>	62	F: ACG CTA GCT ACA GGC TTA A R: ACG CTA CTT GGC TGG TTC A	(22)
Firmicutes	<i>Lactobacillus brevis</i>	52	F: AGA GTT TGA TCC TGG CTC R: ATT ACC GCG GCT GCT GG	(17) (37)
Enterobacteriales	<i>E. coli M15</i>	60	F: ATG GCT GTC GTC AGC TCG T R: CCT ACT TCT TTT GCA ACC CAC T	(18)
<i>E. coli</i>	<i>E. coli M15</i>	61	F: GTT AAT ACC TTT GCT CAT TGA R: ACC AGG GTA TCT AAT CCT GTT	(29)

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Positive control of Total Bacteria was the strain the result was rated with.

742 **Table 2.** Feed, drink and energy intakes of rats fed different diets for 24 weeks, and
 743 residual excreted energy in feces after 20 weeks of intervention.

	STD		HF		HS	
	Mean	SEM	Mean	SEM	Mean	SEM
Feed intake (g/day/100 g body weight)	5.4	0.3	4.1*	0.3	2.7* ^δ	0.2
Drink intake (mL/day/100 g body weight)	7.2	0.3	5.5	0.3	10.7* ^δ	0.3
Total energy intake (kcal/day/100 g body weight)	15.5	0.8	19.5*	1.2	22.8*	0.8
Energy in feces ^a	307	19	362	72	292 ^δ	17

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745 Data are presented as means with their standard errors of the mean, n = 9 per group.

746 Comparisons were performed using one-way ANOVA and Tukey's multiple

747 comparisons test.

748 * $P < 0.05$ vs STD group; ^δ $P < 0.05$ vs HF group.

749 ^a integrated STD signal (ks °C/g) proportional to energy.

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752 **Table 3.** *Fasting plasma glucose and insulin concentration and calculated HOMA-IR.*

	STD		HF		HS	
	Mean	SEM	Mean	SEM	Mean	SEM
<u>week 10</u>						
Fasting glucose (mg/dL)	65.4	1.4	75.7***	2.0	64.8 ^{δδ}	2.7
Insulin (pg/mL)	417.9	71.4	1737***	137.6	584.5 ^{δδδ}	105.8
HOMA-IR	1.9	0.3	9.3***	0.7	2.7 ^{δδδ}	0.5
<u>week 16</u>						
Fasting glucose (mg/dL)	66.0	1.1	70.8*	1.4	59.3 ^{δδ}	3.0
Insulin (pg/mL)	661.2	53.9	1654.8*	331.5	1090.1	203.6
HOMA-IR	3.1	0.2	8.4*	1.8	4.7	1.1

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754 Data are presented as means with their standard errors of the mean, n = 9 per group.
 755 Comparisons were conducted using one-way ANOVA and Tukey's multiple
 756 comparisons test. * $P < 0.05$ and *** $P < 0.001$ vs STD group; δ $P < 0.05$, $\delta\delta$ $P < 0.01$,
 757 $\delta\delta\delta$ $P < 0.001$ vs HF group.
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Table 4. Liver diacylglycerols (DAGs, nmol/g tissue[#]) by LC-MS.

	STD		HF		HS	
	Mean	SEM	Mean	SEM	Mean	SEM
32:0	2332	504	2256	447	5224	2063
34:0	7661	1676	8044	1469	17320	8406
36:0	5598	1213	6116	1124	12040	5659
38:0	296.3	53.3	139.1*	24.9	208.9	32.7
40:0	32.5	5.0	14.3**	14.3	22.0	3.3
32:1	64.3	6.8	69.2	12.7	242.6* ^δ	72.1
32:2	15.1	1.5	14.8	2.4	43.6* ^δ	12.5
34:1	405.7	43.0	540.4	100.1	1499.0* ^δ	427.2
34:2	328.8	39.1	139.6	26.6	414.6 ^δ	111.1
34:3	88.0	10.7	27.0*	6.0	101.7 ^δ	26.7
34:4	17.4	2.3	2.1*	1.4	18.1 ^δ	6.5
36:1	85.6	10.1	111.4	19.3	131.0	31.9
36:2	321.4	35.0	576.4	117.5	1296.0*	380.6
36:3	711.1	86.3	398.9	78.5	681.0	182.1
36:4	381.7	48.8	102.1***	18.7	203.0**	34.4
36:5	50.5	6.3	8.3***	2.6	29.6	8.5
36:6	12.2	2.6	0.0***	0.0	1.7**	1.7
38:1	6.3	1.6	0.0***	0.0	1.6*	1.1
38:2	12.2	1.5	9.7	2.4	17.8	4.7
38:3	18.5	5.1	17.6	4.5	21.0	5.2
38:4	436.5	54.8	325.4	44.2	367.5	41.2
38:5	220.1	25.2	131.8	23.0	170.2	35.3
38:6	227.7	27.3	57.8***	10.3	66.7***	10.5
40:5	171.8	27.1	90.6*	17.9	112.9	16.7
42:5	141.9	22.2	80.6	17.4	80.4	10.2
42:12	12.3	1.8	10.1	1.6	3.5** ^δ	1.3

