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Physiological, metabolic and microbial responses to obesogenic cafeteria diet in rats: The impact of strain and sex

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Abstract

Cafeteria (CAF) diet is known to accurately mimic the human Western diet in modern societies, thereby inducing severe obesity accompanied by drastic alterations on the gut microbiome in animal models. Notably, the dietary impact in the gut microbiota composition might be influenced by genetic factors, thus distinctively predisposing the host to pathological states such as obesity. Therefore, we hypothesized that the influence of strain and sex on CAF-induced microbial dysbiosis leads to distinct obese-like metabolic and phenotypic profiles. To address our hypothesis, two distinct cohorts of male Wistar and Fischer 344 rats, as well as male and female Fischer 344 animals, were chronically fed with a standard (STD) or a CAF diet for 10 weeks. The serum fasting levels of glucose, triglycerides and total cholesterol, as well as the gut microbiota composition, were determined. CAF diet triggered hypertriglyceridemia and hypercholesterolemia in Fischer rats, while Wistar animals developed a marked obese phenotype and severe gut microbiome dysbiosis. Furthermore, CAF diet-induced changes on gut microbiota were related to more profound alterations in body composition of female than male rats. We revealed that distinct rat strains and genders chronically consuming a free-choice CAF diet develop distinct and robust microbiota perturbations. Overall, we showed that genetic background might have a key role in diet-induced obesity, thus distinguishing the suitability of different animal models for future nutritional studies focused on gut microbiota dysbiosis induced by a CAF dietary model.

Key words: Gut microbiota; cafeteria diet; rat strain; sexual dimorphism; diet-induced obesity.

1. Introduction

The gut microbiota plays a crucial role in the maintenance of a healthy metabolic and physiologic phenotype in the host by exerting a wide range of functions such as regulating gut integrity, host immunity and energy homeostasis. Several factors including genetics and environmental conditions are known to impact on the establishment of gut microbes. Among them, diet has been recognised as the main agent influencing the composition and activity of the gut microbial ecosystem [1], while host genetics also have a determinant impact on the microbiome [2,3]. For this reason, recent findings raised the importance of controlling for ethnicity when establishing relationships between gut microbiota alterations and the onset of a disease [4]. In this sense, host genetics might influence gut microbiota responses to environmental challenges, thus distinctively predisposing the host to certain pathological states under a given dietary intervention. Unfortunately, only few studies have examined gut microbial differences among distinct rodent strains and how diet is able to differentially modulate their bacterial ecosystems [5,6].

Sexual dimorphism is characteristic of different common diseases including obesity and related complications [7]. Accordingly, hormonal status exerts an important modulatory role on the gut microbiota-host interaction. For instance, the preventive role of estrogens in the development of obesity-related metabolic disorders such as endotoxemia and low-grade chronic inflammation is tightly mediated by gut microbiome [8]. Overall, the interaction between diet, gut microbiota and host homeostasis lastly depends on the genetic background.

Importantly, changes on dietary habits promote rapid shifts in the gut microbial populations [9]. In this sense, cafeteria (CAF) diet induce severe obesity-associated dysbiosis in rats [10]. Despite that CAF model is known to closely reflect the modern human condition of obesity [11], the influence of genetics in the CAF-induced microbial dysbiosis and metabolic alterations has not been eval-

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uated so far. In such situation, we hypothesized that strain and sex influence on CAF-induced microbial dysbiosis leads to distinct obese-like metabolic and phenotypic profiles. To address this hypothesis, we aimed to characterize the impact of chronic CAF feeding on the gut microbiota composition and host phenotype of distinct rat strains (Wistar or Fischer 344) and sexes (male or female Fischer 344).

2. Materials and methods

2.1. Animal procedures

Twelve 6-weeks-old male Wistar rats, sixteen 7-weeks-old male Fischer 344 (F344) rats and sixteen 7-weeks-old female F344 rats were supplied from Charles River Laboratories (Wilmington, MA, USA). Animals were individually housed in three different temperature-controlled rooms (22°C) according to sex and strain under a 12:12-h light-dark cycle. After 1 week of adaptation with free access to food and water, the animals within each strain and sex were randomly distributed depending on the diet: (A) standard (STD) nonpurified chow diet (Teklad Global 18% Protein Rodent Diet 2018, Harlan, Barcelona, Spain), and (B) CAF diet composed of energy-dense and highly palatable foods including biscuits with pâté and cheese (14–15 g), bacon (5–7 g), and pastries (6–8 g), standard chow pellets *ad libitum* (same pellets offered to STD group), and sugared milk (220 g/L; 50 mL per day). Both STD and CAF had free access to water during all the experimental period. The energetic density and the macronutrient composition of the diets used in this study have been previously characterized by our research group [10] (Supplementary Table 1). The experimental groups were composed as follows: (1) STD diet-fed male Wistar rats (n=6), (2) CAF diet-fed male Wistar rats (n=6), (3) STD diet-fed male F344 rats (n=8), (4) CAF diet-fed male F344 rats (n=8), (5) STD diet-fed female F344 rats (n=8), and (6) CAF diet-fed female F344 rats (n=8). Decision on sample size was made based on previous similar research work. No animals were excluded from the analyses. Food intake and body weight were recorded weekly. Macronutrient intake (g) was calculated as total food intake (grams) x macronutrient content (g)/100. Macronutrient intake of CAF diet-fed groups was calculated as the sum of macronutrient intake from each individual ingredient included in this diet. Total food intake and macronutrient intake are presented in Kcal/d. Food efficiency was calculated as the body weight gain (grams) per each 100 Kcal ingested following the formula: FE. = average daily body weight gain (g)/average daily food intake (kcal) x 100. One week before the end of the study, serum samples were obtained by saphenous venepuncture and processed by centrifugation (2 000 g, 15 min). At 17-weeks-old, Wistar rats were euthanized by exsanguination through cardiac puncture under anaesthesia (pentobarbital sodium, 80 mg/kg body weight), whereas F344 rats were sacrificed by decapitation according to the requirements of independent purposes of this study. Liver; white adipose tissue depots, including mesenteric (MWAT), retroperitoneal (RWAT), inguinal (IWAT) and epididymal (EWAT) or periovarian (OWAT); and cecum were collected, weighed and immediately frozen in liquid nitrogen. All the samples were stored at -80°C until further analyses.

