MULTI-OMICS STRATEGY TO ELUCIDATE THE GUT MICROBIOTA ACTIVITY

Maria Guirro Castellnou

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DOCTORAL THESIS
TARRAGONA 2019

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Multi-omics strategy to elucidate the gut microbiota activity

DOCTORAL THESIS

Supervised by Prof. Lluís Arola Ferrer

and Dr. Núria Canela Canela

Grup de Recerca en Nutrigenòmica

Departament de Bioquímica i Biotecnologia

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Maria Guirro Castellnou

DEPARTAMENT DE BIOQUÍMICA I BIOTECNOLOGIA
Campus Sescelades
C/ Marcel·lí Domingo, 1
43007 Tarragona
Tel. +34 977 55 95 21
Fax +34 977 55 82 32

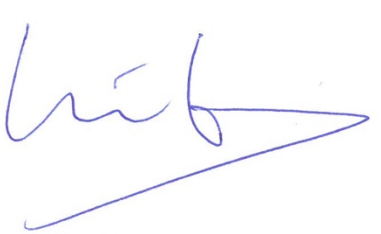
FEM CONSTAR que aquest treball, titulat **Multi-omics strategy to elucidate the gut microbiota activity**, que presenta **Maria Guirro Castellnou** per a l'obtenció del títol de doctorat, ha estat realitzat sota la nostra direcció al Departament de Bioquímica i Biotecnologia de la Universitat Rovira i Virgili i que compleix els requisits per a l'obtenció de la Menció Internacional de Doctorat.

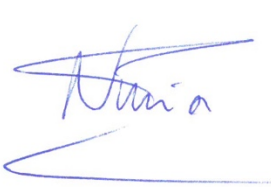
WE STATE that the present study, entitled **Multi-omics strategy to elucidate the gut microbiota activity**, presented by **Maria Guirro Castellnou** for the award of the degree of Doctor, has been carried out under our supervision at the Departament de Bioquímica i Biotecnologia from the Universitat Rovira i Virgili and that is eligible to apply for the International Doctoral Mention.

Tarragona, 6 de Juny de 2019

Els directors de la tesi doctoral

Doctoral thesis supervisors





Dr. Lluís Arola Ferrer

Dr. Núria Canela Canela

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Als meus,

als de casa i als amics.

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Maria Guirro Castellnou

Perquè hi haurà un dia que no podrem més
i llavors ho podrem tot.

Vicent Andrés Estellés

Discovery consists of looking at the same thing
as everyone else and thinking something different.

Albert Szent-Györgyi

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INDEX

SUMMARY

Gut microbiota has become a key player in the pathophysiology of metabolic syndrome and its associated comorbidities, such as hypertension. Treatment of metabolic syndrome is complicated because it is a cluster of disorders; thus, polyphenols can exert beneficial effects to ameliorate metabolic syndrome, especially hesperidin, decreasing the increased levels of blood pressure.

Moreover, some factors such as diet or antibiotics can disrupt the gut microbiota-host equilibrium and trigger a set of detrimental events. To fully comprehend the role of gut microbiota in metabolic syndrome, metaomic sciences have been proposed as the most accurate strategy. A multi-omics strategy combining metagenomics, which provides the taxonomic profile, and metaproteomics, which helps to understand the function in host, is the most adequate approach to study the role of the gut microbiota.

In this regard, this thesis aims to evaluate the role of gut microbiota through a multi-omics strategy in a metabolic syndrome situation. We proposed a cafeteria diet as an accurate model for studying gut microbiota dysbiosis produced by diet. The gut microbiota composition was altered, and its function in the host was modulated; thus, metaproteomics should be preceded by metagenomics to better characterize gut microbiota activity. Moreover, the effectivity of fecal microbiota transplantation was corroborated after a short period of antibiotic depletion, making it an accurate therapy to restore gut microbiota disruption caused by a hypercaloric diet.

Additionally, hesperidin, a polyphenol metabolized by gut microbiota, alters the biodiversity and the functions of the intestinal microbiota,

which are responsible for the amelioration of blood pressure caused by cafeteria diet, and regulates insulin sensitivity and dyslipidemia after chronic administration.

The results of this thesis elucidate the importance of using a multi-omics strategy to explain gut microbiota activity and confirm the significance of the gut microbiota in the maintenance of host homeostasis and energy metabolism.

RESUM

La microbiota intestinal s'ha convertit en un factor clau en la patofisiologia de la síndrome metabòlic i les seves comorbiditats associades tals com la hipertensió. El tractament de la síndrome metabòlica és complicat en ser un conjunt de desordres, així doncs els polifenols, com l'hesperidina, poden tenir efectes beneficiosos, com per exemple, disminuint la pressió arterial.

A més, alguns factors com la dieta o els antibiòtics poden pertorbar l'equilibri microbiota-hoste i desencadenar una sèrie d'esdeveniments perjudicials. Per a comprendre el paper de la microbiota en la síndrome metabòlica, les ciències metaòmiques s'han proposat com l'estratègia més acurada. La combinació de la metagenòmica, que dóna un perfil taxonòmic, i la metaproteòmica, que ajuda a entendre les funcions en l'hoste, és l'aproximació multi-òmica més adequada per a estudiar la microbiota intestinal.

L'objectiu és avaluar el paper de la microbiota mitjançant una estratègia multi-òmica en una situació de síndrome metabòlica. La dieta de cafeteria es va proposar com un model adequat per a estudiar la disbiosi produïda per la dieta. Com a resultat, la composició de la microbiota s'altera i es modula la seva funció en l'hoste, evidenciant que la metaproteòmica hauria d'anar precedida per la metagenòmica per a caracteritzar l'activitat de la microbiota. A més, el transplantament de microbiota fecal es va considerar com una teràpia per a restaurar la disrupció de la microbiota intestinal causada per una dieta hipercalòrica i després d'un període curt d'antibiòtics.

Addicionalment, l'administració crònica de l'hesperidina, un polifenol metabolitzat per la microbiota, altera la biodiversitat i les funcions sent

responsable d'un descens en la pressió arterial, i regula la sensibilitat a la insulina i la dislipèmia causades per la dieta de cafeteria.

Aquests resultats aclareixen la importància d'utilitzar una estratègia multi-òmica per a explicar l'activitat de la microbiota i emfatitzen el paper d'aquesta en el manteniment de la homeòstasis i el metabolisme energètic.

RESUMEN

La microbiota intestinal se ha convertido en un factor clave en la patofisiologia del síndrome metabólico y sus comorbilidades tales como la hipertensión. El tratamiento del síndrome metabólico se complica al ser un conjunto de desórdenes, así que los polifenoles, como la hesperidina, pueden tener efectos beneficiosos, como por ejemplo, disminuyendo la presión arterial.

Además, algunos factores como la dieta o los antibióticos pueden perturbar el equilibrio microbiota-huésped y desencadenar algunos eventos perjudiciales. Para comprender el papel de la microbiota en síndrome metabólico, las ciencias metaómicas se han propuesto como la estrategia más precisa. La combinación de metagenómica, que da un perfil taxonómico, y metaproteómica, que ayuda a entender las funciones en el huésped, es la aproximación más adecuada para a estudiar la microbiota intestinal.

El objetivo es evaluar el papel de la microbiota mediante una estrategia multi-ómica en una situación de síndrome metabólico. La dieta de cafetería se propuso como un modelo adecuado para estudiar la disbiosis producida por la dieta. Como resultado, la composición de la microbiota se altera y se modula la función que desempeña en el huésped, evidenciando que la metaproteómica debería ir precedida por metagenómica para caracterizar la actividad de la microbiota. Además, el trasplante de microbiota fecal se consideró como una terapia para restaurar la disrupción de la microbiota intestinal causada por una dieta hipercalórica y después de un periodo corto de antibióticos.

Adicionalmente, la administración crónica de hesperidina, un polifenol metabolizado por la microbiota, altera la biodiversidad y las funciones siendo responsable de un descenso en la presión arterial, y regula la sensibilidad a la insulina y la dislipemia causadas por la dieta de cafetería.

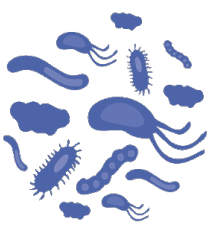
Estos resultados dilucidaron la importancia de utilizar una estrategia multi-òmica para explicar la actividad de la microbiota y enfatizan el papel de ésta en el mantenimiento de la homeóstasis y el metabolismo energético.

ABBREVIATIONS

ABC	Ammonium bicarbonate
ACN	Acetonitrile
ACE	Angiotensin-converting enzyme
Ang	Angiotensin
B/F	Bacteroidetes to Firmicutes ratio
BP	Blood pressure
CAF	Cafeteria
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DC	Sodium deoxycholate
DTT	Dithiothreitol
FA	Formic acid
FASP	Filter-aided sample preparation
FDR	False discovery rate
FFA	Free fatty acids
FMT	Fecal microbiota transplantation
HDL	High-density lipoproteins
HESP	Hesperidin
HFD	High-fat diet
IAA	Iodoacetamide

IGS	InGel-Stacking
IL-6	Interleukin-6
LDL	Low-density lipoproteins
LFD	Low-fat diet
MetS	Metabolic syndrome
MGS	Whole metagenomics sequencing
nHPLC	nano high-performance liquid chromatography
OG	Offgel
OTUs	Operational taxonomic unit
PCA	Principal component analysis
PSM	Peptide spectrum match
RAAS	Renin-aldosterone system
ROS	Reactive oxygen species
SBP	Systolic blood pressure
SCFAs	Short-chain fatty acids
SD	Standard deviation
SDS	Sodium dodecyl sulphate
STD	Standard
TCA	Trichloroacetic acid
TMT	Tandem mass tag
TNF-α	Tumor necrosis factor alpha

VH	Vehicle
WHO	World Health Organization
16Sseq	16S ribosome RNA gene sequencing



INTRODUCTION

INTRODUCTION

1 METABOLIC SYNDROME

Metabolic syndrome (MetS) is characterized by a cluster of metabolic disorders, including abdominal obesity, insulin resistance, dyslipidemia and hypertension, that result from the increasing prevalence of obesity and cardiovascular disease (CVD) [1,2]. In fact, MetS is a heterogeneous disorder with a spectrum of traits that may vary significantly from one affected individual to another. This has led to an attempt to devise criteria that could unify the diagnosis of MetS [3,4].

The diagnostic criteria for MetS have been constantly changing over the last decade, most likely due to its wide range of factors associated with MetS and its increasing prevalence [5]. However, since 2009, there has been a consensus to classify the prevalence of MetS, taking into account the five major aspects involved: waist circumference, blood pressure (BP), glucose fasting levels, triglycerides and high-density lipoprotein (HDL) levels. It is considered that 3 out of 5 risk factors would constitute a diagnosis of MetS [6].

Diabetes, dyslipidemia, hypertension and obesity are associated with MetS, and it is well known that each component of MetS is an independent risk factor for CVD [7–9]. Additionally, obesity is well recognized as a growing epidemic worldwide that affects more than 40% of the population according to the World Health Organization (WHO) [10]. From this population, at least 50% meet the criteria to diagnosis MetS; in other words, a majority of obese people carry the concurrent risk features of an augmented risk of suffering from CVD

[11]. Therefore, in parallel with obesity, MetS is a growing epidemic affecting ~20% of adults in the Western world [8].

The exact cause of MetS is unknown, but the rapidity at which it is expanding in Western society and developed countries betray it as a consequence of complex interactions among genetics, environmental, socioeconomic and dietary factors [12,13].

1.1 Metabolic syndrome pathophysiology

The pathogenic mechanisms of MetS are complex and remain to be fully elucidated. Whether the individual components of MetS represent distinct pathologies or manifestations of a common pathogenic mechanism is still under debate. Currently in the developing world, people with a sedentary lifestyle and extra calorie consumption have a clear excess of energy that is stored as fat. Visceral adiposity has been demonstrated to be a primary trigger for most of the pathways involved in MetS, thus stressing the importance of high caloric intake as a major causative factor [14,15]. From all the proposed mechanisms, insulin resistance, neurohormonal activation and chronic inflammation appear to be the main players in the initiation, progression and transition of MetS to CVD (Figure 1).

Visceral fat has distinct gene expression patterns compared to subcutaneous adipose tissue and is associated with higher insulin resistance and increased levels of low-density lipoprotein (LDL) cholesterol [16]. This high insulin resistance impairs insulin-mediated inhibition of lipolysis, leading to an increase in circulating free fatty acids (FFAs) that further inhibit the antilipolytic effect of insulin, increasing glucose uptake in muscle and liver and inhibiting lipolysis and hepatic gluconeogenesis [17,18]. Augmented FFAs lead to increased triglyceride synthesis and the production of LDL in the liver

and, consequently, a reduction in HDL-cholesterol, which is an indirect consequence of insulin resistance caused by altered lipid metabolism in the liver [19,20].

Additionally, adipokines are secreted by adipose tissue with far-reaching metabolic effects. Leptin controls energy homeostasis, and its levels are increased in obesity and are directly correlated with increased cardiovascular risk. In contrast to leptin, adiponectin is a positive player and is characterized by antiatherogenic properties and promotes insulin sensitivity [21]. In fact, adiponectin has been considered a protector against the development of diabetes and hypertension [22], and its levels are normally reduced in MetS and CVD.

Visceral adiposity is also related to renin-angiotensin-aldosterone system (RAAS) activation through the production of angiotensin II (Ang II), by the activation of angiotensin-converting enzyme (ACE), and serves as an important neurohumoral pathway contributing to MetS development [23]. Ang II activates the generation of reactive oxygen species (ROS), which precipitate a multitude of effects, including LDL oxidation and endothelial injury [24].

Finally, all these pathways culminate in a final inflammatory process that can be chronic. Adipose tissue, which is the origin of inflammation associated with MetS, can induce the secretion of some proinflammatory cytokines [25], such as tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6). TNF-α is known to cause the inactivation of some insulin receptors inducing lipolysis, which increases FFA load and inhibits adiponectin releases [26,27] and IL-6, which increases with body fat and insulin resistance, promotes the

activity of local RAAS pathways and increases fibrinogen levels, resulting in a prothrombotic state [28,29].

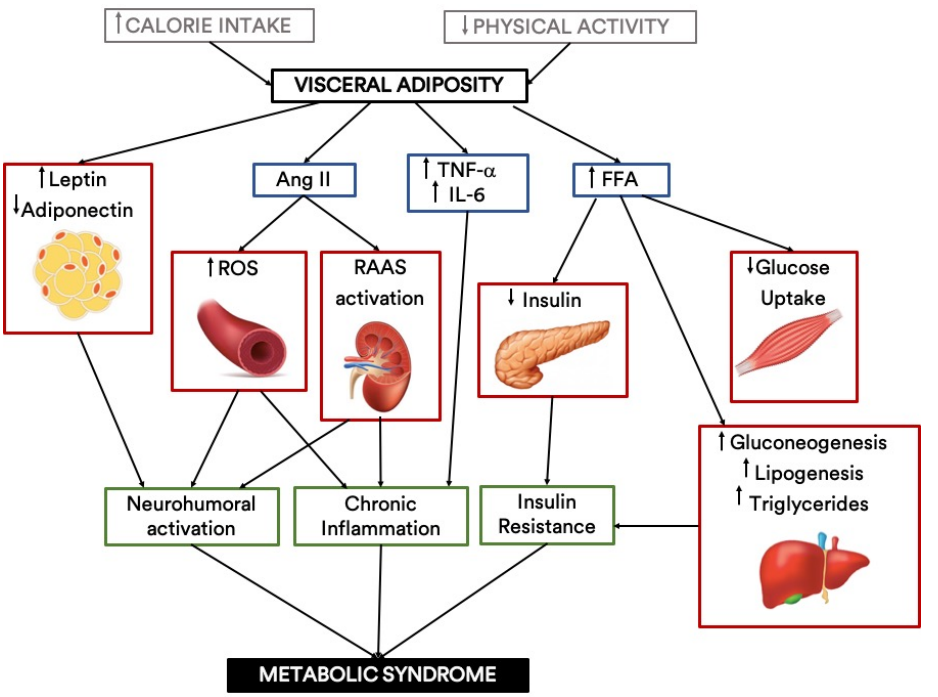


Figure 1. Pathophysiological mechanisms affected in MetS. Ang II, angiotensin II; TNF-α, tumor necrosis factor alpha; IL-6, interleukin-6. Adapted from Rochlani et al. [30]

1.1 Hypertension associated with metabolic syndrome

Hypertension is considered one of the main components of MetS [31], and it is characterized by persistently high BP in arteries. BP is commonly expressed as the ratio of systolic BP (SBP) and diastolic BP (DBP) (SBP/DBP ratio) [32]. Normally, BP levels have narrow distributions with mean values from 120/80 to 139/89 mmHg, with these values being the normal or ideal BP for humans [33,34]. However, when the mean BP recordings are over 140/90 mmHg, they are considered high and a risk factor for CVD development [35]. Moreover, it has been extensively reported that BP is strongly influenced by obesity [36] and insulin resistance [37], which are also two features described in MetS.

Hypertension is currently understood as a multifactorial disease arising from the combined action of many genetic, environmental and behavioral factors. Hence, any change in BP homeostasis is likely to be compensated by feedback or some complementary action to control the mechanisms that regulate BP. When the balance between the factors that tend to increase BP and those that work to normalize it is sufficiently disturbed, essential hypertension results [38]. Therefore, some remarkable insights have been provided that relate dietary salt (NaCl), renal sodium handling and BP. There is evidence linking a high salt intake and the development of hypertension, and when the kidneys are unable to excrete the ingested amount of sodium, BP is increased [38,39].

BP is controlled by a number of complex biochemical pathways. The most important is RAAS, which is known to play a key role in regulating sodium metabolism. Essentially, its regulation is due to ACE function, which generates Ang II by releasing the C-terminal dipeptide His-Leu from angiotensin I (Ang I), a potent vasoconstrictor that controls BP and water-salt equilibrium in the body [40].

In addition to RAAS, the natriuretic peptides induce the excretion of sodium from kidneys regulating the vascular tone, and the endothelium can release the vasoactive substances controlling it. However, the sympathetic nervous system also induces sodium reabsorption, and the immune system can contribute to BP regulation depending on the inflammatory molecules released [32]. There, malfunction or disruption of factors involved in BP control in any component of this integrated system can directly or indirectly lead to modifying BP; whether it is an increased mean or the value varies, over time, it may result in target-organ damage and CVD outcomes [41].

2 GUT MICROBIOTA

Microbiota is defined as the ecological community of commensal, symbiotic and pathogenic microorganisms that live in a specific location; in other words, microbiota represents the bacteria, viruses, fungi, archaea and protozoa that reside at every surface that is in contact with the external environment [42], such as skin, vagina or mouth, but from the vast list of different locations, the gastrointestinal tract is the most popular.

Actually, taking into account each microbiota found in a body, there are approximately 100 trillion microbes existing in largely symbiotic relationships with their hosts and carrying at least 150 times more genes than the entire host genome [43]. The collective of genes that form microbiota are called the microbiome, and this genetic richness enables microbiota to perform important diverse and active activities in the host [44].

A microbiota population can be strongly diverse between locations; for example, the oral bacterial communities of different subjects are more similar than the mouth and skin bacterial communities of a single subject [45]. However, the microbiota varies along different gastrointestinal portions, increasing in quantity and complexity through the intestine [46]. The greatest number of bacteria in the human gastrointestinal tract resides in the large intestine, and the neutral pH and decrease in remnants of pancreatic secretions combined with bile salts facilitates bacterial development. Moreover, the slow transit in the colon favors the proliferation of microorganisms by fermenting the accessible substrates derived from endogenous secretions or just from diet [47].

However, the most studied ecosystem is the gut microbiota, which has recently been considered a 'new revolutionary organ'. Some evidence supports the idea that its metabolic capacity to produce and regulate multiple compounds directly influence systems and vital organs from the host [48].

The gut microbiota comprises three major phyla: Bacteroidetes, Firmicutes and Actinobacteria, including hundreds of species. Firmicutes appears to be the most dominant phyla, with 60% of the population, while Bacteroidetes and Actinobacteria are only 10%, and the rest are represented by other phyla [49–51].

Moreover, some internal and external factors can modulate microbiota populations, such as age, sex, diet, living conditions, antibiotics, health or disease status, among others [43,52]. All these factors can contribute to dysregulating the balance in phyla abundance.

2.1 Factors that modulate gut microbiota

2.1.1 Age

During life, gut microbiota composition is changing constantly, achieving its major richness and diversity and consequently the highest complexity in adult life [52]. Its colonization starts after birth, and the establishment of the bacterial ecosystem in early life is suggested to play a role in the microbial composition and disease susceptibility throughout life. For example, it is known that breast-fed babies have a more heterogeneous microbiota than formula-fed babies [53], and at the end of the first year, the microbiota profile of the baby starts to resemble that of the adult composition. Actinobacteria are dominant in children, whereas Firmicutes and Bacteroidetes predominate in adulthood and are largely stable until later in life, when this stability is reduced [54].

2.1.2 Diet

The gut microbiota interacts with diet to affect host physiology and metabolism, and consequently, an alteration in microbiota composition can strongly affect host health. In fact, the differences among individuals may explain the interindividual variations in response to the same dietary modifications.

Distinctive features have been clearly evidenced across different geographic locations, explained by differing diets. For example, European microbiota was enriched with Firmicutes and Proteobacteria, whereas Actinobacteria and Bacteroidetes were more represented in the African population [55], which could be explained by European diets that are rich in animal proteins, fat and sugar and poor in fiber, contrary to African countries, where vegetarian diets rich in carbohydrates and fiber predominate. Moreover, the fiber content of the diet markedly influences gut microbiota composition by its prebiotic properties, as does the ingestion of foods containing probiotics [56].

However, regarding diet-induced obesity, some controversy exists. What is completely clear is the relationship between diet and microbiota structure and diversity, but there is no consensus with respect to the how they are interrelated. In obese subjects, Bacteroidetes are apparently less predominant than Firmicutes, contrary to normal weight subjects. This situation is reversed when obese subjects lose weight with lower-fat diets [57]. Similarly, mice fed high-fat diets have high levels of Firmicutes and Proteobacteria and small quantities of Bacteroidetes [58]. Many studies have demonstrated differences between gut microbiota biodiversity from lean and obese people, elucidating the term ‘obese microbiota’,

hypothesizing that this profile alone is sufficient to cause obesity [59–62].

Furthermore, microbiota composition varies along the intestine. It is known that the colon is dominated by Firmicutes, which appear to be active in carbohydrate metabolism, whereas Bacteroidetes show activity in a number of functions, such as energy production and conversion as well as amino acid transport and metabolism, in addition to carbohydrate metabolism. Some studies have suggested that the microbiota from the large intestine (mainly colon and cecum) can be differentiated from the composition of several segments from the small intestine [63,64], elucidating the dynamic response from microbiota, especially for changes in nutrient intake.

The complex polysaccharides are degraded by a specialized microbial community, and the released oligosaccharides can in turn be used by other commensal bacteria [52]. In this manner, diet has a crucial influence on intestinal microbial activity.

2.1.3 Antibiotics

Antibiotics are extensively used in the modern world; in fact, they are one of the most common therapies used to treat several bacterial infections. An abusive use of antibiotics can disrupt the microbiota balance. It is widely known that antibiotics not only act to target pathogens but also can affect commensal microorganisms, which are beneficial to host homeostasis [65,66].

The extent of the impact on nontarget microbial populations depends on the particular antibiotic used, which can lead to different states of disease provoking an antibiotic-resistant community of microbes. Normally, one of the characteristics of this state is the decrease in microbiota diversity [67]. Even so, sometimes microbiota composition

cannot be recovered after months of treatment; moreover, after reestablishment, some bacteria persist following colonization and become resistant, allowing foreign microbes to outgrow commensal bacteria and causing permanent changes in microbiota structure and varying disease states (Figure 2) [68,69].

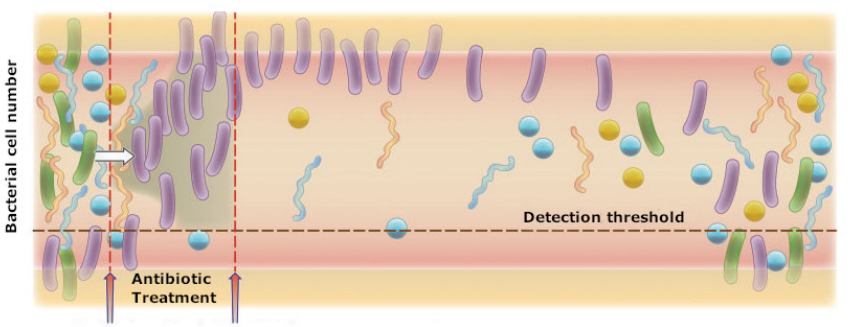


Figure 2. Representation of the impact of antibiotic administration on the bacterial community of the colon. After the onset of treatment, an increase in resistant bacteria (purple rods) can be observed. This increase is due to either a susceptible bacterium (green rods) becoming resistant or resistant bacteria, already present in low levels, increasing in number due to their ability to survive the selective pressure provided by the antibiotic. The acquired resistance is often due to horizontal gene transfer or mutation events (white arrow). As a consequence of treatment, a temporary decrease in diversity can also be seen. Some bacteria may be protected from antibiotic exposure in the mucin layer (yellow shading). Adapted from Jernberg et al. [66].

2.2 Gut microbiota dysbiosis

A healthy microbiota is defined by high diversity and an ability to resist important changes under physiological stress. However, shifts in microbial composition, known as dysbiosis, can destroy these mutualistic relationships and influence host physiology, compromising the health status of the host. Therefore, microbiota associated with disease are defined by lower species diversity, fewer beneficial microbes and/or the presence of pathobionts (any disease-causing microorganism) (Figure 3) [70,71].

During healthy homeostatic conditions, the microbiota is composed of a diversity of organisms that are known to benefit host development and health [72]. However, environmental insults, such as antibiotic use

or diet, can lead to disruptions in the structure of the microbial community. These disruptions can lead to a loss of organisms that are beneficial to the host and a subsequent overgrowth of commensals that have the potential to cause harm, termed pathobionts [73]. Domination of the microbiota by pathobionts can lead to inflammation and pathology. Oftentimes, a total loss of diversity in the microbiota can also influence disease progression or severity and thus also represents a dysbiosis event [74].

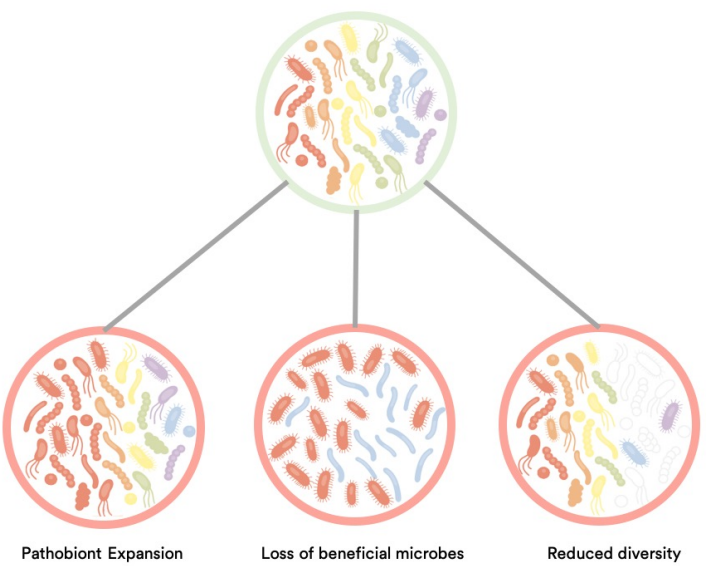


Figure 3. The expansion of pathobionts, a reduced diversity and a loss of beneficial microbes are the principal events that encompass dysbiosis. Adapted from Petersen et al. [71].

The functional role of the microbiota includes a wide range of processes essential to maintaining homeostatic balance, such as nutrient absorption, defense against ingested pathogens and prevention of the translocation of food or antigens into the bloodstream. The disruption of gut microbiota renders the intestine vulnerable to local disease states, having severe consequences to the host [74].

Intestinal dysbiosis has been linked with important diseases, including autoimmune and/or autoinflammatory disorders, such as inflammatory bowel disease, and metabolic disorders, such as obesity, type 2 diabetes, allergies and neurological disorders [75].

