Functional effects of the buckwheat iminosugar D-fagomine on rats with diet-induced prediabetes

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Abbreviations

AMPK, AMP-activated protein kinase

ARA, arachidonic acid

AT, adipose tissue

AUC, area under the curve

BHT, butylated hydroxytoluene

DHA, docosahexaenoic acid

EPA, eicosapentaenoic acid

Fiaf, fasting-induced adipose factor

HDoHE, hydroxydocosahexaenoic acid

HEPE, hydroxyeicosapentaenoic acid

HETE, hydroxyeicosatetraenoic acid

HpEPE, hydroperoxyicosapentaenoic acid

IGT, impaired glucose tolerance

IR, insulin resistance

LTB_4, leukotriene B_4

PGE_2, prostaglandin E_2

qRT-PCR, quantitative real-time PCR

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Abstract

Scope: The goals of this work were to test if D-fagomine, an iminosugar that reduces body weight gain, can delay the appearance of a fat-induced prediabetic state in a rat model and to explore possible mechanisms behind its functional action.

Methods and results: Wistar Kyoto rats were fed a high-fat diet supplemented with D-fagomine (or not; for comparison) or a standard diet (controls) for 24 weeks. The variables measured were: fasting blood glucose and insulin levels; glucose tolerance; diacylglycerols as intracellular mediators of insulin resistance in adipose tissue, liver and muscle; inflammation markers (plasma IL-6 and leptin, and liver and adipose tissue histology markers); eicosanoids from arachidonic acid as lipid mediators of inflammation; and the populations of Bacteroidetes, Firmicutes, Enterobacteriales and Bifidobacteriales in feces. We found that D-fagomine reduces fat-induced impaired glucose tolerance, inflammation markers and mediators (hepatic microgranulomas and lobular inflammation, plasma IL-6, prostaglandin E_2 and leukotriene B_4) while attenuating the changes in the populations of Enterobacteriales and Bifidobacteriales.

Conclusion: D-Fagomine delays the development of a fat-induced prediabetic state in rats by reducing low-grade inflammation. We suggest that the anti-inflammatory effect of D-fagomine may be linked to a reduction in fat-induced overpopulation of minor gut bacteria.

1 Introduction

The World Health Organization estimated that 422 million adults suffered from diabetes in 2014 and 1.5 million deaths could be directly attributed to this pathology in just one year (2012). Most of the population suffering from diabetes is affected by type 2 diabetes (T2D). T2D is preceded by insulin resistance (IR): a reduced capacity to internalize glucose from the bloodstream as a result of insensitivity to insulin that may result from genetic predisposition, physical inactivity and/or obesity in both rats and humans [1]. IR brings about an increase of pancreatic insulin secretion from a greater number or size of pancreatic β-cells, which compensates the low insulin sensitivity. Then, if IR proceeds further into diabetes, a drop in insulin secretion follows, with subsequent increased fasting glucose levels and impaired glucose tolerance (IGT; high glucose levels 2 h after ingestion) as a consequence of a loss and dedifferentiation of pancreatic β-cells [1]. Three main mechanisms have been proposed to explain the pathogenesis of IR in different organs: endoplasmic reticulum stress with activation of the unfolded protein response, ectopic lipid accumulation with impairment of intracellular signaling patterns by particular lipid mediators, and systemic inflammation [2]. More recently, systemic inflammation, IR, and obesity have been linked to shifts in the populations of gut microbiota (gut "dysbiosis") [3].

The major bacterial phyla in distal gut microbiota are Bacteroidetes and Firmicutes. A reduction in the ratio between these two phyla has been related to weight gain by the host [4, 5]. Also, an increase in the population of Enterobacteriales has been associated with diet-induced obesity [6]. A common antecedent that may link dysbiosis, obesity and IR is the induction of plasmatic endotoxemia [7], which may trigger low-grade inflammation and/or changes in energy harvest capacity [8, 9]. Other minor gut bacterial subgroups such

as Lactobacillus and Bifidobacterium may help to maintain host homeostasis [10]. Specifically, high levels of Bifidobacterium reduce diet-induced IR and inflammation [11, 12].

D-Fagomine (1,2-dideoxynojirimycin) is an iminosugar: a carbohydrate analog that includes an endocyclic nitrogen instead of oxygen [13]. D-Fagomine is naturally present in buckwheat (*Fagopyrum esculentum* Moench, Polygonaceae) and can be found in several buckwheat-based foodstuffs such as noodles, pancakes, fried dough, beer, cookies and bread [14]. D-Fagomine lowers post-prandial blood glucose in sucrose/starch loading tests [15] and it reduces elevated plasma insulin concentrations induced by a high-fat high-sucrose diet in the short term (9 weeks) [16].

This study examines the long-term functional effect of D-fagomine on a fat induced prediabetic state and explores possible molecular mechanisms behind its action.

2 Materials and methods

2.1 Animals

A total of twenty-seven male Wistar Kyoto rats from Envigo (Indianapolis, IN, USA), aged 8-9 weeks were used. All the procedures strictly adhered to the European Union guidelines for the care and management of laboratory animals, and were under license from the Catalan authorities (reference no. DAAM7921), as approved by the Spanish CSIC Subcommittee of Bioethical Issues.

2.2 Experimental design: data and sample collection

The rats were kept 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/group): the standard (STD) group, fed a STD diet (2014 Teklad Global 14% Protein) from Envigo; the high-fat (HF) group fed a HF diet (TD.08811 45% kcal Fat) from Envigo; and the group fed the HF diet supplemented with 0.96 g of D-fagomine (> 98% from Bioglane SLNE; Barcelona, Spain) per kg of feed (HF+FG group). The dose of D-fagomine corresponded that used in post-prandial sucrose/starch loading tests (2 mg/g sucrose) [15]. All the groups were fed *ad libitum* with free access to water.

Feed consumption was monitored daily and body weight was measured weekly throughout the experiment. 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.

Fecal samples were collected by abdominal massage at weeks 9, 20 and 24. The energy content of the feces from week 20 was determined by differential scanning calorimetry

(25-600 ºC in an O_2 atmosphere, 10 ºC/min) by means of a thermogravimetric analyzer TGA/SDTA 851e (Mettler Toledo; Columbus, OH, USA) with an integrated SDTA signal.

At weeks 10 and 16, blood samples were collected from the saphenous vein after overnight fasting, and plasma was separated by centrifugation and stored at −80 °C until analysis.

At the end of the experiment, the rats were fasted overnight and anesthetized intraperitoneally with ketamine and xylazine (80 and 10 mg/kg body weight, respectively). Blood was collected by cardiac puncture then plasma was immediately obtained by centrifugation and stored at −80 ºC until analysis. Perigonadal adipose tissue (AT), liver and quadriceps (muscular tissue) were removed, weighed and cut into small pieces. One part of the liver was fixed in 10% formalin for histological analysis. The rest of the liver as well as the muscle and AT samples were washed with 0.9% NaCl solution and stored at −80 ºC for diacylglycerol (DAG) analysis.

2.3. Plasma lipid profile

Plasma total cholesterol, HDL-cholesterol, LDL-cholesterol, and triglycerides were measured using a spectrophotometric method and the corresponding kits from Spinreact (Girona, Spain) as described by Bucolo *et al*. [17, 18]

2.3 Plasma insulin, glucose and oral glucose tolerance test

Plasma insulin levels were measured using Milliplex xMAP multiplex technology on a Luminex xMAP instrument (Millipore, Austin, TX, USA) at weeks 10 and 16. Milliplex

Analyst 5.1 (Vigenetech, Carlisle, PA, USA) software was used for data analysis. The standard curve was generated in the range 69-50,000 pg/mL.