The Animal Ethics Committee of the Technological Unit of Nutrition and Health of EURECAT (Reus, Spain) and the Generalitat de Catalunya approved all procedures (DAAM 8865). The study followed the "Principles of Laboratory Animal Care,", complied with the ARRIVE guidelines and was carried out in accordance with the EU Directive 2010/63/EU for animal experiments.

2.2. Body composition and serum analysis

Fat and lean mass were determined on d 0 and 70 by nuclear magnetic resonance (NMR) using an EchoMRI-700 device (Echo Medical Systems, L.L.C., Houston, TX, USA). Adiposity Index was calculated as the sum of the EWAT, IWAT, MWAT and RWAT depots weight and expressed as percentage of body weight. Serum levels of glucose, triglycerides and total cholesterol were assessed using enzymatic colorimetric kits (QCA, Barcelona, Spain) according to the manufacturer's instructions.

2.3. Assessment of cecal microbiota composition

For microbiota analyses, DNA from the cecal content samples was extracted using a QIAamp DNA stool mini kit (Qiagen Inc., Hilden, Germany) in accordance to the manufacturer's instructions. DNA purity and integrity were evaluated using spectrophotometry (NanoDrop, Thermo Fisher Scientific, MA, USA). V3 and V4 regions from 16S rRNA gene were amplified and purified as described previously [12].

Multiplexed pools were diluted to 50 pM DNA concentration prior to clonal amplification. Clonal amplification and sequencing were performed by employing the Ion 520 and Ion 530 Kit-Chef (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Prepared samples were loaded on Ion 530 chips and sequenced using the Ion S5 System (Life Technologies, Carlsbad, CA, USA). The individual sequence reads were filtered by the Ion Torrent Suit software to remove low-quality and polyclonal sequences and finally analyzed using QIIME [13]. The sequence similarity threshold for both OTU picking and taxonomy assignment was 97%, and Greengenes was utilized as the taxonomy database.

2.4. Statistical analyses

All results are expressed as the mean ± standard error of the mean (SEM). The Grubbs test was applied to remove significant outliers. Two-way ANOVA was applied to evaluate differences on food intake, biometric and serum parameters (Diet and Strain or Diet and Sex, separately). If significant interactions emerged from the two-way ANOVA, differences between STD and CAF diet-fed groups within each sex or strain were assessed by a Student's t-test. The statistical power of the test was 80%, and the two-sided significance level was 0.05. Pearson's correlation analysis was employed to analyse the correlations among variables of interest. Statistical analyses were performed using SPSS Statistics 22 software (SPSS, Inc., Chicago, IL, USA).

For metagenomic analyses, microbial genera present in at least two samples with ≥0.1% abundance were previously selected. Overall dissimilarities of cecal microbiota composition between pairs of groups were assessed using one-way PERMANOVA based on Bray-Curtis distances. The microbial diversity was assessed through the Shannon's diversity index. The contributory role for each factor to the observed differences was assessed using two-way ANOSIM at genus level, with diet nested within strain or sex. The phyla relative abundances were statistically compared using the nonparametric U-Mann Whitney test. Changes on specific microbial genera were assessed through the non-parametric Welch's t-test. *P*-values were adjusted for multiple comparisons according to the Benjamini-Hochberg method with a 5% false discovery rate (FDR). Finally, principal component analyses (PCA) based on genera proportions were performed to identify clusters according to the microbiome composition.

3. Results

3.1. CAF diet promotes obesity independently of rat strain and sex

Food intake, biometric and biochemical parameters in male Wistar and F344 rats are presented in Table 1. The two-way ANOVA revealed that energy intake was increased in both CAF-fed rat strains, although this increment was higher in F344 animals. Accordingly, total food consumption was higher in F344 than in Wistar animals under a CAF feeding (Wistar-CAF: 24.07±0.67 g/d; F344: 33.91±1.19 g/d). These results indicate that the hyperphagia promoted by CAF diet was more evident in F344 than in Wistar rats. Surprisingly, these differences were due to a significantly higher chow intake in F344 than Wistar rats in our free-choice CAF model (Wistar-CAF: 3.22±0.61 g/d, F344-CAF: 10.6±0.37 g/d). These observations were in line with a more pronounced reduction of fibre intake in Wistar than F344 animals when compared to their respective STD-fed controls, although total fibre intake was significantly lower in CAF than STD-fed rats within both strains.

Interestingly, a higher protein intake was observed in CAF-fed F344 rats compared to their STD-fed controls while, on the contrary, protein intake was lower in CAF- than STD-fed Wistar animals. Moreover, a higher increment of simple carbohydrates intake was observed in Wistar than F344 rats when fed with our CAF diet. These results might be mostly explained by differences on sugared milk intake (Wistar-CAF: 47.1±1.43 mL/d, F344-CAF: 42.1±1.60 mL/d), but distinct preferences for different ingredients included in the CAF diet observed between Wistar and F344 might also have an impact on their physiological and microbial responses to this CAF model.

Body weight gain, total fat mass, adipose tissues weights (MWAT, EWAT and IWAT), adiposity index and visceral fat percentage were higher in both F344 and Wistar rats under CAF feeding, whereas body lean mass percentage and lean-to-fat mass ratio were reduced. Interestingly, CAF-fed Wistar rats showed a higher increase in the percentage of RWAT mass than F344 animals when compared to their respective STD-fed controls. Moreover, F344 rats presented a significantly lower body weight gain, percentages of lean mass and visceral fat, and adiposity index than Wistar animals independently of the diet. These results are in accordance

Table 1
Food intake, biometric and serum parameters in male Wistar and Fischer 344 rats fed standard (STD) and cafeteria (CAF) diets.

