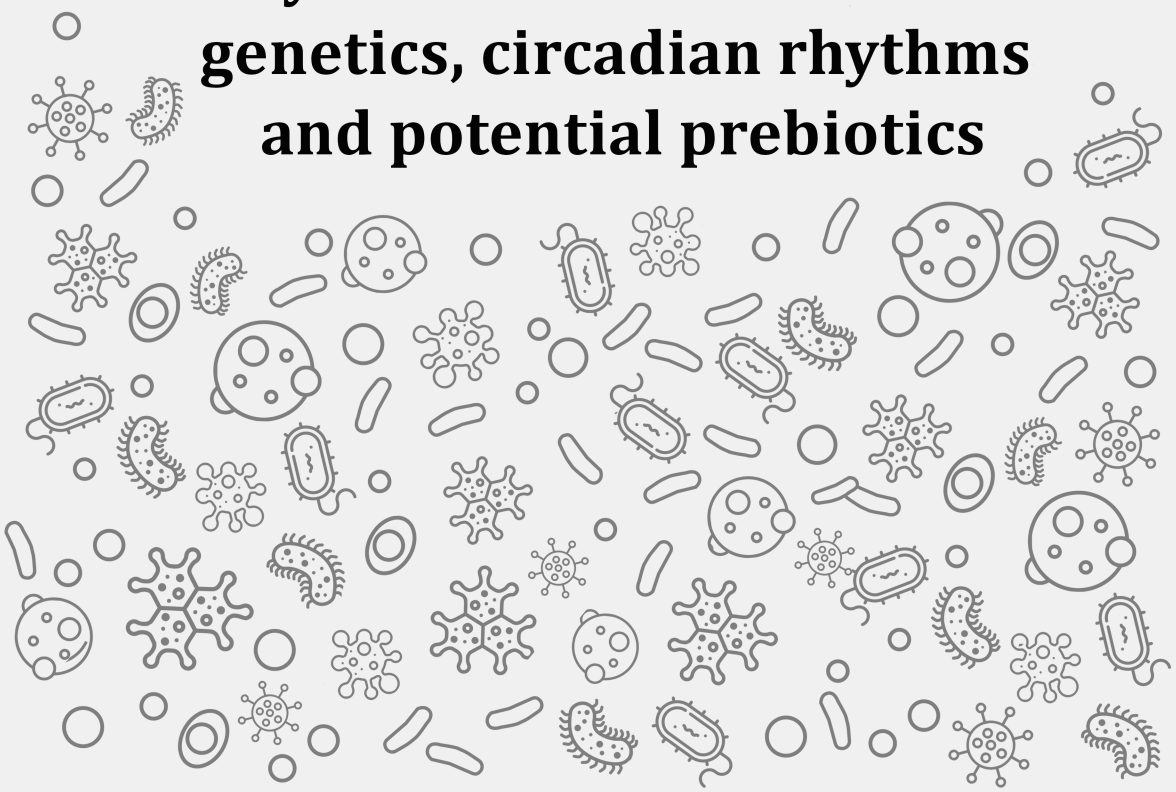
GUT MICROBIOTA DYSBIOSIS IN DIET-INDUCED OBESITY. A FOCUS ON THE INFLUENCE OF GENETICS, CIRCADIAN RHYTHMS AND POTENTIAL PREBIOTICS

Andreu Gual Grau

Gut microbiota dysbiosis in diet-induced obesity. A focus on the influence of genetics, circadian rhythms and potential prebiotics



Andreu Gual Grau

Doctoral Thesis

Department of Biochemistry and Biotechnology

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UNIVERSITAT ROVIRA i VIRGILI

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Doctoral Thesis

Supervised by Prof. Lluís Arola Ferrer and Dr. Noemi Boqué Terré

Nutrigenomics Research Group

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UNIVERSITAT ROVIRA i VIRGILI

TARRAGONA 2019

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FEM CONSTAR que aquest treball, titulat "**Gut microbiota dysbiosis in diet-induced obesity. A focus on the influence of genetics, circadian rhythms and potential prebiotics**", que presenta **Andreu Gual Grau** per a l'obtenció del títol de Doctor, ha estat realitzat sota la nostra direcció al Departament de Bioquímica i Biotecnologia de la Universitat Rovira i Virgili, i que compleix els requisits per a l'obtenció de la Menció Internacional de Doctorat.

HACEMOS CONSTAR que el presente trabajo, titulado "**Gut microbiota dysbiosis in diet-induced obesity. A focus on the influence of genetics, circadian rhythms and potential prebiotics**", que presenta **Andreu Gual Grau** para la obtención del título de Doctor, ha sido realizado bajo nuestra dirección en el Departamento de Bioquímica y Biotecnología de la Universitat Rovira i Virgili, y que cumple con los requerimientos para la obtención de la Mención Internacional de Doctorado.

WE STATE that the present study, entitled "**Gut microbiota dysbiosis in diet-induced obesity. A focus on the influence of genetics, circadian rhythms and potential prebiotics"**, presented by **Andreu Gual Grau** for the award of the degree of Doctor, has been carried out under our supervision at the Department of Biochemistry and Biotechnology from the Universitat Rovira I Virgili, and fulfil the requirements to obtain the International Doctoral Mention.

Tarragona, 9 d'Octubre de 2019 / Tarragona, 9 de Octubre de 2019 / Tarragona, 9th October 2019

Els directors de la tesi doctoral / Los directores de la tesis doctoral / Doctoral Thesis Supervisors

Dr. Lluís Arola Ferrer **Dr. Noemi Boqué Terré**

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A la meva família

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“What we know is a drop,

what we don’t know is an ocean.”

Isaac Newton (1643 – 1727)

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INDEX

SUMMARY

The host and its gut microbiota co-live in a mutually beneficial relationship, while the disruption of this equilibrium has been related to pathological states like obesity. Remarkably, gut microbial dysbiosis has a multi-factorial origin beyond the strong impact of diet. In this thesis, we deeply evaluated the influence of genetics, circadian rhythms and potential prebiotics in obesity from a metagenomic perspective.

Firstly, we characterized the impact of Westernized and semi-purified diets on gut microbiota and host phenotype in Wistar rats, thus identifying cafeteria diet as the most effective dietary model for inducing severe obesity and gut microbiota dysbiosis. Furthermore, the cafeteria diet caused a strong disruption of bile acid metabolism, SCFA production and urinary metabolome. By contrast, semi-purified diets promoted a modest fat accumulation and minor changes on gut microbiome. Then, the influence of strain and gender upon the microbial responses to cafeteria diet was addressed. Wistar rats were more susceptible to cafeteria diet-induced microbial dysbiosis than F344 animals, which was characterized by an increased Bacteroidetes-to-Firmicutes ratio, whereas obesity development similarly occurred in both strains. In addition, F344 females developed more severe alterations on biometric parameters and gut microbiota than males under a cafeteria feeding.

We further evaluated the impact of cafeteria diet and grape seed proanthocyanidins on circadian rhythms of gut microbiota. Whereas the cafeteria diet disturbed the naturally occurring microbial rhythmicity, mainly by altering the cyclical abundance of *Lactobacillus*, grape seed proanthocyanidins, which can be metabolized by intestinal microbes,

restored the microbial signatures of dysbiosis during the active period. Finally, we investigated the prebiotic capacity of several natural ingredients in an *in vitro* fermentation study with human fecal microbiota. Here, pumpkin skin promoted the largest drop in pH and an inulin-like metabolomic profile in normoweight and obese fecal samples, thus revealing for the first time its prebiotic potential.

RESUM

L'hoste i la seva microbiota intestinal conviuen en una relació de benefici mutu, on l'alteració d'aquest equilibri s'associa amb estats patològics com l'obesitat. Més enllà de l'impacte dietètic, la disbiosi microbiana té un origen multifactorial. En aquesta tesi, s'ha avaluat extensament la influencia genètica, els ritmes circadiaris i el potencial prebiòtic d'ingredients naturals en l'obesitat des d'una perspectiva metagenòmica.

Primerament, es va caracteritzar l'impacte de dietes occidentalitzades i semi-purificades sobre la microbiota intestinal i l'hoste en rates Wistar, identificant la cafeteria com el model dietètic d'inducció d'obesitat i disbiosi microbiana més efectiu. A més, aquesta dieta va alterar el metabolisme dels àcids biliars, la producció de SCFA i el perfil metabolòmic urinari. Contràriament, les dietes semi-purificades van causar una acumulació de greix modesta i canvis menors sobre la microbiota. Seguidament, es va estudiar la influencia dels factors genètics sobre la resposta microbiana a la dieta de cafeteria. Les rates Wistar van desenvolupar una disbiosi més pronunciada que les F344, caracteritzada per un increment del rati Bacteroidetes-Firmicutes, mentre que ambdues soques van desenvolupar obesitat. A més, les femelles F344 van presentar alteracions més severes sobre els paràmetres biomètrics i la microbiota que els mascles.

D'altra banda, es va avaluar l'impacte de la cafeteria i les proantocianidines de llavor de raïm sobre els ritmes circadiaris microbians. Mentre que la cafeteria va alterar la ritmicitat del microbioma, causant essencialment una disrupció en l'abundància cíclica

de *Lactobacillus*, les proantocianidines van restaurar els signes de disbiosi microbiana durant el període d'activitat.

Finalment, es va investigar la capacitat prebiòtica d'ingredients naturals en un estudi de fermentació *in vitro* amb microbiota fecal humana. La pell de carbassa va induir la major disminució de pH i un perfil metabolòmic semblant al de la inulina en condicions d'obesitat i normopès, revelant per primer cop el seu potencial efecte prebiòtic.

RESUMEN

El huésped y su microbiota intestinal conviven en una relación de beneficio mutuo, mientras que un equilibrio alterado se asocia con estados patológicos como la obesidad. Más allá del impacto dietético, la disbiosis microbiana tiene un origen multifactorial. En esta tesis, se ha evaluado extensamente la influencia genética, los ritmos circadianos y el potencial prebiótico de ingredientes naturales en la obesidad desde una perspectiva metagenómica.

Primeramente, se caracterizó el impacto de dietas occidentalizadas y semi-purificadas sobre la microbiota intestinal y el huésped en ratas Wistar, identificando la cafetería como el modelo dietético de inducción de obesidad y disbiosis microbiana más efectivo. Además, esta dieta alteró el metabolismo de los ácidos biliares, la producción de SCFA y el perfil metabolómico urinario. Contrariamente, las dietas semipurificadas causaron cambios modestos sobre la grasa corporal y la microbiota. Seguidamente, se estudió la influencia de los factores genéticos sobre la respuesta microbiana a la dieta de cafetería. Las ratas Wistar desarrollaron una disbiosis más pronunciada que las F344, caracterizada por un ratio Bacteroidetes-Firmicutes incrementado, mientras que ambas cepas desarrollaron obesidad. Además, las hembras F344 presentaron alteraciones más severas sobre los parámetros biométricos y la microbiota que los machos.

Por otro lado, se evaluó el impacto de la cafetería y las proantocianidinas de semilla de uva sobre los ritmos circadianos microbianos. Mientras que la cafetería alteró la ritmicidad del microbioma, causando esencialmente una disrupción en la abundancia cíclica de *Lactobacillus*,

las proantocianidinas restauraron los signos de disbiosis microbiana durante el periodo de actividad.

Finalmente, se investigó la capacidad prebiótica de ingredientes naturales en un estudio de fermentación *in vitro* con microbiota fecal humana. La piel de calabaza produjo la mayor disminución de pH y un perfil metabolómico similar al de la inulina en condiciones de obesidad y normopeso, revelando por primera vez su potencial efecto prebiótico.

ABBREVIATIONS

3-HCA	3-hydroxycinnamic acid
AM	*Armillaria mellea*
AMPK	AMP-activated protein kinase
ANOSIM	Analysis of similarities
ANOVA	Analysis of variance
BA	Bile acids
BE	*Boletus edulis*
BMI	Body mass index
BSG	Brewers' spent grain
BSH	Bile salt hydrolase
CA	Cholic acid
CAF	Cafeteria
CDCA	Chenodeoxycholic acid
CG	Cresyl glucuronide
CS	Cresyl sulphate
CVD	Cardiovascular disease
DCA	Deoxycholic acid
DIO	Diet-induced obesity
DMA	Dimethylamine
DMG	Dimethylglycine
DMSO2	Dimethylsulfone
DP	Degree of polymerization

EWAT	Epididymal white adipose tissue
F344	Fischer 344
FDR	False discovery rate
FFA	Free fatty acids
FIAF	Fasting-induced adipose factor
FXR	Farnesoid-X-receptor
GAA	Guanidinoacetic
GCA	Glycocholic acid
GLP	Glucagon-like protein
GPC	Glycerophosphocholine
GPCR	G protein-coupled receptor
GSPE	Grape seed proanthocyanidins extract
HCA	Hierarchical clustering analysis
HDCA	Hyodeoxycholic acid
HDL	High-density lipoprotein
HF	High-fat
HFS	High-fat, high-sucrose
HOMA-IR	Homeostatic model assessment for insulin resistance
HPPA	Hydroxyphenylpyruvic acid
IGN	Intestinal gluconeogenesis
IM	Irish moss
IS	Indoxyl sulfate
IWAT	Inguinal white adipose tissue
LAB	Lactic acid bacteria

LCA	Lithocholic acid
LDL	Low-density lipoprotein
LF	Low-fat
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
MCA	Muricholic acid
MetS	Metabolic Syndrome
MWAT	Mesenteric white adipose tissue
NAG	N-acetyl-glycoproteins
NEFA	Non-esterified fatty acids
NMNA	N-methylnicotinic acid
NMR	Nuclear Magnetic Resonance
NOD	Non-obese diabetic
NOESY	Nuclear overhauser effect spectroscopy
NS	Nutshell
OG	Oxoglutarate
OPC	Olive press cake
OPLS-DA	Orthogonal Projections to Latent Structures Discriminant Analysis
OTU	Operational taxonomic unit
OWAT	Periovarian white adipose tissue
PBS	Phosphate buffer saline
PC	Principal component
PCA	Principal component analysis

PCoA	Principal coordinate analysis
PluS	Plum skin
PS	Pumpkin skin
PYY	Peptide YY
QH	Quinoa husk
RAAS	Renin-angiotensin-aldosterone system
RH	Rice husk
ROS	Reactive oxygen species
RWAT	Retroperitoneal white adipose tissue
SB	Stevia branches
SCFA	Short chain fatty acids
SEM	Standard error of the mean
STD	Standard
TAG	Triacylglycerides
TCA	Tricarboxylic acid cycle
TCDCA	Taurochenodeoxycholic acid
TDCA	Taurodeoxycholic acid
UDCA	Ursodeoxycholic acid
UHPLC	Ultra High Performance Liquid Chromatography
VEH	Vehicle
VLDL	Very low-density lipoprotein
WHO	World Health Organization
ZT	Zeitgeber time

I. INTRODUCTION

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1. OBESITY AS A GLOBAL HEALTH PROBLEM

The World Health Organization (WHO) defines obesity as an abnormal or excessive fat accumulation that may impair health [1], which results from an interaction between lifestyle and genetic susceptibility [2]. The global prevalence of obesity has doubled in more than 70 countries since 1980, accounting for up to 600 million obese adults worldwide in 2015 [3]. If this trend continues, it is estimated that global obesity prevalence increase from 11% to 18% in men and from 13% to 21% in women by 2025 [4]. In addition, obesity constitutes an important risk factor for the development of many other chronic diseases including type II diabetes, hypertension, irritable bowel syndrome and cardiovascular diseases (CVD) [5]. For this reason, the prevention and treatment of obesity have become a global challenge over the past decades. Likewise, several factors are involved in the health outcomes and risks associated with an obese phenotype, including sex, age, ethnicity, socioeconomic standards, exercise levels and dietary habits [2].

Remarkably, fat quantity, distribution and metabolic function are essential to characterize a heterogeneous condition like obesity [6,7]. In this regard, the physiological impact of subcutaneous or abdominal obesity has been clearly distinguished, being visceral fat accumulation a major risk factor for obesity-related metabolic disorders (**Figure 1**). Visceral fat depots are characterized by enlarged and dysfunctional adipocytes that secrete pro-inflammatory biomarkers (i.e. prostaglandins, C-reactive protein and cytokines) contributing to systemic inflammation [8,9]. Inflammatory mediators disrupt the signalling mechanisms of insulin within the adipocytes leading to insulin

resistance [10]. Therefore, the impairment of insulin functions, including the inhibition of lipolysis, promotes a greater non-esterified fatty acids (NEFA) production and secretion from the adipose tissue to the bloodstream [10]. Consequently, NEFAs are build-up in the liver, transformed and accumulated as triglycerides, thus resulting in an increased very-low density lipoprotein (VLDL)-cholesterol production [11–13]. Moreover, excess NEFAs are taken up by skeletal muscle and reesterified into diacylglycerol, which induces a protein kinase C-mediated inhibition of the insulin receptor substrate 1/2, thereby disturbing the insulin-mediated glucose uptake and contributing to insulin resistance [14]. In addition, the reduced secretion of adiponectin from the adipose tissue during obesity, which has an important role in glucose and lipid homeostasis, favours insulin resistance through the inhibition of insulin receptors [15]. Interestingly, hyperinsulinaemia could be misinterpreted as the result of an increased release from the pancreas to compensate NEFAs-induced insulin resistance, whereas NEFAs are also lipotoxic to beta cells causing an impaired insulin production [16]. Therefore, the desensitization of peripheral organs to insulin action leads to an increased glucose accumulation in the blood and to the development of type II diabetes.

The established communication network between visceral adipose tissue, sympathetic nervous system and brain can influence appetite, energy balance, insulin sensitivity, angiogenesis, blood pressure, lipid metabolism and low-grade inflammation. The complex system regulating food intake and body weight involves peripherally synthetized hormones such as leptin and ghrelin, which target specific receptors in the hypothalamus and initiate the host response by promoting changes in

food intake [17]. In this sense, visceral depots act as an active paracrine and endocrine organ that releases adipokines and hormones able to regulate metabolism and inflammation [18]. Particularly, leptin levels increase in proportion to increasing adiposity, thus promoting energy expenditure and decreasing food intake [19]. Interestingly, the increment of leptin expression is likely to be partially explained by hypoxia occurring in the obese state, which favours the differentiation of preadipocytes into leptin-secreting cells [20]. However, the high levels of circulating leptin observed in obese and overweight subjects fails to suppress appetite [21], thereby suggesting a state of leptin resistance [22]. Insulin and leptin resistance at the hypothalamic level decreases satiety and energy expenditure, and thus promotes food intake [23].

In addition, the hyperactivity of hypothalamic-pituitary-adrenal axis results in a cortisol-mediated increment of circulating glucose, food intake and visceral fat deposition [24]. Altogether with the dysregulated suppression of ghrelin secretion in response to stomach fullness, which leads to a continuously favoured food intake and body weight gain [25], there is a failure on the controlling systems of eating behaviour in obesity, thus aggravating the pathology.

Abdominal obesity is an important component of metabolic syndrome (MetS), which is a heterogeneous disorder characterized by a wide range of physiologic and metabolic alterations. An international consensus unified the diagnosis criteria for MetS in 2009, stipulating that MetS is present when at least three out of five of the following risk factors are true: elevated waist circumference (men >102cm; women >88cm), high blood pressure (systolic ≥130mmHg and/or diastolic ≥85mmHg), increased triglycerides (≥1.7mmol/L), reduced HDL cholesterol (men

≤1.0mmol/L; women ≤1.3mmol/L) and increased fasting blood glucose (>5.5mmol/L) [26]. It is well established that each constituent of MetS represent an independent risk factor for CVD [27–31]. Visceral obesity is a better predictive risk factor for mortality and morbidity than BMI or fat percentage, which show a modest association with the incidence of CVD [32–34]. In this sense, the resistant individuals to adiposity-associated cardiometabolic abnormalities are considered 'metabolically healthy' obese [35,36].

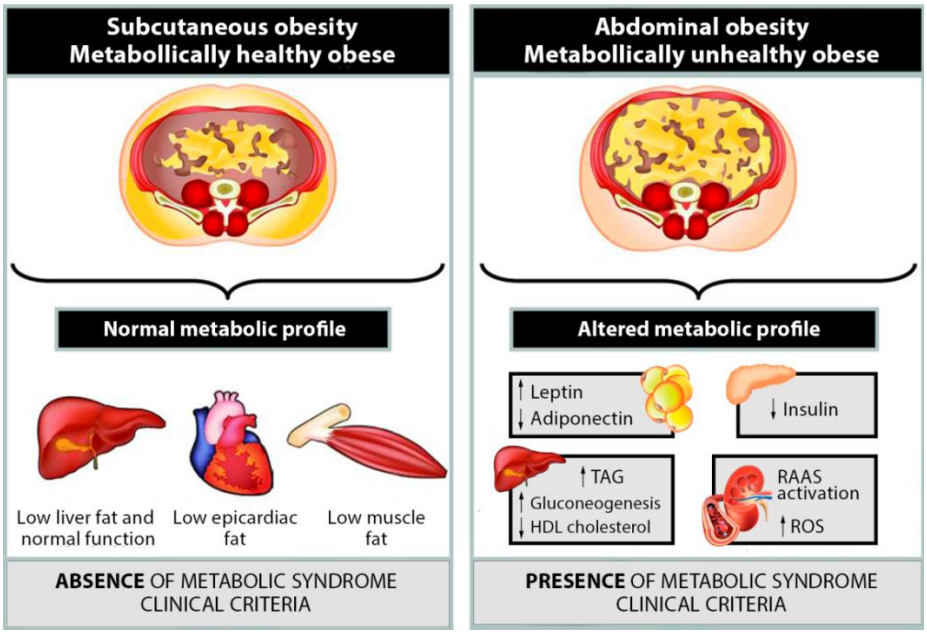

Figure 1. The fat accumulation into insulin-sensitive subcutaneous adipose tissue, which is able to expand through hyperplasia, protects the subject against the development of metabolic syndrome (MetS) under a positive energy balance. However, visceral adipose tissue develops insulin resistance and shows a limited ability to store the energy excess, altogether with vasoconstrictor effects (high blood pressure) and the generation of oxygen reactive species (ROS) promoted by the adipose tissue-mediated activation of renin-angiotensin-aldosterone system (RAAS). The metabolic consequences of excessive visceral adiposity include NEFA-induced insulin resistance, pancreatic lipotoxicity, hepatic fat storage, leptin resistance, and a prothrombotic and inflammatory profile, which are features related to the development of the MetS. Adapted from [37].

2. THE GUT MICROBIOTA

The term microbiota refers to the ecological community of microorganisms, constituted by myriad prokaryotic and eukaryotic organisms including archaea, bacteria, viruses, fungi and protozoans, residing in a particular environment [38]. The earth is dominated by approximately 10^{30} microbial cells forming ecological niches [39], which are largely determined by the environmental conditions [40]. The overlapped microbial organisms living in a specific habitat frequently compete for energy resources [41], while divergent strategies to exploit them allow their co-occurrence avoiding the competition and favouring a diverse community [42]. As result, multiple networks of complex interactions between the own microorganisms inhabiting specific locations exist. Besides to ecological niches, animal and plant evolution has co-occurred in the presence of microbiota, encompassing commensal, parasitic, mutualistic, or even pathogenic interactions with the host [43–45]. In the last years, the exhaustive research looking out the microbiota has been supported by its collective functional role in managing host development, metabolism, immunology and behaviour [46–49].

The human body contains an extensive assortment of microorganisms inhabiting every surface that is exposed to the external environment, namely the skin, the urogenital organs, the respiratory system and the digestive tract, while providing a broad range of functions to the host. In the past decades, it was commonly accepted that bacteria residing in the human body outnumbered human cells by 10 times [50], whereas actually it is accepted that the ratio between somatic and microbial cells is much closer to 1:1 [51,52]. Nevertheless, microbial genes exceed by far

our human genome, existing around 500 – 1000 species of bacteria at any one time [53]. The microbial communities are functionally adapted to their specific niche, presenting important compositional variations across distinct body habitats [54]. In this regard, the microbes inhabiting the human gut, which are known as the gut microbiota, establish the more complex, diverse and abundant community in the body [52]. Along the gastrointestinal tract, microbiota widely varies on its density and composition, being the colon the most colonized organ accounting for approximately 70% of all bacteria in the human body [55]. Despite the enormous biodiversity of gut microbiome, its phylogenetic structure is mainly composed of four major phyla: Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria; where only Firmicutes and Bacteroidetes are estimated to represent the 90% of gut microbiota [56,57]. However, the phylum taxonomic classification is subsequently divided into classes, orders, family, genus and species, thus evidencing the complexity of this microbial ecosystem. Because of the wide-ranged functions that residing microbes exerts in the host, the role of gut microbiota in health and disease has become an important focus for the scientific community in the last years. Particularly, the involvement of gut microbiota in the progression of obesity has been broadly reported [58], and therapeutic strategies targeting microbiota in the prevention and treatment of obesity are constantly emerging.

2.1 Gut Microbiota, Metabolism and Host Homeostasis.

The gut microbiota exerts several functions including vitamin production, protection against pathogens, enhancement of immune system and the fermentation of the non-digestible compounds from the diet [59]. Overall, gut microbiota and host are in a constant and mutually

beneficial crosstalk where intestinal microbes benefit from nutrient supply in a stable environment, while the host takes advantage from the fermentation products such as short-chain fatty acids (SCFA) and vitamins.

Naturally, the gut is the central organ for the digestion and absorption of food, and gut microbiota actively contributes to the enhancement of these processes. In this regard, the digestive functions of the own intestine are concomitant to the microbial action, interacting together to optimize the energy harvest by the host. The gut microbiota is thought to have a role in the development of gut epithelium, partially through the increment of capillaries underlying the villi in the small intestine and the influence on gut motility [60]. Likewise, gut microbiota promotes nutrients uptake and transfer to the liver and adipose tissue, thus regulating energy storage [61]. Importantly, fermentation of non-digestible components of the diet such as complex polysaccharides and certain polyphenols results in valuable microbial products for the maintenance of colonic health and energy homeostasis [62]. Moreover, these bacterial products can act as signalling molecules that regulate the host's metabolism by influencing peripheral tissues such as brain, liver, adipose tissue and muscle [63]. Collectively, gut microbiota plays also an essential role in the regulation of fat storage and energy homeostasis by modulating host genes [64].

2.2 Metabolism and Function of Short-Chain Fatty Acids and Bile Acids.

Non-digestible polysaccharides of edible plants including celluloses, hemicelluloses, pectins, and oligosaccharides such as inulin,

oligofructoses and resistant starches, are not digested by the human gut. These substrates pass intact through the upper part of the intestine, reaching the colon where they could undergo microbial fermentation. The gut microbiota utilize these substrates to obtain energy for growth while they provide the host with end products such as SCFAs (mainly butyrate, propionate and acetate) [65]. Rapidly fermentable non-digestible polysaccharides often lead to the production of lactate by lactic acid-producer bacteria (LAB), whereas the fermentation of protein-derived branched chain amino acids may also contribute to SCFA production [66,67].

The SCFA profiles generated in the gut are determined by the amounts and type of non-digestible polysaccharides consumed, as well as by the gut microbiota activity [68]. The SCFA directly turns into energy harvest for microbial and host's cells [69]. In fact, butyrate was reported as the preferred energy source for isolated rat colonocytes contributing up to 70% of their energy requirements [70], while SCFAs are known to provide nearly 10% of our daily caloric supply [71]. In this sense, germ-free mice compensate their reduced capacity to extract energy from the diet by increasing their food intake [72]. These animals also show a reduced adiposity when compared to their conventional counterparts [61]. In humans, obese people display high amounts of fecal SCFAs, thus suggesting an increased energy harvest from the diet as compared to lean people [73].

In addition to their role as energy sources, SCFAs control several physiological processes including energy expenditure and satiety (**Figure 2**) [74,75]. Most SCFAs are absorbed by the enterocytes, where they may be released into the portal circulation and utilized in

peripheral tissues [76]. Importantly, AMP-activated protein kinase (AMPK) is a sensor of the energetic requirements that promotes catabolism and impedes anabolism in order to balance energy status in the cells. Hence, an increased AMPK activation has been associated with an energy-deprived status occurring in germ-free mice, thus favouring the energy utilization in liver and skeletal muscle and, lastly, protecting them from diet-induced obesity [77,78]. Since the AMPK activation is favoured in germ-free animals, gut microbiota-derived SCFAs also activate AMPK in peripheral tissues, thereby promoting catabolic pathways and energy expenditure [79]. In fact, SCFA mediate the beneficial effects of dietary fiber on the MetS by shifting the metabolism of adipose tissue and liver from lipogenesis to fatty acid oxidation through an AMPK-dependent mechanism [80]. In this sense, the established association between high fecal SCFA concentrations and obesity in humans could be partially due to an insufficient SCFA-mediated AMPK activation, which, in turn, is inhibited by proinflammatory cytokines [81]. Alternatively, it was suggested a less efficient colonic absorption of SCFAs in obesity, although more studies assessing their circulating and fecal levels should be done to corroborate this hypothesis [82].

The SCFAs also act as signalling molecules through the activation of specific G-protein coupled receptors (GPCR) 43 and 41, which are mainly expressed throughout the small intestine and colon and also known as free fatty acid receptors 2 and 3 (FFA2 and FFA3), respectively [68]. However, these receptors are also expressed in a wide range of peripheral tissues suggesting that SCFAs have a specific role beyond the intestine. Upon activation of these receptors, the release of hormones

involved in signalling satiety from the gut to the brain, such as glucagon like peptide-1 (GLP-1) and peptide YY (PYY), is stimulated [83]. Both GLP-1 and PYY are secreted by enteroendocrine L-cells, which are most abundant in the distal part of the small intestine and the colon. Consequently, these hormones activate the insulin secretion while inhibit glucagon in the pancreas, thus leading to an increased utilization of glucose and a reduced cholesterol synthesis in peripheral tissues. Moreover, insulin secretion decelerates the gastric emptying and promotes satiety, while PYY boosts the insulin action on glucose uptake by adipose tissue and muscle [84,85]. Overall, the SCFA-induced production of these anorexigenic hormones by intestinal L-cells inhibit food intake and reduce weight gain [86,87].

The SCFAs reduce the appetite by inducing the expression of leptin in the adipocytes in a GPCR-mediated pathway [88]. In this line, it has been shown that FFA3 KO mice exhibited a reduction of energy expenditure, as also occurred in FFA2 KO mice when fed a high-fat diet [89,90]. Therefore, FFA2 and FFA3 receptors are suggested to play a critical role in managing the energy homeostasis in response to the pool of SCFAs produced in the gut. In this regard, it has been shown that SCFAs increase the oxygen consumption, mitochondrial function, thermogenesis and fat oxidation in rodents [91].

As stated above, the gut microbiota composition also defines the SCFA profile produced in the gut. For instance, LAB like *Lactobacillus* produce SCFAs from the end-products of carbohydrates degradation such as pyruvate, whereas *Bifidobacteria* is able to produce acetate under carbohydrate limitation [92,93]. Therefore, these bacterial genera are considered main targets for dietary stimulation and commonly used as

markers of a healthy gut microbiota. Overall, SCFAs can exert multiple beneficial effects on mammalian metabolism and a healthy gut microbiota composition is a key determinant to its balanced production.

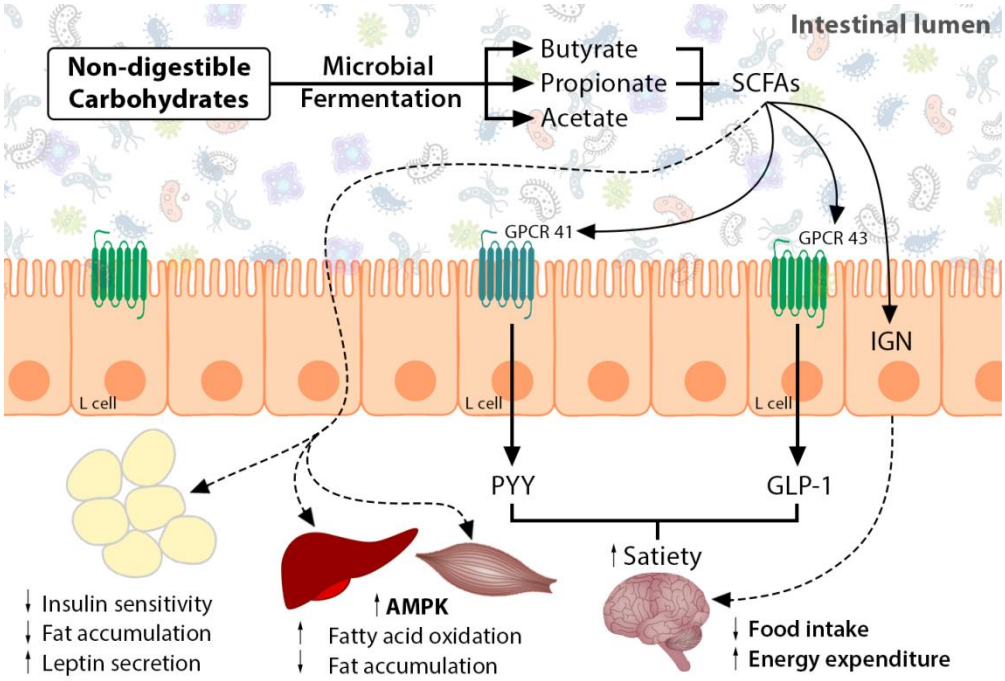

Figure 2. Schematic overview of the SCFA effects on the host metabolism. In the distal gut, SCFA bind to GPCR 41 and GPCR 43 leading to the production of the gut hormones PYY and GLP-1, which signal satiety to the brain, thus reducing food intake and increasing energy expenditure. Intestinal gluconeogenesis (IGN) also promotes satiety by increasing circulating glucose levels. In white adipose tissue, SCFAs decrease insulin sensitivity and decrease fat storage, while there is an increase in leptin production. Leptin also favours the reduction of the appetite. In muscle and liver, SCFAs phosphorylate and activate AMPK, thus inducing fatty acid oxidation.

Bile acids are essential molecules that actively contribute to the emulsification, solubilisation and absorption of dietary lipids reaching the intestine [94]. In hepatocytes, cholesterol is oxidised in a multistep pathway to generate the primary bile acids. In humans, the liver synthetize cholic acid (CA) and chenodeoxycholic acid (CDCA); whereas

CA, CDCA and muricholic acid (MCA), which is a 6-hydroxylated derivative of CDCA, are synthetized by rodents. Subsequently, primary bile acids are conjugated with either taurine or glycine in the liver, which favours their solubility at physiological pH and minimizes passive absorption [95]. Then, bile acids pool is conducted to the gallbladder, where it is stored during the fasting stage. Upon food consumption, the gallbladder is contracted and the bile acids are released into the duodenum. Remarkably, bile acid synthesis is regulated by a feedback loop through the expression and activation of the farnesoid X receptor (FXR) in the epithelial cells of the gut, which is critical for bile acid and lipid homeostasis [96].

Bile salt hydrolases (BSH) are microbial enzymes that cleave the bond between glycine or taurine and the bile acid core. Several genera such as *Lactobacillus* and *Clostridium* are involved in the deconjugation of primary bile acids because of their elevated BSH activity, thus allowing the transformation into secondary bile acids [94]. Deconjugated primary bile acids undergoes dehydroxylation process, which involves a multistep biochemical pathway that is only performed by anaerobic gut bacteria [97]. CA and CDCA, and MCA in rodents, might be 7α – dehydroxylated to form secondary deoxycholic acid (DCA) and lithocholic acid (LCA); whereas ursodeoxycholic acid (UDCA), the 7β-epimer of CDCA, yields LCA. In addition, the oxidation and epimerization of bile acids by gut microbiota result in bile isoforms. For instance, the epimerization of CDCA to UDCA decreases the toxicity of this hydrophobic bile acid for gut microbiota [98]. Importantly, up to 95% of bile acid pool is efficiently reabsorbed into the portal blood circulation to the liver. Therefore, the BSH activity primarily determines the

composition and function of the bile acid pool in the gut, which is involved in several physiological processes including cholesterol and lipid metabolism, epithelial cell homeostasis, intestinal immune function and host circadian rhythms [99,100]. Interestingly, it was shown that BSH-mediated deconjugation of TβMCA, a potent antagonist of the host bile acid receptor FXR, induces significant shifts in bile acid profiles in murine, thereby highlighting the physiological implications of bacterial BSH activity [101].

Bile acids can also act as signalling molecules through specific GPCRs present in enteroendocrine L-cells [102]. The activation of these signalling pathways yields to a wide range of beneficial metabolic effects, such as enhanced insulin sensitivity and energy expenditure [103,104]. Importantly, the activation of bile acid-specific GPCRs relies on the composition of bile acid pool, which, in turn, is strongly determined by the gut microbiota activity. For instance, LCA, DCA and CDCA are natural agonists of TGR5, one of the three sensible members of GPCR superfamily to the action of bile acids [102]. Therefore, external factors altering the phylogenetic structure and activity of gut microbiome are potentially implicated in the disruption of bile acid metabolism, thus impacting host physiological and metabolic status.

Finally, bile acids also shape the composition of gut microbiota by altering the membrane integrity of bile-sensitive bacteria [105]. In this regard, stressors such as high-fat diet leading to abnormal secretion of bile acids may potentially disrupt the gut microbiome composition and function that, in turn, negatively impacts on host lipid metabolism [106].

3. DYSBIOSIS OF GUT MICROBIOTA

The gut microbiota exerts an important role in the maintenance of host homeostasis and it is considered to be at the intersection of diet and human health [107]. In this sense, gut microorganisms and host are under a constant cross-talk that allows mutual benefits. However, gut microbes are continuously exposed to external factors such as diet, antibiotics or stress, which can induce alterations on its composition and, consequently, perturb its interaction with the host. In fact, the ability of microbial community to resist these perturbations or to recover to its original form have been attributed to healthy individuals [108–110]. In contrast, the strong and continuous imbalance in the phylogenetic structure of the gut microbiota, which is known as dysbiosis, has been closely associated with the development of various diseases [111]. This imbalance can be the result of the overall loss of bacterial diversity or changes in specific taxa including a reduction of beneficial microorganisms and the expansion of potentially harmful microbes, which may all occur concurrently [112]. For instance, microbial changes at phylum level altogether with a depleted diversity have been reported in human obesity and inflammatory bowel disease [113,114].

Dysbiosis of gut microbiota leads to a disrupted gut barrier integrity, increasing its permeability and allowing the translocation of bacterial pro-inflammatory toxins such as lipopolysaccharides (LPS). The LPS-induced suppression of fasting-induced adipose factor (FIAF) also favours the accumulation of triglycerides in muscle and adipose tissue [115]. Therefore, microbial dysbiosis actively contributes to the onset of obesity and related chronic low-grade inflammation.

3.1 Gut Microbiota Dysbiosis and Obesity.

Given the increased prevalence of obesity in Western societies [116], the interconnection between dietary patterns, microbiota and obesity development is under constant revision. Further studies have demonstrated that gut microbiota composition differs between lean and obese subjects. For instance, a reduction of bacterial diversity and low Bacteroidetes proportions were found in obese individuals [117]. In this line, another study reported a diminished occurrence of Bacteroidetes in obese subjects when compared to normoweight and anorexic patients, whereas no differences in Firmicutes were described [118]. Moreover, a lower Bacteroidetes-to-Firmicutes ratio was found in obese and overweight subjects as compared to lean ones [119], showing a clear association with an increased BMI [120]. In addition, elevated levels of Firmicutes altogether with depleted Bacteroidetes were also determined in obese children [121]. Therefore, Bacteroidetes-to-Firmicutes ratio has been broadly utilized as a marker of obesity susceptibility. However, significant differences on phylogenetic structure of gut microbiota at phylum level were not found between obese and lean children and adolescents [122,123]. In 2014, a meta-analysis concluded that there was no evidence of a clear trend between the Bacteroidetes-to-Firmicutes ratio and the obese state in humans [124], which was recently corroborated [125]. Altogether, heterogeneous populations, divergent methodologies and insufficient confounder control contribute to discrepancies between studies.

Nevertheless, large differences on the microbiota composition between obese and lean individuals at deeper taxonomic levels have been widely reported in the literature, suggesting that dysbiosis of gut microbiota is

strictly related to the development of obesity. In this regard, the number of gut microbiota genes was revealed as a measure of bacterial richness and it was found associated with the host phenotype. Higher adiposity and insulin resistance, altogether with an increased occurrence of pro-inflammatory genera such as *Bacteroides*, *Parabacteroides* and *Ruminococcus*, among others, were observed in individuals showing low microbial gene counts, whereas anti-inflammatory species such as *Faecalibacterium prausnitzii* were detected in people with high gene counts [126]. Accordingly, individuals with reduced microbial gene richness presented a more pronounced metabolic dysregulation accompanied by low-grade inflammation [127].

Several mechanisms explaining the role of gut microbiota in obesity development have been proposed. Interestingly, obese microbiota was related to an increased capacity to harvest energy from the diet [113]. In this sense, an increased SFCAs production was reported in genetically obese mice, whereas transplantation of gut microbiota from obese humans to germ-free mice resulted in an increased body fat storage when compared to germ-free mice receiving lean microbiota, thus suggesting that obese microbiome has an increased capacity to energy harvest [113]. Moreover, gnotobiotic mice colonized with gut microbiota from conventional mice resulted in increased energy storage even showing a decreased energy intake and raised energy expenditure, which is consistent with a microbiota-mediated rise in energy harvest [61]. Therefore, gut microbiota was postulated to be a crucial contributor to the acquirement of an obese profile. However, similar studies by manipulating the gut microbiota and examining energy harvest have not been done in humans to date.

On the other hand, the beneficial properties of SCFA in the modulation of energy metabolism and food intake, as described above, seem to contrast with the elevated SCFA levels observed in obese individuals [73]. Therefore, the mechanisms explaining the gut microbiota role on obesity should go beyond an increased energy harvest capacity associated with SCFA production. In this regard, the translocation of bacterial products such as LPS through intestinal barrier is regarded as a key mechanism explaining obesity. Moreover, as gut-derived signals are continuously reaching the liver via portal vein, a consistent relationship between microbiota dysbiosis and non-alcoholic fatty liver disease has been reported in obese individuals [128]. Overall, the indirect interaction between microbiota and the metabolic functions in peripheral organs are postulated as the main mechanisms by which microbiota dysbiosis may contribute to the obesity development [129].

3.2 Animal Models of Diet-Induced Obesity and Gut Microbiota.

Different rodent models are used for investigating the role of the gut microbiota in the progression, prevention and treatment of obesity. In addition to genetically obese rodents, diet-induced obesity (DIO) is the most common model employed to evaluate this interaction because of the rapid shift on microbial communities in response to dietary change. In fact, the metabolic pathways and gene expression of the gut microbiota changed in a single day when plant-based high-polysaccharide diet was replaced by a high-fat high-sugar diet [130].

Semi-purified high-fat diets (HF) have been the most extensively dietary model used to induce obesity and investigate the microbiota-related

consequences. The development of HF diet-induced obesity is associated with an enrichment in Firmicutes at the expense of Bacteroidetes altogether with a reduction of bacterial diversity, as broadly reported [131–133]. Furthermore, consistent changes in the community membership of several genera were identified in HF diet-fed rodent when compared to those fed with a low-fat diet (LF) [134]. Here, Carmody *et al.* showed that rats fed with a HF diet showed a dramatic drop in the abundance of *Lactobacillus*, which was previously found to promote the reduction of body weight in both rats and humans [135,136], positively correlate with the lifespan of mice [137], and maintain intestinal barrier integrity [138]. In this regard, different studies have demonstrated that HF diets induce a disruption of intestinal permeability, leading to LPS and bacteria translocation, thus promoting body weight gain, insulin resistance and inflammation [139,140].

Consistently, cafeteria (CAF) diet, based on the free-choice of highly palatable and energy-dense foods with high contents of simple sugars, has emerged as one of the most representative dietary model of the Western populations. Moreover, severe microbiota dysbiosis has been reported after the consumption of a CAF diet, altogether with a decompensated mechanism of satiety that finally leads to exacerbated food consumption (hyperphagia) [141]. This diet robustly reproduces the main features of human MetS including obesity, dyslipidaemia, hypertension and insulin resistance [142].

3.2.1 Sexual Dimorphism of Gut Microbiota.

Sexual dimorphism, defined as the exhibition of gender-dependent responses to a given condition, is characteristic of several common diseases including obesity, metabolic and cardiovascular-related

disorders, which, in turn, are closely associated with imbalanced gut microbial ecosystems [143,144]. In fact, Mestdagh *et al.* showed that germ-free animals do not display sexual dimorphism on the total body fat content, thus suggesting a direct and measurable impact of gut microbiota on the host phenotype [145]. Furthermore, the sexual dimorphism in the establishment and responses of gut microbiota has been recently reviewed [146], thereby supporting that gender is an important factor to consider when examining the gut microbiota-host interactions. In this regard, the comparison between male and female microbiota revealed that puberty-related hormonal changes caused a separation of microbial communities that was not previously observed during the pre-puberty stage in mice [147]. Consistently, sex hormones have been proposed to be responsible, at least in part, for the establishment of the gut microbiota, whereas the mechanisms explaining this observation remain unclear. Supporting these remarks, a testosterone treatment in gonadectomised male mice prevented the significant changes in microbiota composition further observed in untreated controls [148]. In this sense, a significant depletion of *Akkermansia* was revealed in gonadectomised females under a high-fat high-sucrose (HFS) diet as compared to sham controls fed with the same diet. Moreover, the hormonal status was shown to affect the microbiota composition in both chow and HFS diet-fed male mice, whereas this effect was more clear in females under a HFS feeding than under a chow diet, thus revealing that diet impacts on hormonal regulation of gut microbiota [148]. Here, Org *et al.* also showed significant hormone-mediated changes in bile acid profiles. Altogether, since gut microbiota is known to modulate bile acids pool, they provided a microbiome-

mediated mechanism for explaining sex differences in bile acid composition.

In addition, non-obese diabetic (NOD) mice display a high incidence of type I diabetes, being more prevalent in female than in male rodents [149]. Interestingly, the colonization of NOD female germ-free mice with gut microbiota from adult male mice resulted in elevated serum testosterone in the recipients, thereby robustly protecting female recipients from type I diabetes [150]. Thus, hormone levels are regulated by the microbial communities showing protective effects in mice with high genetic risk for disease. Remarkably, a recent study has demonstrated that gut microbiome mediates the preventive effect of estrogens on metabolic endotoxemia and low-grade chronic inflammation, which are recognised as ones of the major causes of MetS [151]. Finally, taxa associated with increased visceral fat, despite showing moderate overlapping at family and genus levels, were not shared at species-level between men and women, potentially suggesting that sexual hormone-sensitive bacteria are responsible for abdominal obesity [152]. Therefore, the involvement of gut microbiota in the onset, progression and establishment of obesity depends on gender as an important modulatory factor on the gut microbiota-host interaction.

4. MICROBIOTA AS A THERAPEUTIC TARGET

4.1 Dietary Modulation of Gut Microbiota.

A sedentary lifestyle and a raised preference for Westernized diets have contributed to increase the prevalence of obesity and related metabolic complications. Dietary patterns have been shown to exert a critical impact on gut microbiota by causing rapid shifts on its composition and function, which has been described in distinct mammalian species [153]. In this sense, the Western diet strongly disturbs the gut microbiota diversity and composition, leading to an increased risk of obesity and CVD. For instance, main features of a Western diet, such as higher energy intake, snacking, and whole milk consumption were negatively associated with microbiota diversity [154]. Interestingly, up to 57% of the phylogenetic structure of the gut microbiome can be explained by dietary changes, whereas genetic background describes around the 12% of the microbiota composition in mice [155]. Therefore, dietary therapy is postulated as one of the most effective tools for the re-establishment of a healthy microbiota as a mechanism to fight against obesity and related disorders.

Long-term diets have been correlated with certain microbial enterotypes, which are defined as the consortia of intertwined bacteria according to their expression patterns [156]. In this sense, *Bacteroides*-dominated enterotype, including *Parabacteroides*, *Lactobacillus*, *Clostridiales* and *Alkaliphilus* genera, is characterized by carbohydrate utilization, proteolytic activity and the production of pro-inflammatory agents, whereas *Prevotella*-dominated enterotype is considered to be mostly anti-inflammatory and it is related to mucin degradation present in the mucosal layer of the gut [157]. *Ruminococcus*-dominated

enterotype is also able to degrade host's mucin although this is the less understood. Accordingly, *Bacteroides* enterotype was associated with a saturated fat and animal protein-based diet, thus contrasting with the relationship found between *Prevotella* cluster and fiber intake [156]. Moreover, animal-based diets containing low fibers and high fats and proteins resulted in an increased abundance of bile-tolerant genera such as *Alistipes*, *Bilophila* and *Bacteroides*, which was accompanied by a decrease in polysaccharides consumers including *Roseburia* and *Ruminococcus bromii*, when compared to plant-based diets in humans [158]. Therefore, different enterotypes employ different routes and substrates to obtain energy from the dietary components available in the colon.

Contrary to the Western diet, the traditional Mediterranean diet has long been associated with an improved health due to its elevated content in mono-unsaturated and poly-unsaturated fatty acids, fibers and vegetable protein (**Figure 3**) [159,160]. Individuals on a Mediterranean diet were characterized by a high colonization by *Lactobacillus*, *Bifidobacterium* and *Prevotella* and low abundance of *Clostridium*, which are associated with an improvement of the lipid profile, weight loss and decreased intestinal inflammation [161]. Indeed, strict vegetarian diet characterized by an elevated fiber intake changed the gut microbiota composition and function, mainly defined by a decrease in *Enterobacteriaceae* and SCFA levels, resulting in a reduced intestinal inflammation in obese individuals [162].

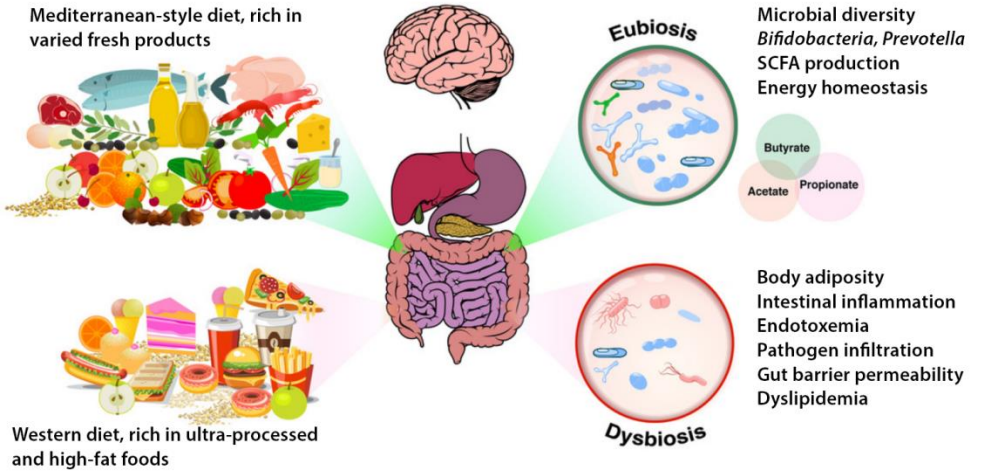

Figure 3. Mediterranean diet, which is rich in fresh produce, including a variety vegetables and fruits, olive oil, nuts and seeds, is known to contribute to high bacterial diversity and to maintain the prevalence of beneficial bacteria (eubiosis), thus favouring SCFA production and health status. On the other hand, dietary patterns rich in ultra-processed foods such as Western diet, with high amounts of refined carbohydrates and fat, are seen to promote the loss of bacterial diversity and the growth of pathogenic microbe (dysbiosis), thus altering gut barrier functions and host homeostasis. Adapted from [163].

In animal models, strong alterations in the phylogenetic structure and diversity of gut microbiome are mainly found under a low-fiber diet, whereas maintaining the fat proportions [138]. These results suggested that low dietary fiber, rather than the total fat content of the diet, is the main responsible for severe alterations of the gut microbiota. Nevertheless, the type of fat in the diet also determinates the characteristics of gut microbiota and, consequently, different fats distinctively contributed to the onset of microbial dysbiosis [139]. Moreover, the excessive intake of a combination of dietary fats and sugars resulted in more harmful effects than fat or sugar alone in the gut microbiota composition and insulin resistance [141]. For instance, Swiss Webster mice fed with a high-butter containing diet exhibited an increased *Desulfovibrio* occurrence altogether with the highest systolic

blood pressure, while mice fed with an extra virgin oil-enriched diet presented the lowest circulating insulin and leptin levels [139]. In fact, the polyphenols contained in olive leaf extracts and olive oil could explain their microbiota-mediated benefits in type II diabetes [140].

4.2 Prebiotics.

In 1995, prebiotics were firstly described as the non-digestible compounds that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, thus improving host health [164]. Over the next years, several definitions have been proposed for prebiotics, while the main concept remains unaltered: prebiotics confer a beneficial physiologic effect on the host through the modulation of microbiota composition and function [165].

4.2.1 Non-Digestible Polysaccharides.

The beneficial effects of dietary fibers on health have been widely described. The high viscosity and "gel-forming" properties of non-digestible polysaccharides were assumed to be the main responsible exerting their effects [166]. Nowadays, we know that the bulking capacity of dietary fibers is not the unique factor explaining its positive correlation with health. The fermentation of non-digestible polysaccharides by the gut microbiota is associated with the proliferation or the depletion of different bacteria and the generation of several end products such as SCFAs, as described above.

Inulin-type fructans were the first type of non-digestible polysaccharides revealing prebiotic capacities in animal models [86,167,168]. In fact, the microbial utilization of these compounds induces changes on the

microbiota composition and function, mainly favouring the growth of beneficial bacteria and promoting a reduction of body weight, fat mass, insulin resistance and food intake in obese rodents [169–171]. Moreover, the modulatory effects of inulin on intestinal microbiota correlated with its degree of polymerization (DP), showing that inulin with lower DP exhibited a better stimulation of beneficial bacteria than those with higher DP [172]. In this sense, it was previously proved that inulin with low DP were fermented more quickly than inulin with high DP in an *in vitro* study, where complex structures were more resistant to hydrolysis [173]. The prebiotic effects of inulin supplementation were also reported in overweight and obese humans by promoting changes on gut microbiota, SCFAs production, fat oxidation and an improved food intake-related behaviour [174–176].

Gut microbiota-derived end products are also able to influence obesity and obesity-related disorders through the modulation of gut-brain axis, specially by regulating the production and secretion of intestinal hormones controlling food intake, which has been broadly reported [86,168,169,176,177]. In this sense, it was reported an increased SCFAs-mediated production of GLP-1 and PYY, both involved in the regulation of body weight and appetite, as the result of the fermentation of chronically supplemented non-digestible polysaccharides in rodents [86,168]. Furthermore, prebiotics were shown to improve tight-junctions integrity in the mucosal layer of the intestine, thus reducing the intestinal permeability and inflammation, which are aggravated in obesity [178,179]. In this regard, improvements in gut barrier functions have been attributed to a microbiota-mediated increment of endogenous

glucagon-like peptide 2 (GLP-2) production, a gut hormone linked to the growth and development of intestinal cells [166,180].

4.2.2 Polyphenols.

The phytochemicals are secondary metabolites produced by plants in response to the constant changing environmental challenges over its evolution. Plant phytochemicals have an important defensive role against stress conditions induced by natural alterations on light and temperature, pathogens, nutrient deficiency and herbivores. Polyphenols, also known as phenolic compounds, are a subclass of phytochemicals chemically characterized as hydroxylated phenyl moieties, and over 8,000 different phenolic structures are currently identified [181].

To date, various polyphenols have shown beneficial properties on human health, including preventive and therapeutic effects for cancer, CVD, diabetes, dyslipidaemia and obesity, among others [182–185]. Most polyphenols are not well absorbed in the small intestine due to its poor bioavailability, reaching the colon and being in direct contact to gut microbiota [186]. Here, they are able to modulate the microbial populations, thus affecting its function and contributing to the improvement of health [187]. Therefore, polyphenols can act as prebiotics by favouring the growth of beneficial species and by increasing their adhesion to intestinal epithelium [187]. Interestingly, the prebiotic effects of polyphenols were suggested to be partially explained by the regulation of SCFA-producing bacteria [188–190]. Moreover, gut microbiota can also metabolize polyphenols into absorbable and bioactive compounds, which are recognized to have anti-inflammatory effects and are able to directly act in peripheral organs

[191]. Inter-individual differences in gut microbiota composition result in different profiles of polyphenol-derived metabolites. However, the polyphenol-converting bacteria belong to the dominant phyla of the human intestine [75].

Along with the well-known antioxidant capacity of polyphenols through the scavenging of reactive species of oxygen (ROS) favoured by their chemical structures [191], a large number of capacities have been described for many of them. In this sense, the modulation of glucose homeostasis, the suppression of adipogenesis and lipid synthesis, the increase of thermogenesis and related energy expenditure and the stimulation of fat oxidation have been identified as beneficial properties [192]. However, most mechanisms of action explaining these effects remain to be elucidated. In this sense, increasing evidence supports that polyphenols exert some of their biological functions by modulating gut microbial balance [193].

The condensed tannins (proanthocyanidins) are derivatives from flavonoids, a class of polyphenolic compounds having significant benefits for human health. The proanthocyanidins are present in flowers, fruits, nuts and seeds of diverse plants protecting them from predators and pathogens. Due to their high DP, some proanthocyanidins are not absorbable, and gut microbiota play an essential role on its degradation into smaller structures that can be absorbed in the large intestine. Therefore, gut microbiota is postulated to partially mediate the beneficial effects of proanthocyanidins as recently reviewed [194]. In addition, proanthocyanidins can also impact on the gut microbiota composition, thus affecting the microbiota-mediated metabolic functions in the host. Some of them have shown health-promoting activities, such

as the inhibition of pathogens and favouring the growth of beneficial bacteria [195]. Different studies evaluating the prebiotic potential of proanthocyanidins revealed a bloom of *Akkermansia* populations concomitantly occurring with the attenuation of DIO in mice [196–198]. Moreover, the supplementation with a grape seed proanthocyanidins extract (GSPE) favoured the occurrence of butyrate-producing species, including *Clostridium XIV* and *Roseburia inulinivorans* in rodents fed with a HF diet [199]. In the same line, an increment of *Lactobacillus* abundance was consistently observed after the administration of commercial GSPE in pigs [189,200]. Altogether, the consumption of proanthocyanidins could confer health benefits by targeting the gut microbiota. However, the limited number of studies describing the proanthocyanidins-microbiota interactions demand for further research to clarify the involvement of specific microorganisms in the health outcomes already established for a wide range of proanthocyanidins [201].

Interestingly, the health benefits of different dietary polyphenols including (–) epigallocatechin-3-gallate from green tea, curcumin, chlorogenic acid, quercetin and resveratrol have been reviewed [192]. Here, the activation of the AMPK system is proposed as the main mechanism explaining the valuable activities of these polyphenols on obesity. The AMPK activation is linked with the downregulation of adipogenic and lipogenic genes, as well as with the upregulation of lipolysis-related genes [202]. In this sense, body weight loss after green tea or black tea supplementation in DIO mice resulted from the AMPK activation, which, in turn, was related to an increased production of SCFA by gut microbiota [203]. Therefore, SCFA-mediated AMPK activation,

which has been previously described [204], highlights the role of gut microbiota in explaining the potential benefits of proanthocyanidins in host health.

5. MICROBIOTA RHYTHMICITY

Circadian rhythms are highly conserved and self-sustained cyclical metabolic patterns of approximately 24 hours exhibited by most organisms such as bacteria, plants and animals [205]. From an evolutionary viewpoint, these rhythms are the result of an adaptation to predictable changes in the environment in order to confer a selective advantage to living organisms. The daily rhythmicity is orchestrated by endogenous circadian clocks that optimize and regulate the cellular functions according to the time of the day. In mammals, central and peripheral clocks are intrinsically involved in the regulation of a broad variety of metabolic and gastrointestinal processes, such as the synthesis, storage and expenditure of energy [206]. The central clock is located in the hypothalamic suprachiasmatic nucleus of the brain and is mainly regulated by light or darkness exposure. The set of core clock genes expressed in this region are the responsible to synchronize the peripheral clocks of the body, which in turn, modulate the expression of clock-controlled genes. In addition to central clock signalling, peripheral clocks are also entrained by behavioural factors including food intake and exercise [206,207]. Circulating levels of glucose, fatty acids and triglycerides, as well as the production and secretion of hormones such as insulin, are under the management of circadian clocks [208,209]. Overall, the internal clocks confer to the organisms the ability to rapidly adapt their metabolism to the changing environmental conditions over the course of the day. However, these synchronized physiological and

metabolic patterns can be also disturbed by external cues, thus altering the internal rhythmicity and homeostasis. For instance, an inadequate sleep pattern, shift work, delayed bedtimes and jet-lag, among others, may cause circadian disruptions [210,211].

In the intestine, the main gateway for energy supply to the human body, most of the physiological functions such as digestion and absorption of dietary components are also regulated by clock genes [212]. In this regard, the misalignment of the intestinal rhythms has been linked to an increased risk of metabolic disorders such as obesity [213]. Interestingly, manipulations of the dark/light cycle promoted an increased body weight gain with no changes on food intake in rodents, thus evidencing the crucial role of central clocks in the management of host physiology [214].

Gut bacteria also exhibit circadian rhythms. Recently, it has been shown that up to 20% of gut microbiota exhibit diurnal oscillations in their relative abundance, thus modulating the activity of the entire microbial ecosystem [214]. These circadian oscillations are regulated by both the central clock and the feeding patterns, thus optimizing the digestion and absorption of dietary nutrients. Importantly, it has been established that host clock genes also influence the microbial rhythms [215]. In this sense, knock-out mice for clock genes, such as Per1/2 and Bmal-1, exhibited a disruption of gut microbiota rhythmicity, leading to dysbiosis and favouring a pro-inflammatory phenotype [214,216]. Furthermore, a four-week period of constant light exposure revealed an increase in *Ruminococcus torques* and a drop in *Lactobacillus johnsonii*, which are respectively involved in the impairment and protection of the intestinal barrier [217]. In the same study, genes promoting beneficial immune

responses were downregulated, while those involved in the production of the endotoxin LPS were upregulated, thus revealing connections between circadian rhythm disturbances and dysfunction of intestinal barrier. For this reason, the importance of microbial rhythms in the maintenance of host health is broadly established (**Figure 4**). In this regard, host transcriptomic oscillations in the intestine and liver are driven, at least in part, by gut microbiome, since germ-free mice or complete depletion of gut microbiota using antibiotics result in altered expression patterns of circadian transcripts in the host [218].