2.3 Gut microbiota metabolic functions in the host

There is a symbiotic relationship between a microbiota and its host. In fact, the gut microbiota acts as a 'metabolic organ' that performs many essential functions to maintain homeostasis within the host [76]. Most microbial products act as signaling molecules and influence the host's metabolism, also affecting the liver and brain, as well as adipose and muscle tissue, which consequently may affect the level of obesity and the associated comorbidities (Figure 4) [77].

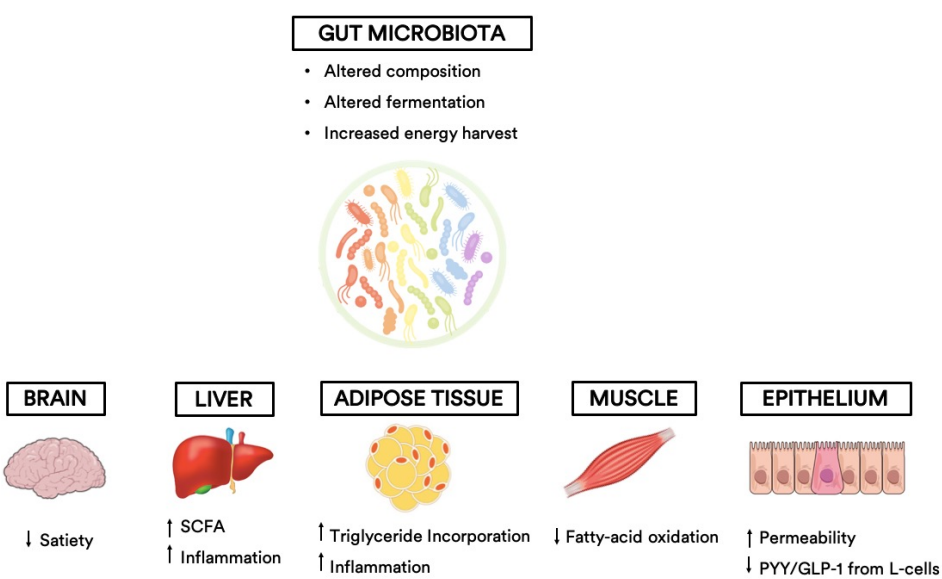


Figure 4. Alteration in gut microbiota composition and metabolic capacity of gut microbiota in obesity promote adiposity and influence metabolic processes in peripheral organs, such as the control of satiety in the brain; the release of hormones from the gut (such as PYY and GLP-1); and the synthesis, storage or metabolism of lipids in the adipose tissue, liver and muscle. Microbial molecules also increase intestinal permeability, leading to systemic inflammation and insulin resistance. Adapted from Tremaroli et al. [77].

Under normal conditions, gut microbiota contributes to the anatomical and physiological intestine structure. These microorganisms constitute

an enormous potential in the gut, playing a wide variety of metabolic functions [50], and all these functions allow the host to utilize many energetic sources. The breakdown of complex indigestible dietary carbohydrates and proteins, the synthesis of some amino acids, the conversion of dietary polyphenolic compounds and the bile acid biotransformation process are some examples of important functions where microbiota is involved [78,79].

2.3.1 Probiotics and prebiotics

Probiotics and prebiotics may exert beneficial effects through changes in microbiota; in fact, favorable modifications of gut microbiota by probiotics and/or prebiotics could be one dietary strategy to consider in some metabolic disorders such as MetS [80].

The definition of probiotics and prebiotics has been modified over the years due to its implication in host health [81].

For probiotics, first, it was stated that they must be viable microorganisms that exert beneficial effects on their host to emphasize their microbial origin [82], but now, the definition formulated by WHO working experts states that probiotics are strictly selected microorganisms that, when administered in adequate amounts, confer a health benefit on the host [83]. While the concept of prebiotics in the past was restricted to carbohydrates, a new definition states that a prebiotic is a non-digestible compound that, through its metabolization by gut microbiota, specifically supports the growth and/or activity of health-promoting bacteria that colonize the gastrointestinal tract [84].

Probiotic characteristics are not associated with the genus or species of a microorganism, but rather the probiotic strains chosen. Probiotics have to meet some requirements associated with the technology used for their production, which means that they have to survive and

maintain their properties throughout the storage and distribution processes [85]; hence, some functional aspects define their survival in the gastrointestinal tract [86]. Moreover, probiotics have numerous advantageous functions in the host, such as the ability to inhabit the microbiota of the organism, ensuring a proper balance between pathogens and the beneficial bacteria required for a normal function of the organism, or the capacity to counteract the activity of pathogenic intestinal microbiota, introduced from contaminated food and/or environment [87].

On the other hand, prebiotics are present in natural products, but they may also be added to food. The purpose of these additions is to improve the nutritional and health value of food, which can serve as a medium to stimulate probiotic growth and effect [88]. Prebiotics are not digested by host enzymes and usually reach the colon without being altered, where they are fermented by bacteria [89]. Hence, prebiotics have enormous potential for modifying the gut microbiota, and their consumption largely affects gut microbiota metabolic activity. However, these modifications in gut microbiota composition occur at the level of individual strains and species and are not easily predicted a priori. Furthermore, the gut environment, especially pH, plays a key role in determining the outcome of interspecies competition [90].

2.3.2 Short-chain fatty acids

Non-digestible or complex carbohydrates are important sources of energy for host and microbial cells. They cannot be degraded in the small intestine. Instead, these carbohydrates, including cellulose, xylans, resistant starch and inulin, are fermented in the colon by microbiota to yield energy for microbial growth and end products such as short-chain fatty acids (SCFAs) [91]. Mainly, butyrate, propionate

and acetate account for 90-95% of SCFAs present in the colon that interact with host metabolism and have profound effects on gut health [77].

Butyrate represents a primary energy source for colonocytes enhancing fatty acid oxidation and thermogenesis by activating key factors in the regulation of energy homeostasis and fat storage [92]. Acetate enters the systemic circulation where it can serve as a cholesterol or fatty acid precursor, and it also reduces appetite by changing the profiles of regulatory neuropeptides through the activation of the Krebs cycle [93]. Propionate enters the portal circulation as a precursor of gluconeogenesis, while it also inhibits liver lipogenesis [94]. Moreover, it is known that SCFAs, along with other metabolites, can enter the portal system to be transported to the liver and can act on resident macrophages and hepatocytes to maintain a healthy liver through the regulation of hepatic metabolism and inflammation. Additionally, SCFAs can regulate insulin in the pancreas, FFA flux from adipocytes, and appetite centers in the brain, providing fuel for the muscle, although this multifaceted role has not been fully described [91], thereby promoting metabolic benefits on body weight and glucose control.

Hence, the increasing focus on the multifaceted roles of SCFAs suggests the relevance they may have in the metabolic control and inflammatory status associated with obesity and its comorbidities.

2.3.3 Dietary polyphenols

Polyphenols are categorized as plant secondary metabolites necessary for the plant to survive in the environment, and normally, they are found bound to sugars and organic acids forming groups

according to their basic structures; they are mainly divided into flavonoids and non-flavonoids [95,96].

Polyphenols have become an intense focus of research due to their potential benefits to health, suggesting beneficial effects such as anticarcinogenic, antiatherogenic, antithrombotic, anti-inflammatory, antiallergenic, anticoagulant, immune modulating, antimicrobial, vasodilatory or analgesic activities [97]. To achieve these health benefits, polyphenols require in situ processing by the gut microbiota to be transformed into a potentially more bioactive, low-molecular-weight metabolite, hence the interest in their physiological function in the gastrointestinal tract [98].

Polyphenol content in the diet is higher than previously believed because of the presence of non-extractable compounds [99]; its poor bioavailability favors the interaction with gut microbes.

Most polyphenols pass through the small intestine without being absorbed, and when they arrive at the colon, microbes can convert those compounds into functional metabolites, developing a two-way interaction between polyphenolic compounds and gut microbiota. First, gut microbiota transforms non-absorbable polyphenols into their metabolites, regulating their bioavailability. Reciprocally, polyphenols act as a modulator of gut microbiota composition through the inhibition of pathogenic bacteria and the stimulation of beneficial bacteria. In that sense, they may act by exerting beneficial effects due to their prebiotic properties [100,101]. Therefore, the gut microbiota is a key player in explaining the variable effects of dietary polyphenols on health [102].

2.3.4 Bile acids

Bile acids are another class of metabolites representing gut microbiota-host co-metabolism, and their chemical composition is significantly influenced by the gut microbial community. Therefore, the microbiota should be considered an essential factor in bile acid homeostasis in the host [103]. Bile acids in the mammalian intestine could be classified into two types according to their origins: primary bile acids, which are generated in the liver and transported to the intestine through the enterohepatic circulation; and secondary bile acids, which come from the modification of gut microbiota from the primary bile acids, and they dominate in the total fecal bile acid pool [104].

Bile acids entering the intestinal lumen come in contact with microbial species that are able to metabolize them to bile salts. Bile salt hydrolases are enzymes present in various microbial species that are responsible for deconjugation, an obligate step in bile salt microbial conversion. This process normally occurs in an anaerobic environment in the small and large intestines [105].

Bile acids play a major role in the digestion of dietary lipids in the gut; thus, an alteration of the balance of microbial species in the gut has the capacity to alter the metabolic potential of this community, altering bile acid signatures and, ultimately, host responses [106].

2.4 Fecal microbiota transplantation

Fecal microbiota transplantation (FMT) is the administration of a solution of fecal material from a healthy donor into the intestinal tract of a recipient to directly change the recipient's microbial composition and confer a health benefit [107,108].

The goal of FMT is to restore host health by increasing the diversity and function of the gut microbiota. Several techniques and routes of FMT have been described, but methods have not yet been standardized [109]. In fact, FMT was primarily used to treat *Clostridium difficile* infections, which are associated with an abusive use of antibiotics [110,111]. After the success of FMT in this type of infection, FMT was considered a strong option to treat other medical conditions in which dysbiosis was postulated to be a contributory factor [112].

From the mounting evidence demonstrating the direct role of gut microbiota in obesity pathogenesis, the gut has emerged as a potential target for therapeutic approaches, and the variances influencing host metabolism through microbial-derived metabolites and substrates have postulated FMT as an effective treatment to palliate obesity and MetS [107,113]. FMT mainly consists of transferring obese-type microbiota or lean-type microbiota to germ-free mice and observing the response [114–116]. In fact, it was demonstrated that the body fat percentage, serum triglycerides and insulin resistance increased in germ-free mice that had fecal content transplanted from lean conventionally raised mice [117], illustrating the role of gut microbiota in obesity.

Moreover, prebiotics and probiotics have been postulated as gut microbiota modulators, and although they are possible therapeutic methods to alter the microbiota as a treatment, FMT has the potential to be a more effective option. The main advantage of FMT over probiotics is the ability to transplant the entire gut microbiota and metabolites from the donor to the recipient [118,119], with the superior capability to ameliorate intestinal dysbiosis over single microbial targets in probiotics [120].

Although the potential of FMT is exciting, there are some limitations, such as donor selection or the lack of optimized methods, which hinder the establishment of FMT as a recurring treatment for metabolic disorders [121].

2.5 Gut microbiota and hypertension

The homeostatic maintenance of BP is a complex process governed by the kidneys and regulated by genetic, environmental and endocrine factors. Recently, the relationship between hypertension and gut microbiota has been elucidated, and it appears that gut microbiota can participate in BP regulation and consequently in hypertension pathogenesis [122–124]. This BP regulation can occur through the secretion of SCFAs. Two new sensory receptors (Olfr78 and Gpr41) have been identified as novel regulators of BP, and both are also receptors for SCFAs [125].

Thus, some studies have demonstrated that gut microbiota richness, diversity and evenness were decreased in spontaneously hypertensive rats and that the Firmicutes and Bacteroidetes ratios increased [124,126]. In fact, it has been suggested that subjects with hypertension exhibit alterations in the relative abundance of 'beneficial' and potentially 'harmful' bacteria compared to healthy subjects [127,128].

Moreover, gut microbiota can influence the production of various hormones, such as serotonin, dopamine, and norepinephrine, which can affect BP. In this sense, gut microbiota metabolites can profoundly affect the cardiovascular system [129].

Probiotics have also been reported to exert beneficial effects in hypertension; specifically, they are inhibitors of ACE activity producing antihypertensive bioactive peptides that are released during protein hydrolysis [130,131]; in fact, in recent years, numerous studies

have shown either moderate or significant reductions in SBP/DBP ratios [132–134].

Altogether, this evidence supports that the antihypertensive activity of probiotics is cross-linked with several different mechanisms, such as improving lipid and triglyceride levels, bile acid deconjugation, and controlling body mass index. In addition, an increase in the absorption of nutrients and reduction in plasma glucose levels may also contribute to the probiotic effect in BP regulation [135].

3 GUT MICROBIOTA AND OMIC SCIENCES

The numbers and diversity of microbes in ecosystems within and around the body are unmatched; these communities of microbes show some characteristics, and they may compete for nutrients, share functional genes, and produce toxins that are lethal to other microbes [136]. The inability to cultivate some of these microorganisms in vitro, to better study their role in the whole microbiome, remains a challenge. Hence, various high-throughput molecular techniques, such as multi-omic sciences, can provide insights into the genomic structure and metabolic potential as well as the activity of complex microbial communities [137].

The addition of 'omics' in a molecular term refers to the large comprehension of a set of molecules as a whole, such as an organism or a cell [138]. When studying microbiota, due to its complexity, the prefix meta- is added to mean 'beyond', making that science more complex.

One of the most common approaches to studying a microbiome is to analyze its constituent microbial genomes through metagenomics [139]. Metagenomics is the study of the genomes present in a given sample, and recently, due to the inclusion of the host environment as part of the microbiome implications and with the interactions between the host and its associated microbial community, metagenomics must be combined with other '-omic' approaches such as metatranscriptomics, metaproteomics or metabolomics [139,140]. By definition, metatranscriptomics provides a snapshot of gene expression levels, metaproteomics sheds light on the active function of the microbial community, and metabolomics completes the picture by

determining which products are being released into the environment [136,137].

3.1 Metagenomics

Metagenomics gives a taxonomic profile of the metagenome, defined as the collective of the microbial genomes present in a given sample. Next-generation sequencing approaches enable the comprehensive analysis of all microbes without the need for cultivation [141]. Two methods are widely used: the sequencing of the 16S ribosome RNA (rRNA) gene (16Sseq) and shotgun metagenomics sequencing (MGS). The first method, 16Sseq, uses the 16S gene, which is highly conserved across bacteria and can be easily amplified. This gene is characterized to have some conserved regions, but there are nine hypervariable sequences distinctive for each species, so a taxonomical estimation can be performed [142]. More precise and more expensive than 16Sseq, MGS is the untargeted sequencing of the whole genome in all the microorganisms in the sample. Moreover, the central role of RNA makes it interesting to also study the gut microbiome using the RNA-sequencing technique, whereby transcripts are converted to cDNA for next-generation sequencing. It is now possible to profile and quantify RNA molecules in any organism [143].

In 16Sseq, sequences are clustered into operational taxonomic units (OTUs) based on similarity. From each cluster, a single sequence is selected as a representative sequence and annotated at the taxonomic levels (phylum, class, order, family, genus and species), inheriting the same annotation within the OTU [144]. However, in MGS, sequences are binned to taxonomic assignments directly based on a reference database, such as BLAST or SEED [145]. Then, the proportion of sequencing reads of a specific taxon to the overall number of reads can be represented as a relative abundance [146].

In addition to relative abundance taxa, two indexes can be calculated to explain microbiota biodiversity: alpha diversity and beta diversity. Alpha diversity measures species richness (number of species) and evenness (species distribution) within a community, and it can be calculated using the Shannon index, Chao index, observed OTUs index and Simpson index; beta diversity represents the composition differences among communities and can be calculated using Bray-Curtis dissimilarity, weighted UniFrac distance and unweighted UniFrac distance [147].

3.2 Metaproteomics

Metaproteomics was coined as the large-scale characterization of the entire protein composition in a microbiota sample at a given point in time [148], and it has provided new features to study complex microbial communities. Some efforts contribute to transforming metaproteomics from a methodological development and small-scale study to a complex approach that provides more extensive coverage of metaproteomes in larger studies, considering metaproteomes as the community of proteins contained in a sample. Moreover, the development of higher resolution mass spectrometers and quantitative proteomics techniques has contributed to the rapid progression in metaproteomics, and the development of efficient bioinformatic tools adapted to the complexity of microbiomes is key to the expansion of metaproteomics in recent years [149–152].

The principal challenge in metaproteomics is to identify and quantify the proteins present in a sample and properly link them to the different microbes found in that sample, increasing its complexity compared to classical proteomics. Nevertheless, there are still many critical issues that need to be addressed in both experimental workflow and bioinformatic aspects of metaproteomics.

A typical metaproteomic workflow consists of four main parts: protein extraction from the microbiome samples, proteolytic digestion, peptide analysis using nano-high-performance liquid chromatography MS/MS (nHPLC-MS/MS) and protein identification and quantification from microbes by searching against a metagenome database [153]. Finally, the protein/peptide information is used for downstream taxonomic and functional analysis (Figure 5). Nevertheless, depending on the biological question asked, the coverage of proteins and microbes needs to be optimized, and fractionation steps have considerably improved microbiome coverage [154,155].

The protein extraction methods will directly impact coverage proteins, and the selection of the method will result in differences in protein recovery, especially the choice of cell lysis type; depending on the cell wall structure, some protocols will be more efficient than others [156,157]. In addition, the efficiency of protein digestion and microbial protein identification can be significantly influenced by other non-microbial components (food debris, inorganic salts, etc.) within the samples. Therefore, some approaches, such as differential centrifugation and protein purification with precipitation, will enrich microbial cells, and protein identification will be performed more accurately [158].

Despite all these efforts in the metaproteomics workflow, the data analysis still remains a challenge [159]. The advances have clearly demonstrated the ability of metaproteomics in examining the functional activity of the microbiome, but there is a high percentage of proteins that are shared by multiple species, and they cannot be specifically assigned further from phylum to genus. In fact, the capacity of metaproteomics depends on the functional annotation of proteins/genes within the reference databases and the bioinformatic

tools, but unfortunately, metagenomics databases are usually poorly annotated [160].

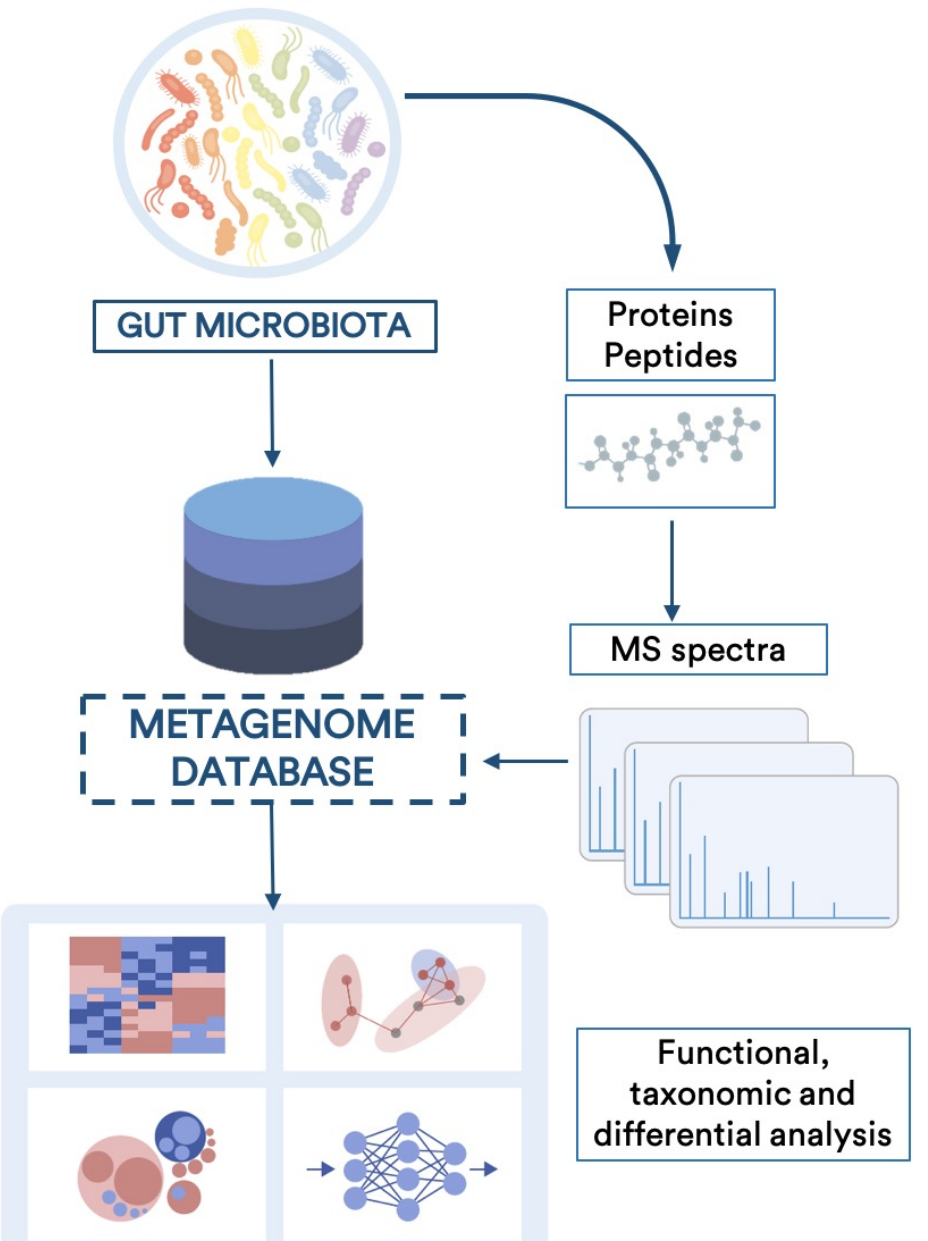


Figure 5. General workflow for metaproteomics. Adapted from Zhang et al. [161].

3.3 Omics data integration

The individual analysis of omic datasets focuses on the community structure and function itself, but the challenge lies in elucidating the large, dynamic, and complex network of interactions between its constituent entities. Hence, integrative multi-omics approaches have been proposed as the most powerful strategy to provide full pictures of the compositions, functions and activities of the interactions [162]. The combination of metagenomics and metaproteomics can improve microbiota knowledge from metagenomics sequencing and go deeper in functional metaproteomics data [163,164]. Moreover, the integration of metabolomics could enhance metaproteomics data and further understand the functions of the microbiome and microbe-microbe interactions with hosts because proteins are the direct players in metabolic pathways that produce metabolites. However, the lack of efficient tools for multi-omics integration hinders reaching this objective and achieving an accurate and proper approach to fully comprehend microbiota-host homeostasis.

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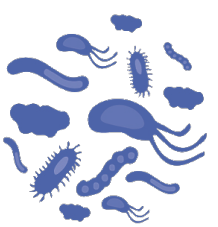


HYPOTHESIS AND OBJECTIVES

HYPOTHESIS AND OBJECTIVES

MetS is one of the most prevalent pathologies in Western societies. It is characterized as a cluster of disorders, mainly metabolic disorders, such as abdominal obesity, hypertension or insulin resistance, which produce irreversible changes into host physiology, affecting an important number of metabolic functions. Its increased prevalence can be caused by several factors, but the most important ones are Western diets and a sedentary lifestyle accompanied by some genetic and environmental factors, which can lead to a more severe prognosis.

The comorbidities associated with MetS make its treatment more complicated; thus, a combination of several disorders should be the most appropriate strategy to treat MetS.

Polyphenols are plant secondary compounds with a massive number of benefits, such as antioxidant properties, improved dyslipidemia, decreased chronic inflammation, increased hypotension or ameliorated insulin sensitivity, among others. Hypertension is the principal cause of CVD development, which is tightly related to MetS.

Moreover, the gut microbiota has acquired some relevance in several pathologies, such as MetS. In fact, the gut microbiota could act as a metabolic organ in the host, regulating, directly or indirectly, numerous important functions.

Several studies have confirmed some patterns in microbiota composition according to diet. It is known that microbiota biodiversity in obese people clearly differs from that in healthy people, elucidating the role of microbiota in MetS. Normally, people who suffer from MetS

conditions, and consequently some of the other comorbidities, have unhealthy lifestyles being one of the possible causes of microbiota disruption. Although the real link between these two factors remains unclear, there are several clues that implicate diet in playing an important role.

We hypothesized that **hesperidin**, a citrus flavanone metabolized by microbiota, **modulates BP through microbiota action when MetS is induced by a hypercaloric diet**. In other words, hesperidin supplementation directly affects the hypertension associated with diet-induced obesity via gut microbiota modulation.

To verify the confidence of this hypothesis, the principal objective of this thesis was **to evaluate whether hesperidin consumption, when obesity and MetS are occurring, can induce alterations in BP through the microbiota and to determine which function will be affected by the changes observed in host physiology.**

Different specific aims were proposed to reach the general objective:

1. **To assess the effect of diet on microbiota biodiversity and functions in an obesity situation (Chapter 1).**

Diet has a major impact on microbiota composition and produces an important number of molecules that are metabolized by microbiota and are essential for maintaining energetic homeostasis. However, to study the effect of diet on microbiota, some specific and sophisticated tools are required due to the sample complexity consisting of several different microorganisms.

Recently, omic sciences have emerged as the most accurate tool to fully comprehend the role of the microbiota in the host. Specifically, metagenomics and metaproteomics are the most relevant. The first

technique uncovers what the microorganisms are, and the second identifies what exactly they are doing in a specific situation.

To date, metagenomics has been used in several studies and has enabled the attribution of particular microbiota patterns, meaning that microbiota diversity is characteristic in specific situations, such as obesity and MetS. Hence, a first study with obese animals was performed to corroborate the impact of diet on microbiota (**Manuscript 1**).

To complement metagenomics, another omic approach was necessary, such as metaproteomics, which is less studied. For this reason, an optimized methodology was required to continue with our objective. Hence, the critical steps in classical proteomics methodologies were optimized to reach the most accurate method to address such complex samples as microbiota (**Manuscript 2**).

2. **To verify the effect of antibiotics on the microbiota and corroborate the role of diet by a FMT (Chapter 2).**

Antibiotics are extensively used to treat bacterial infections and other diseases. However, they have a wide range of actions not only by erasing pathobionts but also by acting on microorganisms that exert beneficial effects on the host. It is known that antibiotics can produce modulation in microbiota even after a short period of antibiotic consumption, leading to microbiota depletion and an alteration of its balance, causing selective dysbiosis. This alteration could be treated by FMT, which can restore microbiota biodiversity and consequently its function on the host. Thus, the microbiota role was corroborated in obese diet-induced animals applying both omics approaches (**Manuscript 3**).

3. **To evaluate the effect of hesperidin as a hypertension treatment through the modulation of microbiota and its function (Chapter 3).**

The gut microbiota is responsible for hydrolyzing some polyphenols into metabolites that are absorbed in the intestine. The differences in microbiota diversity can directly influence its functions, thereby affecting its bioavailability. Hesperidin is a flavanone that is hydrolyzed by microbiota, and its bioactive compound has a beneficial effect in hypertension. To date, few studies have demonstrated its mechanism of action in BP, and none have examined MetS situations induced by a hypercaloric diet. Hence, a new animal study was performed to elucidate the role of hesperidin and to evaluate whether the effect observed on BP was correlated with microbiota alterations. First, the combination of metagenomics and metaproteomics approaches (**Manuscript 4**) was assessed, and second, hesperidin function was studied in MetS situations (**Manuscript 5**).

HIPÒTESIS I OBJECTIUS

La síndrome metabòlica és una de les patologies més prevalents al món occidental. Es caracteritza per ser un conjunt de desordres, bàsicament metabòlics, com l'obesitat abdominal, la hipertensió o la resistència a la insulina, que produeixen canvis irreversibles en la fisiologia afectant greument un important nombre de funcions metabòliques. Aquest augment de la seva prevalença es podria atribuir a diversos factors però els més importants són l'occidentalització de la dieta i l'estil de vida sedentària, ja que acompanyats de factors genètics i ambientals poden desencadenar un pronòstic més o menys greu.

Un dels problemes principals de la síndrome metabòlica és el seu tractament, en ser una malaltia multifactorial amb diverses comorbiditats associades, l'estratègia més adequada és la combinació dels tractaments dels diversos desordres que formen aquest síndrome.