	Wistar		F344		Two-way ANOVA
	STD	CAF	STD	CAF	
Food intake (kcal/d)					
Total energy intake	67.8 ± 2.52	136 ± 3.14*	60.6 ± 0.97	159 ± 3.55*	S*D
Total carbohydrates intake	38.7 ± 1.44	76.7 ± 2.66*	35.2 ± 0.56	92.8 ± 2.71*	S*D
Simple carbohydrates intake	0.00 ± 0.00	67.6 ± 1.34*	0.00 ± 0.00	52.7 ± 2.27*	S*D
Lipid intake	12.2 ± 0.45	46.3 ± 0.85	10.9 ± 0.17	43.7 ± 2.33	D
Protein intake	16.3 ± 0.60	13.8 ± 0.47*	14.6 ± 0.23	18.2 ± 0.52*	S*D
Fiber intake (g/d)	3.18 ± 0.12	0.90 ± 0.09*	2.88 ± 0.05	1.88 ± 0.06*	S*D
Food efficiency	4.93 ± 0.18	3.46 ± 0.10	3.35 ± 0.08	2.24 ± 0.21	S, D
Biometric parameters					
Body weight gain (g)	234 ± 13.3	331 ± 15.3	128 ± 3.29	225 ± 17.1	S, D
Fat mass (%)	11.0 ± 0.87	24.8 ± 1.96	12.0 ± 0.51	24.2 ± 1.73	D
Lean mass (%)	84.2 ± 0.94	70.9 ± 1.94	78.3 ± 0.78	69.7 ± 1.67	S, D
Lean/fat ratio	7.16 ± 0.38	3.00 ± 0.35	6.60 ± 0.25	3.00 ± 0.34	D
Visceral fat (%)	6.93 ± 0.57	12.1 ± 0.66	6.24 ± 0.17	9.66 ± 0.43	S, D
Adiposity Index	9.01 ± 0.75	16.0 ± 1.03	7.63 ± 0.21	12.0 ± 0.63	S, D
MWAT (%)	1.61 ± 0.12	2.78 ± 0.26	1.51 ± 0.08	2.56 ± 0.13	D
RWAT (%)	2.80 ± 0.28	5.25 ± 0.36*	2.26 ± 0.09	3.20 ± 0.16*	S*D
EWAT (%)	2.52 ± 0.26	4.07 ± 0.35	2.43 ± 0.10	3.90 ± 0.24	D
IWAT (%)	2.08 ± 0.20	3.22 ± 0.21	1.34 ± 0.08	2.33 ± 0.26	S, D
Cecum weight (g)	6.55 ± 0.46	4.18 ± 0.29	5.58 ± 0.30	4.36 ± 0.21	D
Serum parameters					
Glucose (mg/dL)	147 ± 12.3	176 ± 10.9	150 ± 5.97	202 ± 10.9	D
Triglycerides (mg/dL)	97.1 ± 23.2	136 ± 12.4	134 ± 8.21	358 ± 24.3*	S*D
Total cholesterol (mg/dL)	73.7 ± 8.65	71.2 ± 2.87	59.0 ± 3.82	94.8 ± 7.29*	S*D

Values: Male Wistar (n=6 per group) and Fischer 344 (n=8 per group) rats were fed with STD or CAF diet. Data are expressed as the mean ± SEM. D, the effect of diet type; S, the effect of strain; S*D, the interaction of strain and diet type (two-way ANOVA, P<.05).
Abbreviations: EWAT, epididymal white adipose tissue; IWAT, inguinal white adipose tissue; MWAT, mesenteric white adipose tissue; RWAT, retroperitoneal white adipose tissue.
* The effect of diet within each strain (Student's t-test, P<.05).

with decreased food efficiency (body weight gain per unit of energy consumed) observed in F344 rats compared to Wistar animals regardless the diet. On the other hand, cecum weight was lower in CAF-fed animals independently of rat strain, which could be explained by the reduced fiber intake. Concerning the metabolic phenotype, both Wistar and F344 rats exhibited higher serum glucose levels under CAF diet. In contrast, hypertriglyceridemia and hypercholesterolemia were only observed in F344 rats consuming a CAF diet.

Alterations on food intake, biometric and biochemical parameters induced by CAF feeding were observed in both male and female F344 rats (Table 2). CAF-fed animals showed an increased intake of total energy, carbohydrates, lipids and proteins as compared to STD-fed counterparts. As expected, energy, macronutrients and fiber intake were lower in females than in males independently of the dietary model. In accordance, cecum weight was lower in females than males regardless the diet, and significantly reduced by CAF feeding independently of sex. Interestingly, fiber intake and cecum weight were positively correlated (Pearson's correlation, r(43)=0.797, P<.001). Despite that energy intake was similarly higher in CAF-fed males and females compared to STD controls (Diet effect), body weight changes were more pronounced in males than females (Diet x Sex interaction), thus suggesting that males were more susceptible to body weight gain under CAF diet. These results were not translated into diet-dependent differences on food efficiency between both genders. However, the percentage of body fat mass, adiposity index and visceral fat was higher in CAF-fed animals independently of sex. Notably, a higher drop on lean mass percentage and a higher increment of RWAT percentage was observed in females than males fed a CAF diet, thus supporting distinct vulnerabilities to diet-induced obesity (DIO). In addition, both male and female F344 rats fed a CAF diet showed higher fasting blood glucose, triglycerides and total cholesterol levels, while hypertriglyceridemia was more pronounced in male than in female F344 rats.

Finally, we conducted correlation analyses between food intake parameters and metabolic and physiological markers including data from all study animals. Total energy intake, carbohydrates intake and lipid intake were positively correlated with body weight gain, body fat mass, adipose tissues weights and circulating TAG, and negatively correlated with body lean mass and cecum size. Simple carbohydrates consumption strongly and positively correlated with visceral fat mass and adiposity index. On the other hand, protein intake was especially associated with high body and liver weights. Fiber intake presented positive correlations with lean body mass and cecum weight, while negatively correlated with body fat mass, visceral fat mass and adiposity index. Overall, food consumption patterns, concomitantly related to distinct animal strains and sexes, were highly related to changes observed at physiological and metabolic levels, as expected.

3.2. CAF diet distinctively altered major phyla and microbial diversity in different rat strains and sexes

Overall, two-way PERMANOVA revealed a significant interaction between strain and diet (P<.001) as factors determining the cecal microbiota composition. Specifically, one-way PERMANOVA revealed that genera populations were significantly altered by CAF

Table 2
Food intake, biometric and serum parameters in male and female Fischer 344 rats fed standard (STD) and cafeteria (CAF) diets.

