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RESEARCH ARTICLE

Fat and not sugar as the determining factor for gut microbiota changes, obesity, and related metabolic disorders in mice

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Abstract

Diet-induced obesity contributes to the development of type 2 diabetes, insulin resistance, metabolic inflammation, oxidative and endoplasmic reticulum (ER) stress. Overall, obesity is associated with deviations in the composition and functionality of the gut microbiota. There are many divergent findings regarding the link between the excessive intake of certain dietary components (i.e., fat and sugar) and obesity development. We therefore investigated the effect of specific diets, with a different content of sugar and fat, in promoting obesity and related comorbidities as well as their impact on microbial load and gut microbiota composition/diversity. C57BL/6J mice were fed either a low-sugar, low-fat control diet (CT), a high-sugar diet (HS), a high-fat, high-sugar diet (HF/HS), or a high-fat diet (HF) for 8 wk. The impact of the different diets on obesity, glucose metabolism, inflammation, and oxidative and ER stress was determined. Diet-induced changes in the gut microbiota composition and density were also analyzed. HF diet-fed mice showed the highest body weight and fat mass gains and displayed the most impaired glucose and insulin profiles. HS, HF/HS, and HF diets differently affected hepatic cholesterol content and mRNA expression of several markers associated with immune cells, inflammation, oxidative and ER stress in several organs/tissues. In addition, HF diet feeding resulted in a decreased microbial load at the end of the experiment. When analyzing the gut microbiota composition, we found that HS, HF/HS, and HF diets induced specific changes in the abundance of certain bacterial taxa. This was not associated with a specific change in systemic inflammatory markers, but HS mice exhibited higher FGF21 plasma levels compared with HF diet-fed mice. Taken together, our results highlight that dietary intake of different macronutrients distinctively impacts the development of an obese/diabetic state and the regulation of metabolic inflammation in specific organs. We propose that these differences are not only obesity-driven but that changes in the gut microbiota composition may play a key role in this context.

NEW & NOTEWORTHY To our knowledge, this study is the first to demonstrate that dietary macronutrients (i.e., sugar and fat) have an impact on fecal bacterial cell counting and quantitative microbiome profiling in mice. Yet, we demonstrate that dietary fat is the determining factor to promote obesity and diabetes progression, and local inflammation in different body sites. These observations can help to disentangle the conundrum of the detrimental effects of fat and sugar in our dietary habits.

dietary fat; dietary sugar; microbial load; microbiota; obesity

INTRODUCTION

The worldwide increase in obesity is a major socioeconomic burden as it is strongly associated with several metabolic comorbidities, including insulin resistance and type 2 diabetes (T2D), among others (1, 2). Promoted by an impaired energy homeostasis and a combination of behavior, genetic, environmental, and physiological factors (3), obesity is primarily characterized by fat mass expansion, accompanied by the development of related metabolic disorders (4). Peripheral organs such as the liver, adipose tissue, and skeletal muscle have critical roles in many physiological processes (i.e., energy, glucose, and lipid metabolism). Along with the peripheral tissues, the central nervous system, and the hypothalamus, in particular, has emerged as a master regulator of whole body energy

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homeostasis (5). However, in obesity, the metabolic functions of those peripheral and central organs are drastically impaired. Indeed, it is well established that obesity is associated with a state of chronic, low-grade inflammation distinguished by the production of several inflammatory cytokines and adipokines (6, 7). Besides inflammation, oxidative and endoplasmic reticulum (ER) stress can also occur in several organs and are recognized as common molecular pathways in the pathogenesis of obesity and diabetes (8, 9). Differences in the levels of gut and liver hormones are also critical in obesity, as they would participate to divergent effects in terms of satiety, insulin sensitivity, and lipid metabolism (10, 11). Over the past decade, accumulating evidence has led the gut microbiota to be considered as one of the key elements contributing to the regulation of host health since it contributes to numerous physiological effects ranging from the modulation of energy homeostasis, glucose/lipid metabolism, and inflammatory processes (12). Many studies in animals and humans have highlighted the importance of diet and dietary components such as the three principal classes of macronutrients (i.e., carbohydrates, fats, and proteins) in influencing the gut microbiota composition and its function (13). A recent study in mice demonstrated that among the different macronutrients, only dietary fat, rather than protein or sugars (i.e., sucrose) increased adiposity (14). In addition, dietary fat was the only macronutrient to affect some hypothalamic hunger pathways (14). However, more in-depth analyses are necessary to better understand the link between dietary intake, the gut microbiome, and host metabolism. In this study, we aimed at investigating how certain macronutrients (i.e., fat and sugar) could differently affect adiposity, glucose metabolism, insulin sensitivity, gut and liver hormones, inflammatory processes, oxidative and ER stress, and gut microbiota composition.

METHODS

Mice and Experimental Design

Fifty male C57BL/6J mice were purchased at the same time and from the same supplier (Janvier Laboratories, Le Genest-Saint-Isle, France) at the age of 7 wk. Mice were housed in a specific pathogen- and opportunistic-free (SOPF) controlled environment (room temperature of 22 ± 2°C, humidity: 55 ± 10%, 12-h daylight cycle, lights off at 6:00 PM) in groups of two mice per cage, with free access to sterile food and sterile water. After an acclimatization period of 1 wk, they were fed a control diet (D12450J; Research Diet; New Brunswick, NJ) and filtered autoclaved water was provided ad libitum. Mice were randomly assigned to one of the four dietary conditions having a different percent of calories in fat and sugars (i.e., sucrose): *1*) CT group (n = 20), fed a low-sugar and a low-fat diet containing 10% calories from fat and 7% calories from sucrose (D12450J; Research Diet; New Brunswick, NJ); *2*) HS group (n = 10), fed a high-sucrose diet containing 10% calories from fat and 17% calories from sucrose (D12450H; Research Diet; New Brunswick, NJ); *3*) HF/HS group (n = 10), fed a high-fat and high-sucrose diet containing 45% calories from fat and 17% calories from sucrose (D12451; Research Diet; New Brunswick, NJ); or *4*) HF group (n = 10), fed a high-fat diet containing 60% calories from fat and 7% calories coming from sucrose (D12492; Research Diet; New Brunswick, NJ). The fiber content (i.e., cellulose) was not diverging between diets, and their chemical composition has been detailed in Supplemental Table S1 (see, https://doi.org/10.6084/m9.figshare.21608445.v1). All mouse experiments were approved by and performed in accordance with the guideline of the local ethics committee (Ethics committee of the Université catholique de Louvain for Animal Experiments specifically approved this study that received the Agreement No, 2017/UCL/MD/005). Housing conditions complied with the rules specified by the Belgian Law of May 29, 2013, regarding the protection of laboratory animals (Agreement No, LA1230314).

Measurements during the Study

Body weight, food, and water intake were recorded three times per week. Cumulative food and water intake were calculated as the sum of daily food/water intake over the entire experiment. Body composition was assessed weekly as previously described (15). Fat and lean mass, and tissue weight were normalized to body weight using the formula: fat mass/body weight × 100%; lean mass/body weight × 100%; tissue weight/body weight × 100%.

Oral Glucose Tolerance Test and Insulin Resistance Index

In the 7th week of the experiment, mice were fasted for 6 h and given an oral glucose load (2 g glucose/kg body wt). Plasma insulin concentration and insulin resistance index were determined as previously described (15).