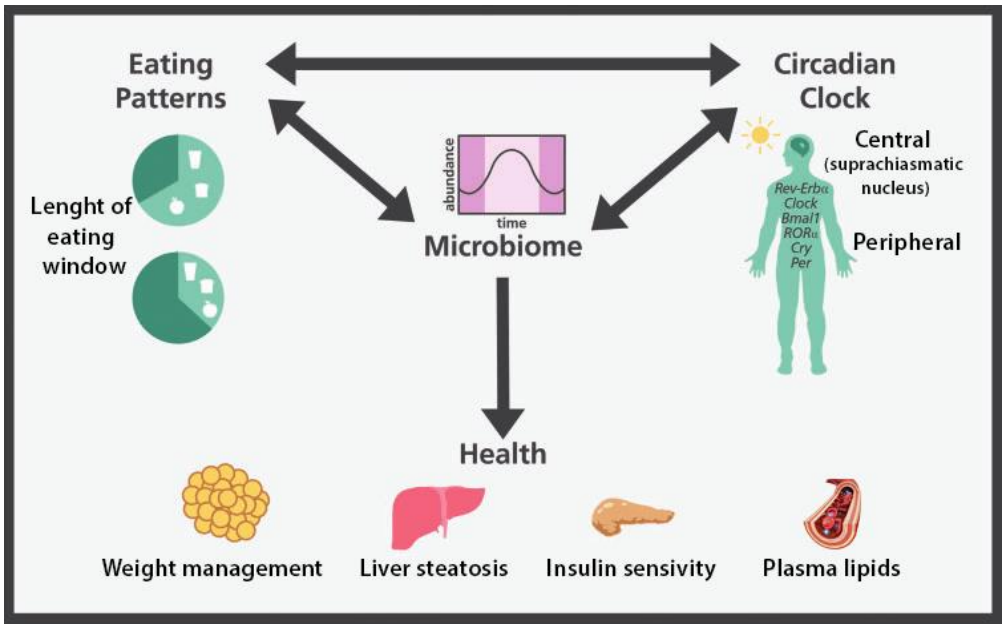

Figure 4. Connections between the internal clock, eating patterns, the microbiome, and health Adapted from [219].

Remarkably, fecal microbiota transplantation from jet-lagged humans showing disrupted microbial oscillations into germ-free mice resulted in the acquirement of obesity and glucose intolerance, whereas it was not induced when transferred from non-jet-lagged individuals [214]. Therefore, intrinsic central clocks strongly control the microbial

rhythmicity, while feeding patterns are thought to be the main contributors to the maintenance or disruption of this feature [220].

Food intake is robustly linked to the establishment of microbial populations according to the daily phase [221]. In this sense, bacteria involved in energy harvest, cell growth processes and DNA repair predominantly colonise the gut during the feeding period, whereas bacteria responsible for detoxification and production of chemotaxis-related metabolites increase during the fasting stage [214]. Moreover, during the fasting periods, when the carbon source to produce SCFAs is limited, the rhythmic changes of gut microbiota composition favours the utilization of host-derived glycans as energy source [217].

Therefore, irregular eating schedules, alcohol consumption or unbalanced diets can disrupt the intestinal circadian rhythms [222,223], as also occurs upon the alteration of host's rhythmicity through changes on dark/light or sleep/wake patterns. The disturbances on microbial rhythms are reflected by altered oscillations of circulating microbiota-produced metabolites such as SCFAs. In this sense, microbial oscillations are perturbed under a high-fat diet in mice, while time-restricted feeding just during the active phase (dark period) partially restores the microbial rhythmicity by reducing obesogenic bacteria and promoting healthy microorganisms [224]. Moreover, microbiota dysbiosis is severely promoted by a high-fat diet in mice with either genetic or environmental impairment of circadian clocks [215,225].

Several metabolic and physiological consequences of an impaired rhythmicity of the gut microbiota have been reported. In this sense, disrupted microbial rhythmicity leads to the establishment of intestinal

dysbiosis, hyperpermeability of gut barrier, altered immune functions, high rates of infection and endotoxemia [217,226,227]. Altogether, desynchronized host and microbial rhythms result in the disturbance of biological functions and a decline in health. However, more research is needed to elucidate the relationships between the rhythmicity of specific members of the microbiota and host phenotypes.

6. REFERENCES

[1] WHO. Obesity : Preventing and managing the global epidemic. World Health Organization: Technical Report Series. WHO Tech Rep Ser No 894 2000:252.

[2] Gadde KM, Martin CK, Berthoud HR, Heymsfield SB. Obesity: Pathophysiology and Management. J Am Coll Cardiol 2018;71:69–84.

[3] Afshin A, Forouzanfar MH, Reitsma MB, Sur P, Estep K, Lee A, et al. Health effects of overweight and obesity in 195 countries over 25 years. N Engl J Med 2017;377:13–27.

[4] Collaboration NRF. Trends in adult body-mass index in 200 countries from 1975 to 2014: A pooled analysis of 1698 population-based measurement studies with 19.2 million participaDi Cesare, Mariachiara Bentham, James Stevens, Gretchen A. Zhou, Bin Danaei, Goodarz Lu, Yuan Bix. Lancet 2016;387:1377–96.

[5] Hruby A, Manson JAE, Qi L, Malik VS, Rimm EB, Sun Q, et al. Determinants and consequences of obesity. Am J Public Health 2016;106:1656–62.

[6] O'Donovan G, Thomas EL, McCarthy JP, Fitzpatrick J, Durighel G, Mehta S, et al. Fat distribution in men of different waist girth, fitness level and exercise habit. Int J Obes (Lond) 2009;33:1356–62.

[7] Lee SW, Son JY, Kim JM, Hwang S-S, Han JS, Heo NJ. Body fat distribution is more predictive of all-cause mortality than overall adiposity. Diabetes Obes Metab 2018;20:141–7.

[8] Huth C, Pigeon É, Riou M-È, St-Onge J, Arguin H, Couillard E, et al. Fitness, adiposopathy, and adiposity are independent predictors of insulin sensitivity in middle-aged men without diabetes. J Physiol Biochem 2016;72:435–44.

[9] Ellulu MS, Patimah I, Khaza'ai H, Rahmat A, Abed Y. Obesity & inflammation: The linking mechanism & the complications. Arch Med Sci 2017;13:851–63.

[10] Sears B, Perry M. The role of fatty acids in insulin resistance. Lipids Health Dis 2015;14:121.

[11] Fabbrini E, Mohammed BS, Magkos F, Korenblat KM, Patterson BW, Klein S. Alterations in adipose tissue and hepatic lipid kinetics in obese men and women with nonalcoholic fatty liver disease. Gastroenterology 2008;134:424–31.

[12] Samuel VT, Shulman GI. Mechanisms for insulin resistance: common threads and missing links. Cell 2012;148:852–71.

[13] Ebbert JO, Jensen MD. Fat depots, free fatty acids, and dyslipidemia. Nutrients 2013;5:498–508.

[14] Smith MM, Minson CT. Obesity and adipokines: effects on sympathetic overactivity. J Physiol 2012;590:1787–801.

[15] Joossens M, Huys G, Cnockaert M, De Preter V, Verbeke K, Rutgeerts P, et al. Dysbiosis of the faecal microbiota in patients with Crohn's disease and their unaffected relatives. Gut 2011;60:631–7.

[16] Tooke JE, Hannemann MM. Adverse endothelial function and the insulin resistance syndrome. J Intern Med 2000;247:425–31.

[17] Pandit R, Beerens S, Adan RAH. Role of leptin in energy expenditure: The hypothalamic perspective. Am J Physiol - Regul Integr Comp Physiol 2017;312:R938–47.

[18] Ibrahim MM. Subcutaneous and visceral adipose tissue: structural and functional differences. Obes Rev 2010;11:11–8.

[19] Hussain Z, Khan JA. Food intake regulation by leptin: Mechanisms mediating gluconeogenesis and energy expenditure. Asian Pac J Trop Med 2017;10:940–4.

[20] Trayhurn P. Hypoxia and adipose tissue function and dysfunction in obesity. Physiol Rev 2013;93:1–21.

[21] Rajala MW, Scherer PE. Minireview: The adipocyte - At the crossroads of energy homeostasis, inflammation, and atherosclerosis. Endocrinology 2003;144:3765–73.

[22] Enriori PJ, Evans AE, Sinnayah P, Cowley MA. Leptin resistance and obesity. Obesity (Silver Spring) 2006;14 Suppl 5:254S-258S.

[23] Belfort-DeAguiar R, Seo D. Food Cues and Obesity: Overpowering Hormones and Energy Balance Regulation. Curr Obes Rep 2018;7:122–9.

[24] Lucassen EA, Cizza G. The Hypothalamic-Pituitary-Adrenal Axis, Obesity, and Chronic Stress Exposure: Sleep and the HPA Axis in Obesity. Curr Obes Rep 2012;1:208–15.

[25] Wang T-N, Chang W-T, Chiu Y-W, Lee C-Y, Lin K-D, Cheng YY, et al. Relationships between changes in leptin and insulin resistance levels in obese individuals following weight loss. Kaohsiung J Med Sci 2013;29:436–43.

[26] Alberti KG, Eckel RH, Grundy SM, Zimmet PZ, Cleeman JI, Donato KA, et al. Harmonizing the metabolic syndrome: a joint interim statement of the Association for the Study of Obesity. Circulation 2009;120:1640–5.

[27] Einarson TR, Acs A, Ludwig C, Panton UH. Prevalence of cardiovascular disease in type 2 diabetes: A systematic literature review of scientific evidence from across the world in 2007-2017. Cardiovasc Diabetol 2018;17.

[28] Csige I, Ujvárosy D, Szabó Z, Lorincz I, Paragh G, Harangi M, et al. The Impact of Obesity on the Cardiovascular System. J Diabetes Res 2018;2018.

[29] Kosmas CE, Martinez I, Sourlas A, Bouza K V., Campos FN, Torres V, et al. High-density lipoprotein (HDL) functionality and its relevance to atherosclerotic cardiovascular disease. Drugs Context 2018;7.

[30] Kjeldsen SE. Hypertension and cardiovascular risk: General aspects. Pharmacol Res 2018;129:95–9.

[31] Wengrofsky P, Lee J, N. Makaryus A. Dyslipidemia and Its Role in the Pathogenesis of Atherosclerotic Cardiovascular Disease: Implications for Evaluation and Targets for Treatment of Dyslipidemia Based on Recent Guidelines. Dyslipidemia [Working Title], IntechOpen; 2019.

[32] Brooks GC, Blaha MJ, Blumenthal RS. Relation of C-Reactive Protein to Abdominal Adiposity. Am J Cardiol 2010;106:56–61.

[33] Kartheuser AH, Leonard DF, Penninckx F, Paterson HM, Brandt D, Remue C, et al. Waist circumference and waist/hip ratio are better predictive risk factors for mortality and morbidity after colorectal surgery than body mass index and body surface area. Ann Surg 2013;258:722–30.

[34] Wei S, Punyanitya M, Jun C, Gallagher D, Albu J, Pi-Sunyer X, et al. Waist circumference correlates with metabolic syndrome indicators better than percentage fat. Obesity 2006;14:727–36.

[35] Després JP, Lemieux I. Abdominal obesity and metabolic syndrome. Nature 2006;444:881–7.

[36] Wildman RP, Muntner P, Reynolds K, McGinn AP, Rajpathak S, Wylie-Rosett J, et al. The obese without cardiometabolic risk factor clustering and the normal weight with cardiometabolic risk factor clustering: prevalence and correlates of 2 phenotypes among the US population (NHANES 1999-2004). Arch Intern Med 2008;168:1617–24.

[37] Després J-P. Body fat distribution and risk of cardiovascular disease: an update. Circulation 2012;126:1301–13.

[38] Christian N, Whitaker BK, Clay K. Microbiomes: Unifying animal and plant systems through the lens of community ecology theory. Front Microbiol 2015;6:869.

[39] Whitman WB, Coleman DC, Wiebe WJ. Prokaryotes: The unseen majority. Proc Natl Acad Sci 1998;95:6578–83.

[40] Pontarp M, Canbäck B, Tunlid A, Lundberg P. Phylogenetic analysis suggests that habitat filtering is structuring marine bacterial communities across the globe. Microb Ecol 2012;64:8–17.

[41] Maire V, Gross N, Börger L, Proulx R, Wirth C, Pontes L da S, et al. Habitat filtering and niche differentiation jointly explain species relative abundance within grassland communities along fertility and disturbance gradients. New Phytol 2012;196:497–509.

[42] Macalady JL, Dattagupta S, Schaperdoth I, Jones DS, Druschel GK, Eastman D. Niche differentiation among sulfur-oxidizing bacterial populations in cave waters. ISME J 2008;2:590–601.

[43] Zilber-Rosenberg I, Rosenberg E. Role of microorganisms in the evolution of animals and plants: the hologenome theory of evolution. FEMS Microbiol Rev 2008;32:723–35.

[44] Brundrett MC. Coevolution of roots and mycorrhizas of land plants. New Phytol 2002;154:275–304.

[45] Van Blerkom LM. Role of Viruses in Human Evolution. Am J Phys Anthropol 2003;37:14–46.

[46] Lee YK, Mazmanian SK. Has the microbiota played a critical role in the evolution of the adaptive immune system? Science (80-) 2010;330:1768–73.

[47] Archie EA, Theis KR. Animal behaviour meets microbial ecology. Anim Behav 2011;82:425–36.

[48] Nicholson JK, Holmes E, Kinross J, Burcelin R, Gibson G, Jia W, et al. Host-gut microbiota metabolic interactions. Science (80-) 2012;336:1262–7.

[49] Ezenwa VO, Gerardo NM, Inouye DW, Medina M, Xavier JB. Animal behavior and the microbiome. Science (80-) 2012;338:198–9.

[50] Savage DC. Microbial Ecology of the Gastrointestinal Tract. Annu Rev Microbiol 1977;31:107–33.

[51] Sender R, Fuchs S, Milo R. Are We Really Vastly Outnumbered? Revisiting the Ratio of Bacterial to Host Cells in Humans. Cell 2016;164:337–40.

[52] Sender R, Fuchs S, Milo R. Revised Estimates for the Number of Human and Bacteria Cells in the Body. PLoS Biol 2016;14.

[53] Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI. The Human Microbiome Project. Nature 2007;449:804–10.

[54] Elizabeth K. C, Christian L. L, Hamady M, Noah F, Jeffrey I. G, Rob K. Bacterial Community Variation in Human Body Habitats Across Space and Time. Science (80-) 2009;46:183–210.

[55] Hillman ET, Lu H, Yao T, Nakatsu CH. Minireview Microbial Ecology along the Gastrointestinal Tract. Microbes Env 2017;32:300–13.

[56] Manimozhiyan A, Shu Y-Z, Arcuri M, Kozlowski M, Wang R, Lam KS, et al. Enterotypes of the human gut microbiome. Nature 2011;473:174–80.

[57] Rinninella E, Raoul P, Cintoni M, Franceschi F, Miggiano G, Gasbarrini A, et al. What is the Healthy Gut Microbiota Composition? A Changing Ecosystem across Age, Environment, Diet, and Diseases. Microorganisms 2019;7:14.

[58] Maruvada P, Leone V, Kaplan LM, Chang EB. The Human Microbiome and Obesity: Moving beyond Associations. Cell Host Microbe 2017;22:589–99.

[59] Bik EM. Composition and function of the human-associated microbiota. Nutr. Rev., vol. 67, 2009.

[60] Stappenbeck TS, Hooper L V., Gordon JI. Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells. Proc Natl Acad Sci U S A 2002;99:15451–5.

[61] Backhed F, Ding H, Wang T, Hooper L V., Koh GY, Nagy A, et al. The gut microbiota as an environmental factor that regulates fat storage. Proc Natl Acad Sci 2004;101:15718–23.

[62] Nyangale EP, Mottram DS, Gibson GR. Gut microbial activity, implications for health and disease: the potential role of metabolite analysis. J Proteome Res 2012;11:5573–85.

[63] Chambers ES, Preston T, Frost G, Morrison DJ. Role of Gut Microbiota-Generated Short-Chain Fatty Acids in Metabolic and Cardiovascular Health. Curr Nutr Rep 2018;7:198–206.

[64] Richards AL, Muehlbauer AL, Alazizi A, Burns MB, Findley A, Messina F, et al. Gut microbiota has a widespread and modifiable effect on host gene regulation. BioRxiv 2018.

[65] Morrison DJ, Preston T. Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. Gut Microbes 2016;7:189–200.

[66] Liu S-Q. Practical implications of lactate and pyruvate metabolism by lactic acid bacteria in food and beverage fermentations. Int J Food Microbiol 2003;83:115–31.

[67] Russell WR, Gratz SW, Duncan SH, Holtrop G, Ince J, Scobbie L, et al. High-protein, reduced-carbohydrate weight-loss diets promote metabolite profiles likely to be detrimental to colonic health. Am J Clin Nutr 2011;93:1062–72.

[68] Alexander C, Swanson KS, Fahey GC, Garleb KA. Perspective: Physiologic Importance of Short-Chain Fatty Acids from Nondigestible Carbohydrate Fermentation. Adv Nutr 2019;10:576–89.

[69] Inoue D, Tsujimoto G, Kimura I. Regulation of energy homeostasis by GPR41. Front Endocrinol (Lausanne) 2014;5.

[70] Roediger WEW. Utilization of Nutrients by Isolated Epithelial Cells of the Rat Colon. Gastroenterology 1982;83:424–9.

[71] Bergman EN. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiol Rev 1990;70:567–90.

[72] Wostmann BS, Larkin C, Moriarty A, Bruckner-Kardoss E. Dietary intake, energy metabolism, and excretory losses of adult male germfree Wistar rats. Lab Anim Sci 1983;33:46–50.

[73] Schwiertz A, Taras D, Schäfer K, Beijer S, Bos NA, Donus C, et al. Microbiota and SCFA in lean and overweight healthy subjects. Obesity 2010;18:190–5.

[74] Freeland KR, Wilson C, Wolever TMS. Adaptation of colonic fermentation and glucagon-like peptide-1 secretion with increased wheat fibre intake for 1 year in hyperinsulinaemic human subjects. Br J Nutr 2010;103:82–90.

[75] Daud NM, Ismail NA, Thomas EL, Fitzpatrick JA, Bell JD, Swann JR, et al. The impact of oligofructose on stimulation of gut hormones, appetite regulation and adiposity. Obesity 2014;22:1430–8.

[76] Koh A, De Vadder F, Kovatcheva-Datchary P, Bäckhed F. From Dietary Fiber to Host Physiology: Short-Chain Fatty Acids as Key Bacterial Metabolites. Cell 2016;165:1332–45.

[77] Bäckhed F, Manchester JK, Semenkovich CF, Gordon JI. Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. Proc Natl Acad Sci U S A 2007;104:979–84.

[78] Donohoe DR, Garge N, Zhang X, Sun W, O'Connell TM, Bunger MK, et al. The microbiome and butyrate regulate energy metabolism and autophagy in the mammalian colon. Cell Metab 2011;13:517–26.

[79] Den Besten G, Van Eunen K, Groen AK, Venema K, Reijngoud DJ, Bakker BM. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. J Lipid Res 2013;54:2325–40.

[80] Den Besten G, Bleeker A, Gerding A, Van Eunen K, Havinga R, Van Dijk TH, et al. Short-chain fatty acids protect against high-fat diet-induced obesity via a pparg-dependent switch from lipogenesis to fat oxidation. Diabetes 2015;64:2398–408.

[81] Viollet B, Horman S, Leclerc J, Lantier L, Foretz M, Billaud M, et al. AMPK inhibition in health and disease. Crit Rev Biochem Mol Biol 2010;45:276–95.

[82] Rahat-Rozenbloom S, Fernandes J, Gloor GB, Wolever TMS. Evidence for greater production of colonic short-chain fatty acids in overweight than lean humans. Int J Obes 2014;38:1525–31.

[83] Chambers ES, Viardot A, Psichas A, Morrison DJ, Murphy KG, Zac-Varghese SEK, et al. Effects of targeted delivery of propionate to the human colon on appetite regulation, body weight maintenance and adiposity in overweight adults. Gut 2015;64:1744–54.

[84] Conterno L, Fava F, Viola R, Tuohy KM. Obesity and the gut microbiota: does up-regulating colonic fermentation protect against obesity and metabolic disease? Genes Nutr 2011;6:241–60.

[85] Moreno-Indias I, Cardona F, Tinahones FJ, Queipo-Ortuño MI. Impact of the gut microbiota on the development of obesity and type 2 diabetes mellitus. Front Microbiol 2014;5.

[86] Cani PD, Dewever C, Delzenne NM. Inulin-type fructans modulate gastrointestinal peptides involved in appetite regulation (glucagon-like peptide-1 and ghrelin) in rats. Br J Nutr 2004;92:521–6.

[87] Batterham RL, Cowley MA, Small CJ, Herzog H, Cohen MA, Dakin CL, et al. Gut hormone PYY(3-36) physiologically inhibits food intake. Nature 2002;418:650–4.

[88] Chambers ES, Viardot A, Psichas A, Morrison DJ, Murphy KG, Zac-Varghese SEK, et al. Effects of targeted delivery of propionate to the human colon on appetite regulation, body weight maintenance and adiposity in overweight adults. Gut 2015;64:1744–54.

[89] Bellahcene M, O'Dowd JF, Wargent ET, Zaibi MS, Hislop DC, Ngala RA, et al. Male mice that lack the G-protein-coupled receptor GPR41 have low energy expenditure and increased body fat content. Br J Nutr 2013;109:1755–64.

[90] Kimura I, Ozawa K, Inoue D, Imamura T, Kimura K, Maeda T, et al. The gut microbiota suppresses insulin-mediated fat accumulation via the short-chain fatty acid receptor GPR43. Nat Commun 2013;4:1829.

[91] Byrne CS, Chambers ES, Morrison DJ, Frost G. The role of short chain fatty acids in appetite regulation and energy homeostasis. Int J Obes (Lond) 2015;39:1331–8.

[92] Pessione E. Lactic acid bacteria contribution to gut microbiota complexity: lights and shadows. Front Cell Infect Microbiol 2012;2:86.

[93] Arun KB, Madhavan A, Reshmitha TR, Thomas S, Nisha P. Short chain fatty acids enriched fermentation metabolites of soluble dietary fibre from Musa paradisiaca drives HT29 colon cancer cells to apoptosis. PLoS One 2019;14.

[94] Ridlon JM, Kang DJ, Hylemon PB, Bajaj JS. Bile acids and the gut microbiome. Curr Opin Gastroenterol 2014;30:332–8.

[95] Chiang JYL. Bile acid metabolism and signaling. Compr Physiol 2013;3:1191–212.

[96] Sinal CJ, Tohkin M, Miyata M, Ward JM, Lambert G, Gonzalez FJ. Targeted disruption of the nuclear receptor FXR/BAR impairs bile acid and lipid homeostasis. Cell 2000;102:731–44.

[97] Ridlon JM, Kang D-J, Hylemon PB. Bile salt biotransformations by human intestinal bacteria. J Lipid Res 2006;47:241–59.

[98] Ridlon JM, Harris SC, Bhowmik S, Kang DJ, Hylemon PB. Consequences of bile salt biotransformations by intestinal bacteria. Gut Microbes 2016;7:22–39.

[99] Joyce SA, Shanahan F, Hill C, Gahan CGM. Bacterial bile salt hydrolase in host metabolism: Potential for influencing gastrointestinal microbe-host crosstalk. Gut Microbes 2015;5:669–74.

[100] Govindarajan K, MacSharry J, Casey PG, Shanahan F, Joyce SA, Gahan CGM. Unconjugated bile acids influence expression of circadian genes: A potential mechanism for microbe-host crosstalk. PLoS One 2016;11.

[101] Joyce SA, MacSharry J, Casey PG, Kinsella M, Murphy EF, Shanahan F, et al. Regulation of host weight gain and lipid metabolism by bacterial bile acid modification in the gut. Proc Natl Acad Sci U S A 2014;111:7421–6.

[102] Kawamata Y, Fujii R, Hosoya M, Harada M, Yoshida H, Miwa M, et al. A G protein-coupled receptor responsive to bile acids. J Biol Chem 2003;278:9435–40.

[103] Thomas C, Gioiello A, Noriega L, Strehle A, Oury J, Rizzo G, et al. TGR5-mediated bile acid sensing controls glucose homeostasis. Cell Metab 2009;10:167–77.

[104] Brighton CA, Rievaj J, Kuhre RE, Glass LL, Schoonjans K, Holst JJ, et al. Bile acids trigger GLP-1 release predominantly by accessing basolaterally located G protein-coupled bile acid receptors. Endocrinology 2015;156:3961–70.

[105] Fiorucci S, Distrutti E. Bile Acid-Activated Receptors, Intestinal Microbiota, and the Treatment of Metabolic Disorders. Trends Mol Med 2015;21:702–14.

[106] Yokota A, Fukiya S, Islam KBMS, Ooka T, Ogura Y, Hayashi T, et al. Is bile acid a determinant of the gut microbiota on a high-fat diet? Gut Microbes n.d.;3:455–9.

[107] Gentile CL, Weir TL. The gut microbiota at the intersection of diet and human health. Science (80-) 2018;362:776–80.

[108] Krogius-Kurikka L, Lyra A, Malinen E, Aarnikunnas J, Tuimala J, Paulin L, et al. Microbial community analysis reveals high level phylogenetic alterations in the overall gastrointestinal microbiota of diarrhoea-predominant irritable bowel syndrome sufferers. BMC Gastroenterol 2009;9:95.

[109] Scanlan PD, Shanahan F, O'Mahony C, Marchesi JR. Culture-independent analyses of temporal variation of the dominant fecal microbiota and targeted bacterial subgroups in Crohn's disease. J Clin Microbiol 2006;44:3980–8.

[110] Delgado S, Suárez A, Mayo B. Identification of dominant bacteria in feces and colonic mucosa from healthy Spanish adults by culturing and by 16S rDNA sequence analysis. Dig Dis Sci 2006;51:744–51.

[111] Ding R xue, Goh WR, Wu R na, Yue X qing, Luo X, Khine WWT, et al. Revisit gut microbiota and its impact on human health and disease. J Food Drug Anal 2019;27:623–31.

[112] Petersen C, Round JL. Defining dysbiosis and its influence on host immunity and disease. Cell Microbiol 2014;16:1024–33.

[113] Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 2006;444:1027–31.

[114] Frank DN, St. Amand AL, Feldman RA, Boedeker EC, Harpaz N, Pace NR. Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. Proc Natl Acad Sci U S A 2007;104:13780–5.

[115] de Velasco P, Ferreira A, Crovesy L, Marine T, das Graças Tavares do Carmo M. Fatty Acids, Gut Microbiota, and the Genesis of Obesity. Biochem. Heal. Benefits Fat. Acids, IntechOpen; 2018.

[116] Sonnenburg JL, Bäckhed F. Diet-microbiota interactions as moderators of human metabolism. Nature 2016;535:56–64.

[117] Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, et al. A core gut microbiome in obese and lean twins. Nature 2009;457:480–4.

[118] Armougom F, Henry M, Vialettes B, Raccah D, Raoult D. Monitoring bacterial community of human gut microbiota reveals an increase in Lactobacillus in obese patients and Methanogens in anorexic patients. PLoS One 2009;4.

[119] Kasai C, Sugimoto K, Moritani I, Tanaka J, Oya Y, Inoue H, et al. Comparison of the gut microbiota composition between obese and non-obese individuals in a Japanese population, as analyzed by terminal restriction fragment length polymorphism and next-generation sequencing. BMC Gastroenterol 2015;15:100.

[120] Koliada A, Syzenko G, Moseiko V, Budovska L, Puchkov K, Perederiy V, et al. Association between body mass index and Firmicutes/Bacteroidetes ratio in an adult Ukrainian population. BMC Microbiol 2017;17:120.

[121] Riva A, Borgo F, Lassandro C, Verduci E, Morace G, Borghi E, et al. Pediatric obesity is associated with an altered gut microbiota and discordant shifts in Firmicutes populations. Environ Microbiol 2017;19:95–105.

[122] Murugesan S, Ulloa-Martínez M, Martínez-Rojano H, Galván-Rodríguez FM, Miranda-Brito C, Romano MC, et al. Study of the diversity and short-chain fatty acids production by the bacterial community in overweight and obese Mexican children. Eur J Clin Microbiol Infect Dis 2015;34:1337–46.

[123] Hu HJ, Park SG, Jang HB, Choi MG, Park KH, Kang JH, et al. Obesity alters the microbial community profile in Korean Adolescents. PLoS One 2015;10.

[124] Walters WA, Xu Z, Knight R. Meta-analyses of human gut microbes associated with obesity and IBD. FEBS Lett 2014;588:4223–33.

[125] Sze MA, Schloss PD. Looking for a signal in the noise: Revisiting obesity and the microbiome. MBio 2016;7.

[126] Le Chatelier E, Nielsen T, Qin J, Prifti E, Hildebrand F, Falony G, et al. Richness of human gut microbiome correlates with metabolic markers. Nature 2013;500:541–6.

[127] Cotillard A, Kennedy SP, Kong LC, Prifti E, Pons N, Le Chatelier E, et al. Dietary intervention impact on gut microbial gene richness. Nature 2013;500:585–8.

[128] Lau LHS, Wong SH. Microbiota, obesity and NAFLD. Adv. Exp. Med. Biol., vol. 1061, Springer New York LLC; 2018, p. 111–25.

[129] Sun L, Ma L, Ma Y, Zhang F, Zhao C, Nie Y. Insights into the role of gut microbiota in obesity: pathogenesis, mechanisms, and therapeutic perspectives. Protein Cell 2018;9:397–403.

[130] Turnbaugh PJ, Ridaura VK, Faith JJ, Rey FE, Knight R, Gordon JI. The effect of diet on the human gut microbiome: A metagenomic analysis in humanized gnotobiotic mice. Sci Transl Med 2009;1.

[131] Palmnäs MSA, Cowan TE, Bomhof MR, Su J, Reimer RA, Vogel HJ, et al. Low-dose aspartame consumption differentially affects gut microbiota-host metabolic interactions in the diet-induced obese rat. PLoS One 2014.

[132] Marques C, Meireles M, Norberto S, Leite J, Freitas J, Pestana D, et al. High-fat diet-induced obesity Rat model: a comparison between Wistar and Sprague-Dawley Rat. Adipocyte 2016.

[133] Jiang T, Gao X, Wu C, Tian F, Lei Q, Bi J, et al. Apple-derived pectin modulates gut microbiota, improves gut barrier function, and attenuates metabolic endotoxemia in rats with diet-induced obesity. Nutrients 2016.

[134] Carmody RN, Gerber GK, Luevano JM, Gatti DM, Somes L, Svenson KL, et al. Diet dominates host genotype in shaping the murine gut microbiota. Cell Host Microbe 2015;17:72–84.

[135] Tanida M, Shen J, Maeda K, Horii Y, Yamano T, Fukushima Y, et al. High-fat diet-induced obesity is attenuated by probiotic strain Lactobacillus paracasei ST11 (NCC2461) in rats. Obes Res Clin Pract 2008;2:I–II.

[136] Kadooka Y, Sato M, Imaizumi K, Ogawa A, Ikuyama K, Akai Y, et al. Regulation of abdominal adiposity by probiotics (Lactobacillus gasseri SBT2055) in adults with obese tendencies in a randomized controlled trial. Eur J Clin Nutr 2010;64:636–43.

[137] Zhang C, Li S, Yang L, Huang P, Li W, Wang S, et al. Structural modulation of gut microbiota in life-long calorie-restricted mice. Nat Commun 2013;4:2163.

[138] Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM, Delzenne NM, et al. Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. Diabetes 2008;57:1470–81.

[139] Cani PD, Knauf C, Iglesias MA, Drucker DJ, Delzenne NM, Burcelin R, et al. Metabolic Endotoxemia Initiates Obesity and Insulin Resistance. Am Diabetes Assoc 2006;55:1484–90.

[140] Lam YY, Ha CWY, Campbell CR, Mitchell AJ, Dinudom A, Oscarsson J, et al. Increased gut permeability and microbiota change associate with mesenteric fat inflammation and metabolic dysfunction in diet-induced obese mice. PLoS One 2012;7:e34233.

[141] Del Bas JM, Guirro M, Boqué N, Cereto A, Ras R, Crescenti A, et al. Alterations in gut microbiota associated with a cafeteria diet and the physiological consequences in the host. Int J Obes (Lond) 2018;42:746–54.

[142] Sampey BP, Vanhoose AM, Winfield HM, Freemerman AJ, Muehlbauer MJ, Fueger PT, et al. Cafeteria diet is a robust model of human metabolic syndrome with liver and adipose inflammation: Comparison to high-fat diet. Obesity 2011;19:1109–17.

[143] Whitacre CC. Sex differences in autoimmune disease. Nat Immunol 2001;2:777–80.

[144] Palmer BF, Clegg DJ. The sexual dimorphism of obesity. Mol Cell Endocrinol 2015;402:113–9.

[145] Mestdagh R, Dumas M-E, Rezzi S, Kochhar S, Holmes E, Claus SP, et al. Gut microbiota modulate the metabolism of brown adipose tissue in mice. J Proteome Res 2012;11:620–30.

[146] Ren C, Sylvia K. Sexual Dimorphism in the Gut Microbiome. IU J Undergrad Res 2018;4:12–6.

[147] Yurkovetskiy L, Burrows M, Khan AA, Graham L, Volchkov P, Becker L, et al. Gender bias in autoimmunity is influenced by microbiota. Immunity 2013;39:400–12.

[148] Org E, Mehrabian M, Parks BW, Shipkova P, Liu X, Drake TA, et al. Sex differences and hormonal effects on gut microbiota composition in mice. Gut Microbes 2016;7:313–22.

[149] Danska JS. Sex matters for mechanism. Sci Transl Med 2014;6:258fs40.

[150] Markle JGM, Frank DN, Mortin-Toth S, Robertson CE, Feazel LM, Rolle-Kampczyk U, et al. Sex differences in the gut microbiome drive hormone-dependent regulation of autoimmunity. Science (80-) 2013;339:1084–8.

[151] Kaliannan K, Robertson RC, Murphy K, Stanton C, Kang C, Wang B, et al. Estrogen-mediated gut microbiome alterations influence sexual dimorphism in metabolic syndrome in mice. Microbiome 2018;6:205.

[152] Min Y, Ma X, Sankaran K, Ru Y, Chen L, Baiocchi M, et al. Sex-specific association between gut microbiome and fat distribution. Nat Commun 2019;10.

[153] Muegge BD, Kuczynski J, Knights D, Clemente JC, González A, Fontana L, et al. Diet drives convergence in gut microbiome functions across mammalian phylogeny and within humans. Science (80-) 2011;332:970–4.

[154] Zhernakova A, Kurilshikov A, Bonder MJ, Tigchelaar EF, Schirmer M, Vatanen T, et al. Population-based metagenomics analysis reveals markers for gut microbiome composition and diversity. Science (80-) 2016;352:565–9.

[155] Zhang C, Zhang M, Wang S, Han R, Cao Y, Hua W, et al. Interactions between gut microbiota, host genetics and diet relevant to development of metabolic syndromes in mice. ISME J 2010;4:232–41.

[156] Wu GD, Chen J, Hoffmann C, Bittinger K, Chen Y, Keilbaugh SA, et al. Long-Term Link with Gut Microbial Enterotypes. Science (80-) 2011;334:105–9.

[157] Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR, et al. Enterotypes of the human gut microbiome. Nature 2011;473:174–80.

[158] David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, et al. Diet rapidly and reproducibly alters the human gut microbiome. Nature 2014;505:559–63.

[159] De Filippis F, Pellegrini N, Vannini L, Jeffery IB, La Storia A, Laghi L, et al. High-level adherence to a Mediterranean diet beneficially impacts the gut microbiota and associated metabolome. Gut 2016;65:1812–21.

[160] Lopez-Legarrea P, Fuller NR, Zulet MA, Martinez JA, Caterson ID. The influence of Mediterranean, carbohydrate and high protein diets on gut microbiota composition in the treatment of obesity and associated inflammatory state. Asia Pac J Clin Nutr 2014;23:360–8.

[161] Singh RK, Chang HW, Yan D, Lee KM, Ucmak D, Wong K, et al. Influence of diet on the gut microbiome and implications for human health. J Transl Med 2017;15.

[162] Kim MS, Hwang SS, Park EJ, Bae JW. Strict vegetarian diet improves the risk factors associated with metabolic diseases by modulating gut microbiota and reducing intestinal inflammation. Environ Microbiol Rep 2013;5:765–75.

[163] Toribio-Mateas M. Harnessing the Power of Microbiome Assessment Tools as Part of Neuroprotective Nutrition and Lifestyle Medicine Interventions. Microorganisms 2018;6:35.

[164] Gibson GR, Roberfroid MB. Dietary Modulation of the Human Colonic Microbiota: Introducing the Concept of Prebiotics. J Nutr 1995;125:1401–12.

[165] Bindels LB, Delzenne NM, Cani PD, Walter J. Towards a more comprehensive concept for prebiotics. Nat Rev Gastroenterol Hepatol 2015;12:303–10.

[166] Alberti KGMM, Gassull MA, Metz GL, Leeds AR. Dietary fibres, fibre analogues, and glucose tolerance: Importance of viscosity. Br Med J 1978;1:1392–4.

[167] Delzenne NM, Cani PD, Daubioul C, Neyrinck AM. Impact of inulin and oligofructose on gastrointestinal peptides. Br J Nutr 2005;93 Suppl 1:S157-61.

[168] Cani PD, Neyrinck AM, Maton N, Delzenne NM. Oligofructose Promotes Satiety in Rats Fed a High-Fat Diet: Involvement of Glucagon-Like Peptide-1. Obes Res 2005;13:1000–7.

[169] Zhang Q, Yu H, Xiao X, Hu L, Xin F, Yu X. Inulin-type fructan improves diabetic phenotype and gut microbiota profiles in rats 2018.

[170] Tan S, Caparros-Martin JA, Matthews VB, Koch H, O'Gara F, Croft KD, et al. Isoquercetin and inulin synergistically modulate the gut microbiome to prevent development of the metabolic syndrome in mice fed a high fat diet. Sci Rep 2018;8.

[171] Zou J, Chassaing B, Singh V, Fythe MD, Kumar V, Gewirtz Correspondence AT. Fiber-Mediated Nourishment of Gut Microbiota Protects against Diet-Induced Obesity by Restoring IL-22-Mediated Colonic Health. Cell Host Microbe 2018;23:41–53.

[172] Zhu L, Qin S, Zhai S, Gao Y, Li L. Inulin with different degrees of polymerization modulates composition of intestinal microbiota in mice. FEMS Microbiol Lett 2017;364.

[173] Roberfroid MB, Van Loo JA, Gibson GR. The bifidogenic nature of chicory inulin and its hydrolysis products. J Nutr 1998;128:11–9.

[174] van der Beek CM, Canfora EE, Kip AM, Gorissen SHM, Olde Damink SWM, van Eijk HM, et al. The prebiotic inulin improves substrate metabolism and promotes short-chain fatty acid production in overweight to obese men. Metabolism 2018;87:25–35.

[175] Vandeputte D, Falony G, Vieira-Silva S, Wang J, Sailer M, Theis S, et al. Prebiotic inulin-type fructans induce specific changes in the human gut microbiota. Gut 2017;66:1968–74.

[176] Hiel S, Bindels LB, Pachikian BD, Kalala G, Broers V, Zamariola G, et al. Effects of a diet based on inulin-rich vegetables on gut health and nutritional behavior in healthy humans. Am J Clin Nutr 2019;109:1683–95.

[177] Hira T, Suto R, Kishimoto Y, Kanahori S, Hara H. Resistant maltodextrin or fructooligosaccharides promotes GLP-1 production in male rats fed a high-fat and high-sucrose diet, and partially reduces energy intake and adiposity. Eur J Nutr 2018;57:965–79.

[178] Kumar SA, Ward LC, Brown L. Inulin oligofructose attenuates metabolic syndrome in high-carbohydrate, high-fat diet-fed rats. Br J Nutr 2016;116:1502–11.

[179] Hamilton MK, Ronveaux CC, Rust BM, Newman JW, Hawley M, Barile D, et al. Prebiotic milk oligosaccharides prevent development of obese phenotype, impairment of gut permeability, and microbial dysbiosis in high fat-fed mice. Am J Physiol Gastrointest Liver Physiol 2017;312:G474–87.

[180] Cani PD, Possemiers S, Van de Wiele T, Guiot Y, Everard A, Rottier O, et al. Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. Gut 2009;58:1091–103.

[181] Tsao R. Chemistry and biochemistry of dietary polyphenols. Nutrients 2010;2:1231–46.

[182] Zhou Y, Zheng J, Li Y, Xu DP, Li S, Chen YM, et al. Natural polyphenols for prevention and treatment of cancer. Nutrients 2016;8.

[183] Aryaeian N, Sedehi SK, Arablou T. Polyphenols and their effects on diabetes management: A review. Med J Islam Repub Iran 2017;31:886–92.

[184] Goszcz K, Duthie GG, Stewart D, Leslie SJ, Megson IL. Bioactive polyphenols and cardiovascular disease: chemical antagonists, pharmacological agents or xenobiotics that drive an adaptive response? Br J Pharmacol 2017;174:1209–25.

[185] Farhat G, Drummond S, Al-Dujaili EAS. Polyphenols and Their Role in Obesity Management: A Systematic Review of Randomized Clinical Trials. Phytother Res 2017;31:1005–18.

[186] Manach C, Williamson G, Morand C, Scalbert A, Rémésy C. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. Am J Clin Nutr 2005;81:230S-242S.

[187] Filosa S, Di Meo F, Crispi S. Polyphenols-gut microbiota interplay and brain neuromodulation. Neural Regen Res 2018;13:2055–9.

[188] Fotschki B, Juśkiewicz J, Jurgoński A, Kołodziejczyk K, Milala J, Kosmala M, et al. Anthocyanins in strawberry polyphenolic extract enhance the beneficial effects of diets with fructooligosaccharides in the rat cecal environment. PLoS One 2016;11.

[189] Williams AR, Krych L, Ahmad HF, Nejsum P, Skovgaard K, Nielsen DS, et al. A polyphenol-enriched diet and Ascaris suum infection modulate mucosal immune responses and gut microbiota composition in pigs. PLoS One 2017;12.

[190] Rothenberg DO, Zhou C, Zhang L. A Review on the Weight-Loss Effects of Oxidized Tea Polyphenols. Molecules 2018;23.

[191] Luca SV, Macovei I, Bujor A, Miron A, Skalicka-Woźniak K, Aprotosoaie AC, et al. Bioactivity of dietary polyphenols: The role of metabolites. Crit Rev Food Sci Nutr 2019:1–34.

[192] Tanabe H, Pervin M, Goto S, Isemura M, Nakamura Y. Beneficial Effects of Plant Polyphenols on Obesity. Obes Control Ther 2017;4:1–16.

[193] Ozdal T, Sela DA, Xiao J, Boyacioglu D, Chen F, Capanoglu E. The reciprocal interactions between polyphenols and gut microbiota and effects on bioaccessibility. Nutrients 2016;8.

[194] Rauf A, Imran M, Abu-Izneid T, Iahtisham-Ul-Haq, Patel S, Pan X, et al. Proanthocyanidins: A comprehensive review. Biomed Pharmacother 2019;116.

[195] Peron G, Sut S, Pellizzaro A, Brun P, Voinovich D, Castagliuolo I, et al. The antiadhesive activity of cranberry phytocomplex studied by metabolomics: Intestinal PAC-A metabolites but not intact PAC-A are identified as markers in active urines against uropathogenic Escherichia coli. Fitoterapia 2017;122:67–75.

[196] Anhê FF, Roy D, Pilon G, Dudonné S, Matamoros S, Varin T V., et al. A polyphenol-rich cranberry extract protects from diet-induced obesity, insulin resistance and intestinal inflammation in association with increased Akkermansia spp. population in the gut microbiota of mice. Gut 2015;64:872–83.

[197] Roopchand DE, Carmody RN, Kuhn P, Moskal K, Rojas-Silva P, Turnbaugh PJ, et al. Dietary polyphenols promote growth of the gut bacterium akkermansia muciniphila and attenuate high-fat diet-induced metabolic syndrome. Diabetes 2015;64:2847–58.

[198] Masumoto S, Terao A, Yamamoto Y, Mukai T, Miura T, Shoji T. Non-absorbable apple procyanidins prevent obesity associated with gut microbial and metabolomic changes. Sci Rep 2016;6.

[199] Liu W, Zhao S, Wang J, Shi J, Sun Y, Wang W, et al. Grape seed proanthocyanidin extract ameliorates inflammation and adiposity by modulating gut microbiota in high-fat diet mice. Mol Nutr Food Res 2017;61.

[200] Choy YY, Quifer-Rada P, Holstege DM, Frese SA, Calvert CC, Mills DA, et al. Phenolic metabolites and substantial microbiome changes in pig feces by ingesting grape seed proanthocyanidins. Food Funct 2014;5:2298–308.

[201] Tao W, Zhang Y, Shen X, Cao Y, Shi J, Ye X, et al. Rethinking the Mechanism of the Health Benefits of Proanthocyanidins: Absorption, Metabolism, and Interaction with Gut Microbiota. Compr Rev Food Sci Food Saf 2019.

[202] Yang CS, Zhang J, Zhang L, Huang J, Wang Y. Mechanisms of body weight reduction and metabolic syndrome alleviation by tea. Mol Nutr Food Res 2016;60:160–74.

[203] Henning SM, Yang J, Hsu M, Lee RP, Grojean EM, Ly A, et al. Decaffeinated green and black tea polyphenols decrease weight gain and alter microbiome populations and function in diet-induced obese mice. Eur J Nutr 2018;57:2759–69.

[204] Den Besten G, Havinga R, Bleeker A, Rao S, Gerding A, Van Eunen K, et al. The short-chain fatty acid uptake fluxes by mice on a guar gum supplemented diet associate with amelioration of major biomarkers of the metabolic syndrome. PLoS One 2014;9:e107392.

[205] Crnko S, Du Pré BC, Sluijter JPG, Van Laake LW. Circadian rhythms and the molecular clock in cardiovascular biology and disease. Nat Rev Cardiol 2019;16:437–47.

[206] Bass J, Takahashi JS. Circadian integration of metabolism and energetics. Science 2010;330:1349–54.

[207] Oosterman JE, Kalsbeek A, la Fleur SE, Belsham DD. Impact of nutrients on circadian rhythmicity. Am J Physiol Regul Integr Comp Physiol 2015;308:R337-50.

[208] Lamia KA, Storch K-F, Weitz CJ. Physiological significance of a peripheral tissue circadian clock. Proc Natl Acad Sci U S A 2008;105:15172–7.

[209] Grimaldi B, Bellet MM, Katada S, Astarita G, Hirayama J, Amin RH, et al. PER2 controls lipid metabolism by direct regulation of PPARγ. Cell Metab 2010;12:509–20.

[210] Möller-Levet CS, Archer SN, Bucca G, Laing EE, Slak A, Kabiljo R, et al. Effects of insufficient sleep on circadian rhythmicity and expression amplitude of the human blood transcriptome. Proc Natl Acad Sci U S A 2013;110.

[211] James SM, Honn KA, Gaddameedhi S, Van Dongen HPA. Shift Work: Disrupted Circadian Rhythms and Sleep—Implications for Health and Well-being. Curr Sleep Med Reports 2017;3:104–12.

[212] Hussain MM, Pan X. Circadian Regulation of Macronutrient Absorption. J Biol Rhythms 2015;30:459–69.

[213] Shi S, Ansari TS, McGuinness OP, Wasserman DH, Johnson CH. Circadian disruption leads to insulin resistance and obesity. Curr Biol 2013;23:372–81.

[214] Thaiss CA, Zeevi D, Levy M, Zilberman-Schapira G, Suez J, Tengeler AC, et al. Transkingdom control of microbiota diurnal oscillations promotes metabolic homeostasis. Cell 2014;159:514–29.

[215] Voigt RM, Summa KC, Forsyth CB, Green SJ, Engen P, Naqib A, et al. The Circadian Clock Mutation Promotes Intestinal Dysbiosis. Alcohol Clin Exp Res 2016;40:335–47.

[216] Liang X, Bushman FD, FitzGerald GA. Rhythmicity of the intestinal microbiota is regulated by gender and the host circadian clock. Proc Natl Acad Sci U S A 2015;112:10479–84.

[217] Deaver JA, Eum SY, Toborek M. Circadian Disruption Changes Gut Microbiome Taxa and Functional Gene Composition. Front Microbiol 2018;9:737.

[218] Thaiss CA, Levy M, Korem T, Dohnalová L, Shapiro H, Jaitin DA, et al. Microbiota Diurnal Rhythmicity Programs Host Transcriptome Oscillations. Cell 2016;167:1495-1510.e12.

[219] Kaczmarek JL, Thompson S V, Holscher HD. Complex interactions of circadian rhythms, eating behaviors, and the gastrointestinal microbiota and their potential impact on health. Nutr Rev 2017;75:673–82.

[220] Parkar SG, Kalsbeek A, Cheeseman JF. Potential Role for the Gut Microbiota in Modulating Host Circadian Rhythms and Metabolic Health. Microorganisms 2019;7.

[221] Mattson MP, Allison DB, Fontana L, Harvie M, Longo VD, Malaisse WJ, et al. Meal frequency and timing in health and disease. Proc Natl Acad Sci U S A 2014;111:16647–53.

[222] Forsyth CB, Voigt RM, Burgess HJ, Swanson GR, Keshavarzian A. Circadian rhythms, alcohol and gut interactions. Alcohol 2015;49:389–98.

[223] Wehrens SMT, Christou S, Isherwood C, Middleton B, Gibbs MA, Archer SN, et al. Meal Timing Regulates the Human Circadian System. Curr Biol 2017;27:1768-1775.e3.

[224] Zarrinpar A, Chaix A, Yooseph S, Panda S. Diet and feeding pattern affect the diurnal dynamics of the gut microbiome. Cell Metab 2014;20:1006–17.

[225] Voigt RM, Forsyth CB, Green SJ, Mutlu E, Engen P, Vitaterna MH, et al. Circadian disorganization alters intestinal microbiota. PLoS One 2014;9.

[226] Castanon-Cervantes O, Wu M, Ehlen JC, Paul K, Gamble KL, Johnson RL, et al. Dysregulation of inflammatory responses by chronic circadian disruption. J Immunol 2010;185:5796–805.

[227] Tognini P, Murakami M, Sassone-Corsi P. Interplay between Microbes and the Circadian Clock. Cold Spring Harb Perspect Biol 2018;10.

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II. HYPOTHESIS AND OBJECTIVES

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HYPOTHESIS AND OBJECTIVES

The inter-relationship between gut microbiota, diet and obesity development has gained significant research interest during the last years. To date, causality or correlation among these concepts is still under debate, although close links have been broadly described in the literature. In this sense, obesity and gut microbiota dysbiosis occurs concomitantly, while diet has been recognised as the major effector on the establishment of a particular microbiome. Remarkably, gut microbiota is implicated in the regulation of several metabolic functions in the host, thus recently becoming a new target for obesity treatment and prevention.

Like obesity, gut microbiota dysbiosis is multi-factorial, and, consequently, the interaction between environmental and genetic conditions determines the onset of a disturbed microbial phenotype. In this sense, dietary habits, antibiotics, age, gender, ethnicity and exercise level constitute main factors leaning the scale between healthy or dysbiotic microbial profile. Usually, obesity is linked to an imbalanced gut microbiota characterized by a loss of diversity and a reduction of beneficial microorganisms at the expense of harmful microbes. In addition, dysbiosis is linked to aberrant microbiota circadian fluctuations, which might be also driven by impaired feeding rhythmicity, thereby linking diet, gut microbiome and circadian rhythms misalignment with increased obesity.

Targeting gut microbiota represents a new avenue for dietary interventions in order to prevent or treating obesity. In fact, the use of prebiotics has been widely related to the rescue or improvement of gut

microbiota activity, thus resulting in beneficial physiologic and metabolic effects on the host. In addition, the potential benefits of grape seed proanthocyanidins for the host have been extensively studied in our research group.

In the present PhD thesis, we **hypothesized** that gut microbiota dysbiosis triggered by diet-induced obesity is conditioned by genetic background and environmental factors, thus distinctively affecting host physiology and metabolism, whereas a dietary intervention with potential prebiotics contributes to the recovery of a balanced gut microbiome and a healthy status.

Therefore, the **principal objective** of this thesis was to examine the impact of diet-induced obesity on gut microbiota by studying the main factors involved in its establishment, and to evaluate the influence of potential prebiotics in the gut microbiota composition and function.

To achieve this purpose, the following specific objectives were raised:

1. **To evaluate and compare the impact of three different dietary models of obesity (cafeteria diet, high-fat diet and high-fat high-sucrose diet) on gut microbiome and to elucidate associations between microbiota alterations and host's metabolic status in Wistar rats (Chapter I).**

   Diet constitutes the main source of utilizable substrates for gut microbiota, thus profoundly influencing its composition and activity. In this sense, Western-style diets in modern societies are accurately reproduced by the cafeteria diet, which is based on the free choice of highly palatable and energy-dense foods. Alternatively, semi-purified

diets, such as HF diets, are most commonly used to evaluate the onset of obesity in rodents. Hence, divergent DIO models might have a distinct impact on the gut microbiome and host phenotype, thus hindering the comparisons between different studies. For this reason, we aimed to characterize and compare the consequences of a chronic exposure to different hypercaloric diets on gut microbiota and obesity development.

2. **To characterize the influence of gender and strain on the dysbiosis of gut microbiome in rats chronically exposed to cafeteria diet, and its relevance on host physiology (Chapter II).**

   Beyond the diet, several intrinsic factors, including rat strain, gender and age, influence the gut microbiota composition. In this sense, inter-individual variability on gut microbiota profiles underlying genetic factors may account for different physiologic and metabolic responses to diet-induced obesity. For instance, sexual dimorphism in obesity, which is mainly attributed to the protective effects of estrogens, could be closely related to distinct microbiome-mediated responses. In this regard, estrogens are known to influence obesity susceptibility in a microbiota-dependent manner.

3. **To investigate the impact of cafeteria diet-induced obesity on gut microbiota rhythmicity in male Fischer 344 rats and to determine the potential benefits of grape seed proanthocyanidins in the recovery of microbial rhythms and host health (Chapter III).**

Due to the cyclical environmental changes, such as dark/light or feeding/fasting periods, most mammals have adapted their metabolism to optimize anabolic and catabolic processes, thus maintaining a balanced homeostasis throughout the day. Most interestingly, the gut microbiota also undergoes diurnal oscillations, which are mainly controlled by central clock machinery and feeding/fasting cycles, thus favouring certain bacteria according to the daily moment. Importantly, gut microbiota also influences the host circadian rhythms in an orchestrated interaction to meet their biological needs. Consequently, the diet-induced disturbances on host-microbial rhythmicity could be contributing to the development of metabolic diseases, such as obesity.

In this scenario, proanthocyanidins have been identified as active compounds able to exert beneficial effects in obesity by promoting satiety and energy expenditure. Since some proanthocyanidins present large degrees of polymerization, their direct absorption in the gut is highly limited. Therefore, the gut microbiota has been proposed to have a determinant role in the mechanisms of action of proanthocyanidins. Moreover, the phylogenetic structure of the microbiome might also be influenced by these compounds, thus suggesting that beneficial effects of proanthocyanidins may be partially mediated by changes on gut microbiota.

4. **To evaluate the potential prebiotic capacity of a wide range of agro-industrial by-products and natural ingredients using an *in vitro* fermentation procedure with human fecal samples from normoweight and obese individuals (Chapter IV).**

The dietary modulation of the gut microbiome has become one major challenge in the treatment and prevention of obesity. Dietary components, such as non-digestible polysaccharides, are utilized by microbes residing in the gut, thereby producing microbial derivatives such as short-chain fatty acids. For instance, the consumption of inulin-type fructans induces the growth of beneficial bacteria, thus promoting an increased short-chain fatty acid production, fat oxidation and a reduced food intake.

The large amount of food wastes generated by the food industry, which negatively impact on the biosphere and countries' economy, claims for an effective strategy of revalorization by benefitting from their nutritional properties. In fact, some authors have reported encouraging prebiotic activities for different industrial by-products such as fruit peel and coffee residues. The *in vitro* screening methods allow performing a broad and rapid evaluation of the prebiotic capacity of natural ingredients and industrial by-products.

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HIPÒTESI I OBJECTIUS

L'interès científic per l'estudi de la relació entre la microbiota intestinal, la dieta i el desenvolupament de l'obesitat ha crescut durant els darrers anys. Tot i que els estrets vincles entre aquests tres conceptes han estat descrits àmpliament a la literatura, actualment encara es discuteix quin és el grau de causalitat o de correlació entre ells. En aquest sentit, l'obesitat i la disbiosi de la microbiota intestinal ocorren simultàniament, mentre que la dieta és reconegut com el principal efector sobre l'establiment d'un determinat microbioma. A més, la microbiota intestinal, que es troba implicada en la regulació de diverses funcions metabòliques de l'hoste, ha esdevingut una nova diana d'actuació per al tractament i la prevenció de l'obesitat.

De la mateixa manera que l'obesitat, la disbiosi de la microbiota intestinal té un origen multi-factorial i, en conseqüència, la interacció entre les condicions ambientals i genètiques determinen el desenvolupament d'un fenotip bacterià alterat. En aquest sentit, els hàbits dietètics, els antibiòtics, l'edat, el sexe, l'ètnia i el nivell d'exercici físic constitueixen els principals factors que decanten la balança entre una microbiota saludable o alterada. Sovint, el desequilibri de la microbiota intestinal que s'observa en condicions d'obesitat es caracteritza per la pèrdua de diversitat i per la reducció dels microorganismes beneficiosos en favor de microbis nocius. A més, la disbiosi s'associa amb una alteració de les fluctuacions circadiàries dels microorganismes que la composen, les quals també es poden veure afectades per canvis severs en els patrons d'alimentació, establint així un vincle entre la dieta, la microbiota intestinal i els ritmes circadiaris i el desenvolupament de l'obesitat.

L'actuació sobre la microbiota intestinal representa una nova via per a les intervencions dietètiques enfocades a prevenir o tractar l'obesitat. De fet, l'ús de prebiòtics s'ha vist àmpliament relacionat amb el rescat o la millora de l'activitat de la microbiota, induint així efectes beneficiosos a nivell fisiològic i metabòlic per a l'hoste. A més, el nostre grup de recerca ha estudiat extensament els beneficis de les proantocianidines procedents de la llavor del raïm sobre l'hoste.

En aquesta tesi doctoral, es va **hipotetitzar** que la disbiosi de la microbiota intestinal en l'obesitat induïda per la dieta està condicionada per factors genètics i ambientals, afectant de manera diferent al metabolisme i la fisiologia de l'hoste, mentre que una intervenció dietètica amb ingredients potencialment prebiòtics contribueix al restabliment d'un microbioma equilibrat i d'un estat saludable.

Per tant, el principal **objectiu** d'aquesta tesi va ser examinar l'impacte de l'obesitat induïda per la dieta sobre la microbiota intestinal a través de l'estudi dels factors implicats en el seu establiment, així com avaluar la influència de prebiòtics potencials sobre la composició i la funció de la microbiota.

Per tal d'assolir aquest objectiu, es van plantejar els següents objectius específics:

1. **Avaluar i comparar l'impacte de tres models dietètics d'obesitat diferents (dieta de cafeteria, dieta alta en greix, dieta alta en greix i sucres) sobre el microbioma i elucidar l'associació entre les alteracions de la microbiota i l'estat metabòlic de l'hoste en rates Wistar (Capítol I).**

La dieta constitueix la principal font de substrats utilitzables per la microbiota intestinal, influenciant profundament la seva composició i activitat. En aquest sentit, les dietes occidentals de les societats modernes són reproduïdes acuradament per una dieta de cafeteria, basada en la lliure elecció d'aliments energèticament densos i altament apetitosos. D'altra banda, les dietes semi-purificades, com les dietes altes en greix, són les que s'utilitzen més comunament per estudiar l'aparició de l'obesitat en rosegadors. Per tant, els diferents models dietètics emprats per induir l'obesitat poden tenir un impacte diferent sobre la microbiota i el fenotip de l'hoste, dificultant doncs la comparació entre estudis.

2. **Caracteritzar la influencia del gènere i la soca animal en la disbiosi de la microbiota intestinal en rates exposades crònicament a una dieta de cafeteria, i la seva rellevància sobre la fisiologia de l'hoste (Capítol II)**

Més enllà de la dieta, diferents factors intrínsecs, incloent la soca, el gènere i l'edat, influencien la composició de la microbiota intestinal. En aquest sentit, la variabilitat interindividual dels perfils microbians que recau en els factors genètics pot dur a diferents respostes fisiològiques i metabòliques davant l'obesitat induïda per la dieta. Per exemple, el dimorfisme sexual de l'obesitat, el qual s'atribueix principalment als efectes protectors dels estrogens, podria estar estretament lligat a les diferents respostes vehiculades pel microbioma. En aquest sentit, els estrogens influencien la susceptibilitat a l'obesitat a través de mecanismes que impliquen la microbiota.

3. Investigar l'impacte de l'obesitat induïda per una dieta de cafeteria sobre la ritmicitat de la microbiota intestinal en rates Fischer 344 i determinar els potencials beneficis d'un extracte de proantocianidines de la llavor del raïm en la recuperació dels ritmes microbians i en la salut de l'hoste (Capítol III)

A causa dels canvis ambientals cíclics, com els períodes de foscor/claror o d'alimentació/dejuni, la majoria dels mamífers han adaptat el seu metabolisme per optimitzar els processos anabòlics i catabòlics, mantenint així un estat d'homeòstasi equilibrat i constant. Encara més interessant és el fet que la microbiota intestinal també pateix oscil·lacions diürnes en la seva composició que són controlades principalment pel rellotge biològic central i pels cicles d'alimentació/dejuni, afavorint així la presència de determinats bacteris en funció del moment del dia. És important destacar que la microbiota intestinal també influeix sobre ritmes circadiaris de l'hoste, establint-se una interacció orquestrada per fer front a les necessitats biològiques. En conseqüència, les pertorbacions induïdes per la dieta en la ritmicitat hoste-microbiota poden contribuir al desenvolupament de malalties metabòliques, com ara l'obesitat. En aquest escenari, les proantocianidines s'han identificat com a compostos actius capaços d'exercir efectes beneficiosos en l'obesitat promovent la sacietat i la despesa energètica. Atès que algunes proantocianidines presenten alts graus de polimerització en la seva estructura, la seva absorció directa a l'intestí és molt limitada. Per tant, s'ha proposat que la microbiota intestinal tingui un paper determinant en els mecanismes d'acció de les proantocianidines. A més, la pròpia composició del microbioma també podria veure's

afectada per aquests compostos, la qual cosa suggereix que els efectes beneficiosos de les proantocianidines podrien estar parcialment mediats per canvis en la microbiota intestinal.

4. **Avaluar la capacitat prebiòtica d'un ampli rang de subproductes de la industria agro-alimentària i d'ingredients naturals mitjançant un protocol de fermentació *in vitro* amb femta humana procedent d'individus normopès i obesos (Capítol IV).**

La modulació dietètica del microbioma intestinal s'ha convertit en un dels principals desafiaments en el tractament i la prevenció de l'obesitat. Els components dietètics, com els polisacàrids no-digeribles, són utilitzats pels microbis residents a l'intestí, produint així derivats microbians com els àcids grassos de cadena curta. Per exemple, el consum de fructans del tipus inulina indueix el creixement de bacteris beneficiosos, afavorint així la producció d'àcids grassos de cadena curta, l'oxidació de greixos i una reducció en la ingesta d'aliments.

La gran quantitat de residus alimentaris generats per la indústria alimentària, que repercuteixen negativament en la biosfera i l'economia dels països, reclama una estratègia efectiva per a la seva revaloració beneficiant-nos de les seves propietats nutricionals. De fet, alguns autors han descrit activitats prebiòtiques encoratjadores per a diferents subproductes industrials com ara les pells de la fruita i els residus del cafè. Els mètodes de cribratge *in vitro* permeten fer una avaluació àmplia i ràpida de la capacitat prebiòtica dels ingredients naturals i dels subproductes industrials.

UNIVERSITAT ROVIRA I VIRGILI
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Andreu Gual Grau

III. RESULTS

UNIVERSITAT ROVIRA I VIRGILI
GUT MICROBIOTA DYSBIOSIS IN DIET-INDUCED OBESITY. A FOCUS ON THE INFLUENCE OF GENETICS, CIRCADIAN RHYTHMS AND POTENTIAL PREBIOTICS.
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CHAPTER I

Impact of Different Hypercaloric Diets on Obesity Features in Rats: A Metagenomics and Metabolomics Integrative Approach.