	Male		Female		Two-way ANOVA
	STD	CAF	STD	CAF	
Food intake (kcal/d)					
Total energy intake	60.6 ± 0.97	159 ± 3.55	38.8 ± 0.60	122 ± 9.35	s, D
Total carbohydrates intake	35.2 ± 0.56	92.8 ± 2.71	22.5 ± 0.35	76.3 ± 6.87	s, D
Simple carbohydrates intake	0.00 ± 0.00	52.7 ± 2.27	0.00 ± 0.00	60.8 ± 6.52	D
Lipid intake	10.9 ± 0.17	43.7 ± 2.33	6.99 ± 0.11	34.4 ± 1.93	s, D
Protein intake	14.6 ± 0.23	18.2 ± 0.52	9.32 ± 0.14	12.0 ± 0.64	s, D
Fiber intake (g/d)	2.88 ± 0.05	1.88 ± 0.06	1.84 ± 0.03	0.80 ± 0.02	s, D
Food efficiency	3.35 ± 0.08	2.24 ± 0.21	2.09 ± 0.08	1.48 ± 0.12	s, D
Biometric parameters					
Body weight gain (g)	128 ± 3.29	225 ± 17.1*	51.1 ± 2.04	109 ± 3.74*	s*D
Fat mass (%)	12.0 ± 0.51	24.2 ± 1.73	15.8 ± 1.26	32.0 ± 0.70	s, D
Lean mass (%)	78.3 ± 0.78	69.7 ± 1.67*	77.7 ± 1.10	62.8 ± 0.75*	s*D
Lean/fat ratio	6.60 ± 0.25	3.00 ± 0.34	5.18 ± 0.47	1.97 ± 0.07	s, D
Visceral fat (%)	6.24 ± 0.17	9.66 ± 0.43	7.81 ± 0.83	13.0 ± 0.29	s, D
Adiposity Index	7.63 ± 0.21	12.0 ± 0.63	8.88 ± 0.88	15.3 ± 0.38	s, D
MWAT (%)	1.51 ± 0.08	2.56 ± 0.13	1.42 ± 0.18	2.45 ± 0.07	D
RWAT (%)	2.26 ± 0.09	3.20 ± 0.16*	1.47 ± 0.22	3.32 ± 0.18*	s*D
EWAT (%)	2.43 ± 0.10	3.90 ± 0.24*	-	-	-
OWAT (%)	-	-	4.76 ± 0.37	7.12 ± 0.23*	-
IWAT (%)	1.34 ± 0.08	2.33 ± 0.26	1.01 ± 0.08	2.19 ± 0.19	D
Cecum weight (g)	5.58 ± 0.30	4.36 ± 0.21	4.17 ± 0.25	2.25 ± 0.23	s, D
Serum parameters					
Glucose (mg/dL)	150 ± 5.97	202 ± 10.9	133 ± 6.18	178 ± 8.45	s, D
Triglycerides (mg/dL)	134 ± 8.21	358 ± 24.3*	76.6 ± 6.95	170 ± 22.7*	s*D
Total cholesterol (mg/dL)	59.0 ± 3.82	94.8 ± 7.29	85.8 ± 2.17	105 ± 5.23	s, D

Values: Male and female Fischer 344 rats (n=8 per group) were fed with STD or CAF diet. Data are expressed as the mean ± SEM. D, the effect of diet type; s, the effect of sex; s*D, the interaction of sex and diet type (two-way ANOVA, P<.05).
Abbreviations: EWAT, epididymal white adipose tissue; IWAT, inguinal white adipose tissue; MWAT, mesenteric white adipose tissue; OWAT, periovarian white adipose tissue; RWAT, retroperitoneal white adipose tissue.
* The effect of diet within each sex (Student's t-test, P<.05).

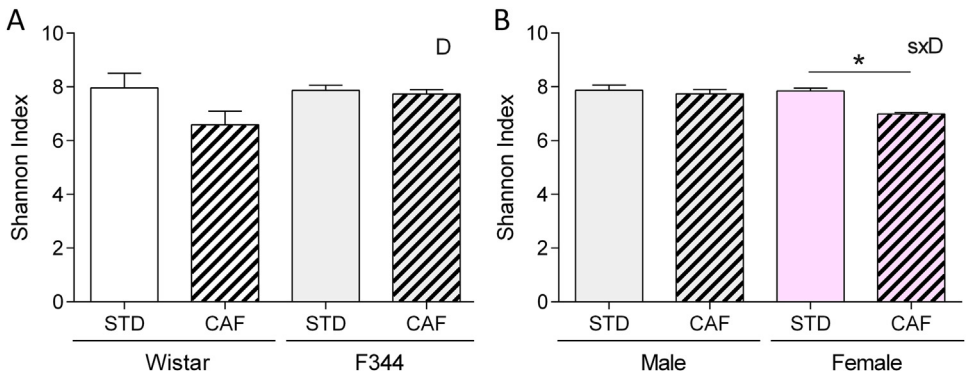

Fig. 1. Differences in alpha-diversity among groups assessed through the Shannon Index. (A) Microbial diversity determined in male Wistar and F344 rats fed with a standard (STD) or a cafeteria (CAF) diet. (B) Microbial diversity determined in F344 male and female rats fed with a STD or a CAF diet. Data are expressed as the mean ± SEM. D, the effect of diet type; sxD, the interaction of sex and diet type (two-way ANOVA, P<.05). * The effect of diet within female rats (Student's t-test, P<.05).

diet within both Wistar (P=.002) and F344 (P<.001) rats (Supplementary Fig. S1), while this diet decreased the bacterial diversity in both Wistar and F344 rats compared to STD diet (two-way ANOVA, P=.04) (Fig. 1A). Interestingly, we identified significant differences in the microbiota composition between F344 and Wistar animals fed with a STD diet (One-way PERMANOVA, P=.003), while bacterial diversity did not differ (Student's t-test, P=.867).

We also revealed an interaction between diet and sex factors within F344 strain (two-way PERMANOVA, P=.02). Microbial populations were significantly altered by CAF diet within both males and females (one-way PERMANOVA, P<.001) (Supplementary Fig. S2). Importantly, bacterial diversity was reduced in female rats under CAF feeding compared to STD-fed counterparts (Student's t-test, P<.001), but not in males (Fig. 1B).