Collection of Fecal Material

For microbial composition analysis, freshly defecated feces were collected after the acclimatization period (*day 0*), and after 1 wk (*day 8*), 4 wk (*day 29*), and 8 wk (*day 56*), and kept on dry ice before storage at −80°C. Briefly, mice were placed in a new clean cage and separated individually. Fresh defecated fecal pellets were collected using clean and sterile forceps in individually labeled sterile tubes, immediately placed on dry ice, and then stored at −80°C until processing. After fecal pellet collection, mice were returned to their respective cage.

Tissue Sampling

At the end of the experimental period and after 6 h of fasting, mice were anesthetized with isoflurane (Forene, Abbott, Queenborough, Kent, UK). Portal vein and vena cava blood were collected, aliquoted, and stored for further analysis as previously described (15). Liver, brown and white adipose tissues (subcutaneous, epididymal, and visceral), muscles (soleus, gastrocnemius; tibialis, and vastus lateralis), and the hypothalamus were precisely dissected, weighed, and immediately snap-frozen in liquid nitrogen and stored at −80°C for further analysis.

Histological Analysis and Immunohistochemistry

A portion of the subcutaneous adipose tissue (SAT) was fixed in 4% paraformaldehyde solution for 24 h at room

temperature. Samples were then immersed in ethanol 100% for 24 h before processing for paraffin embedding and preparation of 5 μm tissue sections and processed as previously described (15). A minimum of five high-magnification fields were analyzed per mouse. Adipocyte size was estimated using a script for Fiji (advanced distribution of ImageJ) that is similar to Adiposoft (16). In brief, first, the red channel of the input image is processed by thresholding. Then, a watershed algorithm is used to segment the adipose cells. The script assumes white adipocytes are unilocular and have a circularly of at least 0.2. Adipocytes on the edges are excluded.

RNA Preparation and Real-Time qPCR Analysis

Total RNA was extracted from collected tissues as previously described (15). cDNA was prepared by reverse transcription of 1 μg total RNA using the Goscript RT Mix OligoDT kit (Promega, Leiden, The Netherlands), and qPCR was performed as previously described (15). The primer sequences for the targeted mouse genes are presented in Supplemental Table S2 (see https://doi.org/10.6084/m9.figshare.21608463.v1).

Biochemical Analyses

After extraction with chloroform-methanol according to a modified Folch method (17, 18), hepatic triglyceride and cholesterol concentrations were measured as previously described (15). Lipid degradation products ensuing from oxidative stress in the liver were evaluated by measuring the concentration of thiobarbituric acid reactive substances (TBARS) as previously described (19). All analysis and samples were run in duplicate. Inflammatory markers and gut hormones were measured in the portal vein blood using ELISA kits (Meso Scale Discovery).

Microbial Load Measurement

Microbial load measurement by flow cytometry was determined in the fecal samples as previously described (15).

Fecal Microbiota Sequencing

Fecal DNA extraction and microbiota profiling by 16S rRNA gene sequencing were performed as previously described (20).

Deriving Quantitative Microbiota Profiles

The quantitative microbiome profiling (QMP) matrix was built as previously described (21) by combining sequencing data and microbial load assessment by flow cytometry. From the 189 fecal sequencing profiles, two were excluded due to insufficient sampling depth. A total of 187 quantitative microbiota profiles were obtained at a sampling depth of 1.44×10^{-7}.

Statistical Analysis

The data are presented as either the means ± SE or as box and Whisker plot (median, minimum-maximum). The statistical significance of difference for the metabolic parameters was evaluated by one-way or two-way ANOVA followed by Tukey's post hoc multiple-comparison test, whereas for the microbial load and the bacterial genera abundances, nonparametric equivalents: Kruskal–Wallis test with Dunn's multiple-comparison test, were used. For the metabolic parameters, only statistically significant differences among HS, HF/HS, and HF groups were reported. The data with a superscript symbol ($^{\$}$HS vs. HF/HS, *HS vs. HF, $^{\#}$HF/HS vs. HF) are significantly different ($^{\$,*,\#}P < 0.05$; $^{\$\$,**,\#\#}P < 0.01$; $^{\$\$\$,***,\#\#\#}P < 0.001$; $^{\$\$\$\$,****,\#\#\#\#}P < 0.0001$). All the analyses were performed using GraphPad Prism version 9.1.2 for Windows (GraphPad Software). The presence of outliers was assessed using the Grubbs test.

Partitioning of Microbiota Variation according to Genotype and Sampling Day

Alpha-diversity index (Observed species, Shannon, and Evenness), and β-diversity indexes (Bray Curtis) were calculated as previously described (15).

Taxa-Metabolic Parameters Associations

Correlations between single taxa quantitative abundances (min 25% prevalence) and significant physiological parameters affected by dietary composition (i.e., sugar, and fat) were assessed by nonparametric Spearman correlation. The strict P value adjustment corresponds to correction over n taxa per n parameters. All statistical tests were subjected to multiple testing corrections (Benjamini–Hochberg method) whenever applicable.

RESULTS

Dietary Fat Promotes Obesity and Impairs Glucose Metabolism

After 8 wk of treatment, HF-fed mice gained the most body weight and fat mass compared with HF/HS-, HS-, and CT-fed mice (Fig. 1, *A* and *B*). According to the highest degree of obesity, lean mass and muscles weight ratio to body weight was decreased in HF-fed mice compared with HF/HS, HS, and CT-fed mice (Fig. 1*C*, Supplemental Fig. S1*A*; see https://doi.org/10.6084/m9.figshare.21608562.v1). Consistently, we found significantly larger adipose tissue depots (SAT: subcutaneous; EAT: epididymal; VAT: visceral) and adipocytes in HF-fed mice (Fig. 1, *D* and *E*). In addition, HF-fed mice exhibited more pronounced impaired glucose tolerance compared with HF/HS-, HS-, and CT-fed mice (Fig. 1, *F* and *G*). This was associated with an increase in plasma insulin levels and glucose-induced insulin secretion in HF-fed mice compared with HS-fed mice (Fig. 1*H*). Both HF/HS- and HF-fed mice developed insulin resistance when compared with HS-fed mice (Fig. 1*I*). All these changes occurred without differences in cumulative food or water intake (Supplemental Fig. S1, *B* and *C*) and of gastrointestinal peptides levels regulating food intake and/or insulin sensitivity such as ghrelin, glucagon-like peptide-1 (GLP-1), and peptide YY (PYY) (Supplemental Fig. S2*A*, see https://doi.org/10.6084/m9.figshare.21608748.v1). The levels of FGF21, a hepatic hormone whose production is associated to sugar consumption (22) were significantly increased in the HS group as compared with HF/HS- and HF-fed mice (Supplemental Fig. S2*B*). These results indicate that fat rather than sugar content in the diet is the driving factor for obesity, impaired glucose metabolism, and insulin sensitivity and that certain nutrients (i.e., sugar) can modulate the production of specific hepatic hormones.

