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

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Running head: Fat, sugar, insulin resistance and microbiota

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ABSTRACT

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

We tested the long-term effects of an excess of fat or sucrose (fructose/glucose) on healthy male Wistar Kyoto (WKY) rats. Fat affects IR and IGT earlier than fructose through low-grade systemic inflammation evidenced by liver inflammatory infiltration, increased levels of plasma interleukin-6, prostaglandin E_2 and reduced levels of protective short-chain fatty acids without triggering hypertension. Increased populations of gut Enterobacteriales and *Escherichia coli* may contribute to systemic inflammation through the generation of lipopolysaccharides. Unlike fat, fructose induces increased levels of diacylglycerols (lipid mediators of IR) in the liver, urine F_2-isoprostanes (markers of systemic oxidative stress) and uric acid, and triggers hypertension. Elevated populations of Enterobacteriales and *E. coli* were only detected in rats given an excess of fructose at the end of the study.

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

Keywords: diabetes, hypertension, microbiota, obesity

INTRODUCTION

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

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

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

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

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

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

MATERIALS AND METHODS

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

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

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

overnight fasting. After week 23, the rats were placed in metabolic cages for urine collection. After week 24, they were fasted overnight and anaesthetized intraperitoneally with ketamine (Merial Laboratorios, Barcelona, Spain) and xylazine (Química Farmacéutica, Barcelona, Spain) at doses of 80 and 10 mg/kg body weight, respectively. Blood was collected by cardiac puncture and plasma was immediately obtained by centrifugation. Perigonadal fat, quadricep muscle and liver samples were collected, washed with 0.9% NaCl solution, weighed and immediately frozen in liquid N_2. All the samples were stored at –80 ºC until analysis.

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

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

Measurement of uric acid. Total urine uric acid was determined by a spectrophotometric method using a uricase/peroxidase kit from BioSystems (Barcelona, Spain) by measuring the absorbance at 520 nm on a SpectraMax M5 spectrophotometer (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=fasting
165 blood glucose in mmol/L times fasting plasma insulin in μU/mL divided by 405 (31).
166 Insulin units (U) were calculated using the conversion 1U = 0.0347 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

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

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

Subpopulations of gut microbiota. The levels of total bacteria and Bacteroidetes, Firmicutes, Enterobacteriales, and *Escherichia coli* were estimated from fecal DNA by 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.

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

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

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

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

Measurement of isoprostanes. F_2-isoprostanes (F_2-IsoPs) were determined in urine samples by LC/ESI–MS/MS following a previously reported procedure (35) with modifications. Urine samples (500 µL) were acidified, β-glucuronidase (90 U/mL) (Sigma, Saint Louis, MI, USA) was added and the mixtures were incubated for 2 h at 37 ºC. After addition of the internal standard [$^2H_4$]15-F_{2t}-IsoP (Cayman, Ann Arbor, MI, USA) (100 µL, 10 µg/L), F_2-IsoPs were purified by SPE. F_2-IsoPs were analyzed using an Agilent 1260 chromatograph fitted with a Mediterranea Sea 18 column (10 cm x 2.1 mm i.d., 2.2 µm particle size) (Teknokroma, Barcelona, Spain) coupled to a 4000 QTRAP mass spectrometer (Applied Biosystems, Foster City, CA, USA). The instrument was operated in the negative-ion mode with a Turbo V source to obtain MS/MS data. Separation was achieved with a binary system consisting of [A] 0.1% aqueous formic acid and [B] 0.1% formic acid in acetonitrile, at 40º C, with an increasing linear gradient (v/v) of [B]: time 0, 10% B; 7 min, 50% B; 7.1 min, 100% B; 8 min, 100% B; 8.1 min, 10% B; and 10 min, 10% B, at a flow rate of 700 µL/min. F_2-IsoPs were detected by MS/MS multiple reaction monitoring (MRM). Calibration curves were prepared using seven matrix-matched standards covering the working concentration range. The LOQ was 0.4 µg/L for 15-F_{2t}-IsoP and 2 µg/L for 5-F_{2t}-IsoP. The results were expressed as ng/mg creatinine, to correct for urine dilution.

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

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

RESULTS

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

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

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

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

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

Liver histology, total liver triacylglycerols and biochemical measurement of liver function. An excess of dietary fat triggered lobular inflammation and microgranulomas, while an excess of fructose did not (Figure 3A). The livers of animals fed the HF diet showed lobular inflammation with lymphoplasmacytic inflammatory infiltration around the blood vessels (e.g. Figure 3C). Conversely, an excess of fructose induced significant and highly localized steatosis (Figure 3A, D) while an excess of dietary fat did not (Figure 3A, C). The levels of total triacylglycerols (μmol/g liver) were STD: 46.8, SEM

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

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

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

Subpopulations of gut microbiota and microbial products. The Bacteroidetes:Firmicutes ratio (Figure 4A) was reduced in HF animals and increased in HS animals compared to STD animals. The proportion of Enterobacteriales and *E. coli* 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 E_2 (PGE_2, 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 LTB_4
368 (leukotriene B_4) 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 HS[1] 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 (OGGT). The rats that
385 consumed and 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 PGE_2 (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 PGE_2 is the predominant
396 prostaglandin in white AT, where it regulates adipose functions (26). PGE_2 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.