At weeks 13 and 21, an oral glucose tolerance test (OGTT) was performed on fasted animals. A solution of glucose (1 g/kg body weight) was administered to the rats by oral gavage. Blood glucose concentration was measured by the enzyme electrode method using an Ascensia ELITE XL blood glucose meter (Bayer Consumer Care AG; Basel, Switzerland) before the experiment and 15, 30, 45, 60, 90 and 120 min after glucose intake. Fasting glucose concentration was measured by the same method at weeks 10 and 16.

2.4 Diacylglycerols in perigonadal adipose tissue, liver and muscle

Frozen samples were weighted and sonicated (SFX150 Sonifier; Emerson Industrial Automation, St. Louis, MO, USA) until total homogenization. DAG extracts were prepared and analyzed using the method described by Simbari *et al.* [19] 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 were loaded into an Acquity UPLC system connected to an LCT Premier orthogonal accelerated time-of-flight mass spectrometer (Waters, Milford, MA, USA), which was operated in positive ESI mode (LC-TOF-MS). 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] at 30 ºC under linear gradient conditions: 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. The flow rate was 0.3 mL/min. Quantification was carried out using the extracted ion chromatogram of each compound, across 50 mDa windows. The linear range was determined by injecting mixtures of internal standards. DGA content was calculated as DAG 16:0, 16:0 equivalents.

2.5 Liver and adipose tissue histology

Fixed liver and AT were dehydrated in alcohol and embedded in paraffin (Panreac Quimica SLU; Barcelona, Spain), then cut into 3 μm thick slices, using a steel knife mounted in a microtome (HM 355S Rotary Microtome; Thermo Fisher Scientific, Waltham, MA, USA). Sections were stained with hematoxylin (hematoxylin solution modified in accordance with Gill III for microscopy; Merck KGaA, Darmstadt, Germany)/eosin (Pharmacy Service of Puerta del Mar Hospital, Cádiz, Spain) then viewed under a light microscope (NIKON Eclipse 80i; NIKON Corporation, Minato, Japan). Variables were graded following the method described by *Taltavull et al.* [20] using observation of the entire field of the tissue preparations. Liver: steatosis, 0 (< 5%), 1 (5%-33%), 2 (33%-66%), or 3 (> 66%); steatosis localization, 0 (absence), 1 (periportal), and 2 (non-zonal); lobular inflammation with lymphoplasmacytic inflammatory infiltration, 0 (absence), 1 (1-2 foci), 2 (2-4 foci), or 3 (> 4 foci); and the presence of microgranulomas, 0 (absence) or 1 (presence). AT: adipocyte hypertrophy, 0 (absence) or 1 (presence); macrophages 0 (absence) or 1 (presence); mast cells, 0 (absence) or 1

(presence); and adipose tissue inflammation with lymphoplasmacytic inflammatory infiltration, 0 (absence) or 1 (presence).

2.6 Biomarkers and lipid mediators of inflammation in plasma

Plasma IL-6 and leptin levels were measured using Milliplex xMAP multiplex technology (Millipore) on a Luminex xMAP instrument.

Lipid mediators derived from arachidonic acid (ARA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were determined in plasma using a method modified from Dasilva *et al.* [21]. Briefly, plasma samples (90 µL) were diluted in cold 0.05% BHT in methanol/water (3:7) (1 mL) and spiked with the internal standard (12HETE-d8, Cayman Chemicals; Ann Arbor, MI, USA). Then, the samples were centrifuged (Avanti J25, Beckman Coulter; Brea, CA, USA) (800 g, 10 min) at 4 ºC. The supernatants were purified by solid-phase extraction.

The LC-MS/MS analyzer used was an Agilent 1260 Series (Agilent; Palo Alto, CA, USA) chromatograph coupled to an LTQ Velos Pro dual-pressure linear ion trap mass spectrometer (Thermo Fisher; Rockford, IL, USA) operated in negative ESI mode. A C18-Symmetry 150 × 2.1 mm inner diameter, 3.5 µm column (Waters) was used with a C18 4 × 2 mm guard cartridge (Phenomenex; Torrance, CA, USA) in the separation step. Samples (10 µL) were eluted with a binary system of 0.02% formic acid in water [A] and in methanol [B]. The gradient was: 1 min, 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. The flow rate was 0.2 mL/min. LC-MS/MS details are provided in Supporting Information Table S1.

2.7 Fecal microbiota

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184 The levels of total bacteria and Bacteroidetes, Firmicutes, Enterobacteriales,
185 Bifidobacteria and Lactobacilliales were estimated from fecal DNA by quantitative real-
186 time PCR (qRT-PCR). DNA was extracted from the feces using QIAamp® DNA Stool
187 Mini Kit from QIAGEN (Hilden, Germany) and its concentration was quantified using a
188 Nanodrop 8000 Spectrophotometer (ThermoScientific; Waltham, MA, USA). All DNA
189 samples were diluted to 20 ng/μL. The qRT-PCR experiments were carried out using a
190 LightCycler® 480 II (Roche; Basel, Switzerland).
191 Each qRT-PCR well was run in triplicate and contained a total of 20 μL: 18 μL of Master
192 Mix (10 μL of 2X SYBR, 1 μL of each (forward and reverse) corresponding primer and
193 6 μL of water) and 2 μL of DNA sample. All reactions were paralleled by analysis of a
194 nontemplate control (water) and a positive control. The primers and annealing
195 temperatures are detailed in Supporting Information Table S2.
196 The qRT-PCR cycling conditions were: 10 s at 95 ºC, then 45 cycles of 5 s at 95 ºC, 30 s
197 at primer-specific annealing temperature (Supporting Information Table S2), and 30 s at
198 72 ºC (extension). Following amplification, to determine the specificity of the qRT-PCR,
199 melting curve analysis was carried out by treatment for 2 s at 95 ºC, 15 s at 65 ºC, and
200 then continuous increase of temperature up to 95 ºC (0.11 ºC/s), with five fluorescence
201 recordings per ºC. The relative DNA abundances for the different genes were calculated
202 from the second derivative maximum of their respective amplification curves (C*p*,
203 calculated in triplicate) by considering C*p* values to be proportional to the dual logarithm
204 of the inverse of the specific DNA concentration, according to the equation:
205 $[DNA_a]/[DNA_b] = 2^{Cpb-Cpa}$ [22]. Total bacteria was normalized as 16S rRNA gene copies
206 per mg of wet feces (copies/mg).
207

208 **2.8 Statistical analysis**

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210 All data manipulation, statistical analysis and figure construction were performed using
211 Graph Pad Prism 5 (Graph Pad Software, Inc., San Diego, CA, USA). The results of the
212 quantitative measurements are expressed as mean values with their standard errors
213 (SEM). Normal distributions and the heterogeneity of data were evaluated and their
214 statistical significance was determined by one- or two-way ANOVA, and Tukey's
215 multiple comparison test was used for mean comparisons. The results from qualitative
216 measurements (histology) are expressed in frequencies (percentage of animals that
217 present the variable, or do not) and their statistical significance was determined using
218 contingency tables and χ^2 statistics. Differences were considered significant when $P <$
219 0.05 and were considered to indicate a tendency when $0.05 < P < 0.1$.

220

3 Results

3.1 Feed intake, body weight, and lipid profile

Feed/energy intake and body weight were monitored throughout the study (Table 1; Supporting Information Fig. S1). Rats fed the two high-energy-dense diets (HF and HF+FG) consumed significantly less feed ($P < 0.05$) and more energy ($P < 0.05$) than those in the STD group (Table 1). Based on feed intake, the mean daily dose of D-fagomine was 2.9 mg/100 g body weight. As observed in previous studies, D-fagomine supplementation did not modify feed intake [16]. The energy excreted, proportional to the SDTA signal obtained by thermal analysis, was similar in both the STD and HF groups and significantly higher ($P < 0.05$) in animals fed HF and D-fagomine. This result may be explained by the inhibitory activity of D-fagomine on intestinal disaccharidases [15], which would result in the excretion of some undigested sucrose.