Firmicutes was the most abundant phylum present in both Wistar (79.3%) and F344 (89.9%) rat strains fed with a STD diet, showing a significantly higher relative abundance in F344 than Wistar group (U-Mann Whitney, P=.005). In contrast, Bacteroidetes relative abundance was significantly higher in Wistar (19.8%) when compared to F344 (8.7%) animals (U-Mann Whitney, P=.003). In this line, Bacteroidetes-to-Firmicutes ratio was higher in Wistar than F344 animals (Student's t-test, P = 0.01) under STD diet. In-

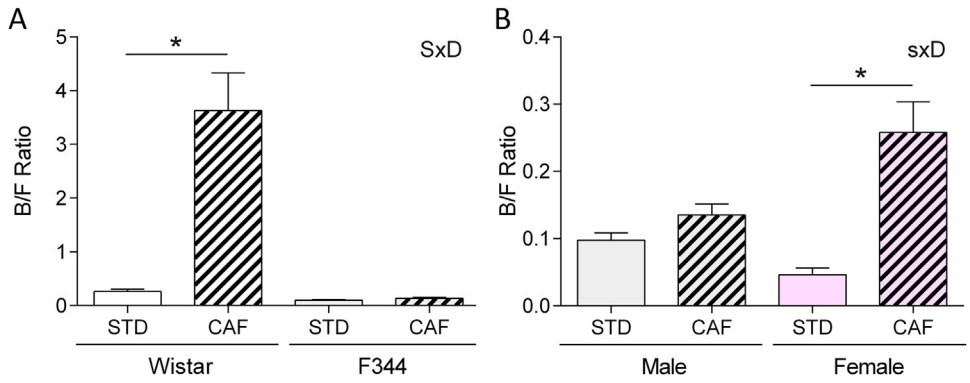

Fig. 2. The two major phyla composing the microbiome expressed as the Bacteroidetes-to-Firmicutes ratio. (A) B/F ratio assessed in male Wistar and F344 rats fed with a standard (STD) or a cafeteria (CAF) diet. (B) B/F ratio assessed in F344 male and female rats fed with a standard STD or a CAF diet. Data are expressed as the mean ± S.E.M. SxD, the interaction of strain and diet type; sxD, the interaction of sex and diet type (two-way ANOVA, $P<.05$). * The effect of diet within male Wistar and female F344 rats (Student's t-test, $P<.05$).

terestingly, CAF-induced increase of Bacteroidetes-to-Firmicutes ratio was 10 times higher in Wistar than F344 rats when compared to their respective controls (Student's t-test, $P=.005$ and $P=.08$, respectively), (Fig. 2A). Thus, our CAF model disrupted the dominance of Firmicutes only in Wistar rats, where Bacteroidetes became the major phylum representing the 68.8% of total bacteria. Concerning sex comparison, the Bacteroidetes-to-Firmicutes was markedly raised after chronic CAF consumption, especially in female F344 animals when compared to their lean counterparts (Student's t-test, $P=.002$) (Fig. 2B).

Verrucomicrobia occurrence was significantly higher in CAF-fed Wistar, but not F344 rats, compared to STD controls (U-Mann Whitney, $P=.01$), whereas Proteobacteria was significantly raised within both Wistar and F344 groups by CAF diet (U-Mann Whitney, $P=.004$ and $P = 0.001$ vs. STD, respectively) (Supplementary Fig. S3A). When assessing the sex contributory role to the CAF-induced changes on these major phyla (Supplementary Fig. S3B), a higher occurrence of Verrucomicrobia (U-Mann Whitney, $P=.03$) was only described in F344 females compared to STD-fed controls, while significant changes on Proteobacteria relative abundance were only identified in F344 males.

We also found that low-abundant phyla representing less than 1% of the microbiome such as Actinobacteria ($P=.02$), Lentisphaerae ($P=.007$) and TM7 ($P=.002$) were higher in F344 compared to Wistar under STD feeding, while Deferribacteres relative abundance was lower in F344 rats ($P=.002$). CAF diet caused a decrease in TM7 ($P<.050$) and Tenericutes ($P<.050$) occurrence, and a raise of Actinobacteria ($P<.010$) in both Wistar and F344 rats. Indeed, a reduced Deferribacteres relative abundance ($P=.004$) was only observed in CAF-fed Wistar animals (U-Mann Whitney test).

Interestingly, up to 14 genera statistically differed between Wistar and F344 rats fed with STD diet (Welch's t-test following FDR correction). Importantly, STD-fed Wistar animals showed higher relative abundances of genera belonging to Bacteroidetes phylum including *Bacteroides* ($P=.02$) and *Prevotella* ($P=.04$), as well as increased *Oscillospira* ($P=.02$) occurrence than STD-fed F344 rats. On the other hand, certain Firmicutes genera such as *Lactobacillus* ($P=.03$), unclassified Lachnospiraceae ($P=.03$) and *Ruminococcus* ($P=.01$) were mainly found in F344 rats.