As DAGs may induce IR independently of inflammation, we measured their levels in liver, muscle and AT. We did not detect any significant increase in the DAG profile in the livers of rats fed HF (Table 4), in agreement with the absence of steatosis (Figure 3C). The only DAGs with elevated levels in the HF group were the monounsaturated 34:1 (muscle), 36:1 (muscle) and 42:1 (AT). As lipid-induced IR occurs earlier in liver than in muscle (28), we concluded that direct impairment of insulin signaling by lipid metabolites would not explain the observed early fat-induced systemic IGT. In contrast, the animals given an excess of fructose presented steatosis around the blood vessels (Figure 3D) and clearly elevated DAGs in the liver (Table 4) without gaining more weight or accumulating more perigonadal AT than those given the STD diet (Figure 1C, E). The levels of total triglycerides were also elevated in the HS group although the differences were not statistically significant probably due to the fact that the steatosis was highly localized. These observations are consistent with hepatic *de novo* lipogenesis from fructose (20). It is becoming evident that different DAGs have a very different impact on cellular signaling (13). While there is no information so far as to what particular DAG species might impair insulin signaling, it has been reported that cellular signaling proteins and receptors such as the human transfer receptor potential C3 (TRPC3) are differentially activated by different DAGs and that DAG 36:2 (1-stearoyl-2-linoleoyl-*sn*-glycerol) is one of the active species (21, 38). The amounts of DAGs 36:2 and 34:1 (putatively 1-palmitoyl-2-oleoyl-*sn*-glycerol) were dramatically increased (3- to 4-fold) in the livers of HS-fed rats (Table 4), which suggests that DAG 36:2 may play a role in late fructose-induced tendency to IR. DAG 34:1, which does not activate the TRCP3 calcium channel (38), may or may not play a role in our model. In muscle, DAG content is much lower than in liver and only the levels of DAG 32:1 were 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 F_2-IsoPs, which are products of free radical-mediated *in vivo*
460 oxidation of ARA (33) different from COX-derived oxidized prostaglandins such as
461 PGE_2, 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.

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

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

CONCLUDING REMARKS

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

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REFERENCES

1. **Buettner R, Parhofer KG, Woenckhaus M, Wrede CE, Kunz-Schughart LA, Scholmerich J, and Bollheimer LC**. Defining high-fat-diet rat models: metabolic and molecular effects of different fat types. *J Mol Endocrinol* 36: 485-501, 2006.

2. **Canfora EE, Jocken JW, and Blaak EE**. Short-chain fatty acids in control of body weight and insulin sensitivity. *Nature Rev Endocrinol* 11: 577-591, 2015.

3. **Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, Neyrinck AM, Fava F, Tuohy KM, Chabo C, Waget A, Delmee E, Cousin B, Sulpice T, Chamontin B, Ferrieres J, Tanti JF, Gibson GR, Casteilla L, Delzenne NM, Alessi MC, and Burcelin R**. Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes* 56: 1761-1772, 2007.

4. **Castro MC, Massa ML, Arbelaez LG, Schinella G, Gagliardino JJ, and Francini F**. Fructose-induced inflammation, insulin resistance and oxidative stress: A liver pathological triad effectively disrupted by lipoic acid. *Life Sci* 137: 1-6, 2015.

5. **D'Orazio SEF, and Collins CM**. The plasmid-encoded urease gene-cluster of the

551 family enterobacteriaceae is positively regulated by urer, a member of the arac family of
552 transcriptional activators. *J Bacteriol* 175: 3459-3467, 1993.
553 6. **Dasilva G, Pazos M, Gallardo JM, Rodríguez I, Cela R, and Medina I**.
554 Lipidomic analysis of polyunsaturated fatty acids and their oxygenated metabolites in
555 plasma by solid-phase extraction followed by LC-MS. *Anal Bioanal Chem* 406: 2827-
556 2839, 2014.
557 7. **Dasilva G, Pazos M, García-Egido E, Pérez-Jiménez J, Torres JL, Giralt M,**
558 **Nogués MR, and Medina I**. Lipidomics to analyze the influence of diets with different
559 EPA:DHA ratios in the progression of Metabolic Syndrome using SHROB rats as a
560 model. *Food Chem* 205: 196-203, 2016.
561 8. **de la Serre CB, Ellis CL, Lee J, Hartman AL, Rutledge JC, and Raybould HE**.
562 Propensity to high-fat diet-induced obesity in rats is associated with changes in the gut
563 microbiota and gut inflammation. *Am J Physiol Gastrointest Liver Physiol* 299: G440-
564 448, 2010.
565 9. **Despres J-P, and Lemieux I**. Abdominal obesity and metabolic syndrome. *Nature*
566 444: 881-887, 2006.
567 10. **DiNicolantonio JJ, and O'Keefe JH**. Hypertension due to woxic white crystals in
568 the diet: should we blame salt or sugar? *Progr Cardiovasc Dis* 59: 219-225, 2016.
569 11. **Donath MY, and Shoelson SE**. Type 2 diabetes as an inflammatory disease. *Nat*
570 *Rev Immunol* 11: 98-107, 2011.
571 12. **Eckel RH, Alberti KGMM, Grundy SM, and Zimmet PZ**. The metabolic
572 syndrome. *Lancet* 375: 181-183, 2010.
573 13. **Eichmann TO, and Lass A**. DAG tales: the multiple faces of diacylglycerol--
574 stereochemistry, metabolism, and signaling. *Cell Mol Life Sci* 72: 3931-3952, 2015.
575 14. **Fabbrini E, Magkos F, Mohammed BS, Pietka T, Abumrad NA, Patterson BW,**