760 Data are presented as means with their standard errors of the mean,
 761 n = 9 per group. Comparisons were conducted using one-way ANOVA
 762 and Tukey's multiple comparisons test.

763 * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs STD group; ^δ $P < 0.05$ vs HF group.

764 [#]Amounts expressed as DAG 16:0, 16:0 equivalents.

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Table 5. Muscle diacylglycerols (DAGs, nmol/g tissue[#]) by LC-MS

	STD		HF		HS	
	Mean	SEM	Mean	SEM	Mean	SEM
32:0	30.8	5.5	29.3	4.1	37.2	5.0
34:0	47.1	14.6	41.2	10.7	47.4	15.5
36:0	10.7	10.4	24.2	8.6	26.3	12.0
38:0	3.8	1.5	2.5	0.9	4.1	1.8
40:0	1.1	0.4	1.2	0.3	1.4	0.3
42:0	0.4	0.2	0.5	0.2	0.7	0.2
32:1	5.9	0.8	9.0	1.5	10.7*	0.8
32:2	1.3	0.3	1.6	0.4	1.9	0.3
34:1	29.3	4.2	52.2*	8.5	54.4*	3.0
34:2	19.2	3.7	12.3	2.3	16.4	1.6
34:3	4.6	0.9	2.4*	0.4	3.4	0.4
36:1	4.0	0.7	8.5*	1.8	4.9	0.5
36:2	15.5	2.9	33.8	8.3	30.9	3.2
36:3	18.9	4.4	13.2	3.2	12.4	2.1
36:4	11.5	2.1	4.6**	0.5	4.9**	0.5
36:5	0.0	0.0	0.0	0.0	0.2* ^{δδ}	0.1
38:1	0.2	0.1	0.3	0.1	0.2	0.1
38:2	0.7	0.3	0.6	0.1	0.8	0.2
38:3	1.7	0.5	0.6*	0.1	0.8	0.2
38:4	10.7	1.7	5.2**	0.5	7.3	1.0
38:5	4.2	0.6	2.7*	0.3	3.2	0.3
38:6	3.5	0.5	2.4	0.5	2.4	0.5
40:5	1.3	0.4	0.1**	0.1	0.6	0.2
42:1	0.1	0.1	0.2	0.1	0.5	0.1

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768 Data are presented as means with their standard errors of the mean,
769 n = 9 per group. Comparisons were conducted using one-way ANOVA
770 and Tukey's tests.

771 * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs STD group; $\delta\delta$ $P < 0.01$ vs HF

772 [#]Amounts expressed as DAG 16:0, 16:0 equivalents.

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Table 6. Adipose tissue diacylglycerols (DAGs, nmol/g tissue[#]) by LC-MS.