Els polifenols són compostos secundaris vegetals amb un gran nombre de propietats atribuïdes com poden ser actuar d'antioxidants, millorar la dislipèmia, disminuir la inflamació crònica, ser hipotensius o millorar la sensibilitat a la insulina, entre d'altres. La hipertensió és una de les principals causes en el desenvolupament de les malalties cardiovasculars, estretament relacionada amb la síndrome metabòlica.

Per altra banda, la microbiota intestinal ha agafat molt de protagonisme en patologies com la síndrome metabòlica. Es creu que podria actuar com un òrgan metabòlic més en l'hoste, regulant un gran nombre de funcions ja sigui de manera directa o indirecta.

De fet, diversos estudis han confirmat patrons en la composició bacteriana de la microbiota segons la dieta, a més s'ha vist que persones obeses presenten una població microbiana que difereix bastant en comparació amb les persones sanes, deixant entreveure que la microbiota en sí podria afavorir el desenvolupament de la malaltia. Normalment, les persones que pateixen síndrome metabòlica i per extensió, alguna de la comorbiditats associades, tendeixen a tenir uns hàbits de vida menys saludables, fet que podria ser una de les causes d'aquests canvis en la microbiota intestinal.

Tot i que encara no s'ha pogut definir quin és el veritable enllaç o mecanisme d'acció que relaciona aquests dos factors, existeixen diverses sospites en les que la dieta jugaria un paper molt important.

La hipòtesis principal plantejada és que **l'hesperidina,** una flavanona present en les fruites cítriques i que es metabolitzada per la microbiota, **modula la pressió arterial a través de la microbiota en una situació de síndrome metabòlica induïda per una dieta hipercalòrica**. És a dir, com la suplementació de l'hesperidina afecta de manera directa la hipertensió associada a un estat d'obesitat induït per una dieta rica en carbohidrats i greixos i que actuaria a través de la modulació de la microbiota intestinal.

Per a comprovar la certesa d'aquesta hipòtesis es va plantejar com a objectiu **avaluar si el consum d'hesperidina en un context d'obesitat i síndrome metabòlica pot induir canvis en la pressió arterial a través de la microbiota i quina és la funció que aquests canvis produiran en l'estat fisiològic de l'hoste**.

Per a poder dur a terme aquest objectiu principal, es van plantejar els següents objectius específics:

1. **Comprovar l'efecte de la dieta en la biodiversitat i funció de la microbiota en una situació d'obesitat (Capítol 1).**

La dieta té un gran impacte sobre la composició de la microbiota i produeix un important nombre de molècules que són metabolitzades per la pròpia microbiota essencials per a mantenir l'homeòstasi energètica. Però per a poder estudiar l'efecte de la dieta en la microbiota es requereixen eines especials i molt sofisticades ja que partim d'una mostra molt complexa formada per una gran quantitat de microorganismes diferents.

Recentment, les ciències òmiques semblen ser les eines més adequades per a entendre el paper de la microbiota en l'hoste. En concret, la metagenòmica i la metaproteòmica serien les dues òmiques més rellevants, ja que la primera ens permet desxifrar qui són aquets microorganismes presents i la segona que estan fent en un context específic.

Fins avui, la metagenòmica ha estat utilitzada en diversos estudis i ha permès atribuir uns patrons concrets de microbiota, és a dir, una població característica per a diferents situacions de malaltia com seria en el cas de l'obesitat o síndrome metabòlica. Per tant, primer de tot es va realitzar un estudi amb animals obesos per a corroborar l'impacte de la dieta en la microbiota (**Manuscrit 1**).

Per a poder complementar la metagenòmica, era necessari dur a terme altres òmiques com la metaproteòmica. Aquesta ciència està molt menys estudiada per tant, era necessària una optimització de la metodologia per a poder continuar amb el nostre objectiu, així que es van optimitzar els diferents punts crítics de la metodologia clàssica de la proteòmica per a obtenir el mètode més adequat per a tractar mostres tant complexes com la microbiota (**Manuscrit 2**).

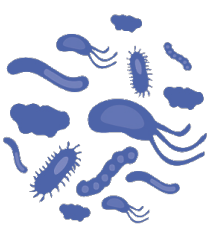
2. **Verificar l'efecte dels antibiòtics en la microbiota i comprovar el paper de la dieta mitjançant un transplantament de microbiota fecal (Capítol 2).**

Els antibiòtics s'utilitzen de manera molt comuna per a les infeccions bacterianes i altres malalties. Però aquests tenen un ampli ventall d'accions, ja que no només actuen per eliminar aquells microorganismes patògens sinó que també impacten sobre aquells que tenen efectes beneficiosos per a l'hoste. Se sap que poden provocar alteracions directament a la microbiota tot i després d'un període curt d'administració, per tant podrien causar un depleció de la microbiota alterant-ne així el balanç i produir una disbiosi selectiva. Una manera de corregir aquesta disbiosi seria fent un transplantament de microbiota fecal, s'ha vist que aquest pot restaurar la biodiversitat i conseqüentment la funció de la microbiota en l'hoste. Així doncs, en un estudi amb animals amb obesitat induïda per una dieta alta en greixos es va avaluar el paper de la microbiota mitjançant la combinació d'ambdues metaòmiques i així corroborar que les eines utilitzades eren les més acurades (**Manuscrit 3**).

3. **Avaluar l'efecte de l'hesperidina com a tractament per la hipertensió a través de la modulació i la funció de la microbiota (Capítol 3).**

La microbiota és responsable de la hidròlisi de certs polifenols en metabòlits que són absorbits a l'intestí. Les diferències en la biodiversitat de la microbiota poden influir directament en la seva funció, de manera que la seva biodisponibilitat quedaria afectada. L'hesperidina és una flavanona que és coneguda per ser hidrolitzada per la microbiota i el seu compost bioactiu té un efecte positiu sobre la hipertensió. Fins ara, pocs estudis han demostrat el seu mecanisme d'acció directa en la pressió arterial i menys en una situació com seria la síndrome metabòlica induïda per una dieta hipercalòrica. Per tant,

un nou estudi amb rates es va plantejar per a poder elucidar el paper de l'hesperidina en aquest context i per intentar avaluar si l'efecte observat sobre la pressió arterial estava correlacionat amb els canvis amb la microbiota. Primer de tot amb la combinació de les dues òmiques principals com la metagenòmica i metaproteòmica (**Manuscrit 4**) i després estudiant la funció de l'hesperidina en el marc de la síndrome metabòlica induïda per la dieta (**Manuscrit 5**).



RESULTS

Chapter 1

To assess the effect of diet on microbiota biodiversity and functions in an obesity situation

Manuscript 1

Alterations in gut microbiota associated with a cafeteria diet and the physiological consequences in the host

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Alterations in gut microbiota associated with a cafeteria diet and the physiological consequences in the host

Josep Maria del Bas[1,2], Maria Guirro[1,3], Noemí Boqué[1,2], Adrià Cereto[1,3], Rosa Ras[1,3], Anna Crescenti[1,2], Antoni Caimari[1,2], Núria Canela[1,3] and Lluís Arola[1,4]

[1] Nutrition and Health Unit, EURECAT-Technology Centre of Catalonia, Reus, Spain;

[2] Nutrition and Health Research Group, EURECAT-Technology Centre of Catalonia, Reus, Spain;

[3] Group of Research on Omic Methodologies (GROM), Joint Unit Universitat Rovira i Virgili—EURECAT-Technology Centre of Catalonia, Centre for Omic Sciences (COS), Unique Scientific and Technical Infrastructures (ICTS), Reus, Spain

[4] Nutrigenomics Research Group, Department of Biochemistry and Biotechnology, Universitat Rovira i Virgili, Tarragona, Spain.

Correspondence: Dr Josep Maria del Bas

ABSTRACT

OBJECTIVE: Gut microbiota have been described as key factors in the pathophysiology of obesity and different components of metabolic syndrome (MetS). The cafeteria diet (CAF)-fed rat is a preclinical model that reproduces most of the alterations found in human MetS by simulating a palatable human unbalanced diet. Our objective was to assess the effects of CAF on gut microbiota and their associations with different components of MetS in Wistar rats.

METHODS: Animals were fed a standard diet or CAF for 12 weeks. A partial least square-based methodology was used to reveal associations between gut microbiota, characterized by 16S ribosomal DNA gene sequencing, and biochemical, nutritional and physiological parameters.

RESULTS: CAF feeding resulted in obesity, dyslipidemia, insulin resistance and hepatic steatosis. These changes were accompanied by a significant decrease in gut bacterial diversity, decreased Firmicutes and an increase in Actinobacteria and Proteobacteria abundances, which were concomitant with increased endotoxemia. Associations of different genera with the intake of lipids and carbohydrates were opposed from those associated with the intake of fiber. Changes in gut microbiota were also associated with the different physiological effects of CAF, mainly increased adiposity and altered levels of plasma leptin and glycerol, consistent with altered adipose tissue metabolism. Also hepatic lipid accretion was associated with changes in microbiota, highlighting the relevance of gut microbiota homeostasis in the adipose–liver axis.

CONCLUSIONS: Overall, our results suggest that CAF feeding has a profound impact on the gut microbiome and, in turn, that these changes may be associated with important features of MetS.

INTRODUCTION

The incidence of metabolic syndrome (MetS) is growing among developed and developing societies because of the increasing prevalence of obesity among all population groups.[1,2] Different preclinical models have been developed in order to study the mechanisms involved in the progression of this complex condition, as well as to define effective treatments.[3,4] In this regard, the cafeteria diet (CAF)-fed rodent has emerged as a valuable model because it develops the main features of MetS, that is, obesity, dyslipidemia, insulin resistance, chronic low-grade inflammation and hypertension.[5,6] Although the exact components of CAF vary among experimenters, the main and common characteristic of this model is based on *ad libitum* access to a highly palatable diet consisting of a blend of commercially available processed food rich in saturated fats and simple carbohydrates with a low fiber content. These components are offered together with a commercial chow that contains all the nutritional requirements for the animals in terms of vitamins, minerals and oligo elements. The high palatability of CAF influences the mechanisms of satiety and the reward system,[7] blunting the natural homeostatic control of energy intake and inducing hyperphagia and therefore exacerbated food consumption, mimicking the mechanisms that lead to obesity in humans under the so-called Western diet.[8,9] Therefore, the different effects of diet-induced MetS have been widely explored by means of the CAF rodent model, either at the physiological, psychological or molecular level, and the effects of different dietary interventions.[5,10,11]

During the last few years, a growing body of evidence has shown that the response to a given diet depends on the gut microbiome of the host among other factors such as the genetic background.[12–16] In fact,

different studies have highlighted important differences that exist in the gut microbiota among subjects from around the world with extremely different diets.[15] The exact mechanisms are still under debate, but it has been widely demonstrated that the gut microbial community is shaped by diet and, at the same time, has a key role in the transformation and absorption of nutrients by the host, such as fibers, fatty acids or amino-acid-derived molecules among others, as well as endogenous metabolites, such as bile acids.[12,17] Therefore, the microbiota determine how different key nutrients and bioactive molecules exert their signaling actions through modulation of hormones and other receptors. At the same time, the microbiota are a source of signaling metabolites, including lipopolysaccharide (LPS), a product of Gram-negative bacteria that has been described as a key trigger of inflammation and its derived alterations.[12,18] The gut microbiome has gained much attention in obesity research.[12,17,19–21] Studies in both preclinical models and human volunteers have shown that obesity and MetS are associated with decreased gut microbial diversity and changes in the equilibrium of certain microbes at different taxonomic levels.[16,22] Thus, fluctuations in the population of four major phyla, that is, *Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria*, among others, have been strongly associated with obesity and its related alterations, such as insulin resistance, dyslipidemia or non alcoholic hepatic steatosis. However, to date, no specific microbiome fingerprint for obesity has been identified.[22]

The clear effects of CAF on different components of MetS and the role of gut microbiota in obesity and its derived pathologies prompted us to hypothesize that CAF can alter the gut microbiome of healthy rats and that these changes may be associated with some physiological alterations related to MetS caused by this specific diet. Therefore, our

objective was to assess the effects of CAF feeding on the gut microbiome and to describe the associations between these changes and alterations of lipid metabolism in the CAF-fed rat. To this aim, we fed Wistar rats with a standard diet (STD) or CAF for 12 weeks. The associations between gut microbiota and nutrient intake and biometric and biochemical parameters were analyzed by means of a sparse partial least square approach. We demonstrate that CAF impacts gut microbiota composition and that these changes may be associated with the intake of specific nutrients and parameters linked to the lipid metabolism of the adipose–liver axis.

MATERIALS AND METHODS

Animals and diets

The Animal Ethics Committee of the University Rovira i Virgili (Tarragona, Spain) approved all the procedures. All animals were housed individually at 22 °C with a light/dark cycle of 12 h (lights on at 0900 hours) and were given free access to food and water. Individual housing was intended to allow an accurate estimation of food intake and to avoid crossed effects on microbiota because of the coprophagia that is usual in rats.

Six-week-old male Wistar rats (Harlan Laboratories, Barcelona, Spain) were randomly distributed to two experimental groups (n = 8) depending on the diet received during 12 weeks: the STD group, which was fed standard chow (Teklad Global 18% Protein Rodent Diet 2018, Harlan, Barcelona, Spain), and the CAF group. Sample size and nutrient compositions of CAF and STD used herein were recently described by Cigarroa *et al.*[10] Blinding was not done. The standard chow used for both diets is designed for young animals but was selected because of its higher content of vitamins and some minerals

in order to guarantee a correct intake of micronutrients by the CAF group.

Body weight and food intake were recorded weekly. For food intake estimation, the weight of chow and of CAF diet components were recorded before and 24h after consumption. To do that, CAF components were confined in a container. After the feeding period, CAF remnants were manually recovered from rat cages and containers and weighted. Nutrient consumption was calculated according to the nutritional composition provided by the manufacturer of each component of the CAF diet or of the chow. For each dietary component, the area under the curve was calculated along the experimental period to report the total intake. Rats were killed after 6 h of fasting in order avoid interferences of the early postprandial state in plasma metabolites and under anesthesia (sodium pentobarbital, 80 mg kg−1 body weight). Blood was collected by cardiac puncture and serum was obtained by centrifugation and stored at − 20 °C until analysis. Cecum, liver, gastrocnemius and soleus muscles, interscapular brown adipose tissue and white adipose tissue depots (retroperitoneal (RWAT), mesenteric (MWAT), epididymal (EWAT) and inguinal (IWAT)) were rapidly removed, weighed, frozen in liquid nitrogen and stored at − 80 °C. Adiposity index was computed as the sum of the EWAT, IWAT, MWAT and RWAT depot weights and expressed as a percentage of the total body weight, as previously described.[23]

Serum analysis

Enzymatic colorimetric kits were used for the determination of serum total cholesterol, triglycerides, glucose and glycerol (QCA, Barcelona, Spain) and non-esterified free fatty acids (WAKO, Neuss, Germany).

Circulating insulin, adiponectin and leptin levels were measured using rat ELISA kits (Merck, Madrid, Spain). Malondialdehyde was determined as previously described.[24]

Lipid extraction and quantification

Lipids were extracted and quantified from the liver (100–120 mg) using methods described previously.23 Briefly, lipids were extracted with 1 ml of hexane/isopropanol (3:2, vol/vol), degassed with nitrogen before left overnight under orbital agitation at room temperature protected from light. After an extraction with 0.3 ml of Na2SO4 (0.47 M), the lipid phase was dried and total lipids quantified gravimetrically before emulsifying as described previously.[25] Triglycerides, cholesterol and phospholipids were assayed with commercial enzymatic kits (QCA).

Microbiota

The genomic bacterial DNA was obtained from 700 to 1000 mg of cecal content of previously snap-frozen cecum with the QIAamp DNA stool kit (Qiagen, Hilden, Germany; cat. no. 51504) following the manufacturer's protocol. Partial 16S ribosomal RNA gene sequences were amplified from 20 ng of extracted DNA using three primer pairs (341F-532R, 515F-806R and 967F-1046R), which target the V3, V4 and V6 regions, respectively. Equimolar pools of each fragment were combined to create the DNA library, which was subjected to a clonal amplification by an emulsion PCR. After an Ion Sphere Particle enrichment process, samples were loaded onto 318 chips and sequenced using the Ion Torrent PGM (Life Technologies, Carisbad, CA, USA). The individual sequence reads were filtered by the PGM software (Life Technologies, Carisbad, CA, USA) to remove low-quality and polyclonal sequences. Those reads were processed using QIIME,[26] selecting only sequences with 150–200 bp and omitting

homopolymers. 16S ribosomal RNA operational taxonomic units (OTUs) were assigned using uclust (497% sequence homology) and a reference data set from GreenGenes (Lawrence Berkeley National Laboratory, Berckeley, CA, USA).

Analysis of plasma LPS

Internal standards, 3-hydroxydecanoic acid, 3-hydroxydodecanoic acid, 3- hydroxytetradecanoic acid and 3-hydroxyhexadecanoic acid (Larodan, Solna, Sweden) and 3-hydroxytetradecanoic acid-2,2,3,4,4-d5 (Sigma, Spain), were added to hydrolased (NaOH) and non-hydrolased samples to estimate total and free hydroxyfatty acids (3OHFA), respectively. After acidification (HCl) and liquid–liquid extraction with chloroform, sample compounds were derivatized with NO-bis-(Trimethylsilyl) trifluoroacetamide and 10% trimethylchlorosilane and 10% of pyridine. Samples were analyzed in a 7890A Series gas chromatograph coupled to a 7000 GCQqQ (Agilent Technologies, Santa Clara, CA, USA). Ionization was performed by electronic impact. LPS content was computed as the sum of nanomoles of individual esterified 3OHFAs (total minus free) divided by 4 to account for the four molecules of 3OHFAs assumed per molecule of LPS.

Statistical methods

Association studies were performed by applying a canonical sparse partial least square discriminant analysis using genus abundances as one of the matrices and four different data sets as the second data matrix in separate analyses using the Mixomics package for R[27] following a previously described methodology.[28] These four independent data sets were absolute values of macronutrient intake (Table 1 and Supplementary Figure 1), biometric parameters as tissue

weights and body weight itself (Table 1 and Supplementary Figure 2), plasma biochemical parameters (Table 1 and Supplementary Figure 3) and liver biochemical parameters (Table 1 and Supplementary Figure 4). Relevance networks were constructed with the software cytoscape 3.0[29] for each analysis following previously described methodologies[30] and for the four analyses simultaneously using the genus identified in all the analyses as the link between networks. With the exception of the microbiome analysis described above, the rest of the statistical analyses were conducted with R commander software for Windows.[31]

RESULTS

Rats fed a CAF for 12 weeks developed obesity and MetS

As expected, CAF was associated with hyperphagia, resulting in a twofold increase in energy intake (Table 1). This was mainly because of a fivefold and a twofold increase in lipid and carbohydrate intake, respectively. Fiber intake and protein intake were reduced by 283% and 33%, respectively. As a result, at the end of the experiment, CAF-fed animals presented a 30% increase in body weight (Table 1) because of a significant 78% increase in the adiposity index. CAF animals presented enlarged pale livers with a higher lipid content than their counterparts (Table 1). Analysis of plasma parameters (Table 1) revealed a clear hypertriglyceridemia accompanied by significantly increased free fatty acids and a statistical tendency toward increased glycemia in CAF animals. It has to be taken into account that glycemia values were unusually high for both groups, likely due to anesthesia, and further results obtained using these values should be interpreted carefully. Both leptin and adiponectin were increased in the CAF group, suggesting alterations in adipose tissue metabolism. Altogether, these results indicated that CAF-induced obesity and hepatic steatosis were

accompanied by altered lipid and glucose metabolism, resembling the human features of MetS.

Table 1. Macronutrient intake during 12 weeks, tissue weights and plasma and liver biochemistry in rats fed a standard chow (STD) or a cafeteria diet (CAF). Mean ± Standard Error of the Mean are represented.

	STD	CAF	*p-value*
Macronutrient intake (AUC)			
energy intake (Kcal/day)	3656±103	8233±352	6.E-09
proteins (g/day)	273±8	205±14	7.E-04
lipids (g/day)	50±1	282±17	3.E-09
carbohydrates (g/day)	580±16	1291±58	1.E-08
fiber (g/day)	34±1	12±2	4.E-08
saturated fatty acids (g/day)	17.1±0.5	147.5±8.4	3.E-10
monounsaturated fatty acids (g/day)	18±1	94±7	2.E-08
polyunsaturated fatty acids (g/day)	3±0	12±1	1.E-06
total fatty acids (g/day)	38±1	254±16	2.E-09
cholesterol (g/day)	0.229±0.006	1.180±0.080	1.E-08
water (g/day)	2834±198	5072±231	4.E-06
Tissue weights (absolute values)			
body weight (g)	453±11	598±20	2.E-05
liver(g)	12±1	16±1	1.E-04
RWAT (g)	17±1	36±2	8.E-07
EWAT (g)	16±1	31±3	9.E-05
IWAT (g)	23±1	63±7	9.E-05
MWAT (g)	10±1	21±2	1.E-04
BAT (g)	0.62±0.06	1.26±0.13	5.E-04
gastrocnemius and soleus (g)	2.63±0.05	2.56±0.05	3.E-01
Tissue weights (relative to body weight)			
liver (%)	2.68±0.06	2.76±0.12	6.E-01
RWAT (%)	3.7±0.2	5.9±0.2	4.E-06
EWAT (%)	3.5±0.2	5.1±0.3	3.E-04
IWAT (%)	5.0±0.2	10.4±0.9	5.E-05
MWAT (%)	2.2±0.1	3.5±0.2	8.E-05
Adiposity index	14.3±0.5	25.0±1.4	5.E-06
BAT (%)	0.14±0.01	0.21±0.02	4.E-03
gastrocnemius and soleus (%)	0.58±0.02	0.43±0.02	7.E-05

Plasma parameters			
triglycerides (mM)	1.7±0.2	3.3±0.3	5.E-04
cholesterol (mM)	2.5±0.2	2.6±0.1	5.E-01
glucose (mM)	11.2±0.7	13.4±0.8	7.E-02
MDA (nM)	257±8	404±82	1.E-01
insulin (nM)	6.6±0.6	8.4±0.6	1.E-01
adiponectin (pg/mL)	3.3±0.2	5.0±0.3	5.E-04
glycerol (mM)	0.14±0.02	0.32±0.02	4.E-05
NEFA (mM)	0.23±0.02	0.33±0.02	7.E-04
Leptin (ng/mL)	19 ±1	106 ±11	1.E-06
Liver biochemistry			
Total lipids (mg/g)	42 ±3	78 ±6	6.E-05
Triglycerides (mg/g)	5.4 ±0.4	11.2 ±0.9	3.E-05
Total cholesterol (mg/g)	2.4 ±0.2	4.7 ±0.4	4.E-05
Phospholipids (mg/g)	10.9 ±0.9	14.6 ±0.7	6.E-03

Abbreviations: BAT, brown adipose tissue; CAF, cafeteria diet; EWAT, epididymal adipose tissue; IWAT, inguinal adipose tissue; MWAT, mesenteric adipose tissue; NEFA, non-esterified fatty acids; RWAT, retroperitoneal white adipose tissue; STD, standard diet. Values are represented as mean ±s.e.m.

Gut microbiota were altered by CAF

Cecum content was obtained and the microbiome was characterized. After analysis, 15 962 OTUs were detected, and their changes between STD and CAF groups were quantified (Supplementary Table 2). Of these OTUs, 5046 (31%) presented statistically significant differences when assessed by means of the false discovery rate, with q-values under 0.05. Analysis of the 27 phyla detected (Figure 1a) revealed that CAF induced a decrease in Firmicutes (q = 0.0003) but not in Bacteroidetes (q = 0.11) relative to the STD-fed animals. The ratio of Bacteroidetes to Firmicutes remained unaltered, though it tended to increase in CAF-fed animals (CAF = 1.26 ± 0.56 and STD = 0.19 ± 0.02; q = 0.08). Moreover, a large increase in Actinobacteria (q = 0.03) and Proteobacteria (q = 0.009) was detected in the CAF group, although Actinobacteria was clearly increased only in five out of eight CAF-fed animals. Together with

these four major phyla, we found a significant 10-fold increase in Cyanobacteria (q = 0.02) and decreased levels of Armatimonadetes (q = 0.02), Deferribacteres (q = 0.003), Fibrobacteres (q = 0.04), Nitrospirae (q = 0.01), SC4 (q = 0.0001), TM7 (q = 0.004) and Tenericutes (q = 0.006). Despite the increase in predominant phyla in animals fed CAF, the alpha diversity, calculated either as the Shannon index, Simpson index or observed OTUs (Figure 1b), was significantly lower (q = 0.002) in the CAF group relative to the STD group. The estimation of beta diversity by Principal Coordinates Analysis (Figure 1c) revealed a clear and statistically significant separation between STD and CAF metagenomes (P = 0.01). Moreover, a hierarchical clustering analysis of the 299 genera quantified (Figure 1d) further confirmed clear differences among metagenomes. Both analyses also show that the differences within CAF-fed animals are much greater than those within their STD counterparts.

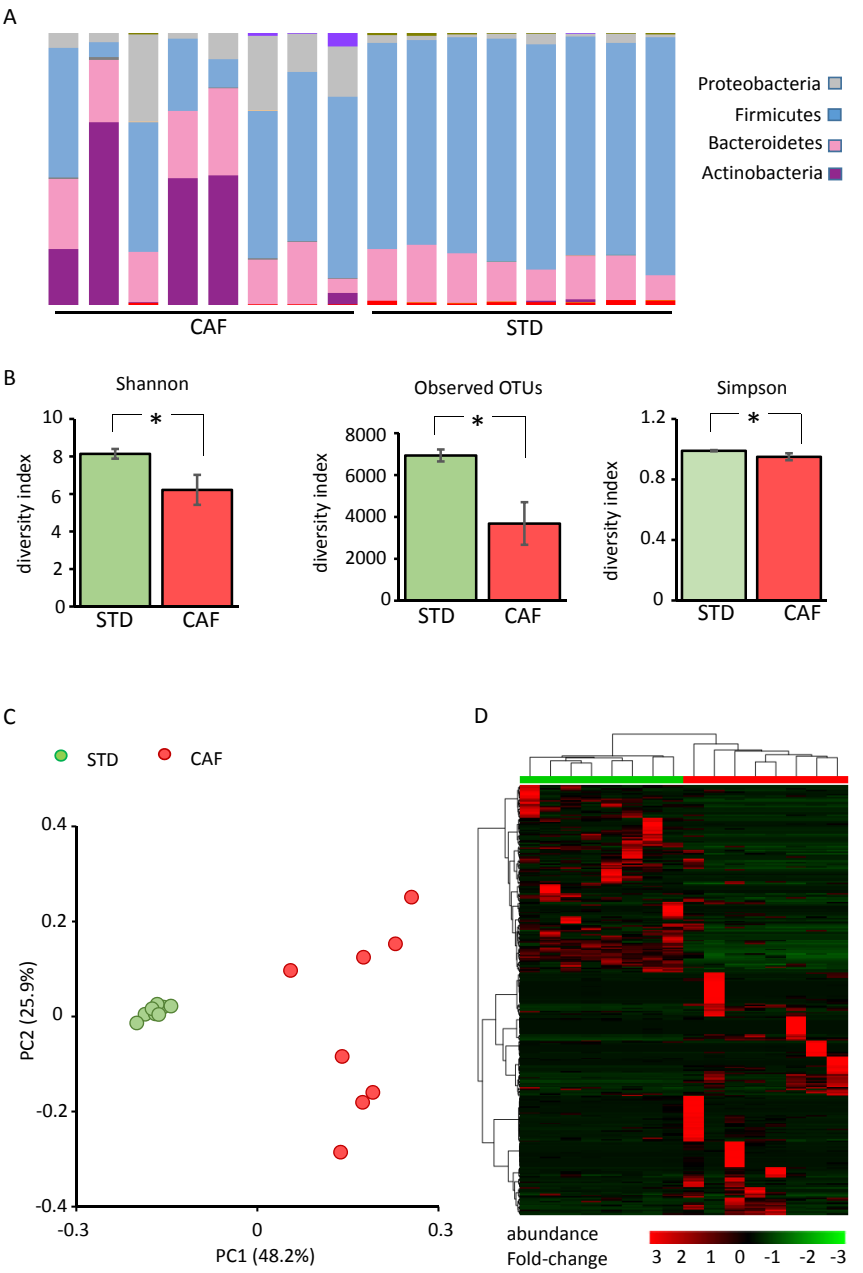

Figure 1. Changes in the metagenome of rats fed a CAF or a STD were assessed by (a) phyla abundance, (b) differences in alpha diversity between CAF- and STD-fed animals calculated by the Shannon, observed OTUs and Simpson indexes; (c) analysis of beta diversity represented by scores of each CAF- and STD-fed animals after principal coordinates analysis; (d) hierarchical clustering analysis for the 16 animals colorized as indicated and the corresponding heatmap depicting the fold-change with respect to the average abundance of each genus. * Denotes a Po0.05 between the highlighted groups by T statistics.