Body weight was similar in all the groups at the beginning (236.1 g, SEM 3.2). After 7 weeks, body weight in the HF group (374.3 g, SEM 10.0) was significantly higher ($P < 0.05$) than in the STD group (320.3 g, SEM 9.1); while the body weight increase in the HF+FG group only reached statistical difference ($P < 0.05$) with respect to the STD group five weeks later: after 12 weeks of diet (Supporting Information Fig. S1). At the end of the study, the HF group gained 29% more weight than those given the STD diet (537.9 g, SEM 15.1 *vs* 416.4 g, SEM 12.9 STD group) while animals supplemented with D-fagomine showed a tendency to gain less weight (20%: 499.9 g, SEM 15.7, $P = 0.06$ *vs* the HF group) (Table 1). The plasma lipid profile presented values within normal ranges with some differences between groups (Supporting Information Table S3).

3.2 Glycemic status

Fasting plasma glucose and insulin were measured at week 10, 16, and at the end of the study (Fig. 1). Fasting glucose levels in the HF group were higher ($P < 0.001$) than those in the STD group (Fig. 1a) already from week 10. D-Fagomine supplementation reduced this increase from week 16 to levels similar to those in the STD group ($P < 0.05$ *vs* the HF group at week 21; Fig. 1a). Fasting glucose levels were below 80 mg/dL in all the groups at all times. Fasting plasma insulin was higher in both groups fed the HF diet at weeks 10 and 16 ($P < 0.05$; Fig. 1b). At the end of the study (week 24), insulin levels in the HF group dropped significantly ($P < 0.05$) while the group supplemented with D-fagomine still presented significantly higher insulin concentrations ($P < 0.01$; Fig. 1b).

The OGTT was performed twice during the study, after 13 and 21 weeks (Fig. 2). In the first test, the levels of postprandial glucose in the HF group were significantly ($P < 0.001$) higher than those in the other two groups (STD and HF+FG) 30, 45 and 60 min after administration, with levels of ≥ 140 mg/dL (Fig. 2a). The area under the curve (AUC) corresponding to the HF group was significantly greater ($P < 0.001$) than that for the STD and HF+FG groups, which presented no significant differences. By the end of the study (week 21, Fig. 2b) plasma glucose concentrations in the group supplemented with D-fagomine were still lower than those in the HF group, but only significantly lower ($P < 0.05$) 30 min after glucose intake. The AUC for the HF+FG and STD groups were not significantly different.

3.3 Biomarkers and lipid mediators of inflammation in plasma

Plasma concentration of IL-6 after 10 and 16 weeks of intervention was higher in animals fed HF diets ($P < 0.05$) than in animals fed the STD diet (Table 2). D-Fagomine showed a tendency ($P = 0.07$) to reduce the levels of plasma IL-6 at week 16. The plasma leptin concentration was higher in both groups fed HF diets ($P < 0.001$).

The levels of ARA-derived pro-inflammatory eicosanoids as well as eicosanoids and docosanoids derived from EPA and DHA, respectively, were measured by LC-MS/MS in plasma samples collected at the end of the study (Table 2). The plasma concentration of PGE_2 significantly increased in the HF group ($P < 0.05$) compared to the STD group. The levels of pro-inflammatory PGE_2 and LTB_4 were similar in the STD and HF+FG groups. No differences were detected in the levels of other eicosanoids or docosanoids (Table 2).

3.4 Diacylglycerols in perigonadal adipose tissue, liver and muscle

IR-related intracellular signaling lipid mediator DAGs were measured in perigonadal AT, liver and muscle by LC-TOF-MS from the samples taken at the end of the study (Fig. 3). There was no increase in the amounts of total DAGs or in some of the relevant structures, namely DAG 34:1 (putatively 1-palmitoyl-2-oleoyl-*sn*-glycerol), DAG 36:2 (putatively 1-stearoyl-2-linoleoyl-*sn*-glycerol) and DAG 38:4 (putatively 1-stearoyl-2-arachidonoyl-*sn*-glycerol) in the HF group compared to the STD one. The levels of total DAGs in AT as well as of DAG 34:1 in AT and DAG 38:4 in AT and muscle were even significantly lower in the HF group. D-Fagomine supplementation did not induce any significant change in the levels of DAGs with respect to the HF group.

3.5 Liver and adipose tissue histology

Steatosis, lobular inflammation and microgranulomas were determined in liver by histology (Fig. 4). Neither high-fat diet induced steatosis to any significant extent. The inflammation and microgranulomas of animals fed HF were significantly higher ($P < 0.001$) than those of the STD and HF+FG groups (Fig. 4e, g). The livers sections obtained from animals in the HF group showed lobular inflammation with lymphoplasmacytic inflammatory infiltration around the blood vessels (e.g. Fig. 4b). In contrast, such infiltration was scarce and smaller in extent in sections from livers pertaining to the group supplemented with D-fagomine (e.g. Fig. 4c).

Adipocytes were larger in the AT of animals fed the HF diets than the STD group (Supporting Information Figure S2a, b, d); while no differences were detected between the HF and HF+FG groups (Supporting Information Figure S2b, c). AT inflammation was not detected in any of the groups (Supporting Information Figure S2g).

3.6 Subpopulations of gut microbiota

The relative proportions of several bacterial groups of the gut microbiota were evaluated at weeks 9 and 24 (Fig. 5). The Bacteroidetes:Firmicutes ratio (Fig. 5a) was significantly reduced ($P < 0.01$) in both high-fat diet groups and the presence of D-fagomine in the diet made no difference. The proportion of Enterobacteriales (Fig. 5b) significantly ($P < 0.05$) increased in the HF group with respect to the STD group. The increase observed in the group supplemented with D-fagomine was not significant. The relative populations of Bifidobacteriales decreased as the animals grew older ($P < 0.01$ *vs* STD week 9; Fig. 5c) and some differences were detected between the groups. The HF diet significantly reduced the population of Bifidobacteriales already at week 9 ($P < 0.05$) independently of supplementation while D-fagomine, which showed a tendency to counteract the age-

319 and diet-related losses of Bifidobacteriales that was only significant ($P < 0.05$) at the end
320 of the intervention (week 24).
321
322

4 Discussion

The present study examines the long-term functional effects of D-fagomine on the preservation of glucose/insulin homeostasis and explores possible mechanisms of action for them in a rat model of diet-induced prediabetes. The prediabetic state was induced in male Wistar Kyoto rats by feeding them a HF diet and the effects of D-fagomine were observed over a period of 24 weeks. In agreement with preceding short-term studies (5 and 9 weeks) in Sprague-Dawley rats [16, 23], D-fagomine partially counteracted the body weight gain induced by the HF diet in the long term (Supporting Information Fig. S1). Plasma insulin levels also increased in animals fed the HF diet after 10 weeks of intervention (Fig. 1). High plasma insulin levels define the first of the five stages of diabetes proposed by Weir and Bonner-Weir for both rats and humans [1]. This compensation stage is characterized by increased overall rates of insulin secretion, via a greater number or size of pancreatic β-cells, in response to loss of insulin sensitivity in tissues. D-Fagomine, which had shown a tendency to reduce insulin levels in the short term [16, 23], did not have an influence on insulin levels after 10 weeks of our intervention (Fig. 1b).

Later on, at the end of the study, the levels of fasting insulin in the HF group (without supplementation) dropped significantly (Fig. 1b), while fasting glucose levels were still moderately high (Fig. 1a). This situation is compatible with the second stage in the diabetes progression, which is characterized by a loss of β-cell mass and disruption of pancreatic function [1]. Animals supplemented with D-fagomine did not seem to reach this second prediabetic stage, as their insulin levels remained high (Fig. 1b) and their fasting glucose levels were similar to those of the STD group (Fig. 1a). The second prediabetic state is also compatible with the IGT recorded in rats fed the HF diet, which already

showed the classic plateau-like prediabetic curve after just 13 weeks. D-Fagomine counteracted this fat-induced IGT pattern as the supplemented rats removed glucose from their blood at a normal rate (Fig. 2a, b). By the end of the study (21 weeks), the AUC for the HF+FG and STD groups were still not significantly different. The evidence presented here, together with our previous observations of the short-term reduction in the early increase of fasting insulin concentration [16, 23], shows that rats supplemented with D-fagomine always seem to be one step behind in the development of diet-induced prediabetes.