576 **Okunade A, and Klein S**. Intrahepatic fat, not visceral fat, is linked with metabolic
577 complications of obesity. *Proc Natl Acad Sci U S A* 106: 15430-15435, 2009.
578 15. **Fak F, Ahrne S, Molin G, Jeppsson B, and Westrom B**. Microbial manipulation
579 of the rat dam changes bacterial colonization and alters properties of the gut in her
580 offspring. *Am J Physiol Gastrointest Liver Physiol* 294: G148-G154, 2008.
581 16. **Franklin SS, and Wong ND**. Hypertension and cardiovascular disease:
582 contributions of the Framingham heart study. *Glob Heart* 8: 49-57, 2013.
583 17. **Haakensen M, Dobson CM, Deneer H, and Ziola B**. Real-time PCR detection of
584 bacteria belonging to the Firmicutes Phylum. *Int J Food Microbiol* 125: 236-241, 2008.
585 18. **Hartman AL, Lough DM, Barupal DK, Fiehn O, Fishbein T, Zasloff M, and**
586 **Eisen JA**. Human gut microbiome adopts an alternative state following small bowel
587 transplantation. *Proc Natl Acad Sci U S A* 106: 17187-17192, 2009.
588 19. **Hatch M, and Vaziri ND**. Enhanced enteric excretion of urate in rats with chronic-
589 renal-failure. *Clin Sci* 86: 511-516, 1994.
590 20. **Havel PJ**. Dietary fructose: implications for dysregulation of energy homeostasis
591 and lipid/carbohydrate metabolism. *Nutr Rev* 63: 133-157, 2005.
592 21. **Hofmann T, Obukhov AG, Schaefer M, Harteneck C, Gudermann T, and**
593 **Schultz G**. Direct activation of human TRPC6 and TRPC3 channels by diacylglycerol.
594 *Nature* 397: 259-263, 1999.
595 22. **Ismail NA, Ragab SH, Elbaky AA, Shoeib AR, Alhosary Y, and Fekry D**.
596 Frequency of Firmicutes and Bacteroidetes in gut microbiota in obese and normal
597 weight Egyptian children and adults. *Arch Med Sci* 7: 501-507, 2011.
598 23. **Janssen AF, and Kersten S**. Potential mediators linking gut bacteria to metabolic
599 health: a critical view. *J Physiol London* 595: 477-487, 2017.
600 24. **Johnson RJ, Nakagawa T, Gabriela Sanchez-Lozada L, Shafiu M, Sundaram**

S, Le M, Ishimoto T, Sautin YY, and Lanaspa MA. Sugar, uric acid, and the etiology of diabetes and obesity. *Diabetes* 62: 3307-3315, 2013.

25. **Kaliannan K, Wang B, Li XY, Kim KJ, and Kang JX**. A host-microbiome interaction mediates the opposing effects of omega-6 and omega-3 fatty acids on metabolic endotoxemia. *Sci Rep* 5: 11276, 2015.

26. **Kimple ME**. Inhibitory G proteins and their receptors: emerging therapeutic targets for obesity and diabetes. *Exp Mol Med* 46: 1-9, 2014.

27. **Klein AV, and Kiat H**. The mechanisms underlying fructose-induced hypertension: a review. *J Hypertens* 33: 912-920, 2015.

28. **Kraegen EW, Clark PW, Jenkins AB, Daley EA, Chisholm DJ, and Storlien LH**. Development of muscle insulin resistance after liver insulin resistance in high-fat-fed rats. *Diabetes* 40: 1397-1403, 1991.

29. **Malinen E, Kassinen A, Rinttila T, and Palva A**. Comparison of real-time PCR with SYBR Green I or 5'-nuclease assays and dot-blot hybridization with rDNA-targeted oligonucleotide probes in quantification of selected faecal bacteria. *Microbiol* 149: 269-277, 2003.

30. **Massey KA, and Nicolaou A**. Lipidomics of oxidized polyunsaturated fatty acids. *Free Radic Biol Med* 59: 45-55, 2013.

31. **Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, and Turner RC**. Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 28: 412-419, 1985.

32. **Mazzali M, Hughes J, Kim YG, Jefferson JA, Kang DH, Gordon KL, Lan HY, Kivlighn S, and Johnson RJ**. Elevated uric acid increases blood pressure in the rat by a novel crystal-independent mechanism. *Hypertension* 38: 1101-1106, 2001.