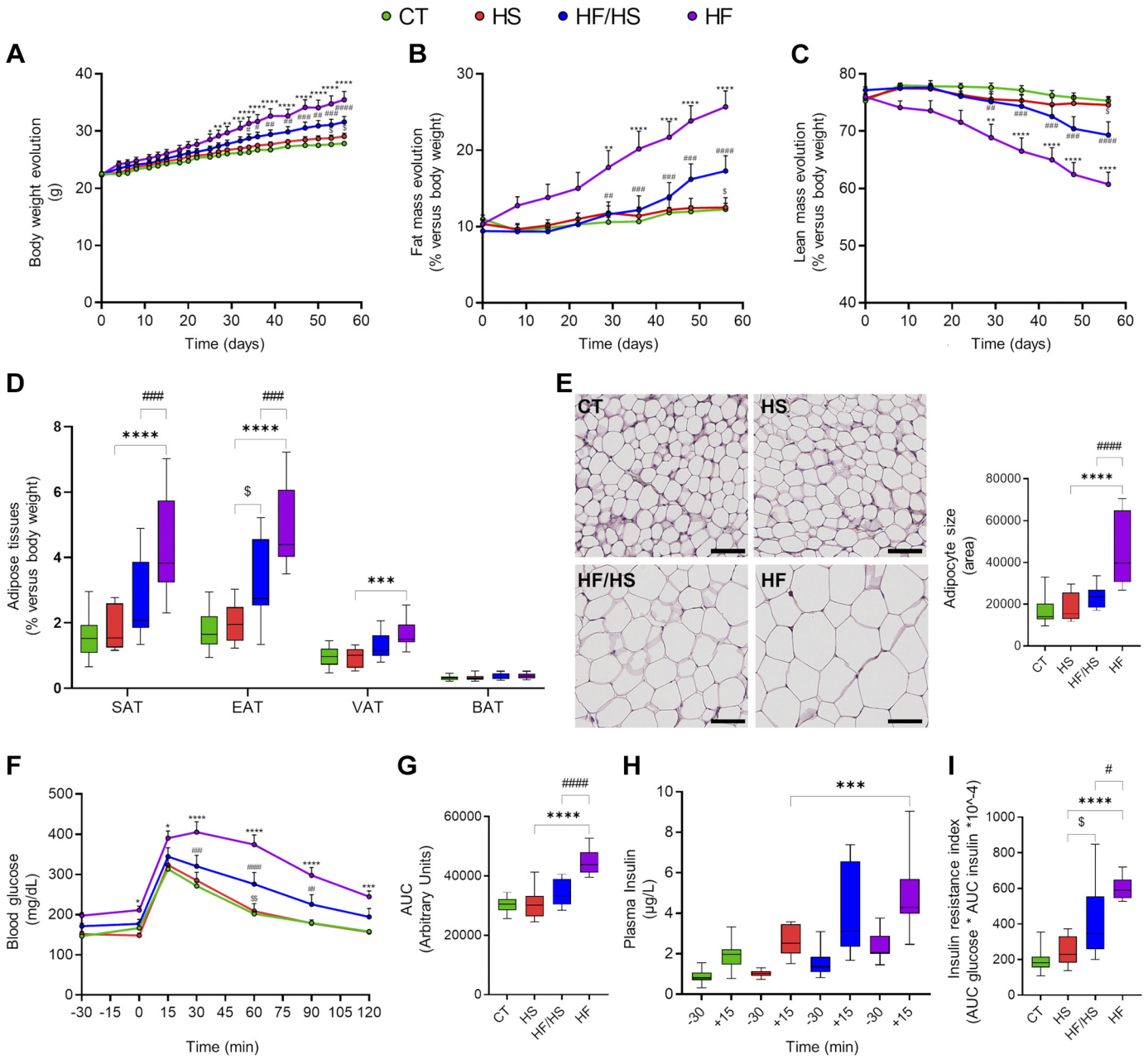

Figure 1. Dietary fat promotes obesity and impairs glucose metabolism. *A*: body weight evolution (g). *B* and *C*: fat mass and lean mass evolution (% body weight) measured by time-domain nuclear magnetic resonance (TD-NMR). *D*: adipose tissues (SAT: subcutaneous; EAT: epididymal; VAT: visceral; BAT: brown) weight (% body weight). *E*: size of the adipocytes in the subcutaneous adipose tissue (SAT). Scale bar, 100 µm; magnification, ×20. *F*: plasma glucose profile (mg/dL) after 2 g/kg glucose oral challenge in freely moving mice and (*G*) the mean area under the curve (AUC) measured between 0 and 120 min after glucose loading. *H*: plasma insulin (µg/L) measured 30 min before and 15 min after glucose loading. *I*: insulin resistance index determined by multiplying the AUC of blood glucose by the AUC of insulin. Green: low-fat control diet (CT) fed mice, red: high-sugar diet (HS) fed mice, blue: high-fat diet (HF)/HS fed mice, and violet: HF fed mice. Data are presented as the means ± SE for *A*–*C* and for *F*, and as box and Whisker plot (Median, minimum-maximum) for *D* and *E* and for *G*–*I*. Data with a superscript symbol ($HS vs. HF/HS, *HS vs. HF, #HF/HS vs. HF) are significantly different ($,*,#$P < 0.05$; $$,**,##$P < 0.01$; ***,###$P < 0.001$; ****,####$P < 0.0001$) (n = 8–18). Data were analyzed using two-way ANOVA followed by Tukey's post hoc test for (*A*–*C*) and (*F*) and according to one-way ANOVA followed by Tukey's post hoc test for *D* and *E* and for *G*–*I*.

Neither Dietary Sugar nor Fat Profoundly Affects Hepatic Markers

The liver plays a critical role in the maintenance of blood glucose and lipid metabolism. Disruption of any of its primary functions can have a major impact on overall metabolic health (23). We therefore investigated whether the altered metabolic features observed can be explained by changes in hepatic lipid accumulation, immune cell recruitment, inflammation, fibrosis, oxidative stress, and endoplasmic reticulum (ER) stress. Along with the increased degree of obesity observed in HF-fed mice, the liver weight ratio to body weight was significantly reduced in these mice as compared with the HS-fed mice (Fig. 2*A*). Hepatic triglycerides

content increased by ~60%–70% in HS-, HF/HS-, and HF-fed mice as compared with CT-fed mice, whereas hepatic cholesterol content was significantly increased in both HS- and HF-fed mice when compared with the HF/HS diet (Fig. 2, *B* and *C*). To further investigate whether hepatic lipid accumulation was also associated with hepatic inflammation, we measured the mRNA expression of markers associated with recruitment of various types of immune cell population (i.e., *Ccl2*, *Adgre1*, *Itgax*, and *Cd68*). The expression of those markers was not significantly affected when comparing the HS-, HF/HS-, and HF-fed mice (Fig. 2*D*). The same was observed for the expression of key receptors involved in the recognition of pathogens-associated molecules patterns of Gram-negative/positive bacteria [i.e., Toll-like receptor (TLR)4, -2, and -5)], and of proinflammatory cytokines (i.e., *Tnf*, and *Il1b*) (Fig. 2*E*). Liver inflammation leads to fibrosis (24), thus, we investigated fibrosis-related genes (i.e., *Col1a1*, and *Tgfb1*). Along with the low inflammatory tone, *Col1a1* and *Tgfb*1 mRNA expression did not change between HS-, HF/HS-, and HF-fed mice (Fig. 2*F*). Since oxidative stress is known to be associated with liver damage and insulin resistance (25), we evaluated markers of hepatic