We next considered through what mechanism or mechanisms D-fagomine exerts this functional metabolic effect. As D-fagomine reduced IGT more dramatically than it reduced body weight gain, we hypothesized that it may delay the development of diabetes in Wistar Kyoto rats by a mechanism that is not directly dependent on lipid accumulation. The results from our DAG analysis and histological study support this explanation. IR has been linked to ectopic fat through the action of DAGs, which are intermediates of lipid metabolism with the capacity to impair intracellular insulin signaling in both liver and muscle [2]. It has been proposed that DAGs interrupt the translocation of the glucose transporter GLUT4 to the plasma membrane by modifying the phosphorylation pattern of the intracellular insulin receptor substrate (IRS) after attaching to protein kinase C [24]. We evaluated total DAGs and the levels of selected molecular species in these two organs as well as in AT (Fig. 3). As there is no information to date as to what particular DAG species might impair insulin signaling, particular DAGs were chosen on the basis of their selective interaction with the cellular PKC–Ca_2^+ signaling network [25]. Systemic IR in our model does not seem to be triggered by DAG-mediated impairment of insulin signaling, as the levels of total and selected DAGs in AT, liver and muscle did not increase in either of the groups fed the high-fat diet (Figure 3); in fact DAG levels were

even lower in some instances. The liver histology supported the hypothesis that direct lipid-mediated loss of insulin sensitivity was probably not a triggering factor of the early prediabetic stage in our model, as significant steatosis was not detected (Fig. 4a, b, c). In contrast, strong lymphocyte infiltration indicated inflammation around the blood vessels (Fig. 4c) which was greatly attenuated in animals supplemented with D-fagomine (Fig. 4d). Lobular inflammation and numbers of microgranulomas were significantly higher in the HF group than in the STD group, while these levels in animals supplemented with D-fagomine were no different from those in the STD group (Fig. 4a). The histology did not detect any sign of inflammation in AT and D-fagomine did not have any observable effect of fat-induced adipocyte hypertrophy (Supporting Information Fig. S3). All these results suggest that D-fagomine has a functional effect on HF diet-induced low-grade systemic inflammation that is independent of lipid accumulation. This hypothesis is supported by our additional measurements of the systemic inflammatory marker IL-6 and its related eicosanoid PGE_2, a strong proinflammatory secondary metabolite from oxidation of ARA catalyzed by cyclooxygenase-2 (COX-2) [26] which induces the production of IL-6 via macrophages [27]. The levels of IL-6 and PGE_2 were significantly higher in the HF group than in the STD group and were significantly reduced in the HF+FG group (Table 2). Also, the levels of LTB_4, another pro-inflammatory ARA-derived metabolite, were significantly lower in the supplemented group compared to the HF group (Table 2). The fact that no differences were detected in the levels of putatively anti-inflammatory EPA- and DHA-derived eicosanoids and docosanoids (Table 2) suggests that D-fagomine exerts its functional effect when inflammation first occurs and not by activating anti-inflammatory pathways.

We next turned to how D-fagomine counteracts fat-induced low-grade inflammation. Gut microbiota may be the answer, or at least part of the answer. Gut dysbiosis is known to

induce endotoxemia and low-grade inflammation in the host [3] through disruption of the intestinal barrier properties and release of pro-inflammatory molecules, such as LPS, into the bloodstream [28]. [26]Chronic subclinical inflammation has been associated with insulin insensitivity [29] and suggested as a link between gut dysbiosis and early IR [7, 11, 28]. We have already suggested that the effect of D-fagomine on body weight gain and glycemic status may be related to a reduction in the overgrowth of gut Enterobacteriales induced by a high-energy-dense diet in the short term (up to 5 weeks) [23]. The results presented here show that this explanation may hold in the long term, as the population of Enterobacteriales experiences experienced no significant changes throughout this study (Fig. 5b). Moreover, D-fagomine also showed a tendency to counteract the reduction in Bifidobacterium induced by the HF diet and age (HF and STD groups at week 24, Fig. 5c). The action of D-fagomine on Bifidobacterium may be connected to its capacity to eliminate Enterobacteriales, as the populations of these subgroups appear to be inversely related [30]. The hypothesis that D-fagomine exerts its anti-inflammatory and anti-diabetic action by balancing the populations of enterobacteria and bifidobacteria is backed by previous observations by others that link enterobacteria to endotoxemia [6] and bifidobacteria to a reduced impact of fat on diet-induced diabetes [11]. Bifidobacteria have also been inversely associated with obesity and age [31].

We also evaluated variations in the populations of Bacteroidetes and Firmicutes: the main two bacterial phyla in the intestinal tract (Fig. 5a). A reduction in the Bacteroidetes:Firmicutes ratio has been related to a shift from lean to fat phenotypes in both rats and humans [9, 32]. Low levels of fasting-induced adipose factor (Fiaf) and phosphorylated AMP-activated protein kinase (AMPK) may be responsible for the lipid accumulation effect associated with changes in gut microbiota [33]. We show here that a HF diet can reduce the Bacteroidetes:Firmicutes ratio concomitantly with a significant

gain in body weight (Fig. 5a, Supporting Information Fig. S1) while D-fagomine does not appear to modify this change (Fig. 5a). This observation confirms that the moderate effect of D-fagomine on weight gain might be associated with the contribution from minor components of gut microbiota (e.g. Enterobacteriales) rather than with changes in the main phyla.

The observation that the effects of D-fagomine on fat-induced changes in Enterobacteriales and Bifidobacteriales were moderate compared to the more dramatic effects on glucose tolerance and inflammation suggests that other putatively proinflammatory microorganisms may be involved. A more thorough examination of the composition of gut microbiota, the gut barrier function and the role of other mediators (e.g. biliary acids) in animals fed HF diets supplemented, or not, with D-fagomine would be the next step to take along this line of enquiry. Thus, D-fagomine may help to shed more light on the complex relationships between gut microbiota and metabolic alterations.

In summary, a very early effect of D-fagomine against fat-induced systemic low-grade inflammation would explain why animals fed D-fagomine are always one step behind in the progression of prediabetes: first against the loss of insulin sensitivity, then against loss of β-cell mass and disruption of pancreatic function. This effect may be attributed, at least in part, to a tendency to counteract the changes induced by a high-fat diet in the populations of gut bacterial subgroups such as Enterobacteriales and Bifidobacteriales.

Author contributions: S.R.-R., I.M., M.R. and J.L.T. conceived and designed the research; S.R.-R. and M.H. supervised and performed the animal intervention, the biometric determinations, the evaluation of glycemic status and the qRT-PCR

experiments; L.A. performed the histology; S.R.-R. and J.C. performed the DAG determinations; G.D. and I.M. determined the lipid mediators of inflammation; S.R.-R. and M.H. analyzed the data; and S.R.-R. and J.L.T. wrote the paper.

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Table 1.- Mean feed and energy intakes, energy excreted in feces and final body weight of WKY rats fed the experimental diets for 24 weeks

	STD		HF		HF+FG	
	Mean	SEM	Mean	SEM	Mean	SEM
Feed intake (g/day/100 g body weight)	4.8	0.7	3.0*	0.7	2.9*	0.5
Energy intake[a] (kcal/day/100 g body weight)	14.3	0.2	17.5*	0.2	19.0*	0.2
Energy in feces (ks ºC/g)[b]	327.0	17.6	381.6	29.5	426.9*	18.4
Body weight at week 24 (g)	416.4	12.9	537.9***	15.1	499.9**†	15.7

* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ *vs* STD group, † $P = 0.06$ *vs* HF group.
[a] estimated as metabolizable energy based on the Atwater factors: 4 kcal/g protein, 9 kcal/g fat, and 4 kcal/g available carbohydrate.
[b] integrated SDTA signal proportional to energy.