33. **Milne GL, Yin H, and Morrow JD**. Human biochemistry of the isoprostane

626 pathway. *J Biol Chem* 283: 15533-15537, 2008.
627 34. **Mobley HL, and Hausinger RP**. Microbial ureases: significance, regulation, and
628 molecular characterization. *Microbiol Rev* 53: 85-108, 1989.
629 35. **Molinar-Toribio E, Pérez-Jiménez J, Ramos-Romero S, Gómez L, Taltavull N,**
630 **Nogués MR, Adeva A, Jaúregui O, Joglar J, Clapés P, and Torres JL**. D-Fagomine
631 attenuates metabolic alterations induced by a high-energy-dense diet in rats. *Food Func*
632 6: 2614-2619, 2015.
633 36. **Mozaffarian D**. Dietary and policy priorities for cardiovascular disease, diabetes,
634 and obesity. A comprehensive review. *Circulation* 133: 187-225, 2016.
635 37. **Muhling M, Woolven-Allen J, Murrell JC, and Joint I**. Improved group-specific
636 PCR primers for denaturing gradient gel electrophoresis analysis of the genetic diversity
637 of complex microbial communities. *ISME J* 2: 379-392, 2008.
638 38. **Nadler A, Reither G, Feng S, Stein F, Reither S, Mueller R, and Schultz C**. The
639 fatty acid composition of diacylglycerols determines local signaling patterns. *Angew*
640 *Chem Int Ed* 52: 6330-6334, 2013.
641 39. **Nascimento AR, Machado M, de Jesus N, Gomes F, Lessa MA, Bonomo IT,**
642 **and Tibirica E**. Structural and functional microvascular alterations in a rat model of
643 metabolic syndrome induced by a high-fat diet. *Obesity* 21: 2046-2054, 2013.
644 40. **Oudot C, Lajoix AD, Jover B, and Rugale C**. Dietary sodium restriction prevents
645 kidney damage in high fructose-fed rats. *Kidney Int* 83: 674-683, 2013.
646 41. **Palmer BF, and Clegg DJ**. Electrolyte and acid-base disturbances in patients with
647 diabetes mellitus. *New Engl J Med* 373: 548-559, 2015.
648 42. **Portune KJ, Benítez-Páez A, Del Pulgar EMG, Cerrudo V, and Sanz Y**. Gut
649 microbiota, diet, and obesity-related disorders-The good, the bad, and the future
650 challenges. *Mol Nutr Food Res* 61: 2017.

651 43. **Robertson RP**. Eicosanoids as pluripotential modulators of pancreatic-islet
652 function. *Diabetes* 37: 367-370, 1988.
653 44. **Samuel VT, and Shulman GI**. Mechanisms for insulin resistance: common threads
654 and missing links. *Cell* 148: 852-871, 2012.
655 45. **Schwiertz A, Taras D, Schafer K, Beijer S, Bos NA, Donus C, and Hardt PD**.
656 Microbiota and SCFA in lean and overweight healthy subjects. *Obesity* 18: 190-195,
657 2010.
658 46. **Simbari F, McCaskill J, Coakley G, Millar M, Maizels RM, Fabriás G, Casas**
659 **J, and Buck AH**. Plasmalogen enrichment in exosomes secreted by a nematode parasite
660 versus those derived from its mouse host: implications for exosome stability and
661 biology. *J Extracell Vesicles* 5: 30741-30741, 2016.
662 47. **Sorensen LB, and Levinson DJ**. Origin and extrarenal elimination of uric-acid in
663 man. *Nephron* 14: 7-20, 1975.
664 48. **Taltavull N, Muñoz-Cortés M, Lluís L, Jové M, Fortuño À, Molinar-Toribio E,**
665 **Torres JL, Pazos M, Medina I, and Nogués MR**. Eicosapentaenoic
666 acid/docosahexaenoic acid 1:1 ratio improves histological alterations in obese rats with
667 metabolic syndrome. *Lipids Health Dis* 13: 31, 2014.
668 49. **Tran LT, Yuen VG, and McNeill JH**. The fructose-fed rat: a review on the
669 mechanisms of fructose-induced insulin resistance and hypertension. *Mol Cell Biochem*
670 332: 145-159, 2009.
671 50. **Weir GC, and Bonner-Weir S**. Five stages of evolving beta-cell dysfunction
672 during progression to diabetes. *Diabetes* 53: S16-S21, 2004.
673 51. **Wong J, Piceno YM, De Santis TZ, Pahl M, Andersen GL, and Vaziri ND**.
674 Expansion of urease- and uricase-containing, indole- and p-cresol-forming and
675 contraction of short-chain fatty acid-producing intestinal microbiota in ESRD. *Am J*

676 *Nephrol* 39: 230-237, 2014.

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; δ $P < 0.05$, δδ $P < 0.01$ and δδδ $P < 0.001$ *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; δ $P < 0.05$, δδ $P < 0.01$ and δδδ $P < 0.001$ *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, PGE_2; D, arachidonic acid (ARA); E, eicosapentaenoic acid (EPA); F
723 docosahexaenoic acid (DHA); F, LTB_4; 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; δ $P < 0.05$ *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; δ $P < 0.05$ *vs* HF group.
735

736
737 **Table 1.** *qRT-PCR primers and conditions*
738

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

739
740 [#] Positive control of Total Bacteria was the strain the result was rated with.
741

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*$^{\delta}$	0.2
Drink intake (mL/day/100 g body weight)	7.2	0.3	5.5	0.3	10.7*$^{\delta}$	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$^{\delta}$	17

744

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; $^{\delta}P < 0.05$ *vs* HF group.
749 [a] integrated STD signal (ks ºC/g) proportional to energy.
750

751

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

	STD		HF		HS	
	Mean	SEM	Mean	SEM	Mean	SEM
week 10						
Fasting glucose (mg/dL)	65.4	1.4	75.7***	2.0	64.8$^{\delta\delta}$	2.7
Insulin (pg/mL)	417.9	71.4	1737***	137.6	584.5$^{\delta\delta\delta}$	105.8
HOMA-IR	1.9	0.3	9.3***	0.7	2.7$^{\delta\delta\delta}$	0.5
week 16						
Fasting glucose (mg/dL)	66.0	1.1	70.8*	1.4	59.3 $^{\delta\delta}$	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

753

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 $P < 0.05$, δδ $P < 0.01$,
757 δδδ $P < 0.001$ *vs* HF group.
758

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*$^{\delta}$	72.1
32:2	15.1	1.5	14.8	2.4	43.6*$^{\delta}$	12.5
34:1	405.7	43.0	540.4	100.1	1499.0*$^{\delta}$	427.2
34:2	328.8	39.1	139.6	26.6	414.6$^{\delta}$	111.1
34:3	88.0	10.7	27.0*	6.0	101.7$^{\delta}$	26.7
34:4	17.4	2.3	2.1*	1.4	18.1$^{\delta}$	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**$^{\delta}$	1.3

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 multiple comparisons test.