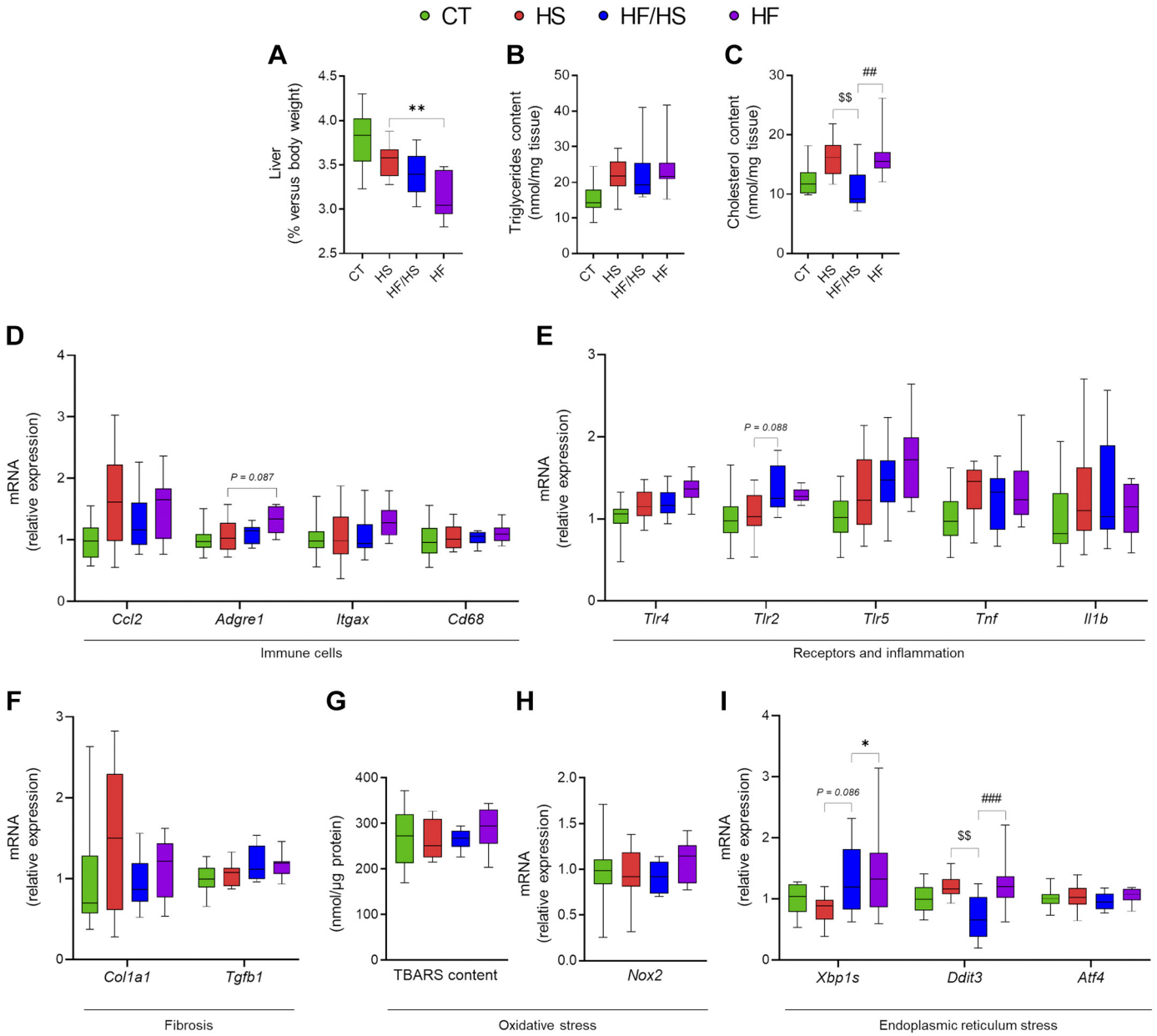

Figure 2. Neither dietary sugar nor fat affect hepatic markers. *A*: liver weight at necropsy (% body weight). *B*: liver triglycerides and (*C*) liver cholesterol content (nmol/mg tissue) measured using a spectrophotometer. mRNA relative expression of immune cells (*D*), receptors and inflammatory cytokines (*E*), fibrosis (*F*), oxidative stress (*H*), and endoplasmic reticulum stress markers measured in the liver by RT-qPCR (*I*). *G*: liver TBARS content measured using a spectrophotometer. Green: low-sugar, low-fat control diet (CT) fed mice, red: high-sugar diet (HS) fed mice, blue: high-fat diet (HF)/HS fed mice, and violet: HF fed mice. Data are presented as box and Whisker plot (Median, minimum-maximum). Data with a superscript symbol ($HS vs. HF/HS, *HS vs. HF, #HF/HS vs. HF) are significantly different (*$P < 0.05$; $$,##$P < 0.01$; ###$P < 0.001$) (n = 8–18). For the mRNA expression, relative units were calculated vs. the mean of the CT fed mice values set at 1. Data were analyzed by one-way ANOVA followed by Tukey's post hoc test.

oxidative stress such as the content of TBARS, and the expression of *Nox2*. No significant differences were observed for these markers (Fig. 2, *G* and *H*). ER stress is also involved in the development of insulin resistance and progression to T2D (8). Therefore, we analyzed well-documented transcriptional ER stress markers [i.e., spliced (s) *Xbp1*, *Ddit3*, and *Atf4*)]. Interestingly, *Xbp1s* mRNA expression was significantly upregulated in HF-fed mice compared with the HS-fed mice (Fig. 2*I*). *Ddit3* was lower in the HF/HS-fed when compared with the HS- and HF-fed mice, whereas the expression of *Atf4* was unchanged between groups (Fig. 2*I*). Altogether, these results highlight that neither sugar nor fat content of the diet profoundly affected the liver markers measured, suggesting that the liver was not the main organ explaining the impaired glucose profile and insulin sensitivity in this study.

Both Dietary Sugar and Fat Influence White Adipose Tissue Markers

Besides the liver, the adipose tissue (AT) also contributes to the regulation of important physiological functions and glucose homeostasis (26, 27). Because the metabolic, endocrine, and inflammatory profile of adipose tissue is depot-dependent (6), we widely explored two different depots (SAT and VAT) representing the two major anatomical sites of white adipose tissue around internal organs. As expected, in the SAT, HF feeding significantly increased the expression of *Ccl2* and *Adgre1*, whereas *Itgax* was upregulated in both HF/HS- and HF-fed mice compared with HS-fed mice (Fig. 3*A*). Along with the increased expression of immune cells markers observed in the SAT, the expression of *Tlr4* significantly increased upon HF diet, whereas the expression of *Tlr2* significantly increased in both HF/HS- and HF-fed mice compared with the HS-fed mice (Fig. 3*B*). No change in *Tlr5* was observed (Fig. 3*B*). Intriguingly, HF/HS-fed mice showed the highest expression of *Tnf* and *Il1b* and this difference was significant as compared with the HS-fed mice (Fig. 3*B*). No significant differences for the same markers were observed in the VAT (Fig. 3, *D* and *E*). ER stress also contributes to inflammation and impaired insulin sensitivity in the adipose tissue (28). We found that *Ddit3* was lower in the HF/HS-fed mice in both SAT and VAT compared with HS- and HF-fed mice (Fig. 3, *C* and *F*). Taken together, these results emphasized that dietary fat alone or in combination with sugar is the driver of adipose tissue inflammation and that a larger fat mass does not reflect higher inflammation when comparing the different diets, thereby suggesting the implication of other factors in this divergent inflammatory tone.