Associations between dietary components and gut metagenome in rats fed STD and CAF

Values of genus abundances and dietary components intake were subjected to sparse partial least squares analysis. The associations with a R^2 coefficient over 0.7 were selected, resulting in 26 genera belonging to the following six phyla: Firmicutes (10 genus), Bacteroidetes (6 genus), Proteobacteria (7 genus), Chloroflexi (1 genus), Deferribacteres (1 genus) and SC4 (1 genus). Regarding dietary components, fiber was associated with 18 genera, carbohydrates with 20 genera, fatty acids and lipids with 22 genera and cholesterol with 23 genera. In turn, protein was only associated with two genera. Remarkably, all Firmicutes were positively associated with fiber intake. The details and directionality of the associations (positive or negative) are detailed in Supplementary Figure 1 and Figure 2. Codes for bacteria are described in Table 2.

Table 2. Bacteria highly associated with physiological parameters in rats fed a Standard chow (STD) and a cafeteria diet (CAF) for 12 weeks.

code	Fold-change	p-value	phylum	class	order	family	genus
bact18	99.79	2.E-02	Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Propionibacterium
bact35	7.78	2.E-04	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides
bact36	UP	1.E-02	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Candidatus Azobacteroides
bact37	13.70	4.E-02	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Paludibacter
bact38	16.12	9.E-04	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae	Parabacteroides
bact41	0.05	1.E-05	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	
bact42	0.07	5.E-04	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotella
bact46	111.80	2.E-03	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	Alistipes
bact47	0.38	5.E-04	Bacteroidetes	Bacteroidia	Bacteroidales	S24-7	
bact50	4.65	8.E-02	Bacteroidetes	Bacteroidia	Bacteroidales	[Paraprevotellaceae]	
bact60	0.01	3.E-06	Chloroflexi	Anaerolineae	S0208		
bact62	0.26	3.E-02	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Anaerofilum

bact 69	0.00	3.E-03	Deferribacteres	Deferribacteres	Deferribacterales	Deferribacteraceae	Mucispirillum
bact 72	DOWN	9.E-05	Firmicutes	Bacilli	Bacillales	Alicyclobacillaceae	
bact 89	0.10	2.E-04	Firmicutes	Bacilli	Lactobacillales	Aerococcaceae	Facklamia
bact 96	25.24	1.E-02	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Pediococcus
bact103	0.04	1.E-10	Firmicutes	Clostridia	Clostridiales		
bact111	0.05	2.E-08	Firmicutes	Clostridia	Clostridiales	Dehalobacteriaceae	Dehalobacterium
bact120	UP	9.E-03	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Epulopiscium
bact135	0.17	5.E-06	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	
bact136	12.99	1.E-02	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Bilophila
bact138	34.01	5.E-03	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Clostridium
bact140	0.11	5.E-07	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillospira
bact142	0.18	3.E-06	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ruminococcus
bact152	74.66	9.E-03	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Phascolarctobacterium
bact164	0.03	8.E-04	Firmicutes	Clostridia	MBA08		
bact180	15.11	8.E-03	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	
bact206	20.70	1.E-04	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Sutterella
bact211	0.00	7.E-05	Proteobacteria	Deltaproteobacteria			
bact216	UP	5.E-02	Lentisphaerae	[Lentisphaeria]	Lentisphaerales	Lentisphaeraceae	Lentisphaera
bact220	0.01	6.E-04	Proteobacteria	Deltaproteobacteria	Myxococcales	0319-6G20	
bact224	0.02	3.E-06	Proteobacteria	Deltaproteobacteria	Thermodesulfobacteriales		
bact229	81.72	7.E-02	Proteobacteria	Gammaproteobacteria	Aeromonadales		
bact231	UP	2.E-01	Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	Zobellella
bact238	UP	5.E-02	Chloroflexi	Ktedonobacteria	Ktedonobacterales	Ktedonobacteraceae	FFCH10602
bact244	3.94	7.E-02	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Morganella
bact261	25.31	7.E-03	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter
bact271	33.23	3.E-02	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Stenotrophomonas
bact273	DOWN	1.E-04	SC4				
bact285	0.10	6.E-03	Tenericutes	Mollicutes	RF39		

Abbreviations: CAF, cafeteria diet; STD, standard diet.

Associations between physiological parameters and metagenome in rats fed STD and CAF

Selection of associations with R2 over 0.7 revealed that, together with Firmicutes, only two more phyla presented negative associations, while the rest were positively associated with white adipose tissues and negatively associated with muscle weight. Proteobacteria were represented by seven genera. Again, associations with muscle were inverse to those shown by adipose tissues. Other represented phyla were Bacteroidetes, Chloroflexi, Lentisphaerae and SC4. IWAT, EWAT and MWAT were the adipose tissues with most associations, with 18, 17 and 16, respectively. RWAT and BAT were only associated with the abundance of 10 genera. Together with the IWAT, the muscle was the most associated tissue with 18 associations (Supplementary Figure 2 and Figure 2).

Concerning the plasma parameters, those showing associations with phyla with a R2 over 0.7 were glucose (three associations), insulin (four associations), triglycerides (seven associations), non-esterified free fatty acid (seven associations) and adiponectin (one association). Surprisingly, glycerol concentration was the biochemical parameter with more associations (18) followed by the hormone leptin (16). Regarding phyla, the most represented was again Firmicutes (nine genus), followed by Bacteroidetes (eight genus) and Proteobacteria (five genus). Other phyla found to be associated with plasma parameters were Chloroflexi (two), Lentisphaerae (one), SC14 (one) and Tenericutes (one). Associations showed a certain degree of specificity. Thus, both glucose and insulin showed positive associations with different phyla, whereas glycerol was negatively associated with Firmicutes and mostly positively (four out of five) associated with Bacteroidetes. Associations of non-esterified free fatty

acids were all positive and reduced to Firmicutes except one with Chloroflexi. Most of the associations of leptin concentration were negative, independently of the phyla (Supplementary Figure 3 and Figure 2).

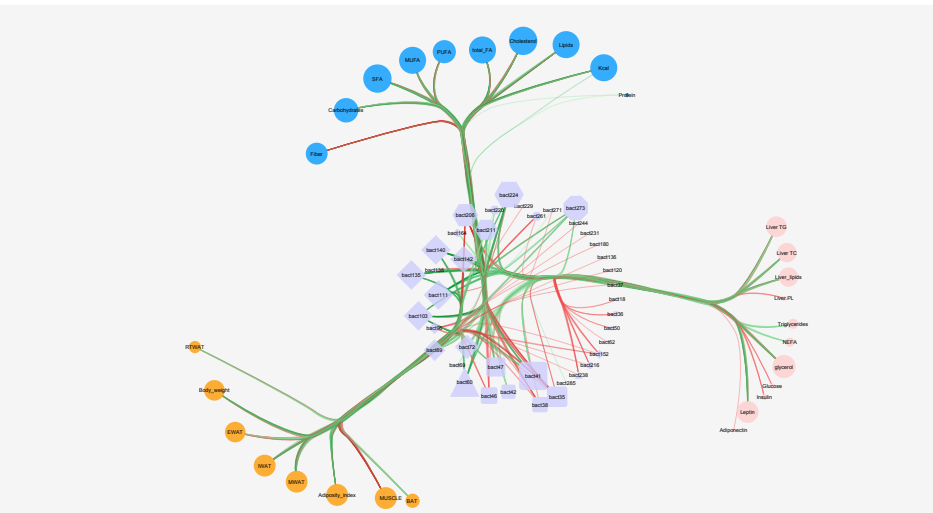

Figure 2. Network of associations between genus and nutritional, physiological and biochemical parameters. Associations with an R240.7 between depicted genus and parameters as reported by sparse partial least squares analysis are represented as the edges of a network. Red edges depict positive associations and green edges indicate negative associations between the connected nodes. Increased intensity denotes higher R2. The size of each node is proportional to the number of connected edges. Genera are coded in Table 2. The form of the node for each genus represents its phylum as diamonds (Firmicutes), squares (Bacteroidetes), triangles (Chloroflexi), hexagons (proteobacteria) and octagons (SC4). Actinobacteria, Deferribacteres and Tenericutes are represented by a circle. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; total FA, total fatty acids; TG, triglycerides, TC, total cholesterol, PL, phospholipids, NEFA, non-esterified fatty acids; RTWAT, retroperitoneal white adipose tissue; EWAT, epididymal adipose tissue; IWAT, inguinal adipose tissue; MWAT, mesenteric adipose tissue; MUS, muscle; BAT, brown adipose tissue.

Liver lipids were mainly associated with the abundance of Firmicutes (seven) and Proteobacteria (four) and, to a lesser extent with Bacteroidetes (two), Chloroflexi (two) and SC14. As has been described for the weight of tissues, the combination of these associations with

the metagenome-macronutrients network (Supplementary Figure 4 and Figure 2) revealed associations between liver lipids and genera not associated with macronutrients.

The 40 genera that were found to be associated with nutrition or physiological parameters and summarized in Table 2 were used as a link to the different physiological parameters, producing the association network shown in Figure 2, which compiles the different results described in this section CAF increased endotoxemia.

The increase in Proteobacteria prompted us to analyze the levels of endotoxemia in another cohort of STD and CAF-fed rats. As shown in Figure 3, all species of 3OFFAs were increased in the plasma of rats fed CAF for 12 weeks, resulting in a statistically significant increase in plasma LPS concentration compared with rats fed STD.

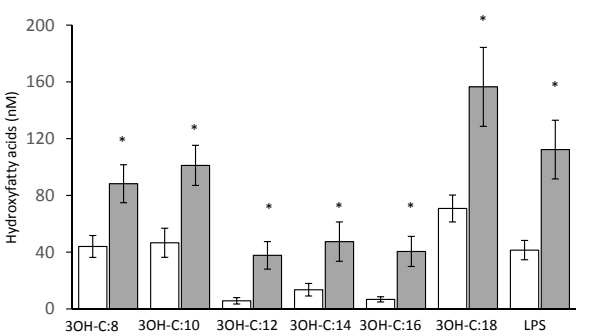

Figure 3. Analysis of LPSs in plasma of rats fed a CAF or a STD. Plasma concentrations of hydroxyl-fatty acids of 8, 10, 12, 14, 16 and 18 carbons were determined and the concentration of total LPS was calculated from their concentration as reported in the Materials and methods section. The results are represented as the mean±s.e.m. of each group. *Denotes a Po0.05 between the CAF and STD groups by T statistics.

DISCUSSION

Different works have shown that obesity involves a reduction in gut species richness in preclinical models and in humans.[12,13,16,32] In Danish

subjects, lower gut bacterial diversity was associated with higher adiposity, insulin resistance and dyslipidemia, key components of MetS.[32] In fact, loss of gut microbial diversity has been proposed as a hallmark of western societies when compared with rural areas around the world.[12] This concept has been explored in mice, and research demonstrates that a Western-like diet with reduced fiber maintained over generations results in an eventual loss of bacterial diversity.[13] Taken together, converging evidence indicates an association between gut microbial diversity and an obesity-prone phenotype. In agreement with this point of view, our results suggest that CAF-induced obesity leads to poorer alpha diversity.

A remarkable number of genera, 15 out of 40, associated with physiological and biochemical alterations in our study were changed by CAF in the direction described by previous works intended to identify the changes that obesity and its related alterations induce in gut microbial communities (summarized in Supplementary Table 2). These positive matches reinforce our findings and support the approach used herein to assess the cross-talk between the gut microbiome and host metabolism. Moreover, of the 40 genera included in the associations network, 25 have not been previously related to obesity to our knowledge. Nevertheless, since this is an association study, which is not addressed to underscore the cause of the associations found, more research assessing the relevance of these genera in the origin of metabolic alterations would shed light on the associations described herein.

At the phylum level, we found that CAF resulted in a clear reduction in Firmicutes. Despite the observation that initial obesity was related to increased Firmicutes abundance and decreased Bacteroidetes to Firmicutes ratio,[20–22] further studies have been unable to detect such

changes in different human cohorts.[19,22,33,34] Moreover, our results agree with a recent study describing a decrease in Firmicutes phylum in rats with obesity induced by different obesogenic diets.[35] Similarly, obesity has been associated with decreased Bacteroidetes,[36,37] but, again, we have not found evidence consistent with this previous observation. Despite these contrasting findings, we show other results that agree with previous works. Thus, we observed a marked increase in Actinobacteria in CAF animals relative to their STD counterparts, and an abundance of this phylum has been previously associated with obesity,[17,19,22] although some genera within this phylum, such as *Bifidobacterium,* might present the opposite behavior.[38] Among the different Actinobacteria detected, only the genus *Propionibacterium* was positively associated with plasma concentrations of insulin and glucose. In agreement with our observations, a study in obese women found similar results, indicating a positive correlation between *Propionibacterium* and glucose homeostasis parameters.[39]

Together with the changes indicated above for Bacteroidetes, Firmicutes and Actinobacteria, Proteobacteria is another predominant phylum in the gut microbiome that was markedly increased by CAF. The positive association between these species and obesity or other diseases has been widely described in different studies, in both preclinical models and human subjects.[20,40–42] Moreover, different families of Proteobacteria have been described as important LPS producers, such as the Enterobacteriaceae family,[43] which was one of the Proteobacteria increased by CAF in the present work. Bacterial LPS has been proposed as a trigger of different metabolic disorders, including increased adiposity and insulin resistance.[44,45] In the present study, we found a large increase in plasma LPS that completely agrees with the role of endotoxemia in the metabolic effects of diet-induced

obesity. Moreover, together with the major phyla described above, we found changes in the abundance of other phyla previously associated with obesity, such as Cyanobacteria,[46] Deferribacteres,[18,47] Fibrobacteres,[48] Tenericutes[37,46] and TM7.[49] Other phyla, such as Nitrospirae and SC4, were also altered between CAF and STD groups; however, to our knowledge, little is known regarding the role of these phyla in obesity.

Our results strongly suggest that the balance among fiber, simple carbohydrate and fat intake has a key role in the effects of diet on gut microbial populations. This result agrees with the relevance of fiber in shaping the gut microbiome.[12,15] In this sense, we found positive associations between fiber intake and a phylum of well-known fermenters, such as Firmicutes. Fermentative bacteria are important for the breakdown of non-digestible carbohydrates leading to the production of short chain fatty acids (SCFAs) that are proposed as key factors in the development and progression of obesity and related alterations, although their exact role is not yet fully understood. On the one hand, it has been proposed that SCFA might represent a salvage pathway of energy derived from non-digestible carbohydrates, as SCFA can be used as precursors of fatty acids by different tissues.[50] Although in our experiment CAF animals presented a decrease in Firmicutes, a reduction in SCFA derived from fermentative bacteria would not have a relevant impact on energy balance, as the energy provided by these metabolites is irrelevant when compared with the exacerbated energy intake induced by the hyperphagia associated with CAF. Contrary to the putative role of SCFA in energy salvage, different studies have demonstrated that these metabolites present a wide array of beneficial actions, increasing fatty acid oxidation and energy expenditure by different mechanisms and leading to reduced

adiposity, ectopic deposition of hepatic fat and insulin resistance.[51] Thus, SCFA can signal directly to different tissues after absorption, activating peroxisome proliferator activated receptors and AMP activated kinase in liver and adipose tissues inducing fatty acid catabolic pathways and reducing body fat content.[51,52] In our case, Firmicutes showed an important number of negative associations with adipose tissue weights and hepatic lipids and, at the same time, positive associations with the intake of fiber. Therefore, it is plausible to hypothesize that the reduced intake of fiber observed in CAF animals resulted in a decrease in fermentative bacteria leading to a reduced production of metabolites, such as SCFA. Indeed, in a recent experiment of our laboratory, we analyzed the levels of SCFA in feces of Wistar rats fed STD and CAF diets for 9 weeks, observing a threefold decrease of SFCA expressed as the sum of acetic, propionic and butyric acids, resulting in a *P*-value under 0.001 when analyzed by Student's *t*-test (data not shown). These results would support our hypothesis. If these changes were also found in blood, could contribute to suboptimal activation of catabolic pathways in the host and, together with other factors such as increased metabolic endotoxemia and exacerbated fat and glucose absorption, could also contribute to increased fat deposition in adipose tissues and liver. In fact, Vrieze *et al.* found that treatment of MetS patients with microbiota from lean subjects results in ameliorated insulin resistance accompanied by an increased population of Firmicutes.[53] Therefore, the associations found between microbiota and tissues such as adipose, liver or muscle might reflect the cross talk between microbiota and the host metabolism mediated by different metabolites and hormones.

Among the different plasma components analyzed, both glycerol and leptin clearly showed the highest number of associations with cecum

bacteria, mainly Firmicutes, Bacteroidetes and Proteobacteria. Both glycerol and leptin are highly related to adipose tissue. Thus, leptin is commonly used as a biomarker of adipose tissue status, whereas glycerol is a well-accepted biomarker of adipose tissue lipolysis.[54,55] As has been discussed above, the host microbiota affect different tissues by different mechanisms, with adipose tissue metabolism being a clear target of this cross talk.[12] Together with the high number of associations found between gut bacteria and adiposity-related parameters, such as adiposity index or the weight of different adipose depots, our results agree with the accepted hypothesis that gut microbiota modulate, in part, adipose tissue physiology. In addition, the association between the microbiome and liver lipids found in this work is remarkable, and previous studies have shown that the gut microbiome can influence liver health via different mechanisms, involving a wide array of microbial metabolites, such as SCFA, bile acids, choline or ethanol, as well as by increasing LPS producing bacteria and consequently endotoxemia.[36,40] In agreement with our work, results from a recent study suggest that analysis of microbiota in human stool samples can be used as a biomarker of non alcoholic fatty liver disease progression.[56] In that work, differences at the phylum level between advanced and mild non alcoholic fatty liver disease were decreased Firmicutes and increased Proteobacteria, changes that have been also identified by our analysis when comparing rats with normal and steatosis livers. Taken together, these results may be related to the known link between adipose tissue remodeling and liver health, as the hepatic metabolism of lipids is highly interconnected with the metabolism of the adipose tissue in order to maintain the homeostasis of lipid metabolism.[57,58] Our association study highlights the relevance of the microbiota on this adipose–liver axis, a putative link that has been explored by other

authors.[40] Nevertheless, more research is needed to clearly define the extent to which the microbiota affect this complex system.

We have demonstrated that CAF modifies markedly the host microbiome. Recent literature suggests that dietary habits have a profound impact on gut microbiome diversity and microbial populations. Our results strongly suggest that the balance between fiber, simple carbohydrates and fats determines the abundance of different genera, which, in turn, can be associated with physiological outcomes. In this sense, we describe clear associations between the alteration of gut microbiota and the physiology of adipose tissues and liver health. Our results reveal that the CAF-fed rat is a useful model for studying diet-induced dysbiosis and may provide further evidence supporting a role of the gut microbiota in obesity and its derived pathologies, such as non alcoholic fatty liver disease or insulin resistance. The associations between specific bacterial genera and physiology require further investigation to assess the relevance of each association and whether these results can be used to design new strategies based on microbiome modulation for improving metabolic dysfunctions.

CONFLICT OF INTEREST

The authors declare no conflict of interest

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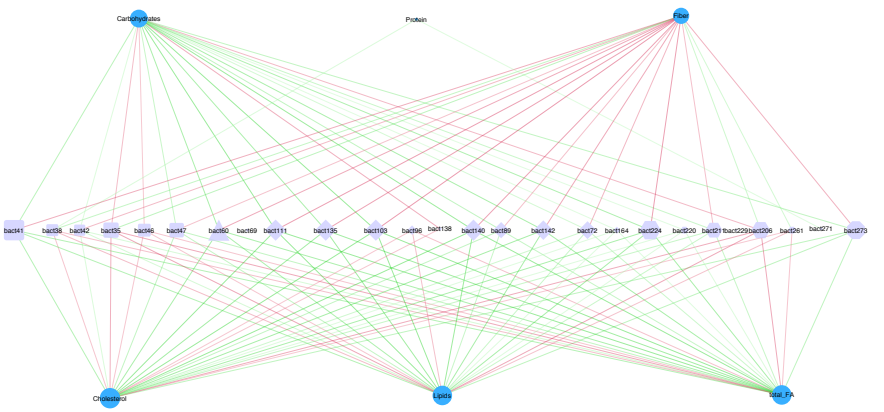
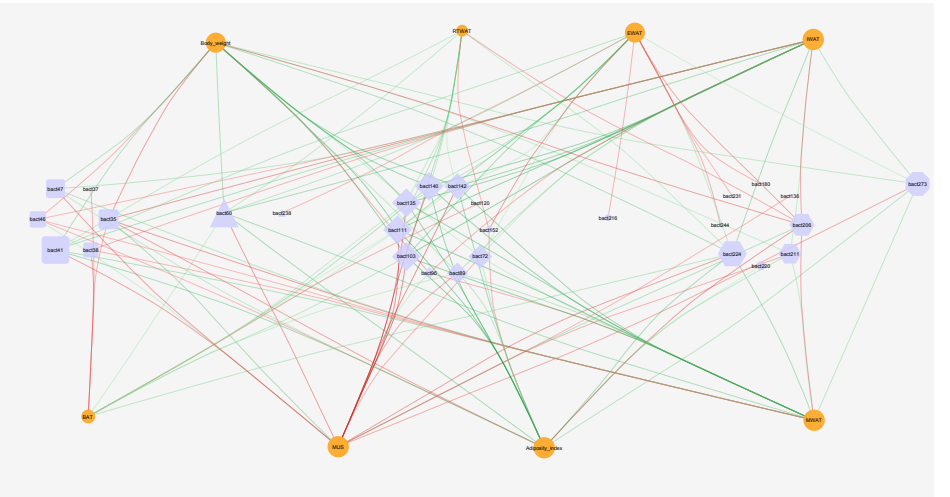
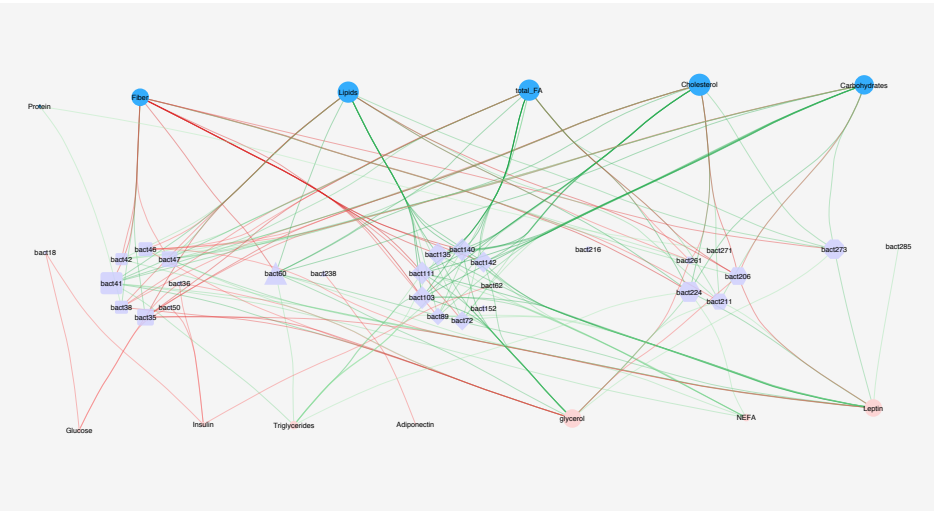
Supplementary Material



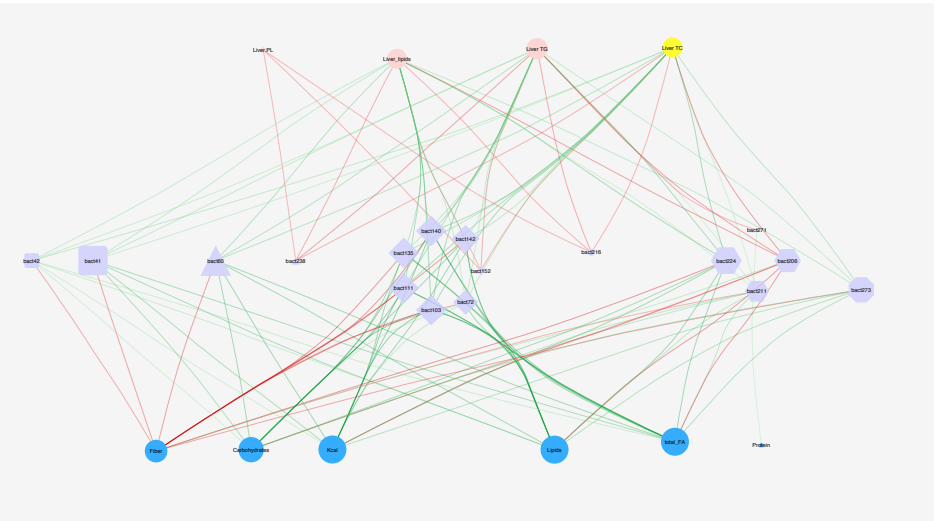
Supplementary figure 1. Network of associations between genus and nutritional parameters. Associations with an R2 > 0.7 between depicted genus and parameters as reported by sPLS analysis are represented as the edges of a network. Red edges depict positive associations and green edges indicate negative associations between the connected nodes. Increased intensity denotes higher R2. The size of each node is proportional to the number of connected edges. Genera are coded as in table 4. The form of the node for each genus represents its phylum as diamonds (Firmicutes), squares (Bacteroidetes), triangles (Chloroflexi), hexagons (proteobacteria) and octagons (SC4). Actinobacteria, Deferribacteres and Tenericutes are represented by a circle.



Supplementary figure 2. Network of associations between genus andphysiological parameters. Associations with an R2 > 0.7 between depicted genus and parameters as reported by sPLS analysis are represented as the edges of a network. Red edges depict positive associations and green edges indicate negative associations between the connected nodes. Increased intensity denotes higher R2. The size of each node is proportional to the number of connected edges. Genera are coded as in table 4. The form of the node for each genus represents its phylum as diamonds (Firmicutes), squares (Bacteroidetes), triangles (Chloroflexi), hexagons (proteobacteria) and octagons (SC4). Actinobacteria, Deferribacteres and Tenericutes are represented by a circle. RTWAT, retroperitoneal white adipose tissue; EWAT, epididymal adipose tissue; IWAT, inguinal adipose tissue; MWAT, mesenteric adipose tissue; MUS, muscle; BAT, brown adipose tissue.



Supplementary figure 3. Network of associations between genus and nutritional and biochemical parameters. Associations with an R2 > 0.7 between depicted genus and parameters as reported by sPLS analysis are represented as the edges of a network. Red edges depict positive associations and green edges indicate negative associations between the connected nodes. Increased intensity denotes higher R2. The size of each node is proportional to the number of connected edges. Genera are coded as in table 4. The form of the node for each genus represents its phylum as diamonds (Firmicutes), squares (Bacteroidetes), triangles (Chloroflexi), hexagons (Proteobacteria) and octagons (SC4). Actinobacteria, Deferribacteres and Tenericutes are represented by a circle. NEFA, non-esterified fatty acids.



Supplementary figure 4. Network of associations between genus and nutritional and hepatic biochemical parameters. Associations with an R2 > 0.7 between depicted genus and parameters as reported by sPLS analysis are represented as the edges of a network. Red edges depict positive associations and green edges indicate negative associations between the connected nodes. Increased intensity denotes higher R2. The size of each node is proportional to the number of connected edges. Genera are coded as in table 4. The form of the node for each genus represents its phylum as diamonds (Firmicutes), squares (Bacteroidetes), triangles (Chloroflexi), hexagons (proteobacteria) and octagons (SC4). Actinobacteria, Deferribacteres and Tenericutes are represented by a circle.

Manuscript 2

Deciphering the functions of gut microbiota in an animal model of obesity using an optimized metaproteomics workflow.