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

$^{\#}$Amounts expressed as DAG 16:0, 16:0 equivalents.

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*$^{\delta\delta}$	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

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; δδ $P < 0.01$ *vs* HF

$^{\#}$Amounts expressed as DAG 16:0, 16:0 equivalents.

774

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

776

	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$^{\delta}$	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$^{\delta}$	0.0

777

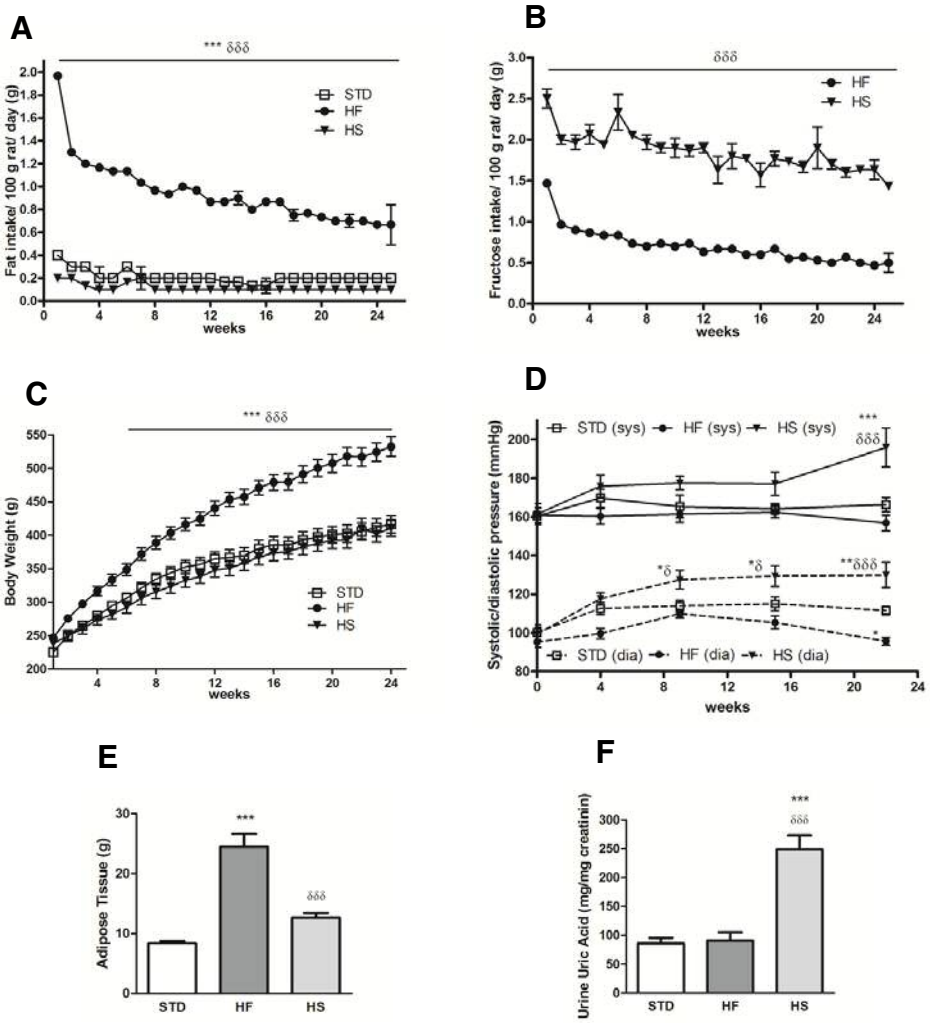
778 Data are presented as means with their standard errors of the mean, n = 9 per group.
779 Comparisons were conducted using one-way ANOVA and Tukey's tests.
780 * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ *vs* STD group; δ $P < 0.05$ *vs* HF
781 [#]Amounts expressed as DAG 16:0, 16:0 equivalents.
782
783