Both Dietary Sugar and Fat Alter Markers in the Gastrocnemius Muscle and in the Hypothalamus

Dysregulation of skeletal muscle metabolism can strongly influence whole body glucose homeostasis and insulin sensitivity. Increased recruitment of immune cells, inflammation, and ER stress has been associated with insulin resistance (28, 29). We therefore investigated the largest muscle in mouse, the gastrocnemius muscle (GAS). We found that *Ccl2* was the only marker significantly upregulated in HF/HS-fed mice compared with HF-fed mice (Fig. 4*A*), whereas the expression of *Tlr5* was lower in the HF-fed mice when compared with the HS-fed mice (Fig. 4*B*). No significant changes were observed for the other markers between the different diets (Fig. 4, *A* and *B*). As in the liver, SAT and VAT, the expression of *Ddit3* was also lower in HF/HS-fed mice compared with the HS- and HF-fed mice (Fig. 4*C*).

The hypothalamus is a key brain area regulating systemic energy and glucose metabolism that can be impaired by inflammation (30, 31). We found that the expression of both *Ccl2* and *Cd45* (also known as *Ptprc*) was significantly increased in HF/HS-fed mice compared with the HF-fed mice (Fig. 4*D*). The same was observed for the expression of both *Tlr4* and *Il1b* (Fig. 4*E*), while the expression of *Tnf* was significantly increased in both HS- and HF/HS-fed mice compared with the HF-fed mice. No significant differences were observed for the other markers (Fig. 4, *D* and *E*). Among the different markers associated with the ER stress and in line with the peripheral tissues discussed earlier, *Ddit3* was lower in HF/HS-fed mice compared with HS- and HF-treated mice (Fig. 4*F*). Taken together, these observations highlight that both dietary sugar and fat influence markers associated with the GAS and the hypothalamic functions, suggesting that these metabolic changes are not obesity-driven but other factors may be involved. Given the presence of a different inflammatory tone in several body sites, we further investigated the systemic inflammatory state through measurement of granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-gamma (IFNγ), interleukin-6 (IL-6), and tumor necrosis factor-α (TNFα) in the plasma. HS diet was the only diet to significantly increase the circulating levels of the proinflammatory cytokine IL-6 when compared with the HF/HS- and HF-fed mice, whereas no significant differences were observed for GM-CSF and TNFα (Supplemental Fig. S2*C*).

Dietary Fat as the Determining Factor for Microbial Load and Gut Microbiota Changes

Dietary components are key factors influencing gut microbiota composition and human health (32, 33). As previously performed (15), we used quantitative microbiome profiling (QMP) and determined the total microbial cell count in feces collected on *days 0*, *8*, *29*, and *56* using flow cytometry. Microbial diversity and load as well as QMPs variation were analyzed in relation to the diverging dietary regimen, as summarized into their fat and sugar content. Microbiota richness and diversity (Observed richness, Shannon diversity, and Evenness) did not significantly correlate with dietary fat or sugar intake (Supplemental Table S3; see, https://doi.org/10.6084/m9.figshare.21608472.v1). When looking at the gut microbiota composition, we first confirmed that microbiota of mice allocated to the different diets were not significantly different at baseline (Adonis test, $R^2 = 0.09$, P value = 0.10, $n = 48$). Analysis of genus-level compositional microbiome diversification as visualized in a principal coordinates analysis (PCoA; Bray–Curtis dissimilarity) revealed that microbiota variation beyond time-associated maturation (9.1% of variation) was significantly driven by dietary composition, with fat content explaining 5.8% of the observed changes ($P = 1e^{-5}$) while dietary sugar contributed another 1.7% (Fig. 5*A*, Supplemental Table S4; see, https://doi.org/10.6084/m9.figshare.21608481.v1).