Maria Guirro, Pol Herrero, Andrea Costa, Andreu Gual-Grau, Adrià Ceretó-Massagué, Adrià Hernández, Helena Torrell, Lluís Arola and Núria Canela.

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Deciphering the functions of gut microbiota in an animal model of obesity using an optimized metaproteomics workflow.

Maria Guirro[1,2], Pol Herrero[2], Andrea Costa[2], Andreu Gual-Grau[1], Adrià Ceretó-Massagué[2], Adrià Hernández[2], Helena Torrell[2], Lluís Arola[1,3] and Núria Canela[2]

[1] Universitat Rovira i Virgili, Biochemistry and Biotechnology Department, Nutrigenomics Research Group, Tarragona, Spain

[2] Eurecat, Centre Tecnològic de Catalunya. Centre for Omic Sciences (COS), Joint Unit Universitat Rovira i Virgili-EURECAT. Unique Scientific and Technical Infrastructures (ICTS), Reus, Spain

[3] Eurecat, Centre Tecnològic de Catalunya. Biotechnological Area, Reus, Spain

Correspondence: Núria Canela

Keywords: Metaproteomics, Microbiota, Metagenomics, Mass Spectrometry

ABBREVIATIONS

ABC: Ammonium Bicarbonate; ACN: Acetonitrile; B/F: Bacteroidetes to Firmicutes Ratio; CAF: cafeteria; DC: Sodium Deoxycholate; DTT: Dithiothreitol; FA: Formic Acid; FASP: Filter-Aided Sample Preparation; FDR: False discovery rate; IGS: InGel-Stacking; IAA: Iodoacetamide; OTU: Operational Taxonomic Unit; PCA: Principal Component Analysis; PSM: peptide spectrum match; SDS: Sodium dodecyl sulphate; SD: Standard Deviation; STD: Standard; TCA: Trichloroacetic acid

ABSTRACT

Metaproteomics has emerged as a new, revolutionary approach to study microbiota functionality, but the lack of consistent studies in this field due to the great complexity of samples has prompted to search new strategies to achieve better metaproteome characterization. Some steps in sample preparation and data analysis procedures are critical for obtaining accurate results, therefore protein extraction buffers, digestion procedures and fractionation steps were tested here. Initially, two lysis buffers were used to improve protein extraction, two common digestion protocols were compared, and fractionation processes were employed at both the peptide and protein levels. The combination of these procedures resulted in five different methodologies; SDS buffer, in-gel digestion and fractionation at the peptide level provided the best results. Finally, the optimized metaproteomics workflow was tested in a real case study with obese rats, in which a metagenomics study was previously performed. Important differences in protein levels were observed between groups that were potentially related to the taxonomical family, indicating that functional processes are modulated by the microbiota. Therefore, in

addition to the necessity of combining different metaomics approaches, an optimized metaproteomics workflow such as the presented in this study is required to obtain a better understanding of the microbiota function.

1. INTRODUCTION

In the last decade, the gut microbiota has been considered one important factor that modulates the health status, with a significant role in maintaining health due to its relation to some noncommunicable diseases [1–3], such as obesity, diabetes, cardiovascular disease, and metabolic syndrome [4]. Moreover, diet, lifestyle and genetic background modulate the gut microbiota, affecting the biodiversity or functional and metabolic activities, resulting in gut dysbiosis [5]. Hence, the need for some specific technical tools to understand the role of the gut microbiota in maintaining the health-disease balance has increased [6].

Recently, metaomic sciences have been developed as the pioneering techniques to understand microbial function in a specific condition; they are a potential source of information to characterize an actual phenotype. To date, most studies have focused on analyzing the bacterial diversity using 16s rRNA sequences to describe the bacterial population and establish a relationship between the host and microbiota ecology. However, these approaches are hampered by a fundamental limitation - the inability to directly measure functional activity [7].

Different omics sciences [8], such as metatranscriptomics, metaproteomics or meta-metabolomics, should be integrated to directly measure the functions of the gut microbiota. Among these sciences, metaproteomics is a “new”, promising methodology that will

enhance the functional study of gut microbiota and will provide a more complete description of the microbiota activity in each specific situation [9]. However, classical proteomics methods must be adapted to address the complexity of the microbiota. Therefore, an optimized workflow is necessary to better maximize the protein coverage and improve protein identification, as well as to relate the identified proteins to the complex microbiota biodiversity.

Some authors have attempted to achieve this goal. Tanca et al. [10] tested different lysis protocols by combining two types of protein digestion methods, and Zhang et al. [11] added a bead-beating step to improve cell lysis but only performed in-solution digestion. Meanwhile, Kolmeder et al. [12] also performed a similar comparison using in-gel and in-solution protein digestion. Despite the promising results from these studies, a consensus regarding the most appropriate methodology for metaproteomic experiments has not been reached. However, research in this field has mainly focused on optimizing lysis or digestion protocols, rather than other crucial steps in sample preparation, such as fractionation or data analysis [13]. For these reasons, the aim of this work is to optimize a complete workflow, including sample preparation, peptide or protein fractionation procedures and the bioinformatics pipeline combining 16s rRNA metagenomics results with metaproteomics data to better decipher the functions of the microbiota.

2. MATERIALS AND METHODS

2.1. Metaproteomics

2.1.1 Reagents

Sodium dodecyl sulfate (SDS), sodium deoxycholate (SDC), ammonium bicarbonate (ABC), dithiothreitol (DTT), iodoacetamide (IAA),

trichloroacetic acid (TCA), acrylamide and formic Acid (FA) were purchased from Sigma-Aldrich (Missouri, USA). Tris-HCl was obtained from Bio-Rad (California, USA) and urea was purchased from PlusOne (GE Healthcare, Illinois, USA). Acetone and acetonitrile (ACN) were obtained from Merck Millipore, (Massachusetts, USA).

2.1.2 Samples

Cecal samples were obtained from rats fed a standard diet (Teklad Global 14% protein Rodent Diet 2014, Harlan, Barcelona) immediately after sacrifice. The totality of cecal content was extracted and mixed homogeneously, before frozen it in liquid N_2 and stored at -80ºC until the assessments of the metaproteomics methodology.

After thawing at 4ºC, approximately 1 g of the cecal content was subjected to differential centrifugation to enrich the microbial cells using the method described by Tanca et al. [14], with minor modifications. Briefly, the sample was resuspended in 10 mL of PBS, vortexed, shaken in a tube rotator for 45 min, and subjected to low-speed centrifugation at 500 g for 5 min at 4ºC. Afterwards, the supernatant was collected and centrifuged at 20000 g for 15 min. The obtained microbial cell pellet was processed with different cell lysis protocols, different digestion procedures and fractionation methodologies. Each method was performed in technical triplicates from the same bacterial pellet.

2.1.3 Cell lysis protocol

The bacterial pellet was resuspended in a small volume of PBS and split into two equal portions (2 methods x 3 replicates). Two different cell lysis methods were tested: (i) SDS-buffer plus bead beating/freeze thawing and (ii) non-SDS-buffer plus sonication on ice.

(i) SDS-buffer method: The pellet was resuspended in 1 mL of SDS-extraction buffer (2% SDS, 100 mM DTT, and 20 mM Tris-HCl, pH 8.8), incubated at 95ºC for 5 min and then subjected to bead beating combined with a freeze-thaw cycle. Two stainless steel balls (3.2 mm diameter) were added before samples were immersed in liquid N_2 and subjected to bead beating for 3 min at medium speed (Bullet Blender, Cultek, Barcelona, Spain); this step was repeated twice.

(ii) Non-SDS-buffer: The bacterial pellet was resuspended in non-SDS extraction buffer (2% Sodium Deoxycholate (DC), 8 M urea, and 50 mM ABC) and alternately sonicated on ice followed by vortexing until the pellet was completely dissolved.

In both lysis methods, samples were centrifuged at 20000 g for 10 min at 4ºC. In the cases where In-Gel digestion was performed, the proteins in the supernatant were precipitated with TCA/acetone overnight. The obtained pellet was resuspended in 6 M urea in 50 mM ABC and the concentration was quantified using the Bradford method prior to the digestion procedure.

2.1.4 Protein digestion procedures

Two different digestion types were tested: (i) Filter-Aided Sample Preparation (FASP) and (ii) In-Gel Stacking (IGS).

(i) FASP digestion: One hundred micrograms of protein extracts obtained using both lysis methods were diluted with 200 µl of a solution containing 8 M urea and 100 mM Tris-HCl, pH 8.8, and loaded in Microcon Ultracel YM-30 filtration devices (Merck Millipore, Massachusetts, USA) and then processed according to a standard FASP protocol [10,15], with minor modifications.

(ii) IGS digestion: One hundred micrograms of proteins extracted with SDS buffer were reduced in 80 mM DTT in 50 mM ABC for 1 hour at 37ºC and alkylated with 80 mM IAA in 50 mM ABC for 30 min at room temperature in the dark. Afterwards, protein extracts were applied to the 1.2-cm wide wells of a conventional SDS-PAGE gel (0.75 mm thick, 4% stacking gel, and 12% resolving gel). The run was stopped as soon as the front had traversed 3 mm of the resolving gel to ensure that the whole proteome was concentrated in the stacking/resolving gel interface. The unseparated protein bands were visualized by Coomassie staining, excised, cut into cubes (2 x 2 mm) and placed in new tubes. The gel pieces were destained in 25 mM ABC and ACN several times and dehydrated by removing all liquid and drying in a SpeedVac. The dried gel pieces were rehydrated in 25 mM ABC, and trypsin was added at a 1:100 trypsin:protein ratio (w/w) (Promega, Madison, WI). The digestion was performed overnight at 37ºC and terminated by the addition of ACN:FA (50:1); the eluate was dried in a SpeedVac before being desalted.

2.1.5 HLB SPE cartridge desalting protocol

Digested proteins were desalted onto HLB SPE cartridge (Waters, Bedford, MA, USA) prior to the nanoLC-MS/MS analysis. Briefly, samples were suspended with 0.1% FA and loaded onto cartridges that had previously been conditioned with 1 mL of a solution containing 80% ACN, 20% water and 0.1% FA, followed by 1 mL of water with 0.1% FA. Afterwards, the cartridges were dried under a vacuum and peptides were eluted twice with 250 µL of 80% ACN, 20% water and 0.1% FA.

2.1.6 Fractionation methodology

Two different fractionation methodologies were employed: (i) fractionation based on the protein size and (ii) fractionation based on the isoelectric point of peptides.

(i) 1D SDS-PAGE Gel (proteins): This procedure was performed as described in the IGS digestion protocol. The main difference was the separation of protein extracts on a conventional SDS-PAGE gel (0.75 mm thick, 4% stacking gel, and 12% resolving gel). The run was terminated when samples were completely separated according molecular weight. Nine separate slices were generated and the digestion was performed as described above.

(ii) OFFGEL (peptides): the digested samples were loaded in a 3100 OFFGEL Fractionator (Agilent Technologies, California, USA) and separated according to the manufacturer's protocol. The 24-lane gel strips with a 3-10 nonlinear pH gradient were rehydrated, and after 15 min, 150 L of each sample was loaded in individual wells. After approximately 24 hours, 24 fractions were collected and combined to be reduced in 9 end fractions for comparison with the other fractionation method.

Finally, the 9 end fractions were desalted using an Oasis HLB SPE cartridge as described above prior to the nanoLC-MS/MS analysis.

2.1.7 nanoLC-MS/MS analysis

Each sample or fraction was loaded on a trap nanocolum (0.01 x 2 cm, 5 µm, Thermo Fisher Scientific) and separated on a C18 reverse-phase (RP) nanocolumn (0.0075 x 12 cm, 3 µm, Nikkyo Technos Co. Ltd., Japan). Chromatographic separation was completed with a 90-min gradient (0-1 min., 2% B isocratic; 1-21 min., 2-20% B; 21-61 min., 20-

35% B; 61-81 min., 35-50% B; 81-86 min., 50-95% B; and 86-90 min., 95% B isocratic). Milli-Q water (0.1% FA) and ACN (0.1% FA) were used as the mobile phase at a flow rate of 300 nl/min.

Mass spectrometry analyses were performed on an LTQ-Orbitrap Velos Pro (Thermo Fisher Scientific) by acquiring an enhanced FT-resolution spectrum (R=30,000 FHMW), followed by a data-dependent MS/MS scan (IT—(CID)MS/MS at 35% NCE) of the ten most intense parent ions with a charge state rejection of one and a dynamic exclusion of 0.5 min.

2.2. Protein identification and statistics

Proteome Discover (version 1.4; Thermo Scientific, Germany) combined with the Mascot search engine (version 2.5) were used to analyze the proteomics data. Proteins were identified using two different databases from UniProt (www.UniProt.org): the *Rattus norvegicus* database (8,003 sequences) and an in-house gut microbiome database (14,480,443 sequences). Two missed cleavages for trypsin digestion and an error of 0.8 Da for the IT-MS/MS fragment ion mass and 10.0 ppm for the FT-MS parent ion mass were allowed. Oxidation of methionine and acetylation of N-termini were set as dynamic modifications, and carbamidomethylation of cysteine was set as a static modification. The false discovery rate (FDR) and protein probabilities were calculated using a fixed peptide spectrum match (PSM) validator and protein abundance, using the average area of the three most abundant peptides for each protein, was used for Label-free quantification (LFQ).

The in-house gut microbiome database was built based on results obtained from the same samples used in previously published metagenomics analyses [16]. For each of the identified taxonomic

families in the metagenomic analyses, all available sequences were retrieved from Uniref100 (UniProt release 2017_06) in order to avoid redundant sequences, totaling 14,480,443 unique protein sequences (Supporting Table 1).

Venn diagrams were constructed with Mass Profiler Professional software v.14.5 (Agilent Technologies), and Metaboanalyst 4.0 was used to perform cluster correlation analyses between protein functional groups and methodologies.

2.3. Study validation

Twelve eight-week-old male Sprague-Dawley rats (Charles River Laboratories, Wilmington, Massachusetts, USA) were housed individually at 22ºC with a light/dark cycle of 12 hours and were provided free access to food and water. Animals were divided into two groups (n=6) and fed different diets: a cafeteria (CAF) [17] or standard chow (STD) diet (Teklad Global 14% protein Rodent Diet 2014, Harlan, Barcelona), for 9 weeks. Cecal samples were obtained immediately after the animals were euthanized, frozen in liquid N_2 and stored at -80ºC until analyses were performed.

The Animal Ethics Committee of the University Rovira i Virgili (Tarragona, Spain) approved the procedures.

2.3.1. Bacterial diversity analysis using partial 16s rRNA gene sequencing

The bacterial genomic DNA was obtained from cecal samples (300 mg) using a QIAamp DNA stool mini kit (Qiagen Inc., Hilden, Germany) according to the manufacturer's protocol. Two variable regions from 16S rRNA gene sequences were amplified by PCR using two primer pairs (341F-532R and 515F-806R) for V3 and V4 respectively. A

mutliplexed mixture of twelve samples was combined to create the DNA library, which was subjected to clonal amplification through emulsion PCR. Prepared samples were loaded on a 530 chip and sequenced using the Ion S5 system (Life Technologies, California, USA).

After sequencing, Ion Torrent Suit software removed the low-quality and polyclonal sequences, and those reads were then analyzed using QIIME 1.9.1 [18]. The analysis included open reference de novo OTU (operational taxonomic unit) picking and clustering (at 97% similarity threshold), taxonomic assignment using blast against GreenGenes database included in QIIME, an alpha-diversity analysis, OTU analysis, species annotation (OTU table), and a beta-diversity analysis.

2.3.2. Metaproteomics analysis

Cecal samples were processed as described in section 2.1.2 to obtain the bacterial pellets, and protein extraction and digestion was performed according to the SDS lysis procedure (section 2.1.3-i) and IGS digestion procedure (section 2.1.4-ii). After lysis and protein digestion, samples were desalted on an HLB SPE column (Waters, Bedford, MA, USA) and dried in a SpeedVac concentrator before labeling with TMT 10-plex (Thermo Fisher Scientific, Germany) according to the manufacturer's instructions. A pool of all samples was labeled with the 126-Tag and included in each batch to normalize all samples and TMT batches. Then, the labeled peptides from each sample were mixed and desalted on HLB SPE columns as described above.

After labeling with TMT 10-plex, OG fractionation was performed as described above. The 24 fractions obtained from each TMT Plex reaction were loaded on a trap nanocolumn (0.01 x 2 cm, 5 μm, Thermo

Fisher Scientific) and separated on a C18 reversed-phase (RP) nanocolumn (0.0075 x 12 cm 3 μm, Nikkyo Technos Co. Ltd., Japan) as described above. Mass spectrometry analyses were performed on a LTQ-Orbitrap Velos Pro (Thermo Fisher Scientific) by acquiring an enhanced FT-resolution spectrum (R=30,000 FHMW), followed by two data-dependent MS/MS scans (IT—(CID)MS/MS at 35% NCE and FT-(HCD)MS/MS R=15,000 FHMW at 40% NCE) of the ten most intense parent ions with a charge state rejection of one and a dynamic exclusion of 0.5 min.

Proteins were identified with Proteome Discover (version 1.4; Thermo Scientific, Germany) combined with the Mascot search engine (version 2.5). Three different databases from UniProt (www.UniProt.org) were used to screen the data and identify proteins: (i) the *Rattus norvegicus* database (8,003 sequences); (ii) an in-house constructed diet database (35,993 sequences) and (iii) an in-house gut microbiome database (27,381,845 sequences). Two missed cleavages for trypsin digestion and an error of 0.02 Da for the FT-MS/MS fragment ion mass, 0.8 Da for the IT-MS/MS fragment ion mass and 10.0 ppm for the FT-MS parent ion mass were allowed. TMT 10plex on lysine and the N-termini were set as quantification modifications, while oxidation of methionine and acetylation of N-termini were set as dynamic modifications and carbamidomethylation of cysteine was set as a static modification. The FDR and protein probabilities were calculated using a fixed PSM validator.

The in-house gut microbiome database was built based on the results obtained in metagenomics methodology and it was constructed with the same criteria as the reported in section 2.2. Briefly, for each of the identified taxonomic families, all matching Uniref100 sequences were retrieved, totaling 27,381,845 sequences.

2.3.3. Data analysis and statistics

For biometric results, GraphPad Prism 6.0 (GraphPad Prism Software Inc.) was used. The results are presented as means ± standard deviations (SD). Differences between groups were analyzed using Student's t-test.

Mass Profiler Professional software v.14.5 (Agilent Technologies) was used to determine the significant changes in metagenomics and protein levels between the different conditions under study. Relative protein abundance calculated from TMT ratios data were log2 transformed and mean centered for a multivariate analysis (principal component analysis, PCA) and univariate statistical analysis (Student's t-test). In addition, a hierarchical clustering analysis was performed on significant results.

3. RESULTS and DISCUSSION

3.1. Evaluating different methodologies to study the gut microbiota

Five different methodologies were tested to determine the optimum protocol for extending the protein coverage in the analysis of microbiota functions. These methods were focused on testing different lysis buffers, different trypsin-based digestion procedures and off-line 2D fractionation procedures both at the protein and peptide levels to expand the number of proteins identified in the microbiome. Finally, all samples were subjected to a C18 reverse-phase high performance nanoliquid chromatography separation coupled to an LTQ-Orbitrap high resolution mass spectrometer (Figure 1). Each procedure was performed in triplicate.

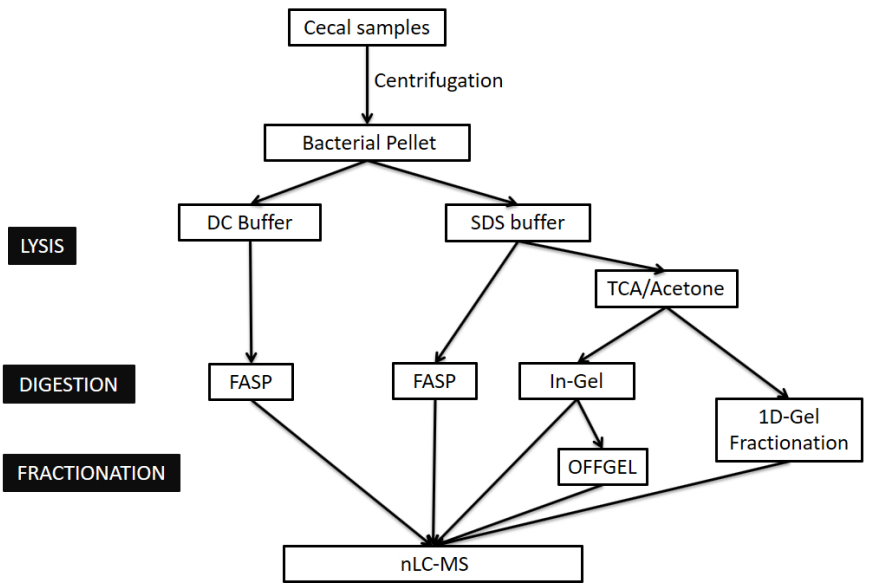

Figure 1: Schematic representation of the different experimental workflows used to compare the extraction buffer, digestion type and fractionation method.

3.1.1. Effects of the lysis buffer on the extraction of gut microbial proteins

Two different lysis buffer protocols were compared, a nondetergent buffer (DC) compatible with MS and SDS-based buffer, both followed by FASP digestion. SDS is not compatible with MS equipment; therefore, the use of a FASP digestion step instead of conventional in-solution digestion would help to completely remove the detergent. Peptides obtained from these two lysis methods were not further fractionated and directly analyzed using nanoLC-(Orbitrap)MS/MS.

As shown in Figure 2, the DC method resulted in the lowest number of proteins identified, only 132, and this number was significantly decreased ($p=0.0095$) compared to the number identified using the SDS-based buffer protocol (2,343 proteins). The DC protocol does not

appear to efficiently disrupt the cell walls, and most of the bacterial proteins present in samples were not released and solubilized in this buffer. In addition, the SDS-FASP protocol remarkably increased the number of peptides and PSM ($p=0.0106$ and $p=0.0105$, respectively) represented in ratios. If we compared the number of identified proteins between both methodologies, SDS identified 2,000 more proteins than DC-FASP; only 50 were identified by both methods. In SDS-FASP, 2,292 proteins were also identified that might have important functions in the microbiota (Figure 3A).

Based on these results, we concluded that the use of the SDS detergent is necessary to increase the number of proteins extracted. Similar results have been reported. For example, Tanca et al. [10] reported that SDS-buffer with in-solution digestion was more effective than Rapigest SF (Waters, Massachusetts, USA), a commercial anionic surfactant that theoretically improves in-solution digestion. Moreover, Zhang et al. [11] tried three buffers, SDS 4%, urea 8 M and nonionic commercially available detergent called B-Per, and after several tests, they concluded that SDS and B-Per resulted in the identification of a significantly greater number of peptides than urea. For this reason, we decided to use the SDS-based lysis buffer in the subsequent experiments.

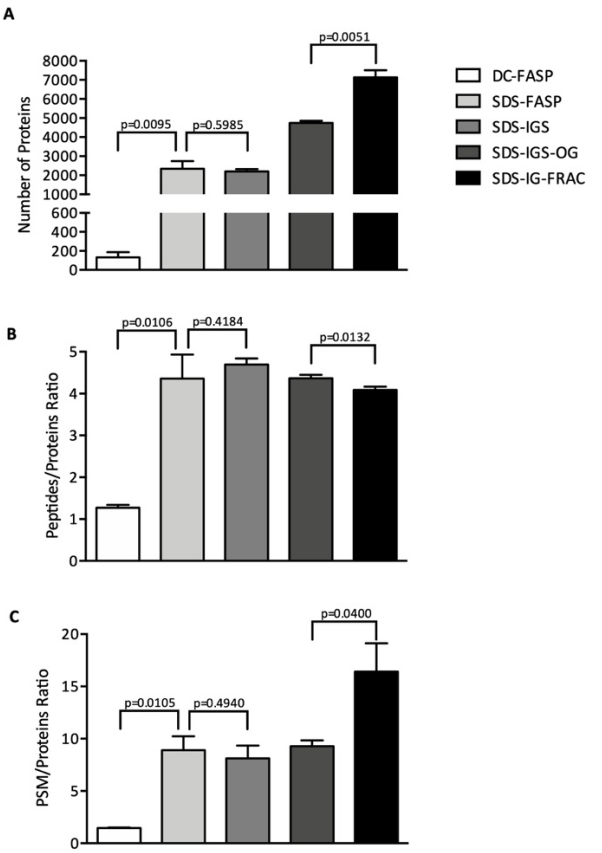

Figure 2: Effects of lysis buffer, digestion type and fractionation method on the gut microbiota. A) Number of proteins identified. B) Ratio of peptide/protein numbers and C) ratio of PSM/protein numbers. The data are presented as the means ± standard deviations (SD). Statistically significant differences between protocols were assessed using unpaired t-tests.

3.1.2. Effects of different digestion protocols combined with SDS lysis buffer

Important drawbacks of FASP digestion have been reported, such as the loss of small proteins due to the use of a 30 kDa cut-off filter or inefficient immobilization of the enzyme in the filter, producing inefficient tryptic digestion [19]. Therefore, in addition to FASP, we decided to explore the use of in-gel stacking digestion (IGS), which is also an easy and widely used digestion procedure in conventional

proteomics that eliminates detergents after protein solubilization and obtains samples compatible with MS analyses.

Similar numbers of proteins (2,343 ± 228 vs 2,199 ± 70), peptide ratios (4.36 ± 0.33 vs 4.70 ± 0.08) and PSM ratios (8.90 ± 0.77 vs 8.11 ± 0.71) were identified between the FASP and IGS procedures, but each of the differences was significant, as shown in Figure 2B and 2C. We also compared the number of proteins that were identified by both methodologies; 673 proteins were shared by both methods (Figure 3B). Some previous studies, such as the study by Kolmeder et al. [12], used a similar in-gel digestion protocol to study the human intestinal microbiota and separated proteins on a 1D-gel by molecular weight. Others performed a 1D-gel tryptic digestion and clearly obtained a sufficient number of proteins to determine the metaproteome functionality [20]. In addition, Northrop et al. [21] performed a similar workflow in an aqueous environment, and the majority of identified proteins were associated with bacteria. The authors also used a BLAST search to attribute a function to each protein identified and observed significant differences between both comparable groups. However, some other studies used FASP to study microbiota functions [14].

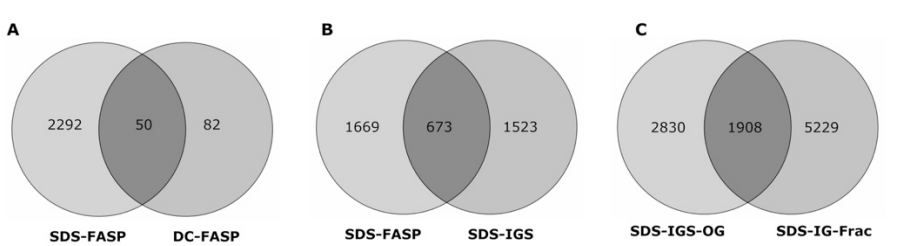

Figure 3: Venn diagrams illustrate unique and common protein identified among the workflows compared in this study. A) SDS-FASP versus DC-FASP. B) SDS-FASP versus SDS-IGS. C) SDS-IGS-OG versus SDS-IG-FRAC.

In our case, both methodologies were similar, in contrast to previous studies. Nevertheless, we decided to pursue the IGS methodology instead of the FASP protocol due to the better reproducibility of the method, and because the SD was always lower in samples fractionated using IGS.

3.1.3. Assessment of the effects of fractionation methodologies on gut microbiota

Fractionation methods are commonly used in shotgun proteomics, particularly for complex samples. These methods are useful to increase the number of identified peptides, resulting in better protein characterization. Metaproteomics samples are extremely complex and contain a mixture of proteomes from a large number of species. Therefore, the assessment of different fractionation methodologies should be particularly interesting and substantially expand proteome characterization.

For this reason, two different fractionation methods were performed before the reversed-phase nanoLC-(Orbitrap)MS/MS analysis to increase the coverage of microbiota proteins. The first is based on the peptide isoelectric point of digested proteins and the other is based on the protein molecular weight before digestion.

The first method was performed similarly to IGS, but an OFFGEL (IGS-OG) method was employed to fractionate the digested peptides. This fractionation method was compared with a 1D-gel fractionation (IG-FRAC), in which proteins were separated according to molecular weight in a 1D SDS-PAGE gel and divided in 9 slices immediately before digestion.