Table 2.- Plasma biomarkers and lipid mediators of inflammation

		STD		HF		HF+FG	
		Mean	SEM	Mean	SEM	Mean	SEM
IL-6 (pg/mL)	wk 10	47.3	19.6	193.9*	26.6	176.2	59.3
	wk 16	44.4	24.4	215.4**	47.4	149.6*†	20.8
Leptin (pg/mL)	wk 24	2444.8	303.7	8511.0**	1389.8	7984.3**	1490.7
Eicosanoids from ARA (ppb)							
PGE_2	wk 24	14.4	1.7	23.3*	2.1	18.4	3.4
LTB_4	wk 24	3.5	0.7	4.4	0.7	2.7^{δ}	0.3
11HETE	wk 24	8.2	1.1	10.2	1.4	7.8	1.1
Eicosanoids from EPA (ppb)							
12HpEPE	wk 24	14606.6	8327.7	3845.3	469.2	5034.2	956.1
12HEPE	wk 24	38.5	1.9	41.8	4.0	39.8	2.2
5HEPE	wk 24	4.4	0.1	4.5	0.1	4.3	0.0
Docosanoids from DHA (ppb)							
17HDoHE	wk 24	16.8	2.7	17.2	1.3	14.2	1.3
11HDoHE	wk 24	14.8	0.0	14.9	0.0	14.8	0.0
4HDoHE	wk 24	11.5	0.6	12.6	0.7	10.7	0.5

* $P < 0.05$, ** $P < 0.01$ *vs* STD group, $^{\delta}$ $P < 0.05$ *vs* HF group, † $P = 0.07$ *vs* HF group

Figure 1 Plasma levels of fasting glucose (a) and insulin (b) in WKY rats fed standard (STD), high-fat (HF), and high-fat supplemented with D-fagomine (HF+FG) diets at weeks 10, 16 and 21 and 24. Concentrations are represented as means with their standard errors. Comparisons were performed 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 group.

Figure 2 Time-course and area under curve (AUC) of plasma glucose concentration after administration of a single dose of glucose (1 g/kg body weight) to WKY rats fed a standard (STD), high-fat (HF), or high-fat supplemented with D-fagomine (HF+FG) diet at week 13 (a) and 21 (b). Values are means with their standard errors. Comparisons were performed using one-way ANOVA and Tukey's tests or two-way ANOVA. ** $P < 0.01$ 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 group.

Figure 3 Levels of DAG 34:1 (a, e, i), 36:2 (b, f, j), 38:4 (c, g, k) and total DAGs (d, h, l) in WKY rats fed a standard (STD), high-fat (HF), or high-fat supplemented with D-fagomine (HF+FG) diet for 24 weeks. Values are means with their standard errors. Comparisons were performed using one-way ANOVA and Tukey's tests. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ *vs* STD group.

Figure 4 Liver histological cuts (20X) stained with hematoxylin-eosin from WKY rats fed a standard (STD) (a), high-fat (HF) (b), or high-fat supplemented with D-fagomine (HF+FG) diet (c) for 24-week histology summary (d). The STD cut (a) shows normal liver anatomy. The HF cut (b) shows lobular inflammation with lymphoplasmacytic inflammatory infiltration (arrows) around blood vessels (red). The HF+FG cut (c) shows slight inflammatory infiltration around a centrilobular vein (red). Values are in frequencies (percentage of animals that present the variable, or do not). Comparisons

were performed using χ^2 statistics. *** $P < 0.001$ *vs* STD group; $^{\delta\delta\delta}$ $P < 0.001$ *vs* HF+FG group.

Figure 5 Excreted intestinal bacteria measured by qRT-PCR and expressed as percentages of total bacteria in fecal samples from WKY rats fed a standard (STD), high-fat (HF), or high-fat supplemented with D-fagomine (HF+FG) diet, after 9 and 24 weeks of nutritional intervention. Values are means with their standard errors. Comparisons were performed using one-way ANOVA and Tukey's tests or two-way ANOVA. * $P < 0.05$ *vs* STD group; $^{\$\$}$ $P < 0.01$ *vs* STD group from week 9.

Figure 1

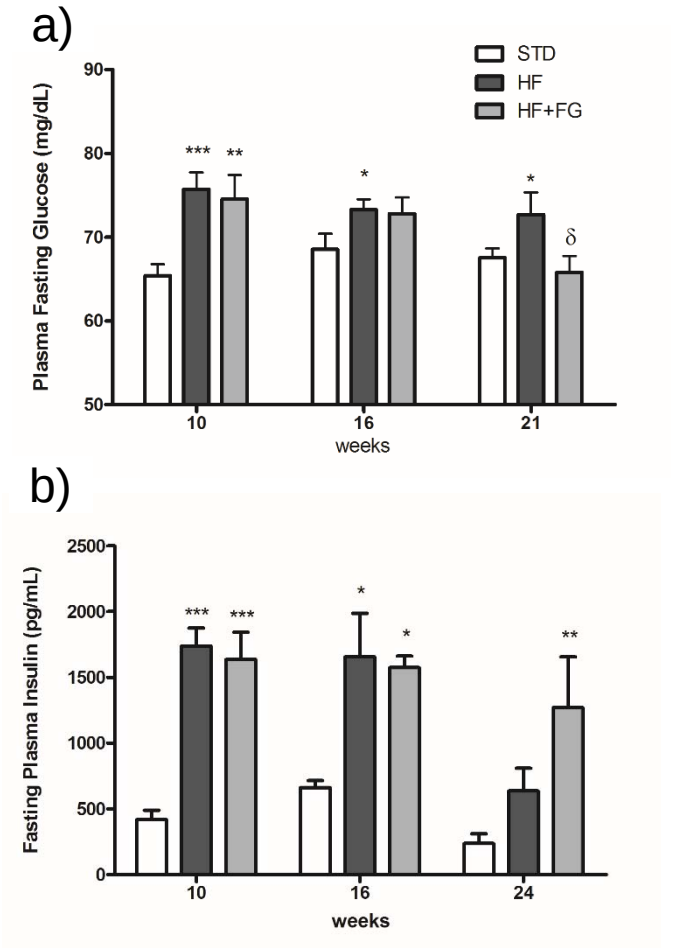
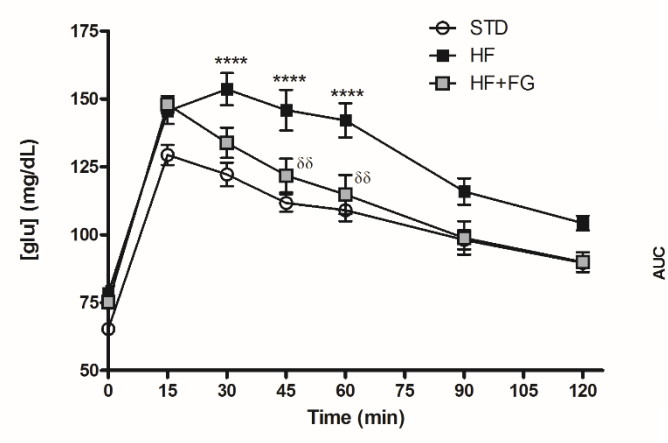
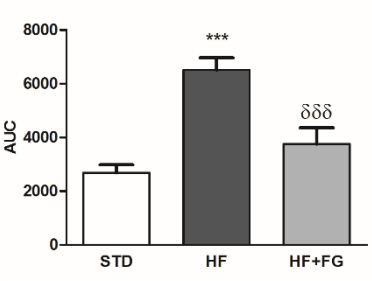

a)
STD
HF
HF+FG
Plasma Fasting Glucose (mg/dL)
90
80
70
60
50
***
**
*
*
δ
10
16
21
weeks
b)
Fasting Plasma Insulin (pg/mL)
2500
2000
1500
1000
500
0
***
***
*
*
**
10
16
24
weeks