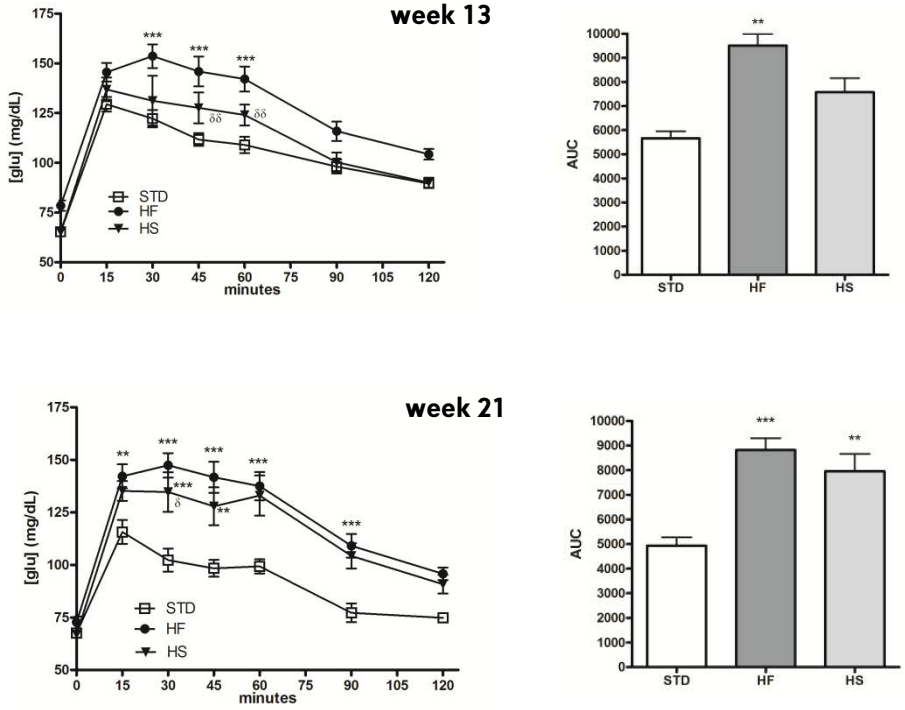
Table 7. *Short-chain fatty acids (SCFAs, mmol/kg) in feces fed after 12 weeks*

	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	$28.0^{\delta\delta}$	5.7
Isobutyric Acid	1.3	0.2	0.2***	0.0	$1.1^{\delta\delta\delta}$	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

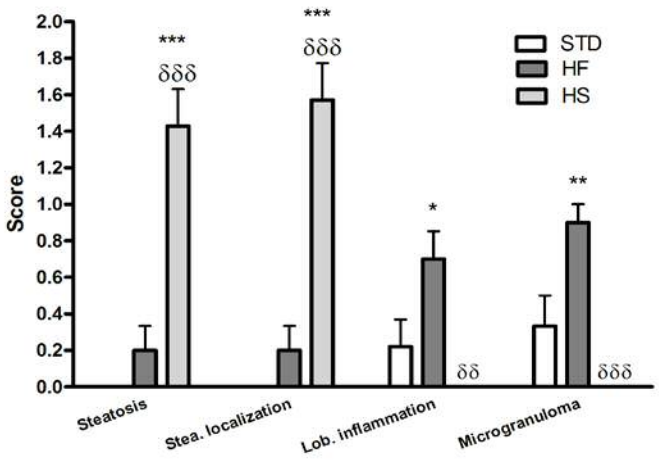
Data are presented as means with their standard errors of the mean, n = 9 per group. Comparisons were conducted using one-way ANOVA and Tukey's multiple comparisons test.

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

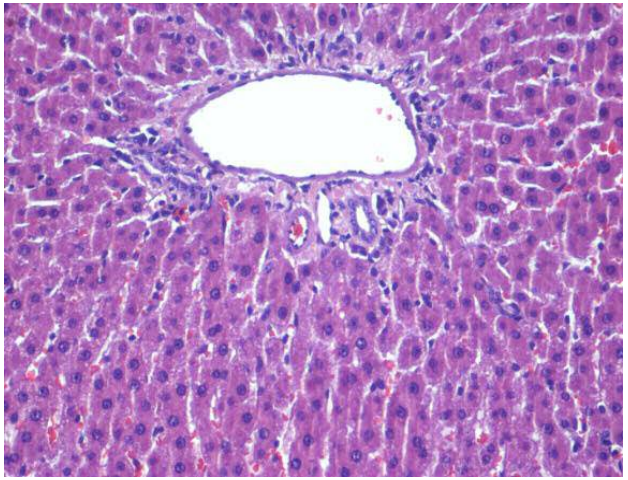

A
*** δδδ
Fat intake/ 100 g rat/ day (g)
STD
HF
HS
weeks
B
δδδ
Fructose intake/ 100 g rat/ day (g)
HF
HS
weeks
C
*** δδδ
Body Weight (g)
STD
HF
HS
weeks
D
STD (sys)
HF (sys)
HS (sys)
***
δδδ
*δ
*δ
**δδδ
*
Systolic/diastolic pressure (mmHg)
STD (dia)
HF (dia)
HS (dia)
weeks
E
Adipose Tissue (g)
***
δδδ
STD
HF
HS
F
Urine Uric Acid (mg/mg creatinin)
***
δδδ
STD
HF
HS


week 13
[glu] (mg/dL)
minutes
STD
HF
HS
AUC
week 21
[glu] (mg/dL)
minutes
STD
HF
HS
AUC

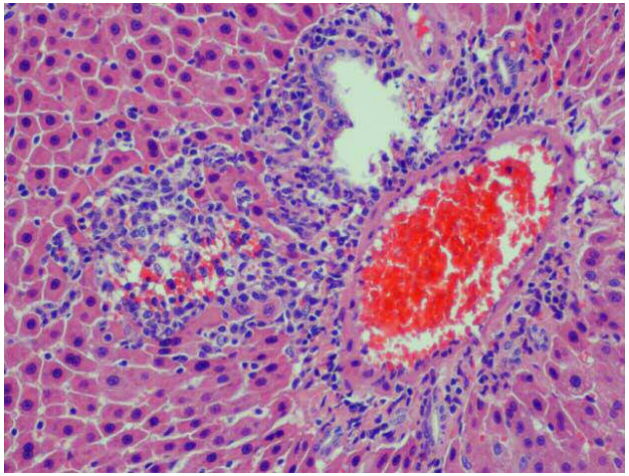
A


Score
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
δδδ
***
δδδ
*
δδ
**
δδδ
STD
HF
HS
Steatosis
Stea. localization
Lob. inflammation
Micrograniloma