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ABSTRACT

Diet is considered a key influencing agent affecting the gut microbiome. Dysbiosis of microbial communities contributes to the development of metabolic diseases such as obesity. We aimed to characterize the physiological, microbial and metabolic changes induced by different obesogenic diets to understand the diet-specific modulation of the host-microbiota co-metabolism in rodents. For this purpose, Wistar rats were fed standard (STD), cafeteria (CAF), low-fat (LF), high-fat (HF) and high-fat high-sucrose (HFS) diets for 10 weeks. The CAF diet strongly induced an obese phenotype accompanied by dyslipidemia, hyperleptinemia, insulin resistance and hepatic steatosis, whereas both HF and HFS diets promoted overweight. Concerning the microbiome, CAF feeding induced a rise of the Bacteroidetes-to-Firmicutes ratio, while few microbial genera were altered in the HF or HFS group. Changes in microbial activity according to dietary treatment were also reflected in the disruption of short-chain fatty acid (SCFA) production and bile acid (BA) metabolism, which were mainly associated with fiber intake. Urinary metabolomics revealed a significant increase in metabolites related to oxidative stress and metabolic inflammation together with an altered excretion of host-microbiota co-metabolites only in the CAF group. Moreover, several associations between metabolic patterns, physiological status and specific microbial communities were described, helping to elucidate the crucial role of the microbiota in host homeostasis. Overall, our study suggests that different hypercaloric dietary models distinctively influence gut microbiota composition and reveals robust and similar clustering patterns concerning both cecal microbiome and urinary metabolome profiles.

Key words: Gut microbiota, urinary metabolome, diet-induced obesity, host-microbiota co-metabolism, hypercaloric diets.

1. INTRODUCTION

Comprehension, prevention and treatment of obesity and related metabolic complications have become a global challenge for researchers over the past decades. Worldwide, the prevalence of obesity has dramatically increased, especially in developed countries [1], together with several obesity-associated common diseases such as type 2 diabetes, hypertension and non-alcoholic fatty liver disease, among others [2]. To explore the mechanisms involved in obesity, animal models of diet-induced obesity (DIO) have been widely used for a long time.

For DIO studies, different obesogenic diets have been employed, including high-fat (HF), high-fat, high-sucrose (HFS), and cafeteria (CAF) [3,4]. The hyperphagia associated with CAF feeding results in severe obesity as occurs in humans under the so-called Western diet [5]. Nevertheless, semi-purified diets are more commonly used in DIO models due to their well-defined nutritional composition. Therefore, investigating and comparing specific diet-linked effects on obesity progression can be useful for further studies.

Recently, the gut microbiota has emerged as a new contributory factor to the development of obesity [6]. Dysbiosis of gut microbial communities and the subsequent altered functions including short-chain fatty acid (SCFA) production, bile acid biotransformation, amino acid synthesis and fermentation of non-digestible substrates [7], have been associated with

the aetiology of obesity [8]. Most studies have shown that HF diets lead to an altered Bacteroidetes-to-Firmicutes ratio in favor of Firmicutes [9], while Western-type diets increase the Bacteroidetes proportion [10]. However, although alterations in the population of major phyla during obesity have been described in several animal and human studies [11,12], a specific microbiome pattern for obesity has not been reported.

Metabolomics can provide a whole picture of the overall health status, reflecting associations between individual phenotypes and metabolic fingerprints, as has been observed in HF- [13] and CAF-fed rodents [14]. Moreover, alterations on the host-microbiome co-metabolism have been related with gut microbial changes in obese humans and animals [15,16].

In this context, our objective was to evaluate and compare three different DIO rat models focusing on the gut microbiome and to describe associations between microbiota changes and metabolic status. To this aim, a multiomics approach involving metagenomics and metabolomics analyses, along with the assessment of physiological parameters, was employed.

2. MATERIALS AND METHODS

2.1 Animal procedures.

Thirty 6-week-old male Wistar rats supplied from Charles River Laboratories (Wilmington, Massachusetts, USA) were housed individually and kept in a temperature-controlled room (22°C) under a 12:12-hour light:dark cycle with free access to food and water. Animals were randomly distributed into five groups (n=6 per group) depending on the diet offered for 10 weeks: (1) a standard chow diet (Teklad Global

18% Protein Rodent Diet 2018, Harlan, Barcelona, Spain) (group STD), (2) a cafeteria diet composed of highly palatable and energy-dense foods such as biscuits with pâté and cheese (14-15 g), bacon (5-7 g), muffins (6-8 g) and sugared milk (220 g/l; 50 mL) together with carrot (6-8 g) and standard chow (group CAF), (3) a semi-purified low-fat diet (D12450K, Research Diets, New Jersey, USA) (group LF), (4) a semi-purified high-fat diet (D12451, Research Diets, New Jersey, USA) (group HF), and (5) a high-fat high-sucrose diet comprised of a semi-purified high-fat diet plus sweetened milk (220g/l; 30 mL) (group HFS). The energetic density and the macronutrient composition of the diets are shown in **Supplementary Table 1**. Body weight and food intake were recorded weekly during the whole experimental procedure. For urine collection, at the ninth week of the study, rats were housed individually in metabolic cages and deprived of food for up to 24 h. Urine samples were collected in vessels containing 1% sodium azide (0.1 mL), filtered into 1.5 mL sterile tubes and immediately frozen at -80°C. At the end of the study, rats were euthanized, and blood was obtained by cardiac puncture under anesthesia (pentobarbital sodium, 80 mg/kg body weight). Serum samples were obtained by centrifugation (2,000 g for 15 min). Liver; white adipose tissue depots, including mesenteric (MWAT), retroperitoneal (RWAT), inguinal (IWAT) and epididymal (EWAT); 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 University Rovira i Virgili (Tarragona, Spain) and the *Generalitat de Catalunya* approved all the procedures (DAAM 8865). The experimental protocol followed the

'Principles of laboratory care' and was carried out in accordance with the European Communities Council Directive (86/609/EEC).

2.2 Body composition and adiposity assessment.

Fat and lean mass were determined on days 0 and 70 by nuclear magnetic resonance (NMR) using an EchoMRI-700™ device (Echo Medical Systems, L.L.C., Houston, USA). Adiposity Index was calculated as the sum of the EWAT, IWAT, MWAT and RWAT depots weight and is expressed as percentage of body weight.

2.3 Serum analysis.

Enzymatic colorimetric kits were used for the determination of serum glucose, triglycerides (TG), total cholesterol (QCA, Barcelona, Spain), high-density lipoprotein (HDL) and very low- and low-density lipoproteins (VLDL/LDL) cholesterol (BioAssay Systems, Hayward, USA) and non-esterified free fatty acids (NEFAs) (WAKO, Neuss, Germany) according to the manufacturers' instructions. Circulating insulin and leptin levels were assessed using ELISA kits (Merck, Darmstadt, Germany). The homeostasis model assessment-estimated insulin resistance (HOMA-IR) was computed as follows: fasting insulin (μU/mL) × fasting glucose (mmol/mL)/22.5 as descried previously [17].

2.4 SCFA quantification.

Each cecal sample was homogenized in 0.1% HCOOH before addition of propionic acid-d_6 as an internal standard, and then a liquid-liquid extraction was accomplished using diethyl ether. The organic phase, which had the extracted SCFAs, was analyzed in a 7890A gas chromatograph coupled to a 7000 triple-quadrupole mass spectrometer

(Agilent Technologies) using a HP-FFAP chromatographic column and helium as mobile phase. A volume of 1 µL was injected at a split rate of 10:1 with an oven program set at an initial temperature of 90°C, which increased to 160°C at 15°C/min, then increased to 240°C at 20°C/min, and held at 240°C for 6 min. Positive chemical ionization using methane as reactant gas and acquisition in SIM mode was applied, acquiring ions of 61 m/z for acetic acid, 75 m/z for propionic acid, 89 m/z for butyric acid and 80 m/z for the internal standard.

2.5 Bile acid profiling and quantification.

The concentration of each bile acid (BA) in the cecal contents was measured using UHPLC-MS/MS as described previously [18]. Briefly, BAs were extracted for each sample and then evaporated and diluted into 1 mL of methanol. Liquid chromatography was performed using a UHPLC 1290 Series (Agilent Technologies, Sta. Clara, CA, USA) with a gradient elution from an EVO C18 column (2.6 µm, 150 mm × 2.1 mm ID; Phenomenex) and maintained at 27°C and a flow rate of 400 µL/min. Ammonium acetate 20 mM and acetonitrile were used as mobile phases for UHPLC-(-ESI)QqQ analysis. MS analysis was performed using a QqQ/MS 6490 Series (Agilent Technologies, Santa Clara, CA, USA) equipped with an ESI probe in negative-ion mode. The bile acids analyzed were cholic acid (CA), α-muricholic acid (α-MCA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), hyodeoxycholic acid (HDCA), ursodeoxycholic acid (UDCA), lithocholic acid (LCA), and some tauro- and glyco-conjugated forms.

2.6 Liver histological analysis.

Frozen liver samples were embedded in Tissue-Tek directly on the mold of the cryostat. Three 12-µm-thick cross-sections from different depths were placed on slides. One milliliter of previously prepared ORO working solution (1.5 parts of 6.25 mg/mL ORO in 99% isopropyl alcohol diluted into one part of distilled water) was added to cover the sections for 20 min. Sections were carefully rinsed with water twice. To visualize cell nuclei, sections were countered with Harris' hematoxylin for 20 sec and rinsed twice for 1 sec each. The slides were mounted with glycerine at 45°C, and coverslips were placed on them.

Images were captured at x400 magnification in six randomly selected fields with a light microscope (LEICA DM 750, camera LEICA ICC50 W). Tissue lipid accumulation was quantified by computer image analysis using ImageJ 1.51j8 software (U. S. National Institute of Health, Bethesha, Maryland, USA) as previously described [19]. Briefly, red-green-blue (RGB) images were color-thresholded to discriminate the lipid droplets, which were displayed by ImageJ as surface area in square micrometers (μm^2) and are presented as integrated density (arbitrary units).

2.7 Urinary metabolic profile.

Urine samples for ^1H NMR spectroscopy were prepared as previously described [20]. Standard nuclear Overhauser effect spectroscopy (NOESY) one-dimensional ^1H NMR spectra were acquired on a 600 MHz Bruker NMR spectroscopy equipped with a 5 mm PABBO probe. For each sample, 4 dummy scans were followed by 256 scans and collected in 64k data points using a spectral width of 16 ppm.

Spectra were manually phased, baseline-corrected and referenced to the 3-trimethylsilyl 1-[2,2,3,3-$^{2}H_4$] propionate (TSP) signal at δ 0.00. ^{1}H NMR spectra (δ 0.2–10.0) were digitized into consecutive integrated spectral regions of equal width (0.00055 ppm). The regions within δ 4.7–6.0, containing the residual water resonance and the urea peak, were removed from all spectra to minimize baseline distortions caused by imperfect water suppression. Each spectrum was normalized to unit area and then aligned using a recursive segment-wise peak alignment algorithm to minimize chemical shift variation.

2.8 Assessment of gut microbiota.

DNA was extracted from the cecal content samples using a QIAamp DNA stool mini kit (Qiagen Inc., Hilden, Germany) according to the manufacturer's instructions. DNA integrity and purity were evaluated using spectrophotometry (NanoDrop, Thermo Fisher Scientific, Massachusetts, USA). 16S rRNA gene amplification and purification were performed as described previously [21].

Three multiplexed pools (4 samples for each pool) were diluted to 26 pM DNA concentration prior to clonal amplification. Clonal amplification and sequencing were performed by employing the Ion PGM™ Hi-Q™ View OT2 Kit and Ion PGM™ Hi-Q™ View Sequencing Kit according to the manufacturer's instructions. Prepared samples were loaded on three 318 chips, respectively, and sequenced using the Ion PGM system (Life Technologies).

After sequencing, the individual sequence reads were filtered by the PGM software to remove low-quality and polyclonal sequences and subsequently analyzed using QIIME [22]. The sequence similarity

threshold for both OTU picking and taxonomy assignment was 97%, and the taxonomy database employed was GreenGenes.

2.9 Statistical analysis.

All results are expressed as the mean ± standard error of the mean (SEM). The Grubbs test was applied to remove significant outliers. Differences between groups were analyzed using one-way analysis of variance (ANOVA) followed by the DMS post hoc test. Body weight progression over time was analyzed using repeated-measures ANOVA. Correlation analyses were performed by Pearson's correlation coefficient. A probability of $p<0.05$ was considered statistically significant. Statistical analyses were performed using SPSS software (SPSS, Inc., Chicago, IL, USA).

For metagenomics analysis, one-way PERMANOVA based on Bray-Curtis distances was employed to assess the dissimilarity of cecal microbiota composition among different diets. Afterwards, the nonparametric Kruskal-Wallis test followed by the Games-Howell post hoc test was performed to elucidate pairwise differences in specific bacteria relative abundance between dietary groups. Statistical analyses were conducted with open-access PAST and STAMP softwares.

Multivariate modeling of metabolomics data was performed in MATLAB (MathWorks) using in-house scripts as previously described [20]. Briefly, it included initially a principal component analysis (PCA) to identify clusters and outliers, followed by pairwise comparisons between diets using an orthogonal projection to latent structures-discriminant analysis (OPLS-DA) to identify discriminatory metabolites. Statistical validation of the OPLS-DA included 1,000 different model

permutations. Colors in the OPLS coefficients plot indicate the correlation coefficient (r^2) between each metabolite and grouping variable, with red indicating strong significance and blue indicating weak significance.

Unsupervised hierarchical clustering analysis (HCA) was performed to identify general patterns of metabolomics and metagenomics variation between samples based on metabolites identified in the OPLS-DA models or bacterial genera present in at least two samples with ≥0.1% abundance, respectively. Data were standardized as *z scores* across samples for each metabolite and microbial genus before clustering so that the mean was 0 and the standard deviation was 1.

To explore the functional correlation between phenotype-defining variables (food intake, biometric measurements, biochemical parameters and urinary metabolome) and the gut microbiome, Pearson's correlation analyses were performed on all the samples grouped together. P-value-adjustment for multiple comparisons was performed according to the Benjamini-Hochberg method considering a 5% false discovery rate.

3. RESULTS

3.1 CAF diet most effectively promotes obesity.

CAF diet-fed animals showed the greatest body weight throughout the study, as revealed by the significant interaction between the type of diet and time (**Figure 1A**). Consistent with those findings, average energy intake was two-fold higher in CAF-fed rats than in STD control rats (**Figure 1B**). HF and HFS groups also presented a higher caloric

intake compared to the corresponding LF control group although to a much lesser extent than CAF-fed rats (**Figure 1B**). As shown in **Figure 1C**, dietary macronutrient composition widely differed between the experimental groups, except for the CAF and HFS groups.

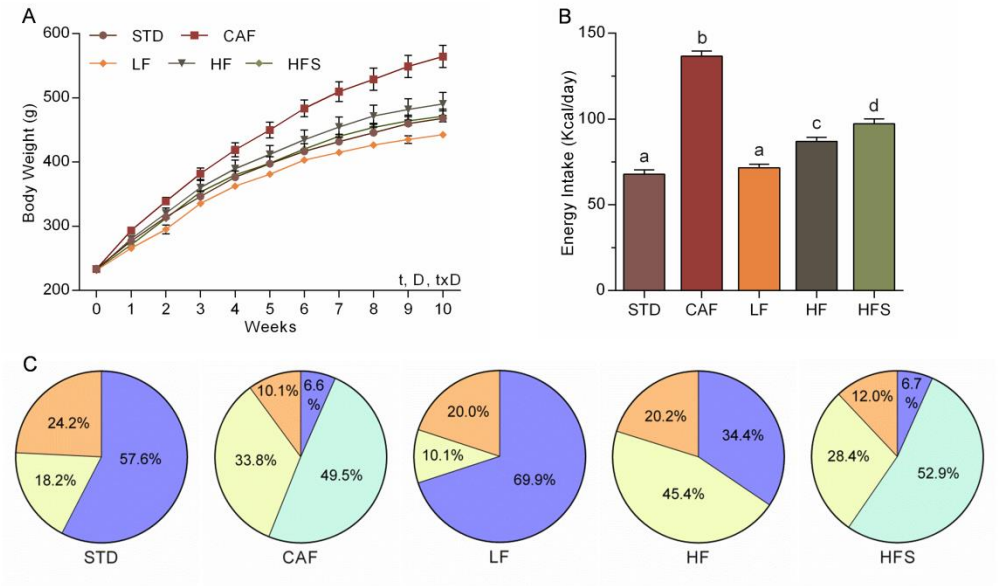

Figure 1. A. Body weight progression for each dietary group for a 10-week period. Data are plotted as the mean ± SEM (n=6) showing: t, time effect; D, diet effect; txD, interaction between time and diet effects (repeated measures ANOVA, $p<0.05$). **B.** Average daily energy intake consumed by animals during the whole experiment. ^{abcd} Mean values with different letters are significantly different (one-way ANOVA followed by DMS post hoc test, $p<0.05$). **C.** Percentage of energy (kcals) derived from each macronutrient within each dietary group showing complex carbohydrates (dark blue), simple carbohydrates (light blue), lipids (yellow) and proteins (orange). STD, standard diet; CAF, cafeteria diet; LF, low-fat diet; HF, high-fat diet; HFS, high-fat high-sugar diet.

Nevertheless, the CAF diet stimulated a larger energy intake from fat and carbohydrates (simple and complex) compared to HFS and the other diets (**Table 1**). Moreover, fiber intake was found significantly reduced under CAF and HFS diets compared with the corresponding control diets

(**Table 1**). In addition, fiber intake was positively associated with colon length (p=0.000, r=0.646) and cecum weight (p=0.000, r=0.862), both parameters being significantly decreased only by the CAF diet (**Table 1**). At the end of the study, and as a result of the hyperphagia promoted by the CAF diet, this group showed the greatest body weight gain, adiposity index and fat mass gain (**Table 1**), whereas HF diet-fed rats increased significantly their body weight gain but not adiposity index or fat mass gain when compared to LF controls. Hence, lean mass gain was reduced by CAF feeding compared to STD control, but not by HF or HFS relative to LF (**Table 1**). Histological analyses of liver samples revealed a significant increase in neutral lipid abundance under CAF, HF or HFS feeding versus STD or LF (**Table 1**).

The analysis of serum parameters (**Table 1**) revealed no significant differences in glucose, NEFAs, TAGs or total cholesterol among the five diet groups. However, animals fed a CAF diet displayed a two-fold increase in insulin compared to the STD group, while no differences were evidenced between the HF or HFS diet and LF controls. HOMA-IR index, a measure of insulin resistance, was significantly higher in the CAF and HF groups compared to the corresponding controls (**Table 1**). In addition, CAF diet consumption caused the development of dyslipidemia by decreasing HDL-cholesterol and increasing VLDL/LDL-cholesterol with respect to STD feeding. The HFS group also showed a significant increase in VLDL/LDL-cholesterol compared to the LF group. In line with the described effects of the different obesogenic diets on adipose tissue weight, leptin was increased only in CAF-fed animals compared to STD controls.

Table 1. Food intake, biometric and serum parameters in rats fed standard (STD), cafeteria (CAF), low-fat (LF), high-fat (HF) or high-fat, high-sucrose (HFS) diets for 10 weeks.

	STD	CAF	LF	HF	HFS	*p-value*
Food intake (kcal/day)						
Total carbohydrates intake	38.7 ± 1.44^{a}	76.7 ± 2.66^{b}	50.8 ± 1.44^{c}	30.3 ± 0.82^{d}	58.4 ± 1.72^{e}	<0.001
Simple carbohydrates intake	0.00 ± 0.00^{a}	67.6 ± 1.34^{b}	0.00 ± 0.00^{a}	15.2 ± 0.41^{c}	52.1 ± 1.82^{d}	<0.001
Lipid intake	12.2 ± 0.45^{a}	46.3 ± 0.85^{b}	7.30 ± 0.21^{c}	40.0 ± 1.08^{d}	27.9 ± 0.78^{e}	<0.001
Protein intake	16.3 ± 0.60^{a}	13.8 ± 0.47^{b}	14.5 ± 0.41^{b}	17.8 ± 0.48^{c}	11.9 ± 0.34^{d}	<0.001
Fiber intake (g/day)	3.18 ± 0.12^{a}	0.90 ± 0.09^{b}	0.89 ± 0.03^{bc}	1.09 ± 0.03^{c}	0.45 ± 0.02^{d}	<0.001
Total food intake (g/day)	21.9 ± 0.81^{a}	65.1 ± 1.89^{b}	18.9 ± 0.53^{a}	18.5 ± 0.50^{a}	50.6 ± 1.97^{c}	0.005
Biometric parameters						
Weight gain (g)	234 ± 13.3^{ab}	331 ± 15.3^{c}	211 ± 6.05^{a}	259 ± 17.1^{b}	239 ± 7.79^{ab}	<0.001
Fat mass (%)	11.0 ± 0.87^{a}	24.8 ± 1.96^{b}	15.4 ± 2.65^{ac}	18.2 ± 3.12^{c}	15.6 ± 1.15^{ac}	0.003
Fat mass gain (%)	3.82 ± 0.67^{a}	17.0 ± 1.38^{b}	7.96 ± 2.02^{ac}	11.1 ± 2.54^{c}	8.35 ± 1.25^{ac}	<0.001
Lean mass (%)	84.2 ± 0.94^{a}	70.9 ± 1.94^{c}	80.1 ± 2.67^{ab}	77.7 ± 3.10^{b}	79.9 ± 1.15^{ab}	0.004
Lean mass gain (%)	-4.18 ± 0.74^{a}	-19.1 ± 0.60^{b}	-8.33 ± 2.19^{a}	-9.73 ± 3.61^{a}	-10.5 ± 0.17^{a}	0.002
Visceral fat (%)	6.93 ± 0.57^{a}	12.1 ± 0.66^{b}	9.16 ± 0.80^{c}	10.0 ± 0.89^{c}	9.51 ± 0.55^{c}	0.001
Adiposity Index	9.01 ± 0.75^{a}	16.0 ± 1.03^{b}	11.7 ± 1.11^{ac}	12.9 ± 1.23^{c}	11.7 ± 0.39^{ac}	0.001
MWAT (%)	1.61 ± 0.12^{a}	2.78 ± 0.26^{b}	2.10 ± 0.26^{abc}	2.30 ± 0.31^{bc}	1.92 ± 0.20^{ac}	0.025

	STD	CAF	LF	HF	HFS	*p-value*
RWAT (%)	2.80 ± 0.28^{a}	5.25 ± 0.36^{b}	3.38 ± 0.28^{ac}	3.70 ± 0.27^{c}	3.82 ± 0.18^{c}	<0.001
EWAT (%)	2.52 ± 0.26^{a}	4.07 ± 0.35^{b}	3.67 ± 0.33^{b}	4.00 ± 0.36^{b}	3.77 ± 0.23^{b}	0.011
IWAT (%)	2.08 ± 0.20^{a}	3.22 ± 0.21^{b}	2.20 ± 0.39^{a}	2.93 ± 0.38^{b}	1.99 ± 0.19^{a}	0.006
Cecum weight (g)	6.55 ± 0.46^{a}	4.18 ± 0.29^{b}	2.88 ± 0.21^{c}	2.58 ± 0.15^{c}	2.91 ± 0.13^{c}	<0.001
Colon length (cm)	21.4 ± 0.65^{a}	19.4 ± 0.55^{b}	17.6 ± 0.71^{b}	18.5 ± 0.62^{b}	18.4 ± 0.83^{b}	0.003
Serum and liver parameters						
Glucose (mg/dL)	147 ± 12.3	176 ± 10.9	163 ± 5.04	198 ± 17.3	184 ± 11.7	0.065
Triglycerides (mg/dL)	97.1 ± 23.2	136 ± 12.4	132 ± 25.9	80.7 ± 17.2	103 ± 22.0	0.287
Total chol (mg/dL)	73.7 ± 8.65	71.2 ± 2.87	61.4 ± 4.14	72.0 ± 8.47	66.2 ± 4.02	0.629
HDL-chol (mg/dL)	60.1 ± 5.92^{a}	41.5 ± 5.81^{b}	40.8 ± 2.95^{b}	47.7 ± 4.71^{ab}	40.4 ± 2.54^{b}	0.031
VLDL/LDL-chol (mg/dL)	14.7 ± 2.77^{a}	28.2 ± 4.18^{b}	14.6 ± 1.03^{a}	16.5 ± 3.82^{ac}	25.5 ± 4.28^{bc}	0.018
NEFAs (mmol/L)	0.32 ± 0.04	0.41 ± 0.05	0.41 ± 0.05	0.36 ± 0.03	0.42 ± 0.04	0.461
Insulin (ng/mL)	16.3 ± 1.57^{a}	26.4 ± 2.51^{b}	19.7 ± 1.07^{ab}	25.5 ± 3.52^{b}	23.0 ± 2.32^{ab}	0.035
Leptin (ng/mL)	8.97 ± 1.37^{a}	34.7 ± 6.32^{b}	16.2 ± 3.78^{ac}	28.4 ± 6.97^{bc}	14.2 ± 1.61^{ac}	0.007
$HOMA_{IR}$	148 ± 19.1^{a}	286 ± 36.9^{bc}	193 ± 14.4^{ac}	320 ± 66.8^{b}	252 ± 7.91^{abc}	0.025
Hepatic lipids (a.u.)	2.11 ± 0.97^{a}	55.1 ± 12.6^{b}	9.54 ± 3.73^{a}	37.5 ± 8.31^{b}	46.2 ± 8.31^{b}	<0.001

Male Wistar rats were fed different diets for 10 weeks. Data are expressed as the mean ± SEM (n=6). ^{abdc} Mean values with different letters are significantly different (one-way ANOVA followed by DMS post hoc test, p<0.05). Chol, cholesterol; EWAT, epididymal white adipose tissue; IWAT, inguinal white adipose tissue; MWAT, mesenteric white adipose tissue; RWAT, retroperitoneal white adipose tissue.

3.2 Gut microbiota is distinctively altered according to dietary treatment.

One-way PERMANOVA analyses revealed pairwise differences on the overall microbial populations. In this sense, gut microbiota composition was strongly altered by CAF diet consumption (p=0.002), while the HFS diet (p=0.008), but not HF (p=0.631), induced significant changes in microbial genera compared to their respective controls. Interestingly, the microbial communities of the LF and STD control groups were significantly different (p=0.002). The effect of the dietary intervention on microbial diversity was determined using Shannon's diversity index within each group at the genus level. As shown in **Figure 2A**, no significant differences among diets were found, although the CAF diet tended to decrease bacterial diversity compared to the STD diet (Student's t-test: p<0.1). Then, a PCoA was performed to evaluate beta-diversity among the five groups (**Figure 2C**). Importantly, PCoA showed a clear separation of STD-fed animals and the other diets through the first principal coordinate (PC1), which explained 18.2% of the overall variation. Moreover, CAF diet-fed rats were differentiated from the rest along the PC2 axis (explaining 18.13% of the overall variation). However, there was no separation among semi-purified feed-based diets (LF, HF and HFS) independently of their composition. Thus, visual differences in bacterial composition assessed by HCA (**Figure 2B**) showed a defined aggrupation for both STD and CAF diets, while the microbial composition of animals fed semi-purified diets did not result in specific clusters according to dietary pattern.

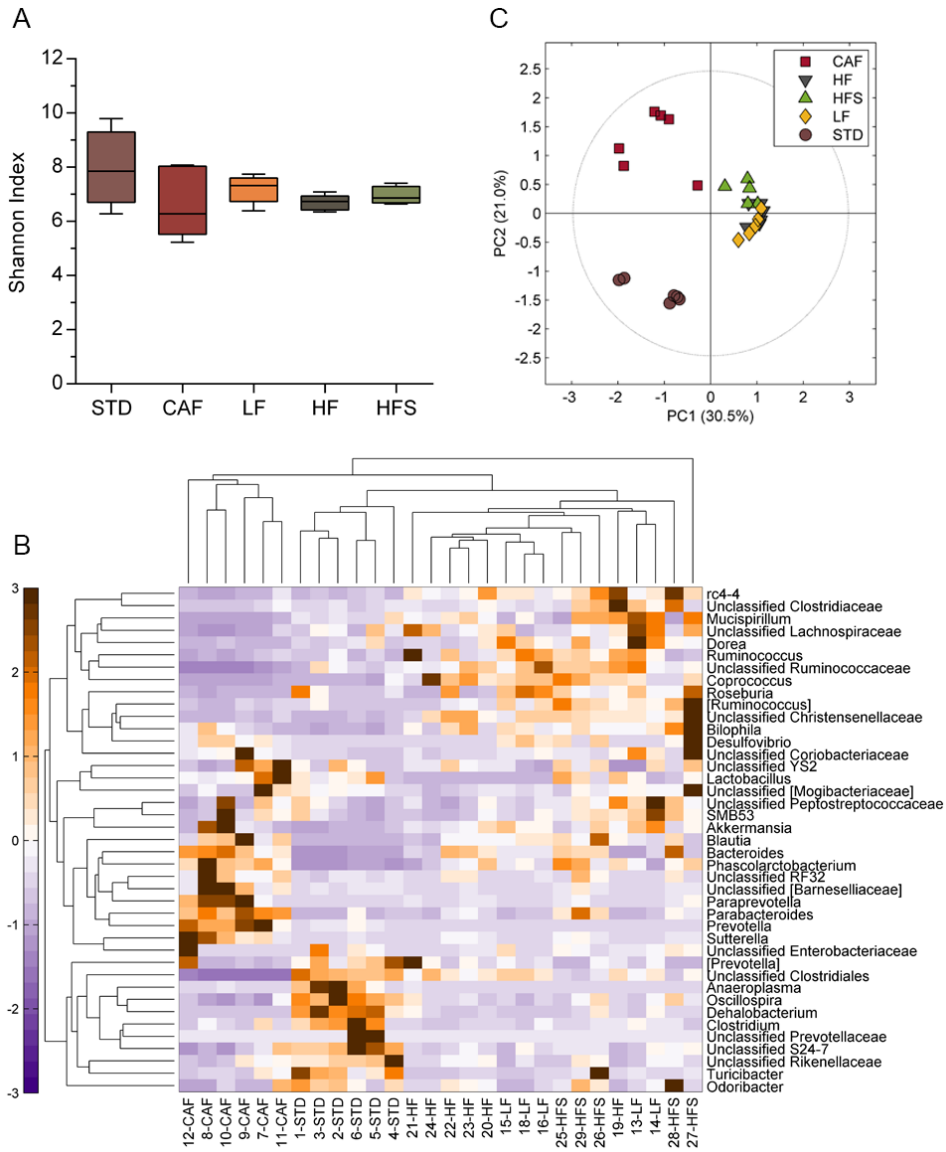

Figure 2. A. Assessment of the alpha-diversity of cecal microbiota using Shannon Index after the consumption of different diets for 10 weeks. **B.** Unsupervised hierarchical clustering analysis of the most representative bacteria identified in cecal samples for all rats. The selected bacteria were those that passed the filtering criteria described in the Materials and methods. Data are standardized as z-scores across samples for each genus before clustering. The clustergram displays the z-scores for each individual and genus colored according to major (red) or minor occurrence (blue). **C.** Analysis of beta diversity of cecal microbiota represented by scores after principal coordinate analysis from unweighted UniFrac distance matrix.

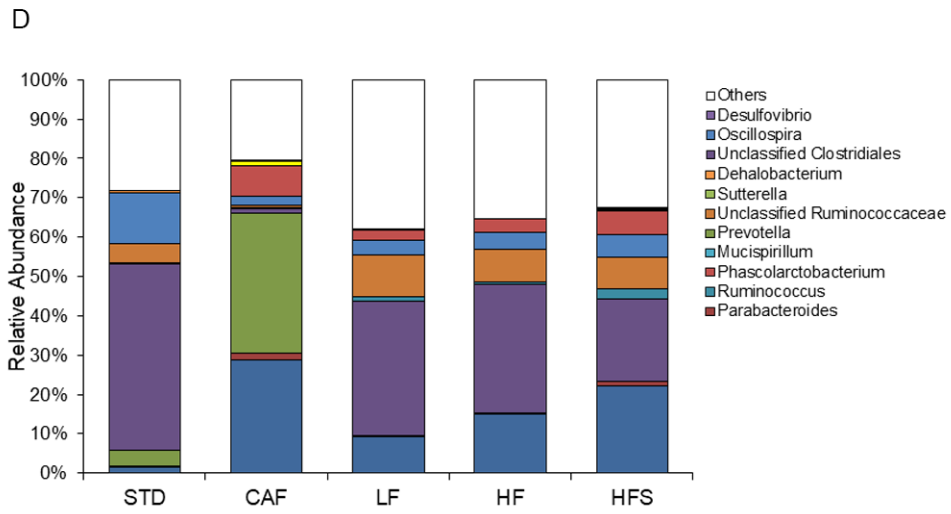

Figure 2. Continued. D. Relative abundance of the most significantly altered genera among groups. STD, standard diet; CAF, cafeteria diet; LF, low-fat diet; HF, high-fat diet; HFS, high-fat, high-sucrose diet.

Hence, the analysis of the relative abundance of the 12 phyla detected revealed that CAF feeding resulted in a drastic reduction of Firmicutes ($p=0.01$), with Bacteroidetes becoming the major phyla ($p=0.01$), compared to STD controls. Therefore, the Bacteroidetes-to-Firmicutes ratio was increased in the CAF group, while this variation was not observed in HF or HFS-fed groups compared to LF controls. Low-abundance phyla (<1%) such as Deferribacteres and Tenericutes were also significantly increased by the CAF diet (**Supplementary Table 2**). Interestingly, there were no significant variations for any phylum among semi-purified feed-based diets.

The relative abundance of significantly altered genera among dietary groups is represented in **Figure 2D** (see **Supplementary Table 3**). Focusing on STD versus CAF, up to 10 genera showed significant variations. The CAF diet induced a decrease in some genera belonging to Firmicutes, such as *Dehalobacterium* ($p<0.01$), *Oscillospira* ($p<0.01$), and

non-identified genera of the Clostridiales order ($p<0.01$) and the Ruminococcaceae family ($p<0.05$). Furthermore, bacteria belonging to Bacteroidetes, such as *Phascolarctobacterium* ($p<0.01$), *Bacteroides* ($p<0.01$), *Parabacteroides* ($p<0.01$) and *Prevotella* ($p<0.05$), were increased in the CAF group. The *Desulfovibrio* and *Mucispirillum* genera were also significantly altered by CAF feeding. In contrast, there was only one significantly increased genus, belonging to the Prevotellaceae family ($p<0.02$), in the HF group relative to LF controls, whereas HFS diet-fed animals showed two overrepresented genera, unclassified Prevotellaceae ($p<0.02$) and *Sutterella* ($p<0.05$), as well as a drop in the occurrence of an unclassified Clostridiales ($p<0.02$) compared to LF. Remarkably, the relative abundance of up to 13 genera was significantly different between STD and LF controls.

3.3 Cecal SCFA profile is only altered by CAF feeding.

SCFAs are primary end-products of fermentation of non-digestible carbohydrates by gut microbiota. The total concentration of SCFAs present in cecal content was strongly decreased in the CAF group, whereas there were no significant differences among semi-purified diets (**Table 2**). This drop in total SCFA content observed in the CAF group was mainly due to a decreased concentration of butyric and acetic acids. Despite this result, the CAF group showed a significant increment of propionic acid compared to STD controls. Interestingly, total SCFAs were also lower in animals under the LF diet compared to STD controls, which was mainly explained by a reduction of cecal acetate and butyrate. As expected, fiber intake and total cecal SCFA content were positively correlated ($p=0.001$, $r=0.886$).

3.4 Bile acid metabolism is altered by high-sucrose diets.

As shown in **Table 2**, total cecal BA concentration was higher in the HFS group, but not in HF, with respect to LF controls, while CAF diet-fed animals also showed greater amounts compared to the STD group. The highest amount of cecal BAs found in the HFS group was mainly explained by the greater concentration of secondary BAs compared to the rest of the diets. Remarkably, total and secondary cecal BAs were also significantly increased in LF versus STD controls. Total BAs were negatively correlated with both fiber ($p=0.000$, $r=0.619$) and protein intake ($p=0.009$, $r=0.479$). Focusing on primary BAs, cholic acid (CA) and α-muricholic acid (α-MCA) remained unaltered among the different diets. However, chenodeoxycholic acid (CDCA) was higher in hypercaloric diets (CAF, HF and HFS) than in their respective controls. In the same way, secondary bile acids such as hyodeoxycholic acid (HDCA), deoxycholic acid (DCA) and lithocholic acid (LCA) were highly increased in the HFS group, whereas only DCA was increased in the CAF and HF groups compared to their respective controls. The excretion of conjugated bile acids was increased under CAF or HFS feeding but not under HF feeding. Likewise, the conjugated primary BAs (glycocholic acid (GCA) and taurochenodeoxycholic acid (TCDCA) were higher in the CAF group, whereas taurocholic acid (TCA) and TCDCA were increased by HFS but not by HF feeding. Among conjugated secondary BAs, only taurodeoxycholic acid (TDCA) was higher in the CAF and HFS than in the STD and LF groups, respectively.

Table 2. Short-chain fatty acids (SCFA) and bile acid (BA) content in cecum from rats fed standard (STD), cafeteria (CAF), low-fat (LF), high-fat (HF) or high-fat, high-sucrose (HFS) diets for 10 weeks.

	STD	CAF	LF	HF	HFS	p-value
Short chain fatty acids (μmol/g)						
Total SCFAs	410 ± 28.1^{a}	139 ± 23.2^{b}	148 ± 18.9^{b}	166 ± 14.8^{b}	113 ± 23.5^{b}	<0.001
Acetic acid	125 ± 14.3^{a}	55.8 ± 3.79^{b}	73.2 ± 11.9^{b}	83.7 ± 4.84^{b}	59.8 ± 13.9^{b}	0.001
Propionic acid	24.8 ± 2.20^{a}	45.6 ± 7.74^{b}	26.9 ± 3.90^{a}	29.1 ± 3.41^{a}	25.9 ± 5.62^{a}	0.032
Butyric acid	259 ± 26.6^{a}	12.7 ± 2.72^{b}	48.2 ± 5.00^{bc}	53.7 ± 7.49^{c}	27.3 ± 5.25^{bc}	<0.001
Bile acids (μg/g)						
Primary bile acids						
Cholic acid	6.87 ± 1.01	241 ± 158	6.81 ± 0.74	16.2 ± 4.34	12.9 ± 1.82	0.119
Chenodeoxycholic acid	21.2 ± 3.79^{a}	60.1 ± 6.07^{b}	29.37 ± 4.03^{a}	57.2 ± 9.54^{b}	85.2 ± 8.29^{c}	<0.001
α-Muricholic acid	0.82 ± 0.12	10.1 ± 7.64	0.89 ± 0.08	2.07 ± 0.53	2.25 ± 0.53	0.296
Total	28.8 ± 4.77	326 ± 180	40.3 ± 6.25	74.5 ± 15.2	118 ± 23. 6	0.121
Secondary bile acids						
Ursodeoxycholic acid	109 ± 10.02^{a}	82.1 ± 33.3^{a}	316 ± 31.6^{b}	239 ± 32.6^{b}	414 ± 40.1^{c}	<0.001
Hyodeoxycholic acid	503 ± 50.8^{ab}	339 ± 188^{a}	840 ± 88.2^{c}	751 ± 38.6^{bc}	1589 ± 129^{d}	<0.001
Deoxycholic acid	286 ± 50.6^{a}	846 ± 89.2^{b}	398 ± 56.7^{a}	654 ± 46.5^{b}	1231 ± 131^{c}	<0.001
Lithocholic acid	52.7 ± 7.21^{ab}	47.8 ± 11.32^{a}	73.8 ± 4.04^{bc}	84.0 ± 7.36^{c}	132 ± 13.91^{d}	<0.001
Total	951 ± 114^{a}	1315 ± 187^{ab}	1628 ± 175^{b}	1701 ± 103^{b}	3365 ± 264^{c}	<0.001

	STD	CAF	LF	HF	HFS	p-value
Conjugated bile acids						
Glycocholic acid	1.97 ± 0.09^{a}	7.00 ± 2.13^{b}	2.02 ± 0.06^{a}	2.19 ± 0.09^{a}	3.44 ± 0.55^{a}	0.009
Taurocholic acid	3.04 ± 0.53^{a}	5.02 ± 1.21^{ab}	2.55 ± 0.24^{a}	3.56 ± 0.39^{a}	7.63 ± 1.99^{b}	0.014
Taurochenodeoxycholic acid	0.05 ± 0.03^{a}	10.11 ± 1.64^{b}	0.98 ± 0.46^{a}	2.99 ± 0.92^{a}	7.53 ± 1.48^{b}	<0.001
Glycodeoxycholic acid	8.31 ± 0.32	9.44 ± 0.46	9.03 ± 0.33	8.71 ± 0.38	8.73 ± 0.36	0.258
Taurodeoxycholic acid	3.30 ± 0.27^{a}	11.5 ± 1.32^{b}	4.26 ± 0.38^{a}	5.81 ± 0.86^{a}	9.33 ± 1.21^{b}	<0.001
Taurolithocholic acid	1.81 ± 0.07	1.96 ± 0.06	1.96 ± 0.06	1.97 ± 0.12	1.95 ± 0.09	0.613
Total	18.7 ± 1.04^{a}	49.2 ± 8.43^{b}	20.5 ± 1.08^{a}	25.0 ± 2.12^{ac}	38.6 ± 3.48^{bc}	<0.001
Secondary/Primary BAs	34.5 ± 2.29^{ac}	14.3 ± 5.45^{b}	41.8 ± 2.17^{c}	26.5 ± 1.26^{a}	31.1 ± 3.46^{a}	<0.001
Total bile acids	998 ± 119^{a}	1691 ± 242^{b}	1689 ± 181^{b}	2141 ± 293^{b}	3522 ± 285^{c}	<0.001

Male Wistar rats were fed different diets for 10 weeks. Data are expressed as the mean ± SEM (n=6). ^{abdc} Mean values with different letters are significantly different (one-way ANOVA followed by DMS post hoc test, $p<0.05$).

3.5 Urinary metabolomics profiles are clustered based on diet and similarly to microbiota patterns.

PCA constructed from urinary metabolic profiles revealed dissimilarities among different dietary groups, as shown in **Figure 3A**. Consistent with the microbiota analyses, the STD group clearly diverged from all of the other groups, whereas CAF-fed animals were also distanced from the rest of animals fed obesogenic diets, revealing specific changes in the metabolome induced by each diet. HCA (**Figure 3B**) of metabolic profiles evidenced two major clusters differentiating the STD group from the rest. CAF-fed animals were also grouped, but rats on semi-purified feed-based diets did not show any clustering pattern based on diet type.

Pairwise OPLS-DA models were constructed to compare the urinary metabolic profiles of rats fed the STD and those fed the CAF diet. A model with strong predictive ability [$Q^2Y = 0.56$; $p=0.010$ (1000 permutations)] was obtained, and the metabolic alterations induced by the CAF diet were shown in the coefficient plot displayed in **Figure 3C**. Animals fed the STD diet excreted greater amounts of tricarboxylic acid (TCA) cycle-related metabolites (citrate, succinate, 2-oxoglutarate (2-OG) and fumarate), microbiota-derived metabolites (hippurate, 3-hydroxycinnamic acid (3-HCA) and 3-hydroxyphenylpropionic acid (3-HPPA)), and other metabolites such as dimethyl sulfone (DMSO2) and taurine. On the other hand, CAF-fed rats excreted higher amounts of metabolites related to choline metabolism (glycerophosphocholine (GPC) and dimethylamine (DMA)), oxidative stress (pseudouridine and 2'-deoxycitidine), uremic toxins (4-cresyl sulphate (4-CS) and 4-cresyl glucuronide (4-CG)) and metabolic inflammation (fucose and N-

acetylglycoproteins (NAG)). Nevertheless, no significant OPLS-DA models were obtained from the LF versus HF or HFS comparison.

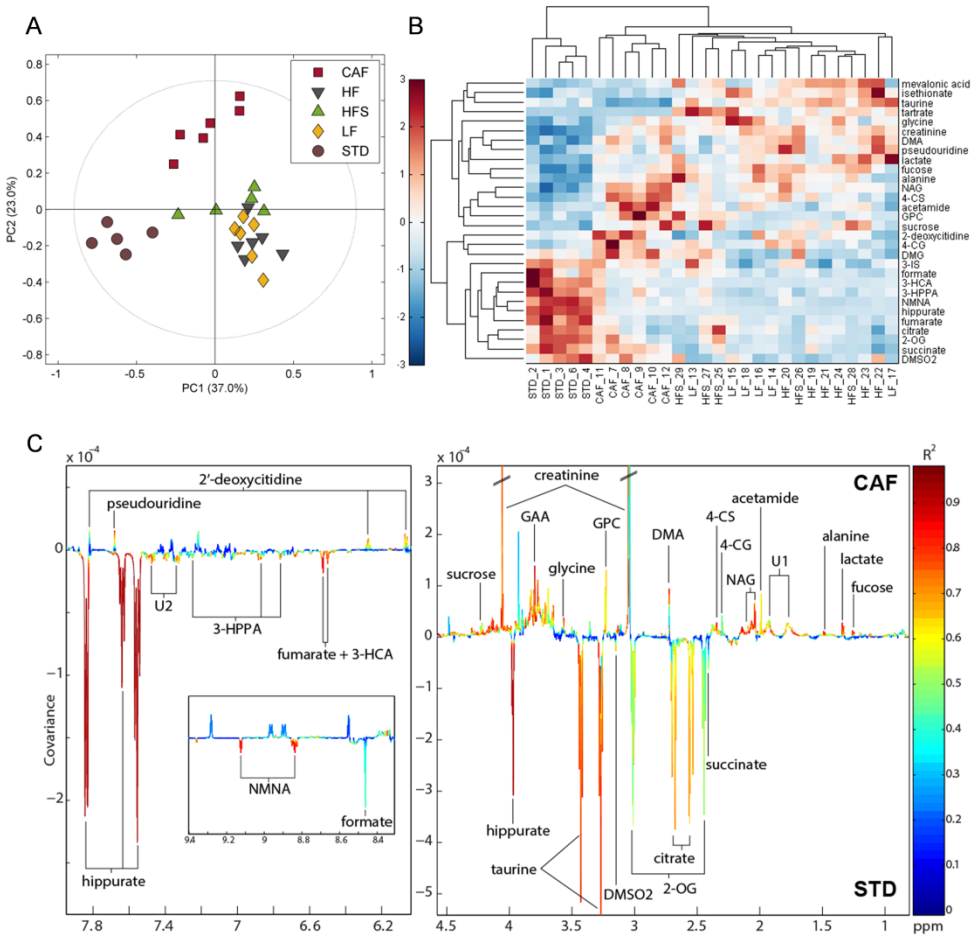

Figure 3. A. Principal component analysis constructed from urine metabolic profiles. **B**. Unsupervised hierarchical clustering analysis of the metabolome in urine samples for all rats. The selected metabolites were those that passed the filtering criteria described in the Materials and methods. Data are standardized as z-scores across samples for each metabolite before clustering. The clustergram displays the z-scores for each individual and metabolite colored according to major (red) or minor presence (blue). **C.** OPLS-DA model constructed from the urinary metabolic profiles. OPLS-DA coefficients indicate the metabolic variation between the cafeteria (CAF) diet-fed group and its respective standard (STD) diet-fed control. DMA, dimethylamine; DMG, dimethylglycine; DMSO2, dimethylsulfone; GAA, guanidinoacetic acid; GPC,

glycerophosphocholine; HF, high-fat diet; HFS, high-fat, high-sucrose diet; LF, low-fat diet; NAG, N-acetyl-glycoproteins; NMNA, N-methylnicotinic acid; PC, principal component; ppm, parts per million; U, unknown metabolite; 2-OG, 2-oxoglutarate; 3-HCA, 3-hydroxycinnamic acid, 3-HPPA, 3-hydroxyphenylpyruvic acid; 3-IS, 3-indoxyl sulfate; 4-CG, 4-cresyl glucuronide; 4-CS, 4-cresyl sulfate.

3.6 Correlation between gut microbiome and urinary metabolome and physiological parameters reveals obesity-defined phenotypes.

Correlation analysis between metabolites, physiological parameters and gut microbiota composition was also performed (**Figure 4**). Concerning parameters defining metabolic status, three main clusters were identified: I) TCA cycle metabolites (2-OG, succinate, citrate, fumarate), microbiota-derived metabolites (hippurate, 3-HCA, 3-HPPA), cecal SCFAs, fiber and protein intake, cecum weight, colon length, etc.; II) metabolites associated with oxidative stress (2'-deoxycitidine), metabolic inflammation (NAG) and choline metabolism (GPC), uremic toxins (4-CS, 4-CG), caloric intake, body weight gain, adiposity index, liver lipids, serum TAG and VLDL/LDL-cholesterol, HOMA-IR and cecal propionate and conjugated BAs; III) taurine, lactate, fucose, pseudouridine, tartrate, cecal total and secondary BA content, and others. Globally, cluster I was positively correlated with *Turicibacter*, *Clostridium*, *Anaeroplasma*, *Dehalobacterium*, *Oscillospira*, and unclassified S24-7 and Clostridiales genera. Among these, unclassified Clostridiales, *Oscillospira* and *Dehalobacterium* were significantly increased in the STD group and showed a positive association with fiber intake. Additionally, cecal SCFA content was positively associated with most of the aforementioned genera.

On the other hand, bacterial communities including *Prevotella*, *Phascolarctobacterium*, *Bacteroides* and *Parabacteroides*, among others, which were increased in the CAF group, were positively correlated with cluster II. Interestingly, *Prevotella*, *Parabacteroides* and *Phascolarctobacterium* were positively associated with biometric measurements defining an obese phenotype, including body weight gain, adiposity index and food intake. Likewise, *Bacteroides* and *Phascolarctobacterium* were negatively correlated with fiber intake and cecal SCFA content, while conjugated BAs were positively associated with these genera. Moreover, unclassified *Clostridiales* genus showed negative associations with up to 16 parameters defining this cluster. Urine metabolites included in cluster II were associated with most of the genera that were mainly prevalent in the CAF group.

Finally, cluster III was defined by some urine metabolites and parameters concerning BA metabolism. Total and secondary BAs were positively correlated with *Ruminococcus*, *Bilophila* and unclassified Christensenellaceae genera. Notably, taurine was negatively associated with *Parabacteroides*, *Prevotella* and *Sutterella*.

Overall, cluster I and cluster II corresponded to the healthiest and the unhealthiest metabolic profiles, respectively.

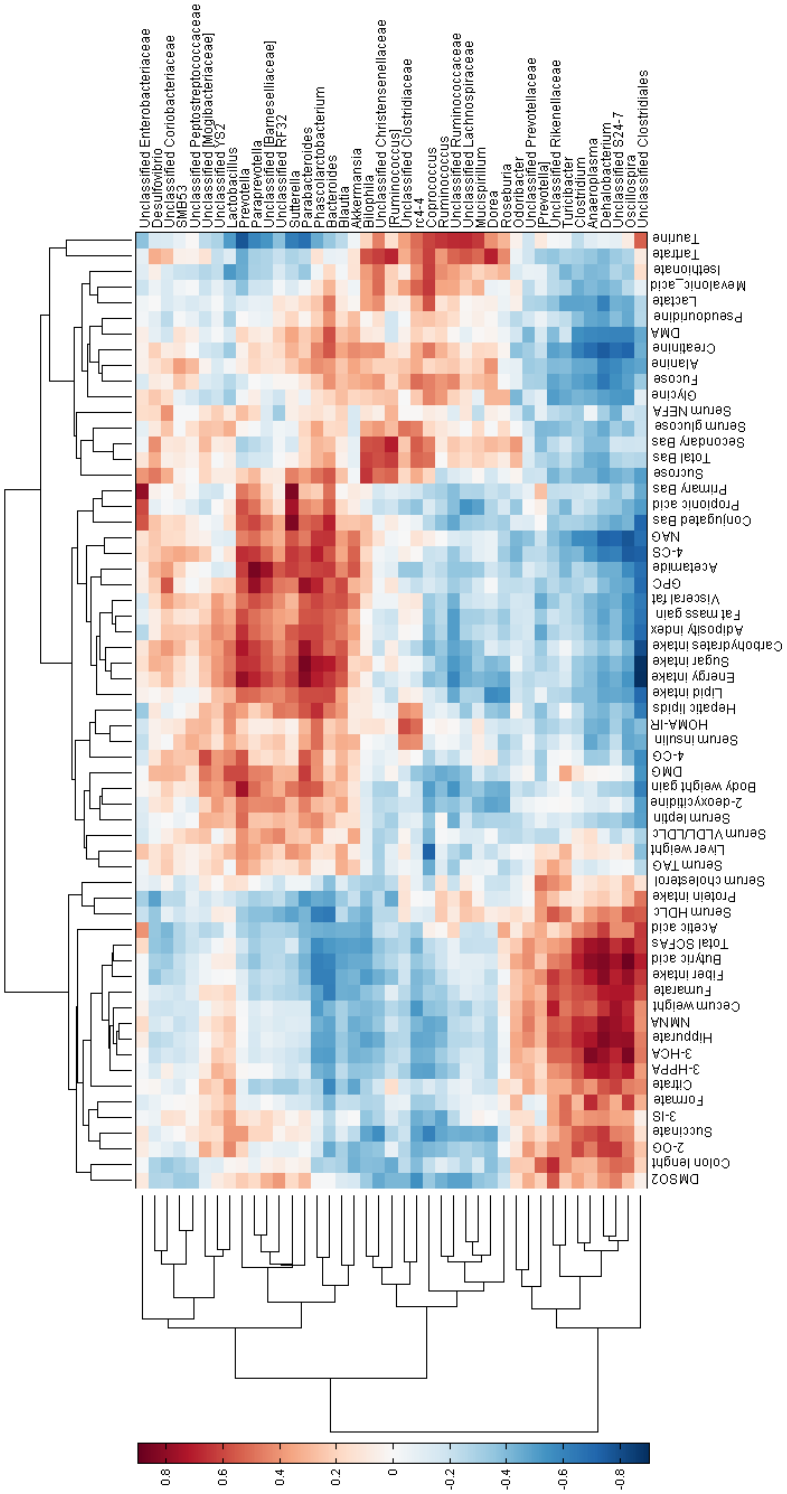

Figure 4. Correlation analysis between gut microbiome and urine metabolites, dietary, biometric and serum parameters assessed by Pearson's correlation coefficient. Positive and negative correlation coefficients are colored red and blue, respectively. The intensity of the color represents the degree of correlation. BAs, bile acids; DMA, dimethylamine; DMG, dimethylglycine; DMSO2, dimethylsulfone; GAA, guanidinoacetic acid; GPC, glycerophosphocholine; NAG, N-acetyl-glycoproteins; NMNA, N-methylnicotinic acid; TAG, triglycerides; U, unknown metabolite; 2-OG, 2-oxoglutarate; 3-HCA, 3-hydroxycinnamic acid; 3-HPPA, 3-hydroxyphenylpyruvic acid; 3-IS, 3-indoxyl sulfate; 4-CG, 4-cresyl glucuronide; 4-CS, 4-cresyl sulfate.

4. DISCUSSION

In this study, we demonstrated that CAF feeding strongly induced an obese phenotype associated with several related disorders, such as insulin resistance, fatty liver, dysbiosis of the gut microbiota and metabolic disruptions. Hyperphagia induced by this diet led to an increased energy intake, favoring an exacerbated body weight gain and adiposity accumulation, as reported before [10]. In this sense, the continuous exposure to highly palatable foods present in the CAF diet has been associated with an undisciplined intake promoted by the activation of the reward system and ultimately affecting feeding behavior [23]. In addition, hyperphagia also reflects the development of leptin resistance in the CAF group by increased circulating leptin levels. Although HF and HFS intake also induced a significant increase in total energy intake, this was much lower than in CAF group, leading to the development of an overweight phenotype. Since HFS and CAF diets had similar nutritional composition, these results suggest that hyperphagic behavior and total energy intake, instead of macronutrient composition, were the main factors responsible for strongly promoting severe obesity in the CAF model in contrast to HF or HFS groups. Overall, in contrast to the literature, HF and HFS groups did not develop obesity, hypertriglyceridemia, leptin resistance or dramatic changes on gut microbiome when compared to LF controls. In this sense, most of studies analyzing the obesogenic effects of semi-purified HF and HFS diets are contrasted to standard chow-fed groups based on higher proportion of dietary fibers [24,25], which could finally lead to highlighted phenotypic differences.

Alterations in gut microbiota have been suggested to contribute to the onset and progression of obesity. In this sense, obesity has been widely associated with a reduction of both microbiome diversity [26] and Bacteroidetes-to-Firmicutes ratio in humans [27] and rodents [28]. Nevertheless, a meta-analysis concluded that there is no consistent evidence regarding these observations, at least in obese humans [29]. In contrast, our study revealed that the CAF diet, but not the HF or HFS, induced an increased Bacteroidetes-to-Firmicutes ratio, which is in accordance with recent findings showing a clear reduction of Firmicutes [10] and an increase in Bacteroidetes [30] in CAF models. Previous findings showed that the occurrence of Firmicutes and Bacteroidetes were promoted by the consumption of lard or milk-derived fat-based diets, respectively [31]. Thus, the conflicting effects of CAF and HF feeding on the Bacteroidetes-to-Firmicutes ratio seem to be explained by the different type of fats used in these diets, mainly lard in HF and milk fat in CAF.

The reported increase in Bacteroidetes in the CAF group was mainly explained by a dramatic drop in the abundance of an unclassified *Clostridiales* (Firmicutes) genus, counteracted by an increase in *Prevotella* and *Bacteroides* (Bacteroidetes) genera. Along these lines, several authors have reported an overpopulation of *Bacteroides* in DIO rodents [30] and in humans eating an animal-based diet [32]. Moreover, we found that *Bacteroides* were negatively correlated with fiber intake, suggesting that these bacteria may be robustly adapted to low-fiber diets due to their high capacity to use host-derived glycans as an energy source [33]. In addition, conjugated BAs were positively correlated with *Bacteroides* abundance, a genus considered highly capable of tolerating

bile salts [34] and biotransforming conjugated into deconjugated forms [35], which could explain its favored occurrence in HF diets fed animals.

Prevotella abundance has been associated with high total carbohydrates and simple sugar intake [36], according to the rise of this genus in the CAF group. Moreover, *Prevotella* has been widely associated with high dietary fiber intake in humans [37], which is in agreement with the near absence of *Prevotella* in the LF, HF and HFS diet-fed groups. On the other hand, *Prevotella*-dominated microbiota has been shown to produce at least 2 times more propionate than a *Bacteroides*-dominated microbiota in humans [38], in line with the association we detected between *Prevotella* and cecal propionate.

The increase in *Sutterella* (Proteobacteria) under HFS feeding was also reported in rats fed a restrictive high-sugar diet [39]. Indeed, *Sutterella* was positively correlated with simple carbohydrates intake, which is in accordance with the numerically greater abundance of this genus in the CAF group.

Taken together, our results indicate that in contrast to HF and HFS, chronic consumption of the CAF diet strongly altered the microbiota composition, which in turn correlated with most of physiological parameters defining a severe obesity profile. Besides the higher energy and nutrient intake induced by CAF diet, previous studies suggested that additives [40] and Maillard reaction products [41] present in industrialized foods, and in the CAF diet, might also be responsible for the dysbiosis of gut microbiota.

BAs exert several biological functions implicated in obesity development, such as regulating lipid and cholesterol metabolism and promoting energy expenditure [42]. Intestinal bacteria are able to transform conjugated primary BAs synthetized in the liver into secondary BAs [43]. In this sense, the decreased cecal secondary-to-primary BA ratio induced by CAF, HF and HFS diets suggested a loss of microbial activity. Moreover, dietary fat content was not the main factor responsible for the increased total cecal BAs observed in the CAF and HFS, since such an increase was not induced in the HF group. Alternatively, we suggest that fiber intake ultimately determines the total content of cecal BAs, as reflected by a negative correlation. Though water-soluble fiber has been widely associated with increased fecal BA excretion, consumption of water-insoluble dietary fibers decreases it [44], supporting our observations. In addition, high-fiber diets may favor a diminished exposure time of fecal bulking to microbial action in the gut [45] resulting in a lower generation of secondary BAs, which represent approximately 95% of total BAs.

On the other hand, high exposure to secondary BAs promotes intestinal epithelium damage, activation of inflammatory processes and increased risk of colon cancer [46]. In this line, obesogenic dietary interventions led to an increased cecal DCA, while LCA, HDCA and UDCA were mainly raised in the HFS diet fed group. Moreover, tauro-conjugated forms, which have been found to increase the solubility of milk fats [47], were boosted in CAF- and HFS-fed animals, also explaining the decreased load of urinary taurine in the CAF group. However, caution must be raised when extrapolating these results to humans due to major differences

concerning bile acids metabolism including synthesis pathway, hydrophobicity index and conjugation ratios, among others [48].

The decrease in total SCFA concentration in the cecum from CAF diet-fed animals may be explained by a lower consumption of dietary fiber [49]. However, all LF, HF and HFS semi-purified feed-based diets contained low amounts of non-fermentable cellulose, explaining the absence of differences in the cecal SCFA profile. Acetate and propionate are the primary end products of the Bacteroidetes phylum, while Firmicutes mainly produces butyrate [50]. This fact is in accordance with the increased cecal levels of propionate observed in the CAF group, whose microbiota is predominantly defined by Bacteroidetes. Furthermore, propionate is thought to play an important role in satiety signaling [51] and reward-based eating behavior. Hence, we suggest that enhanced propionate production could be a microbiota-derived response to counteract overfeeding. Propionate has also shown to inhibit lipolysis and stimulate lipogenesis, leading to major adiposity [52], in line with our results. On the other hand, the decreased butyrate production is suggested to strongly contribute to the resulting unhealthy phenotype. Contrary to our findings, current evidences suggest an increased SCFAs loads in feces from obese humans and genetic animal models of obesity [27,53], showing that dysbiosis of the gut microbiota could favor the growth of SCFA-producing microbial species. It is likely that the limited fiber intake in DIO animal models fails to reproduce this feature. However, rodents have a greater capacity to extract nutrients from the non-digestible foods as compared with humans, mainly due to several morphological divergences in the gut anatomy [54]. Thus, direct

parallels between murine and humans concerning microbiota-derived metabolites must be done carefully.

Several studies have focused on the analysis of the urinary metabolic profile to identify obesity-associated disruptions in host metabolism [55], which in turn may act as an indicator of diet-derived gut microbial perturbations. In our study, the CAF diet, but not the HF or HFS diets, altered the occurrence of several urinary metabolites such as taurine, whose deficiency has been widely associated with the progression of metabolic diseases such as obesity [56]. Indeed, a high urinary removal of TCA cycle intermediates has been shown to reduce energy storage and body lipid accretion [57], which corresponds with the lesser excretion of these metabolites in the CAF group. Accordingly, we have described for the first time a higher urinary excretion of lactate in CAF diet-fed rats, which could indicate a switch to an anaerobic glycolytic pathway.

A wide range of urinary metabolites derived from host-microbial co-metabolism can reflect the diet-induced alterations of gut microbiome. Hippurate has been considered the most important discriminatory metabolite for a lean phenotype [58]. Our results are in accordance with previous works showing a decreased hippurate release in obese rodents and humans [59,60], also supported by a positive correlation with microbial species mostly abundant in the STD group, such as *Clostridium*, *Dehalobacterium*, *Oscillospira* and *Anaeroplasma*. We propose that, in addition to the dietary availability of natural precursors, the production of hippurate could be mediated, at least in part, by the aforementioned bacteria.