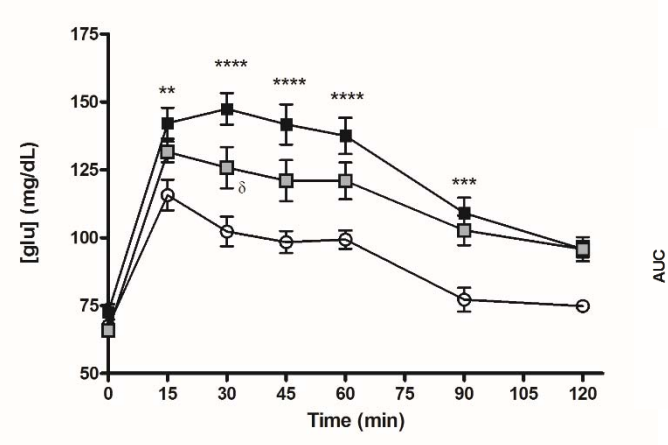
Figure 2

a) week 13





b) week 21



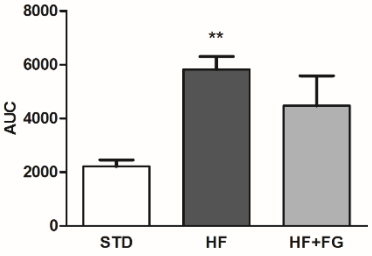


Figure 3

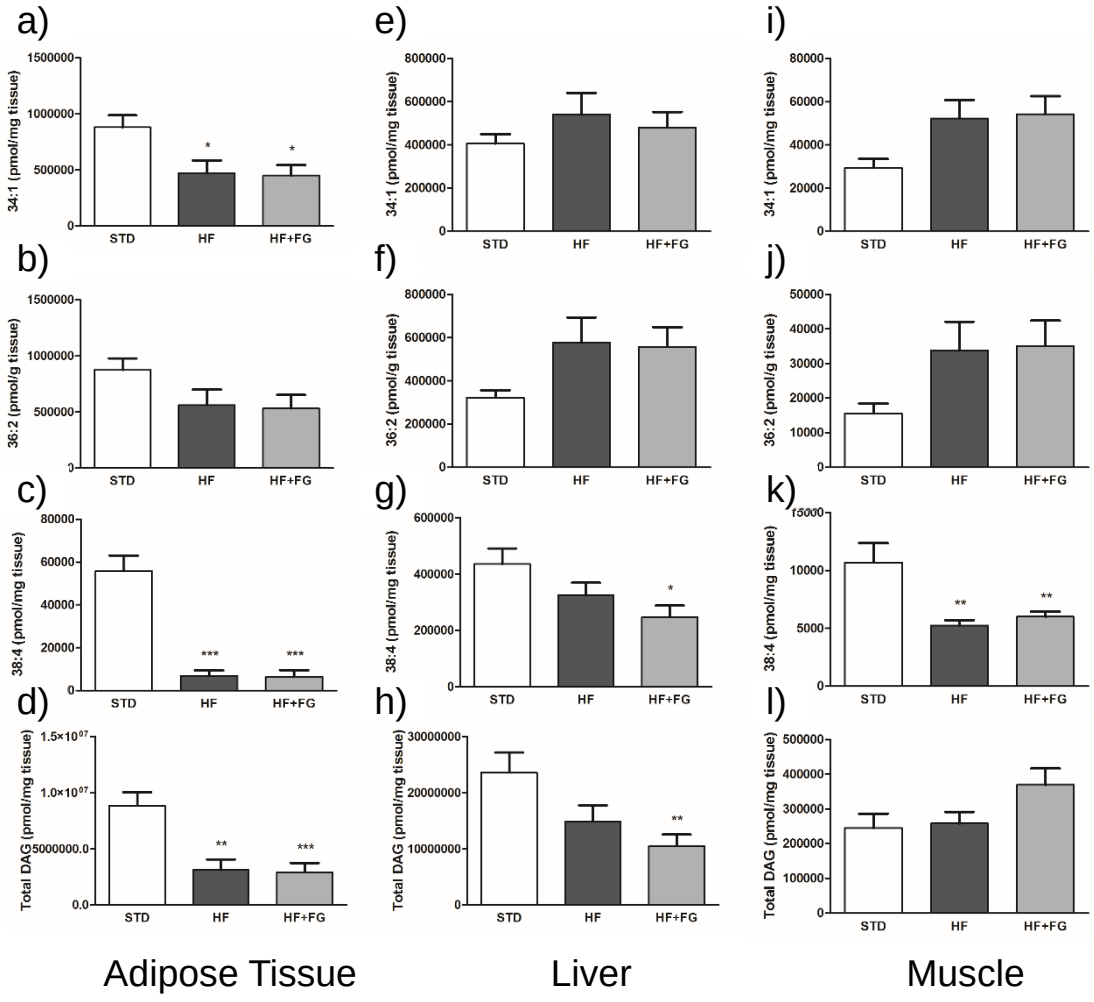
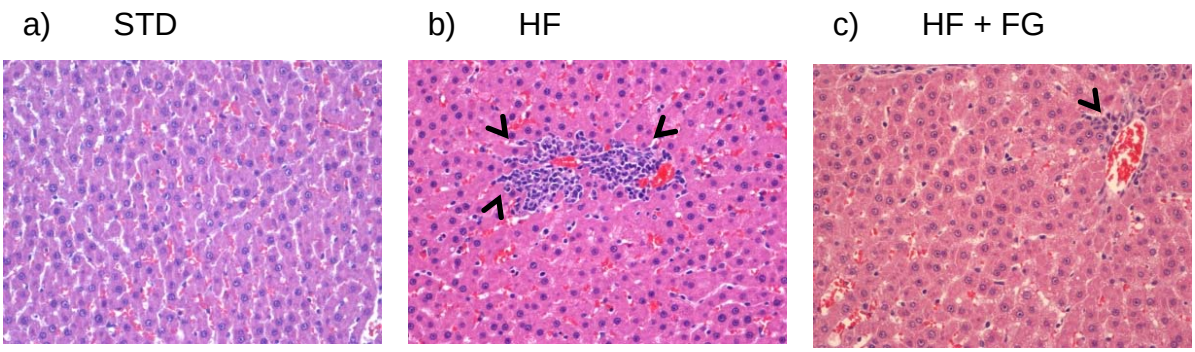

a)
e)
i)
b)
f)
j)
c)
g)
k)
d)
h)
l)
34:1 (pmol/mg tissue)
36:2 (pmol/g tissue)
38:4 (pmol/mg tissue)
Total DAG (pmol/mg tissue)
STD
HF
HF+FG
Adipose Tissue
Liver
Muscle