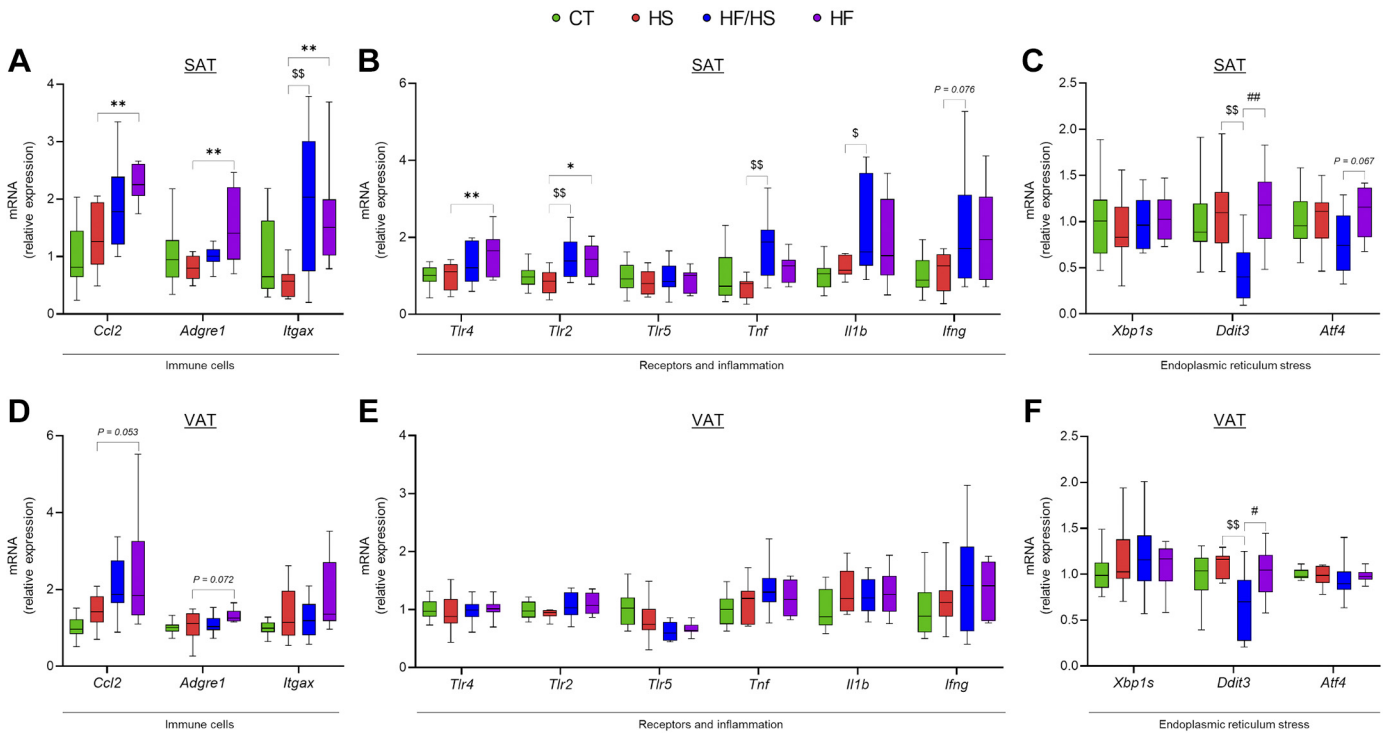

Figure 3. Both dietary sugar and fat influence white adipose tissue markers. mRNA relative expression of immune cells (*A*), receptors and inflammatory cytokines (*B*), and endoplasmic reticulum stress (*C*) markers measured in the subcutaneous adipose tissue (SAT) by RT-qPCR. mRNA relative expression of immune cells (*D*), receptors and inflammatory cytokines (*E*), and endoplasmic reticulum stress (*F*) markers measured in the visceral adipose tissue (VAT) by RT-qPCR. Green: low-sugar, low-fat control diet (CT) fed mice, red: high-sugar diet (HS) fed mice, blue: high-fat diet (HF)/HS fed mice, and violet: HF fed mice. Data are presented as box and Whisker plot (Median, minimum-maximum). Data with a superscript symbol ($HS vs. HF/HS, *HS vs. HF, #HF/HS vs. HF) are significantly different ($,*,#$P < 0.05$; $$,**$P < 0.01$) (n = 9–18). For the mRNA expression, relative units were calculated vs. the mean of the CT fed mice values set at 1. Data were analyzed by one-way ANOVA followed by Tukey's post hoc test.

Moreover, Spearman's rank correlations identified that dietary fat content was negatively associated to microbial load at *day 56* (Fig. 5*B*). In addition, host dietary intake (mainly fat) and to a lesser extent three key metabolic parameters associated with the development of obesity and T2D (i.e., final body weight, final fat mass, and insulin resistance) impacted genera abundances at *day 56* (Fig. 5*C*). An example is the negative correlation between *Akkermansia* (next-generation beneficial microbes) and dietary fat intake. Contrasting analyses of the final microbiome and gene expression profiles (*day 56*), we noted that among the parameters assessed, only dietary fat consumption contributed to explaining genus-level bacterial community variation (3.3%). For gene expression variation, dietary fat was also the main contributor (8.6%), but sugar (8.3%) and the other variables added to a total explanatory power of 23.1% (Fig. 5*D*, Supplemental Table S4). Furthermore, the microbiota and gene expression matrices were strongly correlated (Procrustes, r = 0.48, P = 0.01), suggesting a concerted impact of dietary fat intake. Zooming in on specific bacterial genera abundances at *day 56*, and their correlation to host final physiological status and the expression of several genes significantly affected by diet, allowed us to pinpoint *Romboutsia* and *Parasutterella* as two genera having a strictly positive (*Romboutsia*) or negative (*Parasutterella*) association with obesity, fat mass, adipose tissue weight, adipocyte size, glucose metabolism, and the expression of several genes linked to liver, adipose tissue, and hypothalamic inflammation (Fig. 5*C*, Supplemental Table S5; see, https://doi.org/10.6084/m9.figshare.21608493.v1). Taken together, these results highlighted the importance of dietary fat intake in the modulation of total fecal microbial loads, and compositional modification of the gut microbiota profiles.

DISCUSSION

Because they resemble the genesis of obesity in humans, hypercaloric diets rich in (saturated) fat, sugar, or a combination thereof have classically been used as a mean to induce obesity in rodents [diet-induced obesity (DIO) model)] to understand the complex etiology and consequences of obesity-related disorders. Although studies using high-fat feeding consistently promote obesity by increasing body weight, adipose tissue, insulin resistance, changes in glucose metabolism, hypertension, dyslipidemia, and metabolic syndrome, results with high-carbohydrate diets presented more controversial results (34–39). The discussion was partially put to rest after a massive study by Hu et al. in which 29 different diets and several mouse strains were used to dissect the impact of distinct macronutrients on metabolic disorders. They showed that among the various dietary macronutrients (i.e., carbohydrate, fat, and protein), dietary fat is the major determinant of body fat in mice (14). However, due to a large number of mice used, the authors were not able to further investigate whether dietary components could differently affect metabolic inflammation, oxidative and ER stress, and composition of the gut microbiota independently of an obese