The higher urinary presence of uremic toxins in the CAF group could reflect both a higher intake of its precursors, meaning tyrosine and phenylalanine, or functional changes of gut microbiota. Likewise, the accumulation of 4-CS has been established as a predictor of kidney impairment [61]. Indeed, the increased fucose excretion induced by CAF diet could be an indicator of intestinal inflammation linked to gut microbiota dysbiosis, since some bacteria are able to incorporate fucose into glycans playing an important role in the immune response and metabolic inflammation [62]. Furthermore, the higher excretion of NAG and pseudouridine found in CAF animals has been previously associated with inflammatory processes [63] and oxidative stress [64], respectively.

Finally, adiposity index, visceral fat and body fat mass were higher in LF-fed animals than those in the STD group. We suggest that these physiological differences could be partially explained by the observed dissimilarities of gut microbial communities. In this respect, we also identified diet-dependent clustering patterns concerning both metagenomics and metabolomics profiles, where only STD controls were clearly distanced from the clusters defined by the HF and HFS groups. Altogether, these facts indicate that the evaluation of the impact of DIO highly relies on the selected control diet.

In conclusion, we demonstrated that CAF, HF and HFS diets distinctively modulate host-microbiota co-metabolism in rats. This complex interplay has been evidenced by a strong linkage between specific gut microbial ecosystems and metabolic and physiological phenotypes. Accordingly, robust and similar clusters regarding both microbiome and metabolome profiles were defined based on the dietary interventions. In this sense, the hyperphagic behavior induced by the CAF diet, which is composed of

highly palatable and energy-dense foods, promoted the development of a robust model of severe obesity characterized by the alteration of both cecal microbiota and urinary metabolome. In contrast, HF and HFS diets did not strongly alter the microbial populations, according to the induced overweight phenotypes. Overall, our results reveal diet-specific modulation of the gut microbiome in rats and shed light on the possible role of microbiota alterations on the progression of obesity and related metabolic complications.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

REFERENCES

[1] Morgen CS, Sørensen TIA. Obesity: Global trends in the prevalence of overweight and obesity. Nat Rev Endocrinol 2014;10:513–4.

[2] Singla P, Bardoloi A, Parkash AA. Metabolic effects of obesity: A review. World J Diabetes 2010;1:76–88.

[3] Speakman JR. Use of high-fat diets to study rodent obesity as a model of human obesity. Int J Obes 2019;43:1491–2.

[4] Nilsson C, Raun K, Yan F, Larsen MO, Tang-Christensen M. Laboratory animals as surrogate models of human obesity. Acta Pharmacol Sin 2012;33:173–81.

[5] Sampey BP, Vanhoose AM, Winfield HM, Freemerman AJ, Muehlbauer MJ, Fueger PT, et al. Cafeteria diet is a robust model of human metabolic syndrome with liver and adipose inflammation: Comparison to high-fat diet. Obesity 2011;19:1109–17.

[6] Sun L, Ma L, Ma Y, Zhang F, Zhao C, Nie Y. Insights into the role of gut microbiota in obesity: pathogenesis, mechanisms, and therapeutic perspectives. Protein Cell 2018;9:397–403.

[7] Rowland I, Gibson G, Heinken A, Scott K, Swann J, Thiele I, et al. Gut microbiota functions: metabolism of nutrients and other food components. Eur J Nutr 2017:1–24.

[8] Ussar S, Griffin NW, Bezy O, Fujisaka S, Vienberg S, Softic S, et al. Interactions between gut microbiota, host genetics and diet modulate the predisposition to obesity and metabolic syndrome. Cell Metab 2015;22:516–30.

[9] Everard A, Lazarevic V, Gaïa N, Johansson M, Ståhlman M, Backhed F, et al. Microbiome of prebiotic-treated mice reveals novel targets involved in host response during obesity. ISME J 2014;8:2116–30.

[10] del Bas JM, Guirro M, Boqué N, Cereto A, Ras R, Crescenti A, et al. Alterations in gut microbiota associated with a cafeteria diet and the physiological consequences in the host. Int J Obes 2018;42:746–54.

[11] Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, et al. A core gut microbiome in obese and lean twins. Nature 2009;457:480–4.

[12] Lecomte V, Kaakoush NO, Maloney CA, Raipuria M, Huinao KD, Mitchell HM, et al. Changes in gut microbiota in rats fed a high fat diet correlate with obesity-associated metabolic parameters. PLoS One 2015;10:e0126931.

[13] Kim H, Lee M, Park H, Park Y, Shon J, Liu K-H, et al. Urine and Serum Metabolite Profiling of Rats Fed a High-Fat Diet and the Anti-Obesity Effects of Caffeine Consumption. Molecules 2015;20:3107–28.

[14] Suárez-García S, Del Bas JM, Caimari A, Escorihuela RM, Arola L, Suárez M. Impact of a cafeteria diet & daily physical training on the rat serum metabolome. PLoS One 2017;12:e0171970.

[15] Canfora EE, Meex RCR, Venema K, Blaak EE. Gut microbial metabolites in obesity, NAFLD and T2DM. Nat Rev Endocrinol 2019;15:261–73.

[16] Walker A, Pfitzner B, Neschen S, Kahle M, Harir M, Lucio M, et al. Distinct signatures of host-microbial meta-metabolome and gut microbiome in two C57BL/6 strains under high-fat diet. ISME J 2014;8:2380–96.

[17] Matthews DR, Hosker JP, Rudenski a S, Naylor B a, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 1985;28:412–9.

[18] Hagio M, Matsumoto M, Fukushima M, Hara H, Ishizuka S. Improved analysis of bile acids in tissues and intestinal contents of rats using LC/ESI-MS. J Lipid Res 2009;50:173–80.

[19] Mehlem A, Hagberg CE, Muhl L, Eriksson U, Falkevall A. Imaging of neutral lipids by oil red O for analyzing the metabolic status in health and disease. Nat Protoc 2013;8:1149–54.

[20] Massot-Cladera M, Mayneris-Perxachs J, Costabile A, Swann JR, Franch À, Pérez-Cano FJ, et al. Association between urinary metabolic profile and the intestinal effects of cocoa in rats. Br J Nutr 2017;117:623–34.

[21] Guirro M, Costa A, Gual-Grau A, Mayneris-Perxachs J, Torrell H, Herrero P, et al. Multi-omics approach to elucidate the gut microbiota activity. Metaproteomics and metagenomics connection. Electrophoresis 2018;39:1692–701.

[22] Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods 2010;7:335–6.

[23] De Macedo IC, De Freitas JS, Da Silva Torres IL. The influence of palatable diets in reward system activation: A mini review. Adv Pharmacol Sci 2016;2016:7238679.

[24] Buettner R, Parhofer KG, Woenckhaus M, Wrede CE, Kunz-Schughart LA, Schölmerich J, et al. Defining high-fat-diet rat models: Metabolic and molecular effects of different fat types. J Mol Endocrinol 2006;36:485–501.

[25] Collins KH, Paul HA, Hart DA, Reimer RA, Smith IC, Rios JL, et al. A High-Fat High-Sucrose Diet Rapidly Alters Muscle Integrity, Inflammation and Gut Microbiota in Male Rats. Sci Rep 2016;6.

[26] Le Chatelier E, Nielsen T, Qin J, Prifti E, Hildebrand F, Falony G, et al. Richness of human gut microbiome correlates with metabolic markers. Nature 2013;500:541–6.

[27] Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 2006;444:1027–31.

[28] Zhang C, Zhang M, Pang X, Zhao Y, Wang L, Zhao L. Structural resilience of the gut microbiota in adult mice under high-fat dietary perturbations. ISME J 2012;6:1848–57.

[29] Walters WA, Xu Z, Knight R. Meta-analyses of human gut microbes associated with obesity and IBD. FEBS Lett 2014;588:4223–33.

[30] Kaakoush NO, Martire SI, Raipuria M, Mitchell HM, Nielsen S, Westbrook RF, et al. Alternating or continuous exposure to cafeteria diet leads to similar shifts in gut microbiota compared to chow diet. Mol Nutr Food Res 2017;61.

[31] Devkota S, Wang Y, Musch MW, Leone V, Fehlner-Peach H, Nadimpalli A, et al. Dietary-fat-induced taurocholic acid promotes pathobiont expansion and colitis in Il10-/-mice. Nature 2012;487:104–8.

[32] David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, et al. Diet rapidly and reproducibly alters the human gut microbiome. Nature 2014;505:559–63.

[33] Koropatkin NM, Cameron EA, Martens EC. How glycan metabolism shapes the human gut microbiota. Nat Rev Microbiol 2012;10:323–35.

[34] Wexler HM. Bacteroides: The good, the bad, and the nitty-gritty. Clin Microbiol Rev 2007;20:593–621.

[35] Begley M, Hill C, Gahan CGM. Bile salt hydrolase activity in probiotics. Appl Environ Microbiol 2006;72:1729–38.

[36] Wu GD, Chen J, Hoffmann C, Bittinger K, Chen YY, Keilbaugh SA, et al. Linking long-term dietary patterns with gut microbial enterotypes. Science (80-) 2011;334:105–8.

[37] Schnorr SL, Candela M, Rampelli S, Centanni M, Consolandi C, Basaglia G, et al. Gut microbiome of the Hadza hunter-gatherers. Nat Commun 2014;5:3654.

[38] Chen T, Long W, Zhang C, Liu S, Zhao L, Hamaker BR. Fiber-utilizing capacity varies in Prevotella- versus Bacteroides-dominated gut microbiota. Sci Rep 2017;7.

[39] Liu J-P, Zou W-L, Chen S-J, Wei H-Y, Yin Y-N, Zou Y-Y, et al. Effects of different diets on intestinal microbiota and nonalcoholic fatty liver disease development. World J Gastroenterol 2016;22:7353.

[40] Feng ZM, Li TJ, Wu L, Xiao DF, Blachier F, Yin YL. Monosodium L-glutamate and dietary fat differently modify the composition of the intestinal microbiota in growing pigs. Obes Facts 2015;8:87–100.

[41] Seiquer I, Rubio LA, Peinado MJ, Delgado-Andrade C, Navarro MP. Maillard reaction products modulate gut microbiota composition in adolescents. Mol Nutr Food Res 2014;58:1552–60.

[42] Staels B, Fonseca VA. Bile Acids and Metabolic Regulation. Diabetes Care 2009;32:S237–45.

[43] Hofmann AF, Hagey LR. Bile acids: Chemistry, pathochemistry, biology, pathobiology, and therapeutics. Cell Mol Life Sci 2008;65:2461–83.

[44] Ide T, Horii M, Yamamoto T, Kawashima K. Contrasting effects of water-soluble and water-insoluble dietary fibers on bile acid conjugation and taurine metabolism in the rat. Lipids 1990;25:335–40.

[45] Müller M, Canfora E, Blaak E. Gastrointestinal transit time, glucose homeostasis and metabolic health: modulation by dietary fibers. Nutrients 2018;10:275.

[46] Caimari A, Puiggròs F, Suárez M, Crescenti A, Laos S, Ruiz JA, et al. The intake of a hazelnut skin extract improves the plasma lipid profile and reduces the lithocholic/deoxycholic bile acid faecal ratio, a risk factor for colon cancer, in hamsters fed a high-fat diet. Food Chem 2015;167:138–44.

[47] Devkota S, Chang EB. Interactions between diet, bile acid metabolism, gut microbiota, and inflammatory bowel diseases. Dig. Dis., vol. 33, 2015, p. 351–6.

[48] Li J, Dawson PA. Animal models to study bile acid metabolism. Biochim Biophys Acta - Mol Basis Dis 2018;1865:895–911.

[49] Hugenholtz F, Mullaney JA, Kleerebezem M, Smidt H, Rosendale DI. Modulation of the microbial fermentation in the gut by fermentable carbohydrates. Bioact Carbohydrates Diet Fibre 2013;2:133–42.

[50] Macfarlane S, Macfarlane GT. Regulation of short-chain fatty acid production. Proc Nutr Soc 2003;62:67–72.

[51] Samuel BS, Shaito A, Motoike T, Rey FE, Backhed F, Manchester JK, et al. Effects of the gut microbiota on host adiposity are modulated by the short-chain fatty-acid binding G protein-coupled receptor, Gpr41. Proc Natl Acad Sci 2008;105:16767–72.

[52] Hong YH, Nishimura Y, Hishikawa D, Tsuzuki H, Miyahara H, Gotoh C, et al. Acetate and propionate short chain fatty acids stimulate adipogenesis via GPCR43. Endocrinology 2005;146:5092–9.

[53] Schwiertz A, Taras D, Schäfer K, Beijer S, Bos NA, Donus C, et al. Microbiota and SCFA in lean and overweight healthy subjects. Obesity 2010;18:190–5.

[54] Nguyen TLA, Vieira-Silva S, Liston A, Raes J. How informative is the mouse for human gut microbiota research? Dis Model Mech 2015;8:1–16.

[55] Cho K, Moon JS, Kang JH, Jang HB, Lee HJ, Park SI, et al. Combined untargeted and targeted metabolomic profiling reveals urinary biomarkers for discriminating obese from normal-weight adolescents. Pediatr Obes 2017;12:93–101.

[56] Murakami S. Role of taurine in the pathogenesis of obesity. Mol Nutr Food Res 2015;59:1353–63.

[57] Lillefosse HH, Clausen MR, Yde CC, Ditlev DB, Zhang X, Du ZY, et al. Urinary loss of tricarboxylic acid cycle intermediates as revealed by metabolomics studies: An underlying mechanism to reduce lipid accretion by whey protein ingestion? J Proteome Res 2014;13:2560–70.

[58] Lees HJ, Swann JR, Wilson ID, Nicholson JK, Holmes E. Hippurate: The natural history of a mammalian-microbial cometabolite. J Proteome Res 2013;12:1527–46.

[59] Salek R, Maguire M. A metabolomic comparison of urinary changes in type 2 diabetes in mouse, rat, and human. Physiol Genomics 2007;29:99–108.

[60] Calvani R, Miccheli A, Capuani G, Tomassini Miccheli A, Puccetti C, Delfini M, et al. Gut microbiome-derived metabolites characterize a peculiar obese urinary metabotype. Int J Obes 2010;34:1095–8.

[61] Pinhal CS, Lopes A, Torres DB, Felisbino SL, Rocha Gontijo JA, Boer PA. Time-course morphological and functional disorders of the kidney induced by long-term high-fat diet intake in female rats. Nephrol Dial Transplant 2013;28:2464–76.

[62] Pickard JM, Chervonsky A V. Intestinal Fucose as a Mediator of Host–Microbe Symbiosis. J Immunol 2015;194:5588–93.

[63] Lawler PR, Akinkuolie AO, Chandler PD, Moorthy MV, Vandenburgh MJ, Schaumberg DA, et al. Circulating N-Linked Glycoprotein Acetyls and Longitudinal Mortality Risk. Circ Res 2016;118:1106–15.

[64] Tamura S, Fujioka H, Nakano T, Amuro Y, Hada T, Nakao N, et al. Urinary pseudouridine as a biochemical marker in the diagnosis and monitoring of primary hepatocellular carcinoma. Am J Gastroenterol 1988;83:841–5.

SUPPLEMENTARY MATERIAL

Supplementary table 1. Nutritional composition of the standard (STD), cafeteria (CAF), low-fat (LF), high-fat (HF) and high-fat high-sucrose (HFS) diets used in this study.

	STD	CAF	LF	HF	HFS
Energy density (Kcal/g)	3.1	2.1	3.9	4.7	2.0
Protein (g/100g)	18.6	5.6	19.2	24	7.3
Lipids (g/100g)	6.2	7.9	4.3	24	7.5
Total carbohydrates (g/100g)	44.2	28.0	67.3	41	26.2
Simple carbohydrates (g/100g)	0	17.1	0	20.1	21.6
Total fiber (g/100g)	14.7	1.81	4.7	5.8	1.3

Supplementary table 2. Cecal microbiota composition at phylum taxonomic level corresponding to rats fed a standard (STD), cafeteria (CAF), low-fat (LF), high-fat (HF) or high-fat high-sucrose (HFS) diet for 10 weeks.

Phylum (% relative abundance)	STD	CAF	LF	HF	HFS	p-value	Corrected p-value
Tenericutes	0.11 ± 0.02^{a}	0.00 ± 0.00^{b}	0.01 ± 0.00^{ab}	0.02 ± 0.00^{a}	0.01 ± 0.01^{ab}	<0.001	**<0.001**
Actinobacteria	0.01 ± 0.00	0.05 ± 0.01	0.04 ± 0.01	0.01 ± 0.00	0.04 ± 0.02	<0.001	**0.010**
Bacteroidetes	19.9 ± 2.49^{a}	68.8 ± 6.22^{b}	14.2 ± 2.2^{a}	20.9 ± 3.9^{a}	28.4 ± 4.29^{a}	<0.001	**0.010**
Verrucomicrobia	0.31 ± 0.11^{a}	3.3 ± 1.44^{ab}	3.68 ± 0.58^{b}	1.81 ± 0.27^{b}	1.84 ± 0.40^{ab}	<0.001	**0.010**
Lentisphaerae	0.00 ± 0.00^{a}	0.03 ± 0.01^{ab}	0.01 ± 0.00^{b}	0.04 ± 0.02^{ab}	0.04 ± 0.01^{ab}	0.010	**0.010**
TM7	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	<0.001	**0.010**
Firmicutes	79.3 ± 2.58^{a}	25.7 ± 6.78^{b}	80.9 ± 2.17^{a}	76.3 ± 4.09^{a}	67.5 ± 3.88^{a}	<0.001	**0.010**
Proteobacteria	0.24 ± 0.05^{a}	2.01 ± 0.53^{ab}	0.89 ± 0.11^{b}	0.77 ± 0.18^{ab}	2.00 ± 0.51^{ab}	<0.001	**0.020**
Deferribacteres	0.06 ± 0.01^{a}	0.00 ± 0.00^{b}	0.13 ± 0.05^{ab}	0.11 ± 0.02^{a}	0.12 ± 0.04^{ab}	0.010	**0.020**
Nitrospirae	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.030	**0.040**
Chloroflexi	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.090	0.120
Cyanobacteria	0.06 ± 0.01	0.1 ± 0.03	0.04 ± 0.01	0.05 ± 0.01	0.08 ± 0.01	0.480	0.480
Bacteroidetes-to-Firmicutes Ratio	0.26 ± 0.05^{a}	3.63 ± 0.70^{b}	0.18 ± 0.03^{a}	0.30 ± 0.07^{a}	0.46 ± 0.12^{a}	<0.001	

Cecal microbiota composition corresponding to each dietary group at phylum taxonomic level. Data are expressed as the mean ± SEM (n=6). ^{ab} Values with unlike letters are significantly different among groups after applying Benjamini-Holchberg procedure for controlling a false discovery rate of 5% (Kruskal-Wallis followed by Games-Howell post-hoc test, $p<0.05$). One-way ANOVA followed by DMS post-hoc test ($p<0.05$) was used to evaluate differences in Bacteroidetes-to-Firmicutes ratio among groups.

Supplementary table 3. Cecal microbiota composition at genus taxonomic level corresponding to rats fed a standard (STD), cafeteria (CAF), low-fat (LF), high-fat (HF) or high-fat high-sucrose (HFS) diet for 10 weeks.

Genera (% relative abundance)	STD	CAF	LF	HF	HFS	Corrected p-value
p_;c_;o_;f_;g_	0.04 ± 0.02	0.06 ± 0.05	0.05 ± 0.02	0.03 ± 0.01	0.08 ± 0.03	0.205
p_Actinobacteria;c_Coriobacteriia;o_Coriobacteriales; f_Coriobacteriaceae;g_	0.00 ± 0.00	0.03 ± 0.01	0.02 ± 0.01	0.01 ± 0.00	0.02 ± 0.02	**0.003**
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales; f_Bacteroidaceae;g_Bacteroides	1.49 ± 0.17^{a}	28.87 ± 3.24^{b}	9.39 ± 1.77^{a}	15.0 ± 3.71^{ab}	22.3 ± 4.70^{ab}	**0.002**
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales; f_Porphyromonadaceae;g_Parabacteroides	0.20 ± 0.09^{a}	1.69 ± 0.17^{b}	0.19 ± 0.02^{a}	0.30 ± 0.08^{a}	0.95 ± 0.22^{ab}	**0.003**
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales; f_Prevotellaceae;g_	0.12 ± 0.07^{ab}	0.00 ± 0.00^{ab}	0.00 ± 0.00^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	**0.010**
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales; f_Prevotellaceae;g_Prevotella	4.13 ± 0.92^{a}	34.8 ± 5.67^{b}	0.08 ± 0.02^{c}	0.02 ± 0.00^{c}	0.09 ± 0.03^{ac}	**0.003**
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales; f_Rikenellaceae;g_	2.75 ± 0.79	1.01 ± 0.29	0.66 ± 0.15	0.89 ± 0.16	1.06 ± 0.19	**0.021**
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales; f_S24-7;g_	8.08 ± 1.08^{a}	2.84 ± 1.16^{ab}	2.96 ± 0.50^{b}	2.51 ± 0.27^{b}	3.37 ± 0.18^{ab}	**0.011**
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales; f_[Barnesiellaceae];g_	0.05 ± 0.01	0.64 ± 0.22	0.01 ± 0.00	0.02 ± 0.01	0.12 ± 0.04	**0.005**
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales; f_[Odoribacteraceae];g_Odoribacter	0.06 ± 0.01	0.03 ± 0.02	0.02 ± 0.00	0.03 ± 0.01	0.08 ± 0.03	0.050
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales; f_[Paraprevotellaceae];g_Paraprevotella	0.01 ± 0.00	1.16 ± 0.32	0.04 ± 0.01	0.07 ± 0.02	0.19 ± 0.06	**0.004**
p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales; f_[Paraprevotellaceae];g_[Prevotella]	2.99 ± 0.68	1.02 ± 0.83	0.92 ± 0.22	2.06 ± 0.92	0.28 ± 0.23	**0.018**
p_Cyanobacteria;c_4C0d-2;o_YS2;f_;g_	0.06 ± 0.01	0.12 ± 0.05	0.04 ± 0.01	0.05 ± 0.01	0.08 ± 0.01	0.398
p_Deferribacteres;c_Deferribacteres;o_Deferribacterales; f_Deferribacteraceae;g_Mucispirillum	0.06 ± 0.01^{a}	0.00 ± 0.00^{b}	0.13 ± 0.05^{ab}	0.11 ± 0.02^{a}	0.12 ± 0.04^{ab}	**0.017**
p_Firmicutes;c_Bacilli;o_Lactobacillales; f_Lactobacillaceae;g_Lactobacillus	2.58 ± 0.58	4.57 ± 1.79	0.64 ± 0.34	0.79 ± 0.55	2.35 ± 0.67	**0.027**
p_Firmicutes;c_Bacilli;o_Turicibacterales; f_Turicibacteraceae;g_Turicibacter	0.09 ± 0.02	0.02 ± 0.01	0.01 ± 0.00	0.00 ± 0.00	0.05 ± 0.04	**0.017**
p_Firmicutes;c_Clostridia;o_Clostridiales; f_;g_	47.4 ± 2.44^{a}	0.78 ± 0.20^{b}	34.0 ± 2.03^{c}	32.7 ± 2.94^{cd}	21.2 ± 2.20^{d}	**0.002**

Genera (% relative abundance)	STD	CAF	LF	HF	HFS	Corrected p-value
p_Firmicutes;c_Clostridia;o_Clostridiales; f_Christensenellaceae;g_	0.01 ± 0.00^{a}	0.01 ± 0.01^{a}	0.08 ± 0.01^{b}	0.09 ± 0.02^{b}	0.14 ± 0.04^{ab}	**0.003**
p_Firmicutes;c_Clostridia;o_Clostridiales; f_Clostridiaceae;g_	0.04 ± 0.01	0.04 ± 0.02	0.12 ± 0.04	0.22 ± 0.13	0.24 ± 0.08	0.088
p_Firmicutes;c_Clostridia;o_Clostridiales; f_Clostridiaceae;g_Clostridium	0.09 ± 0.02	0.02 ± 0.01	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	**0.026**
p_Firmicutes;c_Clostridia;o_Clostridiales; f_Clostridiaceae;g_SMB53	0.28 ± 0.11	0.74 ± 0.31	0.77 ± 0.28	0.29 ± 0.10	0.68 ± 0.08	0.204
p_Firmicutes;c_Clostridia;o_Clostridiales; f_Dehalobacteriaceae;g_Dehalobacterium	0.43 ± 0.06^{a}	0.00 ± 0.00^{b}	0.03 ± 0.02^{b}	0.04 ± 0.02^{b}	0.05 ± 0.03^{b}	**0.003**
p_Firmicutes;c_Clostridia;o_Clostridiales; f_Lachnospiraceae;g_	6.52 ± 1.19	2.12 ± 0.79	11.4 ± 3.18	10.3 ± 1.91	7.48 ± 1.60	**0.027**
p_Firmicutes;c_Clostridia;o_Clostridiales; f_Lachnospiraceae;g_Blautia	0.01 ± 0.00^{a}	0.46 ± 0.14^{ab}	0.19 ± 0.03^{b}	0.16 ± 0.03^{b}	0.35 ± 0.10^{ab}	**0.005**
p_Firmicutes;c_Clostridia;o_Clostridiales; f_Lachnospiraceae;g_Coprococcus	1.35 ± 0.46^{a}	0.46 ± 0.12^{a}	5.92 ± 0.77^{b}	7.46 ± 1.51^{ab}	6.86 ± 1.37^{ab}	**0.003**
p_Firmicutes;c_Clostridia;o_Clostridiales; f_Lachnospiraceae;g_Dorea	0.11 ± 0.02	0.04 ± 0.00	0.42 ± 0.08	0.11 ± 0.02	0.18 ± 0.04	**0.003**
p_Firmicutes;c_Clostridia;o_Clostridiales; f_Lachnospiraceae;g_Roseburia	0.18 ± 0.07^{ab}	0.05 ± 0.01^{a}	0.37 ± 0.06^{b}	0.14 ± 0.06^{ab}	0.27 ± 0.07^{ab}	**0.011**
p_Firmicutes;c_Clostridia;o_Clostridiales; f_Lachnospiraceae;g_[Ruminococcus]	0.48 ± 0.12^{a}	0.40 ± 0.09^{a}	1.28 ± 0.15^{b}	0.63 ± 0.11^{ab}	2.55 ± 0.57^{ab}	**0.002**
p_Firmicutes;c_Clostridia;o_Clostridiales; f_Peptococcaceae;g_rc4-4	0.10 ± 0.02^{a}	0.09 ± 0.04^{a}	0.34 ± 0.03^{b}	0.50 ± 0.12^{ab}	0.63 ± 0.12^{ab}	**0.005**
p_Firmicutes;c_Clostridia;o_Clostridiales; f_Peptostreptococcaceae;g_	0.43 ± 0.14	0.71 ± 0.28	0.81 ± 0.33	0.50 ± 0.20	0.71 ± 0.10	0.566
p_Firmicutes;c_Clostridia;o_Clostridiales; f_Ruminococcaceae;g_	4.86 ± 0.81^{a}	0.66 ± 0.31^{b}	10.54 ± 1.99^{a}	8.21 ± 1.18^{a}	8.04 ± 0.87^{a}	**0.003**
p_Firmicutes;c_Clostridia;o_Clostridiales; f_Ruminococcaceae;g_Oscillospira	12.8 ± 1.17^{a}	2.62 ± 0.99^{b}	3.80 ± 0.51^{b}	4.39 ± 0.61^{b}	5.81 ± 0.97^{b}	**0.003**
p_Firmicutes;c_Clostridia;o_Clostridiales; f_Ruminococcaceae;g_Ruminococcus	1.54 ± 0.40^{a}	0.93 ± 0.37^{a}	7.68 ± 1.12^{b}	6.40 ± 2.03^{ab}	3.84 ± 0.99^{ab}	**0.005**
p_Firmicutes;c_Clostridia;o_Clostridiales; f_Veillonellaceae;g_Phascolarctobacterium	0.01 ± 0.01^{a}	7.67 ± 1.10^{b}	2.53 ± 0.57^{ac}	3.32 ± 0.86^{ab}	5.96 ± 0.98^{bc}	**0.004**
p_Firmicutes;c_Clostridia;o_Clostridiales; f_[Mogibacteriaceae];g_	0.04 ± 0.00	0.06 ± 0.03	0.03 ± 0.00	0.02 ± 0.00	0.07 ± 0.03	0.405

Genera (% relative abundance)	STD	CAF	LF	HF	HFS	Corrected p-value
p_Proteobacteria;c_Alphaproteobacteria; o_RF32;f_;g_	0.03 ± 0.01	0.23 ± 0.09	0.02 ± 0.00	0.05 ± 0.02	0.09 ± 0.04	**0.045**
p_Proteobacteria;c_Betaproteobacteria;o_Burkholderiales; f_Alcaligenaceae;g_Sutterella	0.05 ± 0.01^{a}	1.04 ± 0.33^{ab}	0.04 ± 0.01^{a}	0.11 ± 0.04^{ab}	0.20 ± 0.03^{b}	**0.003**
p_Proteobacteria;c_Deltaproteobacteria;o_Desulfovibrionales; f_Desulfovibrionaceae;g_Bilophila	0.08 ± 0.02^{a}	0.44 ± 0.12^{ab}	0.62 ± 0.07^{b}	0.61 ± 0.14^{ab}	1.17 ± 0.36^{ab}	**0. 007**
p_Proteobacteria;c_Deltaproteobacteria;o_Desulfovibrionales; f_Desulfovibrionaceae;g_Desulfovibrio	0.00 ± 0.00^{a}	0.21 ± 0.05^{b}	0.19 ± 0.07^{ab}	0.00 ± 0.00^{a}	0.49 ± 0.25^{ab}	**0.003**
p_Proteobacteria;c_Gammaproteobacteria;o_Enterobacteriales; f_Enterobacteriaceae;g_	0.03 ± 0.02	0.05 ± 0.04	0.02 ± 0.01	0.01 ± 0.00	0.02 ± 0.01	0.387
p_Tenericutes;c_Mollicutes;o_Anaeroplasmatales; f_Anaeroplasmataceae;g_Anaeroplasma	0.06 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	**0.030**
p_Verrucomicrobia;c_Verrucomicrobiae;o_Verrucomicrobiales; f_Verrucomicrobiaceae;g_Akkermansia	0.31 ± 0.11^{a}	3.35 ± 1.43^{ab}	3.69 ± 0.58^{b}	1.82 ± 0.27^{ab}	1.85 ± 0.44^{ab}	**0.006**

Cecal microbiota composition corresponding to each dietary group at genus taxonomic level. Data are expressed as the mean ± SEM (n=6). ^{abcd} Values with unlike letters are significantly different among groups after applying Benjamini-Holchberg procedure for controlling a false discovery rate of 5% (Kruskal-Wallis followed by Games-Howell post-hoc test, $p<0.05$).

CHAPTER II

Influence of Strain and Gender on the Gut Microbiota Composition and Obesity Development in Cafeteria Diet-Fed Rats.

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In preparation

UNIVERSITAT ROVIRA I VIRGILI
GUT MICROBIOTA DYSBIOSIS IN DIET-INDUCED OBESITY. A FOCUS ON THE INFLUENCE OF GENETICS, CIRCADIAN RHYTHMS AND POTENTIAL PREBIOTICS.
Andreu Gual Grau

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, dietary impact in the gut microbiota composition might be vastly influenced by genetic factors, thus distinctively predisposing the host to pathological states such as obesity. For instance, the gut microbiota-host interactions depend on hormonal status, which contributes to the establishment of diseases-associated sexual dimorphisms. Therefore, we aimed to characterize the strain and gender influence on the CAF diet-induced alterations of gut microbial ecosystems. For this purpose, two distinct cohorts of male Wistar and Fischer 344 rats, as well as male and female Fischer 344 animals, were chronically exposed to a STD or a CAF diet. In this study, we revealed that strain-specific microbial dysbiosis promoted by the CAF diet could be influenced by external factors such as food intake, thus revealing an absence of relevant microbial alterations in Fischer 344 that was related to the higher chow consumption as compared to Wistar rats. Furthermore, sex-dependent changes on gut microbiota, which were unrelated to differences in food intake according to gender and suggested to be mediated by estrogens, prompted the acquisition of an exacerbated obese phenotype in female rats under a CAF diet. Overall, our study revealed that CAF diet triggered a more pronounced microbiota dysbiosis in Wistar than in F344 rats, whereas females were more susceptible to the disruption of gut microbiota and alterations on host phenotype than males after the CAF feeding.

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

1. INTRODUCTION

The gut microbiota activity is highly related to the metabolic and physiologic phenotype of the host, actively contributing to the maintenance of energy homeostasis. Importantly, a wide range of factors are known to contribute to the configuration of gut microbiota including genetic and environmental factors. Whereas diet has been proposed as the main agent influencing the composition and activity of the gut microbial ecosystem [1], host genetics are known to have a determinant impact on the microbiome [2,3]. Recent findings raise the importance of controlling for ethnicity when establishing a relationship between changes on gut microbiota and the onset of a disease [4]. In fact, genetic differences in the gut microbiota composition may account for distinct metabolic and physiologic responses to diet-induced obesity. Several animal models have been used to elucidate the crucial role of the microbiome in the onset of diet-induced obesity. For instance, diet-induced microbial shifts were similarly reported between Wistar and Sprague-Dawley rats under a high-fat diet, which were observed in parallel with a distinguished obese phenotype [5]. Interestingly, high-fat diet was previously shown to alter the gut microbiota independently of obesity [6].

Furthermore, sexual dimorphism is characteristic of different common diseases including obesity and related metabolic complications, which has been recently reviewed [7]. Accordingly, hormonal status exerts an important modulatory role on the gut microbiota-host interaction. In this regard, the preventive effects of estrogens in obesity-related metabolic disorders such as endotoxemia and low-grade chronic inflammation were recently shown to be

mediated by gut microbiome [8]. Overall, the interaction between diet, gut microbiota and host homeostasis seems to underlie the genetic conditions.

In **Chapter I**, cafeteria (CAF) diet has been identified as the most robust diet to induce severe obesity-associated dysbiosis in rats [9]. Despite that CAF diet is known to closely reflect the modern human condition of obesity [10], the influence of genetics in the CAF diet-induced dysbiosis have not been evaluated so far. Here, we hypothesized that the profound alterations of gut microbial ecosystems promoted by the CAF diet are conditioned, at least in part, by rat strain and gender. In order to address this hypothesis, we assessed the cecal microbiota composition of Fischer 344 (F344) and Wistar rats after a continuous feeding with standard chow or CAF diet. Furthermore, the influence of the gender on microbial and physiologic alterations was also evaluated within both male and female F344 rats after the chronic consumption of a CAF diet.

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, Massachusetts, USA). Animals were housed in three different temperature controlled rooms (22ºC) according to gender and strain under a 12:12-hour light-dark cycle. After one week of adaptation to the facilities with free access to food and water, the animals within each strain and sex were randomly distributed into two groups depending on the diet: (1) a standard chow diet (Teklad Global 18% Protein Rodent Diet 2018, Harlan, Barcelona, Spain) (STD), and (2) a CAF diet composed of energy-dense and highly palatable foods including biscuits with

pâté and cheese, bacon, pastries and sugared milk (220 g/l; 50 mL) *ad libitum*. Food intake and body weight were recorded weekly during the whole experimental procedure. One week before the end of the study, body composition was assessed by nuclear magnetic resonance (NMR) using an EchoMRI-700^{TM} device (Echo Medical Systems, L.L.C., Houston, USA). Serum samples were obtained by saphenous venepuncture and processed by centrifugation (2,000 g for 15 min). When rats were 17-weeks-old, Wistar rats were euthanized by exsanguination through cardiac puncture under anesthesia (pentobarbital sodium, 80 mg/kg body weight). 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.

2.2 Assessment of biochemical parameters and gut microbiota.

Serum levels of glucose, triglycerides and total cholesterol were assessed using enzymatic colorimetric kits (QCA, Barcelona, Spain) according to the manufacturer's instructions. Cecal microbiota was assessed as previously described in the Materials and Methods section of the **Chapter I**.

2.3 Statistical analysis.

All results are expressed as the mean ± standard error of the mean (SEM). The Grubbs test was applied to remove significant outliers. A two-way ANOVA was applied to study the influence these factors on food intake, biometric and serum parameters. Differences between STD and

CAF diet-fed groups were assessed by Student's t-test within each strain or gender. A probability of $p < 0.05$ was considered statistically significant. Statistical analyses were performed using SPSS Statistics 22 software (SPSS, Inc., Chicago, IL, USA).

For metagenomic analyses, 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. Moreover, the contributory role for each factor to the observed differences was tested using two-way analysis of similarities (ANOSIM) at genus level, with diet nested within strain or gender. Here, microbial genera present in at least two samples with ≥0.1% abundance were previously selected. Data of relative abundances were used to construct a Bray-Curtis similarity matrix, on which the ANOSIM was based. Therefore, changes on specific cecal microbes were elucidated 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 the genera proportions were performed in order to identify clusters according to the microbiome composition.

3. RESULTS AND DISCUSSION

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

Food intake, biometric and biochemical parameters from Wistar and F344 rats fed with STD or CAF diets are shown in **Table 1**. The two-way

ANOVA revealed that CAF diet induced an increased energy intake within both strains when compared to STD diet, although this increase was higher in F344 rats. In the same way, the carbohydrate and protein intake was higher in F344 than in Wistar rats fed with a CAF diet, while the increased consumption of simple carbohydrates was more pronounced in Wistar than in F344 rats under a CAF feeding. Importantly, CAF diet promoted a higher increment in total food consumption (grams) in F344 than Wistar animals (data not shown). These results indicate that the hyperphagia promoted by CAF diet was more evident in F344 than in Wistar rats. In this regard, a greater acute leptin response to intravenous glucose and a higher fasting leptin concentrations, altogether with a lack of responsiveness on satiety, was assessed in obese versus lean F344 rats [11], thus suggesting a proneness to leptin resistance. Moreover, the leptin response was also higher in F344 than in Sprague-Dawley rats [11], as similarly observed in another study showing higher fasting leptin levels in F344 compared to Lou rats [12], supporting the previous suggestion. The exacerbated hyperphagia observed in the present study is consistent with the discussed leptin resistant phenotype of F344 rats.

Interestingly, CAF diet was associated with a reduced protein intake in Wistar animals, while it was raised in F344 rats when compared to their respective STD diet-fed controls. Furthermore, the decrease of fiber intake in CAF diet-fed animals was more pronounced in Wistar animals. These results were mainly due to a strongly diminished chow intake in Wistar rats when processed and highly palatable foods were available (Wistar-CAF: 3.22 ± 0.61 g/day, F344-CAF: 10.6 ± 0.37 g/day), whereas total energy obtained from the rest of the ingredients, excluding chow,

did not differed between both strains (Wistar-CAF: 126 ± 2.31 kcal/day; F344-CAF: 127 ± 4.18 kcal/day). Therefore, strain-dependent differences on energy intake were mainly determined by divergent chow consumption under a CAF feeding. However, cecum weight was decreased in CAF diet-fed animals regardless the rat strain, which could be the consequence of a reduced fiber intake. In this sense, a rise of cecum weight was previously observed with increased fiber concentrations [13,14], probably due to greater amounts of non-fermentable fibers passing through this fermentative organ.

The effects of the CAF diet on biometric parameters, including increased body weight gain, total fat mass, adipose tissues weights (MWAT, EWAT and IWAT), adiposity index and visceral fat percentage, altogether with a decreased body lean mass percentage and lean-to-fat mass ratio, were all found to occur in both F344 and Wistar rats. Therefore, CAF diet-fed F344 animals showed a higher energy intake than Wistar rats that was not accompanied by an exacerbated obese phenotype. Accordingly, a large difference in the food efficiency was assessed between the two strains where F344 group was less efficient in body weight gain than Wistar animals regardless the diet type. In this regard, factors affecting the energy expenditure such as physical activity, thermogenesis and basal metabolism could be contributing to the differences observed between both strains [15]. Interestingly, the CAF diet led to an increased percentage of RWAT mass within the Wistar compared to F344 animals. 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 also in accordance with the decreased food efficiency identified in F344 rats.

The biochemical measurements revealed that CAF diet induced an increment of serum glucose levels independently of the strain. In contrast, hypertriglyceridemia and hypercholesterolemia were only induced in F344 rats consuming a CAF diet. Therefore, the highest energy intake observed in CAF diet-fed F344 rats led to an obese profile associated with drastic metabolic alterations in this group. Overall, distinct diet-induced obesity profiles were generated by our CAF dietary model according to rat strain.

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
	STD	**CAF**	**STD**	**CAF**	**ANOVA**
Food intake (kcal/day)					
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/day)	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

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; SxD, the interaction of strain and diet type (two-way ANOVA, $p < 0.05$). * The effect of diet within each strain (Student's t-test, $p < 0.05$). EWAT, epididymal white adipose tissue; IWAT, inguinal white adipose tissue; MWAT, mesenteric white adipose tissue; RWAT, retroperitoneal white adipose tissue.

The comparison between male and female F344 rats fed STD or CAF diet concerning food intake, biometric and biochemical parameters is shown in **Table 2**. The CAF diet was associated with an increased total energy intake, also showing a raised caloric contribution from carbohydrates, proteins and lipids in both males and females. As expected, energy and macronutrients intake was lower in females than in males, independently of the dietary model. However, high-palatable sugar-containing milk was almost entirely consumed by all the animals, which explained the absence of the gender effect, on the consumption of simple carbohydrates. Moreover, a reduced fiber intake and cecum weight were observed in CAF diet-fed groups, with males showing higher values of these measurements than females regardless the type of the diet. Thus, these observations reinforce the previously discussed relationship between the consumption of non-digestible substrates and the cecum weight.

Despite that CAF diet promoted a raised energy intake in both genders similarly, body weight gain was distinct in male and female F344 rats, where males were more susceptible to body weight changes promoted by this diet. Conversely, previous studies reported similar increases in body weight gain induced by CAF feeding in both male and female rodents [16,17], although divergent dietary composition, length of feeding, strains and species could explain these differences. In addition, the dramatic body weight gain in males could be the consequence of a lower energy expenditure compared to females as previously reported in mice [18].

CAF feeding induced an increased percentage of body fat mass, adiposity index and visceral fat within both male and female rats. Female rats

showed an increased fat accretion as compared to males independently of the diet consumed. This fact is in accordance with the increased lipid storage and low rate of fat mobilization in females to preserve them in case of pregnancy [19]. Furthermore, sex-dependent responses to CAF diet on body weight gain do not seem to be explained by differences on adiposity. Contrary to our results, high-fat diet has been previously shown to induce a greater fat accumulation in female than in male mice [20]. Despite this, the significant interaction between gender and diet indicated that the drop on lean mass and the increase of RWAT percentages linked to CAF feeding were more pronounced in female than in male, thus supporting a sex-dependent vulnerability to diet-induced obesity.

Concerning serum parameters, CAF diet promoted an increase in fasting blood glucose, triglycerides and total cholesterol levels within both males and females, while the diet-induced hypertriglyceridemia was more pronounced in male than in female F344 rats. Likewise, it is well-known that men compared with women [21,22] have greater fasting serum concentration of triglycerides (TAG), which is attributed to an increased efficiency on TAG removal from the circulation in women [23], thus supporting our results. Altogether, despite that changes on body composition after the CAF feeding were markedly observed in females, the exacerbated increment in serum lipids suggested a metabolically unhealthier profile in male than in female rats.

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/day)					
Total energy intake	60.6 ± 0.97	159 ± 3.55	38.8 ± 0.60	122 ± 9.35	G, D
Total carbohydrates intake	35.2 ± 0.56	92.8 ± 2.71	22.5 ± 0.35	76.3 ± 6.87	G, 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	G, D
Protein intake	14.6 ± 0.23	18.2 ± 0.52	9.32 ± 0.14	12.0 ± 0.64	G, D
Fiber intake (g/day)	2.88 ± 0.05	1.88 ± 0.06	1.84 ± 0.03	0.80 ± 0.02	G, D
Food efficiency	3.35 ± 0.08	2.24 ± 0.21	2.09 ± 0.08	1.48 ± 0.12	G, D
Biometric parameters					
Body weight gain (g)	128 ± 3.29	225 ± 17.1*	51.1 ± 2.04	109 ± 3.74*	G*D
Fat mass (%)	12.0 ± 0.51	24.2 ± 1.73	15.8 ± 1.26	32.0 ± 0.70	G, D
Lean mass (%)	78.3 ± 0.78	69.7 ± 1.67*	77.7 ± 1.10	62.8 ± 0.75*	G*D
Lean/fat ratio	6.60 ± 0.25	3.00 ± 0.34	5.18 ± 0.47	1.97 ± 0.07	G, D
Visceral fat (%)	6.24 ± 0.17	9.66 ± 0.43	7.81 ± 0.83	13.0 ± 0.29	G, D
Adiposity Index	7.63 ± 0.21	12.0 ± 0.63	8.88 ± 0.88	15.3 ± 0.38	G, 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*	G*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	G, D
Serum parameters					
Glucose (mg/dL)	150 ± 5.97	202 ± 10.9	133 ± 6.18	178 ± 8.45	G, D
Triglycerides (mg/dL)	134 ± 8.21	358 ± 24.3*	76.6 ± 6.95	170 ± 22.7*	G*D
Total cholesterol (mg/dL)	59.0 ± 3.82	94.8 ± 7.29	85.8 ± 2.17	105 ± 5.23	G, D

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; G, the effect of gender; GxD, the interaction of gender and diet type (two-way ANOVA, $p < 0.05$). * The effect of diet within each gender (Student's t-test, $p < 0.05$). 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.

3.2 Strain and gender-dependent modulation of the gut microbiota by CAF feeding.

Overall, two-way PERMANOVA revealed a significant interaction between strain and diet ($p < 0.001$) as factors that were directly involved in the changes of the cecal microbiota. Concerning each rat strain, one-way PERMANOVA revealed that the bacterial proportions at genus level were significantly altered by the consumption of a CAF diet within both Wistar ($p = 0.002$) and F344 ($p < 0.001$) rats (**Supplementary Figure 1**), while this diet decreased the bacterial diversity measured by Shannon index regardless the strain (two-way ANOVA, $p = 0.042$), as presented in **Figure 1A**. Interestingly, we identified significant differences between F344 and Wistar animals fed with a chow diet regarding the microbiota composition at genus level (One-way PERMANOVA, $p = 0.003$) that was not accompanied by dissimilarities on bacterial diversity (Student's t-test, $p = 0.867$). Exploring the sex influence on diet-induced alterations of the microbiome, two-way PERMANOVA within F344 strain revealed an interaction between diet and gender factors ($p = 0.015$). Therefore, microbial taxa were distinctively influenced by diet according to the gender. Indeed, one-way PERMANOVA revealed that microbial populations were significantly altered by CAF diet within both males ($p < 0.001$) and females ($p < 0.001$) (**Supplementary Figure 2**). Importantly, we also observed that CAF feeding was able to significantly decrease the bacterial diversity in females (Student's t-test, $p < 0.001$), but not in males rats (**Figure 1B**).

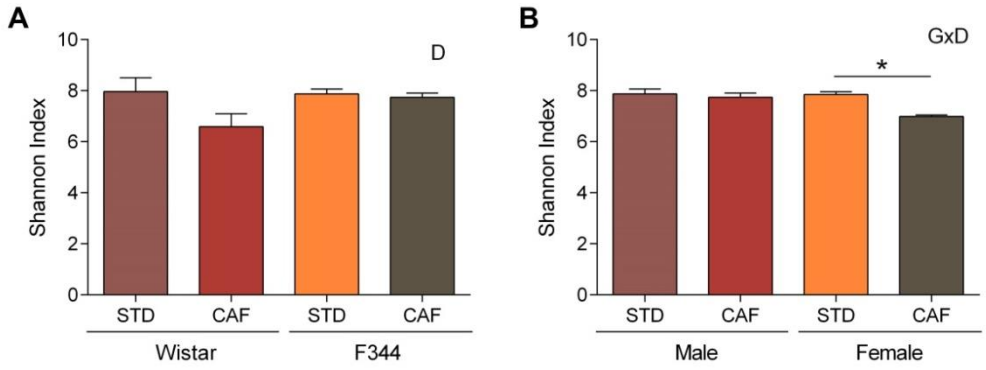


Figure 1. Differences in alpha-diversity among groups assessed through the Shannon Index. **A**. Microbial diversity determined in male Wistar and F344 rats fed with a standard (STD) or a cafeteria (CAF) diet. **B**. Microbial diversity determined in F344 male and female rats fed with a STD or a CAF diet. Data are expressed as the mean ± SEM. D, the effect of diet type; GxD, the interaction of gender and diet type (two-way ANOVA, $p< 0.05$). * The effect of diet within female rats (Student's t-test, $p < 0.05$).

Westernized diet has been shown to have a greater negative impact upon the gut microbiota diversity than an increased body mass index (BMI) in humans [24], postulating diet as one of the major factors contributing to the dysbiosis of microbiome. In this sense, CAF diet induced a drop on bacterial diversity regardless the differences on adiposity between both strains. Moreover, the absence of changes in bacterial diversity according to rat strains under a STD diet was previously reported in mice [2]. On the other hand, the metabolic effects of a high-fat diet were more pronounced in Wistar compared to Sprague-Dawley rats, while a similar dietary modulation of the gut microbiota was described [25]. Contrary to these findings, the distinctive alterations of the microbiota promoted by our CAF model in F344 and Wistar rats were contrasted by the absence of differences on biometric parameters. Thus, CAF diet-induced microbial changes did not reflect specific physiological outcomes according to rat strain. However, metabolic effects such as

hypertriglyceridemia, which was highly pronounced in F344 rats, and hypercholesterolemia, which was only identified in F344 animals, could be associated with the distinct changes on the microbiome promoted by the CAF feeding. Interestingly, a previous study indicated that the gut microbiome can explain a substantial proportion of the variation of blood lipid levels, independent of age, sex, BMI, and genetics [26].

We also reported that bacterial diversity was significantly modulated by diet in a gender-dependent way, showing for the first time that F344 females, but not males, were susceptible to changes on this feature after a prolonged feeding with a CAF diet. Initial evidence of the influence of sex steroids on the composition of the gut microbiome showed a positive correlation between urinary estrogen levels and microbial diversity [27], while no estrogen differences were assessed in CAF diet-fed female rats when compared to lean controls [28]. On the other hand, castrated male mice, with the consequent absence of sex steroids, showed alterations in intestinal microbiota when fed a high-fat diet [29]. However, a reduced diversity was reported in ovariectomized female mice receiving estrogen implants under a high-fat diet [30], which is in line with the observed reduction of bacterial diversity in CAF diet-fed female rats only. Altogether, it is suggested that changes on microbial diversity are primarily explained by an interaction between the diet and sex steroids, although further research is needed to better understand this relationship. In addition, despite that total simple carbohydrate intake did not differ between CAF diet-fed male and female rats, a proportionally greater sugar consumption was elucidated in females in relation to their body weight, which could contribute to the onset of a more pronounced microbial dysbiosis as compared to males. In this

regard, the consumption of high-sucrose diets has been previously shown to promote a loss of microbial diversity [31], thus supporting our results. Overall, a loss of microbiota diversity and changes on bacterial populations has been reported for diet-induced obesity in rodents [32], whereas we showed that dietary modulation of the microbial diversity is gender-dependent. In addition, gut microbiota composition was significantly but distinctively altered by the CAF diet in a strain- and sex-dependent manner.

3.3 CAF diet strongly alters the Bacteroidetes-to-Firmicutes ratio in Wistar and female F344 rats.

The relative abundance of identified phyla between groups was statistically compared using the non-parametric U-Mann Whitney test. The analysis of the microbiota composition revealed that Firmicutes was the most abundant phylum present in both Wistar (79.3%) and F344 (89.9%) rats fed with a standard chow, showing a greater relative abundance within the F344 group ($p = 0.005$). In contrast, Bacteroidetes occurrence was significantly higher in Wistar (19.8%) when compared to F344 (8.7%) animals ($p = 0.003$). In this line, Wistar rats showed a higher Bacteroidetes-to-Firmicutes ratio than F344 animals (Student's t-test, $p = 0.014$) when both groups were under a chow diet. Interestingly, CAF diet-induced increase in the Bacteroidetes-to-Firmicutes ratio was 10 times higher in Wistar (Student's t-test, $p = 0.005$) than in F344 rats (Student's t-test, $p = 0.08$), as shown in **Figure 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. Moreover, **Figure 2B** shows that the consumption of a CAF diet

markedly increased this ratio in female F344 animals when compared to their respective controls (Student's t-test, p = 0.002).

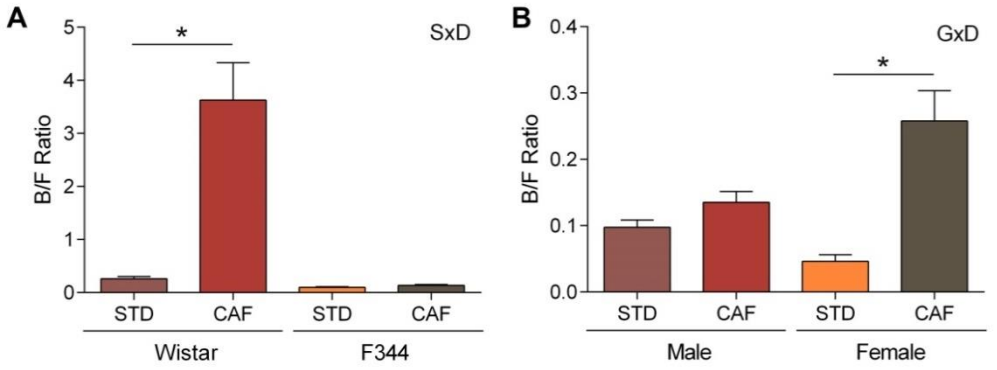


Figure 2. The two major phyla composing the microbiome expressed as the Bacteroidetes-to-Firmicutes ratio. **A**. B/F ratio assessed in male Wistar and F344 rats fed with a standard (STD) or a cafeteria (CAF) diet. **B**. B/F ratio assessed in F344 male and female rats fed with a standard STD or a CAF diet. Data are expressed as the mean ± S.E.M. SxD, the interaction of strain and diet type; GxD, the interaction of gender and diet type (two-way ANOVA, $p < 0.05$). * The effect of diet within male Wistar and female F344 rats (Student's t-test, $p < 0.05$).

As previously discussed, CAF diet triggers a substantial impact on phenotypic and metabolic parameters, finally leading to severe obesity [33,34]. The CAF-promoted robust alterations of microbial taxa were also correlated with the parameters defining the resulting obese profile, as previously described [9]. Contrary to our findings in rats, healthy human gut microbiota has been widely characterized by an elevated Bacteroidetes-to-Firmicutes ratio [35–37], with Firmicutes prevailing in obese individuals. Thus, this ratio has been postulated to act as a promising biomarker of obesity susceptibility [38]. However, a meta-analysis revealed that the ratio changes between obese and lean individuals were not statistically significant overall, thus concluding that

this feature should not be considered for distinguishing lean and obese human gut microbiota across populations [39].

Here, we observed an increment of Bacteroidetes-to-Firmicutes ratio in CAF diet-induced obese male Wistar rats, which is in agreement with previous studies using similar dietary models [40,41]. However, this ratio was not altered in male F344 rats, although a less pronounced but significant increment was observed in females, which consumed higher amounts of sucrose per kg of body weight than males. Therefore, we elucidated a sex-dependent alteration of Bacteroidetes-to-Firmicutes ratio by the CAF diet within F344 strain for the first time. In this scenario, as there is no evidence on a clear relationship between Bacteroidetes-to-Firmicutes ratio and obesity risk, the complex modulation of obesity by diet and the influence of gut microbiome need to be further analyzed.

3.4 Major phyla are profoundly affected by CAF feeding in F344 females.

Altogether with the described strain-dependent alterations in the two major phyla, CAF diet also induced an increment of Verrucomicrobia occurrence in Wistar rats ($p = 0.010$), as well as a tendency to increase ($p = 0.090$) in F344. On the other hand, Proteobacteria were significantly raised by the CAF diet within both Wistar and F344 groups ($p < 0.010$), as shown in **Figure 3A**. When assessing the gender contributory role to the CAF-induced changes on major phyla (**Figure 3B**), a higher occurrence of Verrucomicrobia ($p = 0.027$) was only described in F344 females compared to standard controls, while significant changes on

Proteobacteria relative abundances were only assessed in F344 males fed with a CAF diet compared to their lean counterparts.

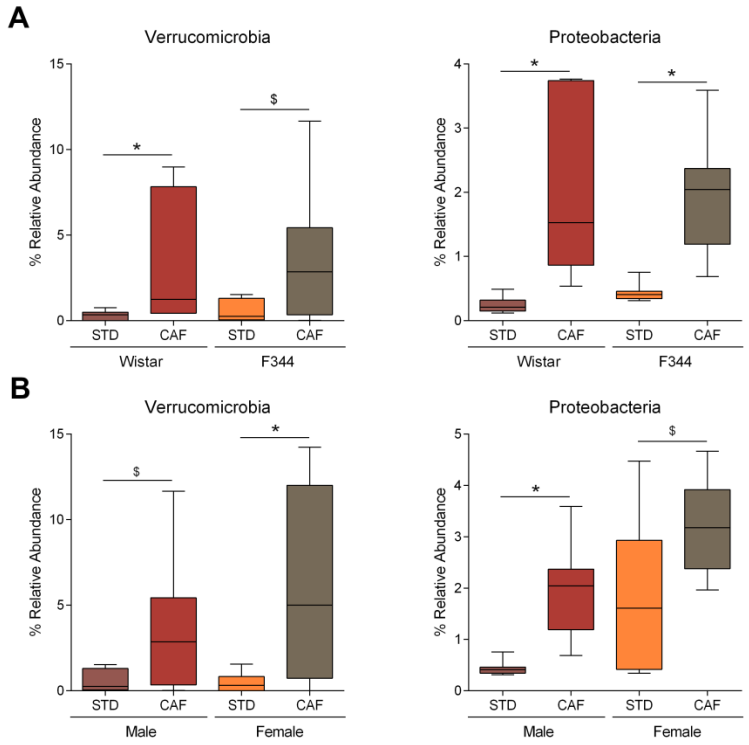


Figure 3. Differences on the relative abundances of Verrucomicrobia and Proteobacteria within male F344 and Wistar animals (**A**) or within F344 male and female rats (**B**) fed with a standard (STD) or a cafeteria (CAF) diet. $Tendency or *significant differences assessed through Welch's t-test after Benjamini-Holchberg FDR correction considering corrected p-values less than 0.10 or 0.05, respectively.

Interestingly, the influence of a HF diet and gender to gut microbial alterations in F344 rats has been recently investigated. At phylum level, HF diet administered to young F344 rats significantly reduced Firmicutes and raised Verrucomicrobia proportions independently of sex influence [42]. Nevertheless, we showed that CAF diet led to a significant increase of Verrucomicrobia phylum only within F344

females. Thus, altogether with the changes on bacterial diversity and Bacteroidetes-to-Firmicutes ratio, it seems that female F344 rats are highly susceptible to the microbiota dysbiosis induced by our CAF model.

We also found that low-abundant phyla representing less than 1% of the microbiome such as Actinobacteria ($p = 0.020$), Lentisphaerae ($p = 0.007$) and TM7 ($p = 0.002$) were higher in F344 compared to Wistar under a chow feeding, while a lower Deferribacteres abundance was observed in F344 rats ($p = 0.002$). Concerning both Wistar and F344 strains, CAF diet caused a decrease in TM7 ($p < 0.050$) and Tenericutes ($p < 0.050$) occurrence, while Actinobacteria ($p < 0.010$) was raised. Indeed, a reduction of Deferribacteres ($p = 0.004$) was induced by the CAF diet only within Wistar strain. In accordance with our results, an extensive metagenomic study discovered higher Actinobacteria proportion in obese than lean individuals [43]. However, no significant changes on Actinobacteria occurrence has been assessed in female F344 rats under a CAF feeding.

3.5 Rat strain has a great impact on microbial communities under a chow diet.

Concerning microbiome composition, up to 14 genera statistically differed between Wistar and F344 rats fed with standard chow. Importantly, Wistar animals had a higher relative abundance of genera belonging to Bacteroidetes phylum including *Bacteroides* ($p = 0.017$) and *Prevotella* ($p = 0.037$), as well as an increased *Oscillospira* ($p = 0.022$) occurrence. On the other hand, genera belonging to Firmicutes such as *Lactobacillus* ($p = 0.027$), unclassified Lachnospiraceae ($p = 0.034$) and *Ruminococcus* ($p = 0.014$) were mainly found in F344 rats.

The degradation of complex polysaccharides taking place in the large intestine finally generates fermentation by-products such as short chain fatty acids (SCFA). The differences found in bacterial communities between standard chow-fed Wistar and F344 rats involves taxa that actively contributes to these fermentative processes. For instance, Lachnospiraceae and Ruminococcaceae families were described to have a crucial role in polysaccharide degradation, likely to be enriched in high energy extraction groups [44]. On the other hand, *Bacteroides* can be rapidly adapted to low-fiber rich diets due to their capacity to utilize host glycans [45]. Moreover, the reported rise of *Lactobacillus* in F344 rats, which is a well-known genus for its health promoting properties [46–48] and found increased after a weight loss program in adolescents [49], is in accordance with its previously reported drop in Wistar compared to Sprague-Dawley rats under a chow diet [25]. Overall, we suggest that phenotypic, metabolic and functional changes on the gut microbiota induced by CAF diet are determined, at least in part, by the pre-established strain-specific microbial populations.

3.6 CAF distinctively modulates the genera abundance according to rat strain.

Two-way ANOSIM revealed strong influences of both strain ($R = 0.876$, $p < 0.001$) and diet ($R = 0.801$, $p < 0.001$) factors on the differences found at genus level after the consumption of a CAF diet. Nevertheless, some of the changes induced by the CAF diet on microbial populations were similarly described for both rat strains, as presented in **Figure 4**. In this sense, we detected a highly reduced occurrence of unclassified Clostridiales ($p < 0.010$), *Oscillospira* ($p < 0.010$) and *Dehalobacterium* ($p < 0.050$), accompanied by an increment of

Bacteroides ($p < 0.050$), *Parabacteroides* ($p < 0.050$), *Prevotella* ($p < 0.050$) and *Phascolarctobacterium* ($p < 0.050$) relative abundances in CAF-fed rats compared to the respective standard chow-fed groups. *Bacteroides* proportion reached the 28.0% of total bacteria, mainly in detriment of unclassified Clostridiales that was reduced from 47.5% to 6.3% of overall population, in CAF diet-fed Wistar rats. Otherwise, *Bacteroides* represented only 1% of total bacteria after CAF feeding in F344 animals, while unclassified Clostridiales were decreased in a lesser extent (from 54.5% to 34.1%) than in Wistar rats. On the other hand, whereas *Prevotella* occurrence was found increased up to 0.42% in CAF-fed F344 animals, Wistar rats presented a strong increment of this genus (reaching the 34.7% of total bacteria).