3.3. CAF feeding induced profound changes on genera abundance, especially in Wistar rats

Two-way ANOSIM revealed strong influences of both strain ($R = 0.876$, $P<.001$) and diet ($R = 0.801$, $P<.001$) on the establishment of microbial populations at genus level. Nevertheless, some of these diet-induced alterations were similarly described for both Wistar and F344 rat strains (Supplementary Fig. S4). In this sense, we detected a highly reduced occurrence of unclassified Clostridiales ($P<.010$), *Oscillospira* ($P<.010$) and *Dehalobacterium* ($P<.050$), accompanied by an increment of *Bacteroides* ($P<.050$), *Parabacteroides* ($P<.050$), *Prevotella* ($P<.050$) and *Phascolarctobacterium* ($P<.050$) in both Wistar and F344 CAF-fed rats compared to their respective STD-fed groups. In Wistar animals chronically exposed to CAF diet, *Bacteroides* relative abundance was higher (STD: 1.5%; CAF: 28.0%), essentially to the detriment of unclassified Clostridiales (STD: 47.5%; CAF: 6.3%). However, *Bacteroides* represented only 1% of total bacteria in CAF-fed F344 rats, whereas unclassified Clostridiales relative abundance was not as reduced as in Wistar rats (STD:54.5%; CAF: 34.1%), thus evidencing that dietary changes on microbial abundances strongly depended on rat strain. On the other hand, *Prevotella* occurrence increased in CAF-fed animals up to 0.42% (in F344) and 34.7% (in Wistar) compared to their STD-fed controls. Interestingly, several genera were only influenced by the dietary intervention within a particular strain (Supplementary Fig. S5). For instance, CAF diet significantly decreased Bacteroidales S24-7 ($P=.02$) and unclassified Ruminococcaceae ($P=.02$) only in Wistar animals. On the other hand, only F344 rats showed an increased occurrence of unclassified Lachnospiraceae ($P=.05$), *Roseburia* ($P=.05$), *Desulfovibrio* ($P=.05$), *Blautia* ($P=.04$), *Anaerostipes* ($P=.04$), *Ruminococcus* ($P=.03$), *Bilophila* ($P=.03$), *Allobaculum* ($P=.009$) and rc4-4 ($P=.002$) under a CAF diet, while *Odoribacter* ($P = .02$) relative abundance was decreased.

Principal component analysis (PCA) revealed that CAF distinctively influenced the cecal microbiota composition at the genus level according to strain. The first and second principal components explained up to 48.1% of the overall variation (PC1= 26.4%, PC2=21.7%), where groups were clearly separated according to strain and diet (Fig. 3A).

3.4. CAF diet induced distinct gut microbiota alterations in male and female F344 animals

Both diet and sex significantly contributed to the establishment of gut microbiota profiles in F344 rats as revealed by two-way ANOSIM analysis (Sex: $R=0.273$, $P=.003$; Diet: $R=0.758$, $P<.001$). Remarkably, CAF diet increased S24-7 relative abundance in females ($P=.005$), and caused a more dramatic drop of unclassified Clostridiales ($P<.001$) in female than male rats. Similar to our observations in male F344 rats, F344 females also showed raised rel-

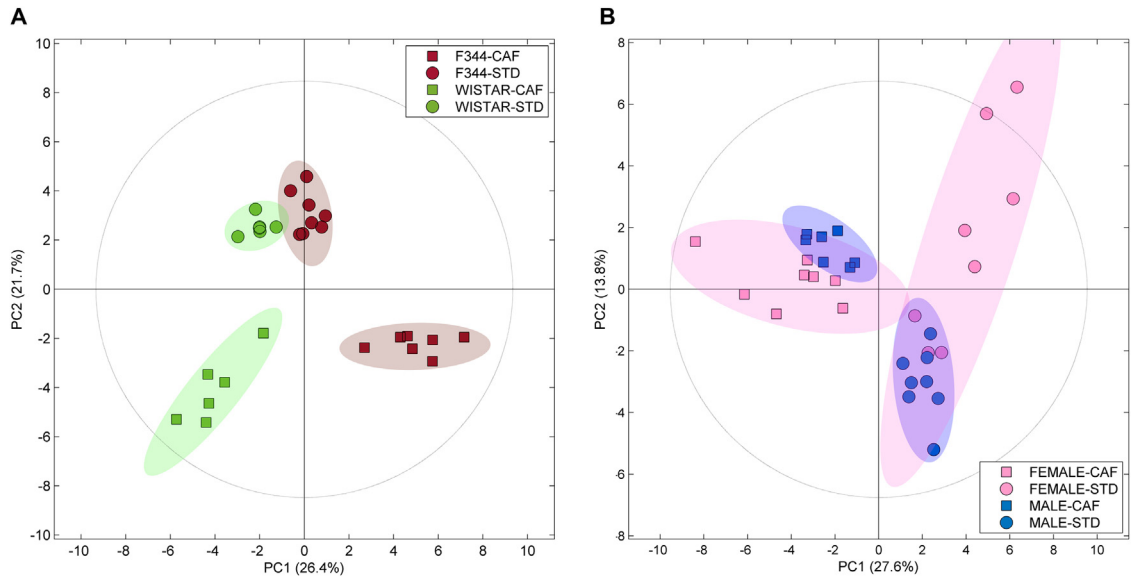

Fig. 3. Principal component analyses constructed from the relative abundance of genera representing more than 1% of bacteria at least in two samples. Circular and squared dots represent standard (STD) and cafeteria (CAF) diet-fed animals, respectively. Different colors discriminate between (A) Wistar (green) and F344 (red) rat strains, or between (B) male (blue) and female (pink) F344 rats. PC, principal component. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ative abundances of *Blautia* (*P*=.05), *Parabacteroides* (*P*=.03), *Phascolarctobacterium* (*P*=.02) and *rc4-4* (*P*=.02) when chronically exposed to a CAF diet. However, previously described changes on *Oscillospira* and *Ruminococcus* occurrences in male rats were not identified in females (Supplementary Fig S6).

Focusing on sex differences of gut microbiota composition, PCA revealed that genera were mainly influenced by diet, but showing an overlapped clustering between genders (Fig. 3B). Up to 41.4% of the overall variation was explained by both the first and the second principal components (PC1=26.7%, PC2=13.8%). Here, males and females belonging to the same dietary group (STD or CAF) were partially overlapped, even though males clustered closer to each other than females in both diets.

4. Discussion

In this study we showed that the alterations on physiologic and metabolic phenotypes induced by the chronic intake of an obesogenic CAF diet might vary according to rats' sex and strain. It is critical to understand that diet plays a major role in the development and regulation of host's health status, and therefore any differences in diet consumption between groups could be partially responsible for the changes observed. In our scenario, differences on food consumption might be related to genetic factors (*i.e.,* male rats normally eat more than females), thus generating controversy about the proper interpretation of the results. For this reason, both genetic factors (strain or sex) and dietary differences have been carefully pointed out when drawing conclusions.