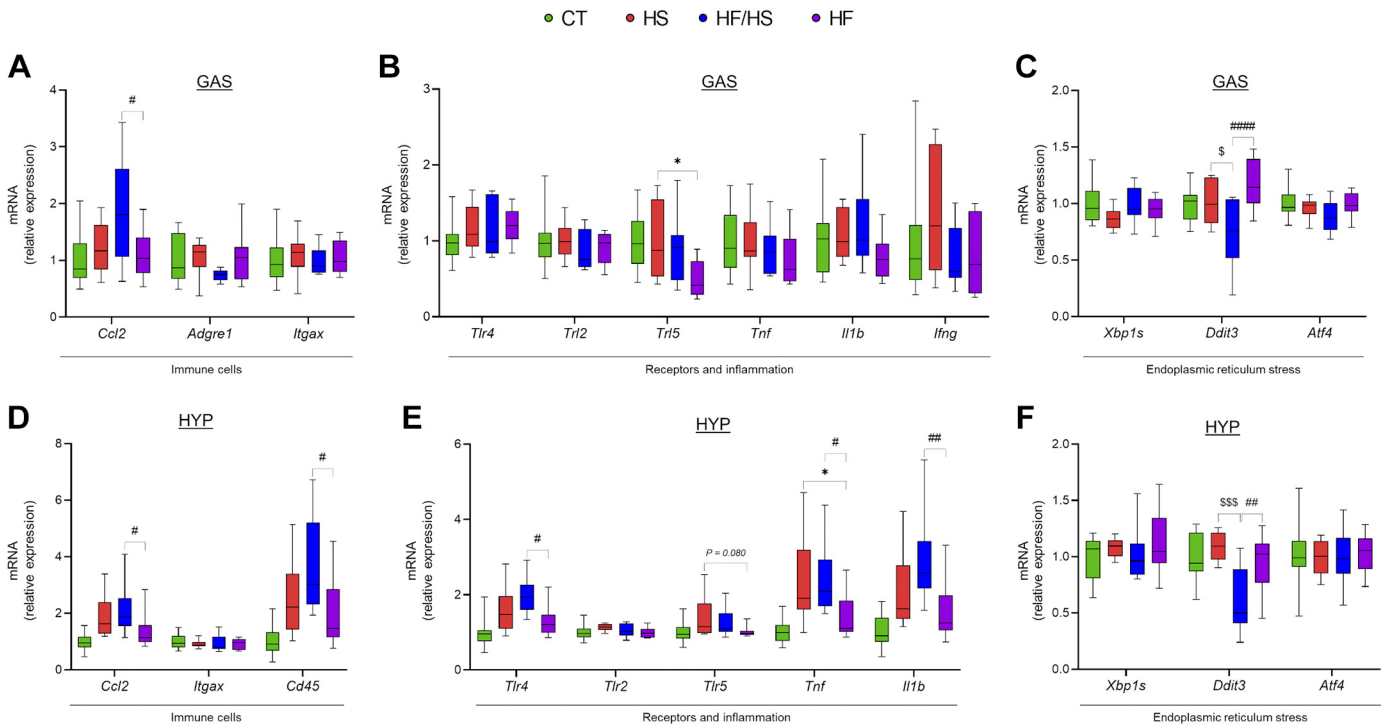

Figure 4. Both dietary sugar and fat alter markers in the gastrocnemius muscle and in the hypothalamus. mRNA relative expression of immune cells (*A*), receptors and inflammatory cytokines (*B*), and endoplasmic reticulum stress (*C*) markers measured in the gastrocnemius muscle (GAS) by RT-qPCR. mRNA relative expression of immune cells (*D*), receptors and inflammatory cytokines (*E*), and endoplasmic reticulum stress (*F*) markers measured in the hypothalamus (HYP) by RT-qPCR. Green: low-sugar, low-fat control diet (CT) fed mice, red: high-sugar diet (HS) fed mice, blue: high-fat diet (HF)/HS fed mice, and violet: HF fed mice. Data are presented as box and Whisker plot (Median, minimum-maximum). Data with a superscript symbol ($HS vs. HF/HS, *HS vs. HF, #HF/HS vs. HF) are significantly different ($,*,#$P < 0.05$; ##$P < 0.01$; $$$$P < 0.001$; ####$P < 0.0001$) ($n$ = 8–19). For the mRNA expression, relative units were calculated vs. the mean of the CT fed mice values set at 1. Data were analyzed by one-way ANOVA followed by Tukey's post hoc test.

state. In the present study, we investigated the effect of only three different diets high in sugar (HS), fat (HF), and a combination of both (HF/HS) in one mouse strain (i.e., C57BL/6). Although this represents a limitation of the current study with respect to the study of Hu et al. (14), we were able to decipher how diets having a different percentage of dietary macronutrients (i.e., sugars and fats) could induce changes in the metabolic parameters mentioned earlier. As previously described, all diets led to a different evolution in terms of body weight, fat and lean mass, and glucose and insulin profiles but we also found quite distinctive metabolic features. Our data revealed that metabolic dysfunctions were not only determined by body fat content and other factors such as changes in the composition of the gut microbiota or in the microbial load might be implicated in overall health.

At the level of the liver, we observed that the severity of lipid accumulation depended on the type of diet with all hypercaloric diets increasing triglycerides content, but only the HS- and HF-diets increasing cholesterol content. Of note, lipid content was not associated with differences in neither inflammatory tone nor oxidative stress. Although HF/HS- and particularly HF diets have previously been shown to result in increased inflammation and impaired hepatic metabolism (7, 40, 41), growing evidence suggests that diet-driven modifications of the gut microbiota may affect liver homeostasis, possibly explaining some of the discrepancies between studies. Of note, we found a higher level of the hepatokine fibroblast growth factor 21 (FGF21), which has been shown to contribute to suppressed consumption of simple sugars that was higher in HS-treated mice (22). Our findings are in line with this paper suggesting the existence of a liver-to-brain hormonal axis likely representing a negative feedback loop via an elevated hepatic FGF21 production during sucrose ingestion (22).

At the level of adipose tissues, we found that mice displaying an increase in fat mass (HF/HS and HF) had the highest inflammatory tone. Several factors (e.g., gut-derived substances, dietary components, and microbes) can contribute to this inflammatory state (42), and among them microbes have emerged as an important player (12). Indeed, depletion of the gut microbiota through antibiotics treatment in obese and diabetic mice was associated with reduced adiposity and adipose inflammation, and improved glucose homeostasis (43–45). In addition, recent human studies demonstrated the presence of certain bacteria and bacterial DNA in several adipose tissue depots in obesity and T2D (46, 47), thereby suggesting a role for bacteria in promoting adipose tissue inflammation.

When looking at skeletal muscle, we did not observe any signs of inflammation except for a significant upregulation of *Mcp1* in the HF/HS group. In the hypothalamus, however, we observed that the combination of fat and sugar was more deleterious than dietary fat alone in promoting recruitment of immune cells and inflammation. Several studies have already suggested that HS-, HF/HS-, and HF diets can promote