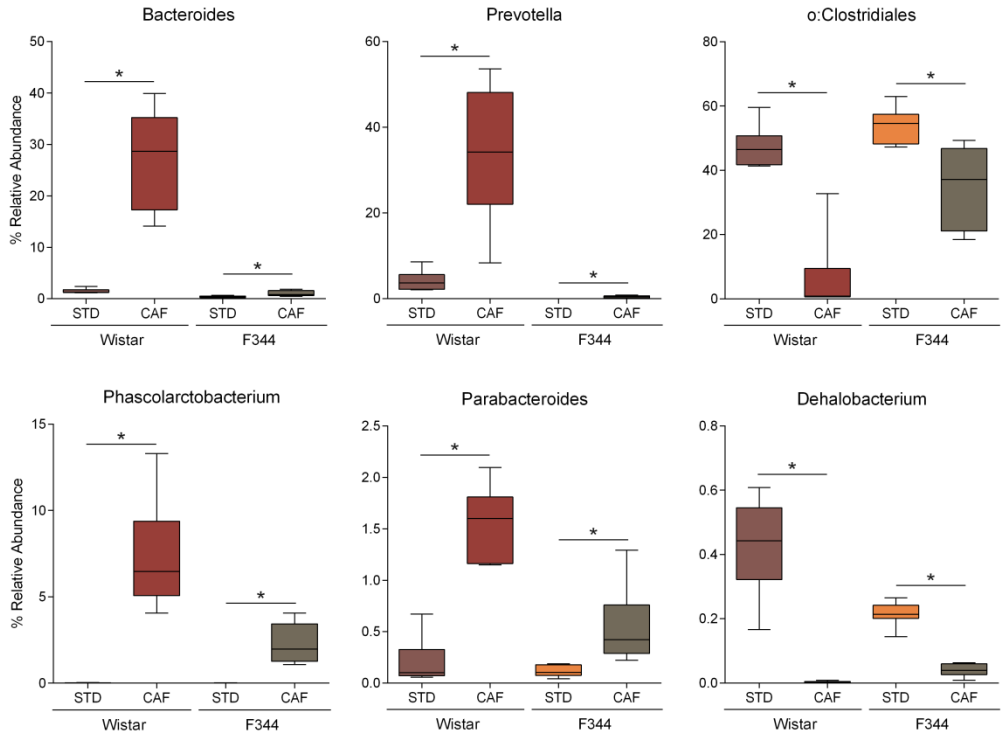


Figure 4. Relative abundance of the main genera similarly influenced by the cafeteria (CAF) diet within both Wistar and F344 rat strains. *Significant differences assessed through Welch's t-test after Benjamini-Holchberg FDR correction considering corrected p-values < 0.05.

Accordingly, a raised proportion of *Bacteroides* have been found in obese individuals, positively correlating with BMI [50]. Despite this fact, an inverse relationship between *Bacteroides* and BMI has been also observed [51], so further studies are necessary to elucidate the role of this genera in human obesity. Importantly, *Bacteroides* abundance was associated with the long-term consumption of a diet rich in animal protein, sugar, starch, and fat and low in fiber [52]. In rodents, an increased occurrence of *Bacteroides* has been determined as a response to a high-sucrose diet [31]. Here, we demonstrated that *Bacteroides*

became a major genus in Wistar, but not in F344 rats, after CAF feeding, which was supported by the highest increase in the consumption of simple sugars observed in this strain. Strong differences on the CAF diet-induced rise of Bacteroides occurrence advertises of the complex interaction between the genotype and diet in the establishment of the gut microbiota. On the other hand, *Bacteroides* have been identified as the one of the main bacterial taxa involved in the conversion of cholesterol into coprostanol in the intestine [53]. Given the inverse relationship between serum cholesterol levels and coprostanol/cholesterol ratio in human feces [54], we suggest that the low abundance of *Bacteroides* detected in CAF diet-fed F344 rats (1%) could be associated with the developed hypercholesterolemia, a feature that was absent in CAF diet-fed Wistar rats showing high *Bacteroides* occurrence (27%).

The reduction of Firmicutes phyla associated to the consumption of a CAF diet was mainly explained by a dramatic drop of an unclassified genus from Clostridiales order in both Wistar and F344 rats. These results are in accordance with the literature discussing that HF diet feeding reduces the occurrence of unclassified Clostridiales, which had an inverse correlation with body fat mass, in rats [55]. In addition, a diminution of uncultured Clostridiales was also described for obese compared to non-obese subjects [56]. Thus, the exacerbated modulation of both *Bacteroides* and unclassified Clostridiales in diet-induced obese Wistar rats suggested that this strain is more susceptible than F344 to the impact of a CAF diet. In addition, we demonstrated that *Prevotella* genus was favoured by the consumption of a sugar-rich hypercaloric and obesogenic diet such as CAF in both F344 and Wistar rats. Interestingly,

the CAF-promoted increment in this genus was especially pronounced in Wistar rats, where *Prevotella* became of the most abundant genera representing up to 34.7% of the bacterial population. Accordingly, *Prevotella* has been previously linked to the consumption of simple sugars [52] and its relative abundance was found reduced in old F344 rats under HF diet [42]. Then, we hypothesized that dietary sugar content is a determinant factor describing a bloom in *Prevotella* proportion. However, no clear evidences supporting this hypothesis were found on F344 rats, where *Prevotella* increased in a marginal way. On the other hand, *Prevotella* was previously found increased after a chronic exposure to a protein deficient diet in mice [57], suggesting that the lowest protein intake observed in CAF diet-fed Wistar rats could be also favouring this genus. Overall, the strain-dependent modulation of cecal microbiome by CAF diet was evident within the most representative above-mentioned genera, while further studies are need to better understand the specific role of each genera in CAF-induced obesity.

Oscillospira decreased from 6.8% to 2.6% and from 12.9% to 2.2% in CAF diet-fed F344 and Wistar rats, respectively. *Oscillospira* species were found strongly increased in hosts consuming fresh green fields [58] and hypothesized to be adapted to primarily utilize plant polysaccharides [59]. Then, the observed *Oscillospira* reduction could be linked to the decreased fiber content of our CAF model compared to the standard chow. Moreover, *Oscillospira* has been widely linked to the leanness and lower BMI in humans, which is in accordance with our results [56,59,60].

Akkermansia was detected as the main genus belonging to the Verrucomicrobia phylum within both Wistar and F344 strains. In this

sense, despite the higher occurrence of Verrucomicrobia and the numerically higher relative abundance of *Akkermansia* in CAF diet-fed Wistar rats compared to its respective controls, no significant differences were found for this genus. However, previous studies have reported an increase of *Akkermansia* in rats fed with high-fat diet compared to low-fat diet-fed rats [61], as well as in mice fed with a protein deficient diet [57]. In the last years, the role of *A. Muciniphila* in regulating host health has been widely declared [62,63], showing a negative correlation with obesity and related metabolic disorders [64]. Thus, despite that species identification was not reached in this study, CAF diet could be probably modulating other *Akkermansia* genus than *A. Muciniphila*.

Interestingly, several genera were only influenced by the dietary intervention within a particular strain (**Figure 5**). Thus, the CAF diet significantly decreased Bacteroidales S24-7 ($p = 0.023$) and unclassified Ruminococcaceae ($p = 0.022$) only in Wistar animals when compared to their respective controls. The Bacteroidales family S24-7 is considered a core component of the normal mouse microbiome [65] with the capacity to digest both complex carbohydrates and host-derived glycans [66]. This genus was also previously found increased in mice fed with a high-starch diet rich in plant polysaccharides [67]. Thus, the higher reduction of fiber intake in Wistar compared to F344 animals under the CAF feeding could finally explain the disfavoured occurrence on Bacteroidales S24-7 only observed in Wistar rats. On the other hand, Ruminococcaceae family, linked to the utilization of plant polysaccharides, was also found reduced in HF-diet fed mice [68], thus supporting our observations in Wistar animals.

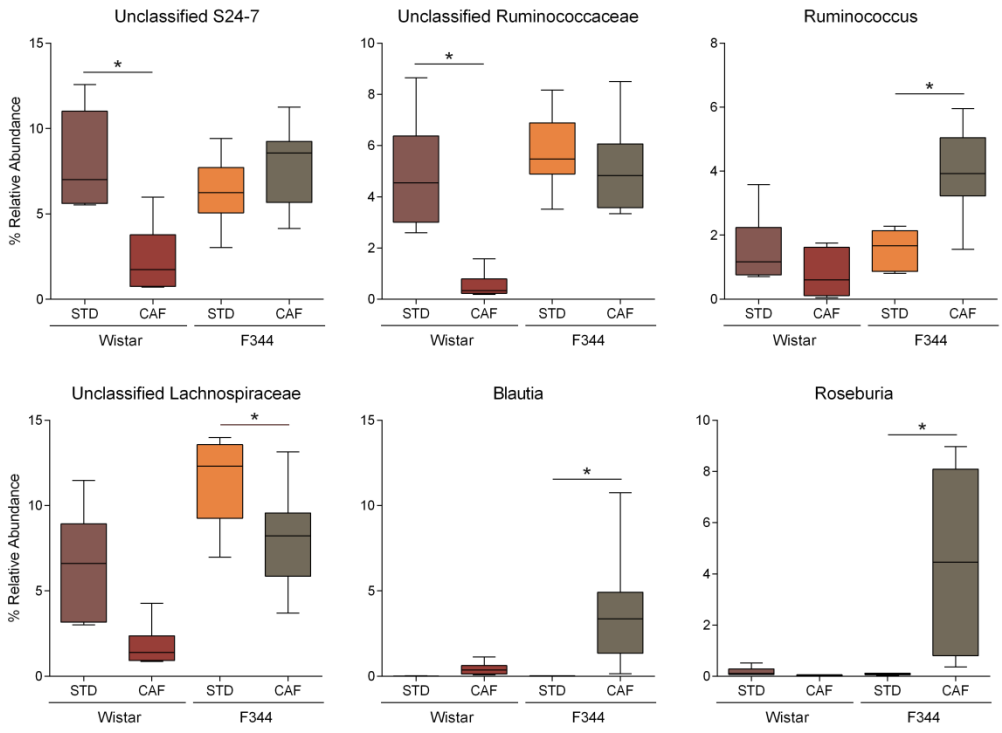


Figure 5. Relative abundance of the main genera distinctively influenced by the cafeteria (CAF) diet within Wistar or F344 rat strains. *Significant differences assessed through Welch's t-test after Benjamini-Holchberg FDR correction considering corrected p-values < 0.05.

Only F344 rats showed an increased occurrence of unclassified Lachnospiraceae (p = 0.049), *Roseburia* (p = 0.048), *Desulfovibrio* (p = 0.046), *Blautia* (p = 0.043), *Anaerostipes* (p = 0.041), *Ruminococcus* (p = 0.025), *Bilophila* (p = 0.025), *Allobaculum* (p = 0.009) and rc4-4 (p = 0.002) after the CAF consumption, while *Odoribacter* (p = 0.018) was decreased. Interestingly, Lachnospiraceae-related microbes such as *Roseburia*, *Blautia*, *Dorea* and *Coprococcus* were associated with an increased risk of obesity; individuals showing high occurrence of these genera had elevated BMI, waist circumference and blood pressure [69]. Recently, a clear association has been identified between *Blautia*, *Dorea*

and *Ruminococcus* and obesity-related plasma metabolites in humans [70], pointing to the potential role of these Lachnospiraceae-belonging genera in obesity development. In this sense, *Blautia* and *Ruminococcus* have been found to be associated with metabolic syndrome and weight gain in humans [71]. Alternatively, a negative correlation between body weight gain and *Blautia* abundance was reported in mice [72], whereas opposite results were later published [73]. Therefore, the literature concerning *Blautia* and obesity or metabolic syndrome is equivocal to date and conclusions must be carefully discussed.

Roseburia spp. are butyrate-producer bacteria considered an essential member for a beneficial flora, even proposed to act as health markers [74]. Our results showed an exacerbated CAF diet-induced increase of *Roseburia* exclusively in F344 rats, which seems to be discordant with the reduced *Roseburia* abundance in obese humans and diet-induced obese mice [75,76]. Interestingly, *Roseburia* was repeatedly found raised in high-fat diet-fed Sprague-Dawley rats [77,78]. Altogether, it is suggested that genotype is a determinant factors on the occurrence of *Roseburia* in diet-induced obesity. Ultimately, we hypothesize that the lower decrease of fiber intake during the CAF feeding in F344 compared to Wistar rats could be favouring a strain-specific upregulation of *Roseburia* as a microbiota-derived response to CAF diet.

Principal component analysis (PCA) revealed that CAF distinctively influenced the cecal microbiota composition at the genus level according to strain and dietary group. 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 (F344 and Wistar rats were prominently located at the right and at the left,

respectively) and diet (STD and CAF diets were positioned at the top and at the bottom, respectively), as presented in **Figure 6**.

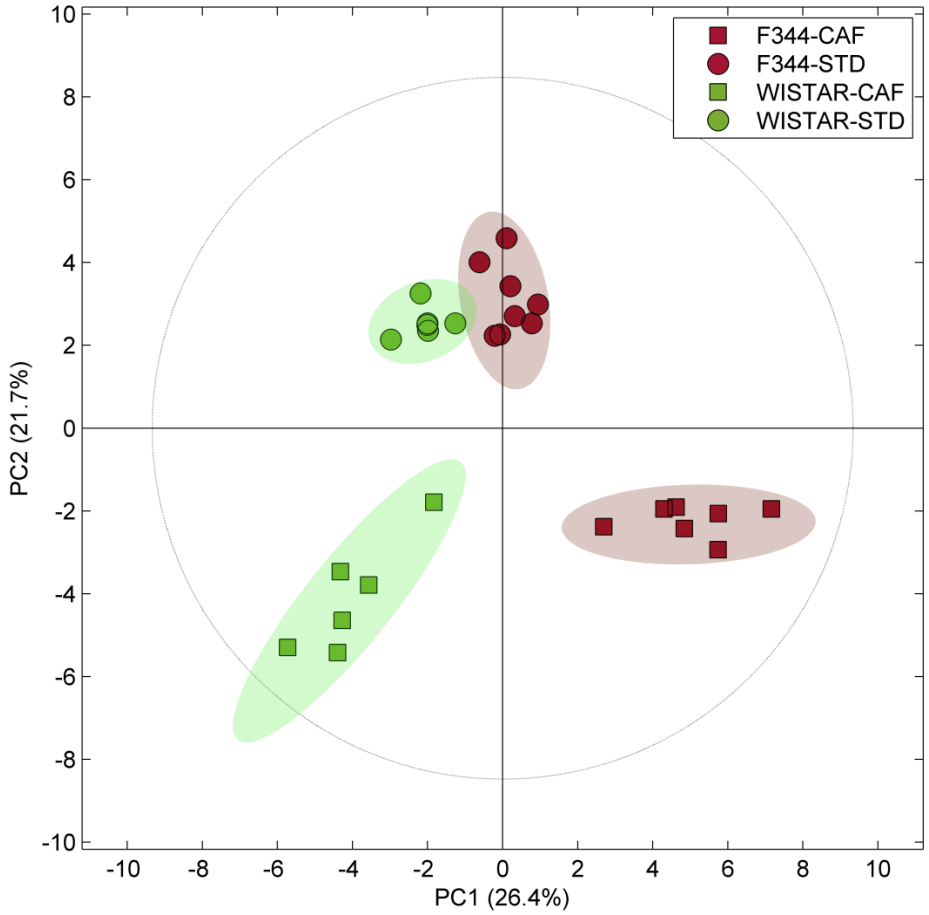


Figure 6. Principal component analysis constructed from the relative abundance of genera representing more than 1% of bacteria at least in two samples. Circular and squared dots represent standard (STD) and cafeteria (CAF) diet-fed animals, respectively. Different colours discriminate between Wistar (green) and F344 (red) rat strains. PC, principal component.

3.7 Gender also influences the CAF diet-driven microbiome perturbations.

Dietary modulation of cecal microbiome was also influenced by gender within F344 rats, as revealed by two-way ANOSIM analysis

(Gender: R = 0.273, p = 0.003; Diet: R = 0.758, p < 0.001). Remarkably, CAF diet led to an increased proportion of S24-7 (p = 0.005), as well as caused a more dramatic drop of unclassified Clostridiales (p < 0.001), in female rats. In line with the CAF-induced microbial variations observed in male F344 rats, females also showed raised portions of *Blautia* (p = 0.049), *Parabacteroides* (p = 0.033), *Phascolarctobacterium* (p = 0.023) and *rc4-4* (p = 0.024) after the chronic consumption of a CAF diet. However, previously described changes on *Oscillospira* and *Ruminococcus* occurrences in male rats were not identified in females, as presented in **Figure 7**. Interestingly, the depletion of *Oscillospira* abundance in CAF diet-fed males, a genus linked to lower BMI [60], altogether with the increment of unclassified S24-7 in females, described as a relevant genus to human health [66], suggests a healthier microbial phenotype in female than in male F344 rats. In addition, the positive correlation between *Ruminococcus* genera and circulating lipid levels reported in humans [79,80], was also found in our study (Pearson's correlation coefficient, r(31) = 0.378, p = 0.036). Therefore, we showed that an increased serum TAG concentrations and *Ruminococcus* abundance co-occurred in male, but not in female, rats after a chronic CAF feeding, thereby partially explaining the sex-dependent differences on metabolic profiles. However, caution must be taken when comparing the microbial signatures of obesity between genders and species due to the multi-factorial influences on the host microbiota co-metabolism [81]. Overall, while an elevated Bacteroidetes-to-Firmicutes ratio and a drop on bacterial diversity in F344 females indicated a marked microbial dysbiosis, the analysis of the occurrences of specific genera suggested a less detrimental response to the cafeteria feeding when compared to F344 males.

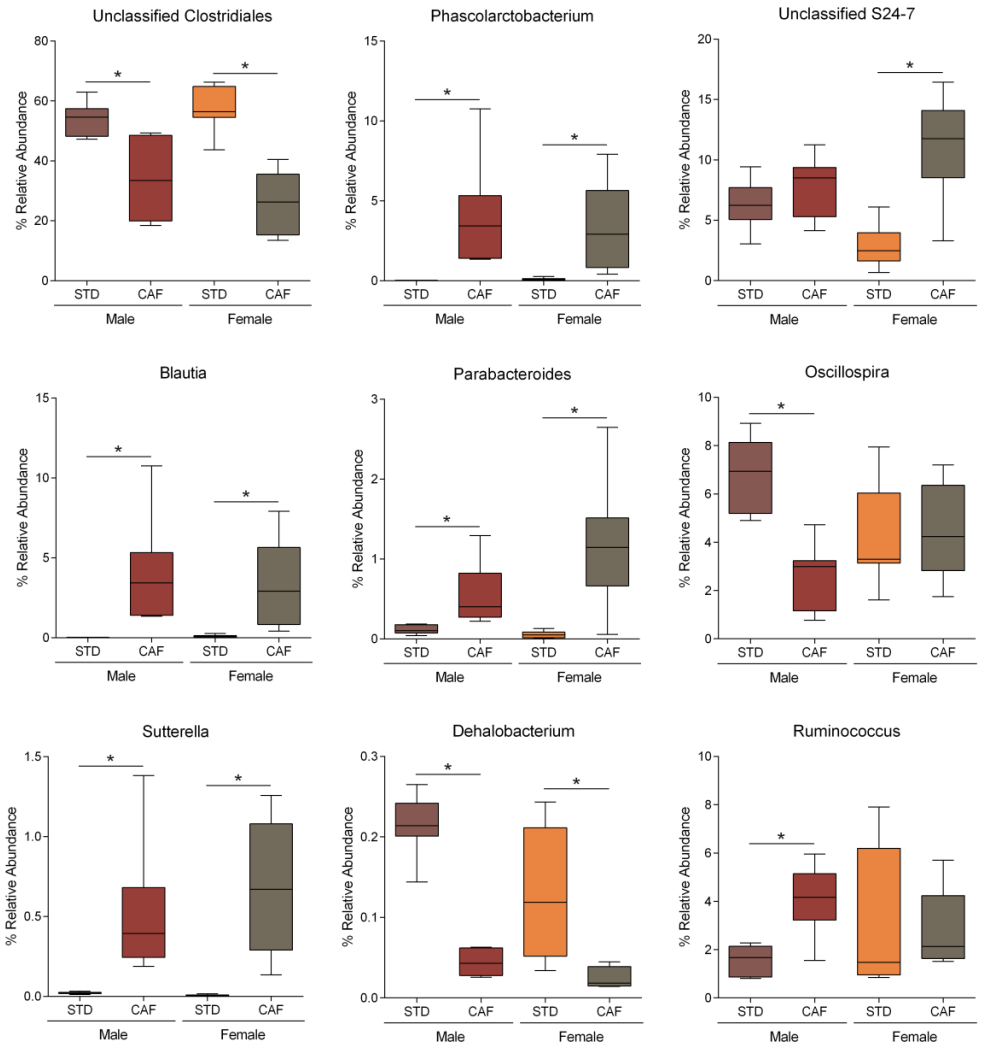


Figure 7. Relative abundance of the main genera influenced by the cafeteria (CAF) diet within male or female F344 rats. *Significant differences were assessed through Welch's t-test after Benjamini-Holchberg FDR correction considering corrected p-values < 0.05.

Focusing on gender-dependent dietary effects, PCA revealed that genera were mainly influenced by diet, also showing marginal clustering according to gender (**Figure 8**). 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 along the axis, even though males showed a more homogeneous clustering than females in both cases. In accordance with our data, it has been previously shown that the dietary effects on the composition of gut microbiota are dependent on sex-specific interactions in mice [82].

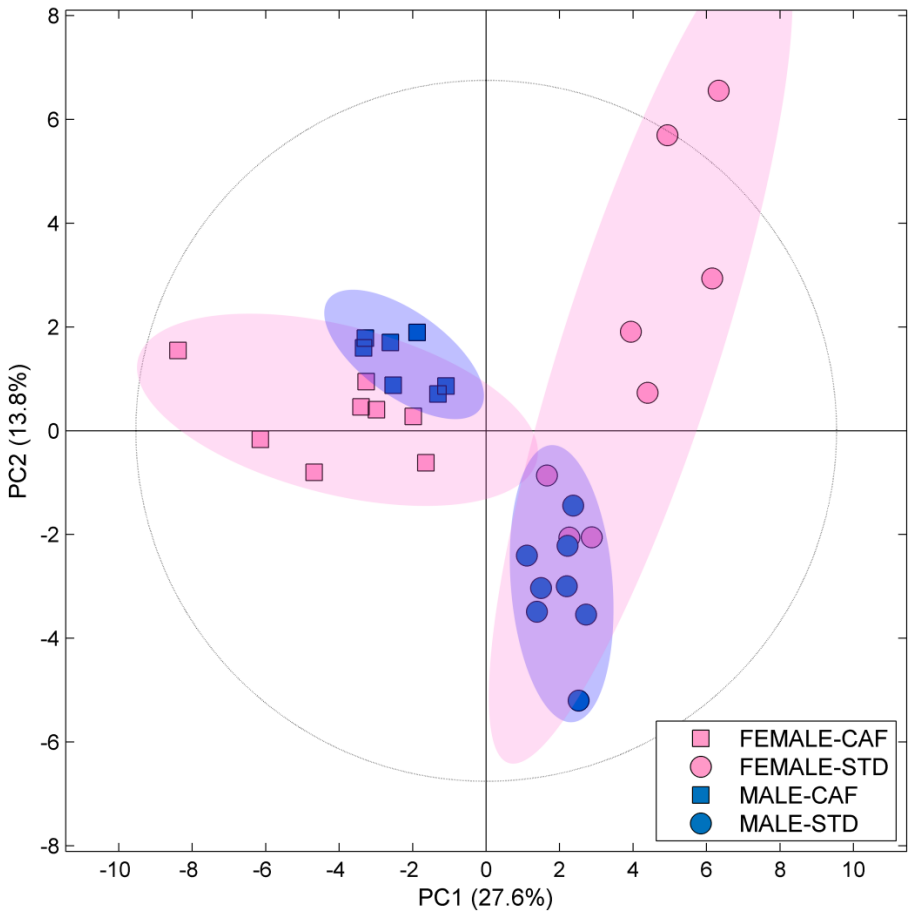


Figure 8. Principal component analysis constructed from the relative abundance of genera representing more than 1% of bacteria at least in two samples. Circular and squared dots represent standard (STD) and cafeteria (CAF) diet-fed animals, respectively. Different colours discriminate between male (blue) and female (pink) F344 rats. PC, principal component.

CONCLUDING REMARKS

In the present study, we observed a significant contributory role of both intrinsic (strain and gender) and external (diet) factors to the gut microbiota composition in rats. The influence of nutritional interventions on gut microbiota has been widely explored previously, showing that changes on dietary habits promote rapid shifts in the microbial populations in the gut, thus postulating diet as the main factor determining the phylogenetic structure of the microbiome [83]. Nevertheless, distinct host genetics may have a crucial impact on gut microbiota response to 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 [5,84]. Interestingly, recent studies showed that host genetic distance is a strong predictor of the gut microbiota composition between different populations of wild mice [85], and that microbiome returned back to the former composition when mice were separated from their parental lines after co-housing [86]. In fact, differences in gut microbiota may result in divergent metabolic responses to dietary changes, thus distinctively predisposing the animals to pathological states such as obesity.

In our study, Wistar rats showed a more pronounced CAF diet-induced gut microbiota dysbiosis than F344 animals, despite their lower total energy intake. This could be explained by their lower chow intake, which is the main source of protein and fiber. For instance, we revealed a strong increase in the prevalence of obesity-related taxa such as *Bacteroides* in Wistar animals. Altogether, strain-specific preferences to the ingredients included in the CAF, a free choice-based dietary model,

are crucial to explain the differences on food intake, gut microbiota dysbiosis and host physiology according to rat strain. Therefore, diet could be acting as a confounder factor in our study by masking the strain influence on gut microbial changes, which should be assessed along with a similar food intake.

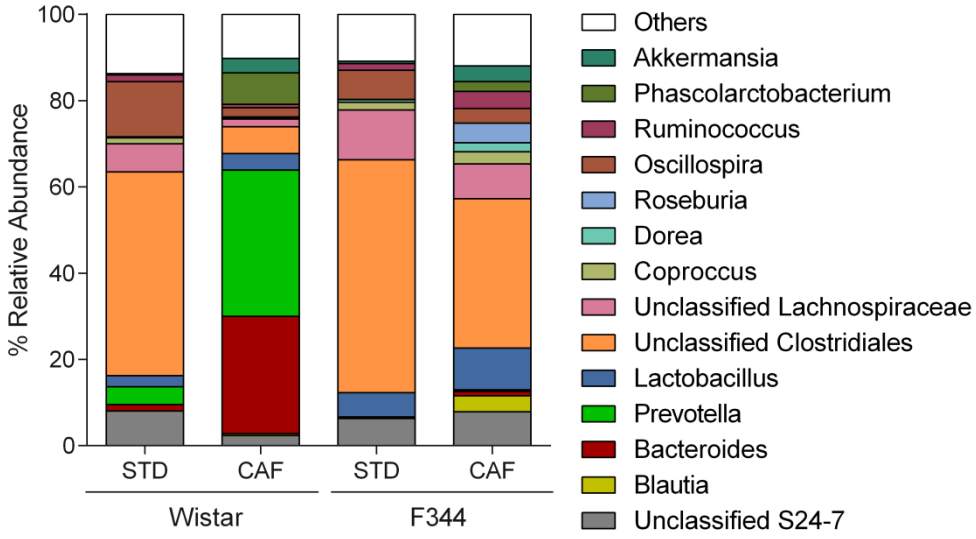
Diet-induced microbial changes were markedly observed in both male and female F344 rats. However, CAF diet more pronouncedly impacted the gut microbiome in female animals, as revealed by the altered microbial diversity and Bacteroidetes-to-Firmicutes only in females fed with a CAF diet. Importantly, CAF diet similarly increased food intake in both males and females, thus indicating that the gender-dependent alterations of gut microbiota induced by the CAF diet were not explained by changes on food intake. Alternatively, the effects of the CAF diet might be conditioned by the complex interaction between estrogens, bile acids and gut microbiota. In this sense, gut microbiota is known to affect the systemic levels, potency and activity of estrogen metabolites by facilitating its reabsorption [87], whereas estrogens have been shown to exert a control upon the bile acid metabolism and their biological effects [88,89]. In this regard, bile acids also contribute to sexual dimorphism of gut microbiota in a farnesoid-X-receptor-dependent manner [90]. Hence, the CAF diet-induced microbial dysbiosis in females is suggested to strongly disrupt the hormonal control exerted by estrogens upon bile acid metabolism, which, in turn, may contribute to alter the microbial ecosystem.

Despite that CAF diet-induced microbial alterations at genus taxonomic level seemed to be less detrimental in female than in males, females showed a higher increase of RWAT weight and a greater decrease of

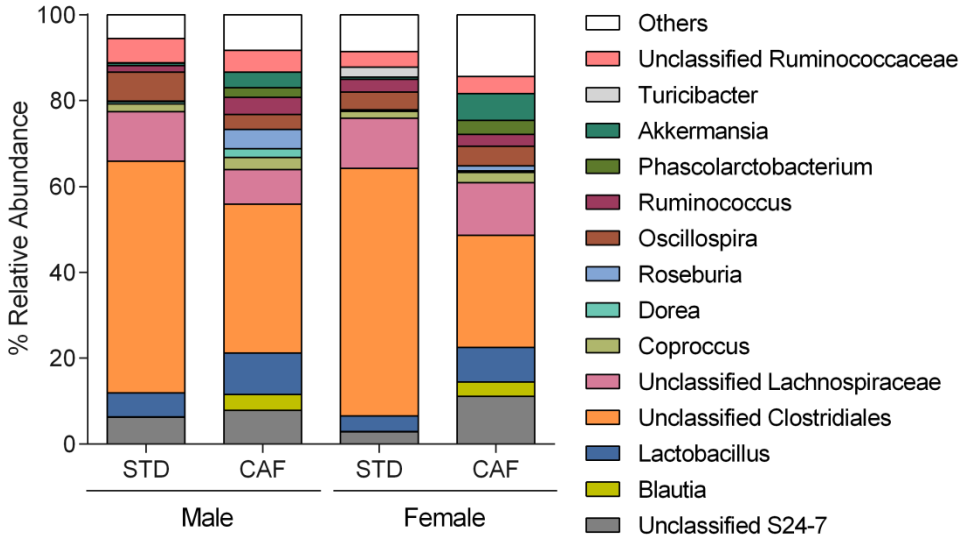
body lean mass than males, which was consistent with alterations on microbial diversity and Bacteroidetes-to-Firmicutes ratio. Moreover, males developed an unhealthier metabolic profile than females, which was correlated with an increased *Ruminococcus* occurrence. Overall, diet-induced shapes on gut microbiota composition underlie the gender influence in F344 rats, where estrogens play an important role in the maintenance of bile acid metabolism and gut microbiota.

In conclusion, our results incorporate non-previously available information about the intrinsic differences of microbial populations between Wistar and F344 rats, as well as examine the strain and sex-dependent modulatory role of the CAF diet. Here, we demonstrated that CAF, a Western-like energy-dense and highly palatable diet, distinctively altered the gut microbiome according to rat strain, although these strain-specific alterations could be influenced by environmental factors such as food intake. Therefore, the evaluation of a dietary intervention on the gut microbiome and host response should not to be directly extrapolated among different rat strains because of the described bias related to intrinsic or external factors. Furthermore, CAF diet also influences the microbiota composition in a gender-dependent manner, whereas diet is the factor that preferably, but not uniquely, defines the resulting phylogenetic profile. In conclusion, our hypotheses have been confirmed, showing that robust microbiota perturbations promoted by the CAF diet depend on rat strain and gender factors.

SUPPLEMENTARY INFORMATION



Supplementary Figure 1. Relative abundance of the most representative genera in male Wistar and F344 rats fed with a standard (STD) or a cafeteria (CAF) diet.



Supplementary Figure 2. Relative abundance of the most representative genera in F344 rats fed with a standard (STD) or a cafeteria (CAF) diet.

REFERENCES

[1] Moschen AR, Wieser V, Tilg H. Dietary factors: Major regulators of the Gut's microbiota. Gut Liver 2012;6:411–6.

[2] Elderman M, Hugenholtz F, Belzer C, Boekschoten M, van Beek A, de Haan B, et al. Sex and strain dependent differences in mucosal immunology and microbiota composition in mice. Biol Sex Differ 2018;9:26.

[3] Santos-Marcos JA, Haro C, Vega-Rojas A, Alcala-Diaz JF, Molina-Abril H, Leon-Acuña A, et al. Sex Differences in the Gut Microbiota as Potential Determinants of Gender Predisposition to Disease. Mol Nutr Food Res 2019;63.

[4] Brooks AW, Priya S, Blekhman R, Bordenstein SR. Gut microbiota diversity across ethnicities in the United States. PLoS Biol 2018;16:e2006842.

[5] Marques C, Meireles M, Faria A, Leite J, Freitas J, Norberto S, et al. High-fat diet-induced obesity Rat model: a comparison between Wistar and Sprague-Dawley Rat. Adipocyte 2015;5:11–21.

[6] Hildebrandt MA, Hoffmann C, Sherrill-Mix SA, Keilbaugh SA, Hamady M, Chen Y-Y, et al. High-fat diet determines the composition of the murine gut microbiome independently of obesity. Gastroenterology 2009;137:1716-24.e1-2.

[7] Ren C, Sylvia K. Sexual Dimorphism in the Gut Microbiome. IU J Undergrad Res 2018;4:12–6.

[8] Kaliannan K, Robertson RC, Murphy K, Stanton C, Kang C, Wang B, et al. Estrogen-mediated gut microbiome alterations influence sexual dimorphism in metabolic syndrome in mice. Microbiome 2018;6:205.

[9] Gual-Grau A, Guirro M, Mayneris-Perxachs J, Arola L, Boqué N. Impact of different hypercaloric diets on obesity features in rats: a metagenomics and metabolomics integrative approach. J Nutr Biochem 2019;71:122–31.

[10] Sampey BP, Vanhoose AM, Winfield HM, Freemerman AJ, Muehlbauer MJ, Fueger PT, et al. Cafeteria diet is a robust model of human metabolic syndrome with liver and adipose inflammation: Comparison to high-fat diet. Obesity 2011;19:1109–17.

[11] Levy JR, Lesko J, Krieg RJ, Adler RA, Stevens W. Leptin responses to glucose infusions in obesity-prone rats. Am J Physiol Metab 2000;279:E1088-96.

[12] Abdoulaye D, Wetzler S, Goubern M, Helies JM, Fromentin G, Tomé D, et al. Comparison of energy balance in two inbred strains of rats: Fischer F344 prone to obesity and Lou rats resistant to obesity. Physiol Behav 2006;87:245–50.

[13] Schroeder N, Marquart LF, Gallaher DD. The role of viscosity and fermentability of dietary fibers on satiety- and adiposity-related hormones in rats. Nutrients 2013;5:2093–113.

[14] Moen B, Henjum K, Måge I, Knutsen SH, Rud I, Hetland RB, et al. Effect of dietary fibers on cecal microbiota and intestinal tumorigenesis in azoxymethane treated A/J Min/+ mice. PLoS One 2016;11:e0155402.

[15] Speakman JR. Measuring energy metabolism in the mouse - theoretical, practical, and analytical considerations. Front Physiol 2013;4:34.

[16] Lalanza JF, Caimari A, Del Bas JM, Torregrosa D, Cigarroa I, Pallàs M, et al. Effects of a post-weaning cafeteria diet in young rats: Metabolic syndrome, reduced activity and low anxiety-like behaviour. PLoS One 2014;9:e85049.

[17] Morais Mewes J, Rodrigues Silva Gasparin F, Yoshida T, Amâncio Daniel da Silva M, Raquel Marçal Natali M, Francisco Veiga Bizerra P, et al. The Role of Mitochondria in Sex-Dependent Differences in Hepatic Steatosis and Oxidative Stress in Response to Cafeteria Diet-Induced Obesity in Mice. Nutrients 2019;11:1618.

[18] Hwang LL, Wang CH, Li TL, Chang SD, Lin LC, Chen CP, et al. Sex differences in high-fat diet-induced obesity, metabolic alterations and learning, and synaptic plasticity deficits in mice. Obesity 2010;18:463–9.

[19] Palmer BF, Clegg DJ. The sexual dimorphism of obesity. Mol Cell Endocrinol 2015:402, 113–9.

[20] Medrikova D, Jilkova ZM, Bardova K, Janovska P, Rossmeisl M, Kopecky J. Sex differences during the course of diet-induced obesity in mice: Adipose tissue expandability and glycemic control. Int J Obes 2012;36:262–72.

[21] Johnson JL, Slentz CA, Duscha BD, Samsa GP, McCartney JS, Houmard JA, et al. Gender and racial differences in lipoprotein subclass distributions: The STRRIDE study. Atherosclerosis 2004;176:371–7.

[22] Lemieux I, Pascot A, Lamarche B, Prud'homme D, Nadeau A, Bergeron J, et al. Is the gender difference in LDL size explained by the metabolic complications of visceral obesity? Eur J Clin Invest 2002;32:909–17.

[23] Olefsky J, Farquhar JW, Reaven GM. Sex difference in the kinetics of triglyceride metabolism in normal and hypertriglyceridaemic human subjects. Eur J Clin Invest 1974;4:121–7.

[24] Davis SC, Yadav JS, Barrow SD, Robertson BK. Gut microbiome diversity influenced more by the Westernized dietary regime than the body mass index as assessed using effect size statistic. Microbiologyopen 2017;6.

[25] Marques C, Meireles M, Norberto S, Leite J, Freitas J, Pestana D, et al. High-fat diet-induced obesity Rat model: a comparison between Wistar and Sprague-Dawley Rat. Adipocyte 2015;5:1–11.

[26] Fu J, Bonder MJ, Cenit MC, Tigchelaar EF, Maatman A, Dekens JAM, et al. The gut microbiome contributes to a substantial proportion of the variation in blood lipids. Circ Res 2015;117:817–24.

[27] Flores R, Shi J, Fuhrman B, Xu X, Veenstra TD, Gail MH, et al. Fecal microbial determinants of fecal and systemic estrogens and estrogen metabolites: A cross-sectional study. J Transl Med 2012;10:253.

[28] Sagae SC, Menezes EF, Bonfleur ML, Vanzela EC, Zacharias P, Lubaczeuski C, et al. Early onset of obesity induces reproductive deficits in female rats. Physiol Behav 2012;105:1104–11.

[29] Harada N, Hanaoka R, Horiuchi H, Kitakaze T, Mitani T, Inui H, et al. Castration influences intestinal microflora and induces abdominal obesity in high-fat diet-fed mice. Sci Rep 2016;10:23001.

[30] Acharya KD, Gao X, Bless EP, Chen J, Tetel MJ. Estradiol modulates gut microbiota in female ob/ob mice fed a high fat diet. BioRxiv 2019.

[31] Kong C, Gao R, Yan X, Huang L, Qin H. Probiotics improve gut microbiota dysbiosis in obese mice fed a high-fat or high-sucrose diet. Nutrition 2019;60:175–84.

[32] Turnbaugh PJ, Bäckhed F, Fulton L, Gordon JI. Diet-Induced Obesity Is Linked to Marked but Reversible Alterations in the Mouse Distal Gut Microbiome. Cell Host Microbe 2008;3:213–23.

[33] del Bas JM, Guirro M, Boqué N, Cereto A, Ras R, Crescenti A, et al. Alterations in gut microbiota associated with a cafeteria diet and the physiological consequences in the host. Int J Obes 2018;42:746–54.

[34] Mariné-Casadó R, Domenech-Coca C, del Bas JM, Bladé C, Arola L, Caimari A. Intake of an Obesogenic Cafeteria Diet Affects Body Weight, Feeding Behavior, and Glucose and Lipid Metabolism in a Photoperiod-Dependent Manner in F344 Rats. Front Physiol 2018;9:1639.

[35] Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, et al. A core gut microbiome in obese and lean twins. Nature 2009;457:480–4.

[36] Ferrer M, Ruiz A, Lanza F, Haange SB, Oberbach A, Till H, et al. Microbiota from the distal guts of lean and obese adolescents exhibit partial functional redundancy besides clear differences in community structure. Environ Microbiol 2013;15:211–26.

[37] Bervoets L, Van Hoorenbeeck K, Kortleven I, Van Noten C, Hens N, Vael C, et al. Differences in gut microbiota composition between obese and lean children: A cross-sectional study. Gut Pathog 2013;5:10.

[38] Sivamaruthi BS, Kesika P, Suganthy N, Chaiyasut C. A Review on Role of Microbiome in Obesity and Antiobesity Properties of Probiotic Supplements. Biomed Res Int 2019;11:e7940.

[39] Walters WA, Xu Z, Knight R. Meta-analyses of human gut microbes associated with obesity and IBD. FEBS Lett 2014;588:4223–33.

[40] Lecomte V, Kaakoush NO, Maloney CA, Raipuria M, Huinao KD, Mitchell HM, et al. Changes in gut microbiota in rats fed a high fat diet correlate with obesity-associated metabolic parameters. PLoS One 2015;10:e0126931.

[41] Kaakoush NO, Martire SI, Raipuria M, Mitchell HM, Nielsen S, Westbrook RF, et al. Alternating or continuous exposure to cafeteria diet leads to similar shifts in gut microbiota compared to chow diet. Mol Nutr Food Res 2017;61.

[42] Lee SM, Kim N, Yoon H, Nam RH, Lee DH. Microbial changes and host response in F344 rat colon depending on sex and age following a high-fat diet. Front Microbiol 2018;9.

[43] Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, et al. A core gut microbiome in obese and lean twins. Nature 2009;457:480–4.

[44] Flint HJ, Bayer EA, Rincon MT, Lamed R, White BA. Polysaccharide utilization by gut bacteria: Potential for new insights from genomic analysis. Nat Rev Microbiol 2008;6:121–31.

[45] Koropatkin NM, Cameron EA, Martens EC. How glycan metabolism shapes the human gut microbiota. Nat Rev Microbiol 2012;10:323–35.

[46] Lee SY, Sekhon SS, Ko JH, Kim HC, Kim SY, Won K, et al. Lactic acid bacteria isolated from kimchi to evaluate anti-obesity effect in high fat diet-induced obese mice. Toxicol Environ Health Sci 2018;10:11–6.

[47] Delzenne NM, Neyrinck AM, Bäckhed F, Cani PD. Targeting gut microbiota in obesity: Effects of prebiotics and probiotics. Nat Rev Endocrinol 2011;7:639–46.

[48] Qiao Y, Sun J, Xia S, Li L, Li Y, Wang P, et al. Effects of different Lactobacillus reuteri on inflammatory and fat storage in high-fat diet-induced obesity mice model. J Funct Foods 2015;14:424–34.

[49] Santacruz A, Marcos A, Wärnberg J, Martí A, Martin-Matillas M, Campoy C, et al. Interplay between weight loss and gut microbiota composition in overweight adolescents. Obesity 2009;17:1906–15.

[50] Ppatil D, Pdhotre D, Gchavan S, Sultan A, Jain DS, Lanjekar VB, et al. Molecular analysis of gut microbiota in obesity among Indian individuals. J Biosci 2012;37:647–57.

[51] Schwiertz A, Taras D, Schäfer K, Beijer S, Bos NA, Donus C, et al. Microbiota and SCFA in lean and overweight healthy subjects. Obesity 2010;18:190–5.

[52] Wu GD, Chen J, Hoffmann C, Bittinger K, Chen YY, Keilbaugh SA, et al. Linking long-term dietary patterns with gut microbial enterotypes. Science (80-) 2011;334:105–8.

[53] Gérard P, Lepercq P, Leclerc M, Gavini F, Raibaud P, Juste C. Bacteroides sp. strain D8, the first cholesterol-reducing bacterium isolated from human feces. Appl Environ Microbiol 2007;73:5742–9.

[54] Sekimoto H, Shimada O, Makanishi M, Nakano T, Katayama O. Interrelationship between serum and fecal sterols. Jpn J Med 1983;22:14–20.

[55] Kang JY, Kim HN, Chang Y, Yun Y, Ryu S, Shin H, et al. Gut microbiota and physiologic bowel 18F-FDG uptake. EJNMMI Res 2017;7:72.

[56] Verdam FJ, Fuentes S, De Jonge C, Zoetendal EG, Erbil R, Greve JW, et al. Human intestinal microbiota composition is associated with local and systemic inflammation in obesity. Obesity 2013;21.

[57] Navarro G, Sharma A, Dugas LR, Forrester T, Gilbert JA, Layden BT. Gut microbial features can predict host phenotype response to protein deficiency. Physiol Rep 2018;6:e13932.

[58] Mackie RI, Aminov RI, Hu W, Klieve A V., Ouwerkerk D, Sundset MA, et al. Ecology of Uncultivated Oscillospira Species in the Rumen of Cattle, Sheep, and Reindeer as Assessed by Microscopy and Molecular Approaches. Appl Environ Microbiol 2003;69:6808–15.

[59] Tims S, Derom C, Jonkers DM, Vlietinck R, Saris WH, Kleerebezem M, et al. Microbiota conservation and BMI signatures in adult monozygotic twins. ISME J 2013;7:707–17.

[60] Goodrich JK, Waters JL, Poole AC, Sutter JL, Koren O, Blekhman R, et al. Human genetics shape the gut microbiome. Cell 2014;159:789–99.

[61] Zhong Y, Marungruang N, Fåk F, Nyman M. Effects of two whole-grain barley varieties on caecal SCFA, gut microbiota and plasma inflammatory markers in rats consuming low- and high-fat diets. Br J Nutr 2015;113:1558–70.

[62] Zhou K. Strategies to promote abundance of Akkermansia muciniphila, an emerging probiotics in the gut, evidence from dietary intervention studies. J Funct Foods 2017;33:194–201.

[63] Zhang T, Li Q, Cheng L, Buch H, Zhang F. Akkermansia muciniphila is a promising probiotic. Microb Biotechnol 2019.

[64] Derrien M, Belzer C, de Vos WM. Akkermansia muciniphila and its role in regulating host functions. Microb Pathog 2017;106:171–81.

[65] Seedorf H, Griffin NW, Ridaura VK, Reyes A, Cheng J, Rey FE, et al. Bacteria from diverse habitats colonize and compete in the mouse gut. Cell 2014;159:253–66.

[66] Nielsen LK, Daly JN, Morrison M, Wood DLA, Lachner N, Cooper MA, et al. Genomic characterization of the uncultured Bacteroidales family S24-7 inhabiting the guts of homeothermic animals. Microbiome 2016;4:36.

[67] Sinani H, Schloss PD, Koropatkin NM, Lesniak NA, Baxter NT. The Glucoamylase Inhibitor Acarbose Has a Diet-Dependent and Reversible Effect on the Murine Gut Microbiome. MSphere 2019;4:1–12.

[68] Daniel H, Gholami AM, Berry D, Desmarchelier C, Hahne H, Loh G, et al. High-fat diet alters gut microbiota physiology in mice. ISME J 2014;8:295–308.

[69] de la Cuesta-Zuluaga J, Corrales-Agudelo V, Velásquez-Mejía EP, Carmona JA, Abad JM, Escobar JS. Gut microbiota is associated with obesity and cardiometabolic disease in a population in the midst of Westernization. Sci Rep 2018;8:11356.

[70] Ottosson F, Brunkwall L, Ericson U, Nilsson PM, Almgren P, Fernandez C, et al. Connection between BMI-Related Plasma Metabolite Profile and Gut Microbiota. J Clin Endocrinol Metab 2018;103:1491–501.

[71] Lippert K, Kedenko L, Antonielli L, Kedenko I, Gemeier C, Leitner M, et al. Gut microbiota dysbiosis associated with glucose metabolism disorders and the metabolic syndrome in older adults. Benef Microbes 2017;8:545–56.

[72] Yang J, Bindels LB, Munoz RRS, Martínez I, Walter J, Ramer-Tait AE, et al. Disparate metabolic responses in mice fed a high-fat diet supplemented with maize-derived non-digestible feruloylated oligoand polysaccharides are linked to changes in the gut microbiota. PLoS One 2016;11:e146144.

[73] Danneskiold-Samsøe NB, Andersen D, Radulescu ID, Normann-Hansen A, Brejnrod A, Kragh M, et al. A safflower oil based high-fat/high-sucrose diet modulates the gut microbiota and liver phospholipid profiles associated with early glucose intolerance in the absence of tissue inflammation. Mol Nutr Food Res 2017;61.

[74] Tamanai-Shacoori Z, Smida I, Bousarghin L, Loreal O, Meuric V, Fong SB, et al. Roseburia spp.: A marker of health? Future Microbiol 2017;12:157–70.

[75] Aguirre M, De Souza CB, Venema K. The gut microbiota from lean and obese subjects contribute differently to the fermentation of arabinogalactan and inulin. PLoS One 2016;11.

[76] Neyrinck AM, Possemiers S, Verstraete W, De Backer F, Cani PD, Delzenne NM. Dietary modulation of clostridial cluster XIVa gut bacteria (Roseburia spp.) by chitin-glucan fiber improves host metabolic alterations induced by high-fat diet in mice. J Nutr Biochem 2012;23:51–9.

[77] Liu J-P, Zou W-L, Chen S-J, Wei H-Y, Yin Y-N, Zou Y-Y, et al. Effects of different diets on intestinal microbiota and nonalcoholic fatty liver disease development. World J Gastroenterol 2016;22:7353.

[78] Luo Q, Cheng D, Huang C, Li Y, Lao C, Xia Y, et al. Improvement of colonic immune function with soy isoflavones in high-fat diet-induced obese rats. Molecules 2019.

[79] Lahti L, Salonen A, Kekkonen RA, Salojärvi J, Jalanka-Tuovinen J, Palva A, et al. Associations between the human intestinal microbiota, Lactobacillus rhamnosus GG and serum lipids indicated by integrated analysis of high-throughput profiling data. PeerJ 2013;2013:e32.

[80] Vojinovic D, Radjabzadeh D, Kurilshikov A, Amin N, Wijmenga C, Franke L, et al. Relationship between gut microbiota and circulating metabolites in population-based cohorts. BioRxiv 2019:617431.

[81] Franklin CL, Ericsson AC. Microbiota and reproducibility of rodent models. Lab Anim (NY) 2017;46:114–22.

[82] Org E, Mehrabian M, Parks BW, Shipkova P, Liu X, Drake TA, et al. Sex differences and hormonal effects on gut microbiota composition in mice. Gut Microbes 2016;7:313–22.

[83] David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, et al. Diet rapidly and reproducibly alters the human gut microbiome. Nature 2014;505:559–63.

[84] Ericsson AC, Akter S, Hanson MM, Busi SB, Parker TW, Schehr RJ, et al. Differential susceptibility to colorectal cancer due to naturally occurring gut microbiota. Oncotarget 2015;6:33689–704.

[85] Suzuki TA, Phifer-Rixey M, Mack KL, Sheehan MJ, Lin D, Bi K, et al. Host genetic determinants of the gut microbiota of wild mice. Mol Ecol 2019;28:3197–207.

[86] Korach-Rechtman H, Freilich S, Gerassy-Vainberg S, Buhnik-Rosenblau K, Danin-Poleg Y, Bar H, et al. Murine Genetic Background Has a Stronger Impact on the Composition of the Gut Microbiota than Maternal Inoculation or Exposure to Unlike Exogenous Microbiota. Appl Environ Microbiol 2019;85.

[87] Cross TWL, Kasahara K, Rey FE. Sexual dimorphism of cardiometabolic dysfunction: Gut microbiome in the play? Mol Metab 2018;15:70–81.

[88] Chico Y, Fresnedo O, Botham K, Lacort M, Ochoa B. Regulation of bile acid synthesis by estradiol and progesterone in primary cultures of rat hepatocytes. Exp Clin Endocrinol Diabetes 1996;104:137–44.

[89] De Bari O, Wang TY, Liu M, Portincasa P, Wang DQH. Estrogen induces two distinct cholesterol crystallization pathways by activating ERα and GPR30 in female mice. J Lipid Res 2015;56:1691–700.

[90] Sheng L, Jena PK, Liu HX, Kalanetra KM, Gonzalez FJ, French SW, et al. Gender Differences in Bile Acids and Microbiota in Relationship with Gender Dissimilarity in Steatosis Induced by Diet and FXR Inactivation. Sci Rep 2017;7:1748.

CHAPTER III

Disruption of Microbial Rhythmicity in Rats Fed with a Cafeteria Diet and Time-Dependent Effect of Grape Seed Proanthocyanidins Extract (GSPE) Supplementation.

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In preparation

UNIVERSITAT ROVIRA I VIRGILI
GUT MICROBIOTA DYSBIOSIS IN DIET-INDUCED OBESITY. A FOCUS ON THE INFLUENCE OF GENETICS, CIRCADIAN RHYTHMS AND POTENTIAL PREBIOTICS.
Andreu Gual Grau

ABSTRACT

The synchronization between host circadian rhythms and gut microbial ecosystem is essential for maintaining a mutually beneficial cross-talk and host homeostasis. In a healthy state, the abundance of gut microbes oscillates throughout the course of the day, thus optimizing their metabolic role. Since nutrient type and availability regulates the clock machinery, we hypothesized that cafeteria (CAF) diet strongly disrupts the microbial rhythms, thereby contributing to obesity development. In addition, some beneficial effects of proanthocyanidins on obesity are meditated by their interaction with circadian rhythms and gut microbiota. Consequently, we hypothesized that the supplementation with a grape seed proanthocyanidins extract (GSPE) restores the microbial rhythmicity under a CAF feeding. To address these hypotheses, the cecal microbiota composition was assessed during both light/resting (ZT3) and dark/active (ZT15) phases in F344 rats fed with standard (STD) or CAF diets. Indeed, CAF diet-fed groups were diurnally or nocturnally supplemented with vehicle or GSPE and gut microbiota was characterized at ZT3 or ZT15, respectively. In this study, we revealed circadian variations in the gut microbial populations in STD diet-fed rats, which were mainly characterized by an oscillatory abundance of *Lactobacillus*. Importantly, CAF diet induced-obesity was linked to a strong disruption of microbial rhythmicity, since no differences on the occurrence of microbial genera were detected between both resting and active phases. Despite that GSPE supplementation was not able to restore microbial rhythms at genus level; the rise of Bacteroidetes-to-Firmicutes ratio promoted by the CAF diet during the dark phase was effectively counteracted, thereby

revealing that GSPE was able to manage, at least in part, the time-dependent CAF diet-induced alterations in the gut microbiota. Overall, our results shed light on the possible role of GSPE in the regulation of microbial rhythms, thus postulating a new microbiome-based mechanism explaining its described beneficial effects on obesity.

Key words: Gut microbiota, cafeteria diet, circadian rhythms, active phase, resting phase, grape seed proanthocyanidins, diet-induced obesity.

1. INTRODUCTION

Animals and plants have developed complex mechanisms to adapt their metabolism, physiology and behaviour to the cyclical and continuous variations in the environment. The synchronization of biological processes with these external changes constitute the circadian rhythms, which confers a selective advantage to living organisms by optimizing their metabolic efficiency [1]. The chronic disruption of the circadian rhythms has been linked to the development of a wide range of diseases including diabetes and obesity [2].

Importantly, the gut microbiota also undergoes diurnal oscillations, which are mainly controlled by host's clock genes and feeding patterns, thus determining specific host-microbiota interactions according to the daily phase [3]. In turn, circadian fluctuations of the gut microbiota modulate the activity of peripheral clocks through the production of metabolites. In fact, obesity and related metabolic disorders have also been broadly associated with disturbances in the gut microbiota composition [4].

Drastic dietary changes in the time of consumption and the type of food may alter the clock machinery. For instance, the consumption of a high-fat diet induced changes in the relative abundance of cyclic bacteria in mice [5]. However, the impact of a cafeteria (CAF) diet on the microbiome rhythmicity has not been yet evaluated. Therefore, we hypothesized that the chronic consumption of a highly appetitive and energy-dense CAF diet entails the disruption of microbial rhythms, thus disrupting peripheral clocks and contributing to the acquisition of an obese phenotype.

The recovery of the microbiome functionality using prebiotics has been extensively evaluated in obese rodents and humans [6–8]. In this sense, several studies have revealed the prebiotic potential of polyphenols in metabolic and cardiovascular diseases in animal models [9–12]. Recent studies have also demonstrated that some polyphenols, mainly resveratrol and proanthocyanidins, interact with the circadian rhythms by normalizing the altered expression of clock genes occurring during obesity and cardiovascular complications [13,14]. For instance, the disturbances of hepatic clock genes exhibited by diet-induced obese rats were counteracted by a chronic administration of low doses of grape seed proanthocyanidins extract (GSPE) [15]. Therefore, the varied beneficial effects described for proanthocyanidins are suggested to underlie the regulation of both circadian rhythms and gut microbiota. Thus, we hypothesized that GSPE, which has been widely described as a valuable functional food by our research group [16–18], is able to restore the CAF diet-induced alterations on microbial rhythms.

To elucidate the first hypothesis, we aimed to characterize the microbiota composition of male F344 rats fed with a standard chow or a

CAF diet during both the light/resting and the dark/active phases. In this sense, differences in the microbiota composition between diurnal and nocturnal conditions were evaluated within each diet, whereas the time of day-dependent changes on the microbiome promoted by the CAF diet were investigated.

In order to address the second hypothesis, we aimed to evaluate the compositional differences of the gut microbiome from CAF diet-fed rats treated with vehicle or GSPE during either diurnal or nocturnal stages.

2. MATERIALS AND METHODS

2.1 Experimental procedures.

Forty-eight 7-weeks-old male Fischer 344 (F344) rats were supplied from Charles River Laboratories (Wilmington, Massachusetts, USA). Animals were housed in temperature controlled rooms (22ºC) under a 12:12-hour light-dark cycle. After one week of adaptation to the facilities with free access to food and water, the animals were randomly distributed into six groups (n = 8 per group) depending on the diet, treatment and daily phase. Diets employed in this study consisted of a standard chow diet (Teklad Global 18% Protein Rodent Diet 2018, Harlan, Barcelona, Spain) (STD) or a cafeteria diet composed of energy-dense and highly palatable foods including biscuits with pâté and cheese, bacon, pastries and sugared milk (220 g/l) (CAF). The treatment consisted of GSPE, which was kindly provided by Les Dérives Résiniques et Terpéniques (Dax, France), and orally administered at doses of 25 mg/kg of body weight every day during the last 4 weeks of the study. The composition of the GSPE has been previously characterized [19].

Those groups that did not receive the GSPE treatment were supplemented with a vehicle consisting of condensed milk diluted with water in a 1:1 proportion. Daily phase was evaluated depending on the Zeitgeber time (ZT), starting the light/resting phase at ZT0. The treatment was administered at ZT0 or ZT12 to the corresponding groups (ZT0: light or resting phase; ZT12: dark or active phase). Importantly, cecum samples for metagenomic analyses from ZT0 and ZT12 groups were obtained at ZT3 and ZT15, respectively, at the end of the study.

Therefore, animals were divided into six different groups: (1) STD diet-fed rats diurnally supplemented with vehicle and sacrificed during the resting/light phase (STD-V-ZT3); (2) STD diet-fed rats nocturnally supplemented with vehicle and sacrificed during the active/dark phase (STD-V-ZT15); (3) CAF diet-fed rats diurnally supplemented with vehicle and sacrificied during the resting/light phase (CAF-V-ZT3); (4) CAF diet-fed rats diurnally supplemented with GSPE and sacrificed during the resting/light phase (CAF-GSPE-ZT3); (5) CAF diet-fed rats nocturnally supplemented with vehicle and sacrificed during the active/dark phase (CAF-V-ZT15); and (6) CAF diet-fed rats nocturnally supplemented with GSPE and sacrificed during the active/dark phase (CAF-GSPE-ZT15). Animals supplemented with vehicle were used to evaluate the impact of the CAF diet depending on the daily phase. Furthermore, we investigated the daily phase-dependent effects of GSPE supplementation in CAF diet-fed rats. Food intake and body weight were recorded weekly during the whole experimental procedure. One week before the end of the study, body composition was assessed by nuclear magnetic resonance (NMR) using an EchoMRI-700™ device (Echo Medical Systems, L.L.C., Houston, USA). Serum samples were obtained by saphenous venepuncture and

processed by centrifugation (2,000 g for 15 min). At the end of the experimental procedure, 17-weeks-old rats were sacrificed by decapitation according to the ZT, as described above. Liver, white adipose tissue depots including mesenteric (MWAT), retroperitoneal (RWAT), inguinal (IWAT) and epididymal (EWAT), and cecum were collected, weighed and immediately frozen in liquid nitrogen. All the samples were stored at -80°C until further analyses.

Cecal microbiota was analyzed as previously described in the Materials and Methods section of the **Chapter I**. The assessment of biochemical and biometric parameters, as well as the statistical analysis of the results, were performed as described in Materials and Methods section of **Chapter II**.

3. RESULTS AND DISCUSSION

3.1 Cafeteria diet distinctively alters host physiology according to Zeitgeber time.

The comparison of food intake, biometric and biochemical parameters between ZT3 and ZT15 male F344 rats fed with STD or CAF diet is shown in **Table 1**. As expected, CAF diet promoted an increase in total energy intake regardless the daily phase as compared to STD diet-fed animals. In fact, the consumption of the CAF diet resulted in a higher energy intake from carbohydrates, proteins and lipids, while fiber intake was lower, which is in accordance with a reduction of the cecum weight when compared to STD controls. Moreover, CAF diet induced a drop in food efficiency independently of the daily phase. The CAF diet-induced alterations on food intake led to the development of an obese profile

within both ZT3 and ZT15 groups. In this sense, CAF diet promoted changes in all the biometric parameters defining the obese rat phenotype, including an increment of body weight gain and fat mass, adiposity index and visceral fat percentage, accompanied by a reduction on body lean mass.

Surprisingly, differences according to daily phase (light or dark conditions) were identified in visceral fat, adiposity index, and percentages of RWAT and IWAT, being lower in ZT3 (resting phase) compared to Z15 (active phase) independently of the diet. In accordance, the circadian organization of the metabolic processes is optimized throughout the day by increasing food intake and energy harvest during the active phase (ZT15) and mobilizing fat stores in order to sustain basal metabolic activity during the resting phase (ZT3) [20]. In this regard, leptin is one of the major regulators of fat deposition and utilization in adipose tissue, acting as an appetite inhibitor and energy expenditure promoter [21], and positively correlating with the amount of stored body fat in both humans and rodents [22,23]. Leptin levels, altogether with lipid storage, exhibits striking circadian patterns in both gene expression and protein secretion, which is retained even in altered metabolic states such as obesity [24,25]. Leptin achrophase (maximum expression) occurs during the night phase in humans, which might be associated to a lower degree of fat accumulation and higher mobilization during the sleep phase [26]. Therefore, the lower adiposity during the resting phase reported in our study, which occurred independently of the diet, could be explained by an increased leptin expression and fat utilization. Alternatively, the major food intake occurring during the dark

phase may contribute to the increased fat deposition observed in this period.

However, no differences on body fat and lean mass percentages were observed depending on the daily phase. In this sense, visceral fat, adiposity index and percentages of white adipose tissues were measured at the corresponding daily phase when rats were sacrificed, while body composition was assessed at the same moment of the day for every group, thus normalizing the obesogenic effect of the CAF feeding regardless the daily phase. On the other hand, an increased body weight gain was reported during the light phase (ZT3) regardless of the diet, which was due to the inclusion of a couple of low body weight animals in the CAF diet-fed ZT3 group that finally reached a similar final body weight that the rest of rats in this group.