The samples were fractionated in nine fragments in both methodologies to ensure a comparable number of acquired spectra in MS.

In this case, the number of identified proteins was considerably different (p=0.0051) between both methodologies, 4,737 ± 62 proteins in IGS-OG and 7,137 ± 217 in IG-FRAC. Although the peptide ratios were similar (4.37 ± 0.04 and 4.10 ± 0.05, respectively), the values were significantly different (p=0.0132) (Figure 2B), and PSM ratios differed remarkably (p=0.0400; 9.27 ± 0.32 and 16.41 ± 1.56, respectively) (Figure 2C).

Moreover, only 1,908 proteins were identified after both IGS-OG and IG-FRAC, but 2,830 and 5,229 unique proteins were identified, respectively. These differences could be due to the use of different fractionation methods (Figure 3C).

Up to our knowledge, few studies applying fractionation methodologies in metaproteomics have been previously reported in the literature but any of them compare more than one methodology within the same set of samples [22,23]. In more conventional proteomics fields, greater proteome coverage is achieved when fractionation methods are employed, producing a greater number of proteins, peptides and PSM, and therefore more confidence in protein identification and quantification is obtained. In our case, peptides and PSM were represented as peptides/protein and PSM/protein ratios to better compare and understand the changes on protein coverage between the different protocols employed due to the high number of identified proteins.

Another factor to consider is the reproducibility of the technique. Fractionation in 1D gels is less robust than the OG technique, as

evidenced by the SD. The peptide and PSM ratios and its associated SD were similar between both techniques, but in terms of the protein number, the SD for IG-FRAC was increased considerably.

Based on the results, both fractionation methods substantially increased the number of identified peptides and proteins compared to non-fractionation-based methodologies. However, the choice of the most appropriate fractionation methodology will depend on the application area or study. Thus, if the in-depth characterization of a unique sample is needed, a 1D gel seems to be the better option, while for quantitative comparative metaproteomics studies (both for labeled and label-free approaches), OG is the better option due to its reproducibility [24,25].

3.2. Assessment of the functions of gut microbiota proteins using different extraction methodologies

In addition to the pros and cons of various technical procedures regarding protein and peptide numbers and identification, the effects of the five different protocols on predicting metaproteome functions were also compared. First, we assessed whether the proteins have a specific function related to microbiome; therefore, we clustered the proteins in functional groups. These results are shown in Figure 4.

In each case, the majority of proteins had a known function, of which proteins related to energy metabolism were the most frequently represented/abundant; the distribution of proteins in each functional group was similar across all methods (Table 1).

Table 1. Summary of number and functions of protein identified in each methodology tested

	DC-FASP	SDS-FASP	SDS-IG-Frac	SDS-IGS	SDS-IG-OG	Significance
Total number of proteins	132	2343	7137	2199	4738	* $ ±
Number of proteins with function (%)	108 (81.8)	2150 (91.8)	5165 (72.4)	2005 (91.2)	4257 (89.9)	* $ ±
Number of proteins without functions (%)	24 (18.2)	193 (8.2)	1972 (27.6)	194 (8.8)	481 (10.1)	* ±
Number of proteins in each group of functions (%)	DC-FASP	SDS-FASP	SDS-IG-Frac	SDS-IGS	SDS-IG-OG	Significance
Amino acid transport and metabolism	3 (2.3)	75 (3.2)	299 (4.2)	75 (3.4)	270 (5.7)	* ±
Carbohydrate transport and metabolism	9 (6.8)	159 (6.8)	276 (3.9)	124 (5.6)	307 (6.5)	* $ ±
Cellular functions	8 (6.1)	118 (5.0)	395 (5.5)	132 (6.0)	255 (5.4)	* $ ±
Coenzyme and cofactor metabolism	1 (0.8)	25 (1.1)	75 (1.1)	21 (1.0)	67 (1.4)	* ±
General DNA function	2 (1.5)	55 (2.3)	168 (2.4)	59 (2.7)	156 (3.3)	* ±
General energy metabolism	41 (31.1)	1093 (46.6)	2050 (28.7)	956 (43.5)	1749 (36.9)	* $ ±
Ion transport and metabolism	8 (6.1)	195 (8.3)	626 (8.8)	254 (11.6)	617 (13.0)	* $
Lipid transport and metabolism	0 (0.0)	1 (0.0)	8 (0.1)	2 (0.1)	8 (0.2)	
Nucleotide metabolism	2 (1.5)	63 (2.7)	116 (1.6)	56 (2.5)	103 (2.2)	* $ ±
Transcription	1 (0.8)	3 (0.1)	14 (0.2)	3 (0.1)	6 (0.1)	±
Translation	31 (23.5)	350 (14.9)	1111 (15.6)	316 (14.4)	704 (14.9)	* $ ±
Other functions	2 (1.5)	13 (0.6)	27 (0.4)	7 (0.3)	15 (0.3)	* $ ±

Results are represented in mean and percentage. Significance was calculated using Student's t-test. *$± p<0.05. * DC-FASP versus SDS-FASP; $ SDS-FASP versus SDS-IGS; ± SDS-IG-OG versus SDS-IG-Frac.

In addition, a hierarchical clustering analysis was performed using the protein abundance to assess if a functional group was specifically enriched. As expected, the lysis buffer produced the most important differences between groups, and two different groups were clearly observed based on the detergent used. The functions observed for proteins obtained using the DC-FASP protocol differed from the proteins obtained using the SDS-based lysis protocol, except for lipid transport and metabolism, which were similar (Figure 4). Furthermore, the functions of the identified proteins were not substantially affected by the different digestion and fractionation procedures.

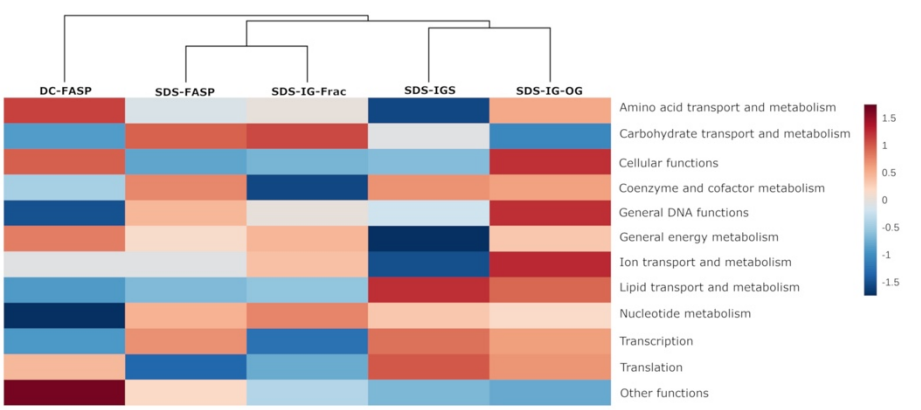

Figure 4: Effects of the various protocols on the distributions of GO functional categories. Each row indicates a cluster of GO functional categories, and each column indicates a sample. Log- and z-score transformed LFQ intensities of functional categories were used for the two-dimensional hierarchical clustering analysis. Samples are represented as an average of each triplicate. Column clustering was calculated based on the Euclidean distance.

No previous study has compared fractionation methodologies, and thus information about how this type of protocol would affect the functions of the identified proteins is unavailable. However, some studies have compared different types of digestion protocols and observed that FASP and in-gel digestion produced similar results in terms of localization or function [10]. Additionally, Cerdó et al. [26]

performed in-gel digestion and covered the main functions involved in energy metabolism, finding several differences between their groups of human samples.

3.3. Study validation: Microbiota diversity and the metaproteome is affected by obesity.

After testing and analyzing each methodology, the SDS-IGS-OG protocol was tested in an animal model. Hence, the workflow chosen was able to be verified in a specific condition, namely, obesity, where the gut microbiota has been described as a factor implicated in its etiology and related comorbidities, influencing energy extraction from the diet, lipid metabolism, the immunological response and endocrine functions. Sprague-Dawley rats were fed the CAF diet for 9 weeks to induce obesity. After this period, the body weight and fat mass were significantly increased compared to the STD-fed group ($p<0.0001$) (Figure 5), confirming previous findings [27,28].

In this case, this animal study was employed as a merely descriptive manner to test the protocol chosen and justify why was better to use this instead another one. In fact, another study performed in parallel, with a more complex experimental design studying the decrease of systolic blood pressure related to Hesperidin supplementation, has already been published by the same authors applying the developed methodology presented here [29].

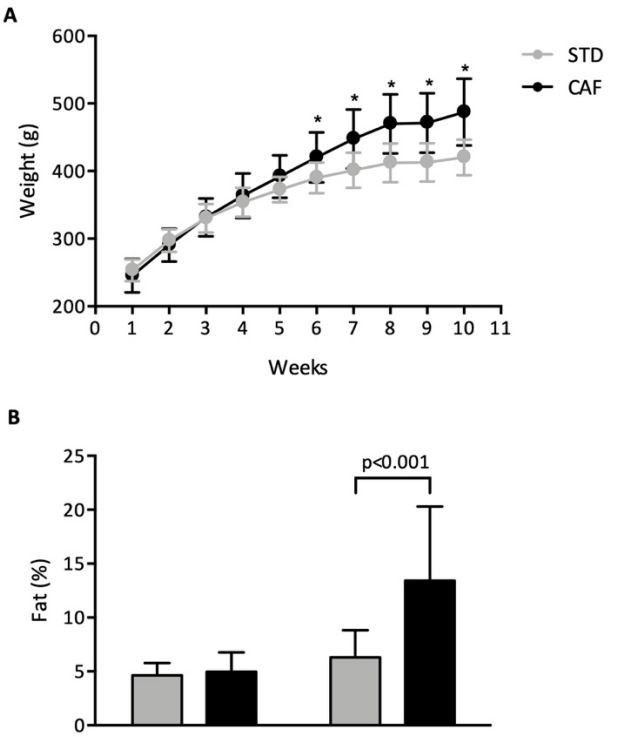

Figure 5: A) Measurement of body weight during the 10 week study and B) percentage of body fat measured at the first and last week of the study. *p<0.05 calculated using Student's t test for the comparison between the CAF and STD groups.

Therefore, the metaproteomic approach was combined with metagenomics (through partial 16s rRNA gene sequencing) in cecal samples to assess the effect of obesity on the microbiota composition and function. Recently, similar studies have described a disruption of the microbiota in rats fed the cafeteria diet, as evidenced by changes in the bacterial composition [16,29]. We postulated that the metagenomic analysis would be an accurate method for validating our metaproteomic workflow.

The metagenomics analysis revealed slight differences in bacterial composition between the STD group and CAF group (Figure 6A). If we compared the groups at the phylum level, the Bacteroidetes to Firmicutes ratio (B/F) was not significantly different between the two

groups (STD 0.333; CAF 0.346; p=0.351), although some significant differences were found at the family level that are consistent with a previous study [29].

After the metagenomics analysis, metaproteomics was able to be performed accurately because real connections were assessed using the obtained bacterial biodiversity at family taxonomical level to generate a metaproteomic database. One thousand four hundred forty-three proteins were identified. Of these proteins, 598 were selected as more confident proteins by filtering at a 50% frequency in each group of samples. Statistical analyses using an unpaired t-test and PCA were performed. Finally, a clear separation in the PCA was observed for 16 significantly differentially expressed proteins ($p<0.001$) (Figure 6A).

GO functions were attributed to these 16 proteins and related to the taxonomical family to understand changes in microbiota functions. These proteins have some important metabolic functions involved in ATP metabolism or TCA cycle. Figure 6B provides a detailed list of the functions affected, and most of the proteins are involved in energy metabolism and tightly correlated with obesity and its associated comorbidities, showing a remarkable difference between the two groups.

As shown in the present study, the microbiota diversity and its functions are affected by obesity, consistent with a previous study [29]. Alterations in microbiota composition agree with reported studies, but the application of a metaproteomics workflow adds valuable information about the functions of the bacterial community.

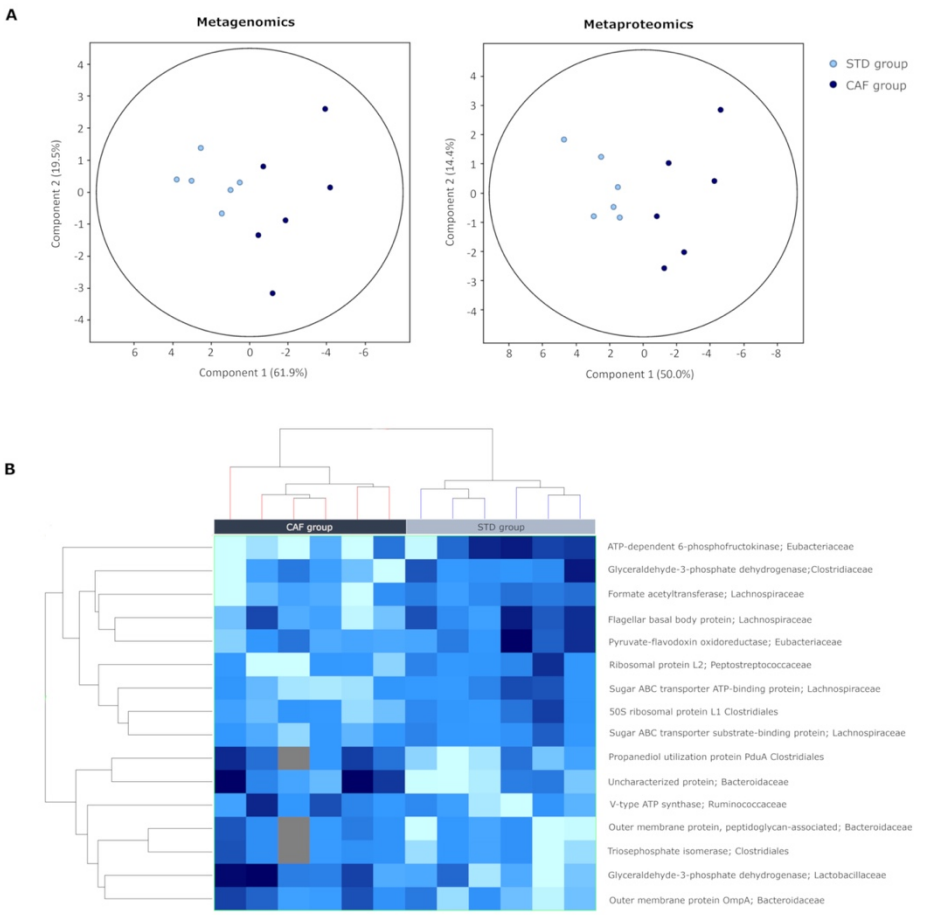

Figure 6: A) PCA of up- and downregulated families and proteins identified by metagenomics and metaproteomics, respectively, showing the differences between the STD group and the CAF group. The two first components are shown with the percentage of variance that they explain. Columns correspond to individual samples. B) The sixteen identified proteins that were significantly different between groups and their taxonomic families are shown, along with the differences found in each group. Each column represents a sample.

4. CONCLUDING REMARKS

The application of metaproteomics in gut microbiota studies improves our understanding of the functions of the complex ecosystem that interacts with the host. However, a consensus and the optimization of critical steps in the methodology for gut metaproteomic studies are required. The procedure used in the present study made a major

contribution to improving our knowledge of the roles of the microbiota.

We drew some conclusions from the essential steps evaluated during the experimental procedure. 1) SDS-based lysis buffer is needed to extract a broader range of proteins, 2) in-gel digestion is the most reproducible method and substantially increases functional coverage and 3) fractionation is crucial to obtain an in-depth characterization of the metaproteome. Although a combined metagenomics-metaproteomics database is also an important factor to consider, a non-accurate data analysis can address the identification of false positives. Thus, every metaproteomics study should be preceded by a metagenomics analysis to overcome these limitations, as it is demonstrated by the findings from our real case study providing sufficient evidence for the correlation of the microbiota with obesity and consequently the link between metagenomics and metaproteomics.

This study provides novel insights into metaproteomics studies by reviewing the general procedures used. Nevertheless, this research field is new and further studies are needed to continuously improve and deciphering the real interactions between the microbiota and host.

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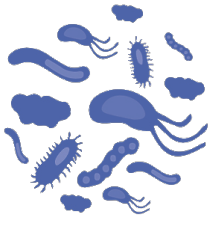

Chapter 2

To verify the effect of antibiotics on the microbiota and corroborate the role of diet by a FMT

UNIVERSITAT ROVIRA I VIRGILI
MULTI-OMICS STRATEGY TO ELUCIDATE THE GUT MICROBIOTA ACTIVITY
Maria Guirro Castellnou

Manuscript 3

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Obesity-induced gut microbiota dysbiosis can be ameliorated by fecal microbiota transplantation: a multiomics approach.

Maria Guirro[1,2], Andrea Costa[2], Andreu Gual-Grau[1], Pol Herrero[2], Helena Torrell[2], Núria Canela[2] and Lluís Arola[1,3]

[1] Universitat Rovira i Virgili, Biochemistry and Biotechnology Department, Nutrigenomics Research Group, Tarragona, Spain

[2] Eurecat, Centre Tecnològic de Catalunya. Centre for Omic Sciences (COS), Joint Unit Universitat Rovira i Virgili-EURECAT. Unique Scientific and Technical Infrastructures (ICTS), Reus, Spain

[3] Eurecat, Centre Tecnològic de Catalunya. Biotechnological Area, Reus, Spain

Correspondence: Dr. Núria Canela

Abstract

Obesity and its comorbidities are currently considered an epidemic, and the involved pathophysiology is well studied. Recently, the gut microbiota has emerged as a new potential therapeutic target for the treatment of obesity. Diet and antibiotics are known to play crucial roles in changes in the microbiota ecosystem and the disruption of its balance; therefore, the manipulation of gut microbiota may represent a strategy for obesity treatment. Fecal microbiota transplantation, during which fecal microbiota from a healthy donor is transplanted to an obese subject, has aroused interest as an effective approach for the treatment of obesity. To determine its success, a multiomics approach was used that combined metagenomics and metaproteomics to study microbiota composition and function.

To do this, a study was performed in rats that evaluated the effect of a hypercaloric diet on the gut microbiota, and this was combined with antibiotic treatment to deplete the microbiota before fecal microbiota transplantation to verify its effects on gut microbiota-host homeostasis. Our results showed that a high-fat diet induces changes in microbiota biodiversity and alters its function in the host. Moreover, we found that antibiotics depleted the microbiota enough to reduce its bacterial content. Finally, we assessed the use of fecal microbiota transplantation as an obesity therapy, and we found that it reversed the effects of antibiotics and reestablished the microbiota balance, which restored normal functioning and alleviated microbiota disruption.

Introduction

Obesity is defined as a disequilibrium in energy balance and is currently a global health problem in Western societies, where its prevalence has increased considerably in recent years. Obesity triggers a vast number of comorbidities associated with hypertension, cardiovascular disease, and diabetes, as well as other conditions [1]. It is widely known that obesity is affected by numerous factors, such as diet, lifestyle and genetic background [2], and recently it has been shown to be related to gut microbiota [3], which have been implicated in energy homeostasis and metabolic functions [4]. Moreover, the same factors that affect obesity can modulate gut microbiota composition, and the function of the gut microbiota will be affected by factors involved in gut microbiota-host equilibrium [5].

Several diet-induced animal models of obesity can be used to explore the mechanisms involved in obesity. There are different obesogenic diets that can be employed. One example of these diets is the semi-purified high-fat diet [6,7]. These types of diets are more commonly used in these models due to their well-defined nutritional composition [8–10].

Alterations in the gut microbiota composition have been shown to result in an imbalance that leads to dysbiosis, which likely will have dramatic effects on the maintenance of health [11]. Fecal microbiota transplantation (FMT) is a new and straightforward therapy that manipulates the gut microbiota by transferring healthy donor microbiota into an existing disrupted gut microbial ecosystem. This therapy can be an effective approach to obesity treatment [12,13]. Even though FMT has some limitations, several studies have tested its effectiveness and have demonstrated an improvement of some

comorbidities associated not only with obesity [14] but also with other noncommunicable diseases [15–18].

Animal models have been increasingly employed to investigate the role and function of gut microbiota, and there have been several studies where mice that were fed a high-fat diet showed a clear disruption in their microbiota composition [19,20]. Such changes in the microbiota due to diet can modulate important metabolic functions, including fat storage [21]. Among the different animal models available, germ-free (GF) mice represent the model that is most used to study the interaction between hosts and their microbiota, and it is also the preferred option for FMT studies. However, GF mice have less body fat in comparison with wild-type mice, even if they consume more food [22,23], and as a result, they are not the most realistic model for obesity-induced studies. In addition, these animals must be bred in sterile environments, and conducting these kinds of experiments requires skilled personnel and a special infrastructure. Thus, gut microbiota depletion by using a cocktail with a combination of broad-spectrum antibiotics [24] is an accessible alternative to the use of GF mice to study the role of microbiota in the host [18].

Nonetheless, the effect of FMT on hosts has hardly been studied due to its novelty, and specific tools are needed to comprehend these effects. Recently, multiomics approaches have been proposed as the most accurate methods for the study of the complexity of the gut microbiota and its environment [4,21,25]. Metagenomics, which provides a taxonomical profile of the biodiversity present in each experimental condition, and metaproteomics, which is focused on the characterization of the whole proteome to reveal its functionality in

the host [5,26,27], are the most promising omics strategies that could be used to reveal the role of gut microbiota.

Hence, the aim of this study were to investigate the role of the gut microbiota with a multiomic approach that combined metagenomics and metaproteomics to determine the effects of a dietary intervention consisting of two different diets (a low-fat diet (LFD) and a high-fat diet (HFD)), to assess the effects of antibiotics on gut microbiota depletion and to corroborate the effectiveness of FMT in rats fed a HFD.

Materials and methods

Animals

Forty eight-week-old male Wistar rats (Charles River Laboratories, Massachusetts, USA) were housed individually at 22ºC with a light/dark cycle of 12 hours and were given access to food and water *ad libitum* during the experiment. After one week of adaptation, the animals were divided into five groups (n=8). For 9 weeks, two groups were fed a LFD (10% fat, 70% carbohydrate, and 20% protein; D12450K, Research Diets, New Brunswick, USA) or a HFD (45% fat, 35% carbohydrate, and 20% protein; D12451, Research Diets, New Brunswick, USA). Two other groups were also fed either a LFD or a HFD for 11 weeks, and during the last 2 weeks, they were given antibiotic treatment (ABS). The last group was fed a HFD for 14 weeks; at 10 and 11 weeks the rats received antibiotic treatment, and during the last three weeks (12-14), they received FMTs from the LFD group (Fig 1).

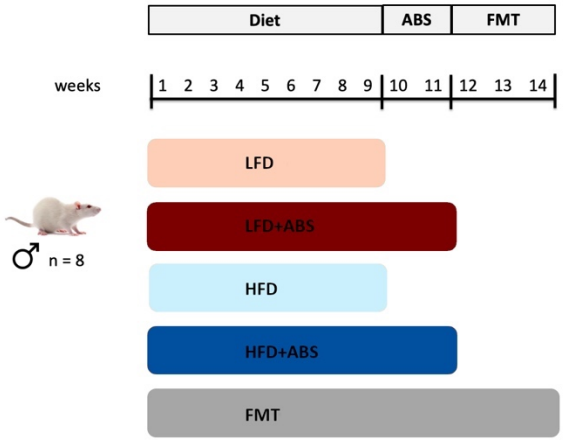

Fig 1. Schematic representation of the experimental design. LFD, Low-Fat Diet; HFD, High-Fat Diet; ABS, Antibiotics; FMT, Fecal Microbiota Transplantation.

Body weight and food intake were measured weekly throughout the study. The body fat mass was determined on weeks 1, 9, 11 and 14 by nuclear magnetic resonance (NMR) using an EchoMRI-700™ device (Echo Medical Systems, L.L.C., Houston, USA).

Cecal samples were obtained immediately after the animals were sacrificed and were frozen in liquid nitrogen and stored at -80ºC until the analyses were performed.

The Animal Ethics Committee of the University of Rovira i Virgili (Tarragona, Spain) approved all procedures.

Antibiotic treatment and Fecal Microbiota Transplants.

ABS was started 9 weeks after being fed either diet, and the cocktail of antibiotics used consisted of 0.5 g/L vancomycin (Sigma-Aldrich, UK) and 1 g/L neomycin, metronidazole and ampicillin (Sigma-Aldrich, UK). The water flasks were supplemented with the antibiotic cocktail. The mixture was freshly prepared every day, and the animals were given free access to it.

The FMTs were performed for 3 weeks; cecal content from LFD-fed rats was administered by oral gavage for four consecutive days during the first week, two alternating days during week 2 and three days before the rats were sacrificed. Omeprazole (20 mg/kg) was administered by oral gavage 4 hours before each FMT.

Metagenomics analysis

DNA extraction and 16S rRNA gene amplification and purification

DNA was extracted from 300 mg cecal samples using a QIAamp DNA Stool Mini Kit (Qiagen Inc., Hilden, Germany) according to the manufacturer's instructions. The DNA purity and integrity were assessed using spectrophotometry (NanoDrop, Thermo Fisher Scientific, Massachusetts, USA).

Two variable regions (V3 and V4) in the 16S rRNA gene were amplified by PCR as described previously [21].

Ion Torrent sequencing and taxonomic assignments

A multiplexed mixture of twenty DNA samples was diluted to a concentration of 60 pM prior to clonal amplification. The Ion 520 & Ion 530 Kit-Chef (Life Technologies, California, USA) was employed for template preparation and sequencing according to the manufacturer's instructions. The prepared samples were loaded on an Ion 530 chip and sequenced using the Ion S5 system (Life Technologies, California, USA).

After sequencing, the Ion Torrent Suite software package was used to remove the low-quality and polyclonal sequences, and the remaining reads were then analyzed using QIIME [28]. The analysis included OTU (operational taxonomic unit) clustering, alpha diversity analysis, OTU analysis and species annotation (OTU table), and beta diversity

analysis. The OTU table, which indicates the number of reads per sample per OTU, was used for the subsequent statistical analysis.

Metaproteomics analysis

The metaproteomics methodology was conducted as described previously [21,29] with minor modifications.

Cell lysis and protein digestion

Briefly, 300 mg of stool sample was subjected to differential centrifugation to collect the microbial cells, and the obtained bacterial pellet was suspended in SDS-extraction buffer (2% SDS, 100 mM DTT, and 20 mM Tris-HCl pH 8.8), incubated at 95°C and subjected to a bead beating process (Bullet Blender, Cultek, Barcelona, Spain) combined with freeze-thawing cycles. The proteins were purified by TCA/acetone precipitation, and 75 μg of protein from each sample was reduced and alkylated, loaded onto a polyacrylamide gel and digested overnight at 37°C with trypsin (Promega, Wisconsin, USA) at an enzyme-to-protein ratio of 1:100.

Peptide TMT 10plex labeling

The digested proteins were desalted with an HLB SPE column before labeling with TMT 10plex reagent (Thermo Fisher Scientific, Massachusetts, USA) according to the manufacturer's instructions. To normalize the samples and the TMT batches, all samples were pooled and labeled with a 126-tag, and the pooled sample was included in each batch. Then, the labeled peptides from each sample were mixed together and desalted again with an HLB SPE column.

Peptide fractionation

The pooled samples were fractionated by isoelectric focusing with an Off-Gel Fractionator (OG) (Agilent Technologies, California, USA) and 24-well IPG strips (with a nonlinear gradient from pH 3 to pH 10) according to the manufacturer's protocol. After fractionation, each of the 24 fractions was desalted again with an HLB column (Waters, Massachusetts, USA) prior to nanoLC-Orbitrap MS/MS analysis.

nanoLC-Orbitrap MS/MS analysis.

The 72 fractions obtained from the OG fractionation (3 TMT x 24 fractions) were loaded on a trap nanocolumn (0.01 x 2 cm, 5 µm; Thermo Fisher Scientific, Massachusetts, USA) and separated with a C-18 reversed-phase (RP) nanocolumn (0.0075 x 12 cm 3 µm; Nikkyo Technos Co. LTD, Japan). The chromatographic separation was performed with a 90-min gradient that used Milli-Q water (0.1% FA) and ACN (0.1% FA) as the mobile phase at a rate of 300 nl/min.