Globally, similar obese phenotypes were observed within both rat strains despite that energy intake was higher in CAF diet-fed F344 than Wistar rats. However, only F344 animals developed hypertriglyceridemia and hypercholesterolemia. Long-term sugar consumption is known to have a major impact on rising triglycerides levels in plasma. In our study, F344 animals consumed lower amounts of simple carbohydrates than their Wistar counterparts under a CAF diet, suggesting that sugar intake was not responsible for the increment in triglycerides, especially observed in F344 rats. On the other hand, abnormal high complex carbohydrates intake, representing around 58% of total energy consumption in CAF diet-fed F344 rats, might induce a rise in blood triglycerides, a phenomenon known as carbohydrate-induced hypertriglyceridemia that may occur when carbohydrates represent >55% of energy intake [14]. Conversely, selective genetic conditions (*i.e.,* different metabolic adaptation capacities to dietary challenges) might determine the establishment of disparate phenotypes even under the same dietary model, as previously reported [15]. In addition, distinct diet-induced microbiota alterations should be also considered given its important role in the maintenance of host homeostasis as further discussed. On the other hand, energy intake was increased by the consumption of a CAF diet similarly in both sexes. Nevertheless, CAF-induced changes on body composition were more markedly observed in females than males, while an exacerbated increment in serum lipids of male rats indicated the establishment of a metabolically unhealthier profile in males than females. Therefore, F344 males and females, as well as male Wistar and F344 rats, distinctively responded to the same dietary model, which could be partially explained by differences on pre-established gut microbiota or even to divergent microbial responses to this challenging dietary model. However, it is crucial to point out that particular microbial changes induced by our free-choice CAF diet might be the response to distinct food preferences and macronutrients intake intrinsically linked to each strain or gender. Beyond the action of gut microbiota and differences in food consumption, several factors could have contributed to distinct metabolic and phenotypic responses to the same dietary model including differences in hormonal states, basal energy expenditure, physical activity levels, nutrients absorption and utilization or overall host metabolism. Particularly, we took a deep look into the role of gut microbiota, whose composition and activity might be distinctively altered when CAF diet is chronically offered to Wistar, F344, male or female rats.

Bacterial diversity was highly altered by our CAF diet within both F344 and Wistar rats, independently of both genetic background and phenotype. In this sense, the CAF-induced reduction of microbial diversity seems not to be linked to certain strain-specific metabolic alterations such as hypertriglyceridemia (only observed

in F344 rats). Despite that the gut microbiome can explain a substantial proportion of blood lipid levels [16], bacterial diversity has not been identified as a crucial contributor to this feature. Additionally, differences in macronutrient consumption between rat strains did not seem to modify the chronic impact of CAF diet in reducing bacterial diversity. On the other hand, we showed for the first time that CAF diet more pronouncedly altered the bacterial diversity and Bacteroidetes-to-Firmicutes ratio in female than male F344 rats. In this situation, reduced fiber consumption was observed in CAF diet-fed males and females compared to their lean counterparts. However, fiber intake was still much lower in females than males under CAF diet, even positively correlating with reduced cecum sizes, which could help to explain these differences in microbial diversity. In this scenario, the complex interaction between estrogens, bile acids and gut microbiota could be also potentially involved. The gut microbiota is known to affects the systemic levels, potency and activity of estrogens metabolites by facilitating its reabsorption [17]. Hence, CAF diet, and lastly microbial dysbiosis, might strongly disrupt the hormonal control that estrogens exert upon bile acid metabolism. In such situation, dysregulation of bile acids metabolism may contribute to alter the own microbial ecosystem, thus entering in a vicious circle. Alternatively, the proportionally greater sugar consumption observed in CAF diet-fed females in relation to their body weight could contribute to the onset of a deeper microbial dysbiosis than males, where high-sucrose diets are widely known to promote a loss of microbial diversity [18].

Healthy human gut microbiota has been widely characterized by an elevated Bacteroidetes-to-Firmicutes ratio [19,20], with Firmicutes prevailing in obese individuals. Thus, this ratio has been postulated to act as a promising biomarker of obesity susceptibility [21]. However, a meta-analysis concluded that this ratio do not differ between obese and lean people [22], thus evidencing that the evaluation of microbiota profiles at lower phylogenetic levels (genera, species) are essential to decipher its association with obesity. In our study, the Bacteroidetes-to-Firmicutes ratio markedly raised in CAF-fed Wistar rats, as previously reported [23], while this increase was not significant in CAF-fed F344 animals. Once exploring microbial genera, the important CAF diet-induced increment of obesity-related taxa such as *Bacteroides,* mainly in Wistar rats, might be due to a lower chow intake, the main dietary source of protein and fiber, and a higher sucrose intake in Wistar than F344 rats. Accordingly, *Bacteroides* abundance in rodents is linked to the long-term consumption of a diet rich in animal protein, sugar, and fat and low in fiber [24] and of a high-sucrose diet [18]. Interestingly, *Bacteroides* have been identified as one of the main bacterial taxa involved in the intestinal conversion of cholesterol into coprostanol [25]. Given the inverse relationship between serum cholesterol levels and coprostanol/cholesterol ratio in human feces [26], we hypothesize that the low relative abundance of *Bacteroides* in CAF diet-fed F344 rats (1%) could partially explain the development of hypercholesterolemia, a feature that was absent in CAF-fed Wistar rats showing an elevated *Bacteroides* occurrence (27%). On the other hand, *Prevotella* was specially favoured by CAF diet in Wistar animals. This genus has been linked to the consumption of simple sugars [24] and protein deficient diet in mice [27], while showing a reduced occurrence in rats fed low sugar-containing high-fat (HF) diets [28]. In line with previous studies [24], we have reported a bloom in *Prevotella* especially in Wistar rats consuming higher amounts of simple carbohydrates under CAF feeding than F344 rats. In addition, the reduced protein intake observed in CAF-fed Wistar rats, could also be favouring *Prevotella* prevalence.