In addition, the diminution of lean-to-fat ratio promoted by the CAF diet was more pronounced during the resting (ZT3) phase than during the active (ZT15) phase. These results are mostly explained by a high lean-to-fat ratio in STD diet-fed rats supplemented with vehicle during the resting phase, whereas CAF diet-fed rats showed similar values. Thus, we suggest that vehicle (condensed milk:water) delivery during the resting phase promotes the energy storage in fat deposits, thus counteracting the leptin-mediated energy expenditure during this period. In this regard, the consumption of rapidly absorbable carbohydrates contained in the condensed milk were previously related to an insulin-meditated suppression of fatty acids release from adipose tissue accompanied by an increment of fat storage [27]. Moreover, food intake and host's circadian rhythms are directly involved in managing the activity of the gut microbiome [28]. Therefore, it is hypothesized that perturbations of

circadian clocks by the CAF diet led to disturbances on the rhythmicity of gut microbiota, which could be indirectly implicated in the observed differences on host physiology between dark and light phases.

Concerning biochemical parameters, the CAF diet induced hypertriglyceridemia, hyperglycaemia and hypercholesterolemia independently of the daily period. Serum glucose levels were raised during the active phase as compared to ZT3 group regardless of the diet type, which is in accordance with the daily rhythmicity exhibited in fasting blood glucose in both mice and rats [29,30], showing a peak at the beginning of the dark phase [30]. In this sense, it was suggested that the hepatic intracellular clock may play a crucial role in the fluctuation of blood glucose because clock genes-deficient mice does not exhibit such rhythmicity [30]. Overall, the influence of diurnal and nocturnal conditions on the biometric and biochemical outcomes has been reported, whereas major indicators of hyperphagia and obesity induced by the CAF diet were observed regardless the daily phase.

Table 1. Food intake, biometric and serum parameters in male Fischer 344 rats during resting (ZT3) or active (ZT15) phases fed with a standard (STD) or a cafeteria (CAF) diet.

	ZT3		ZT15		Two-way ANOVA
	STD	CAF	STD	CAF	
Food intake (kcal/day)					
Total energy intake	60.6 ± 0.97	159 ± 3.55	60.6 ± 1.79	151 ± 5.53	D
Total carbohydrates intake	35.2 ± 0.56	92.8 ± 2.71	35.2 ± 1.04	87.9 ± 3.34	D
Simple carbohydrates intake	0.00 ± 0.00	52.7 ± 2.27	0.00 ± 0.00	46.6 ± 2.27	D
Lipid intake	10.9 ± 0.17	43.7 ± 2.33	10.9 ± 0.32	45.6 ± 2.00	D
Protein intake	14.6 ± 0.23	18.2 ± 0.52	14.5 ± 0.43	17.4 ± 0.42	D
Fiber intake (g/day)	2.88 ± 0.05	1.88 ± 0.06	2.87 ± 0.08	1.76 ± 0.03	D
Food efficiency	3.35 ± 0.08	2.24 ± 0.21	3.17 ± 0.19	2.00 ± 0.07	D
Biometric parameters					
Body weight gain (g)	128 ± 3.29	225 ± 17.1	121 ± 8.00	189 ± 4.16	t,D
Fat mass (%)	12.0 ± 0.51	24.2 ± 1.73	15.0 ± 0.58	23.5 ± 0.81	D
Lean mass (%)	78.3 ± 0.78	69.7 ± 1.67	78.2 ± 0.54	70.2 ± 0.91	D
Lean/fat ratio	6.60 ± 0.25	3.00 ± 0.34*	5.26 ± 0.24	3.03 ± 0.14*	t*D
Visceral fat (%)	6.24 ± 0.17	9.66 ± 0.43	7.19 ± 0.16	10.1 ± 0.24	t,D
Adiposity Index	7.63 ± 0.21	12.0 ± 0.63	9.14 ± 0.26	12.9 ± 0.37	t,D
MWAT (%)	1.51 ± 0.08	2.56 ± 0.13	1.83 ± 0.05	2.70 ± 0.19	D
RWAT (%)	2.26 ± 0.09	3.20 ± 0.16	2.53 ± 0,09	3.46 ± 0.14	t,D
EWAT (%)	2.43 ± 0.10	3.90 ± 0.24	2.65 ± 0.12	4,12 ± 0.09	D
IWAT (%)	1.34 ± 0.08	2.33 ± 0.26	1.95 ± 0.18	2.79 ± 0.38	t,D
Cecum weight (g)	5.58 ± 0.30	4.36 ± 0.21	5.28 ± 0.38	3.54 ± 0.34	D
Serum parameters					
Glucose (mg/dL)	150 ± 5.97	202 ± 10.9	159 ± 2.69	238 ± 13.6	t,D
Triglycerides (mg/dL)	134 ± 8.21	358 ± 24.3	144 ± 10.4	399 ± 23.6	D
Total cholesterol (mg/dL)	59.0 ± 3.82	94.8 ± 7.29	60.4 ± 3.18	100 ± 8.99	D

Male F344 rats (n = 8 per group) were fed with STD or CAF diet and sacrificed at ZT3 or ZT15. Data are expressed as the mean ± SEM. D, the effect of diet type; t, the effect of daily phase; txD, the interaction of daily phase and diet type (two-way ANOVA, $p< 0.05$). * The effect of diet within each ZT group (Student's t-test, $p < 0.05$). EWAT, epididymal white adipose tissue; IWAT, inguinal white adipose tissue; MWAT, mesenteric white adipose tissue; RWAT, retroperitoneal white adipose tissue.

On the other hand, we evaluated the effect of a chronic supplementation with dietary doses of GSPE in male F344 rats fed with a CAF diet. Here, we investigated the differences on food intake, biometric measurements and biochemical parameters according to the treatment with vehicle or GSPE during the light/resting or dark/active phases, as presented in **Table 2**.

Total energy intake, as well as carbohydrate and lipid consumption, was unaltered by the GSPE supplementation under a CAF feeding. However, the GSPE-treated rats showed a reduced protein and fiber intake with respect to the vehicle-treated groups in both daily phases, which could be due to a lower standard chow consumption (ZT3-VEH: 10.6 ± 0.37 g/day; ZT3-GSPE: 7.55 ± 0.28 g/day; ZT15-VEH: 8.58 ± 0.44 g/day; ZT15-GSPE: 7.65 ± 0.29 g/day). Even so, highly appetitive ingredients included in the CAF diet were similarly consumed (data not shown), finally showing no significant differences on total energy intake. However, GSPE supplementation tended to decrease energy intake during the resting phase (ZT3 groups) (Student's t-test, $p = 0.07$), which, in turn, was predominantly explained by a reduced chow intake. In this sense, Ibars *et al.* demonstrated that the chronic and diurnal administration of dietary doses of GSPE reduced the total caloric intake in rats fed with a CAF diet [31]. In addition, we also observed a lower cecum weight in GSPE-supplemented groups, which was in accordance with a reduced total fiber intake.

Moreover, the GSPE treatment consistently reduced the body weight gain induced by CAF diet regardless the daily phase. However, this body weight gain limiting effect of GSPE, which is supported by a previous study using pharmacological doses in CAF diet-fed rats [32], was not

accompanied by a significant reduction on adiposity; thus it could not be taken as a beneficial effect for obesity. Altogether, the lowering effects of GSPE supplementation on protein and fiber consumption, mainly due to a reduced chow intake, might explain the lower body weight gain, which was accompanied by a numerically lower lean-to-fat mass ratio as compared to vehicle-supplemented groups. For this reason, the differences observed in feeding behaviour might be masking the previously described beneficial properties of GSPE in our study.

Surprisingly, body weight gain and cecum weight were lower in the groups supplemented during the active phase. A possible explanation to this outcome may underlie the water:milk solution used as vehicle, which is suggested to be not completely utilized when administered during the resting phase, thus contributing to an increased energy storage and body weight gain.

No treatment effects were described for biochemical parameters such as blood glucose, triglycerides or total cholesterol levels. Interestingly, fasting glucose levels were higher during the active phase (ZT15), which is in accordance with the previously discussed increment of serum glucose at the beginning of this period [30]. Overall, the CAF diet-promoted alterations on physiological, biometric and biochemical parameters were not counteracted by the chronic administration of low doses of GSPE.

Table 2. Food intake, biometric and serum parameters in vehicle- and GSPE-treated male Fischer 344 rats during resting (ZT3) or active (ZT15) phases during 4 weeks under a cafeteria (CAF) feeding.

	ZT3		ZT15		Two-way
	VEH	GSPE	VEH	GSPE	ANOVA
Food intake (kcal/day)					
Total energy intake	159 ± 3.55	147 ± 5.07	151 ± 5.53	148 ± 3.59	*n.s*
Total carbohydrates intake	92.8 ± 2.71	85.3 ± 3.19	87.9 ± 3.34	85.6 ± 2.55	*n.s*
Simple carbohydrates intake	52.7 ± 2.27	48.0 ± 3.37	46.6 ± 2.27	46.3 ± 1.60	*n.s*
Lipid intake	43.7 ± 2.33	45.1 ± 1.91	45.6 ± 2.00	45.2 ± 1.04	*n.s*
Protein intake	18.2 ± 0.52	16.6 ± 0.49	17.4 ± 0.42	16.7 ± 0.37	T
Fiber intake (g/day)	1.88 ± 0.06	1.56 ± 0.07	1.76 ± 0.03	1.65 ± 0.07	T
Food efficiency	2.24 ± 0.21	2.09 ± 0.11	2.00 ± 0.07	1.97 ± 0.10	*n.s*
Biometric parameters					
Body weight gain (g)	225 ± 17.1	193 ± 7.16	189 ± 4.16	182 ± 6.64	t,T
Fat mass (%)	24.2 ± 1.73	25.4 ± 1.16	23.5 ± 0.81	24.4 ± 1.11	*n.s*
Lean mass (%)	69.7 ± 1.67	68.4 ± 0.98	70.2 ± 0.91	69.2 ± 0.99	*n.s*
Lean/fat ratio	3.00 ± 0.34	2.73 ± 0.15	3.03 ± 0.14	2.89 ± 0.17	*n.s*
Visceral fat (%)	9.66 ± 0.43	9.91 ± 0.37	10.1 ± 0.24	10.1 ± 0.31	*n.s*
Adiposity Index	12.0 ± 0.63	12.2 ± 0.46	12.9 ± 0.37	13.2 ± 0.47	*n.s*
MWAT (%)	2.56 ± 0.13	2.45 ± 0.14	2.70 ± 0.19	2.59 ± 0.21	*n.s*
RWAT (%)	3.20 ± 0.16	3.42 ± 0.14	3.46 ± 0.14	3.32 ± 0.13	*n.s*
EWAT (%)	3.90 ± 0.24	4.04 ± 0.15	4.12 ± 0.09	4.22 ± 0.21	*n.s*
IWAT (%)	2.33 ± 0.26	2.33 ± 0.17	2.79 ± 0.38	3.07 ± 0.32	*n.s*
Cecum weight (g)	4.36 ± 0.21	3.63 ± 0.19	3.54 ± 0.34	3.22 ± 0.18	t,T
Serum parameters					
Glucose (mg/dL)	202 ± 10.9	203 ± 8.07	238 ± 13.6	217 ± 12.5	t
Triglycerides (mg/dL)	358 ± 24.3	389 ± 57.0	399 ± 23.6	419 ± 23.4	*n.s*
Total cholesterol (mg/dL)	94.8 ± 7.29	93.0 ± 4.18	100 ± 8.99	103 ± 7.94	*n.s*

Male F344 rats (n = 8 per group) under a CAF feeding were supplemented with vehicle or GSPE during the last 4 weeks of the study and sacrificed at ZT3 or ZT15. Data are expressed as the mean ± SEM. t, the effect of daily phase; T, the effect of treatment; txT, the interaction of daily phase and treatment (two-way ANOVA, $p< 0.05$). * The effect of treatment within each ZT group (Student's t-test, $p < 0.05$). EWAT, epididymal white adipose tissue; IWAT, inguinal white adipose tissue; MWAT, mesenteric white adipose tissue; RWAT, retroperitoneal white adipose tissue.

3.2 Daily oscillations of cecal microbiome are altered by cafeteria diet.

The assessment of gut microbiota composition in male F344 rats fed with a STD or a CAF diet within the light (ZT3) or dark (ZT15) phases revealed that diet significantly contributed to changes on the microbial phylogenetic structure at genus level (two-way PERMANOVA, $p < 0.001$) (**Supplementary Figure 1**). Importantly, one-way PERMANOVA showed that gut microbiome composition was significantly different between ZT3 and ZT15 groups when animals were fed with a STD diet ($p = 0.011$), but not with a CAF diet ($p = 0.107$). In this sense, circadian fluctuations in the composition of the microbiome have been extensively reviewed in the last years [33,34]. These changes on microbiota configuration are associated with time of the day-specific tasks carried by out by the microbes including detoxification and chemotaxis during the resting phase, and cell growth, DNA repair and energy harvest during the active phase [28]. Therefore, microbiome dynamically changes in response to nutrient availability, thus highlighting a particular metabolic role performed by the whole community. Interestingly, these daily oscillations, which are displayed in both rodents and humans independently on a particular microbiome, rely upon the host's internal circadian clocks [35]. Furthermore, the entire gut microbial communities, particularly its generated metabolites such as short-chain fatty acids (SCFA), are considered key agents affecting the expression of host's circadian clock genes. For instance, high-fat diet was shown to induce an altered hepatic expression of clock genes in mice, whereas this feature was not observed in the absence of gut microbiota [36].

Altogether, among the wide variety of metabolic and behavioural functions attributed to clock genes, they are known to rapidly adapt the host-microbiota metabolism to the changing environmental conditions such as the nutrient availability [37]. In turn, microbiota-derived metabolites such as SCFAs contribute to the regulation of the energy homeostasis and eating behaviour [38], thus resulting in a complex interactive network between diet, gut microbiota and host's circadian rhythms.

In the present study, while gut microbiome composition differed between both phases in rats fed with a STD diet, we showed that CAF feeding abolished the microbial circadian rhythmicity. Thus, we suggested that the CAF diet-induced alterations on gut microbial rhythms and host's circadian clocks led to the chronodisruption of feeding patterns, thus favouring hyperphagia and its deleterious consequences on metabolic health.

Focusing on each daily phase, CAF diet was able to change the gut microbial composition of both ZT3 ($p < 0.001$) and ZT15 ($p < 0.001$) groups when compared to their respective STD diet-fed controls (One-way PERMANOVA), without impacting on the bacterial diversity at any daily phase, as revealed by Shannon Index (**Figure 1**). In this sense, bacterial diversity is robustly maintained over the course of the day within both STD and CAF diet-fed groups despite the alteration of microbial rhythmicity by the CAF feeding. In this sense, a recent study concluded that the alteration of microbial rhythms has no influence on the bacterial diversity in humans and rats [39]. Thus, caution must be taken when assessing the diminution of alpha-diversity as an

unavoidable consequence of the disruption of microbial rhythmicity linked to metabolic diseases.

On the other hand, gut microbiome of vehicle- or GSPE-treated rats under a CAF feeding was characterized within both light and dark phases. Despite that no treatment-induced differences on the genera composition were identified within neither ZT3 nor ZT15 groups, GSPE tended to modulate the microbial community only within the active phase (one-way PERMANOVA, $p = 0.054$) (**Supplementary Figure 2**). In this sense, we postulate that GSPE supplementation could be able to restore the CAF diet-induced disruption on gut microbial composition when administered during the dark phase, but not during the resting phase. Moreover, two-way ANOVA revealed a time x treatment interaction describing changes on bacterial diversity ($p = 0.029$).Therefore, the GSPE-induced changes on Shannon's diversity index largely depended on the time of administration, despite that no significant variations in bacterial diversity were observed within each daily phase (Student's t-test). As recently published, a chronic administration of pharmacological doses of GSPE (500 mg/kg body weight) caused a depletion of bacterial alpha-diversity in STD diet-fed rats [40]. However, our results revealed that dietary doses of GSPE did not promote a diminution on alpha-diversity even when rats were under a long-term CAF feeding. In summary, F344 rats consistently maintain the diversity of gut microbiota despite the consumption of a CAF diet and the GSPE supplementation regardless the daily phase.

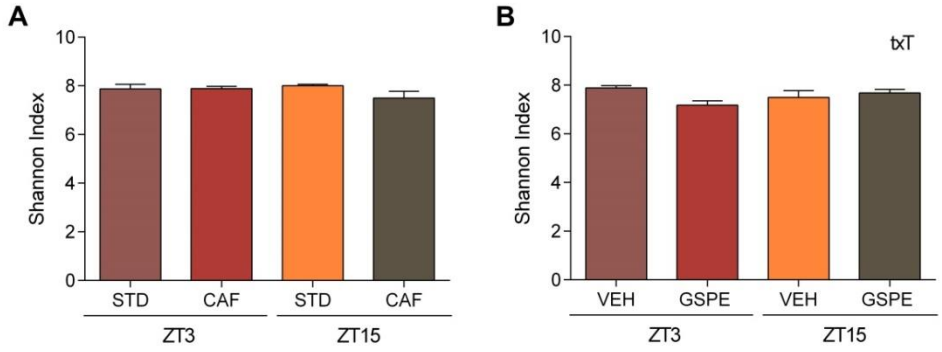


Figure 1. Differences in alpha-diversity among groups assessed through the Shannon Index. **A**. Microbial diversity determined during the resting (ZT3) or during the active (ZT15) phase in male F344 rats fed with a standard (STD) or a cafeteria (CAF) diet. **B.** Microbial diversity determined during the resting (ZT3) or during the active (ZT15) phase in vehicle- and GSPE-treated animals fed with a CAF diet. Data are expressed as the mean ± SEM. txT, the interaction of time and treatment (two-way ANOVA, $p< 0.05$). * The effect of diet or treatment within each daily phase (Student's t-test, $p < 0.05$).

3.3 Time-dependent alterations of the main taxa at phylum level.

Firmicutes was identified as the most abundant phylum, representing up to 89.9% and 83.0% of the total bacteria within both ZT3 and ZT15 groups fed with a STD diet, respectively. Interestingly, while no significant differences on the main bacterial taxa were found between daily phases in rats under a STD feeding, Lentisphaerae (a low-abundant phylum representing less than 0.01% of the total bacteria) was significantly decreased in ZT15 compared to ZT3 group ($p = 0.004$). *Victivallis vadensis* is the unique bacterial specie within the phylum of Lentisphaerae that has been isolated from the gastrointestinal tract in humans [41,42], which is in accordance with our observations at genus level, although its impact on the host remains to be determined. Here, we reported that circadian variations of this unexplored phylum in STD diet-

fed rats were disrupted by the CAF feeding, showing no differences on its relative abundance between light and dark phases ($p = 0.629$).

Two-way ANOVA revealed a significant interaction between time and diet type ($p = 0.018$) on the modulation of Bacteroidetes-to-Firmicutes ratio, which was significantly incremented during the dark phase, but not during the light phase, by the CAF diet when compared to its respective STD diet-fed group (Student's t-test, $p = 0.021$), as shown in **Figure 2**. In accordance with this result, we showed for the first time that the CAF diet disrupts the diurnal rhythmicity of the main phyla. As discussed above, host's circadian clock genes regulate the food consumption that, in turn, is the primary driver for the changes in microbiota composition [43]. In addition, the intestinal microbiome is interconnected with the host's circadian rhythms, that in turn modulate the metabolic pathways related to anabolism and catabolism [35]. Therefore, we suggest that the continuous availability and consumption of energy-dense and highly appetitive foods markedly disrupted the host-microbiota oscillations by altering microbial communities. Accordingly, it has been shown that time-restricted feeding partially restores the microbiome fluctuations over time in diet-induced obese rodents [44].

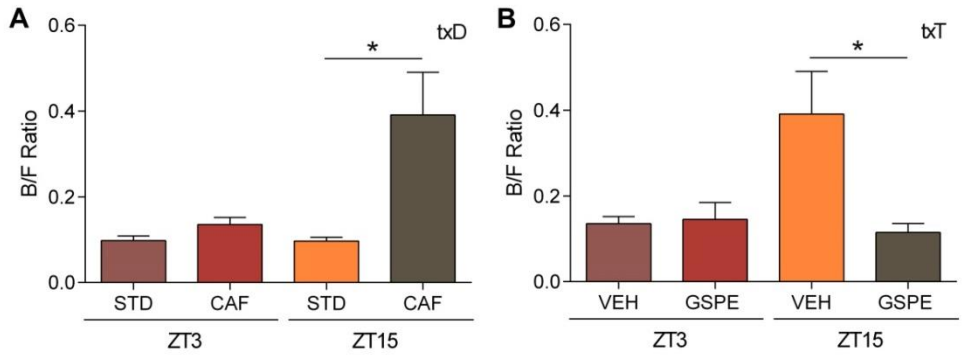


Figure 2. The two major phyla composing the microbiome expressed as the Bacteroidetes-to-Firmicutes ratio. **A**. B/F ratio assessed during the resting (ZT3) or during the active (ZT15) phase in both standard (STD) and cafeteria (CAF) diet-fed rats. **B**. B/F ratio assessed in vehicle- and GSPE-treated animals fed with a CAF diet within both ZT3 and ZT15 groups. Data are expressed as the mean ± SEM. txD, the interaction of time and diet type; txT, the interaction of time and treatment (two-way ANOVA, $p< 0.05$). * The effect of diet or treatment within each daily phase (Student's t-test, $p < 0.05$).

On the other hand, during the dark phase (ZT15), GSPE tended to rise the Firmicutes abundance (VEH: 65.7 ± 6.01, GSPE: 80.6 ± 2.83, $p = 0.059$), while Bacteroidetes tended to drop (VEH: 22.2 ± 4.43, GSPE: 11.3 ± 2.50, $p = 0.093$), while no significant changes were observed during the light phase (ZT3) (data not shown). Whereas GSPE supplementation at high doses led to an increment of Firmicutes abundance [40], we demonstrated that dietary doses of GSPE tended to reproduce this feature in male F344 rats even under a CAF feeding. Concerning the modulation of the Bacteroidetes-to-Firmicutes ratio, we reported a significant interaction between time and treatment factors (two-way ANOVA $p = 0.033$). Consistently, this ratio was significantly reduced in GSPE-treated group during the dark phase as compared to ZT15 controls (Student's t-test, $p = 0.046$). These results suggest that a chronic administration of GSPE was able to reverse the time-dependent

alterations of the main taxa induced by the CAF diet. Despite that compositional changes of gut microbiota in GSPE-supplemented rats were not linked with improvements on obesity-related parameters in this study, the beneficial effects of chronic and acute administration of dietary and high doses of GSPE in diet-induced obese rodents have widely been reported in the literature. In this regard, the chronic administration of elevated doses of GSPE (500 mg/kg body weight) modulated the gene expression patterns in adipose tissue, liver and muscle, leading to an increased fatty acid oxidation and a reduced amounts of white adipose tissue under a high-fat high-sugar diet [45]. Interestingly, dietary doses of GSPE (25 mg/kg body weight) administered to animals fed with a CAF diet normalized major regulators of lipids metabolism in liver [46], improved glucose homeostasis and insulin resistance [18], increased the thermogenic capacity and mitochondrial functions [47] and protected from diet-induced intestinal alterations [16]. However, consistent differences on animal strains, length of dietary intervention and duration of treatment may contribute to explain the absence of beneficial physiologic effects previously attributed to GSPE. In our metagenomic study, we demonstrated that low doses of GSPE were able to restore the daily oscillations of the main bacterial taxa altered by a CAF diet in F344 rats, which could be partially linked to the GSPE-mediated metabolic responses against diet-induced obesity described in the literature.

Importantly, we found up to 7 different phyla altered by the CAF feeding compared to STD controls within each ZT3 and ZT15 groups. As presented in **Figure 3**, Firmicutes ($p < 0.050$), Proteobacteria ($p < 0.010$), Tenericutes ($p < 0.050$) and TM7 ($p < 0.010$) were similarly

altered by CAF diet in both dark and light phases. The depletion of Firmicutes, Tenericutes and TM7 relative abundances induced by our CAF model was counteracted by a significant rise of Proteobacteria population ($p < 0.050$).

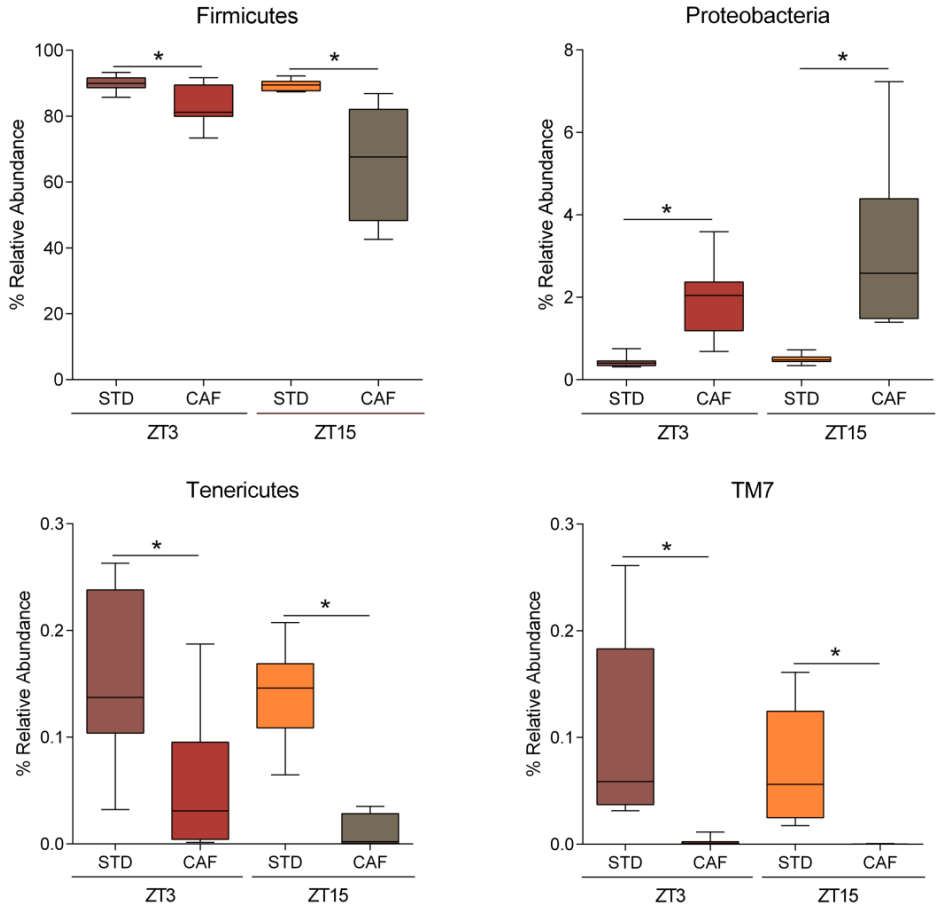


Figure 3. Relative abundance of the main phyla influenced by the cafeteria (CAF) diet within both light (ZT3) and dark (ZT15) phases in F344 rats. *Significant differences assessed through Welch's t-test after Benjamini-Holchberg FDR correction considering corrected p-values < 0.05.

Altogether with the rise of Bacteroidetes induced by the CAF diet uniquely detected in during the dark phase, CAF diet promoted a significant increment of Actinobacteria abundance during the resting

phase (ZT3), whereas no daily differences were detected between ZT3 and ZT15 groups fed with a STD diet ($p = 0.318$). Therefore, it is suggested that the response of this phylum to the dietary intervention is daily phase-dependent, whereas its maintained abundance in STD diet-fed rats across daily phases was disrupted by the CAF feeding. *Bifidobacterium* is the most studied genus of the Actinobacteria phylum, showing a high versatility in terms of degrading dietary polysaccharides and inhibiting the growth of pathogens in both humans and rodents [48–50]. However, an unclassified Coriobacteriaceae, instead of *Bifidobacterium*, was the main responsible for the observed CAF diet-induced rise of Actinobacteria during the light phase only. High Coriobacteriaceae abundance was also reported in obesity [51] and after partial sleep deprivation [52], being the sleep timing a factor that profoundly contributes to the host's rhythmicity [53]. Therefore, we suggest that high occurrences of Actinobacteria, which was detected during the resting phase, are linked to the CAF diet-induced disturbances on microbial rhythmicity.

The above reported changes on Lentisphaerae abundance in STD diet-fed rats according to daily phase were also induced by the CAF feeding. In this regard, the increased Lentisphaerae occurrence during the resting phase in STD diet-fed rats was depleted after the consumption of the CAF diet ($p = 0.013$), hence contributing to the disruption of the microbiome rhythmicity.

3.4 CAF diet distinctively alters gut microbiota according to daily phase.

In this study, both daily phase ($p = 0.018$, $R = 0.152$) and diet ($p < 0.001$, $R = 0.550$) significantly contributed to the differences on microbial populations as revealed by two-way ANOSIM, with the dietary factor explaining most of these changes. Importantly, global differences between both light and dark phases in animals fed with a STD diet were mainly explained by variations on the *Lactobacillus* occurrence. Here, we reported that *Lactobacillus* genus was significantly more abundant during the resting (ZT3) than during the active (ZT15) phase, representing up to 5.62% and 1.89% of total bacteria, respectively. In accordance with these results, certain *Lactobacillus* species were found to decline during the active/dark phase and increase during the resting/light phase in mice [35]. In addition, cyclical oscillations of *Lactobacillus* were disturbed under an *ad libitum* high-fat diet, while time-restricted feeding restored its diurnal fluctuations resembling to that observed in the regular chow-fed mice [5]. Because of gut microbes utilize the complex carbohydrates to generate SCFAs and lactate (lactic acid bacteria such as *Lactobacillus* spp.), an oscillatory production according to the circadian abundance of microbial producers should be expected. Furthermore, every other day fasting in mice resulted in a shift in the gut microbiome leading to an elevation of the fermentation products such as acetate and lactate [54]. In our study, *Lactobacillus* genus was identified as the most abundant lactic acid bacteria, therefore suggested to be the major lactate producer in the gut. Recent literature support an increasing evidence for lactate acting as a linkage between neuronal activity and metabolism [55], as well as suggested to be the

preferred fuel for human brain [56,57]. Therefore, an hypothetical rhythmicity of lactate production in our study, which has already been observed in the cecum of mice [58], could be actively contributing to the maintenance of brain functions essentially during the resting period, although further research is needed to understand the role of a rhythmic *Lactobacillus* abundance and microbiota-derived lactate in the host metabolism.

Alternatively, the gut microbiome of rats fed with a CAF diet did not show differences on its phylogenetic structure at genus level between both light and dark phases, which is in accordance with previous suggestions postulating diet as a determinant factor in the daily oscillations of gut microbiome [59]. Therefore, CAF diet is suggested to strongly disrupt the daily microbiota oscillations at genus level, as discussed above regarding the phyla composition. In addition, when compared to STD diet-fed animals, CAF rats displayed changes on genera abundance according to the daily phase, although some relevant genera were similarly altered between groups in both active and resting phases, as presented in **Figure 4**. In this regard, an unclassified genus belonging to the Clostridiales order was significantly decreased within both CAF diet-fed ZT3 ($p = 0.003$) and ZT15 ($p = 0.019$) groups compared to their respective lean controls. In the same way, CAF diet induced a drop on *Dehalobacterium* abundance independently of the daily phase. In contrast, other Firmicutes-belonging genera such as *Blautia*, *Phascolarctobacterium* and *rc4-4*, altogether with *Bacteroides* (Bacteroidetes) and *Sutterella* (Proteobacteria), were indistinctively raised by CAF feeding within both light and dark phases.

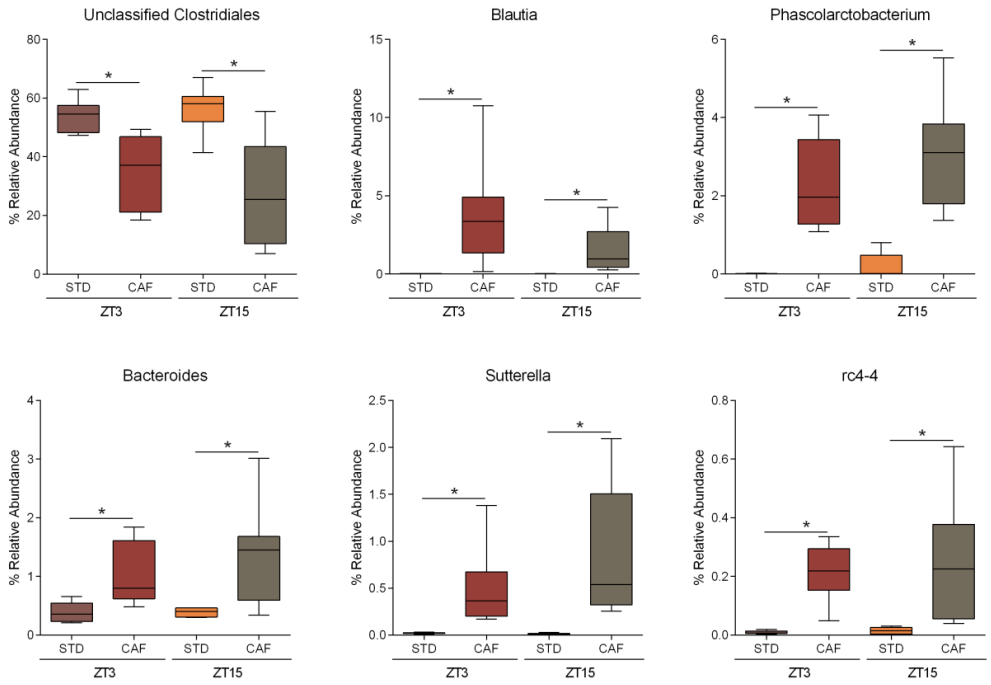


Figure 4. Relative abundance of the main genera similarly influenced by the cafeteria (CAF) diet within both light (ZT3) and dark (ZT15) phases in F344 rats. *Significant differences assessed through Welch's t-test after Benjamini-Holchberg FDR correction considering corrected p-values < 0.05.

In this scenario, CAF diet seems to drive a consistent alteration on gut microbiome that is maintained throughout the day. However, some of the bacterial genera were only altered within the active or resting phase in the CAF diet-fed groups, suggesting that this dietary model also induces time of the day-specific alterations of the microbial ecosystem. Among them, genera belonging to Firmicutes phylum comprising *Roseburia* (p = 0.045), *Allobaculum* (p = 0.043) and *Ruminococcus* (p = 0.012) were increased, altogether with a diminution of *Oscillospira* (p = 0.048) abundance, in CAF diet-fed rats during the resting phase, but not during the active phase, as shown in **Figure 5**. Commonly, most of the studies evaluating the diet-induced obesity consequences on the

microbiota composition collect the fecal or cecum samples for metagenomic analyses during the light/resting period of the animals. Therefore, diet-induced microbiome alterations observed in ZT3 group are subjected to be contrasted with the recent literature. However, several factors such as rat strain, dietary model and feeding duration difficult the direct comparison between our results and those obtained in other studies. Interestingly, *Ruminococcus* genus was previously found enriched in mice and obese-prone rats fed with a high-fat diet [60,61]. In contrast, the reported enrichment in *Roseburia* induced by the CAF diet during the light phase is in contrast with the literature describing a dropped abundance of this genus in high-fat diet fed mice [62]. In this regard, butyrate-producers such as *Roseburia* spike in response to available substrates after food intake and decrease as the energy source is depleted [63]. Therefore, higher *Roseburia* abundance in CAF diet-fed rats during the resting phase could be in alignment with an increased food intake during this period by the CAF group.

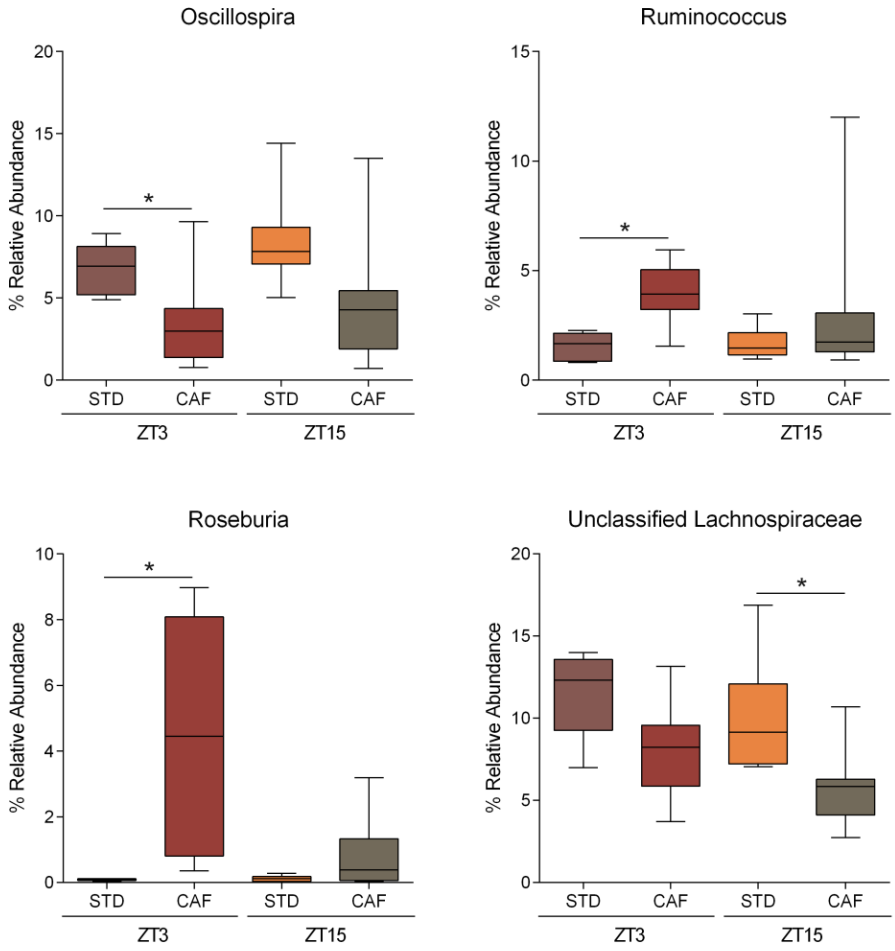


Figure 5. Relative abundance of the main genera distinctively influenced by the cafeteria (CAF) diet according to light (ZT3) or dark (ZT15) phase in F344 rats. *Significant differences assessed through Welch's t-test after Benjamini-Holchberg FDR correction considering corrected p-values < 0.05.

In the ZT3 group, we also reported that CAF diet caused a significant rise of *Parabacteroides* (p = 0.045; Bacteroidetes phylum), and *Desulfovibrio* and *Bilophila* (p = 0.043, p = 0.049; Proteobacteria phylum) abundances, as previously presented in **Chapter II**. On the other hand, CAF diet-fed rats showed a significantly increased *Butyricimonas* (p = 0.042; Bacteroidetes phylum) occurrence during the active/dark phase (ZT15),

but not during the light phase, while the occurrences of *Odoribacter* (p = 0.046) and genera belonging to Firmicutes phyla such as unclassified Lachnospiraceae (p = 0.046) and *Lactococcus* (p = 0.049) were dropped.

Overall, we demonstrated that several specific genera levels were distinctively modulated by the CAF diet according to the daily phase, thus reflecting that microbial oscillations naturally occurring under a STD diet were profoundly altered by the CAF feeding. In conclusion, the daily phase actively contributes to the diet-induced changes of the microbiota composition, resulting to be determinant for final conclusions and, consequently, being considered a factor that should be taken into account in further microbiome-related studies.

In line with the absent differentiation between cecal microbial populations in CAF diet-fed rats treated with GSPE compared to those treated with the vehicle (one-way PERMANOVA, p > 0.05), no significant changes on specific genera were identified at any daily phase.

3.5 Principal component analysis.

The clustering of the samples by diet and daily phase was investigated through a Principal Component Analysis (PCA), revealing that diet factor played a key role in the distribution of samples along the two principal components (PC). Globally, PCA described up to 46.2% of the overall variation, where rats fed with a STD or CAF diet were separately represented along the first PC axis (31.8%) as shown in **Figure 6**. Furthermore, cecal microbiota samples from CAF diet-fed rats exhibited a high inter-individual variability than STD controls that, in turn, is suggested to be the main consequence of the free-choice based dietary model defining the CAF feeding. On the other hand, PC2

described the 14.4% of the overall variation, where samples from active/dark and resting/light phases were mostly located in the lower and upper parts, respectively. Microbiota samples from STD diet-fed rats robustly clustered within both light and dark phases, but significant differences between the two populations, mostly explained by changes on Lactobacillus abundances, were identified, as discussed above. In this way, the microbiota composition of CAF diet-fed rats was overlapped in the right side according to the lack of statistical differences on their microbiota composition. Therefore, CAF diet was able to strongly modulate the microbial ecosystems within both light and dark phases in male F344 rats, while disrupting time of the day-dependent divergences on the phylogenetic structure of the microbiome.

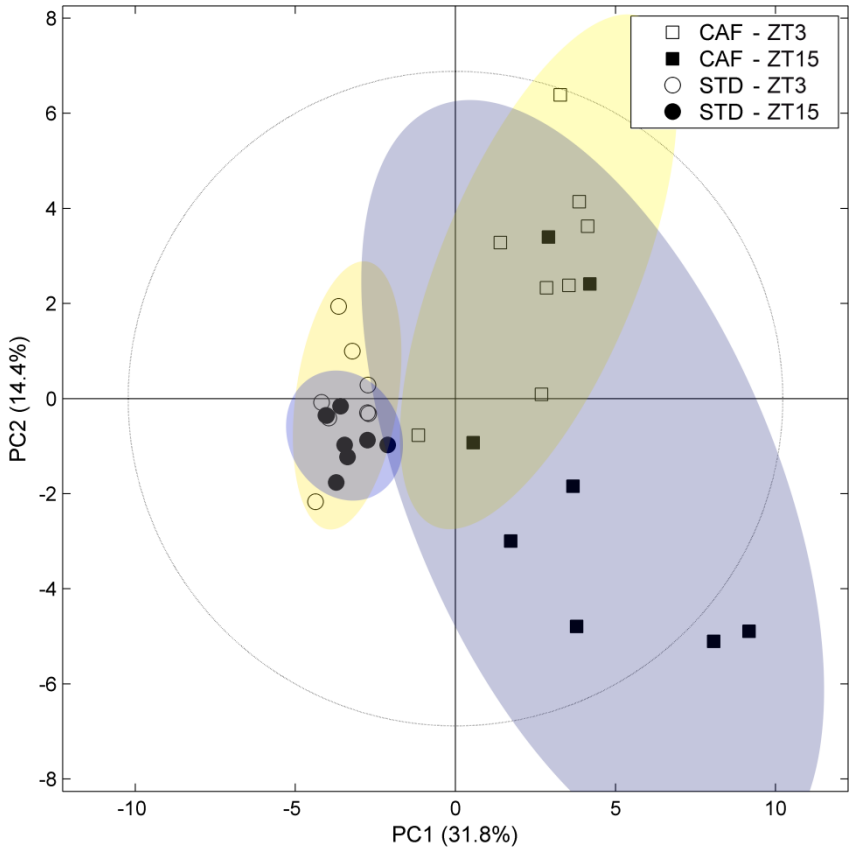


Figure 6. Principal component analysis constructed from the relative abundance of genera representing more than 1% of bacteria at least in two samples. Circular and squared dots represent standard (STD) and cafeteria (CAF) diet-fed animals, respectively. Black and white dots discriminate between active/dark and resting/light daily phases, respectively. PC, principal component.

On the other hand, the effect of a chronic supplementation with low doses of GSPE in CAF diet-fed rats on gut microbiota composition did not show a particular clustering with respect to vehicle-treated rats, as shown in **Figure 7**. In light of the non-significant changes on the microbiota populations revealed by one-way PERMANOVA, the 34.0% of the overall variation explained by the two principal components (PC1:

20.9%; PC2: 13.1%) of the PCA was not able to group the samples by the treatment received.

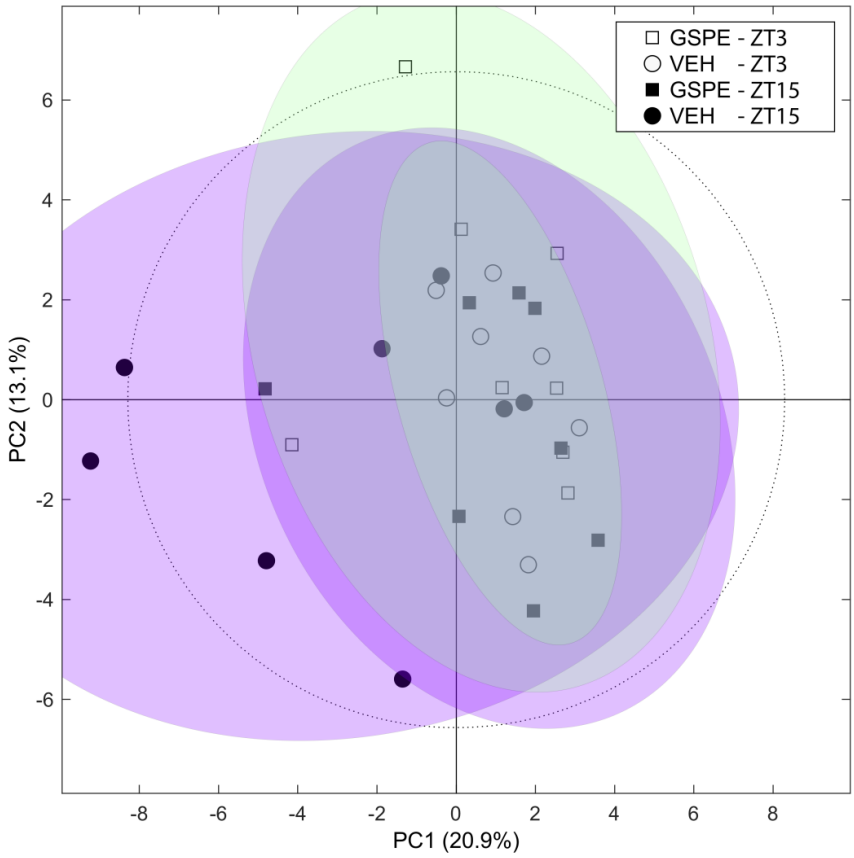


Figure 7. Principal component analysis constructed from the relative abundance of genera representing more than 1% of bacteria at least in two samples. Circular and squared dots represent the vehicle- and GSPE-treated animals under a cafeteria (CAF) feeding, respectively. Black and white dots discriminate between active/dark and resting/light daily phases, respectively. PC, principal component.

4. CONCLUDING REMARKS

In this study, we observed that gut microbiota composition at genus level differed between diurnal and nocturnal stages, suggesting a

naturally occurring microbial rhythmicity when animals were fed under a chow diet. In this sense, it is well known that rodents with *ad libitum* access to normal chow show cyclical feeding patterns, preferably consuming their diets during the night-time (active phase) [64]. According to this feature, cyclical fluctuations in the relative abundance of Bacteroidetes and Firmicutes were also observed in chow-fed mice, while alpha-diversity was shown to fluctuate with the time of the day by rising during feeding periods and falling with fasting [5]. However, we did not detect changes on major phyla or alpha-diversity between dark and light phases in STD diet-fed rats. In this sense, the peaks of abundance of cyclical phyla might not correspond to our ZT points, thereby limiting the detection of circadian changes in our study. On the other hand, diet-induced obesity, as well as the disruption of circadian rhythms by a continuous exposure to light, are related to a decreased bacterial diversity [65,66]. However, a similar study analysing the changes on gut microbiota composition during light or dark phase in mice fed with a STD or a HFD reported an absence of regular oscillations in Shannon's diversity index independently of the diet [67], which is in accordance with our results. Furthermore, HFD-fed mice showed an increased Bacteroidetes-to-Firmicutes ratio during the resting stage [67]. Contrary, we revealed that CAF diet strongly increased this ratio during the active phase, but not during the resting phase, in F344 rats. Since no changes were detected in the Bacteroidetes-to-Firmicutes ratio during the resting phase, it is postulated that the effects of the CAF diet, mainly detected during the active feeding period, are not constant throughout the day. In this sense, nutrient availability exerts an important control over the rhythms of microbial ecosystem in the gut as previously stated [68].

Interestingly, the divergent composition of gut microbiota at genus level between light (ZT3) and dark (ZT15) phases in STD diet-fed rats was not observed in the CAF diet-fed groups, thus indicating that CAF diet was able to strongly disrupt the microbial rhythmicity. Remarkably, the changing abundance pattern observed for *Lactobacillus* in STD diet-fed rats was not reported in the animals consuming a CAF diet. In this regard, it was shown that time-restricted feeding restores the loss of cyclical rhythmicity in *Lactobacillus* abundance under a HFD feeding in mice [5]. This genus mediates the deconjugation of bile acids due to its bile salt hydrolase (BSH) activity [69]. Moreover, the conjugated tauro-beta-muricholic acid (TβMCA) is known to act as a potent antagonist of intestinal FXR in rodents [70]. In this sense, a decreased BSH activity, which was previously related to anti-obesity effects in mice [71], favours the intestinal FXR inhibition that, in turn, was shown to prevent obesity-related metabolic complications [72]. In line with these observations, elevated *Lactobacillus* populations in obese and diabetic subjects have been reported [73]. However, an increased intestinal BSH activity was demonstrated to significantly reduce weight gain, LDL cholesterol and liver triglycerides in mice fed chow or high-fat diets [71], in contrast with the connection between FXR agonists and aggravated disorders in lipid metabolism in obese mouse models previously shown [74]. Hence, contradictory evidences on the role of intestinal FXR in obesity and related metabolic complications claim for further investigation. In addition, several *Lactobacillus* species including *L. paracasei*, *L. rhamnosus* and *L. acidophilus* have shown prebiotic activities in rodents, even attenuating obesity comorbidities [75,76]. In our study, CAF diet-induced disturbances on cyclical *Lactobacillus* abundance reflected the circadian desynchronization of microbe-host crosstalk, thus possibly

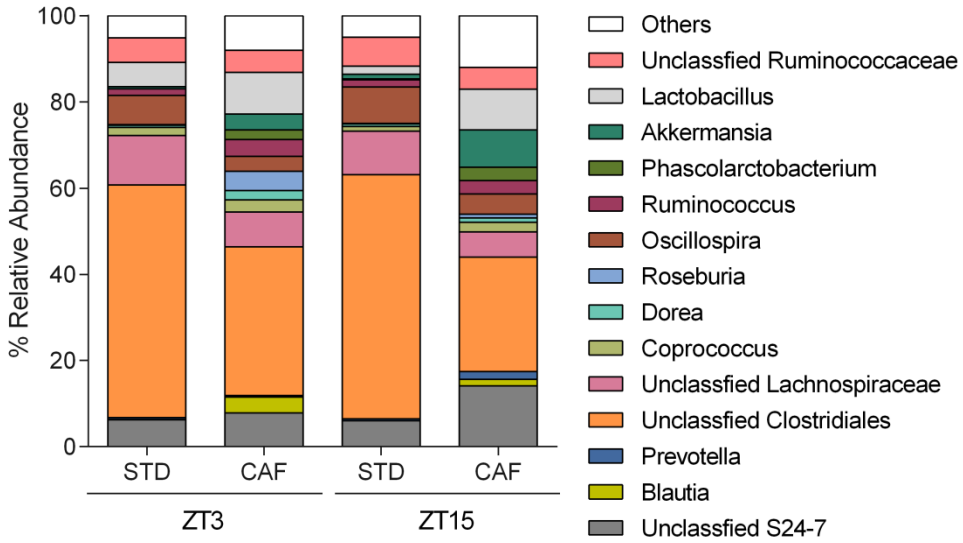
affecting bile acid metabolism and, in turn, contributing to the disruption of lipid metabolism and host adiposity. Moreover, we showed that the dietary effects on gut microbiota composition highly depended on the time of the day.

On the other hand, dietary doses of GSPE did not promote the recovery of microbial oscillations under a CAF feeding in male F344 rats. It is important to note that both rat strain and gender factors also influence the CAF diet-induced changes on gut microbiota, as previously shown in **Chapter II**. Therefore, the absence of changes on specific genera in response to the GSPE treatment in this study could be conditioned by multiple factors. In this sense, since both fiber and protein are actively utilized by the gut microbiota, the decreased consumption of these macronutrients observed in GSPE-supplemented rats, mainly due to a reduced chow intake, could be conditioning the GSPE effects in host physiology and gut microbiome. In our study, satiating properties previously described for GSPE seemed to suppress chow intake only, while highly palatable and energy-dense foods were similarly consumed in both vehicle and GSPE-treated groups. In addition, the decreased body weight gain observed in GSPE-treated animals was not accompanied by a reduced adiposity, thereby suggesting a loss of lean mass as revealed by a numerically lower lean-to-fat mass ratio. Therefore, preventive effects of GSPE in body weight gain must not be discussed as a beneficial outcome for diet-induced obesity. Moreover, the lower fiber and protein intake observed in GSPE-treated animals could be ablating their potential benefits for host health.

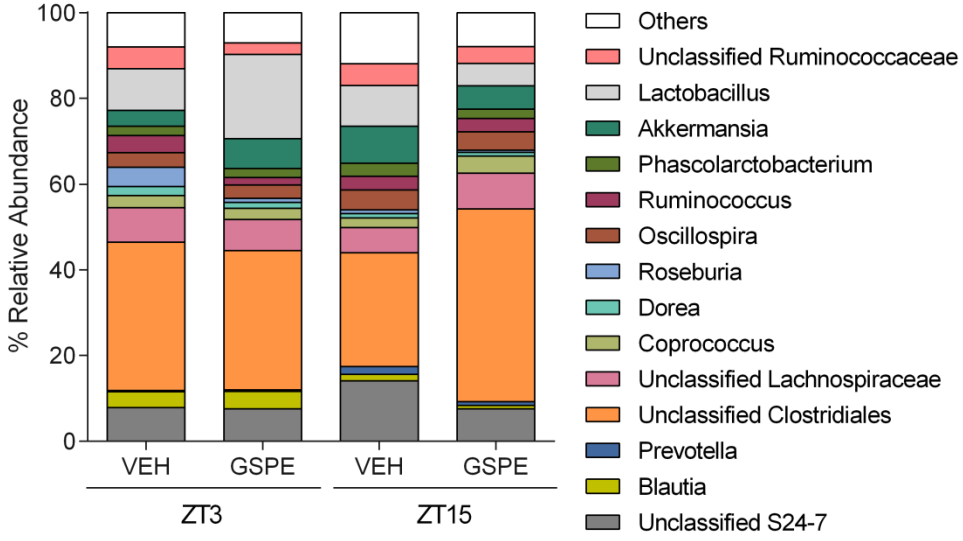
Nevertheless, we demonstrated that dietary doses of GSPE were able to prevent the CAF diet-promoted increase of Bacteroidetes-to-Firmicutes

ratio during the active phase. In a previous study [77], Firmicutes were shown to be negatively associated with adipose tissue weights and hepatic lipids, and positively correlated with fiber intake. Therefore, GSPE seems to counteract the CAF diet-induced reduction of Firmicutes phylum, which is characterized by butyrate-producing bacteria from non-digestible components of the diet [78], thereby increasing the bacterial fermentation capacity and the SCFA production during the active phase. Furthermore, pharmacological doses of GSPE were shown to up-regulate the expression of intestinal GLP-1 and PYY, thus promoting satiety and energy expenditure [79]. In fact, enteroendocrine colonic L-cells produce these regulatory hormones in response to microbial-derived SCFA [80]. Hence, the preventive effects of GSPE against CAF diet-induced alterations in the main phyla are hypothesized to favour the gut microbiota-mediated secretion of satiety signalling hormones during the active phase in a SCFA-dependent manner. In addition, the modulatory role of high doses of GSPE on the gut microbiota of STD diet-fed rats was reported [40], thereby suggesting that GSPE exerts its above discussed beneficial effects on obesity through the management of the microbiota-host co-metabolism. Supporting the time-dependent effects of GSPE on the modulation of gut microbiota observed in our study, the GSPE administered during the active phase, but not during the resting phase, demonstrated to enhance mitochondrial oxidation in the liver in a previous study [81]. Then, GSPE distinctively affect host metabolism and gut microbiota according to the time of administration, beneficially altering the microbial ecosystem when supplemented during the dark phase in CAF diet-fed rats. Further research is needed to accurately elucidate the prebiotic potential of dietary doses of GSPE in the management of obesity.

SUPPLEMENTARY INFORMATION



Supplementary B 1. Relative abundance of the most representative genera in F344 rats fed with a standard (STD) or a cafeteria (CAF) diet and sacrificed at ZT3 (light/resting phase) or ZT15 (dark/active phase).



Supplementary Figure 2. Relative abundance of the most representative genera in F344 rats fed with a cafeteria (CAF) diet and sacrificed at ZT3 (light/resting phase) or ZT15 (dark/active phase) according to diurnal or nocturnal supplementation with GSPE, respectively.

REFERENCES

[1] Paschos GK, FitzGerald GA. Circadian Clocks and Metabolism: Implications for Microbiome and Aging. Trends Genet 2017;33:760–9.

[2] Depner CM, Stothard ER, Wright KP. Metabolic consequences of sleep and circadian disorders. Curr Diab Rep 2014;14:507.

[3] Voigt RM, Forsyth CB, Green SJ, Engen PA, Keshavarzian A. Circadian Rhythm and the Gut Microbiome. Int. Rev. Neurobiol., 2016, p. 193–205.

[4] Lazar V, Ditu LM, Pircalabioru GG, Picu A, Petcu L, Cucu N, et al. Gut microbiota, host organism, and diet trialogue in diabetes and obesity. Front Nutr 2019;6:21.

[5] Zarrinpar A, Chaix A, Yooseph S, Panda S. Diet and feeding pattern affect the diurnal dynamics of the gut microbiome. Cell Metab 2014;20:1006–17.

[6] Salazar N, Dewulf EM, Neyrinck AM, Bindels LB, Cani PD, Mahillon J, et al. Inulin-type fructans modulate intestinal Bifidobacterium species populations and decrease fecal short-chain fatty acids in obese women. Clin Nutr 2015;34:501–7.

[7] Duan M, Sun X, Ma N, Liu Y, Luo T, Song S, et al. Polysaccharides from Laminaria japonica alleviated metabolic syndrome in BALB/c mice by normalizing the gut microbiota. Int J Biol Macromol 2019;121:996–1004.

[8] Sun X, Duan M, Liu Y, Luo T, Ma N, Song S, et al. The beneficial effects of Gracilaria lemaneiformis polysaccharides on obesity and the gut microbiota in high fat diet-fed mice. J Funct Foods 2018;46:48–56.

[9] Masumoto S, Terao A, Yamamoto Y, Mukai T, Miura T, Shoji T. Non-absorbable apple procyanidins prevent obesity associated with gut microbial and metabolomic changes. Sci Rep 2016;6:31208.

[10] Etxeberria U, Arias N, Boqué N, Macarulla MT, Portillo MP, Martínez JA, et al. Reshaping faecal gut microbiota composition by the intake of trans-resveratrol and quercetin in high-fat sucrose diet-fed rats. J Nutr Biochem 2015;26:651–60.

[11] Qiao Y, Sun J, Xia S, Tang X, Shi Y, Le G. Effects of resveratrol on gut microbiota and fat storage in a mouse model with high-fat-induced obesity. Food Funct 2014;5:1241–9.

[12] Chen ML, Yi L, Zhang Y, Zhou X, Ran L, Yang J, et al. Resveratrol attenuates trimethylamine-N-oxide (TMAO)-induced atherosclerosis by regulating TMAO synthesis and bile acid metabolism via remodeling of the gut microbiota. MBio 2016;7:e02210-02215.

[13] Sun L, Wang Y, Song Y, Cheng X-R, Xia S, Rahman MRT, et al. Resveratrol restores the circadian rhythmic disorder of lipid metabolism induced by high-fat diet in mice. Biochem Biophys Res Commun 2015;458:86–91.

[14] Ribas-Latre A, Del Bas JM, Baselga-Escudero L, Casanova E, Arola-Arnal A, Salvadó M-J, et al. Dietary proanthocyanidins modulate melatonin levels in plasma and the expression pattern of clock genes in the hypothalamus of rats. Mol Nutr Food Res 2015;59:865–78.

[15] Ribas-Latre A, Baselga-Escudero L, Casanova E, Arola-Arnal A, Salvadó MJ, Arola L, et al. Chronic consumption of dietary proanthocyanidins modulates peripheral clocks in healthy and obese rats. J Nutr Biochem 2015;26:112–9.

[16] Gil-Cardoso K, Ginés I, Pinent M, Ardévol A, Arola L, Blay M, et al. Chronic supplementation with dietary proanthocyanidins protects from diet-induced intestinal alterations in obese rats. Mol Nutr Food Res 2017;61.

[17] Serrano J, Casanova-Martí À, Gil-Cardoso K, Blay MT, Terra X, Pinent M, et al. Acutely administered grape-seed proanthocyanidin extract acts as a satiating agent. Food Funct 2016;7:483–90.

[18] Montagut G, Bladé C, Blay M, Fernández-Larrea J, Pujadas G, Salvadó MJ, et al. Effects of a grapeseed procyanidin extract (GSPE) on insulin resistance. J Nutr Biochem 2010;21:961–7.

[19] Margalef M, Pons Z, Iglesias-Carres L, Arola L, Muguerza B, Arola-Arnal A. Gender-related similarities and differences in the body distribution of grape seed flavanols in rats. Mol Nutr Food Res 2016;60:760–72.

[20] Cipolla-Neto J, Amaral FG, Afeche SC, Tan DX, Reiter RJ. Melatonin, energy metabolism, and obesity: A review. J Pineal Res 2014;56:371–81.

[21] Rosenbaum M, Leibel RL. Role of leptin in energy homeostasis in humans. J Endocrinol 2014;223:T83–96.

[22] Maffei M, Halaas J, Ravussin E, Pratley RE, Lee GH, Zhang Y, et al. Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. Nat Med 1995;1:1155–61.

[23] Considine R V, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW, Nyce MR, et al. Serum immunoreactive-leptin concentrations in normal-weight and obese humans. N Engl J Med 1996;334:292–5.

[24] Kalra SP, Bagnasco M, Otukonyong EE, Dube MG, Kalra PS. Rhythmic, reciprocal ghrelin and leptin signaling: new insight in the development of obesity. Regul Pept 2003;111:1–11.

[25] Bray MS, Young ME. Circadian rhythms in the development of obesity: potential role for the circadian clock within the adipocyte. Obes Rev 2007;8:169–81.

[26] Gómez Abellán P, Gómez Santos C, Madrid JA, Milagro FI, Campion J, Martínez JA, et al. Site-specific circadian expression of leptin and its receptor in human adipose tissue. Nutr Hosp 2011;26:1394–401.

[27] Hall KD. A review of the carbohydrate-insulin model of obesity. Eur J Clin Nutr 2017;71:323–6.

[28] Thaiss CA, Levy M, Korem T, Dohnalová L, Shapiro H, Jaitin DA, et al. Microbiota Diurnal Rhythmicity Programs Host Transcriptome Oscillations. Cell 2016;167:1495–510.

[29] La Fleur SE, Kalsbeek A, Wortel J, Buijs RM. A suprachiasmatic nucleus generated rhythm in basal glucose concentrations. J Neuroendocrinol 1999;11:643–52.

[30] Ando H, Ushijima K, Shimba S, Fujimura A. Daily fasting blood glucose rhythm in male mice: A role of the circadian clock in the liver. Endocrinology 2016;157:463–9.

[31] Ibars M, Ardid-Ruiz A, Suárez M, Muguerza B, Bladé C, Aragonès G. Proanthocyanidins potentiate hypothalamic leptin/STAT3 signalling and Pomc gene expression in rats with diet-induced obesity. Int J Obes 2017;41:129–36.

[32] Ginés I, Gil-Cardoso K, Serrano J, Casanova-Martí À, Blay Mt, Pinent M, et al. Effects of an intermittent grape-seed proanthocyanidin (GSPE) treatment on a cafeteria diet obesogenic challenge in rats. Nutrients 2018;10:315.