Mass spectrometry analyses were performed on a LTQ-Orbitrap Velos Pro (Thermo Fisher Scientific, Massachusetts, USA) by acquiring an enhanced FT-resolution spectrum (R=30,000 FHMW) followed by the data-dependent FT-MS/MS scan events (FT-(HCD)MS/MS (R=15,000 FHMW at 35% NCE) from the ten most intense parent ions with a charge state rejection of one and a dynamic exclusion of 0.5 min.

The 24 raw data files for each TMT-plex were analyzed by multidimensional protein identification technology (MudPIT) using Proteome Discoverer software v.1.4.0.288 (Thermo Fisher Scientific, Massachusetts, USA). For protein identification, all MS and MS/MS spectra were analyzed using the Mascot search engine (version 2.5), which was set up to search two different SwissProt databases based

on (i) *Rattus norvegicus* (8,003 sequences) and (ii) an in-house metagenomics database created from metagenomics results at the family level using the Uniref100 sequence identity to reduce the database size and avoid false positive findings (23,768,352 sequences). Two missed cleavage sites were allowed by assuming trypsin was used for digestion, and an error of 0.02 Da for the FT-MS/MS fragment ion mass and of 10.0 ppm for the FT-MS parent ion mass was allowed. For TMT-10plex analysis, lysine and the N-termini were set as quantification modifications, while methionine oxidation and the acetylation of N-termini were set as dynamic modifications, and carbamidomethylation of cysteine was set as a static modification. The false discovery rate (FDR) and protein probabilities were calculated by a fixed PSM validator.

For protein quantification, the ratios between each TMT label and the 126-TMT label were used and normalized based on the protein median.

Statistical Analysis

To determine the significant metagenomic and protein changes between the different conditions under study, the Mass Profiler Professional Software v.14.5 (Agilent Technologies, Massachusetts, USA) was used. The data were log2 transformed and mean-centered for the multivariate analysis (Principal Component Analysis, PCA) and the univariate statistical analysis (Student's t-test).

Results and discussion

Effect of diet, ABS and FMT on body weight and fat mass.

During the nine-week period, the HFD group exhibited a significant increase in body weight after week 5 ($p<0.001$), and the body fat mass measured by NMR in this group was also significantly higher. During

antibiotic administration (from 10 to 11 weeks), both parameters were decreased but remained significantly different between the different dietary groups ($p < 0.001$). Nonetheless, when the FMTs began, the animals recovered their body weight, and their body mass content increased again (Fig 2). Similar results have been reported in other studies in which rats were fed a commercial diet with a high fat content [8,30]; moreover, when an antibiotic treatment is administered, normally the animals exhibit reductions in appetite and food intake, and a loss of body weight occurs [31–33]. This could be an explanation for the fact that the rats lost body weight during antibiotic treatment.

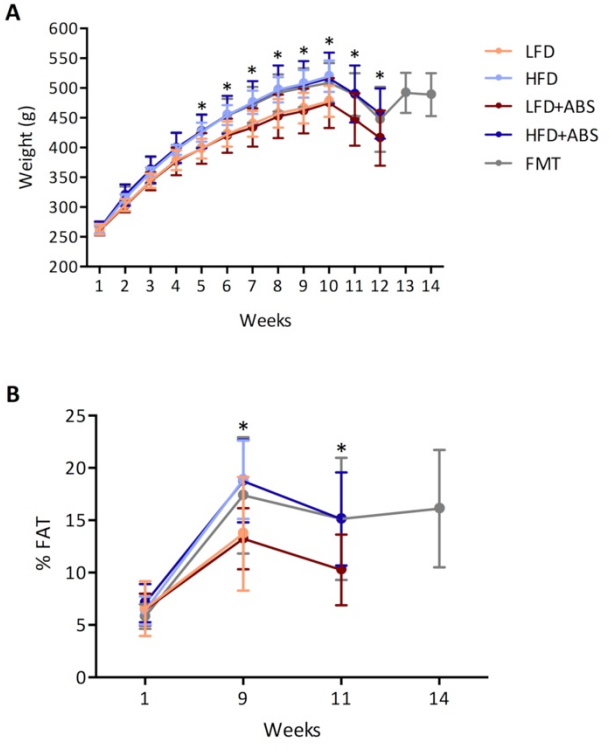

Fig 2. A) Measurement of body weight during the 14 weeks of study and B) the percentage of body fat measured at weeks 1, 9, 11 and 14. *p<0.05, calculated using ANOVA.

Changes in microbiota biodiversity and functionality caused by diet.

To assess the impact of diet on gut microbiota, metagenomics and metaproteomics analyses were performed in cecal samples obtained immediately after sacrifice that were divided into two equal portions for use in both omics approaches.

The metagenomics analysis was performed using NGS Ion Torrent Technology. The sequencing runs produced a total of 49,106,850 reads that were filtered for quality, and 25,535,562 were obtained for the QIIME analysis. From these reads, a total of 17,220 OTUs were obtained for the V3 and V4 regions of the 16S rRNA gene sequence that were used to analyze the relative abundances and diversity in the microbiota at different taxonomical levels.

The two dominant phyla were Bacteroidetes (14.6 – 44.5%) and Firmicutes (52.6 – 84.1%) in both groups, which is in line with the results of previous studies [21,34]. The Bacteroidetes to Firmicutes ratio (B/F) was significantly increased ($p=0.023$) in the HFD group (B/F=0.393) compared to the LFD group (B/F=0.294). No differences were found with regard to phylum level or alpha diversity or the Shannon and Simpson indexes, but some comparisons could be made at the family level, and a clear separation between the groups was observed (Fig 3). In total, the abundances of 9 family taxa were significantly different between the LFD- and HFD-fed rats (Table 1); 7 out of 9 were from the Firmicutes phylum, and 6 were from the Clostridiales order. Even the abundance of Clostridiales was not very high (52.0 – 89.8%), and Clostridiales was probably the order most affected by diet and was responsible for gut microbiota disruption. Similar results have been reported before that have shown clear

differences in microbiota composition when a diet rich in fat is administered [19–21,35] and that the Firmicutes phylum, including the Clostridiales order, represents the taxon with the most changes in terms of microbiota composition.

Table 1: Families with significant differences in abundance between the LFD and HFD groups. Metagenomics analysis

Family	Phylum	LFD (%)	HFD (%)	Reg.	FC	p-value
Coriobacteriaceae	Actinobacteria	0.011	0.007	down	-2.44	0.029
Streptococcaceae	Firmicutes	0.026	0.012	down	-2.67	0.005
Christensenellaceae	Firmicutes	0.196	0.116	down	-1.86	0.004
Clostridiaceae	Firmicutes	0.268	0.088	down	-5.07	0.008
Dehalobacteriaceae	Firmicutes	0.017	0.142	up	5.48	0.017
Peptostreptococcaceae	Firmicutes	0.188	0.109	down	-6.47	0.007
Veillonellaceae	Firmicutes	2.750	1.706	down	-10.86	0.028
Mogibacteriaceae	Firmicutes	0.046	0.019	down	-2.46	0.001
Desulfovibrionaceae	Proteobacteria	0.810	0.462	down	-1.66	0.039

Reg.: Regulations; FC: Fold-Change

In addition to metagenomics, metaproteomics was performed to assess the impact of diet on microbiota function. A total of 72 fractions were analyzed, and 1598 bacterial proteins were identified. By filtering these proteins on the basis of their being present in at least 50% of samples from at least one of the groups, 415 were selected due to greater confidence.

To assess the impact of diet, the differences between the LFD and HFD groups were identified, which showed that 22 and 11 proteins were up-regulated in the HFD group and the LFD group, respectively

(Table 2); most of these proteins were involved in metabolic functioning and played roles in numerous biological processes, such as the TCA and ATP metabolic pathways. The differences in all 33 of these proteins allowed us to perfectly separate both groups (Fig 3).

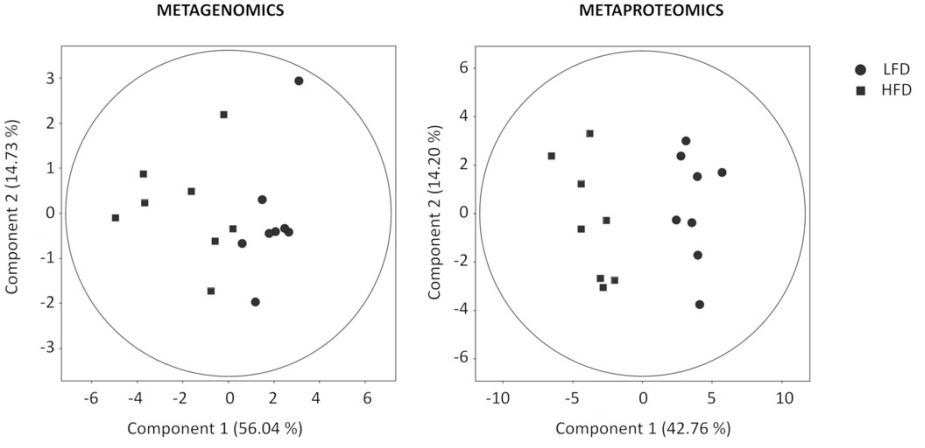

Fig 3. A) PCA of the family OTU abundance and the proteins that were identified that shows the separation between the LFD and HFD groups. The first two components are shown along with the percent variance that is explained by each. The points correspond to the individual samples.

Moreover, each protein was assigned to a family taxon, and if we compare the phyla and orders corresponding to the significant proteins we found, we find that all such proteins were derived from Firmicutes and Clostridiales, which is in line with the metagenomics analysis. However, the correlation between the metagenomics and metaproteomics analyses was not complete at the family taxa level.

Few studies have used similar experimental approaches that can corroborate our results. Only a single previous study carried out in our laboratory assessed the functionality of microbiota [21], and other studies performed in mice have explored the changes in the main functions of the microbiota resulting from a high-fat diet [19]. In both

cases, the majority of the functions affected by microbiota were involved in important metabolic functions.

The microbiota composition was disrupted by antibiotic treatment.

After the assessment of diet, a metagenomics analysis was performed in each dietary group after ABS treatment to verify microbiota disruption, and the results were compared to those from groups without ABS administration. The LFD group was compared to the LFD+ABS group, and the HFD group was compared to the HFD+ABS group.

The actual OTU abundance is shown in Fig 4A, which shows that the majority of the bacterial content in the cecal microbiota was depleted by antibiotic treatment and also indicates that the bacterial content was restored by FMT.

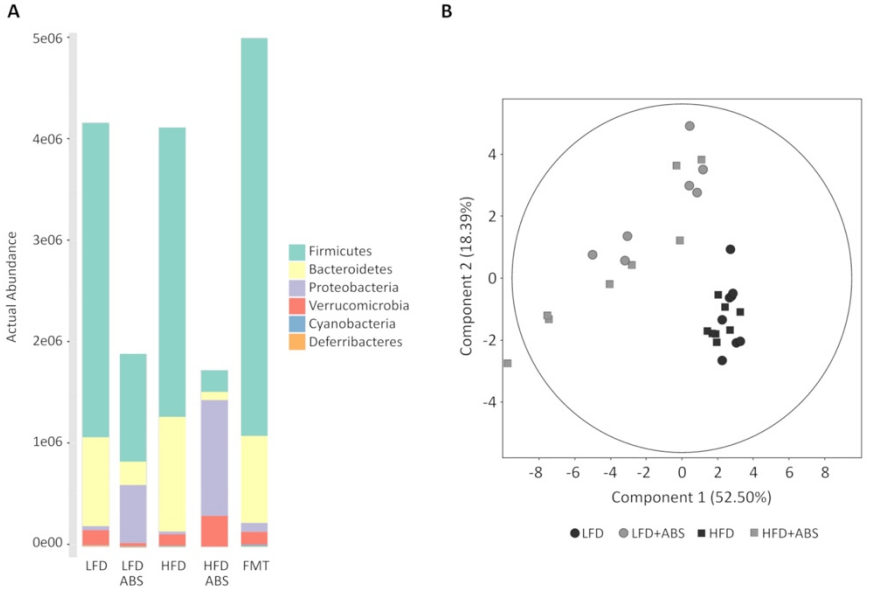

Fig 4. A) Differences in the actual OTU abundances among the different groups at the phylum taxon levels. B) PCA of the differences between the groups treated with and without ABS. The first two components are shown along with the percentages of variance that they explain. The points correspond to individual samples.

Regarding the metagenomics results in the LFD groups, a decrease in both Bacteroidetes (LFD 21.2%; LFD+ABS 10.5%) and Firmicutes (LFD 74.0%; LFD+ABS 45.9%) phyla were observed in the LFD+ABS group, whereas Proteobacteria (LFD 1.0%; LFD+ABS 38.9%; p=0.007) represented the dominant phylum after antibiotic treatment. The B/F ratio was significantly decreased (LFD B/F=0.294; LFD+ABS B/F=0.216; p=0.039); furthermore, the alpha diversity based on observed OTUs and the Shannon and Simpson indexes was not significant (p=0.192, p=0.095, and p=9.982, respectively).

In contrast to the LFD-fed rats, in the HFD-fed rats no significant differences were found for the B/F ratio (HFD B/F=0.393; HFD+ABS B/F=0.382; p=0.110), although similar results due to antibiotic treatment occurred in the LFD+ABS group, which resulted in dramatic decreases in both phyla (Bacteroidetes, HFD 26.4%, HFD+ABS 8.70%; Firmicutes, HFD 70.5%, HFD+ABS 26.5%) and an enormous increase in Proteobacteria (HFD 0.6%; HFD+ABS 59.1%; p<0.001). Moreover, the alpha diversity decreased significantly in the HFD+ABS group compared to the HFD group (observed OTUs p=0.011; Shannon index p=0.009; Simpson index p=0.011).

The results found in both ABS groups were consistent with those of previous studies where a similar cocktail of ABS was administered to wild-type mice, which caused the relative abundances of Bacteroidetes and Firmicutes to decrease, while the abundances of Proteobacteria and Cyanobacteria increased [24,33]. These results could be explained by differences in antibiotic effectiveness against bacteria from different phyla. As can be seen, the antibiotics had stronger activity against bacteria from the Bacteroidetes and Firmicutes phyla and are the reason why changes in the abundance of

Proteobacteria were observed in these particular groups, although the bacterial DNA quantity was considerably lower in the ABS samples.

In addition, the relative abundances were compared at the family level for both the LFD and HFD groups. In the LFD group comparison, distinct differences were found in 18 family taxa, most of which are in the Firmicutes and Proteobacteria phyla, whereas in the HFD groups distinct differences were found for 15 family taxa among the Bacteroidetes, Firmicutes and Proteobacteria phyla (Table 3 and Fig 4B). A total of 10 families were found to be in common in rats fed both diets: *S24-7* from Bacteroidetes; *Enterococcaceae*, *Streptococcaceae*, *Christensenellaceae, Lachnospiraceae, Peptococcaceae* and *Ruminococcaceae* from Firmicutes; *Alcaligenaceae*, *Enterobacteriaceae* and *Pseudomonadaceae* from Proteobacteria. All of these were regulated equally in the ABS groups compared to the respective non-ABS groups. Similar changes in microbiota composition were found in several previous studies of chronic antibiotic exposure [36–38] that showed a significant reduction of bacterial richness and diversity and corroborated the effectiveness of gut microbiota depletion for FMT studies.

FMT restored microbiota biodiversity and functionality.

To assess the use of FMT as a possible treatment for overweight or obesity caused by dietary habits, FMT was performed by transplanting cecal microbiota content from LFD rats to HFD rats previously depleted by ABS treatment. The metagenomics analysis revealed that Bacteroidetes (LFD 21.2%, HFD 26.4%, FMT 18.0%) and Firmicutes bacteria (LFD 74.0%, HFD 70.5%, FMT 77.0%) were restored after FMT to levels similar to those found prior to antibiotic treatment, and consequently Proteobacteria (LFD 1.0%, HFD 0.6%, FMT 2.0%) were

decreased considerably. As can be observed, the B/F ratio was more similar between the LFD (B/F=0.294) and FMT (B/F=0.233) groups since no significant differences were observed (p=0.109), whereas the B/F ratio was significantly different in the HFD group (B/F=0.393, p=0.011). Regarding alpha diversity, some differences were found in terms of the observed OTUs and the Shannon and Simpson indexes (p=0.001, p<0.001, and p<0.001, respectively) in the FMT groups compared to both the LFD and the HFD group (one-way ANOVA). These differences could be ameliorated by prolonging the FMT treatment in future studies, but these differences were quite small.

Additionally, a decision was made to determine whether some differences could be found at the family level. After one-way ANOVA, the abundances of 10 families were found to be significantly different between two of the three experimental groups, as shown in Table 4. However, Tukey post hoc tests revealed that the abundances of only 3 families were significantly different between the LFD and HFD groups (*Clostridiaceae, Christensenellaceae* and *Mogibacteriaceae)* and were not significantly different when the FMT group was compared to the LFD group. Moreover, these three families were equally regulated in the LFD and FMT groups when compared to the HFD group. This indicates greater similarity between the FMT and LFD groups than between either of these groups and the HFD group. PCA and hierarchical cluster analysis (HCA) based on the relative abundances of these 3 families was performed to assess the similarities between the LFD, HFD and FMT groups. As shown in Fig 5A and B, the FMT group was more similar to the LFD group than the HFD group even when the rats were fed a hypercaloric diet during and after ABS and FMT treatment.

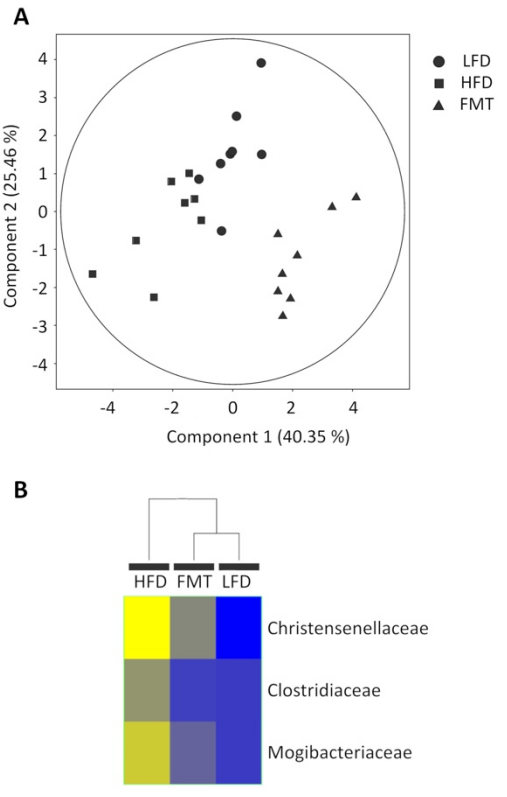

Fig 5. A) PCA of OTU abundance. The first two components are shown along with the percentage of variance that they explain. The points correspond to individual samples. B) Hierarchical clustering analysis of the three significant families in the LFD, HFD and FMT groups.

Furthermore, metaproteomics was performed to assess the disruption of microbiota functionality due to diet and FMT. To assess whether there were some differences between these three groups, one-way ANOVA was performed, and a total of 235 proteins were found to be different in one of the groups. These proteins allowed us to perfectly separate these three groups during PCA (Fig 6A). Of these 235 proteins, 155 and 194 were different in the FMT group compared to the LFD and HFD groups, respectively. Moreover, 21 proteins were significantly different between the LFD and HFD groups, and this could also explain the differences between these groups and the FMT

group. As seen, the greater number of significant proteins in the FMT group compared to the LFD and HFD groups may be due to the low level of biodiversity observed in the FMT group, as previously described.

Subsequently, each of these proteins was assigned to a taxonomical family to correlate them with the metagenomics results, but only the Clostridiaceae family showed significant differences based on both omics approaches (Table 5). However, if we made the same comparison based on the order taxon, the majority of families that showed significant differences were from the Clostridiales order and the Firmicutes phylum, as was observed when only the LFD and HFD groups were compared.

To more deeply understand the function of the whole gut microbiome, GO functions were attributed to each protein, and the 15 most highly represented activities are shown in Fig 6B. The most highly represented functions in each group were represented by proteins involved in the structure of the ribosome [GO:0003735], translation [GO:0006412], the cytoplasm [GO:0005737] and ATP binding [GO:0005524]. These four GO functions were slightly increased in the HFD group, whereas ATP binding and cytoplasmic proteins were decreased in the FMT group compared to both the HFD and LFD groups. Additionally, proteins representing integral components of the membrane [GO:0016021] were increased in the FMT group compared to the other groups.

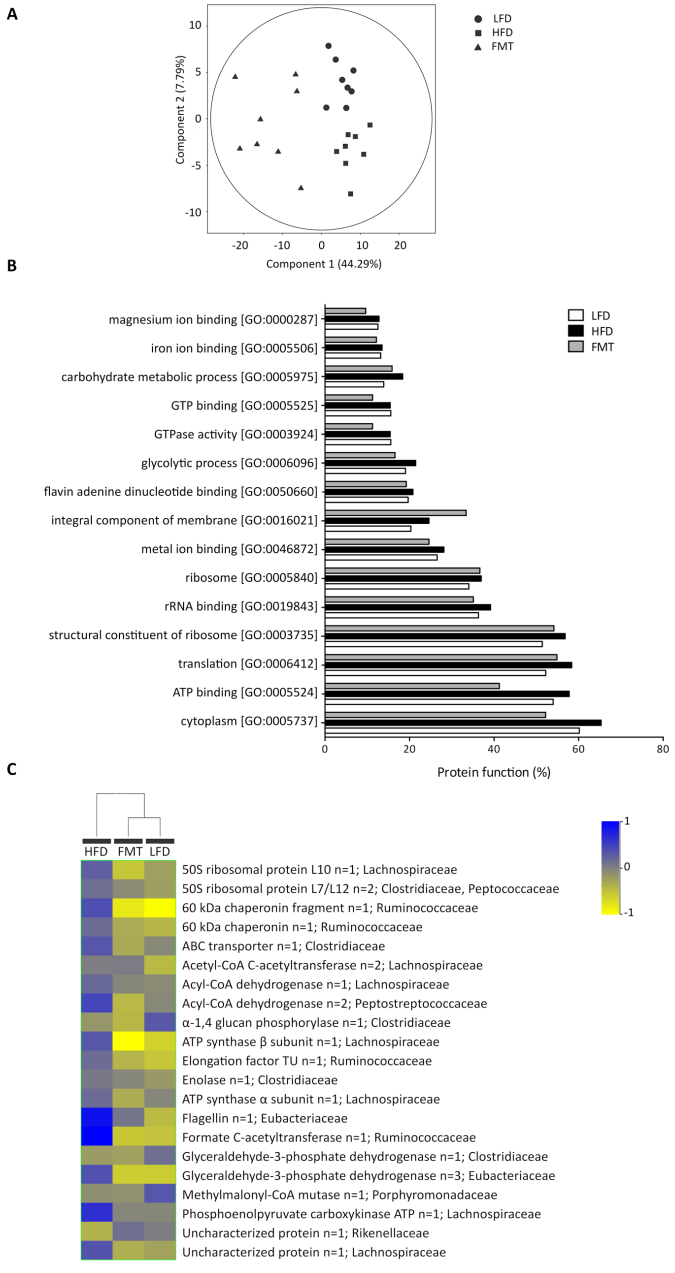

Fig 6. A) PCA of up- and downregulated proteins showing the separation between the three groups. The first two components are shown along with the percentages of variance that they explain. The points correspond to individual samples. B) Percentages of proteins that represent the 15 most abundant protein functions according to Gene Ontology (GO) terms in the LFD, HFD and FMT groups. C) Hierarchical clustering analysis of 21 significant proteins in the LFD, HFD and FMT groups.

Based on their metabolic functions, the majority of these proteins were involved in important metabolic pathways such as those involved in ATP/energy metabolism or glycolysis. A total of 8 proteins were equally regulated in both the LFD and FMT groups compared to the HFD group, and important proteins such as Acyl-CoA dehydrogenase and phosphoenolpyruvate carboxykinase, both from the Lachnospiraceae family, were found. Furthermore, two significant proteins, enolase (n=1) and 50S ribosomal protein L7/L12 (n=2), were from Clostridiaceae, whereas the other significant proteins were not clearly correlated with the metagenomics results. It needs to be taken into account that protein databases can be more accurate for certain families than others, and this can affect the taxonomical assignment. These results were represented in the PCA and the HCA (Fig 6AC), where it can be observed that the FMT group clusters closer to the LFD group than the HFD group, indicating that the functionality of gut microbiota after FMT is more similar to that of healthy donor microbiota.

Additionally, a correlation analysis between the metagenomics and metaproteomics results was performed. As shown in Fig 7, the profile that was obtained was very similar in terms of the correlation between the metagenomics and metaproteomics results for the relative abundance of the three significant families that contained all of the significant proteins. These results can be explained by the similarities between these three families, since they are from the same order (Clostridiales) and phylum (Firmicutes). Moreover, clustering of the proteins that belong to the same family or families that are taxonomically related is also observed. Overall, one of the most interesting proteins in our study was glyceraldehyde-3-phosphate dehydrogenase from Clostridiaceae, which has a moderate positive

correlation (r=0.5) with the metagenomics results. The slope for this correlation was > 1.3 (metagenomics vs metaproteomics), which indicates that the abundance of this protein and, therefore, its function was diminished in the HFD group vs the LFD and FMT groups. Glyceraldehyde-3-phosphate dehydrogenase was found to control NAD-dependent glycolytic activity in some Clostridium species. It is known that Clostridium species are gram-positive obligate anaerobes and typically perform butyric acid fermentation that is carried out during the exponential growth phase, and this generates acetate and butyrate as the main fermentation products from glucose. In this sense, butyrate products could be considered short-chain fatty acids (SCFAs) produced by gut microbiota [39,40].

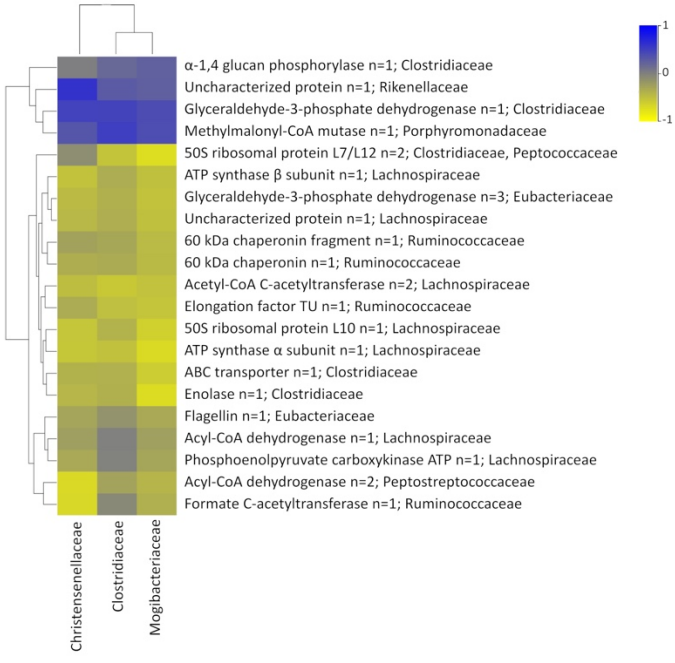

Fig 7. Significant correlations between families and proteins in the LFD, HFD and FMT groups.

Finally, the corroboration of our findings was challenging due to the low number of studies that have been published that have assessed the relationship between metaproteomics and hosts, and even fewer have examined this after FMT. Nevertheless, a study that was performed in rats fed a diet rich in fat and rats fed a chow diet found that Firmicutes were presumed to benefit from a high-fat diet [41], which is contrary to our findings that showed that the abundance of Firmicutes was reduced in the HFD group. However, these differences depend on the part of the colon that is analyzed, as shown in this paper. Moreover, another study performed in pigs found that proteins involved in carbohydrate metabolism showed the most changes in HFD animals, but proteins involved in this process were not changed in our study [42].

Conclusions

The gut microbiota is essential for maintaining health and has a primary role in metabolism and homeostasis, and its alteration during obesity is a problem that needs to be addressed. Our results applied a combination of metagenomics and metaproteomics approaches to confirm some previous observations: (i) the diet can alter the biochemical composition of the gut microbiota either by shifting the phylotype composition or the activity of bacterial cells; (ii) antibiotics disrupt microbiota biodiversity; (iii) FMT is effective in recolonizing the gut microbiota and in restoring some metabolic functions. When testing these three microbiota modulation strategies, different changes were observed in the bacterial metaproteome, demonstrating that every single change in the host environment can affect microbiota function. In addition to results observed over a short-term period of time [16,18], these findings show that a HFD has a major impact on the

mouse cecal microbiota that extends beyond compositional changes to major alterations in bacterial physiology, and FMT can be considered a new strategy to treat obesity.