	STD		HF		HS	
	Mean	SEM	Mean	SEM	Mean	SEM
32:0	217.3	30.4	143.4	41.6	137.1	28.6
34:0	50.1	6.2	63.2	21.7	21.1	4.2
38:0	1.0	0.2	0.7	0.3	0.4	0.1
40:0	0.4	0.1	0.3	0.1	0.2	0.1
42:0	0.0	0.0	0.9	0.4	0.0	0.0
32:1	258.3	54.6	281.9	89.9	210.0	48.4
32:2	207.8	44.5	122.3	50.9	70.8	18.4
32:3	26.8	6.9	10.7	5.0	4.2*	1.1
34:1	880.9	106.0	471.6*	112.7	457.8*	81.0
34:2	1338.0	164.7	400.6***	127.2	436.9***	85.7
34:3	1337.0	153.3	449.2***	125.3	404.2***	83.3
34:4	41.7	10.3	6.2***	3.1	4.3***	1.1
36:1	93.8	11.5	213.3	74.7	39.9 ^δ	8.6
36:2	874.1	103.3	560.4	139.0	465.2*	83.4
36:3	1337.0	153.2	449.2***	125.3	404.2***	83.3
36:4	41.8	3.1	8.5***	2.0	7.3***	1.0
36:5	120.1	20.0	9.2***	4.3	4.8***	1.6
36:6	2.7	0.6	0.1***	0.1	0.0***	0.0
38:1	4.5	1.2	2.5	2.1	0.5	0.4
38:2	38.4	5.7	18.2	8.9	11.1*	3.0
38:3	77.7	11.1	13.2***	6.5	9.3***	2.5
38:4	55.7	7.3	6.9***	2.5	3.2***	0.5
38:5	63.1	9.8	11.9***	4.3	8.5***	1.8
38:6	2.5	0.3	0.4***	0.1	0.3***	0.0
40:3	0.3	0.1	0.2	0.1	0.0**	0.0
40:5	2.6	0.4	0.1***	0.1	0.0***	0.0
42:1	0.2	0.1	1.0*	0.3	0.2 ^δ	0.0

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Data are presented as means with their standard errors of the mean, n = 9 per group.

Comparisons were conducted using one-way ANOVA and Tukey's tests.

* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs STD group; $\delta P < 0.05$ vs HF

[#]Amounts expressed as DAG 16:0, 16:0 equivalents.

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785 **Table 7.** Short-chain fatty acids (SCFAs, mmol/kg) in feces fed after 12 weeks

	STD		HF		HS	
	Mean	SEM	Mean	SEM	Mean	SEM
Acetic Acid	310.9	61.1	149.5*	17.6	265.5	45.6
Propionic Acid	27.4	6.2	3.1**	0.6	28.0 ^{δδ}	5.7
Isobutyric Acid	1.3	0.2	0.2***	0.0	1.1 ^{δδδ}	0.1
Butyric Acid	17.6	3.3	6.2	1.6	23.1 ^δ	5.8
Isovaleric Acid	1.0	0.3	0.4	0.1	1.0	0.2
Valeric Acid	0.7	0.1	0.4	0.1	1.1 ^δ	0.2
TOTAL SCFAs	356.9	66.1	159.4*	19.1	287.2	55.3

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787 Data are presented as means with their standard errors of the mean, n = 9 per group.

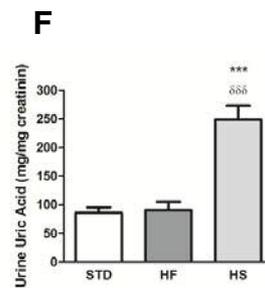
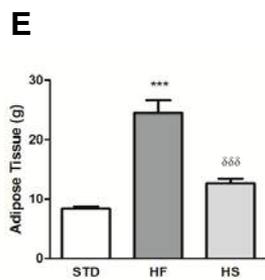
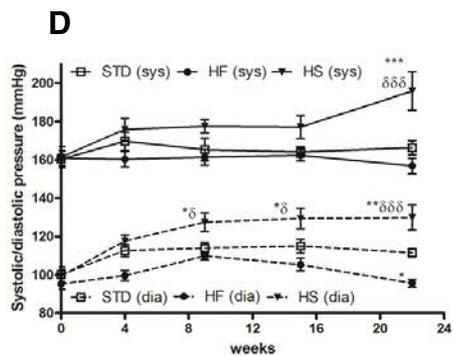
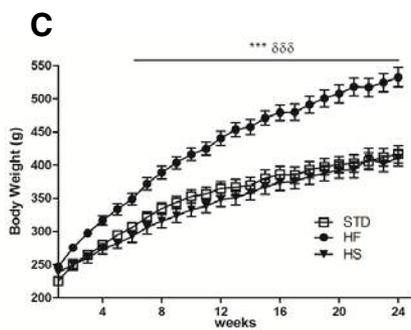
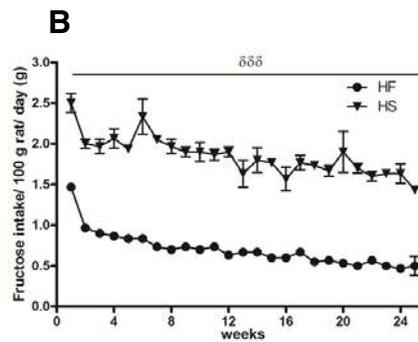
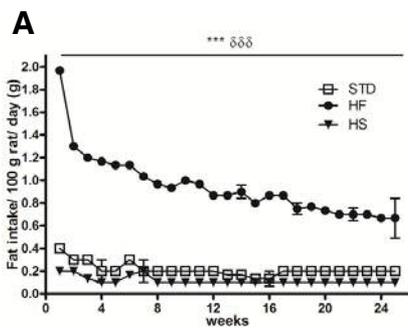
788 Comparisons were conducted using one-way ANOVA and Tukey's multiple