[33] Liang X, Bushman FD, FitzGerald GA. Rhythmicity of the intestinal microbiota is regulated by gender and the host circadian clock. Proc Natl Acad Sci 2015.

[34] Reinke H, Asher G. Crosstalk between metabolism and circadian clocks. Nat Rev Mol Cell Biol 2019.

[35] Thaiss CA, Zeevi D, Levy M, Zilberman-Schapira G, Suez J, Tengeler AC, et al. Transkingdom control of microbiota diurnal oscillations promotes metabolic homeostasis. Cell 2014.

[36] Leone V, Gibbons SM, Martinez K, Hutchison AL, Huang EY, Cham CM, et al. Effects of diurnal variation of gut microbes and high-fat feeding on host circadian clock function and metabolism. Cell Host Microbe 2015;17:681–9.

[37] Challet E. The circadian regulation of food intake. Nat Rev Endocrinol 2019;15:393–405.

[38] Koh A, De Vadder F, Kovatcheva-Datchary P, Bäckhed F. From Dietary Fiber to Host Physiology: Short-Chain Fatty Acids as Key Bacterial Metabolites. Cell 2016;165:1332–45.

[39] Zhang SL, Bai L, Goel N, Bailey A, Jang CJ, Bushman FD, et al. Human and rat gut microbiome composition is maintained following sleep restriction. Proc Natl Acad Sci U S A 2017;114:E1564-71.

[40] Casanova-Martí À, Serrano J, Portune KJ, Sanz Y, Blay MT, Terra X, et al. Grape seed proanthocyanidins influence gut microbiota and enteroendocrine secretions in female rats. Food Funct 2018;9:1672–82.

[41] Claesson MJ, Jeffery IB, Conde S, Power SE, O'connor EM, Cusack S, et al. Gut microbiota composition correlates with diet and health in the elderly. Nature 2012;488:178–84.

[42] van Passel MWJ, Kant R, Palva A, Lucas S, Copeland A, Lapidus A, et al. Genome sequence of Victivallis vadensis ATCC BAA-548, an anaerobic bacterium from the phylum Lentisphaerae, isolated from the human gastrointestinal tract. J Bacteriol 2011;193:2373–4.

[43] Challet E. Interactions between light, mealtime and calorie restriction to control daily timing in mammals. J Comp Physiol B Biochem Syst Environ Physiol 2010.

[44] Hatori M, Vollmers C, Zarrinpar A, DiTacchio L, Bushong EA, Gill S, et al. Time-restricted feeding without reducing caloric intake prevents metabolic diseases in mice fed a high-fat diet. Cell Metab 2012.

[45] Iris G, Katherine G-CJ, Serrano; Àngela C-MM 5 L, Ximena TM, Teresa B, Anna A. Long-lasting effects of proanthocyanidins in rat prevent cafeteria diet effects 2 by limiting adipose tissue accrual Long-lasting effects of GSPE limiting WAT accrual Montserrat Pinent n.d.

[46] Baselga-Escudero L, Pascual-Serrano A, Ribas-Latre A, Casanova E, Salvadó MJ, Arola L, et al. Long-term supplementation with a low dose of proanthocyanidins normalized liver miR-33a and miR-122 levels in high-fat diet-induced obese rats. Nutr Res 2015.

[47] Pajuelo D, Díaz S, Bladé C, Arola-Arnal A, Fernández-Iglesias A, Arola L, et al. Chronic dietary supplementation of proanthocyanidins corrects the mitochondrial dysfunction of brown adipose tissue caused by diet-induced obesity in Wistar rats. Br J Nutr 2012;107:170–8.

[48] Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, et al. Metabolic endotoxemia initiates obesity and insulin resistance. Diabetes 2007.

[49] Salvini F, Riva E, Salvatici E, Boehm G, Jelinek J, Banderali G, et al. A Specific Prebiotic Mixture Added to Starting Infant Formula Has Long-Lasting Bifidogenic Effects. J Nutr 2011.

[50] Wang M, Chen Y, Wang Y, Li Y, Zheng H, Ma F, et al. The effect of probiotics and polysaccharides on the gut microbiota composition and function of weaned rats. Food Funct 2018;9:1864–77.

[51] Zhang H, DiBaise JK, Zuccolo A, Kudrna D, Braidotti M, Yu Y, et al. Human gut microbiota in obesity and after gastric bypass. Proc Natl Acad Sci 2009;106:2365–70.

[52] Benedict C, Vogel H, Jonas W, Woting A, Blaut M, Schürmann A, et al. Gut microbiota and glucometabolic alterations in response to recurrent partial sleep deprivation in normal-weight young individuals. Mol Metab 2016;5:1175–86.

[53] Archer SN, Oster H. How sleep and wakefulness influence circadian rhythmicity: Effects of insufficient and mistimed sleep on the animal and human transcriptome. J Sleep Res 2015;24:476–93.

[54] Li G, Xie C, Lu S, Nichols RG, Tian Y, Li L, et al. Intermittent Fasting Promotes White Adipose Browning and Decreases Obesity by Shaping the Gut Microbiota. Cell Metab 2017.

[55] Riske L, Thomas RK, Baker GB, Dursun SM. Lactate in the brain: an update on its relevance to brain energy, neurons, glia and panic disorder. Ther Adv Psychopharmacol 2017.

[56] Smith D, Pernet A, Hallett WA, Bingham E, Marsden PK, Amiel SA. Lactate: A preferred fuel for human brain metabolism in vivo. J Cereb Blood Flow Metab 2003.

[57] Hashimoto T, Tsukamoto H, Takenaka S, Olesen ND, Petersen LG, Sørensen H, et al. Maintained exercise-enhanced brain executive function related to cerebral lactate metabolism in men. FASEB J 2018.

[58] Tahara Y, Yamazaki M, Sukigara H, Motohashi H, Sasaki H, Miyakawa H, et al. Gut Microbiota-Derived Short Chain Fatty Acids Induce Circadian Clock Entrainment in Mouse Peripheral Tissue. Sci Rep 2018.

[59] Kaczmarek JL, Thompson S V., Holscher HD. Complex interactions of circadian rhythms, eating behaviors, and the gastrointestinal microbiota and their potential impact on health. Nutr Rev 2017;75:673–82.

[60] Kim KA, Gu W, Lee IA, Joh EH, Kim DH. High Fat Diet-Induced Gut Microbiota Exacerbates Inflammation and Obesity in Mice via the TLR4 Signaling Pathway. PLoS One 2012.

[61] Duca FA, Sakar Y, Lepage P, Devime F, Langelier B, Doré J, et al. Replication of obesity and associated signaling pathways through transfer of microbiota from obese-prone rats. Diabetes 2014.

[62] Neyrinck AM, Possemiers S, Verstraete W, De Backer F, Cani PD, Delzenne NM. Dietary modulation of clostridial cluster XIVa gut bacteria (Roseburia spp.) by chitin-glucan fiber improves host metabolic alterations induced by high-fat diet in mice. J Nutr Biochem 2012.

[63] Parkar S, Kalsbeek A, Cheeseman J. Potential Role for the Gut Microbiota in Modulating Host Circadian Rhythms and Metabolic Health. Microorganisms 2019;7:E41.

[64] Liu Z, Huang M, Wu X, Shi G, Xing L, Dong Z, et al. PER1 phosphorylation specifies feeding rhythm in mice. Cell Rep 2014;7:1509–20.

[65] Lozupone CA, Stombaugh JI, Gordon JI, Jansson JK, Knight R. Diversity, stability and resilience of the human gut microbiota. Nature 2012;489:220–30.

[66] Deaver JA, Eum SY, Toborek M. Circadian disruption changes gut microbiome taxa and functional gene composition. Front Microbiol 2018;9.

[67] Li S, Qi C, Zhu H, Yu R, Xie C, Peng Y, et al. Lactobacillus reuteri improves gut barrier function and affects diurnal variation of the gut microbiota in mice fed a high-fat diet. Food Funct 2019;10:4705–15.

[68] Thaiss CA, Zeevi D, Levy M, Segal E, Elinav E. A day in the life of the meta-organism: Diurnal rhythms of the intestinal microbiome and its host. Gut Microbes 2015;6:137–42.

[69] Jiang J, Hang X, Zhang M, Liu X, Li D, Yang H. Diversity of bile salt hydrolase activities in different lactobacilli toward human bile salts. Ann Microbiol 2010;60:81–8.

[70] Sayin SI, Wahlström A, Felin J, Jäntti S, Marschall H-U, Bamberg K, et al. Gut microbiota regulates bile acid metabolism by reducing the levels of tauro-beta-muricholic acid, a naturally occurring FXR antagonist. Cell Metab 2013;17:225–35.

[71] Li F, Jiang C, Krausz KW, Li Y, Albert I, Hao H, et al. Microbiome remodelling leads to inhibition of intestinal farnesoid X receptor signalling and decreased obesity. Nat Commun 2013;4:2384.

[72] Jiang C, Xie C, Lv Y, Li J, Krausz KW, Shi J, et al. Intestine-selective farnesoid X receptor inhibition improves obesity-related metabolic dysfunction. Nat Commun 2015;6:10166.

[73] Ignacio A, Fernandes MR, Rodrigues VAA, Groppo FC, Cardoso AL, Avila-Campos MJ, et al. Correlation between body mass index and faecal microbiota from children. Clin Microbiol Infect 2016;22:258.e1-8.

[74] Prawitt J, Abdelkarim M, Stroeve JHM, Popescu I, Duez H, Velagapudi VR, et al. Farnesoid X receptor deficiency improves glucose homeostasis in mouse models of obesity. Diabetes 2011;60:1861–71.

[75] Wang J, Tang H, Zhang C, Zhao Y, Derrien M, Rocher E, et al. Modulation of gut microbiota during probiotic-mediated attenuation of metabolic syndrome in high fat diet-fed mice. ISME J 2015;9:1–15.

[76] Yadav H, Jain S, Sinha PR. Antidiabetic effect of probiotic dahi containing Lactobacillus acidophilus and Lactobacillus casei in high fructose fed rats. Nutrition 2007;23:62–8.

[77] Del Bas JM, Guirro M, Boqué N, Cereto A, Ras R, Crescenti A, et al. Alterations in gut microbiota associated with a cafeteria diet and the physiological consequences in the host. Int J Obes 2018;42:746–54.

[78] Rivière A, Selak M, Lantin D, Leroy F, De Vuyst L. Bifidobacteria and butyrate-producing colon bacteria: Importance and strategies for their stimulation in the human gut. Front Microbiol 2016;7.

[79] Ginés I, Gil-Cardoso K, Terra X, Blay Mt, Pérez-Vendrell AM, Pinent M, et al. Grape Seed Proanthocyanidins Target the Enteroendocrine System in Cafeteria-Diet-Fed Rats. Mol Nutr Food Res 2019;63:e1800912.

[80] Chambers ES, Preston T, Frost G, Morrison DJ. Role of Gut Microbiota-Generated Short-Chain Fatty Acids in Metabolic and Cardiovascular Health. Curr Nutr Rep 2018;7:198–206.

[81] Ribas-Latre A, Baselga-Escudero L, Casanova E, Arola-Arnal A, Salvadó MJ, Bladé C, et al. Dietary proanthocyanidins modulate BMAL1 acetylation, Nampt expression and NAD levels in rat liver. Sci Rep 2015;5:10954.

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CHAPTER IV

Prebiotic Potential of Natural Ingredients in Lean and Obese Microbiota: an *In Vitro* Fermentation Study.

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ABSTRACT

The global prevalence of obesity and related metabolic complications is continuously increasing, and the gut microbiota has been targeted to address this challenge. The consumption of prebiotics may help to prevent or mitigate obesity. In addition, the food industry generates large amounts of discarded residues that could be revalorized immediately, thereby reducing their negative economic and ecological impacts. In this study, we aimed to evaluate the prebiotic capacity of a broad range of natural ingredients including industrial by-products. For this purpose, the selected candidates including food skins, husks, shells, trimming residues, mosses and mushrooms were subjected to *in vitro* fermentation with fecal microbiota obtained from lean and obese adults. The pH changes, gas production and SCFA profiles observed during fermentation were evaluated as markers of microbial activity. Our results indicated that pumpkin skin was the most promising ingredient to be revaluated as a new prebiotic compound, showing pH-lowering effects accompanied by increased gas and SCFA production over time. Furthermore, we also demonstrated a high prebiotic potential for brewers' spent grain (BSG), whereas certain mushrooms such as *Armillaria mellea* and *Boletus edulis*, Irish moss, plum skin and quinoa husk, also exhibited prebiotic properties. Importantly, differential SCFA profiles were identified between obese and lean microbiota after 24 h fermentation, with major propionate production being observed in obese microbiota. Potential prebiotic candidates showed similar fermentation rates between both lean and obese conditions, suggesting that fermentative processes were mostly substrate-dependent. In conclusion, this study reveals for the first time that pumpkin skin shows

a relevant prebiotic potential when fermented with both lean and obese microbiota. To validate our results, the prebiotic effects of our most promising candidates should be further evaluated *in vivo* in animal models.

Key words: prebiotic ingredients, industrial by-products, lean microbiota, obese microbiota, *in vitro* screening, fermentation, obesity.

1. INTRODUCTION

The microbial communities living to mammals' gastrointestinal tract provide a wide range of essential metabolites that hosts are not able to synthetize on their own [1]. Our microbiome takes advantage of the dietary components that reach the large intestine, such as indigestible plant polysaccharides, which are primarily transformed into short chain fatty acids (SCFA) [2,3]. These bacterial products have been related to multiple beneficial effects on mammalian energy metabolism, mainly through the signalling of satiety and the enhancement of energy expenditure, whereas a healthy gut microbiota composition is a key determinant to its balanced production [4,5]. In this scenario, both microbes and host co-live in a mutually beneficial symbiosis. However, exogenous events, such as stress, drugs and diet, can alter the phylogenetic structure and function of gut microbiota [6,7], leading to the impairment of host homeostasis.

In recent years, the gut microbiota has been identified as a key element in the aetiology of obesity [8]. Accordingly, microbiota actively contribute to host metabolic efficiency by enhancing energy harvest from the diet [9]. In this sense, the lack of gut microbiota prevents the

development of diet-induced obesity in rodents [10], whereas obesity-resistant germ-free mice become obese after receiving a microbiota transplant [11]. Although human studies have shown controversial results concerning the contributory role of specific phyla to the obese phenotype, changes in the prevalence of certain species and the diversity of bacterial communities between lean and obese individuals have been widely described [12–14], highlighting the importance of the intestinal microbiome in obesity progression. Thus, the dietary modulation of the gut microbiome has become one of the major targets in the prevention of metabolic diseases [15]. In this regard, the prebiotic potential of some dietary fibers, which are the main substrates for gut microbiota, has been reported by several researchers [16,17]. For instance, inulin-type fructans favour the growth of beneficial bacteria, SCFA production, fat oxidation and reduced food intake during overweight and obesity [18–20].

Every year, the food industry generates large amounts of food wastes or by-products that negatively impact the biosphere because of their poor oxidative and biological stability [21], representing one major concern around the world. Currently, the circular economy is emerging as a new sustainable approach to confront the substantial economic losses for manufacturing industries related to waste production [22]. In this model, the demand to optimize the impact of industrial by-products on society is addressed through the management and revalorization of food wastes by benefitting from their nutritional properties. In this sense, some food by-products such as husks, skins, seeds, shells and trimming residues are mostly composed by highly valuable components, such as

polysaccharides, proteins, fibers, phytochemicals and bioactive compounds.

In vitro methods for screening untapped prebiotic candidates enable a simple and rapid evaluation of its impact on the ecosystem and the microbial community response. Several publications have obtain encouraging results in evaluating the potential prebiotic effects of different industrial by-products, such as coffee wastes [23], fruit peels [24], soybean Okara [25] and Aloe Vera mucilage [26], using *in vitro* fermentation procedures. In this study, we aimed to evaluate the prebiotic capacity of a wide range of industrial by-products in both normoweight and obese conditions using an *in vitro* fermentation procedure with human fecal samples. To this end, several industrial residues including husks, shells, skins, grains and branches, as well as other potentially miscellaneous candidates, were tested.

2. MATERIALS AND METHODS

2.1 Sample preparation.

The industrial by-products used in this screening study included nutshell (mixed varieties including Alcalde, Onteniente and Baldo II, Carcagente, Cerdá, Escribá and Villena, Tarragonès, Tarragona, Spain), rice husk (Montsianell variety, *Oryza sativa*, El Montsià, Tarragona, Spain), brewers' spent grain (BSG) (white IPA-derived residues, Alcover, Tarragona, Spain), pumpkin skin (*Curcubita maxima*, La Pobla de Montornès, Tarragona, Spain), olive press cake (OPC) (Arbequina variety, *Oleo europea*, Tarragonès, Tarragona, Spain), stevia branches (*Stevia rebaudiana bertoni*, Balaguer, Lleida, Spain), quinoa husk

(*Quenopodium quinoa*, Almenar, Lleida, Spain), and plum skin (*Prunus domestica*, Lleida, Spain). We also tested the potential prebiotic activity of some miscellaneous ingredients including fungus (*Boletus edulis*, Ripollès, Girona, Spain; *Armillaria mellea*, Solsonès, Lleida, Spain) and Irish moss (*Chondrus crispus*; A Coruña, Galicia, Spain). All products were accurately selected according to their nutrient composition, essentially pointing up the fiber content shown in the literature, and the products were obtained from local manufacturers as detailed above. Inulin (Orafti® HP, BENEO GmbH, Germany) and cellulose (C6288, Merck, Spain) were used as positive and negative controls, respectively. All samples were stored at -80ºC upon arrival until they were manually grounded, crushed with a grinder and lyophilised. Dry samples were stored at room temperature, properly protected from light and preserved from moisture in a desiccator.

2.2 In vitro digestion.

Prior to the fermentation procedure, an *in vitro* gastrointestinal digestion was conducted to simulate the mammalian conditions after food intake, which was performed as previously described [27] with slight modifications. Briefly, lyophilised samples were diluted with pH 7.0-controlled 1X phosphate buffer saline (PBS) at a 3:50 (weight-to-volume) ratio and subsequently heated to 37ºC for 10 min. During the entire procedure, samples were maintained under magnetic stirring and temperature-controlled conditions (37ºC). The oral phase was simulated by adding 50 µL of α-amylase from human saliva (A1031, Merck, Spain) and incubating for 15 min. To reproduce the gastric phase, the pH was set to 2.0 ± 0.1 with HCl, 250 µL of 1 mg/mL pepsin from porcine gastric mucosa (P7012, Merck, Spain) was added and the solution was

incubated for 30 min. During the intestinal phase, pH was readjusted to 6.9 ± 0.1 with NaOH before adding 1 mL of 50 mg/mL pancreatin from porcine pancreas (P7545, Merck, Spain) and incubated for 3 h. Samples were transferred into dialysis membranes with a 1000 Da cut off (Spectra/Por 1000 Da MWCO Dialysis Tubing, Spectrum Laboratories Inc., UK) as previously described [28]. The dialysis procedure was performed to reflect the absorption of low-molecular-weight compounds before reaching the large intestine during digestion. After 24 h under constant water circulation, the digestion residues were lyophilised and kept into a desiccator at room temperature until they were used for *in vitro* fermentation.

2.3 Donors and fecal sample collection.

Fecal samples from male adult volunteers between 30 and 50 years-old were collected for *in vitro* fermentation. Subjects were excluded if they met any of the following criteria: recent use of antibiotics or nutraceutics, chronic alcohol intake, and presence of gastrointestinal pathologies. All procedures were approved by the Ethics Committee on Clinic Investigation from the Hospital Sant Joan, Reus, Spain. Six adult volunteers were included in the study, including three normoweight (BMI = 20-25 kg/m^2) and three obese (BMI = 30-35 kg/m^2) individuals. Donors were previously instructed to defecate directly into a sterile recipient and quickly placed into an anaerobic bag system (Bio-Bag type C, BD, Germany) including an anaerobic sachet to ensure the maintenance of a CO_2 atmosphere. The samples were immediately refrigerated and brought to the laboratory within the next 12 h. Once obtained, fecal samples were pooled according to groups (normoweight

or obese), and the inoculum was freshly prepared for *in vitro* fermentation as described below.

2.4 Fermentation protocol.

The *in vitro* fermentation experiment was performed under strict sterile conditions at 37ºC and mostly maintained an anaerobic atmosphere during the entire procedure. The fermentation media was composed of 2 g peptone water (CM0009, Oxoid, United Kingdom), 2 g yeast extract (Y4250, Merck, Spain), 100 mg sodium chloride (S5886, Merck, Spain), 40 mg potassium phosphate dibasic (121512, PanReac AppliChem, Germany), 40 mg potassium phosphate monobasic (131509, PanReac AppliChem, Germany), 10 mg magnesium sulphate heptahydrate (230391, Merck, Spain), 10 mg calcium chloride dehydrate, 2 g sodium bicarbonate (S5761, Merck, Spain), 2 mL Tween 80 (P4780, Merck, Spain), 0.5 g bile salt (B8631, Merck, Spain) and 50 mg hemin (H9039, Merck, Spain). The pH was set to 7.0 ± 0.1 before adding 4 mL of 0.025% resazurin (R7017, Merck, Spain), which acts as an indicator of anaerobic conditions through colour change. The fermentation media was autoclaved, and 10 µL of vitamin K1 (v3501, Merck, Spain) was added prior to starting the experimental procedure. Fifty milligrams of each digestion-derived product was placed into sterile Hungate tubes (2047-16125, Bellco Glass Inc., USA) with rubber and screw caps. Then, 4 mL of media along with 100 µL of oxyrase (30620059-1, bioWORLD, USA), which removes the oxygen from the environment, was added at each fermentation tube and kept in an anaerobic atmosphere for 30 min at 37ºC for its hydration. To prepare the inoculums, pooled fecal samples were diluted into PBS 0.1 M at 10% (w/v), maintained at 37ºC under a flux of CO_2 gas to ensure anaerobic conditions, and finally filtered

through a sterile 1 mm nylon mesh (FMNY 250, Filter Lab, Spain). Once the residues were properly hydrated, 1 mL of inoculum was added to each tube using a syringe, going through its rubber cap. Triplicates for each condition were maintained in a shaking water bath at 37ºC for 0 h, 4 h or 24 h until 200 µL of copper sulphate (209198, Merck, Spain) at 2.75 mg/mL was added to inhibit further fermentation.

2.5 In situ determinations.

Total gas volume was measured by inserting an empty graduated syringe through the rubber cap and measuring the plunger displacement after 24 h of fermentation. At the corresponding time point, all reactions were inhibited as described above and 400 µL per tube were aliquoted into 1.5 mL sterile tubes and frozen at -80ºC for further metabolomic analysis. Finally, pH was measured with a pH Meter (PHS-3D, Shanghai San-Xin Instrumentation, Inc., China) previously calibrated.

After *in vitro* fermentation, the supernatants obtained were subjected to metabolomic analysis using nuclear magnetic resonance 1H (1H NMR). Three hundred µL of the fermented solution was centrifuged to eliminate particulates (21000 g, 25 min, 4°C). Then, 400 µL of 0.05 M PBS buffer in D_2O (pH 7.2, TSP 0.7 mM) was added, vigorously vortexed, sonicated until complete homogenization and centrifuged (14000 g, 5 min, 25ºC). For NMR measurement, 600 µL of the upper phase was placed into a 5 mm O.D. NMR tube. 1H NMR spectra were recorded at 300 K on an Avance III 600 spectrometer (Bruker, Germany) operating at a proton frequency of 600.20 MHz using a 5 mm PBBO gradient probe. One-dimensional (1D) 1H pulse experiments were carried out using the nuclear Overhauser effect spectroscopy (NOESY- Noesypr1d pulse

programme in Bruker). Solvent presaturation with an irradiation power of 160 uW was applied during recycling delay (5 s) and mixing time (100 ms). The 90° pulse length was calibrated for each sample and varied from 10.31 to 11.12 ms. The spectral width was 9.6 kHz (16 ppm), and a total of 256 transients were collected into 64 k data points for each 1H spectrum. The exponential line broadening applied before Fourier transformation was 0.3 Hz. The frequency domain spectra were manually phased and baseline-corrected using Topspin software (v. 3.2, Bruker). Metabolite identification and quantification were performed using HMDB, AMIX 3.9 (Bruker) and Chenomx ® software spectra databases.

2.6 Statistical analysis.

All results are expressed as the mean ± standard error of the mean (SEM). Because the fermentation procedure was not performed on all samples at the same time, the negative control was properly used to correct the pH measurements and SCFA results, which were expressed as the relative variation within each time point. To assess significant differences regarding the relative variation of the measurements between ingredients and our positive control (inulin), statistical analyses were based on repeated measures ANOVA within each condition (normoweight or obese microbiota). In the case of a significant ingredient x time interaction, the main effects within each time point were studied through one-way ANOVA followed by Sidak post hoc test. A probability of $p < 0.05$ was considered to be statistically significant. Statistical analyses were performed using SPSS 22 software (SPSS, Inc., Chicago, IL, USA). Principal component analysis (PCA) was performed

through the multivariate modelling of metabolomics data in MATLAB (MathWorks) using in-house scripts as previously described [29].

3. RESULTS

3.1 pH changes and total gas production.

Overall, the pH changes were closely related to those observed in gas production. Repeated measures ANOVA revealed a significant ingredient x time interaction ($p < 0.01$) explaining the pH variation within both normoweight and obese conditions (**Figure 1**). After 4 h of fermentation and focusing on normoweight samples, pumpkin skin induced the highest pH decrease (18%), followed by quinoa husk, plum skin and BSG (16%, 15% and 11%, respectively). In this instance, other substrates such as *Armillaria mellea* and *Boletus edulis* caused a slight drop in pH that was significantly higher than that promoted by nutshell, rice husk and Irish moss, which showed the largest pH values. However, these values were all significantly different from those obtained with inulin, which induced a decrease in pH of 29%. Importantly, pumpkin skin triggered the highest pH decrease (17%) in obese fecal samples after 4 hours of fermentation, showing no significant differences when compared to inulin (23%). In addition, substantial pH-lowering effects were also attributed to BSG (12%), plum skin (10%) and quinoa husk (9%).

After 24 h of fermentation, the pH decreased up to 32% and 29% when inulin was included as a fermentable substrate in normoweight and obese conditions, respectively (**Figure 1**). Overall, the pH-lowering effects observed in normoweight fecal samples at 4 h were maintained

after 24 h by pumpkin skin, plum skin and BSG ingredients. In addition, other ingredients showing marginal pH-lowering effects at 4 h drove an important decrease in pH at the end time point, such as *Armillaria mellea*, *Boletus edulis* and Irish moss, within both normoweight and obese conditions. However, nutshell, rice husk, OPC and stevia branches did not induce changes in pH during the entire fermentation period within either normoweight or obese individuals.

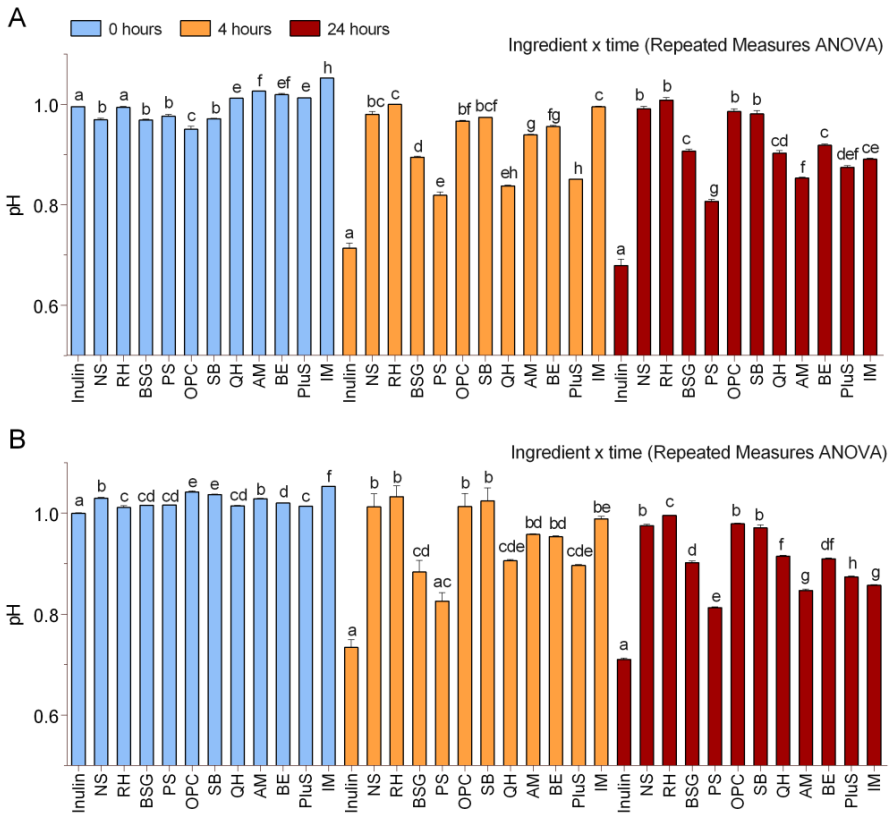

Figure 1. Normalized pH values of the fecal slurries after 0 h, 4 h and 24 h of *in vitro* fermentation using fecal inoculums from lean (A) and obese (B) individuals. The pH measurements obtained from ingredient fermentation were properly corrected by negative controls within the same time point and expressed as the relative pH variation. Data are expressed as the mean ± SEM (n = 3). Ingredient x time interactions were assessed using repeated measures

ANOVA. The main effects were evaluated and different letters represent significant differences among ingredients within each time point (one-way ANOVA followed by Sidak post hoc test). AM. *Armillaria mellea*; BE, *Boletus edulis*; BSG, brewers' spent grain; IM, Irish moss (*Chondrus crispus*); NS, nutshell, OPC, olive press cake; PluS, plum skin; PS, pumpkin skin; QH, quinoa husk; RH, rice husk; SB, stevia branches.

In vitro fermentation of fecal samples from both normoweight and obese individuals induced gas production at the end of the incubation period (24 h), which acted as an indicator of particular fermentation rates depending on the tested substrates, as shown in **Figure 2**. Prominently, *Armillaria mellea* and pumpkin skin showed the highest fermentation rate within normoweight conditions, reaching 42.2% and 34.7% of the total amount of gas produced by the positive control and significantly differing from the rest of the ingredients. Moreover, quinoa husk, *Boletus edulis* and BSG produced 18.6%, 16.7% and 13.4% of the total gas volume generated by inulin, respectively, showing no significant differences between them. In contrast, nutshell generated significantly lower amounts of gas than the other substrates, whereas rice husk, OPC, stevia branches and Irish moss did not promote gas production after 24 h of fermentation. Similarly, the highest gas production within the obese condition was observed for pumpkin skin (33.1%), *Armillaria mellea* (27.9%), *Boletus edulis* (25.3%), BSG (23.9%), plum skin (17.3%) and quinoa husk (13.6%) after 24 h (**Figure 2**). Finally, Irish moss and nutshell were identified as the ingredients inducing the lowest gas production, while no gas generation was detected when rice husk, OPC or stevia branches were used as fermentable substrates, as previously described within normoweight conditions.

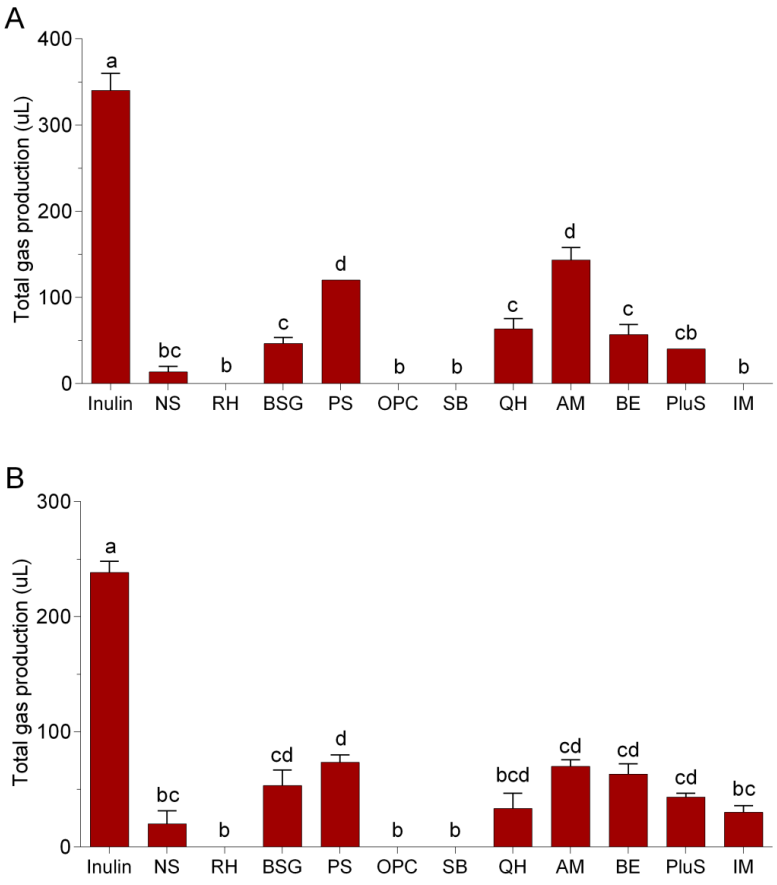

Figure 2. Absolute gas production measured after 24 h of in vitro fermentation using fecal inoculums from lean (A) and obese (B) individuals. Data are expressed as the mean ± SEM (n = 3). Different letters represent significant differences among ingredients (one-way ANOVA followed by Sidak post hoc test). AM. Armillaria mellea; BE, Boletus edulis; BSG, brewers' spent grain; IM, Irish moss (Chondrus crispus); NS, nutshell, OPC, olive press cake; PluS, plum skin; PS, pumpkin skin; QH, quinoa husk; RH, rice husk; SB, stevia branches.

3.2 SCFA production.

The relative amounts of SCFAs produced after 0 h, 4 h and 24 h are presented in **Table 1**. Overall, we found ingredient-dependent significant variations in the SCFA butyrate, propionate and acetate production over

time in both normoweight and obese conditions ($p < 0.001$, repeated measures ANOVA).

Focusing on normoweight samples (**Table 1.1**), pairwise comparisons revealed that pumpkin skin, BSG and stevia branches promoted a similar generation of total SCFAs than inulin at 4 h, although it was not maintained at the end time point (24 h). Surprisingly, pumpkin skin and BSG induced a greater increase in butyrate production than inulin after 4 h, denoting significant differences. In addition, while BSG fermentation resulted in a lower butyrate concentration after 24 h, pumpkin skin maintained an elevated butyrate formation emerging as the only ingredient showing no significant differences when compared to inulin. On the other hand, stevia branches were the unique ingredient presenting an increment of butyrate similar to that induced by our positive control after 4 h of fermentation.

Inulin fermentation did not yield propionate production after 4 or 24 h. Most of the ingredients also showed a lack of propionate formation during the first 4 h of fermentation, whereas nutshell, stevia branches and pumpkin skin did not differ from inulin at the end of the study. Other ingredients including *Armillaria mellea*, *Boletus edulis* and Irish moss, significantly differed from the inulin control because of an increase in propionate generation at 24 h. Furthermore, the concentration of acetate followed an inulin-like pattern only when pumpkin skin was used as a fermentable substrate, showing acetate overproduction within the first 4 h of fermentation. However, this effect was not maintained after 24 h, where acetate production induced by all of the ingredients significantly differed from that promoted by inulin.

Table 1.1. SCFA production after 0 h, 4 h and 24 h of *in vitro* fermentation of the lean faecal microbiota.

		Inulin	Nutshell	Rice husk	BSG	Pumpkin skin	OPC	Stevia branches	Quinoa husk	*A. mellea*	*B. edulis*	Plum skin	Irish moss
Butyrate	0h	1.0 ± 0.0	0.9 ± 0.0	1.0 ± 0.0	1.3 ± 0.0*	0.9 ± 0.1	1.2 ± 0.0	1.0 ± 0.0	1.1 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	0.7 ± 0.0*	1.0 ± 0.0
	4h	2.1 ± 0.1	1.1 ± 0.0*	1.1 ± 0.0*	2.9 ± 0.1*	3.3 ± 0.1*	1.1 ± 0.2*	1.7 ± 0.0	1.3 ± 0.1*	1.2 ± 0.0*	1.1 ± 0.1*	1.2 ± 0.1*	1.4 ± 0.1*
	24h	3.5 ± 0.2	1.1 ± 0.0*	1.0 ± 0.0*	2.5 ± 0.1*	3.2 ± 0.1	1.1 ± 0.1*	1.4 ± 0.1*	1.6 ± 0.0*	1.6 ± 0.0*	1.3 ± 0.0*	1.3 ± 0.0*	1.2 ± 0.0*
Propionate	0h	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.1 ± 0.0	0.9 ± 0.1	1.0 ± 0.0	0.9 ± 0.0	1.0 ± 0.0	1.1 ± 0.0	1.5 ± 0.0*	1.0 ± 0.0	1.1 ± 0.0
	4h	0.8 ± 0.0	1.1 ± 0.0	1.1 ± 0.1	1.2 ± 0.0*	1.2 ± 0.0*	1.1 ± 0.2	1.3 ± 0.0*	0.8 ± 0.0	1.0 ± 0.1	1.3 ± 0.1*	0.9 ± 0.1	1.3 ± 0.1*
	24h	0.8 ± 0.0	1.0 ± 0.0	1.1 ± 0.0*	1.0 ± 0.0*	0.9 ± 0.0	1.1 ± 0.1*	1.0 ± 0.0	1.1 ± 0.0*	1.5 ± 0.0*	1.5 ± 0.0*	1.1 ± 0.0*	1.6 ± 0.0*
Acetate	0h	1.1 ± 0.0	1.1 ± 0.0	1.0 ± 0.0	1.1 ± 0.0	1.0 ± 0.1	1.1 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.1	1.1 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
	4h	3.3 ± 0.1	1.2 ± 0.1*	1.1 ± 0.1*	2.2 ± 0.0*	2.8 ± 0.0	1.2 ± 0.2*	1.6 ± 0.1*	1.5 ± 0.1*	1.2 ± 0.1*	1.3 ± 0.1*	1.5 ± 0.1*	1.3 ± 0.1*
	24h	2.5 ± 0.0	1.1 ± 0.0*	1.0 ± 0.0*	1.5 ± 0.0*	1.6 ± 0.1*	1.0 ± 0.0*	1.2 ± 0.1*	1.6 ± 0.0*	1.6 ± 0.1*	1.6 ± 0.0*	1.6 ± 0.0*	1.6 ± 0.1*
Total SCFA	0h	1.0 ± 0.0	0.9 ± 0.0	1.0 ± 0.0	1.2 ± 0.0	0.9 ± 0.1	1.1 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.1	1.1 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
	4h	1.8 ± 0.0	1.1 ± 0.0*	1.1 ± 0.1*	1.8 ± 0.0	2.0 ± 0.0	1.2 ± 0.2*	1.4 ± 0.0	1.2 ± 0.1*	1.2 ± 0.1*	1.2 ± 0.1*	1.3 ± 0.1*	1.3 ± 0.1*
	24h	1.9 ± 0.0	1.0 ± 0.0*	1.1 ± 0.0*	1.5 ± 0.0*	1.5 ± 0.1*	1.0 ± 0.0*	1.1 ± 0.1*	1.4 ± 0.0*	1.5 ± 0.0*	1.4 ± 0.0*	1.3 ± 0.0*	1.5 ± 0.0*

Data is presented as the mean ± SEM, and normalized by the negative control (cellulose) at the same time point (n = 3 per group). Repeated Measures ANOVA revealed ingredient x time interaction in all cases. Main effects within each time were examined using one-way ANOVA followed by Sidak post-hoc test. * Significant differences compared to inulin at each time point ($p < 0.05$). BSG, brewers' spent grain; OPC, olive press cake.

In obese fecal samples, BSG, pumpkin and plum skins, quinoa husk and mushrooms increased the total SCFA production after 4 h of fermentation, following an inulin-like pattern. After 24 h, *Armillaria mellea*, *Boletus edulis* and Irish moss maintained an elevated generation of SCFAs, showing no significant differences with respect to inulin, as presented in **Table 1.2**. At 4 h, five ingredients, including BSG, pumpkin skin, Irish moss, rice husk and stevia branches followed an inulin-like butyrate generation. Although the rise of butyrate levels after 24 h in the inulin group was not reproduced by any ingredient, a numerically greater butyrate production was observed with pumpkin skin, BSG, *Armillaria mellea*, *Boletus edulis* and plum skin compared to less effective ingredients such as nutshell, rice husk, OPC and stevia branches at 24 h.

In contrast to that observed in fecal samples from normoweight individuals, inulin fermentation of obese microbiota led to a rise of propionate production. Moreover, BSG, pumpkin skin, quinoa husk and plum skin promoted the generation of propionate paralleled to inulin after 24 h. The same ingredients also induced the production of acetate after 4 and 24 h, presenting no differences with inulin.

Table 1.2. SCFA production after 0 h, 4 h and 24 h of *in vitro* fermentation of the obese faecal microbiota.

		Inulin	Nutshell	Rice husk	BSG	Pumpkin skin	OPC	Stevia branches	Quinoa husk	*A. mellea*	*B. edulis*	Plum skin	Irish moss
Butyrate	0h	1.0 ± 0.0	0.9 ± 0.0	1.0 ± 0.1	1.2 ± 0.0*	1.1 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.1 ± 0.0	1.1 ± 0.0	1.0 ± 0.0	0.8 ± 0.0*	1.0 ± 0.0
	4h	1.2 ± 0.2	1.0 ± 0.1*	1.1 ± 0.1	1.4 ± 0.2	1.4 ± 0.2	1.0 ± 0.2*	1.1 ± 0.1	1.8 ± 0.0*	1.5 ± 0.0*	1.5 ± 0.0*	1.7 ± 0.0*	1.4 ± 0.0
	24h	2.3 ± 0.0	1.0 ± 0.1*	1.1 ± 0.0*	1.6 ± 0.0*	1.8 ± 0.0*	1.2 ± 0.0*	1.2 ± 0.0*	1.7 ± 0.1*	1.7 ± 0.0*	1.6 ± 0.0*	1.5 ± 0.0*	1.4 ± 0.0*
Propionate	0h	1.0 ± 0.0	1.0 ± 0.0	1.1 ± 0.0	1.1 ± 0.0	1.1 ± 0.0	1.1 ± 0.0	1.0 ± 0.0	1.1 ± 0.0	1.1 ± 0.0	1.4 ± 0.1*	1.1 ± 0.0	1.1 ± 0.1
	4h	1.4 ± 0.0	1.0 ± 0.0*	1.1 ± 0.0*	1.2 ± 0.0*	1.1 ± 0.0*	1.1 ± 0.0*	1.1 ± 0.0*	1.2 ± 0.0	1.2 ± 0.0*	1.3 ± 0.1	1.3 ± 0.0	1.1 ± 0.1*
	24h	1.5 ± 0.1	1.0 ± 0.1*	1.1 ± 0.0*	1.3 ± 0.0	1.3 ± 0.0	1.1 ± 0.0*	1.2 ± 0.0*	1.3 ± 0.1	1.8 ± 0.0*	1.8 ± 0.0*	1.4 ± 0.0	1.9 ± 0.0*
Acetate	0h	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.1 ± 0.0*	1.1 ± 0.0	1.1 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.1 ± 0.0*	1.0 ± 0.0	1.0 ± 0.0
	4h	1.4 ± 0.0	1.0 ± 0.0*	1.1 ± 0.0*	1.4 ± 0.0	1.4 ± 0.1	1.1 ± 0.0*	1.1 ± 0.0*	1.4 ± 0.0	1.1 ± 0.0*	1.2 ± 0.0*	1.4 ± 0.0	1.0 ± 0.0*
	24h	1.4 ± 0.1	1.1 ± 0.0*	1.1 ± 0.0*	1.3 ± 0.0	1.3 ± 0.0	1.1 ± 0.0*	1.3 ± 0.0	1.5 ± 0.1	1.7 ± 0.0*	1.7 ± 0.0*	1.6 ± 0.1	1.6 ± 0.0
Total SCFA	0h	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.1 ± 0.0*	1.1 ± 0.0	1.1 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.1 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
	4h	1.4 ± 0.0	1.0 ± 0.0*	1.1 ± 0.0*	1.3 ± 0.0	1.3 ± 0.0	1.1 ± 0.0*	1.1 ± 0.0*	1.4 ± 0.0	1.2 ± 0.0	1.3 ± 0.0	1.4 ± 0.0	1.1 ± 0.0*
	24h	1.7 ± 0.1	1.0 ± 0.1*	1.1 ± 0.0*	1.4 ± 0.0*	1.5 ± 0.0*	1.1 ± 0.0*	1.2 ± 0.0*	1.5 ± 0.1*	1.7 ± 0.0	1.6 ± 0.0	1.5 ± 0.0*	1.6 ± 0.0

Data is presented as the mean ± SEM, and normalized by the negative control (cellulose) at the same time point (n = 3 per group). Repeated Measures ANOVA revealed ingredient x time interaction in all cases. Main effects within each time were examined using one-way ANOVA followed by Sidak post-hoc test. * Significant differences compared to inulin at each time point ($p < 0.05$). BSG, brewers' spent grain; OPC, olive press cake.

3.3 Clustering Analysis.

Metabolomic techniques allowed for the wide-range identification and quantification of metabolites resulting from *in vitro* fermentation in a comprehensive approach, thereby revealing particular metabolomic profiles. PCAs constructed from metabolomic profiles were useful to identify clusters within each condition after 4 and 24 hours of *in vitro* fermentation (**Figure 3**). These analyses revealed that inulin generated the most dissimilar metabolic pattern at every time point within both conditions. Concerning normoweight fecal samples, PCA explained up to 59% of the overall variation (PC1: 32.5%; PC2: 26.5%) after 4 hours of fermentation as shown in **Figure 3A**. Among all the ingredients tested, quinoa husk, plum and pumpkin skins, *Armillaria mellea* and BSG shaped the closest clusters to positive control samples. Consistently, ingredients showing no relevant impact on pH or gas production such as nutshell, rice husk, OPC and stevia branches were clustered together nearby to cellulose. Indeed, the clustering patterns after 24 h of fermentation, where 43.8% and 20.8% of the total variation were explained by PC1 and PC2, respectively, revealed that pumpkin skin exhibited the most inulin-like displacement along the PC2 axis (**Figure 3B**).

Fermentation of obese fecal samples revealed several clusters after 4 h, as presented in **Figure 3C**, where PC1 and PC2 explained up to 38.7% and 29.7% of the overall dissimilarities, respectively. According to the clustering obtained within the normoweight individuals, pumpkin skin and inulin closely clustered at this time point, revealing a highly similar metabolic pattern after fermentation. Moreover, BSG was also associated with both pumpkin skin and inulin along the PC1, while *Boletus edulis* clustered separately throughout the PC2. As also observed within

normoweight samples, such ingredients as nutshell, stevia branches, rice husk and OPC, which showed no significant variation in fermentation rates compared to cellulose, clustered alongside the negative control. After 24 h of fermentation, the positive control clearly differed from the rest of the ingredients, showing only close distances with the metabolic profiles generated after using the pumpkin skin as a fermentable source (**Figure 3D**). In this case, 34.5% of the overall variation explained by PC1 defined substantial differences regarding the fermentable susceptibility of ingredients, where the presence of mostly non-fermentable substrates was intensified on the left side. Finally, 28.8% of the total dissimilarities explained by PC2 elucidated that the metabolic profiles generated by *Boletus edulis* strongly differed from the positive control.

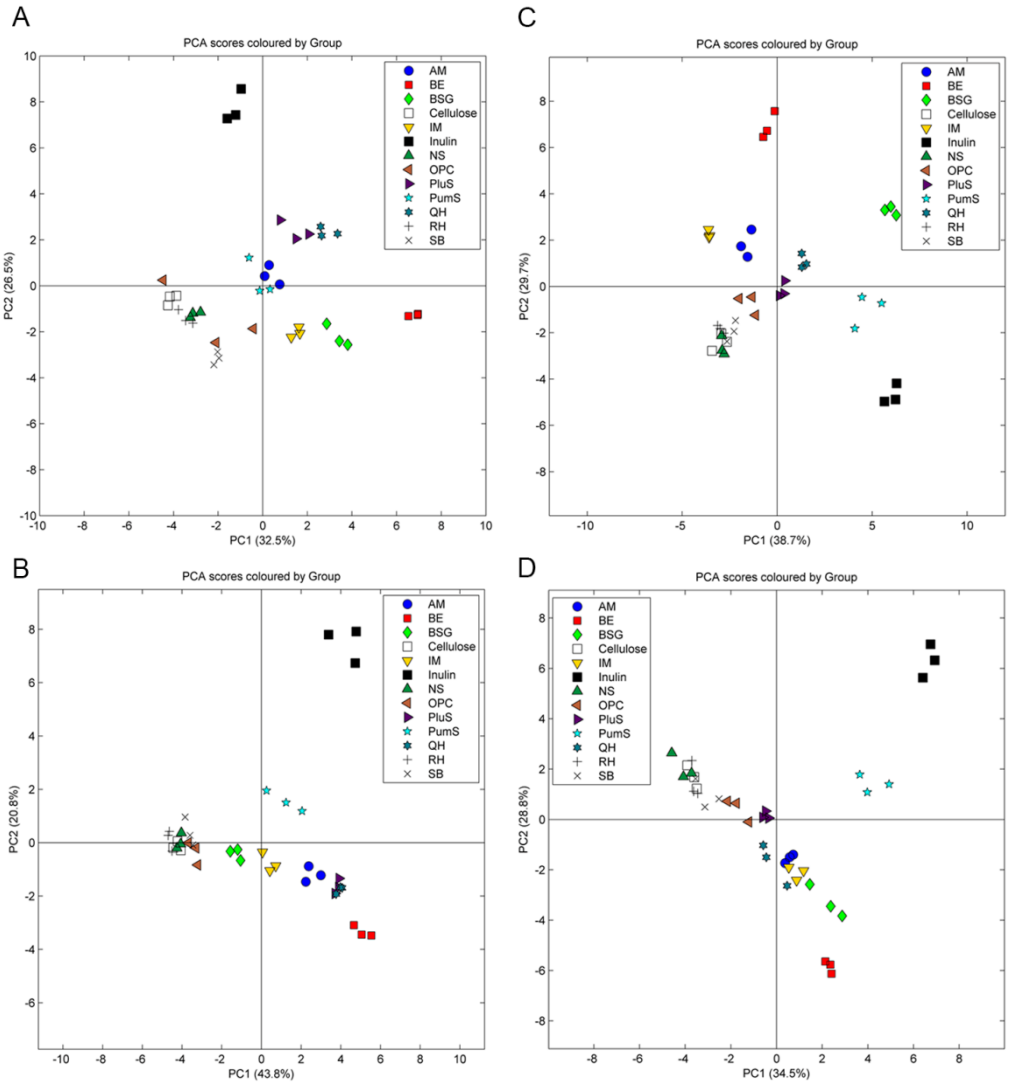

Figure 3. Principal component analysis constructed from the fecal slurries metabolic profiles. Clustering analyses included all the ingredients tested and both positive (inulin) and negative (cellulose) controls. *In vitro* fermentations with fecal inoculum from lean individuals at 4 h (**A**) and at 24 h (**B**), as well as from obese individuals at 4 h (**C**) and at 24 h (**D**) are included. AM. *Armillaria mellea*; BE, *Boletus edulis*; BSG, brewers' spent grain; IM, Irish moss (*Chondrus crispus*); NS, nutshell (NS), OPC, olive press cake; PC, principal component (axis); PluS, plum skin; PumS, pumpkin skin; QH, quinoa husk; RH, rice husk; SB, stevia branches.

4. DISCUSSION

Using a validated procedure for the *in vitro* fermentation of human fecal samples, we investigated the pH changes, gas production and metabolomic profiles generated by distinct ingredients. Gut microbial fermentation of indigestible plant polysaccharides produces a wide range of acidic end metabolites, such as SCFAs and lactate. Then, changes in pH can provide relevant information regarding the fermentation rate occurring in a system, while gas production is concomitant with fermentative processes acting as the main marker of complex carbohydrate utilization by gut microbiota. Moreover, metabolomic techniques revealed the specific contributory role of each ingredient on the resulting metabolic profiles after being exposed to fecal microbiota.

According to the literature [30], inulin induced an important drop in pH and a high gas production after being fermented with both normoweight and obese microbiota, whereas cellulose is known to be poorly fermented by the gut microbiota. In humans, a lower colonic pH has been suggested to play a determinant influence on the microbiota composition and function by favouring the occurrence of butyrate-producing bacteria such as *Roseburia spp.* [31]. In fact, this genus was found to be increased in subjects consuming high amounts of indigestible polysaccharides [32]. Otherwise, an increment in the populations of *Bacteroides spp.*, which is robustly adapted to low-fiber diets [33] and previously found increased in diet-induced obese rodents [34], was reported when the pH increased from 5.5 to 6.5, highly correlating with a an increment of propionate production [35]. Thus, the pH-lowering effect, which is linked to the SCFA production as a result of microbial fermentation of dietary compounds, contributes to the overall microbiota composition and, in

turn, is prevents the overgrowth of pathogenic bacteria, such as *Enterobacteriaceae* and *Clostridia* [36]. In this regard, pumpkin skin, which caused the largest drop in pH and promoted butyrate production, is suggested to be preferably utilized by the favoured butyrogenic bacteria. Concerning this ingredient, high mucilage content was previously assessed in pumpkin skin peel extract [37]. In parallel, mucilage-containing plants have been shown to improve the glycaemic and lipid control in diabetic patients [38]. The prebiotic properties of different mucilage sources have been elucidated in bacterial cultures by favouring the growth of beneficial bacteria and promoting SCFA production [26,39,40].

On the other hand, we hypothesized that insoluble fibers, which are highly present in pumpkin skin [41], could partially mediate the prebiotic-like effects described for this ingredient. For many years, insoluble fibers have been considered inert bulking compounds unable to be utilized by gut microorganisms [42]. However, it has been stated that insoluble fibers should not be considered to be non-fermentable carbohydrates [43], whereas a current revision concluded that "insoluble" and "soluble" fiber classification may not be sufficient to explain fermentation performances [44]. In this sense, insoluble fibers more effectively suppressed high-fat diet-induced obesity than soluble fibers in mice, which was accompanied by an active modulation of gut microbiota [45], thereby supporting our hypothesis. Furthermore, although pumpkin oils have been widely studied, showing beneficial effects on dyslipemia in mice [46], and pumpkin seed mixtures showed anti-atherogenic, hypolipidaemic and immunomodulatory effects in rats [47], the prebiotic potential of pumpkin skin has not been previously

evaluated. In this sense, the demonstrated acetogenic effect of pumpkin skin, together with a significant rise in butyrate and propionate production in normoweight and obese microbiota, respectively, reveal for the first time its strong prebiotic potential in both lean and obese subjects.

Among the rest of the ingredients, the mushrooms *Armillaria mellea* and *Boletus edulis* showed noticeable pH lowering effects together with high gas and propionate production under lean and obese conditions, which are suggested to be promising ingredients with prebiotic activity. In this sense, many species of mushrooms have been previously reported to be a potential source of dietary fiber with high beta-glucan content [48], which were linked to several metabolic and physiological benefits, such as cholesterol lowering effects [49]. The resulting SCFA production from the fermentation of beta-glucans in the large intestine, particularly propionate, was suggested to directly mediate its described hypocholesterolaemic effects [50]. Interestingly, BSG, a source of beta-glucans, and pumpkin skin fermentation also led to the generation of propionate within obese conditions. Propionate production is postulated to be a microbiota-derived response due to its anti-obesity properties [51,52]. In this sense, propionate can induce intestinal gluconeogenesis, which is related to the suppression of the appetite through signalling pathways [53], conferring protection to the host from diet-induced obesity as previously shown [54]. Moreover, BSG also contains noteworthy amounts of arabinoxylans [55], which have been shown to impact the gut microbial ecosystems and stimulate the production of SCFAs [56]. In this instance, the potential prebiotic effects of BSG were also explained by a high butyric acid production, although it was lower

than inulin. Consistently, previous studies have shown an association between the consumption of arabinoxylans and the stimulation of butyrate-producing bacteria [57]. Clinical interventions have demonstrated that the dietary intake of fermentable carbohydrates is linked to changes in gut microbial ecosystems, thus enhancing the production of butyrate and reporting beneficial consequences to the host [58–60]. For instance, butyrate has been widely shown to combat distal inflammatory bowel diseases by the inhibition of proinflammatory cytokines [61] and to promote the apoptosis of colon carcinogenic cells [62]. In this sense, recent literature has attributed prebiotic properties to BSG, such as an increased cecal and fecal bacterial diversity [63] and an active modulation of microbiota composition towards a higher butyric acid formation [64]. Altogether, our observations were validated by the clustering obtained from metabolomic profiles, thereby confirming recent studies attributing prebiotic capacities to BSG [57,65] and introducing pumpkin skin as a promising candidate.

On the other hand, the diminution of pH induced by Irish moss (*Chondrus crispus*) in the obese condition, which was accompanied by gas and SCFA production, suggests a prebiotic potential for this ingredient. In this regard, chronic supplementation with *C. crispus* triggered an increase in the beneficial bacteria *Bifidobacterium* accompanied by high concentrations of fecal SCFAs in normoweight rats [66]. Likewise, the *C. crispus*-enriched diet also increased the production of SCFAs in layer hens [67]. Overall, whereas previous studies support our results, we demonstrated that *C. crispus* was able to mediate prebiotic-like effects in microbiota samples from obese individuals. In contrast, the absence of pH or gas production during the fermentation period observed in

nutshell, rice husk, OPC and stevia branches incubations suggested either a depressed bioavailability or a low concentration of fermentable compounds contained in these ingredients. Other ingredients tested in this study, such as quinoa husk and plum skin, slightly modulated the production of the main SCFAs, despite the significant differences found when compared to inulin. These observations were accompanied by a moderate decrease in pH and a favoured gas production within both obese and normoweight conditions. Thus, although no clear evidence was found concerning a prebiotic activity attributable to these ingredients, further research on these substrates would be optimal to validate our results.

The gut microbiota from lean and obese subjects have been previously shown to differentially contribute to the fermentation of certain fibers [68], suggesting that the utilization rate of dietary fibers strongly depends on the phylogenetic structure of the microbiome. Our study revealed that the highest gas production was observed within normoweight conditions, suggesting that microbiota from lean individuals predominantly favoured the fermentation of our tested substrates. In this respect, microbiota from obese subjects has been associated with enhanced energy extraction from the diet [11,69], whereas a previous study showed no differences in gas production induced by inulin between lean and obese microbiota [70]. Additionally, the pH-lowering effects induced by inulin under both normoweight and obese conditions suggested that fermentation was similarly occurring. Therefore, caution must be taken when interpreting total gas production as an absolute indicator of fermentation in this study. The ingredients promoted a similar pattern of gas production and pH-lowering effects

within both lean and obese microbiota, indicating that fermentation is mostly substrate-dependent.

Inulin, BSG and pumpkin skin induced an early increase in total SCFA levels, especially within normoweight fecal samples. Noticeably, relative butyrate amounts induced by these ingredients were higher in lean than obese individuals, which is in accordance with enhanced butyrate production after inulin fermentation by lean microbiota [68]. Thus, it is suggested that the intrinsic microbiota composition of lean subjects could favour the beneficial effects of highly fermentable substrates. Moreover, although butyrate was the main end-product of inulin fermentation, differential patterns concerning the generation of acetate and propionate were also observed between both lean and obese subjects. Hence, the increase in propionate production found after early inulin fermentation under obese conditions contrasted with the predominant formation of acetate in fecal samples from lean individuals. This finding is in accordance with a favoured propionate production previously described for obese microbiota in humans [68,71]. Furthermore, an increased generation of propionate in obese compared to normoweight fecal inoculums was also previously observed after *in vitro* fermentation of fibers [72]. While *Firmicutes* are considered butyrate-producers, propionate is the main product of microbial species belonging to *Bacteroidetes* [31]. Moreover, as discussed above, the growth of propiogenic bacteria is favoured by less acidic pH, which is mainly found in the distal parts of the large intestine, where non-digestible substrates are limited. Taken together, the results of this study suggest that an increased propionate production in obese fermentation is associated with a predominant propiogenic bacterial ecosystem,

which, in turn, could be explained by different reasons: a lower consumption of non-digestible dietary compounds, upper pH conditions, and a microbiota-derived response to obesity. Accordingly, increased propionate production was mainly detected in obese but not in normoweight microbiota fermentation. Therefore, while fermentation rates have been proposed to be substrate-dependent, we also suggest that SCFA profiles generated by inulin and potential prebiotics highly rely on the intrinsic microbiota composition and function. However, further studies are needed to elucidate the critical role of the lean or obese microbiome in the fermentation of non-digestible compounds.

Overall, the present *in vitro* fermentation study evaluated a wide range of ingredients that could potentially show prebiotic activity within both lean and obese human microbiota. Pumpkin skin revealed an inulin-like response of the microbiota within both lean and obese conditions, showing that this by-product could be considered one of the most promising ingredients to be revaluated as prebiotic foods. Furthermore, we reported a relevant prebiotic capacity for BSG defined by inulin-like SCFA production in obese and normoweight fecal microbiota. Other ingredients, such as *Armillaria mellea* and *Boletus edulis*, followed by Irish moss, plum skin and quinoa husk, revealing substantial increments in the fermentation rates and changes in SCFA production, are also proposed to become potential prebiotics. The comparison between obese and lean microbiota fermentations suggested that fermentation rates were substrate-dependent, while SCFA profiles strongly relied on microbial action. In this sense, obese microbiota preferably produced propionate in detriment of butyrate, suggesting a higher butyrogenic activity in lean microbiota. Finally, the prebiotic potential described for

these industrial by-products can contribute to their revalorization, thereby alleviating the negative impact of food industry wastes on the ecosystems and countries' economies. Future *in vivo* studies are needed to further explore the fermentation mechanisms, the prebiotic capacities and the health benefits linked to these ingredients, especially with regard to obesity treatment and prevention.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

REFERENCES

[1] Cani PD, Van Hul M, Everard A, Rastelli M, Lefort C, Depommier C. Microbial regulation of organismal energy homeostasis. Nat Metab 2019;1:34–46.

[2] Henningsson Å, Björck I, Nyman M. Short-chain fatty acid formation at fermentation of indigestible carbohydrates. Näringsforskning 2012;45:165–8.

[3] Makki K, Deehan EC, Walter J, Bäckhed F. The Impact of Dietary Fiber on Gut Microbiota in Host Health and Disease. Cell Host Microbe 2018;23:705–15.

[4] Freeland KR, Wilson C, Wolever TMS. Adaptation of colonic fermentation and glucagon-like peptide-1 secretion with increased wheat fibre intake for 1 year in hyperinsulinaemic human subjects. Br J Nutr 2010;103:82–90.

[5] Daud NM, Ismail NA, Thomas EL, Fitzpatrick JA, Bell JD, Swann JR, et al. The impact of oligofructose on stimulation of gut hormones, appetite regulation and adiposity. Obesity 2014;22:1430–8.

[6] Karl PJ, Hatch AM, Arcidiacono SM, Pearce SC, Pantoja-Feliciano IG, Doherty LA, et al. Effects of psychological, environmental and physical stressors on the gut microbiota. Front Microbiol 2018;9.

[7] Di Cagno R, Saarela M, Flint HJ, Graf D, Fåk F, Nyman M, et al. Contribution of diet to the composition of the human gut microbiota. Microb Ecol Heal Dis 2015;26.