Figure 4


a) STD
b) HF
c) HF + FG

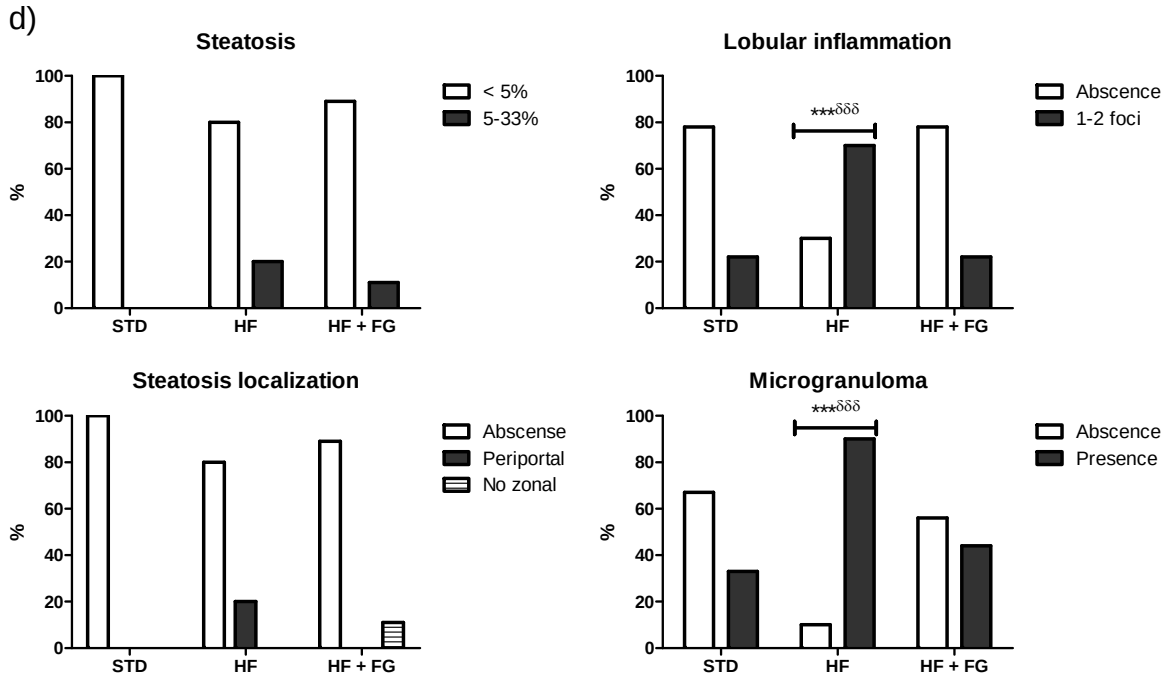
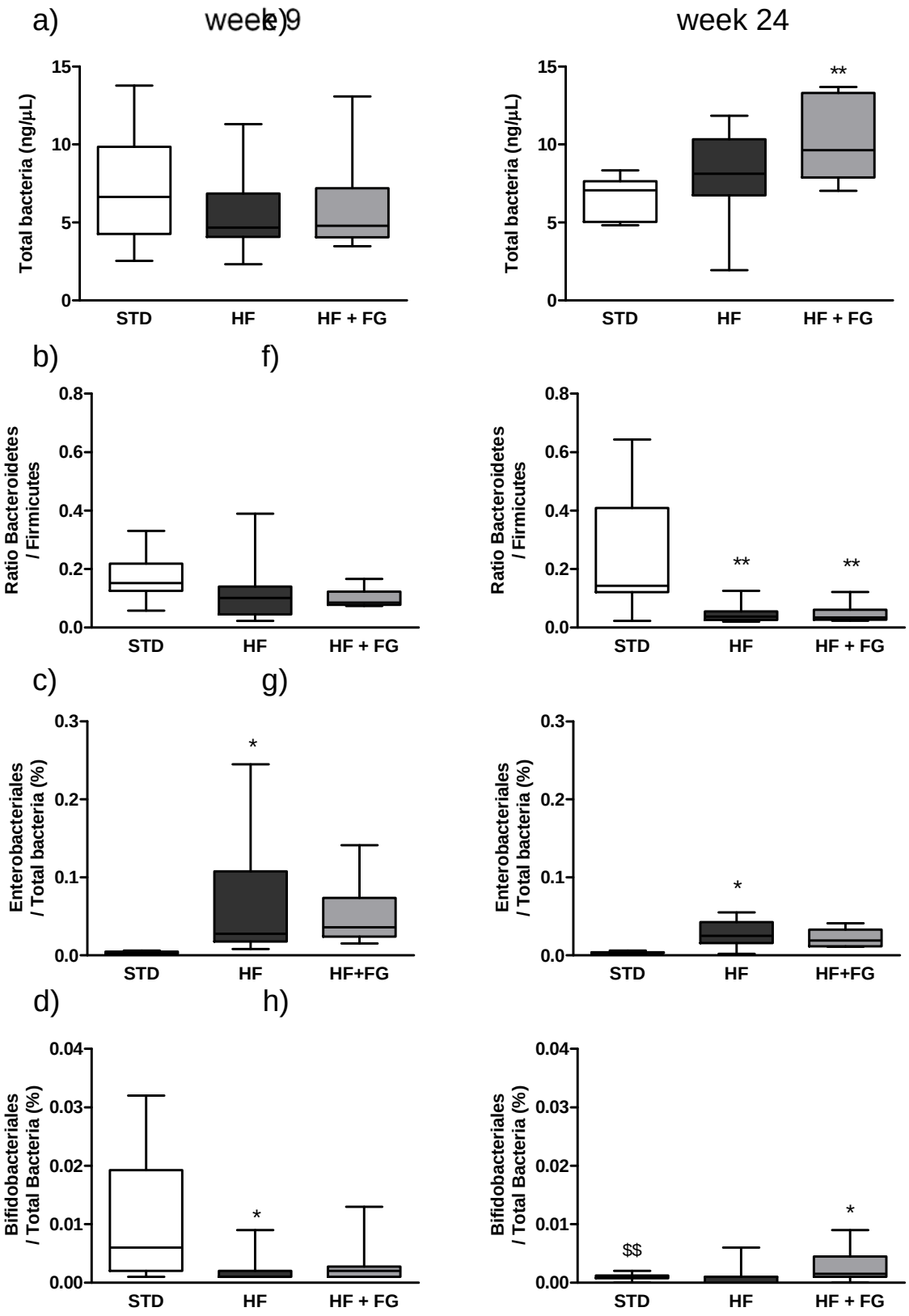

d)
Steatosis
100
80
60
40
20
0
%
STD
HF
HF + FG
< 5%
5-33%
Lobular inflammation
***δδδ
Abscence
1-2 foci
Steatosis localization
Abscense
Periportal
No zonal
Microgranuloma
***δδδ
Abscence
Presence

Figure 5


a)
week 9
week 24
Total bacteria (ng/µL)
15
10
5
0
STD
HF
HF + FG
**
b)
f)
Ratio Bacteroidetes / Firmicutes
0.8
0.6
0.4
0.2
0.0
**
c)
g)
Enterobacteriales / Total bacteria (%)
0.3
0.2
0.1
0.0
*
HF+FG
d)
h)
Bifidobacteriales / Total Bacteria (%)
0.04
0.03
0.02
0.01
0.00
$$

Supporting information

Table S1.- LC-MS/MS experimental details

Compound	Retention Time (min)	MS/MS parameters	
		Collision energy (eV)	Quantification transition (*m/z*)
PGE_2	9.09	20	351→315
LTB_4	13.79	27	335→195
11HETE	22.09	30	319→167
12HpEPE	17.80	25	333→315
12HEPE	18.72	27	317→179
5HEPE	20.47	25	317→255
17HDoHE	21.94	27	343→245
11HDoHE	23.20	27	343→149
4HDoHE	23.64	27	343→281

The identification of the lipid mediators was done with the help of the full ion product spectra recorded in the range from 90 to 400 *m/z* units. To corroborate the identification and to quantify the analytes, the most intense and selective MS/MS transitions, obtained after direct infusion of individual standard solutions (5 µg/mL, 20 µL/min), were chosen. The linear dynamic range was determined by individual standards for each identified compound.