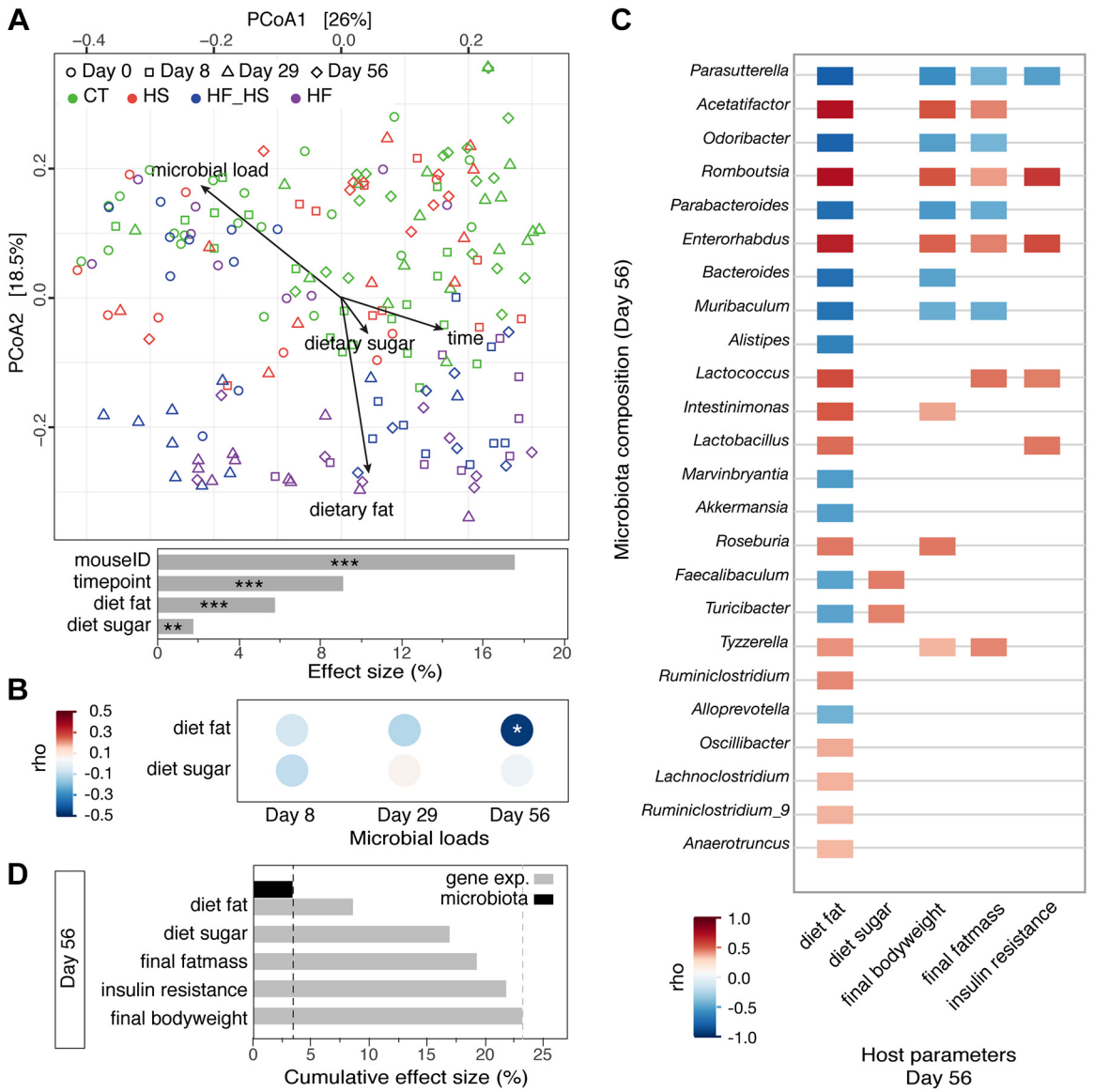

Figure 5. Dietary fat as the determining factor for microbial load and gut microbiota changes. *A*: genus-level fecal microbiome community variation and microbial load, represented by a principal coordinates analysis (PCoA, Bray-Curtis dissimilarity, $n = 187$), with colors and symbols representing mouse groups and timepoints, respectively. Arrows correspond to a post hoc fit of the variables on the ordination. Barplot representation of the nonredundant contribution of inter-individual variation, time-associated maturation, and dietary fat and sugar intake in explaining microbiota variation (stepwise dbRDA). *B*: Spearman's rank correlations between dietary fat or sugar intake and fecal microbial load at the different timepoints. *C*: Spearman's rank correlations between final microbiota composition (genera abundances at *day 56*) and host dietary intake and final metabolic status ($n = 43$; only significant correlation after multiple testing correction are represented). *D*: barplot representation of the cumulative contribution of dietary intake and host final metabolic status in explaining microbiota variation (dbRDA, Bray–Curtis dissimilarity), or host gene expression variation (dbRDA, Euclidean distance). The dashed lines represent the total explanatory power achieved with all five variables. *A* and *B*: tests significance (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

hypothalamic inflammation (48–50). However, it is still unclear how macronutrients can differently promote such inflammation. As a matter of fact, we did not find specific changes in circulating inflammatory markers, suggesting more of an organ-specific inflammatory tone rather than a systemic inflammation. We have recently shown the causal role of obesity-driven gut microbiota changes in the dysregulation of the dopaminergic reward system and the hedonic food intake (51). Thus, we may speculate that changes in the gut microbiota composition may explain the different phenotypes observed in our study.

Besides divergent inflammatory responses induced by the different diets, a common observation among the different tissues mentioned earlier was the downregulation of *Ddit3* (also known as Chop), a marker of ER stress, in HF/HS-fed mice. Given the well-documented intertwined link between ER stress, obesity, and diabetes (8), further studies are required to clarify this metabolic path.

The gut microbiota has been implicated in the regulation of the several metabolic functions mentioned earlier and dietary components influence its composition and functionality (21). Our results revealed that changes in the total fecal

microbial loads, recently proposed to be associated with inflammatory bowel disease and obesity in humans, are at least partially dietary-driven, further confirming our previous findings observed in genetically obese and diabetic mice fed with a low-fat and a low-sugar diet (15). A notable descriptor of the microbiota often associated with poor health is a reduced bacterial diversity (52, 53). In the current study, however, microbial diversity was not affected by either dietary fat or sugar intake, suggesting that instead changes in specific bacterial genera may explain the host phenotypes' progression. Indeed, analysis revealed that many specific bacterial genera were affected, mostly by dietary fat and to a lesser extent by dietary sugar and obesity-related host metabolic parameters. For example, we found that *Romboutsia* was positively correlated with dietary fat, and with a range of obesity-related host metabolic parameters. Previous studies in humans found its quantity to be increased in obese subjects and to be positively correlated with body weight and fat intake ratios (54, 55). Conversely, *Parasutterella* was negatively correlated with dietary sugar, obesity, and several markers associated with glucose metabolism and inflammation. Animal and human studies have shown a significant reduction of *Parasutterella* in response to HF-diet treatment and metabolic phenotypes including hypothalamic inflammation (56, 57). A recent study in vitro and in vivo provided new insight into the mechanisms through which *Parasutterella* may act as a beneficial bacterium and its ability to influence host health (58). Of note, *Akkermansia* was negatively correlated to fat intake. This is interesting since its supplementation in both mice and humans was previously linked to better health outcomes (59, 60). Hence, we may not exclude that dietary fat-associated lower quantity of this bacterium, combined together with a perturbation of the entire microbial community may contribute to our phenotype.

In conclusion, though the underlying mechanisms by which macronutrient composition influence host physiology remain unclear, indirect effects due to changes in gut microbiota composition may be involved. Identifying key bacteria, understanding their role, and learning how to target them may be an effective complementary approach to diet and exercise in the treatment of obesity and related metabolic disorders.

SUPPLEMENTAL DATA

Supplemental Table S1: https://doi.org/10.6084/m9.figshare.21608445.v1.
Supplemental Table S2: https://doi.org/10.6084/m9.figshare.21608463.v1.
Supplemental Table S3: https://doi.org/10.6084/m9.figshare.21608472.v1.
Supplemental Table S4: https://doi.org/10.6084/m9.figshare.21608481.v1.
Supplemental Table S5: https://doi.org/10.6084/m9.figshare.21608493.v1.
Supplemental Table S6: https://doi.org/10.6084/m9.figshare.21608499.v1.
Supplemental Fig. S1: https://doi.org/10.6084/m9.figshare.21608562.v1.
Supplemental Fig. S2: https://doi.org/10.6084/m9.figshare.21608748.v1.

DATA AVAILABILITY

All data generated or analyzed during this study are included in this published article and its supplementary information files. The raw amplicon sequencing data analyzed in this study have been deposited in the EMBL-EBI European Nucleotide Archive (ENA) under accession number PRJEB53668 (https://www.ebi.ac.uk/ena/browser/view/PRJEB53668). The processed quantitative microbiota matrix is provided in Supplemental Table S6 (see https://doi.org/10.6084/m9.figshare.21608499.v1).

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DISCLOSURES

P. D. Cani was cofounder of The Akkermansia company and Enterosys. P. D. Cani is the owner of patents concerning the use of bacteria on health. None of the other authors has any conflicts of interest, financial or otherwise, to disclose.

Patrice Cani is an editor of *American Journal of Physiology-Endocrinology and Metabolism* and was not involved and did not have access to information regarding the peer-review process or final disposition of this article. An alternate editor oversaw the peer-review and decision-making process for this article.

AUTHOR CONTRIBUTIONS

F.S., M.V.H., and P.D.C. and conceived and designed research; F.S., A.d.W.d, P.P., and M.V.H. performed experiments; F.S., S.V.-S., G.F., M.V.H., and P.D.C., analyzed data; F.S., S.V.-S., G.F., M.V.H., and P.D.C., interpreted results of experiments; F.S., S.V.-S., and G.F. prepared figures; F.S., M.V.H., and P.D.C. drafted manuscript; F.S., S.V.-S., G.F., A.d.W.d., P.P., N.M.D., A.E., J.R., M.V.H., and P.D.C. edited and revised manuscript; F.S., S.V.-S., G.F., A.d.W.d., P.P., N.M.D., A.E., J.R., M.V.H., and P.D.C. approved final version of manuscript.

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