[8] Kobyliak N, Virchenko O, Falalyeyeva T. Pathophysiological role of host microbiota in the development of obesity. Nutr J 2016;15.

[9] Wichmann A, Allahyar A, Greiner TU, Plovier H, Lundén GÖ, Larsson T, et al. Microbial modulation of energy availability in the colon regulates intestinal transit. Cell Host Microbe 2013;14:582–90.

[10] Bäckhed F, Manchester JK, Semenkovich CF, Gordon JI. Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. Proc Natl Acad Sci 2007;104:979–84.

[11] Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 2006;444:1027–31.

[12] Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, et al. A core gut microbiome in obese and lean twins. Nature 2009;457:480–4.

[13] Ignacio A, Fernandes MR, Rodrigues VAA, Groppo FC, Cardoso AL, Avila-Campos MJ, et al. Correlation between body mass index and faecal microbiota from children. Clin Microbiol Infect 2016;22:258.e1-258.e8.

[14] Million M, Maraninchi M HM, Armougom F, Richet H CP, et al., Raccah D, Vialettes B, Raoult D. Obesity-associated gut microbiota is enriched in Lactobacillus reuteri and depleted in Bifidobacterium animalis and Methanobrevibacter smithii. Int J Obes 2012;36:817–25.

[15] Okubo H, Nakatsu Y, Kushiyama A, Yamamotoya T, Matsunaga Y, Inoue M-K, et al. Gut Microbiota as a Therapeutic Target for Metabolic Disorders. Curr Med Chem 2018;25:984–1001.

[16] Flint HJ, Scott KP, Duncan SH, Louis P, Forano E. Microbial degradation of complex carbohydrates in the gut. Gut Microbes n.d.;3:289–306.

[17] Rossi M, Corradini C, Amaretti A, Nicolini M, Pompei A, Zanoni S, et al. Fermentation of fructooligosaccharides and inulin by bifidobacteria: a comparative study of pure and fecal cultures. Appl Environ Microbiol 2005;71:6150–8.

[18] Hiel S, Bindels LB, Pachikian BD, Kalala G, Broers V, Zamariola G, et al. Effects of a diet based on inulin-rich vegetables on gut health and nutritional behavior in healthy humans. Am J Clin Nutr 2019;109:1683–95.

[19] Vandeputte D, Falony G, Vieira-Silva S, Wang J, Sailer M, Theis S, et al. Prebiotic inulin-type fructans induce specific changes in the human gut microbiota. Gut 2017;66:1968–74.

[20] van der Beek CM, Canfora EE, Kip AM, Gorissen SHM, Olde Damink SWM, van Eijk HM, et al. The prebiotic inulin improves substrate metabolism and promotes short-chain fatty acid production in overweight to obese men. Metabolism 2018;87:25–35.

[21] Thompson LA, Darwish WS. Environmental Chemical Contaminants in Food: Review of a Global Problem. J Toxicol 2019;2019.

[22] Zuin VG, Ramin LZ. Green and Sustainable Separation of Natural Products from Agro-Industrial Waste: Challenges, Potentialities, and Perspectives on Emerging Approaches. Top Curr Chem 2018;376.

[23] López-Barrera DM, Vázquez-Sánchez K, Loarca-Piña MGF, Campos-Vega R. Spent coffee grounds, an innovative source of colonic fermentable compounds, inhibit inflammatory mediators in vitro. Food Chem 2016;212:282–90.

[24] Tejada-Ortigoza V, Garcia-Amezquita LE, Kazem AE, Campanella OH, Cano MP, Hamaker BR, et al. In Vitro Fecal Fermentation of High Pressure-Treated Fruit Peels Used as Dietary Fiber Sources. Molecules 2019;24.

[25] Pérez-López E, Cela D, Costabile A, Mateos-Aparicio I, Rupérez P. In vitro fermentability and prebiotic potential of soyabean Okara by human faecal microbiota. Br J Nutr 2016;116:1116–24.

[26] Gullón B, Gullón P, Tavaria F, Alonso JL, Pintado M. In vitro assessment of the prebiotic potential of Aloe vera mucilage and its impact on the human microbiota. Food Funct 2015;6:525–31.

[27] Minekus M, Alminger M, Alvito P, Ballance S, Bohn T, Bourlieu C, et al. A standardised static in vitro digestion method suitable for food-an international consensus. Food Funct 2014;5:1113–24.

[28] Noack J, Timm D, Hospattankar A, Slavin J. Fermentation profiles of wheat dextrin, Inulin and partially hydrolyzed guar gum using an in Vitro digestion pretreatment and in Vitro batch fermentation system model. Nutrients 2013;5:1500–10.

[29] Massot-Cladera M, Mayneris-Perxachs J, Costabile A, Swann JR, Franch À, Pérez-Cano FJ, et al. Association between urinary metabolic profile and the intestinal effects of cocoa in rats. Br J Nutr 2017;117:623–34.

[30] Holscher HD. Dietary fiber and prebiotics and the gastrointestinal microbiota. Gut Microbes 2017;8:172–84.

[31] Walker AW, Duncan SH, Carol McWilliam Leitch E, Child MW, Flint HJ. pH and peptide supply can radically alter bacterial populations and short-chain fatty acid ratios within microbial communities from the human colon. Appl Environ Microbiol 2005;71:3692–700.

[32] Duncan SH, Belenguer A, Holtrop G, Johnstone AM, Flint HJ, Lobley GE. Reduced dietary intake of carbohydrates by obese subjects results in decreased concentrations of butyrate and butyrate-producing bacteria in feces. Appl Environ Microbiol 2007;73:1073–8.

[33] Koropatkin NM, Cameron EA, Martens EC. How glycan metabolism shapes the human gut microbiota. Nat Rev Microbiol 2012;10:323–35.

[34] Gual-Grau A, Guirro M, Mayneris-Perxachs J, Arola L, Boqué N. Impact of different hypercaloric diets on obesity features in rats: a metagenomics and metabolomics integrative approach. J Nutr Biochem 2019.

[35] Musso G, Gambino R, Cassader M. Interactions Between Gut Microbiota and Host Metabolism Predisposing to Obesity and Diabetes. Annu Rev Med 2011;62:361–80.

[36] den Besten G, van Eunen K, Groen AK, Venema K, Reijngoud D-J, Bakker BM. The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. J Lipid Res 2013.

[37] Bahramsoltani R, Farzaei MH, Abdolghaffari AH, Rahimi R, Samadi N, Heidari M, et al. Evaluation of phytochemicals, antioxidant and burn wound healing activities of Cucurbita moschata Duchesne fruit peel. Iran J Basic Med Sci 2017;20:798–805.

[38] Ziai SA, Larijani B, Fakhrzadeh H, Dastpak A, Bandarian F, Rezai A, et al. Study of Psyllium (Plantago ovata L.) effects on diabetes and lipidemia in the Iranian type II diabetic patients. J Med Plants 2004;4:33–42.

[39] Guevara-Arauza JC, de Jesús Ornelas-Paz J, Pimentel-González DJ, Rosales Mendoza S, Soria Guerra RE, Paz Maldonado LMT. Prebiotic effect of mucilage and pectic-derived oligosaccharides from nopal (Opuntia ficus-indica). Food Sci Biotechnol 2012;21:997–1003.

[40] Mueller M, Čavarkapa A, Unger FM, Viernstein H, Praznik W. Prebiotic potential of neutral oligo- and polysaccharides from seed mucilage of Hyptis suaveolens. Food Chem 2017;221:508–14.

[41] Butke W, Marques M, Santos R, Aparecida Do Amaral L, Dos Santos E, D N. Addition of pumpkin skin flour in pizza changes the physicochemical and sensory acceptability of children. Int J Dev Res 2018;8:21409–15.

[42] Wong JMW, De Souza R, Kendall CWC, Emam A, Jenkins DJA. Colonic health: Fermentation and short chain fatty acids. J Clin Gastroenterol 2006;40:235–43.

[43] Brotherton CS. Insoluble fiber and intestinal microbiota metabolism. J Gastroenterol 2015;50:491.

[44] Wang M, Wichienchot S, He X, Fu X, Huang Q, Zhang B. In vitro colonic fermentation of dietary fibers: Fermentation rate, short-chain fatty acid production and changes in microbiota. Trends Food Sci Technol 2019;88:1–9.

[45] Li X, Guo J, Ji K, Zhang P. Bamboo shoot fiber prevents obesity in mice by modulating the gut microbiota. Sci Rep 2016;6.

[46] Morrison MC, Mulder P, Stavro PM, Suárez M, Arola-Arnal A, Van Duyvenvoorde W, et al. Replacement of dietary saturated fat by PUFA-rich pumpkin seed oil attenuates non-alcoholic fatty liver disease and atherosclerosis development, with additional health effects of virgin over refined oil. PLoS One 2015;10:e0139196.

[47] Barakat LAA, Mahmoud RH. The antiatherogenic, renal protective and immunomodulatory effects of purslane, pumpkin and flax seeds on hypercholesterolemic rats. N Am J Med Sci 2011;3:411–7.

[48] Sari M, Prange A, Lelley JI, Hambitzer R. Screening of beta-glucan contents in commercially cultivated and wild growing mushrooms. Food Chem 2017;216:45–51.

[49] Bai J, Ren Y, Li Y, Fan M, Qian H, Wang L, et al. Physiological functionalities and mechanisms of β-glucans. Trends Food Sci Technol 2019;88:57–66.

[50] Hughes SA, Shewry PR, Gibson GR, McCleary B V., Rastall RA. In vitro fermentation of oat and barley derived β-glucans by human faecal microbiota. FEMS Microbiol Ecol 2008;64:482–93.

[51] Al-Lahham SH, Peppelenbosch MP, Roelofsen H, Vonk RJ, Venema K. Biological effects of propionic acid in humans; metabolism, potential applications and underlying mechanisms. Biochim Biophys Acta - Mol Cell Biol Lipids 2010;1801:1175–83.

[52] Ruijschop RMAJ, Boelrijk AEM, te Giffel MC. Satiety effects of a dairy beverage fermented with propionic acid bacteria. Int Dairy J 2008;18:945–50.

[53] De Vadder F, Kovatcheva-Datchary P, Goncalves D, Vinera J, Zitoun C, Duchampt A, et al. Microbiota-generated metabolites promote metabolic benefits via gut-brain neural circuits. Cell 2014;156:84–96.

[54] Chambers ES, Viardot A, Psichas A, Morrison DJ, Murphy KG, Zac-Varghese SEK, et al. Effects of targeted delivery of propionate to the human colon on appetite regulation, body weight maintenance and adiposity in overweight adults. Gut 2015;64:1744–54.

[55] Lynch KM, Steffen EJ, Arendt EK. Brewers' spent grain: a review with an emphasis on food and health. J Inst Brew 2016;122:553–68.

[56] Neyrinck AM, Possemiers S, Druart C, van de Wiele T, de Backer F, Cani PD, et al. Prebiotic effects of wheat Arabinoxylan related to the increase in bifidobacteria, roseburia and bacteroides/prevotella in diet-induced obese mice. PLoS One 2011;6.

[57] Teixeira C, Prykhodko O, Alminger M, Fåk Hållenius F, Nyman M. Barley Products of Different Fiber Composition Selectively Change Microbiota Composition in Rats. Mol Nutr Food Res 2018;62.

[58] Majid HA, Emery PW, Whelan K. Faecal microbiota and short-chain fatty acids in patients receiving enteral nutrition with standard or fructo-oligosaccharides and fibre-enriched formulas. J Hum Nutr Diet 2011;24:260–8.

[59] Venkataraman A, Sieber JR, Schmidt AW, Waldron C, Theis KR, Schmidt TM. Variable responses of human microbiomes to dietary supplementation with resistant starch. Microbiome 2016;4.

[60] Baxter NT, Schmidt AW, Venkataraman A, Kim KS, Waldron C, Schmidt TM. Dynamics of Human Gut Microbiota and Short-Chain Fatty Acids in Response to Dietary Interventions with Three Fermentable Fibers. MBio 2019;10.

[61] Ogawa H, Iimura M, Eckmann L, Kagnoff MF. Regulated production of the chemokine CCL28 in human colon epithelium. Am J Physiol Liver Physiol 2004;287:G1062–9.

[62] Yu DCW, Waby JS, Chirakkal H, Staton CA, Corfe BM. Butyrate suppresses expression of neuropilin I in colorectal cell lines through inhibition of Sp1 transactivation. Mol Cancer 2010;9.

[63] Maukonen J, Aura AM, Niemi P, Raza GS, Niemelä K, Walkowiak J, et al. Interactions of Insoluble Residue from Enzymatic Hydrolysis of Brewer's Spent Grain with Intestinal Microbiota in Mice. J Agric Food Chem 2017;65:3748–56.

[64] Teixeira C, Prykhodko O, Alminger M, Fåk Hållenius F, Nyman M. Barley Products of Different Fiber Composition Selectively Change Microbiota Composition in Rats. Mol Nutr Food Res 2018;62:1701023.

[65] Sajib M, Falck P, Sardari RRR, Mathew S, Grey C, Karlsson EN, et al. Valorization of Brewer's spent grain to prebiotic oligosaccharide: Production, xylanase catalyzed hydrolysis, in-vitro evaluation with probiotic strains and in a batch human fecal fermentation model. J Biotechnol 2018;268:61–70.

[66] Liu J, Kandasamy S, Zhang J, Kirby CW, Karakach T, Hafting J, et al. Prebiotic effects of diet supplemented with the cultivated red seaweed Chondrus crispus or with fructo-oligo-saccharide on host immunity, colonic microbiota and gut microbial metabolites. BMC Complement Altern Med 2015;15:279.

[67] Kulshreshtha G, Rathgeber B, Stratton G, Thomas N, Evans F, Critchley A, et al. Feed supplementation with red seaweeds, Chondrus crispus and Sarcodiotheca gaudichaudii, affects performance, egg quality, and gut microbiota of layer hens. Poult Sci 2014;93:2991–3001.

[68] Aguirre M, De Souza CB, Venema K. The gut microbiota from lean and obese subjects contribute differently to the fermentation of arabinogalactan and inulin. PLoS One 2016;11.

[69] Backhed F, Semenkovich CF, Hooper L V., Gordon JI, Koh GY, Ding H, et al. The gut microbiota as an environmental factor that regulates fat storage. Proc Natl Acad Sci 2004;101:15718–23.

[70] Sarbini SR, Kolida S, Gibson GR, Rastall RA. In vitro fermentation of commercial α-gluco-oligosaccharide by faecal microbiota from lean and obese human subjects. Br J Nutr 2013;109:1980–9.

[71] Schwiertz A, Taras D, Schäfer K, Beijer S, Bos NA, Donus C, et al. Microbiota and SCFA in lean and overweight healthy subjects. Obesity 2010;18:190–5.

[72] Yang J, Keshavarzian A, Rose DJ. Impact of Dietary Fiber Fermentation from Cereal Grains on Metabolite Production by the Fecal Microbiota from Normal Weight and Obese Individuals. J Med Food 2013;16:862–7.

IV. GENERAL DISCUSSION

UNIVERSITAT ROVIRA I VIRGILI
GUT MICROBIOTA DYSBIOSIS IN DIET-INDUCED OBESITY. A FOCUS ON THE INFLUENCE OF GENETICS, CIRCADIAN RHYTHMS AND POTENTIAL PREBIOTICS.
Andreu Gual Grau

GENERAL DISCUSSION

Over the last decade, human microbiota has been defined as a new revolutionary organ constituted by trillions of cells that exhibits exceptional attributes. By containing 150 more genes than the host, the microbiome exerts a wide variety of metabolic functions and significantly contributes to the maintenance of the body homeostasis [1,2]. Despite that microbial niches are extensively scattered throughout biological cavities in the body, most research have focused on the study of gut microbiota. Due to its noteworthy importance in the digestion of dietary compounds reaching the large intestine, along with the subsequent production of a broad spectrum of absorbable and functional metabolites, the gut microbiota has been strongly related to the promotion of health. Consequently, abnormalities in the composition and activity of gut microbial ecosystems have been shown to contribute to the development of several diseases, including obesity [3].

The evidence linking the gut microbiota with the genesis of obesity is based on various mechanisms including an enhanced energy harvest from the diet, a decreased *fiaf*-mediated inhibition of lipoprotein lipase (LPL) leading to an increased adiposity [4], a reduced fatty acid oxidation in peripheral tissues through the down-regulation of AMPK activity [5], an impaired bile acid metabolism [6], changes in satiety-regulating hormones [7], and an increased gut barrier permeability leading to LPS-mediated low-grade inflammation that, in turn, may alter food intake regulation [8,9]. Remarkably, gut microbiota is at the intersection between environmental factors and host. In this sense, external cues, particularly diet, are considered the main effectors on the establishment of microbial phenotypes [10].

Dysbiosis, as the persistent imbalance in the phylogenetic structure of the gut microbiota, has been repeatedly reported in DIO animal models [11–14], whereas a specific microbiome pattern for obesity has not been reported to date. In this context, the first aim of this thesis was to characterize and compare the impact of three different dietary models of DIO -including CAF, HF and HFS diets- on gut microbiota and host phenotype in rats, as well as to describe associations between microbial changes and metabolic status (**Chapter I**).

High-fat or high-energy diets are the most common dietary models used to evaluate the obesity-associated microbial phenotype in experimental animals. However, food overconsumption and a rapid onset of obesity and related metabolic complications are strongly promoted by free-choice dietary models such as the CAF diet, which are known to accurately reflect the typical Western-style diets in modern societies [11]. In our study, the increased body weight gain observed in HF and HFS groups, which is mainly explained by an increased total energy intake, was not accompanied by a significant higher adiposity, thus suggesting the development of an overweight phenotype. In contrast, the undisciplined and imbalanced consumption of the CAF diet, which was composed of highly palatable and energy-dense foods, promoted severe obesity and gut microbiota dysbiosis. In this sense, the slightly reduced microbial diversity uniquely induced by the CAF diet was in accordance with previous observations in both obese humans and rodents [15–17]. However, all of three hypercaloric diets were suggested to induce a loss of microbial activity, as revealed by the decreased transformation from primary to secondary bile acids, thus altering bile acid pool, whose maintenance is essential for an optimal regulation of microbial populations, glucose and lipid metabolism [18]. Relevantly, the dysbiosis of gut microbiota was also reflected

by increased urinary markers of metabolic inflammation and oxidative stress in the CAF diet-fed rats, but not in the HF or HFS groups. In this regard, the dietary interventions triggered similar clusters concerning both gut microbiome and urinary metabolome, where diets based on semi-purified ingredients nearly assembled, thus evidencing the close relationship between microbiota dysbiosis and metabolic disruption, which mostly occurred in the CAF diet-fed group.

Some meta-analyses have concluded that there is no consistence linking the Bacteroidetes-to-Firmicutes ratio with an obese profile, at least in humans [19,20]. In our study, both obesity development and an increment of the Bacteroidetes-to-Firmicutes ratio were observed in CAF diet-fed rats, but not in HFS group, although CAF and HFS diets had a similar macronutrient composition. Therefore, it is postulated that hyperphagia, which was uniquely promoted by CAF diet, altogether with the presence of additives and Maillard reaction products present in industrialized foods, are the main responsible for the disruption of the gut microbial ecosystem. Indeed, the differences in the type of ingredients present at each diet could also explain the conflicting effects between CAF and semi-purified high-energy diets on gut microbiota dysbiosis. For instance, the consumption of milk-derived fat-based diets was previously demonstrated to increase the occurrence of Bacteroidetes [21], thus supporting our observations in CAF diet-fed rats. In addition, the reduction of Firmicutes, which are known as positive fermenters containing the major butyrate-producing species [22], in favour of Bacteroidetes phylum might also contributes to the reduced breakdown of non-digestible carbohydrates and SCFA production. Altogether, total food intake, macronutrient composition and the presence of non-energetic components

such as additives are suggested to directly and strongly impair the homeostasis of gut microbiota and host phenotype.

The link between SCFAs, microbiota dysbiosis and the development of obesity-related complications is a key feature to contextualize our results. In this sense, non-digestible carbohydrates reaching the large intestine are primarily metabolized by gut microbes, thereby generating SCFAs that are involved in the regulation of energy homeostasis [23]. Relevantly, the low and similar content of total fiber contained in both low-fat and high-fat semi-purified diets could explain the lack of differences in gut microbiota and total SCFAs amounts among these dietary models. In contrast, the overconsumption of highly appetitive foods in detriment to chow feed in CAF diet-fed rats, which was the unique source of fiber in the STD controls, was associated with the decreased SCFA production. Then, SCFA production highly relied on the amount of dietary fermentable substrates reaching the large intestine. The decreased generation of SCFAs could lead to the impairment of energy homeostasis and satiety control, thus contributing to increase fat deposition in both adipose tissue and liver, as previously stated [24,25]. Accordingly, the lower consumption of dietary fiber and the consequently reduced SCFAs production observed in LF diet-fed animals when compared to STD group might be associated with the observed higher visceral fat in these animals, which constitutes a central component of MetS. For this reason, the selection of matched control diets is crucial for a proper physiological and metabolic evaluation of DIO.

The beneficial effects of SCFA have been broadly accepted. However, human obesity is associated with a high SCFA concentration in feces [26]. Here, the scientific interpretation of high fecal SCFA amounts observed in obesity is limited because of overall SCFA turnover including absorption and peripheral

metabolism has not been explored [27]. Moreover, an enhanced adherence to the Mediterranean diet was associated with an elevated SCFA production in humans [28]. In our study, CAF feeding resulted in lower fecal SCFA amounts, which was explained by the reduced fiber consumption in favour of energy-dense foods. Therefore, CAF DIO in animal models fails to reproduce the increased SCFA concentrations observed in human obesity [30]. In this regard, the higher capacity to extract nutrients from the non-digestible foods in rodents than humans may contribute to enlarge the differences on SCFA production [31]. For this reason, a direct parallel between DIO animal models and human obesity concerning microbiota-derived metabolites must be done carefully. Remarkably, our study demonstrated that CAF diet promoted robust changes on gut microbiota that, in turn, correlated with most of the physiologic, biochemical and metabolomic markers of severe obesity, including a drop on cecal SCFA levels.

Interestingly, CAF diet promoted a higher propionate production, which is known to down-regulate appetite by promoting the secretion of PYY and GLP-1 via GCPR [29], thus suggesting a microbiota-derived response to counteract overfeeding. However, the increased propionate production in CAF group did not compensate the dramatic drop on butyrate and acetate levels, thus suggesting a compositional switch of gut microbiota from a butyrogenic to a propiogenic environment.

Beyond the recognised influence of diet on gut microbiota composition, the relevancy of host genetics in the microbiome should not be underestimated. Whereas human genetics have a minor role in determining microbiome composition under a shared common environment [32], the knowledge of environmental and genetic interactions contributing to microbial dysbiosis is essential for developing adequate strategies to combat obesity. In this sense, a

study using more than 100 strains of mice revealed strong relationships between genotype and gut microbiota plasticity after a HFS feeding [33]. In addition, the sex-dependent configuration of the gut microbiota may be potentially contributing to the established sexual dimorphism in the onset of several common diseases, including obesity [32]. In this line, gut microbiota dysbiosis was found more pronounced in women than in men at high BMI [34]. Accordingly, a recently proposed microbiome-based mechanism elucidated the important role of sex hormones in the sexual dimorphism for MetS in mice [35]. Altogether, the microbial signatures and metabolic consequences of DIO may rely upon genetic factors. However, genetic influences in the CAF diet-induced microbiota alterations have not been previously evaluated. For this reason, the second aim of this thesis was to investigate the impact of strain and gender factors on the physiologic disturbances and microbiota dysbiosis promoted by the CAF diet (**Chapter II**). Hence, we carried out a study using both Wistar and F344 rats chronically exposed to STD or CAF diet in order to examine the role of strain in DIO, whereas sex-specific physiologic and microbial signatures were examined in both male and female F344 rats under STD or CAF feeding.

One of the most interesting findings of this study was that while the acquirement of an obese profile was identified in both Wistar and F344 rats, CAF diet-induced changes in the gut microbiota composition were highly dependent on the rat strain. In this regard, Bacteroidetes-to-Firmicutes ratio was profoundly altered by the CAF diet in Wistar, but not in F344 rats, which is in line with the inconsistent relationship between this ratio and obesity [20]. Moreover, gut microbiota dysbiosis was more pronounced in Wistar than in F344 rats under a CAF feeding when evaluating bacterial genera. For instance, we revealed a strong increase in the prevalence of taxa involved in

the utilization of animal fat (*Bacteroides*) and simple carbohydrates (*Prevotella*) in CAF diet-fed Wistar animals. Here, we examined the differences on food intake as a possible causative factor explaining these divergent results. Importantly, the greater total energy intake observed in CAF diet-fed F344 as compared to Wistar rats was mainly due to an increased chow consumption, which represented the main source of protein and fiber, whereas the consumption of energy-dense foods were similar in both Wistar and F344 animals. This feature is suggested to result in a less detrimental impact of CAF diet on the gut microbiome of F344 rats, which, for instance, presented a higher occurrence of beneficial butyrate-producing genera such as *Roseburia* as compared to CAF diet-fed Wistar animals. Moreover, the higher increase of *Prevotella* abundance in Wistar than F344 rats under a CAF feeding was in accordance with a deficient protein intake, as previously reported [37]. Therefore, food intake could act as a confounder factor by masking the strain-dependent gut microbial changes in response to the CAF diet. Beyond the dietary influence on the establishment of gut microbial phenotype, it is also widely established that gut microbiota affects the feeding behaviour through the regulation of hormones controlling energy homeostasis [38]. Therefore, our results might be explained by bidirectional interactions between feeding behaviour and gut microbiota composition, which are particular of each rat strain.

Focusing on the gender-dependent response to the CAF diet, a greater dietary impact on host biometric parameters was described in female than male animals, whereas the development of an unhealthy metabolic profile was more pronounced in male rats. In previous studies, a diminished body fat mass was determined in female mice when compared to males after a HFD feeding, which was in line with the protective role of ovarian hormones against fat

accumulation [39], and with the lower susceptibility of females to HFD-induced obesity [40]. In addition, ovariectomy eliminated the protection against body weight gain in female mice [40]. However, a previous study revealed a higher increment of adipose tissue weights in female than in male Wistar rats under a CAF feeding accompanied by a decreased adiponectin production [41], which is involved in the fatty acids breakdown [42]. In our study, the higher percentage of body fat mass found in females regardless the diet is in accordance with an improved lipid storage and low fat mobilization as compared to male rats [39,43]. Interestingly, these features seemed to be magnified by the consumption of the CAF diet, where females showed a greater decrease in the body lean mass percentage and a higher increase in RWAT as compared to males. Therefore, it is like the preventive effects of estrogens in response to HFD do not occurred under the CAF feeding, which is suggested to be lastly driven by the disruption of gut microbiota. Interestingly, a greater adipose tissue expandability and enlargement of adipocytes were suggested to protect females from obesity-associated metabolic disorders [44,45], which is in accordance with the less detrimental impact of the CAF diet on circulating triglycerides in females than males.

In accordance to our results, we suggest that the CAF diet-induced dysbiosis, which was more evident in female than in male F344 rats, altered the balanced homeostasis between estrogens, bile acids and gut microbiome. In this sense, the disrupted hormonal control exerted by estrogens upon bile acid metabolism, which, in turn, affects the gut microbiota composition, is postulated to contribute to the microbial dysbiosis predominantly observed in female F344 rats. In addition, the higher vulnerability of male rats to CAF diet-induced body weight gain and metabolic alterations was in accordance with a previous study in HFD-fed rats [46]. Notably, different diets, time of exposure

and animal models makes difficult the direct comparison with other studies. Here, we conclude that CAF diet-induced changes on gut microbiota composition in F344 rats depend on the gender influence, where estrogens play an important role in the regulation of bile acid metabolism and, consequently, in the establishment of the microbial community.

To continue exploring the impact of obesity on gut microbiota and host physiology, we aimed to evaluate the CAF diet-induced disturbances on microbial rhythmicity, and to examine the prebiotic potential of dietary doses of GSPE, equivalent to human consumption of proanthocyanidins in a normal diet [47,48], in counteracting microbiota dysbiosis and host physiologic and metabolic disruptions (**Chapter III**).

Gut microbiota is known to present circadian fluctuations throughout the day, thereby cyclically altering the relative abundance of commensal bacteria to optimize its functions according to the daily phase [49]. Despite some authors reported daily fluctuations in the occurrence of Bacteroidetes and Firmicutes phyla [50], we were unable to detect these changes in STD diet-fed F344 rats. As a possible explanation to this controversy, it is suggested that our ZT points could not correspond to the peaks of abundance of cyclical phyla, thus limiting the detection of its rhythmic behaviour in our study. Nevertheless, we reported significant differences on the overall gut microbiota composition between light/resting and dark/active phases in STD diet-fed F344 rats, which were mainly explained by a changing abundance of *Lactobacillus* genus. Our results were in accordance with previous studies showing an increased abundance of certain *Lactobacillus* species during the light/resting phase [51]. In this sense, lactate, the main bacterial product of this genus and considered the preferred fuel for brain [52], could be also presenting a rhythmic production, as previously observed in mice [53], thus contributing to the

maintenance of brain functions during the resting period, when the dietary energy supply decreases.

Most importantly, we revealed that CAF diet was able to disrupt the microbiota rhythmicity, since no differences on gut microbiota composition of CAF diet-fed animals were observed between resting and active phases. Therefore, the alterations on microbial rhythms could be implicated in the CAF impact on host physiology. Interestingly, the CAF diet promoted distinct microbial disturbances according to daily phase, thus emphasizing the importance of the time of the day when evaluating the effect of a dietary intervention on gut microbiota. In this regard, CAF diet increased the Bacteroidetes-to-Firmicutes ratio during the active phase, but not during the resting phase, in F344 rats. This feature is suggested to result from the continuous availability and consumption of energy-dense and highly appetitive foods, thus disrupting the maintenance of major phyla observed in STD diet-fed rats throughout the ZT points. On the other hand, it is also suggested that CAF dietary components reaching the distal part of the intestine predominantly occurred during the active phase, thus highly favouring the Bacteroidetes prevalence at this ZT point.

At deep taxonomic levels, *Oscillospira* was significantly depleted, while *Roseburia* and *Ruminococcus* were increased, by the CAF diet during the resting phase only. For instance, this increment in *Roseburia* is postulated as a microbial adaptation to the diurnal food intake occurring in CAF diet-fed rats. In line with these outcomes, we propose for the first time that the CAF diet disrupts the diurnal rhythmicity of the gut microbiome, which is essential for the maintenance of host homeostasis [54].

Unfortunately, the chronic supplementation with dietary doses of GSPE, which was previously shown to modify the gut microbiota composition in rodents [55,56], was unable to restore the CAF diet-induced disruption of gut microbiota at genus level. However, we demonstrated that GSPE prevented the CAF diet-promoted increase in Bacteroidetes-to-Firmicutes ratio during the active phase, thus positively influencing host-microbiota co-metabolism. Interestingly, high doses of GSPE were previously shown to regulate appetite by favouring the secretion of intestinal GLP-1 and PYY [57], which, in turn, are managed by gut microbiota-derived SCFAs [58]. Therefore, it is postulated that GSPE effects on major phyla, mainly by promoting the occurrence of fermentative Firmicutes, could favour the secretion of satiety regulatory hormones in a SCFA-mediated manner. Altogether, the GSPE-mediated prebiotic effects were highlighted during the active phase, whereas its previously described influence on host metabolism is postulated to be partially driven through the management of gut microbiome.

Importantly, and in contrast to our expectations, the consumption of fiber and protein was reduced in GSPE-treated rats regardless the daily phase, which was mainly explained by a decreased chow intake. In this sense, the prebiotic potential of GSPE in counteracting CAF diet-induced dysbiosis at genus level could be masked by an altered protein and fiber intake in the GSPE-supplemented groups. Moreover, the protective role of GSPE against body weight gain observed in our study was not accompanied by a reduced adiposity, and thus, might not be discussed as a beneficial effect for DIO. Therefore, further research is needed to accurately elucidate the microbiota-mediated effects of GSPE in the management of obesity.

Once we had exhaustively evaluated the impact of DIO on gut microbiota, we wanted to go further. From a comprehensive view of dietary therapy as the

most effective tool to modulate gut microbiota, especially in the field of obesity-related dysbiosis, we focused on the research of untapped and potentially valuable prebiotic ingredients. For this purpose, we performed an *in vitro* fermentation study using a wide range of industrial by-products and natural ingredients to evaluate their prebiotic capacity in human fecal samples from normoweight and obese individuals (**Chapter IV**).

In this study, the prebiotic capacity of the ingredients was evaluated through the measurement of pH-lowering effect, gas generation, and SCFA production, altogether with an integrative approach of the metabolomic profiles resulting from the *in vitro* fermentation. Importantly, the favouring effects of inulin-type fructans on the growth of beneficial bacteria, SCFA production, fat oxidation and control of food intake in obesity, prompted us to utilize this compound as a positive control to evaluate the prebiotic capacity of our substrates [59,60]. First of all, we revealed distinct fermentative capacities of gut microbiota from obese and normoweight individuals. In this sense, obese microbiota preferably generated propionate, which has been previously found increased in obese individuals as a microbiota-derived response due to its anti-obesity properties [61], instead of acetate. This result is in accordance with a higher acetate-to-propionate ratio found in lean compared to obese volunteers reported in the literature [30]. Interestingly, acetate is able to cross the blood-brain barrier and reduce appetite [62], and to improve glucose and lipid metabolism in white adipose tissue [63]. Thus, SCFA profiles highly relied on the intrinsic microbiota composition and function, suggesting a major prevalence of propiogenic bacteria in obese conditions.

Relevantly, we observed that *in vitro* fermentation occurred in a substrate-dependent manner. In this sense, we showed for the first time the strong prebiotic potential of pumpkin skin. This ingredient exerted an inulin-like

effect on fermentation measurements, including the decrease of pH, gas generation and SCFA production. Hence, pumpkin skin is postulated as one of the most promising ingredients to be potentially revaluated as a prebiotic food. In addition, we confirmed recent studies attributing prebiotic activities to BSG [64,65], showing that SCFA production is favoured by both lean and obese microbiota fermentation. In this sense, it is suggested that BSG potential benefits are mediated by its high content in arabinoxylans and, subsequently, to the stimulation of butyrate-producing bacteria, as previously shown [66]. Despite that the prebiotic potential of these ingredients should be further evaluated *in vivo* in animal models, we successfully identified prebiotic candidates including pumpkin skin, BSG, *Armillaria mellea* and *Boletus edulis*, Irish moss, quinoa husk and plum skin.

Altogether, the implication of gut microbiota on DIO has been extensively evaluated in this thesis. First, we reported that CAF diet, in contrast to HF and HFS diets, robustly impacts on the physiologic, metabolic and microbial profiles of rats, thus promoting the development of severe obesity and microbiota dysbiosis. Moreover, the CAF diet-induced changes on gut microbiota are influenced by rat strain, thus evidencing that direct extrapolation of DIO effects on gut microbiome between different rat strains must be carefully done. On the other hand, the CAF diet-induced dysbiosis is suggested to strongly disrupt the hormonal control exerted by estrogens in female F344 rats, which was in line with a higher increase in the percentage of RWAT and a higher decrease of body lean mass as compared to males. In addition, GSPE was able to counteract the CAF diet-promoted disturbances on the abundances of the main phyla during the active phase, although no evidences supporting the recovery of gut microbial rhythmicity, which was found disrupted by the CAF feeding, were obtained. Finally, we have

addressed the potential prebiotic capacity of previously untapped natural ingredients, thus revealing for the first time that pumpkin skin, among others, is readably fermented by the gut microbiota and promotes the production of beneficial SCFAs. Overall, although more studies are necessary to elucidate the mechanisms underlying the diet-induced disruption of microbiota-host co-metabolism, this thesis provides a detailed evaluation of gut microbial signatures related to DIO in rats.

REFERENCES

[1] Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature 2010;464:59–65.

[2] Wu ZA, Wang HX. A Systematic Review of the Interaction Between Gut Microbiota and Host Health from a Symbiotic Perspective. SN Compr Clin Med 2019;1:224–35.

[3] Ding R xue, Goh WR, Wu R na, Yue X qing, Luo X, Khine WWT, et al. Revisit gut microbiota and its impact on human health and disease. J Food Drug Anal 2019;27:623–31.

[4] Bäckhed F, Ding H, Wang T, Hooper L V., Gou YK, Nagy A, et al. The gut microbiota as an environmental factor that regulates fat storage. Proc Natl Acad Sci U S A 2004;101:15718–23.

[5] Bäckhed F, Manchester JK, Semenkovich CF, Gordon JI. Mechanisms underlying the resistance to diet-induced obesity in germ-free mice. Proc Natl Acad Sci U S A 2007;104:979–84.

[6] Lin H, An Y, Tang H, Wang Y. Alterations of Bile Acids and Gut Microbiota in Obesity Induced by High Fat Diet in Rat Model. J Agric Food Chem 2019;67:3624–32.

[7] Cani PD, Lecourt E, Dewulf EM, Sohet FM, Pachikian BD, Naslain D, et al. Gut microbiota fermentation of prebiotics increases satietogenic and incretin gut peptide production with consequences for appetite sensation and glucose response after a meal. Am J Clin Nutr 2009;90:1236–43.

[8] Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, et al. Metabolic endotoxemia initiates obesity and insulin resistance. Diabetes 2007;56:1761–72.

[9] Amar J, Burcelin R, Ruidavets JB, Cani PD, Fauvel J, Alessi MC, et al. Energy intake is associated with endotoxemia in apparently healthy men. Am J Clin Nutr 2008;87:1219–23.

[10] Chassaing B, Vijay-Kumar M, Gewirtz AT. How diet can impact gut microbiota to promote or endanger health. Curr Opin Gastroenterol 2017;33:417–21.

[11] Murphy EF, Cotter PD, Healy S, Marques TM, O'Sullivan O, Fouhy F, et al. Composition and energy harvesting capacity of the gut microbiota: Relationship to diet, obesity and time in mouse models. Gut 2010;59:1635–42.

[12] Lecomte V, Kaakoush NO, Maloney CA, Raipuria M, Huinao KD, Mitchell HM, et al. Changes in gut microbiota in rats fed a high fat diet correlate with obesity-associated metabolic parameters. PLoS One 2015;10:e0126931.

[13] Jiang T, Gao X, Wu C, Tian F, Lei Q, Bi J, et al. Apple-derived pectin modulates gut microbiota, improves gut barrier function, and attenuates metabolic endotoxemia in rats with diet-induced obesity. Nutrients 2016;8.

[14] Hildebrandt MA, Hoffmann C, Sherrill-Mix SA, Keilbaugh SA, Hamady M, Chen Y-Y, et al. High-fat diet determines the composition of the murine gut microbiome independently of obesity. Gastroenterology 2009;137:1712–6.

[15] Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, et al. A core gut microbiome in obese and lean twins. Nature 2009;457:480–4.

[16] Everard A, Lazarevic V, Gaïa N, Johansson M, Ståhlman M, Backhed F, et al. Microbiome of prebiotic-treated mice reveals novel targets involved in host response during obesity. ISME J 2014;8:2116–30.

[17] Del Bas JM, Guirro M, Boqué N, Cereto A, Ras R, Crescenti A, et al. Alterations in gut microbiota associated with a cafeteria diet and the physiological consequences in the host. Int J Obes (Lond) 2018;42:746–54.

[18] Kuno T, Hirayama-Kurogi M, Ito S, Ohtsuki S. Reduction in hepatic secondary bile acids caused by short-term antibiotic-induced dysbiosis decreases mouse serum glucose and triglyceride levels. Sci Rep 2018;19:1253.

[19] Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR, et al. Enterotypes of the human gut microbiome. Nature 2011;473:174–80.

[20] Walters WA, Xu Z, Knight R. Meta-analyses of human gut microbes associated with obesity and IBD. FEBS Lett 2014;588:4223–33.

[21] Devkota S, Wang Y, Musch MW, Leone V, Fehlner-Peach H, Nadimpalli A, et al. Dietary-fat-induced taurocholic acid promotes pathobiont expansion and colitis in Il10-/- mice. Nature 2012;487:104–8.

[22] Flint HJ, Scott KP, Duncan SH, Louis P, Forano E. Microbial degradation of complex carbohydrates in the gut. Gut Microbes n.d.;3:289–306.

[23] Chambers ES, Preston T, Frost G, Morrison DJ. Role of Gut Microbiota-Generated Short-Chain Fatty Acids in Metabolic and Cardiovascular Health. Curr Nutr Rep 2018;7:198–206.

[24] Nishina PM, Freedland RA. Effects of propionate on lipid biosynthesis in isolated rat hepatocytes. J Nutr 1990;120:668–73.

[25] Sahuri-Arisoylu M, Brody LP, Parkinson JR, Parkes H, Navaratnam N, Miller AD, et al. Reprogramming of hepatic fat accumulation and "browning" of adipose tissue by the short-chain fatty acid acetate. Int J Obes 2016;40:955–63.

[26] Goffredo M, Mass K, Parks EJ, Wagner DA, McClure EA, Graf J, et al. Role of gut microbiota and short chain fatty acids in modulating energy harvest and fat partitioning in youth. J Clin Endocrinol Metab 2016;101:4367–76.

[27] Klancic T, Reimer RA. Gut microbiota and obesity: Impact of antibiotics and prebiotics and potential for musculoskeletal health. J Sport Heal Sci 2019.

[28] Garcia-Mantrana I, Selma-Royo M, Alcantara C, Collado MC. Shifts on gut microbiota associated to mediterranean diet adherence and specific dietary intakes on general adult population. Front Microbiol 2018;9:890.

[29] Morrison DJ, Preston T. Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. Gut Microbes 2016;7:189–200.

[30] Schwiertz A, Taras D, Schäfer K, Beijer S, Bos NA, Donus C, et al. Microbiota and SCFA in lean and overweight healthy subjects. Obesity 2010;18:190–5.

[31] Nguyen TLA, Vieira-Silva S, Liston A, Raes J. How informative is the mouse for human gut microbiota research? DMM Dis Model Mech 2015;8:1–16.

[32] Pradhan AD. Sex differences in the metabolic syndrome: implications for cardiovascular health in women. Clin Chem 2014;60:44–52.

[33] Parks BW, Nam E, Org E, Kostem E, Norheim F, Hui ST, et al. Genetic control of obesity and gut microbiota composition in response to high-fat, high-sucrose diet in mice. Cell Metab 2013;17:141–52.

[34] Haro C, Rangel-Zúñiga OA, Alcalá-Díaz JF, Gómez-Delgado F, Pérez-Martínez P, Delgado-Lista J, et al. Intestinal Microbiota Is Influenced by Gender and Body Mass Index. PLoS One 2016;11:e0154090.

[35] Kaliannan K, Robertson RC, Murphy K, Stanton C, Kang C, Wang B, et al. Estrogen-mediated gut microbiome alterations influence sexual dimorphism in metabolic syndrome in mice. Microbiome 2018;6:205.

[36] Wu GD, Chen J, Hoffmann C, Bittinger K, Chen YY, Keilbaugh SA, et al. Linking long-term dietary patterns with gut microbial enterotypes. Science (80-) 2011;334:105–8.

[37] Navarro G, Sharma A, Dugas LR, Forrester T, Gilbert JA, Layden BT. Gut microbial features can predict host phenotype response to protein deficiency. Physiol Rep 2018;6:e13932.

[38] Kim JS, de La Serre CB. Diet, gut microbiota composition and feeding behavior. Physiol Behav 2018;192:177–81.

[39] Palmer BF, Clegg DJ. The sexual dimorphism of obesity. Mol Cell Endocrinol 2015:402, 113–9.

[40] Hong J, Stubbins RE, Smith RR, Harvey AE, Nũez NP. Differential susceptibility to obesity between male, female and ovariectomized female mice. Nutr J 2009;8.

[41] Ribot J, Rodríguez AM, Rodríguez E, Palou A. Adiponectin and resistin response in the onset of obesity in male and female rats. Obesity 2008;16:723–30.

[42] Kadowaki T, Yamauchi T, Kubota N, Hara K, Ueki K, Tobe K. Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome. J Clin Invest 2006;116:1784–92.

[43] Toth MJ, Gardner AW, Arciero PJ, Calles-Escandon J, Poehlman ET. Gender differences in fat oxidation and sympathetic nervous system activity at rest and during submaximal exercise in older individuals. vol. 59. 1998.

[44] Medrikova D, Jilkova ZM, Bardova K, Janovska P, Rossmeisl M, Kopecky J. Sex differences during the course of diet-induced obesity in mice: Adipose tissue expandability and glycemic control. Int J Obes 2012;36:262–72.

[45] Amengual-Cladera E, Lladó I, Gianotti M, Proenza AM. Sex differences in the effect of high-fat diet feeding on rat white adipose tissue mitochondrial function and insulin sensitivity. Metabolism 2012;61:1108–17.

[46] Taraschenko OD, Maisonneuve IM, Glick SD. Sex differences in high fat-induced obesity in rats: Effects of 18-methoxycoronaridine. Physiol Behav 2011;103:308–14.

[47] Zamora-Ros R, Andres-Lacueva C, Lamuela-Raventós RM, Berenguer T, Jakszyn P, Barricarte A, et al. Estimation of dietary sources and flavonoid intake in a Spanish adult population (EPIC-Spain). J Am Diet Assoc 2010;110:390–8.

[48] Wang Y, Chung S-J, Song WO, Chun OK. Estimation of daily proanthocyanidin intake and major food sources in the U.S. diet. J Nutr 2011;141:447–52.

[49] Li Y, Hao Y, Fan F, Zhang B. The Role of Microbiome in Insomnia, Circadian Disturbance and Depression. Front Psychiatry 2018;9:669.

[50] Liang X, Bushman FD, FitzGerald GA. Rhythmicity of the intestinal microbiota is regulated by gender and the host circadian clock. Proc Natl Acad Sci U S A 2015;112:10479–84.

[51] Thaiss CA, Zeevi D, Levy M, Zilberman-Schapira G, Suez J, Tengeler AC, et al. Transkingdom control of microbiota diurnal oscillations promotes metabolic homeostasis. Cell 2014;159:514–29.

[52] Smith D, Pernet A, Hallett WA, Bingham E, Marsden PK, Amiel SA. Lactate: A preferred fuel for human brain metabolism in vivo. J Cereb Blood Flow Metab 2003.

[53] Tahara Y, Yamazaki M, Sukigara H, Motohashi H, Sasaki H, Miyakawa H, et al. Gut Microbiota-Derived Short Chain Fatty Acids Induce Circadian Clock Entrainment in Mouse Peripheral Tissue. Sci Rep 2018;8.

[54] Voigt RM, Forsyth CB, Green SJ, Mutlu E, Engen P, Vitaterna MH, et al. Circadian disorganization alters intestinal microbiota. PLoS One 2014;9:e97500.

[55] Liu W, Zhao S, Wang J, Shi J, Sun Y, Wang W, et al. Grape seed proanthocyanidin extract ameliorates inflammation and adiposity by modulating gut microbiota in high-fat diet mice. Mol Nutr Food Res 2017;61.

[56] Casanova-Martí À, Serrano J, Portune KJ, Sanz Y, Blay MT, Terra X, et al. Grape seed proanthocyanidins influence gut microbiota and enteroendocrine secretions in female rats. Food Funct 2018;9:1672–82.

[57] Ginés I, Gil-Cardoso K, Terra X, Blay Mt, Pérez-Vendrell AM, Pinent M, et al. Grape Seed Proanthocyanidins Target the Enteroendocrine System in Cafeteria-Diet-Fed Rats. Mol Nutr Food Res 2019;63:e1800912.

[58] Christiansen CB, Gabe MBN, Svendsen B, Dragsted LO, Rosenkilde MM, Holst JJ. The impact of short-chain fatty acids on GLP-1 and PYY secretion from the isolated perfused rat colon. Am J Physiol Gastrointest Liver Physiol 2018;315:G53–65.

[59] Liu F, Prabhakar M, Ju J, Long H, Zhou H-W. Effect of inulin-type fructans on blood lipid profile and glucose level: a systematic review and meta-analysis of randomized controlled trials. Eur J Clin Nutr 2017;71:9–20.

[60] Singh A, Zapata RC, Pezeshki A, Reidelberger RD, Chelikani PK. Inulin fiber dose-dependently modulates energy balance, glucose tolerance, gut microbiota, hormones and diet preference in high-fat-fed male rats. J Nutr Biochem 2018;59:142–52.

[61] Arora T, Sharma R, Frost G. Propionate. Anti-obesity and satiety enhancing factor? Appetite 2011;56:511–5.

[62] Frost G, Sleeth ML, Sahuri-Arisoylu M, Lizarbe B, Cerdan S, Brody L, et al. The short-chain fatty acid acetate reduces appetite via a central homeostatic mechanism. Nat Commun 2014;5:3611.

[63] Kimura I, Ozawa K, Inoue D, Imamura T, Kimura K, Maeda T, et al. The gut microbiota suppresses insulin-mediated fat accumulation via the short-chain fatty acid receptor GPR43. Nat Commun 2013;4:1829.

[64] Teixeira C, Prykhodko O, Alminger M, Fåk Hållenius F, Nyman M. Barley Products of Different Fiber Composition Selectively Change Microbiota Composition in Rats. Mol Nutr Food Res 2018;62:e1701023.

[65] Sajib M, Falck P, Sardari RRR, Mathew S, Grey C, Karlsson EN, et al. Valorization of Brewer's spent grain to prebiotic oligosaccharide: Production, xylanase catalyzed hydrolysis, in-vitro evaluation with probiotic strains and in a batch human fecal fermentation model. J Biotechnol 2018;268:61–70.

[66] Knudsen KEB, Lærke HN, Hedemann MS, Nielsen TS, Ingerslev AK, Nielsen DSG, et al. Impact of diet-modulated butyrate production on intestinal barrier function and inflammation. Nutrients 2018;10:1499.

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V. CONCLUSIONS

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CONCLUSIONS

The main conclusions of this thesis are:

1. The intake of CAF, HF and HFS diets distinctively alters gut microbial ecosystem, metabolic status and host phenotype in male Wistar rats, leading to robust and similar clusters on intestinal microbiome and urinary metabolomic profiles according to the dietary intervention.

2. CAF diet promotes severe gut microbiota dysbiosis, disrupts bile acid metabolism and SCFA production, and triggers an increased urinary excretion of uremic toxins and metabolites associated with oxidative stress and inflammation.

3. Semi-purified feed-based diets result in overweight phenotypes characterized by modest body fat accumulation, without promoting relevant alterations on the gut microbial ecosystem and urinary metabolome.

4. Wistar rats are more susceptible to CAF diet-induced dysbiosis of gut microbiota than F344 rats, while strain-dependent differences on chow intake might act as a confounder factor.

5. The negative impact of the CAF diet on body lean mass and RWAT weight, as well as on gut microbiota composition, is more pronounced in female than male F344 rats.

6. Consumption of the CAF diet disrupts the rhythmicity of gut microbiota in male F344 rats mainly by altering the cyclical *Lactobacillus* abundance, whereas the diet-induced alterations on the main phyla are

more pronounced during the active phase than during the resting phase.

7. Daily supplementation with dietary doses of GSPE (25 mg/kg body weight) counteracts the CAF diet-promoted increment of Bacteroidetes-to-Firmicutes ratio during the active phase, without inducing beneficial effects for the host's metabolic and physiologic status.

8. The *in vitro* fermentation of pumpkin skin, brewers' spent grain, *Armillaria mellea*, *Boletus edulis*, Irish moss, plum skin and quinoa husk by human fecal microbiota decreases the pH and triggers the generation of gas and SCFAs, thus disclosing a prebiotic potential for these natural ingredients.

9. Pumpkin skin promotes the largest drop in pH and the highest butyrate production within both normoweight and obese conditions, generating the closest metabolomic profiles to those derived from inulin fermentation.

10. Gut microbiota from obese individuals favours the generation of propionate *in vitro*, whereas total SCFA, butyrate and acetate production is mostly favoured when fermentable substrates are exposed to a lean microbiome.

CONCLUSIONS

Les principals conclusions d'aquesta tesi són:

1. El consum d'una dieta CAF, HF i HFS altera de manera diferent l'ecosistema bacterià, l'estat metabòlic i el fenotip de l'hoste en rates Wistar mascle, revelant una agrupació consistent i similar dels perfils microbià intestinal i metabolòmic urinari d'acord amb la intervenció dietètica.

2. La dieta CAF indueix una disbiosi severa de la microbiota intestinal, altera el metabolisme dels àcids biliars i la producció de SCFA, i desencadena un increment de l'excreció urinària de toxines urèmiques i metabòlits associats amb l'estrès oxidatiu i la inflamació.

3. Les dietes semi-purificades indueixen un fenotip de sobrepès caracteritzat per una modesta acumulació de greix corporal, sense promoure alteracions rellevants en l'ecosistema bacterià i en el metaboloma urinari.

4. Les rates Wistar són més susceptibles a la disbiosi de la microbiota intestinal induïda per la dieta CAF, si bé el diferent consum de pinso entre soques pot actuar com un factor de confusió.

5. L'impacte negatiu de la dieta CAF sobre la massa magra corporal i el pes del RWAT, així com sobre la composició de la microbiota intestinal, és més pronunciat en femelles F344 que en mascles.

6. El consum d'una dieta CAF interromp la ritmicitat de la microbiota intestinal en rates F344 mascle, essencialment per l'alteració de

l'abundància cíclica de *Lactobacillus*, mentre que les alteracions induïdes per la dieta sobre els principals fílum són més pronunciades durant la fase activa que durant la fase de repòs.

7. La suplementació diària amb dosis dietètiques de GSPE (25 mg/kg pes corporal) neutralitza l'increment del rati Bacteroidetes/Firmicutes induït per la dieta CAF durant la fase activa, sense induir efectes beneficiosos sobre l'estat fisiològic i metabòlic de l'hoste.

8. La fermentació *in vitro* de la pell de carbassa, el bagàs de cervesa, *Armillaria mellea*, *Boletus edulis,* la molsa d'Irlanda, la pell de pruna i la pellofa de quinoa amb microbiota fecal humana indueix una reducció del pH i desencadena la formació de gas i SCFAs, revelant així un potencial prebiòtic per a aquests ingredients naturals.

9. La pell de carbassa produeix la major disminució de pH i la major producció de butirat tant en condicions de normopès com d'obesitat, generant el perfil metabolòmic més semblant al que se'n deriva de la fermentació de la inulina.

10. La microbiota intestinal d'individus obesos afavoreix la generació de propionat a nivell *in vitro*, mentre que la producció de SCFA totals, butirat i acetat es troba afavorida quan els substrats són fermentats per la microbiota procedent d'individus normopès.

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Encara em veig els quatre asseguts en fila i de cara a la paret, al laboratori principal, quan amb prou feines ens coneixíem i tots teníem la nostra feina. A partir d'aquí, i amb la confiança del dia a dia, ha sorgit una generosa relació entre amics. A l'Héctor, el meu segon company de tesi, amb qui s'ha forjat una excepcional complicitat, per tots els riures, i converses que hem compartit, gràcies. Al Roger, perquè sempre he pogut comptar amb tu pel que fos, i perquè en aquest racó del laboratori s'hi treballa millor amb bona companyia. A la Maria, companya d'estabulari i publicacions, per suportar aquests tres personatges cada dia amb bon humor, per rebre pacientment els meus missatges *random*, i per totes les mostres de suport, gràcies per ser-hi. A l'Eugènia, per la bona sintonia que aportaves en aquest grupet durant els primers, i no per això menys important, mesos de tesi. Gràcies a tots quatre per haver fet d'aquesta una experiència inolvidable.

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A totes i cadascuna de les persones que heu format part d'aquest camí,

GRÀCIES

LIST OF PUBLICATIONS

Papers included in this thesis

Gual-Grau A, Guirro M, Mayneris-Perxachs J, Arola L, Boqué N. Impact of different hypercaloric diets on obesity features in rats: a metagenomics and metabolomics integrative approach. Published in The Journal of Nutritional Biochemistry. 2018; 71:122-131.

Gual-Grau A, Guirro M, Crescenti A, Boqué N, and Arola L. Prebiotic Potential of Natural Ingredients in Lean and Obese Microbiota: an In Vitro Fermentation Study. Submitted to Plos One.

Gual-Grau A, Guirro M, Boqué N, Arola L. Influence of Strain and Gender on the Gut Microbiota Composition and Obesity Development in Cafeteria Diet-Fed Rats. In preparation.

Gual-Grau A, Guirro M, Arola L, Boqué N. Disruption of Microbial Rhythmicity in Rats Fed with a Cafeteria Diet and Time-Dependent Effect of Grape Seed Proanthocyanidins Extract (GSPE) Supplementation. In preparation.

Other papers

Guirro M, Herrero P, Costa A, **Gual-Grau A**, Ceretó Massagué A, Hernández A, Torrell H, Arola L, Canela N. Comparison of metaproteomics workflows for deciphering the functions of gut microbiota in an animal model of obesity. Published in Journal of Proteomics. 2019; 209:103489.

Guirro M, Costa A, **Gual-Grau A**, Herrero P, Torrell H, Canela N, Arola L. Effects from diet-induced gut microbiota dysbiosis and obesity can be ameliorated by fecal microbiota transplantation: A multiomics approach. Published in Plos One. 2019; 14(9):e0218143.

Guirro M, Costa A, **Gual-Grau A**, Mayneris-Perxachs J, Torrell H, Herrero P, Canela N Arola L. Multi-omics approach to elucidate the gut microbiota activity: Metaproteomics and metagenomics connection. Published in Electrophoresis. 2018;39: 1692–1701.

Guirro M, **Gual-Grau A**, Gibert-Ramos A, Alcaide JM, Canela N, Arola L, Mayneris-Perxachs J. Protective effects of hesperidin against the Metabolic Syndrome induced by a cafeteria diet in rats. Submitted to Molecular Nutrition and Food Research.

Serrano J, Casanova-Martí À, **Gual-Grau A**, Pérez-Vendrell AM, Blay MT, Terra X, Ardévol A, Pinent M. A specific dose of grape seed-derived proanthocyanidins to inhibit body weight gain limits food intake and increases energy expenditure in rats. Published in European Journal of Nutrition. 2017; 56(4):1629-1636.

LIST OF CONFERENCE PAPERS

Is the Fountain of (Brain) Youth in the Gut Microbiome? Impact of Fecal Microbiota Transplantation from Young to Aged Mice on Brain, Behavior and Immunity. 2019, Ireland. M. Boehme, K.E. Guzzetta, T.F.S. Bastiaanssen, M. van de Wouw, S. Spichak, **A. Gual-Grau**, G.M. Moloney, L. Olavarria-Ramirez, P. Fitzgerald, N. Ritz, E. Morillas, M. Jaggar, C.S.M. Cowan, A. Walsh, F. Crispie, F. Donoso Vazquez, E. Halitzki, M. Neto, M. Sicchetti, J.S. Cruz-Pereira, A.V. Golubeva, P.D. Cotter, O. O'Leary, T.G. Dinan, J.F. Cryan. *Poster in preparation*.

The impact of age-associated microbiota on neuroimmunity, physiology, and behavior in germ-free mice. 2019, Ireland. K.E. Guzzetta, M. Boehme, **A. Gual-Grau**, T.F.S. Bastiaanssen, S. Spichak, E. Morillas, M. Jaggar, J.S. Cruz-Pereira, M. van de Wouw, G.M. Moloney, N. Ritz, T.G. Dinan, J.F. Cryan. *Poster in preparation*.

IWBBIO 2018, 6th International Work-Conference on Bioinformatics and Biomedical Engineering. 2018. Spain. Elucidation of microbiota activity by a multi-omic approach. Guirro M, Costa A, **Gual-Grau A**, Mayneris-Perxachs, Torrell H, Herrero P, Canela N, Arola L. Poster Presentation.

XVII Congreso de la Sociedad Española de Nutrición (SEÑ) y X Jornada de l'Associació Catalana de Ciències de l'Alimentació (ACCA). 2018, Spain. The intestinal microbiota composition is influenced by circadian rhythms in Fischer 344 rats. **Gual-Grau A**, Boqué N, Bladé C, Arola L. Potser presentation.

NuGO Week. Molecular Nutrition-Understanding how food influences health. 2017. Bulgaria. Hesperidin modulates hypertension and urine metabolism in rats fed with a cafeteria diet. Guirro M, **Gual-Grau A**, Mayneris-Perxachs J, Canela N, Arola L. Poster Presentation.

NuGO Week. Molecular Nutrition-Understanding how food influences health. 2017. Bulgaria. Urine metabolomics profiling reveals specific metabolic alterations in rats fed with different obesogenic diets. **Gual-Grau A**, Guirro M, Mayneris-Perxachs J, Boqué N, Arola L. Poster Presentation.

14th French-Spanish Meeting of the Conferences on Trans-Pyrenean Investigations in Obesity and Diabetes. 2017, Spain. Urine metabolomics profiling reveals specific metabolic alterations in rats fed with different obesogenic diets. **Gual-Grau A**, Guirro M, Mayneris-Perxachs J, Boqué N, Arola L. Oral presentation.

IX Seminario Sobre Alimentación y Estilos de Vida Saludables. 2017. Spain. Comparación del perfil metabolómico urinario inducido por diferentes dietas obesogénicas en ratas Wistar. **Gual-Grau A**, Guirro M, Mayneris-Perxachs J, Boqué N, Arola L. Poster Presentation.

VLAG course The Intestinal Microbiome and Diet in Human and Animal Health, 4th edition. 2017. The Netherlands. Setting a diet-induced obesity reference model for gut microbiota studies in rats. **Gual-Grau A**, Guirro M, Boqué N, Arola L. Poster Presentation.

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GUT MICROBIOTA DYSBIOSIS IN DIET-INDUCED OBESITY. A FOCUS ON THE INFLUENCE OF GENETICS, CIRCADIAN RHYTHMS AND POTENTIAL PREBIOTICS.
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The host and its gut microbiota co-live in a mutually beneficial relationship, while the disruption of this equilibrium has been related to pathological states like obesity. Remarkably, gut microbial dysbiosis has a multi-factorial origin beyond the strong impact of diet. In this thesis, we deeply evaluated the influence of genetics, circadian rhythms and potential prebiotics in obesity from a metagenomic perspective.

Firstly, we characterized the impact of Westernized and semi-purified diets on gut microbiota and host phenotype in Wistar rats, thus identifying cafeteria diet as the most effective dietary model for inducing severe obesity and gut microbiota dysbiosis. Furthermore, the cafeteria diet caused a strong disruption of bile acid metabolism, SCFA production and urinary metabolome. By contrast, semi-purified diets promoted a modest fat accumulation and minor changes on gut microbiome. Then, the influence of strain and gender upon the microbial responses to cafeteria diet was addressed. Wistar rats were more susceptible to cafeteria diet-induced microbial dysbiosis than F344 animals, which was characterized by an increased Bacteroidetes-to-Firmicutes ratio, whereas obesity development similarly occurred in both strains. In addition, F344 females developed more severe alterations on biometric parameters and gut microbiota than males under a cafeteria feeding.

We further evaluated the impact of cafeteria diet and grape seed proanthocyanidins on circadian rhythms of gut microbiota. Whereas the cafeteria diet disturbed the naturally occurring microbial rhythmicity, mainly by altering the cyclical abundance of Lactobacillus, grape seed proanthocyanidins, which can be metabolized by intestinal microbes, restored the microbial signatures of dysbiosis during the active period.

Finally, we investigated the prebiotic capacity of several natural ingredients in an in vitro fermentation study with human fecal microbiota. Here, pumpkin skin promoted the largest drop in pH and an inulin-like metabolomic profile in normoweight and obese fecal samples, thus revealing for the first time its prebiotic potential.