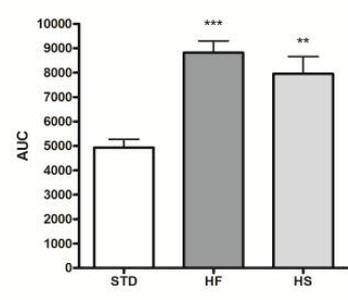
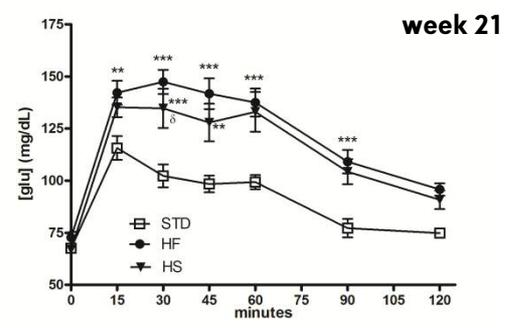
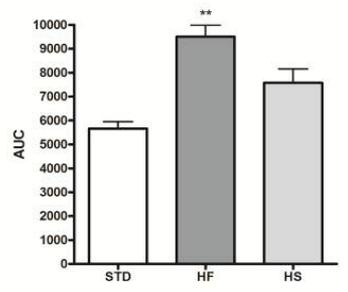
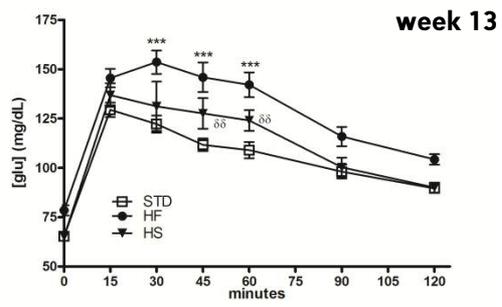
789 comparisons test.