Table S2.- qRT-PCR primers and conditions

Target bacteria	Positive control	Annealing temperature (ºC)	Sequence (5'-3')	Reference
Total Bacteria	[a]	65	F: ACT CCT ACG GGA GGC AGC AGT	[1]
			R: ATT ACC GCG GCT GCT GGC	
Bacteroidetes	*Bacteroides fragilis*	62	F: ACG CTA GCT ACA GGC TTA A	[2]
			R: ACG CTA CTT GGC TGG TTC A	
Firmicutes	*Lactobacillus brevis*	52	F: AGA GTT TGA TCC TGG CTC	[3]
			R: ATT ACC GCG GCT GCT GG	[4]
Enterobacteriales	*Escherichia coli* M15	60	F: ATG GCT GTC GTC AGC TCG T	[1]
			R: CCT ACT TCT TTT GCA ACC CAC T	
Bifidobacteriales	*Bifidobacterium longum*	55	F: CTC CTG GAA ACG GGT GG	[5]
			R: GGT GTT CTT CCC GAT ATC TAC A	

[a] Positive control of total bacteria was the strain with which the result was rated.

Table S3 Plasma lipid profile (mg/dL) in rats supplemented fed HF diet and supplemented with D-fagomine for 24 weeks.

	STD		HF		HF+FG	
	Mean	SEM	Mean	SEM	Mean	SEM
Cholesterol	135.2	3.7	139.4	5.1	150.4*	3.4
HDL-cholesterol	48.7	1.1	47.3	1.3	50.3	1.2
LDL-cholesterol	23.2	1.6	24.1	1.3	29.4*$^{\delta}$	1.1
Triglycerides	63.1	5.6	92.4**	7.1	116.4*	16.4

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

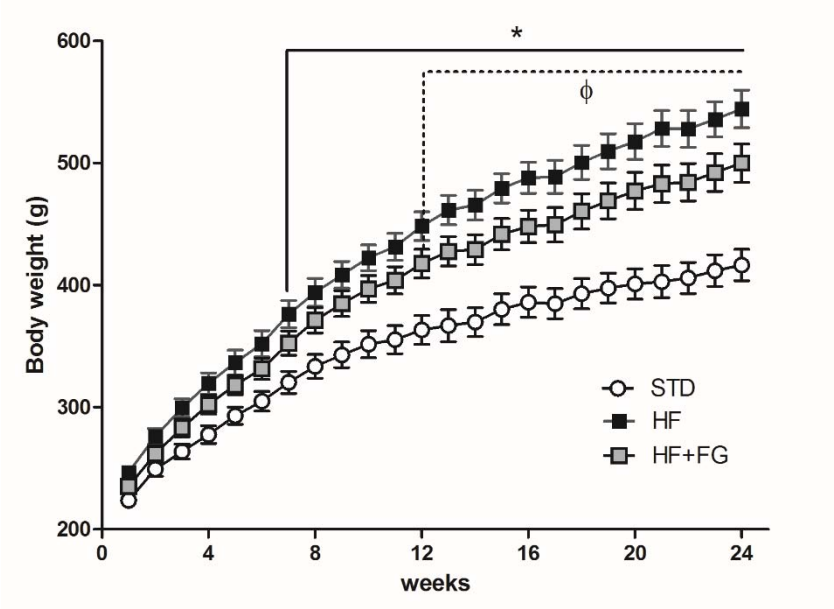


Figure S1.- Body weight in rats fed a standard (STD, ○), high-fat (HF, ■), or high-fat supplemented with D-fagomine (HF+FG, □) diet for 24 weeks. Data are presented as means with their standard errors. Comparisons were performed using the two-way ANOVA test. *$P < 0.05$ HF *vs* STD group, φ $P < 0.05$ HF+FG *vs* STD group.

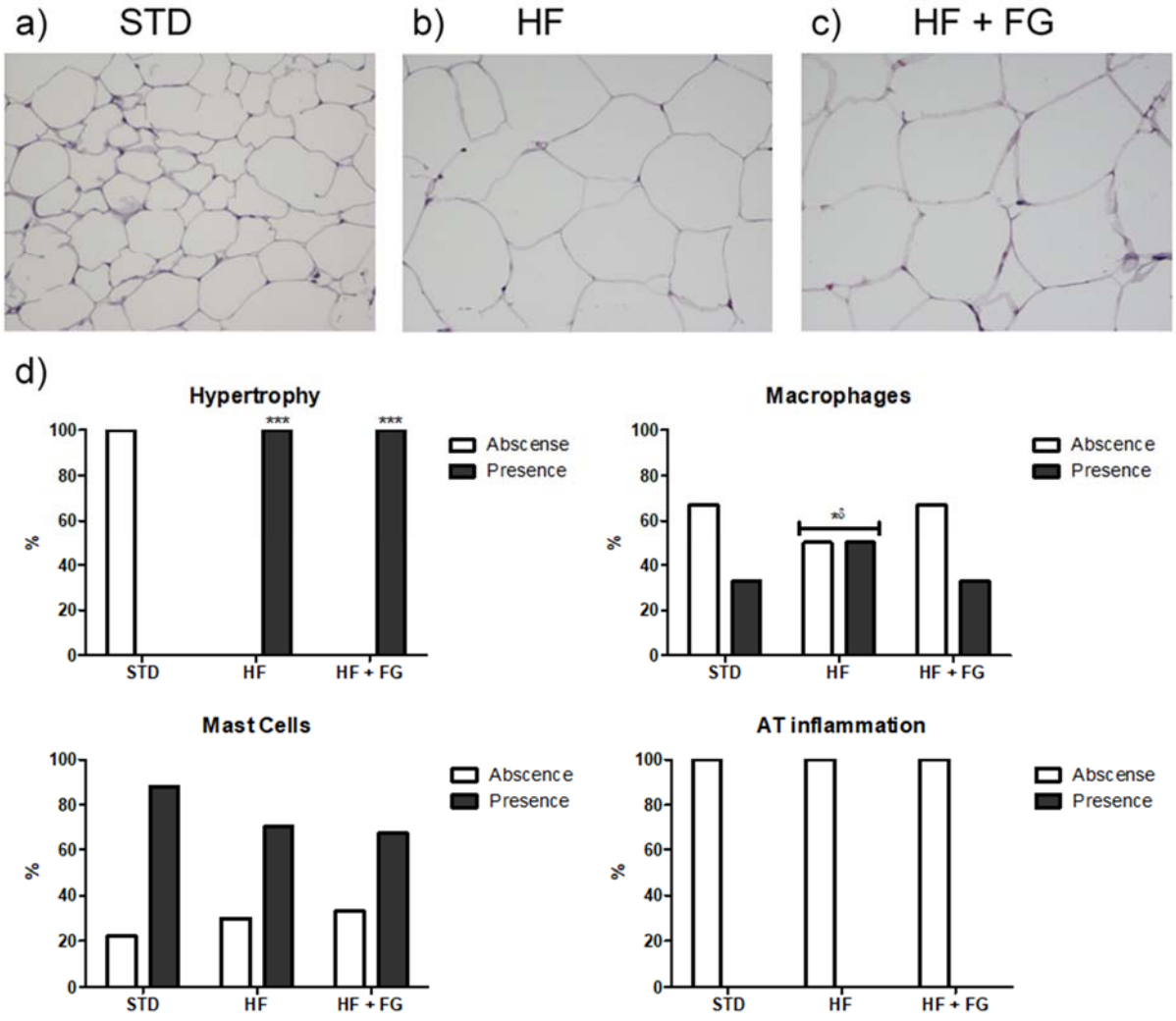


Figure S2.- Adipose tissue histological cuts (20X) stained with hematoxylin-eosin from WKY rats fed a standard (STD) (a), high-fat (HF) (b), or high-fat supplemented with D-fagomine (HF+FG) diet (c) for 24 weeks and histology summary (d). Values are in frequencies (percentage of animals that present or not the variable). Comparisons were performed using χ^2 statistics. * $P < 0.05$ and *** $P < 0.001$ *vs* STD group; $^{\delta}$ $P < 0.05$ *vs* HF+FG group.**Supporting References**

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