The reduction of Firmicutes in CAF-fed animals was mainly due to the dramatic drop of an unclassified Clostridiales in both Wistar and F344 rats, as previously shown in HF diet-fed rats [29] and in people with obesity [30]. In parallel, our results suggest that the low fiber content present in our CAF diet partially drives the strain-independent depletion of *Oscillospira* abundance, which primarily utilizes plant polysaccharides as energy source [31]. In light of our suspicions, both unclassified Clostridiales and *Oscillospira* abundance were positively correlated with fiber intake (Pearson's correlation, $r(43)=0.654$, $P<.001$; $r(43)=0.636$, $P<.001$; respectively). Other less abundant genera distinctively altered by CAF diet according to rat strain included: unclassified S24-7, a core component of the normal mouse microbiome with the capacity to digest both complex carbohydrates and host-derived glycans [32] and found increased in mice fed with a high-starch diet rich in plant polysaccharides [33]; unclassified Ruminococcaceae, linked to the utilization of plant polysaccharides and decreased in HF diet-fed mice [34]; *Roseburia*, a butyrate-producer bacteria considered an essential member for a beneficial flora, even proposed to act as a health marker [35]; and Lachnospiraceae-related microbes such as *Roseburia, Blautia, Dorea* and *Coprococcus*, associated with an increased risk of obesity, elevated BMI, waist circumference and blood pressure [36]. Particularly, a clear association was identified between *Blautia, Dorea* and *Ruminococcus* and obesity-related plasma metabolites in humans [37], pointing to the potential role of these Lachnospiraceae-belonging genera in obesity development.

Finally, the role of *Akkermansia Muciniphila* in regulating host health has been widely declared, showing a negative correlation with obesity and related metabolic disorders [38,39]. However, previous studies reported an increase of *Akkermansia* in HF diet-induced obese rats [40]. In our study, its abundance was not altered after CAF feeding. Therefore, discriminating between the dietary impact and health status (*i.e.,* lean vs obese) on the composition of gut microbiome could help to understand the role of *Akkermansia*, which remains uncertain and should be further studied. These results altogether suggest that strain seems to be a determinant factor to the establishment of gut microbiota dysbiosis in DIO models, since we have seen that Wistar rats were more susceptible than F344 rats to the impact of a CAF diet on gut microbiota.

The role of sex differences in shaping gut microbial communities has been previously reported [41]. In our study, we evaluated the sex differences observed in gut microbiome due to chronic CAF feeding. Our results support the establishment of an unhealthier microbial phenotype in males than females F344 rats. In this sense, the term "unhealthy microbiota" refers to microbial populations that have widely observed in the development, establishment or course of pathology, such as obesity. For instance, the occurrence of beneficial bacteria such as *Oscillospira* was strongly depleted in males, while unclassified S24-7 abundance was increased in females after a chronic exposure to CAF diet. In addition, we found a positive correlation between *Ruminococcus* abundance and circulating lipid levels (Pearson's correlation, $r(26)=0.720$, $P<.001$), as previously reported in humans [42,43]. Particularly, we found that both hypertriglyceridemia and high *Ruminococcus* abundance co-occurred in male, but not in female rats, under CAF feeding, thereby possibly explaining the differences observed on their metabolic profiles. Importantly, while an elevated Bacteroidetes-to-Firmicutes ratio and a drop on bacterial diversity observed in F344 females might be indicating a marked microbial dysbiosis, the analysis of specific genera occurrences suggested a less detrimental response to the CAF feeding in females compared to F344 males.

Given the nature of our dietary model (free-choice based diet), the differences in both chow and CAF ingredients intake, as well as total food consumption, observed between Wistar and F344 animals constitute a limitation when discriminating between dietary or genetic contributions to the onset of microbial dysbiosis un-

der a CAF feeding. However, gut microbiota from male and female F344 rats was distinctively altered by CAF diet even in the absence of differences on food preferences. Therefore, variations in choice of CAF ingredients and their intake seem unlikely to be the main cause of such strong and distinctive alterations of gut microbial ecosystems observed in our study. Further studies using pair-fed animal models could help to validate our findings. On the other hand, we chose to individually house the rats to control CAF diet intake, which allowed correlations analyses between food intake data and physiologic, metabolic or microbial outcomes. However, social isolation can lead to increased stress levels that has been linked to changes in gut permeability, gut motility and secretion of hormones, such as corticosterone, which can lastly affect the gut microbiota composition [44,45]. Individual housing avoided any potential interference of other animals on the gut microbiota and metabolism due to their coprophagic habits, which would have made difficult to interpret the results of our study. Finally, our study design aimed to identify the presence or the absence of strain and sex (genetic background) influence on host and microbial responses to CAF diet. However, the absence of additional groups such as female Wistar rats partially limits the characterization of the exact influence of strain and sex factors to each diet-induced microbial variation. Further explorations of these contributions are encouraged, especially in this these particular animal models spready used in the field of nutrition and obesity.

In conclusion, our results provide non-previously available information about the intrinsic differences of gut microbial populations between Wistar and F344 rats, and demonstrate that feeding a CAF diet, a Western-like energy-dense and highly palatable diet, to different rat strains and sexes distinctively alters the gut microbiome and host phenotype. F344 rats developed hypertriglyceridemia and hypercholesterolemia, while Wistar animals developed a marked obese phenotype and severe gut microbiome dysbiosis under CAF feeding. CAF-induced changes on gut microbiota suggested the establishment of an unhealthier microbial phenotype in males than females F344 rats. Importantly, strain- and sex-specific preferences to the ingredients included in the CAF diet, a free choice-based dietary model, are crucial to explain the differences on food intake, gut microbiota dysbiosis and host phenotype. Overall, diet is the factor that preferably, but not uniquely, determines the microbial phylogenetic profiles and its impact on host phenotype, while robust microbiota perturbations promoted by our CAF model are distinct depending on rat strain and sex (even if partially explained by differences in food choices). Remarkably, researchers must take into account that distinct genders and strains might have different food preferences when including free-choice diets (*i.e.,* CAF diet), thus finally impacting on the study results. Our results should be considered in future studies evaluating the impact of diets on the gut microbiota composition in animal models, especially to prevent global statements that might be only validated under certain conditions (*i.e.,* specific strain or sex).

Declaration of competing interest

The authors declare that there are no conflicts of interest.

CRediT authorship contribution statement

Andreu Gual-Grau: Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Maria Guirro:** Methodology, Formal analysis, Investigation, Writing – original draft. **Noemí Boqué:** Conceptualization, Validation, Data curation, Writing – original draft, Writing – review & editing, Supervision, Project administration. **Lluís Arola:** Conceptualization, Validation, Supervision, Project administration, Funding acquisition, Writing – review & editing.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2023.109338.

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