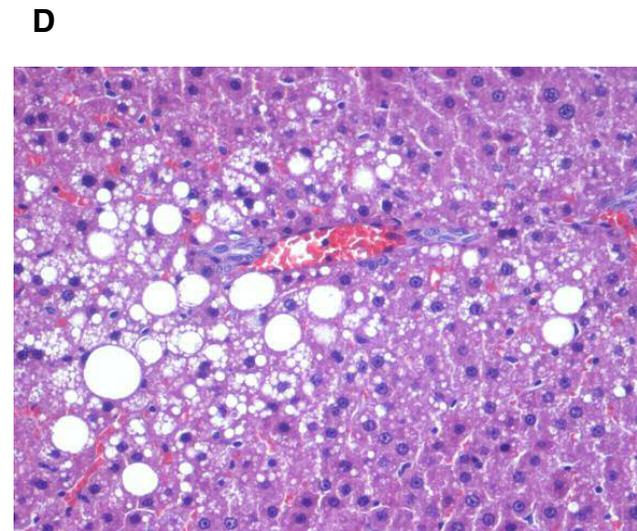
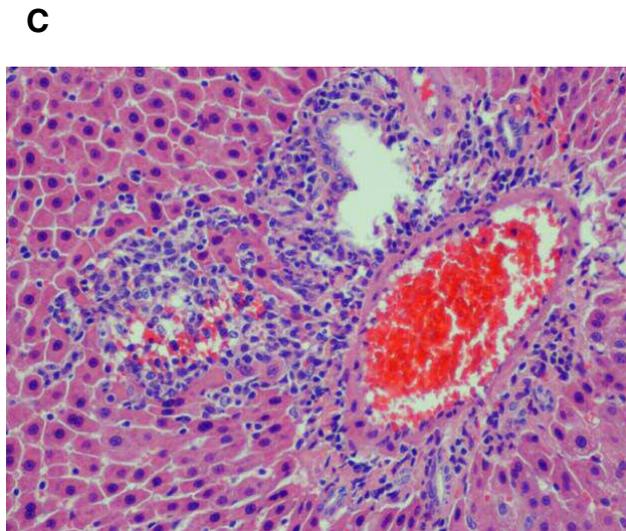
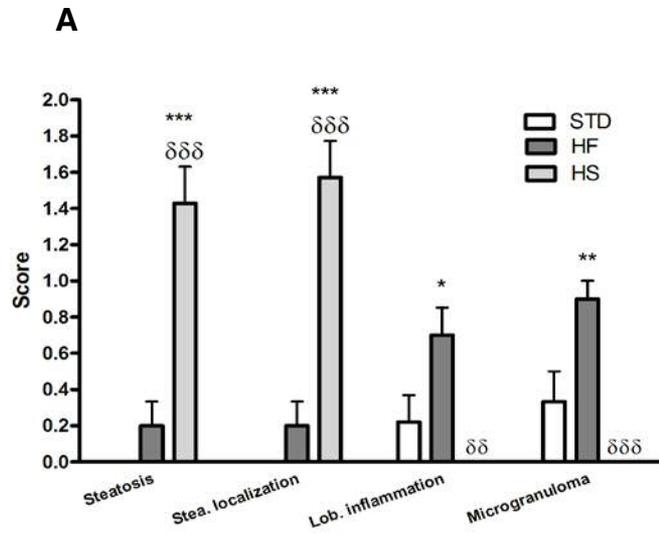
790 * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs STD group; δ $P < 0.05$, $\delta\delta$ $P < 0.01$ and $\delta\delta\delta$

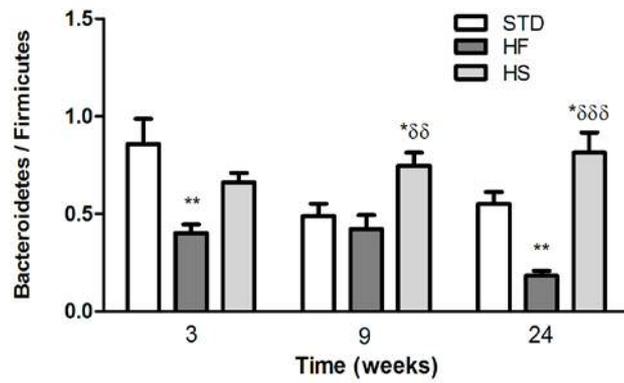
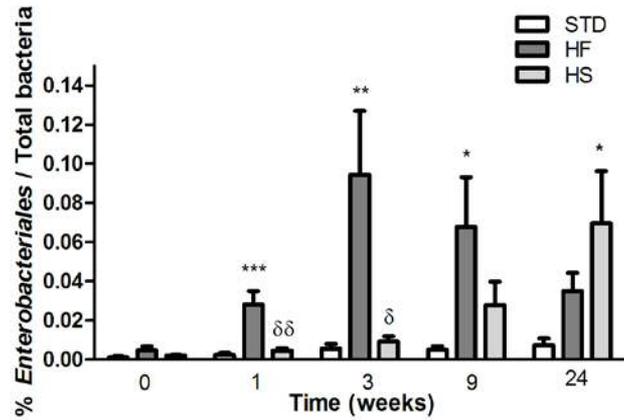
791 $P < 0.001$ vs HF group.

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