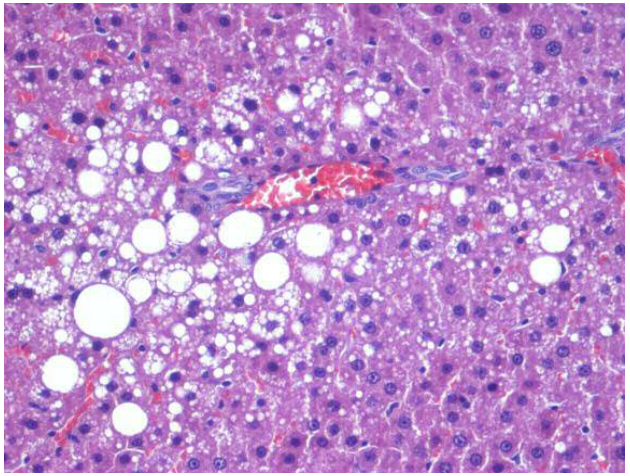
B

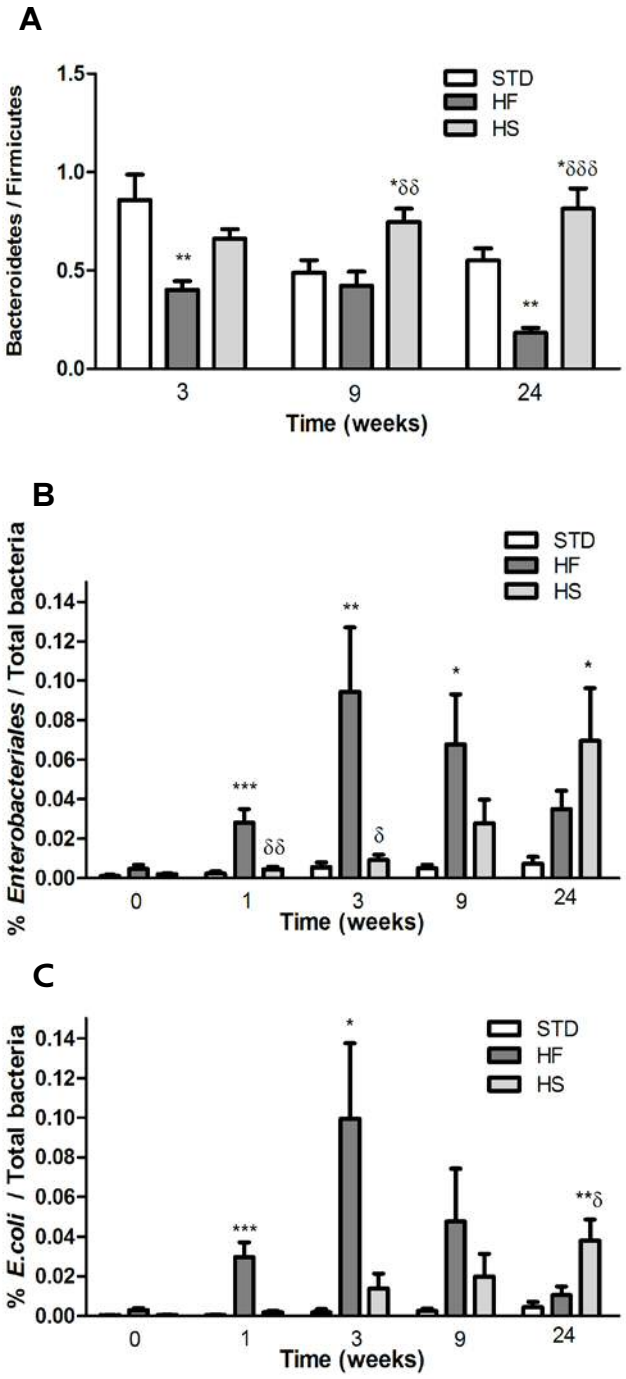


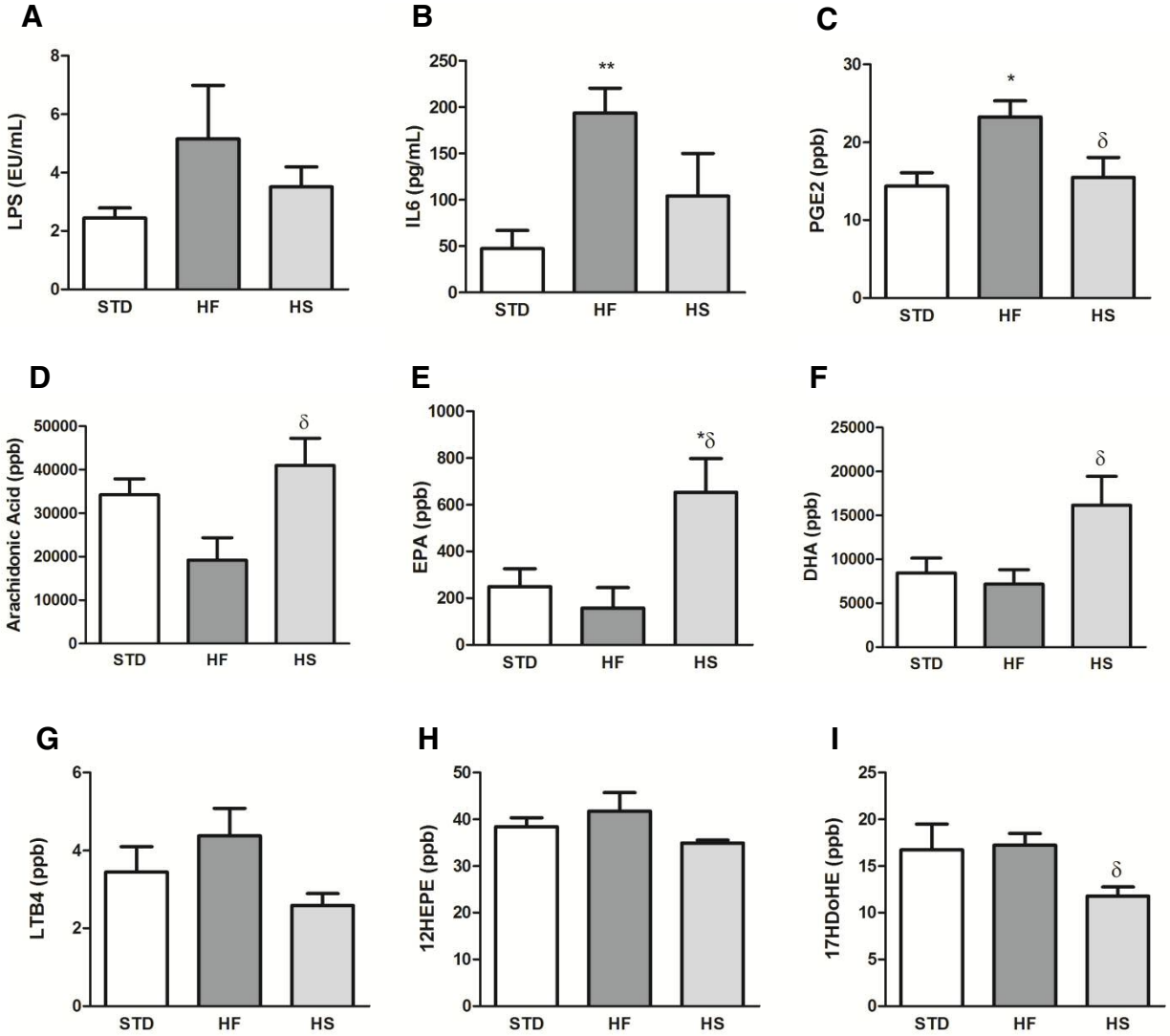
C

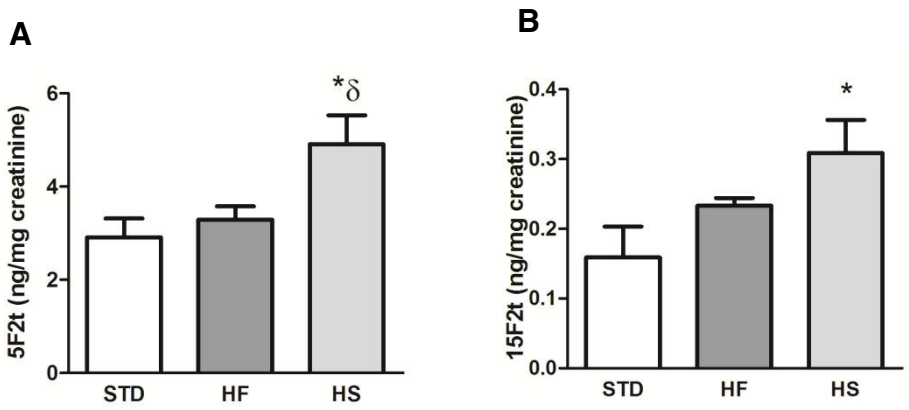


D




A
Bacteroidetes / Firmicutes
1.5
1.0
0.5
0.0
STD
HF
HS
**
*δδ
**
*δδδ
3
9
24
Time (weeks)
B
% Enterobacteriales / Total bacteria
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
STD
HF
HS
***
δδ
**
δ
*
*
0
1
3
9
24
Time (weeks)
C
% E.coli / Total bacteria
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
STD
HF
HS
***
*
**δ
0
1
3
9
24
Time (weeks)


A
LPS (EU/mL)
8
6
4
2
0
STD
HF
HS
B
IL6 (pg/mL)
250
200
150
100
50
0
**
STD
HF
HS
C
PGE2 (ppb)
30
20
10
0
*
δ
STD
HF
HS
D
Arachidonic Acid (ppb)
50000
40000
30000
20000
10000
0
δ
STD
HF
HS
E
EPA (ppb)
1000
800
600
400
200
0
*δ
STD
HF
HS
F
DHA (ppb)
25000
20000
15000
10000
5000
0
δ
STD
HF
HS
G
LTB4 (ppb)
6
4
2
0
STD
HF
HS
H
12HEPE (ppb)
50
40
30
20
10
0
STD
HF
HS
I
17HDoHE (ppb)
25
20
15
10
5
0
δ
STD
HF
HS


A
B
5F2t (ng/mg creatinine)
15F2t (ng/mg creatinine)
*δ
*
STD
HF
HS
0
2
4
6
0.0
0.1
0.2
0.3
0.4