Moreover, this study reaffirms that metaproteomics should be a complementary tool used along with metagenomics and that combining the results of both approaches can result in the improved characterization of cecal microbiota.

Table 2. Proteins significantly up- or downregulated between the LFD and HFD groups. Metaproteomics analysis.

Protein	Pathway	Family	Reg.	FC	p-value
60 kDa chaperonin Fragment n=1	ATP/energy metabolism	Ruminococcaceae	up	6.16	0.002
60 kDa chaperonin n=1	ATP/energy metabolism	Ruminococcaceae	up	2.14	0.004
ABC transporter n=1	ATP/energy metabolism	Clostridiaceae	up	1.90	0.018
ATP synthase subunit beta n=1	ATP/energy metabolism	Lachnospiraceae	up	3.65	0.002
sn-glycerol-3-phosphate import ATP-binding protein UgpC n=1	ATP/energy metabolism	Eubacteriaceae	down	-3.75	0.040
Flagellin n=1	Bacteria	Eubacteriaceae	up	7.72	0.002
TonB-linked outer membrane. SusC/RagA family protein n=1	Bacteria	Bacteroidaceae	up	2.21	0.012
TonB-linked outer membrane. SusC/RagA family protein n=6	Bacteria	Porphyromonadaceae, Bacteroidaceae	down	-1.78	0.028
Alpha-1.4 glucan phosphorylase n=1	CARB Metabolism	Clostridiaceae	down	-2.07	<0.001
Maltose-binding periplasmic proteins/domains n=1	CARB Metabolism	Clostridiaceae	up	1.83	0.007
Phosphoglucomutase/phosphomanno mutase. C-terminal domain protein n=1	CARB Metabolism	Bacteroidaceae	up	1.52	0.028
Elongation factor Tu Fragment n=1	Cellular Division	Neisseriaceae	down	-1.84	0.012
Elongation factor Tu n=1	Cellular Division	Ruminococcaceae	up	2.52	0.006

Elongation factor Tu n=12	Cellular Division	Bacteroidaceae	up	1.54	0.030
Formate C-acetyltransferase n=1	Glycolysis	Ruminococcaceae	up	10.22	<0.001
Glyceraldehyde-3-phosphate dehydrogenase n=1	Glycolysis	Clostridiaceae	down	-1.62	0.014
Glyceraldehyde-3-phosphate dehydrogenase n=3	Glycolysis	Eubacteriaceae	up	3.78	0.005
Dissimilatory sulfite reductase B n=1	Iron metabolism	Desulfovibrionaceae	down	-1.72	0.024
Uncharacterized protein Fragment n=1	Non	Microbacteriaceae	down	-2.46	0.043
Uncharacterized protein n=1	Non	Rikenellaceae	down	-1.66	0.004
Uncharacterized protein n=1	Non	Bacteroidaceae	up	2.03	0.017
Uncharacterized protein n=1	Non	Desulfovibrionaceae	down	-1.52	0.048
Uncharacterized protein n=1	Non	Lachnospiraceae	up	2.50	0.001
30S ribosomal protein S8 n=1	Ribosomal/translation	Lachnospiraceae	down	-2.12	0.034
50S ribosomal protein L1 n=2	Ribosomal/translation	Prevotellaceae	up	1.71	0.008
50S ribosomal protein L10 n=1	Ribosomal/translation	Lachnospiraceae	up	2.07	0.016
50S ribosomal protein L4 n=7	Ribosomal/translation	Bacteroidaceae	up	1.78	0.027
50S ribosomal protein L7/L12 n=2	Ribosomal/translation	Clostridiaceae, Peptococcaceae	up	1.66	0.001
Acetyl-CoA C-acetyltransferase n=2	TCA	Lachnospiraceae, Clostridiaceae	up	1.74	0.032
Acyl-CoA dehydrogenase n=1	TCA	Lachnospiraceae	up	1.53	0.014

Acyl-CoA dehydrogenase n=2	TCA	Peptostreptococcaceae	up	2.37	0.001
Methylmalonyl-CoA mutase n=1	TCA	Porphyromonadaceae	down	-2.00	0.007
Phosphoenolpyruvate carboxykinase ATP n=1	TCA	Lachnospiraceae	up	3.18	0.008

Table 3: Families with significant differences in abundance between the diet-only groups and the respective diet-plus-ABS groups. Metagenomics analysis

Familiy	Phylum	LFD (%)	LFD+ ABS (%)	HFD (%)	HFD+ ABS (%)	Reg.	FC	p-value
Propionibacteriaceae	Actinobacteria	0.000	0.011			up[a]	8.39[a]	0.044[a]
Bacteroidaceae	Bacteroidetes			15.513	1.383	down[b]	-9.47	0.002[b]
Rikenellaceae	Bacteroidetes			1.451	0.226	down[b]	-6.50	0.007[a] 0.012[b]
S24-7	Bacteroidetes	5.312	2.320	4.086	1.007	down[a,b]	-3.24[a] 4.31[b]	0.005[a]
Odoribacteraceae	Bacteroidetes	0.064	0.036			down[a]	-4.84[a]	0.025[a]
Paraprevotellaceae	Bacteroidetes			5.193	0.463	down[b]	-9.71[b]	0.008[b]
Deferribacteraceae	Deferribacteres			0.100	0.014	down[b]	-8.43	0.008[b]
Staphylococcaceae	Firmicutes	0.000	0.011			up[a]	12.03[a]	<0.001[a]
Enterococcaceae	Firmicutes	<0.001	0.130	<0.001	0.014	up[a,b]	115.51[a] 29.05[b]	<0.001[a,b]
Streptococcaceae	Firmicutes	0.026	17.981	0.012	1.655	up[a,b]	212.53[a] 162.04[b]	<0.001[a,b]
Christensenellaceae	Firmicutes	0.196	0.002	0.116	0.016	down[a,b]	-2.83[a] -5.75[b]	0.016[a] 0.013[b]
Dehalobacteriaceae	Firmicutes			0.142	0.014	down[b]	-10.19[b]	0.020[b]
Lachnospiraceae	Firmicutes	14.395	7.887	10.707	1.346	down[a,b]	-2.79[a] -6.92	0.018[a] 0.001[b]
Peptococcaceae	Firmicutes	0.702	0.271	0.569	0.049	down[a,b]	-4.64[a] -10.88[b]	0.001[a] 0.004[b]
Peptostreptococcaceae	Firmicutes	0.188	0.099			down[a]	-2.75[a]	0.027[a]

Ruminococcaceae	Firmicutes	16.887	9.349	19.415	2.126	down[a.b]	-2.57[a] -7.18[b]	0.010[a] 0.001[b]
Mogibacteriaceae	Firmicutes	0.046	0.026			down[a]	-3.01[a]	0.019[a]
Alcaligenaceae	Proteobacteria	0.070	1.418	0.084	10.011	up[a.b]	10.99[a] 8.30[b]	0.029[a] 0.028[b]
Enterobacteriaceae	Proteobacteria	0.057	19.103	0.053	41.999	up[a.b]	227.03[a] 194.87[b]	<0.001[a,b]
Pasteurellaceae	Proteobacteria	0.010	0.005			down[a]	-2.53[a]	0.031[a]
Moraxellaceae	Proteobacteria	0.001	0.141			up[a]	28.33[a]	0.024[a]
Pseudomonadaceae	Proteobacteria	0.001	8.852	0.002	15.388	up[a,b]	1171.54[a] 3125.62[b]	<0.001[a,b]
Anaeroplasmataceae	Tenericutes	0.011	0.159			up[a]	16.32[a]	0.021[a]

Reg.: Regulations; FC: Fold-Change

a) LFD versus LFD+ABS

b) HFD versus HFD+ABS

Table 4. Families with significant differences in abundance based on ANOVA between the LFD, HFD and FMT groups. Metagenomics analysis.

Family	Phylum	LFD (%)	HFD (%)	FMT (%)	Reg. FMT/LFD	FC FMT/LFD	Reg. FMT/HFD	FC FMT/HFD	Reg. HFD/LFD	FC HDF/LFD	p-value
Coriobacteriaceae	Actinobacteria	0.011	0.007	0.014	up	1.01	up	2.46	down	-2.44	0.036
Prevotellaceae	Bacteroidetes	0.259	0.391	0.033	down	-11.02	down	-17.47	up	1.59	<0.001
Odoribacteraceae	Bacteroidetes	0.064	0.001	0.000	down	-219.21	down	-80.82	down	-2.71	<0.001
Paraprevotellaceae	Bacteroidetes	2.923	5.193	0.089	down	-50.24	down	-98.25	up	1.96	<0.001
Deferribacteraceae	Deferribacteres	0.210	0.100	0.002	down	-60.29	down	-45.95	down	-1.31	<0.001
Christensenellaceae	Firmicutes	0.196	0.116	0.197	down	-1.03	up	1.80	down	-1.86	0.008
Clostridiaceae	Firmicutes	0.268	0.088	0.130	down	-2.68	up	1.89	down	-5.07	0.021
Mogibacteriaceae	Firmicutes	0.019	0.046	0.036	down	-1.36	up	1.81	down	-2.46	0.007
Erysipelotrichaceae	Firmicutes	0.049	0.021	0.137	up	2.57	up	5.90	down	-2.30	0.002
Desulfovibrionaceae	Proteobacteria	0.810	0.462	1.537	up	1.95	up	3.24	down	-1.66	0.001

Reg.: Regulations; FC: Fold-Change

Table 5. Proteins significantly up- or downregulated between the LFD, HFD and FMT groups. Metaproteomics analysis.

Protein	Pathway	Family	Reg. FMT/LFD	FC FMT/LFD	Reg. FMT/HFD	FC FMT/HFD	Reg. HFD/LFD	FC HFD/LFD	p-value
60 kDa chaperonin Fragment n=1	ATP/energy metabolism	Ruminococcaceae	up	1.22	down	-5.05	up	6.16	0.023
60 kDa chaperonin n=1	ATP/energy metabolism	Ruminococcaceae	up	1.11	down	-1.94	up	2.14	0.039
ABC transporter n=1	ATP/energy metabolism	Clostridiaceae	down	-1.36	down	-2.59	up	1.90	0.001
ATP synthase subunit beta n=1	ATP/energy metabolism	Lachnospiraceae	down	-1.53	down	-5.61	up	3.65	<0.001
ATP synthase subunit alpha n=1	ATP/energy metabolism	Lachnospiraceae	down	-1.42	down	-1.99	up	1.40	<0.001
Flagellin n=1	Bacteria	Eubacteriaceae	up	1.98	down	-3.91	up	7.72	0.003
Alpha-1.4 glucan phosphorylase n=1	CARB Metabolism	Clostridiaceae	down	-2.82	down	-1.36	down	-2.07	<0.001
Elongation factor Tu n=1	Cellular Division	Ruminococcaceae	up	1.18	down	-2.13	up	2.52	0.044
Enolase n=1	Glycolysis	Clostridiaceae	up	1.18	down	-1.19	up	1.40	0.029

Formate C-acetyltransferase n=1	Glycolysis	Ruminococcaceae	down	-1.06	down	-10.79	up	10.22	<0.001
Glyceraldehyde-3-phosphate dehydrogenase n=1	Glycolysis	Clostridiaceae	down	-1.70	down	-1.05	down	-1.62	0.017
Glyceraldehyde-3-phosphate dehydrogenase n=3	Glycolysis	Eubacteriaceae	down	-1.02	down	-3.85	up	3.78	0.011
Methylmalonyl-CoA mutase n=1	Minerals Metabolism	Porphyromonadaceae	down	-2.05	down	-1.03	down	-2.00	0.015
Uncharacterized protein n=1	Non	Rikenellaceae	up	1.20	up	2.00	down	-1.66	<0.001
Uncharacterized protein n=1	Non	Lachnospiraceae	down	-1.12	down	-2.81	up	2.50	<0.001
50S ribosomal protein L10 n=1	Ribosomal/ Translation	Lachnospiraceae	down	-1.44	down	-2.99	up	2.07	0.001
50S ribosomal protein L7/L12 n=2	Ribosomal/ Translation	Clostridiaceae. Peptococcaceae	up	1.19	down	-1.39	up	1.66	0.020
Acetyl-CoA C-acetyltransferase n=2	TCA	Lachnospiraceae. Clostridiaceae	up	1.80	up	1.03	up	1.74	0.023

Acyl-CoA dehydrogenase n=1	TCA	Lachnospiraceae	up	1.05	down	-1.46	up	1.53	0.010
Acyl-CoA dehydrogenase short-chain specific n=2	TCA	Peptostreptococcaceae	down	-1.57	down	-3.72	up	2.37	<0.001
Phosphoenolpyruvate carboxykinase ATP n=1	TCA	Lachnospiraceae	up	1.00	down	-3.16	up	3.18	0.002

Reg.: Regulations; FC: Fold-Change

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UNIVERSITAT ROVIRA I VIRGILI
MULTI-OMICS STRATEGY TO ELUCIDATE THE GUT MICROBIOTA ACTIVITY
Maria Guirro Castellnou

Chapter 3

To evaluate the effect of hesperidin as a hypertension treatment through the modulation of microbiota and its function

Manuscript 4

Multi-omics approach to elucidate the gut microbiota activity: Metaproteomics and metagenomics connection

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Multi-omics approach to elucidate the gut microbiota activity: Metaproteomics and metagenomics connection

Maria Guirro[1], Andrea Costa[2], Andreu Gual-Grau[1], Jordi Mayneris-Perxachs[3], Helena Torrell[2], Pol Herrero[2], Núria Canela[2], Lluís Arola[1,3]

[1] Nutrigenomics Research Group, Biochemistry and Biotechnology Department, Rovira i Virgili University, Tarragona, Spain

[2] Centre for Omic Sciences, Joint Unit Universitat Rovira I Virgili-EURECAT Technology Centre of Catalonia, Unique Scientific and Technical Infrastructures, Reus, Spain

[3] Nutrition and Health Technological Unit, EURECAT-Technology Centre of Catalonia, Reus, Spain

Correspondence: Dr Núria Canela

Keywords: Hesperidin, Metagenomics, Metaproteomics, Microbiota

Abbreviations: B/F, Bacteroidetes to Firmicutes ratio; CAF, cafeteria; CVD, cardiovascular disease; HESP, hesperidin; OTU, operational taxonomic unit; SBP, systolic blood pressure; STD, standard; VH, vehicle

Abstract

Over the last few years, the application of high-throughput meta-omics methods has provided great progress in improving the knowledge of the gut ecosystem and linking its biodiversity to host health conditions, offering complementary support to classical microbiology. Gut microbiota plays a crucial role in relevant diseases such as obesity or cardiovascular disease (CVD), and its regulation is closely influenced by several factors, such as dietary composition. In fact, polyphenol-rich diets are the most palatable treatment to prevent hypertension associated with CVD, although the polyphenol–microbiota interactions have not been completely elucidated. For this reason, the aim of this study was to evaluate microbiota effect in obese rats supplemented by hesperidin, after being fed with cafeteria or standard diet, using a multi meta-omics approaches combining strategy of metagenomics and metaproteomics analysis. We reported that cafeteria diet induces obesity, resulting in changes in the microbiota composition, which are related to functional alterations at proteome level. In addition, hesperidin supplementation alters microbiota diversity and also proteins involved in important metabolic pathways. Overall, going deeper into strategies to integrate omics sciences is necessary to understand the complex relationships between the host, gut microbiota, and diet.

1 Introduction

In recent years, omics sciences have been presented as a holistic solution to understanding the health and disease equilibrium by measuring a huge number of biomolecules at the same time. Although genomics, transcriptomics, proteomics, and metabolomics analyses allowed the discovery of relationships between genes, enzymes, and effectors, the combination of multiple omics strategies and bioinformatics tools to integrate different levels of regulation has definitively impulse systems biology and biomedical research by offering a comprehensive, structured, and interactive overview of biological mechanisms.

Nonetheless, for the study of very complex biological models, such as the gut microbiota, multi meta-omics approaches, such as metagenomics and metaproteomics, have emerged as very promising strategies in deciphering the biological relationship between the bacterial community and host system [1]. The gut microbiota is a complex and dynamic community of bacteria, fungi, viruses, and archaea that colonize the gastrointestinal tract. The gut microbiota has emerged as a fascinating “new organ” that plays multiple roles in the modulation of physiological host functions, homeostasis, disease, maintaining immune and metabolic responses, and protecting against pathogens [2–4].

In this context, metagenomics analyzes the genomic content of all microorganisms present in an ecosystem to characterize their biodiversity in each experimental condition, whereas metaproteomics focuses on the characterization of the whole microbial proteome to reveal which proteins or enzymes are up- or downregulated to better understand their functionality [5].

There are numerous factors that can influence the gut microbiota integrity, such as diet, lifestyle, antibiotics, and genetic background. Alterations in the gut bacterial composition can be associated with variations in some functional genes and metabolic activities, resulting in dysbiosis [6], which can contribute to the development of obesity, diabetes, or cardiovascular disease (CVD) and their associated complications, such as hypertension or dyslipidemia [7–9]. Among them, hypertension is the most prevalent risk factor for CVD, and its relationship with the gut microbiota and diet has already been reported [9].

Dietary supplements, or nutraceuticals, have been used extensively as a natural treatment for CVD and related diseases as they exhibit antioxidant, anti-inflammatory, or hypotensive effects [10–12]. In particular, hesperidin, a flavanone glycoside that is found abundantly in citrus fruits, is metabolized by the gut microbiota to its aglycone form, hesperetin [13, 14], which is known to have cardioprotective effects [15], and some studies already demonstrated its hypotensive effects in spontaneously hypertensive rats (SHR) [16, 17]. Roowi et al. [18] reported that phenolic acids excreted in urine after the ingestion of orange juice indicate that the hesperetin released through colonic bacteria-mediated deglycosylation is catabolized producing 3-hydroxyphenylhydracrylic acid, 3-hydroxyphenylacetic acid, 3-methoxy-4-hydroxyphenylhydracrylic acid, dihydroferulic acid, and 3-hydroxyhippuric acid. Other example is the transformation of quercetin and rutin to 3,4-dihydrophenylacetic acid, which is a metabolite of the neurotransmitter dopamine with anticancer, anti-inflammatory, cardioprotective, and neuroprotective properties [19]. Thus, phenolic acids or bacterial fermentation products from polyphenol compounds may exert some of the effects observed.

For this reason, in the present study, a multi meta-omics study is proposed combining metagenomics and metaproteomics for comprehensive evaluation of the role of the microbiota in modulating the effect of the flavanone hesperidin as a protective factor in hypertension generated by a high-caloric diet since the link between gut microbiota and host metabolism modulated by hesperidin to health outcomes still remains a challenge.

2. Materials and methods

2.1. Animals and diets

Twenty-four 8-wk-old male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA) were housed individually at 22°C with a light/dark cycle of 12 h and were given free access to food and water. After 1 wk of adaptation, animals were divided into two groups (n = 12) depending on the diet, cafeteria (CAF), or standard chow (STD) diet (Teklad Global 14% protein Rodent Diet 2014, Harlan, Barcelona), that they received for 9 wk. After this period, each diet group (n = 6) was supplemented either with low-fat condensed milk as a vehicle (VH, n = 6) or hesperidin dissolved with low-fat condensed milk (HESP 100 mg/kg, n = 6) for 8 wk. Body weight and food intake were measured weekly during the whole study, and systolic blood pressure (SBP) was recorded every week during treatment. Stool samples were obtained just before animals were sacrificed, frozen in liquid nitrogen, and stored at −80°C until analyses were performed.

The Animal Ethics Committee of the University Rovira i Virgili (Tarragona, Spain) approved the procedures.

2.2. Metagenomics analysis

2.2.1. DNA extraction and quality assessment

DNA was extracted from the stool samples using a QIAamp DNA stool mini kit (Qiagen Inc., Hilden, Germany) according to manufacturer's instructions. DNA purity and integrity were assessed using spectrophotometry (NanoDrop, Thermo Fisher Scientific, MA, USA).

2.2.2. 16S rRNA gene amplification and purification

Two regions (V3, V4) of the 16S rRNA gene were PCR amplified using the primer pair 341F-532R (5'-CCTACGGGRSGCAGCAG-3'; 5'-ATTACCGCGGCTGCT-3'), which targets the V3 region and the primer pair 515F-806R (5'-GTGCCAGCMGCCGCGGTAA-3'; 5'-GGACTACHVGGGTWTCTAAT-3'), which targets the V4 region. Coordinates are based on the 16S rRNA K-gene of Escherichia coli strain 12 substr. MG1655. These primers need to be redesigned to include each 5' adaptor sequence used in the preparation protocol of the Ion Torrent sequencing library and also a barcode sequence of ten bases for sample differentiation.

Each 12.5 µL PCR reaction mixture consisted of genomic DNA (50 ng), 6.75 µL AmpliTaq Gold 360 Master Mix (Applied Biosystems, CA, USA) and 0.75 µL of each primer (5 µM). PCR was performed in Veriti Thermal Cycler (Applied Biosystems) with the following cycling conditions: 5 min at 95°C, 30 cycles of 30 s at 95°C, 30 s at 55°C, and 90 s at 72°C, ending with 2 min at 72°C.

PCR products were analyzed using 2% agarose gel electrophoresis and further DNA band purification was performed with NucleoSpin (Macherey-Nagel, Berlin, Germany).

The Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA) and the associated Agilent DNA 7500 Reagent kit (Agilent Technologies) were used to determine the quality, length, and concentration of the libraries needed for the sequencing procedure. Once individual libraries were created, they were mixed in equimolar amounts.

2.2.3. Ion Torrent sequencing

The multiplexed mixture of 24 samples was diluted to a 60 pM DNA concentration prior to clonal amplification. The Ion 520 & Ion 530 Kit-Chef (Life Technologies, CA, USA) was employed for template preparation and sequencing according to the manufacturer's instructions. Prepared samples were loaded on a 530 chip and sequenced using the Ion S5 system (Life Technologies).

After sequencing, Ion Torrent Suit software removed the low quality and polyclonal sequences, and those reads were then analyzed using QIIME [20]. The analysis included operational taxonomic unit (OTU) clustering, alpha-diversity analysis, OTU analysis and species annotation (OTU table), and beta-diversity analysis. The OTU table, which gives the number of reads per sample per OTU, was used in the subsequent statistical analysis.

2.3. Metaproteomics analysis

2.3.1. Cell lysis and protein digestion

Approximately 300 mg of stool samples was subjected to differential centrifugation to collect microbial cells according to Tanca et al. [21] with minor modifications. Briefly, each stool sample was mixed with 10 mL PBS in a tube rotator for 45 min and subjected to low-speed centrifugation (500 g for 5 min), and the supernatant was collected. Then, it was ultracentrifuged at 20 000 g for 15 min (Ultracentrifuge,

Beckman-Coulter, CA, USA), and the obtained bacterial pellet was suspended in SDS-extraction buffer (2% SDS, 100 mM DTT, 20 mM Tris-HCl pH 8.8), incubated at 95ºC and subjected to a bead-beating process (Bullet Blender, Cultek, Barcelona, Spain) combined with freeze–thawing cycles for cell lysis and protein solubilization. Finally, proteins were purified by TCA/acetone precipitation before digestion.

Then, 50 µg of protein from each sample were reduced with 4 mM DTT for 1 h at 37ºC and alkylated with 8 mM iodoacetamide for 30 min at 25ºC in the dark, loaded onto a polyacrylamide gel (only staking process) to remove the detergents and digested overnight at 37ºC with trypsin (Promega, WI, USA) at an enzyme-to-protein ratio of 1:100.

2.4. Peptide 10-plex TMT labeling

Digested proteins were desalted on an HLB SPE column (Waters, Bedford, MA, USA) using 80% acetonitrile, 20% water, and 0.1% formic acid for elution and dried in a Speed-Vac concentrator before labeling with TMT 10-plex (Thermo Fisher Scientific) following the manufacturer's instructions. To normalize all the samples and TMT batches, a pool of all the samples was labeled with a 126-Tag and included in each batch. Then, the labelled peptides from each sample were mixed together and desalted on HLB SPE columns as was done before.

2.5. Peptide fractionation

Pooled samples were fractionated by isoelectric focusing on an Off-Gel fractionator from Agilent Technologies through 24-well IPG strips (nonlinear gradient from pH 3 to 10) according to the manufacturer's protocol. After fractionation, each of the 24 fractions was desalted

again on HLB columns (Waters) and resuspended in 50 µL of 0.1% formic acid for nanoLC-(Orbitrap) MS/MS analysis.

2.6. NanoLC-(Orbitrap) MS/MS analysis

The 96 fractions obtained from the Off-Gel fractionation (4 TMT × 24 fractions) were loaded on a trap nano-column (0.01 × 2 cm, 5 µm, Thermo Fisher Scientific) and separated on a C-18 reverse phase (RP) nano-column (0.0075 × 12 cm 3 µm, Nikkyo Technos Co. Ltd, Japan). Chromatographic separation was performed with a 90 min gradient with Milli-Q water (0.1% FA) and ACN (0.1% FA) as the mobile phase at 300 nL/min.

Mass spectrometry analyses were performed on a LTQ-Orbitrap Velos Pro (Thermo Fisher Scientific) by acquiring an enhanced FT-resolution spectrum (R = 30 000 FHMW) followed by two data-dependent MS/MS scan events (IT-(CID)MS/MS at 35% NCE and FT-(HCD)MS/MS (R = 15 000

FHMW at 40% NCE)) from the most intense ten parent ions with a charge state rejection of 1 and a dynamic exclusion of 0.5 min.

The 24 raw data files of each TMT plex were analyzed by multidimensional protein identification technology (MudPIT) using Proteome Discoverer software v.1.4.0.288 (Thermo Fisher Scientific). For protein identification, all the MS and MS/MS spectra were analyzed using the Mascot search engine (version 2.5) and was set up to search three different Swiss-Prot databases, as follows: (i) Rattus norvegicus (8003 sequences); (ii) in-house constructed diet database (35 993 sequences); and (iii) in-house metagenomics data base created from the metagenomics results at the family level and using the Uniref100 sequence identity to reduce the database size and avoid

false positive findings (27 381 845 sequences). Two missed cleavages were allowed assuming trypsin digestion and an error of 0.02 Da for the FT-MS/MS fragment ion mass, 0.8 Da for the IT-MS/MS fragment ion mass, and 10.0 ppm for the FT-MS parent ion mass. TMT10- plex on lysine and the N-termini were set as quantification modifications, while oxidation of methionine and acetylation of N-termini were set as dynamic modifications, and carbamidomethylation of cysteine was set as a static modification. The false discovery rate (FDR) and protein probabilities were calculated by a Fixed PSM validator.

For protein quantification, the ratios between each TMT label against the 126 TMT labels were used and normalized based on the protein median.

2.7. Statistical analysis

To find the significant metagenomic and protein changes between the different conditions under study, Mass Profiler Professional software v.14.5 (Agilent Technologies) was used. Data were Log2 transformed and mean centered for multivariate analysis (principal component analysis (PCA)) and univariate statistical analysis (Student's t-test).

3. Results and discussion

3.1. Cafeteria diet induces obesity and hypertension, and hesperidin decreases SBP in obese rats

In Sprague-Dawley rats, the CAF diet caused (i) a significant increase in body weight after 6 wk ($p < 0.001$); (ii) higher contents of body fat mass in the obese rat group at 8 wk as measured by NMR; (iii) adiposity index changes related to fat mass significantly ($p = 0.003$) increased between dietary groups in CAF-fed rats; (iv) and finally, a higher SBP in the rats fed with the CAF diet. All these results have been

reported before in similar studies, with rats fed either with the CAF diet or a high-fat diet [22–26].

SBP was measured weekly, and a decrease of 89% in obese rats treated with hesperidin was detected, equaling SBP levels of normal weight rats. The results clearly show that hesperidin reduces the SBP in the CAF group, which is in agreement with a previously published study that claims that hesperetin, the gut-related active metabolite of hesperidin, seems to be responsible for hypotensive activity in SHR [16]. Moreover, there is other evidence of the beneficial vascular effects of hesperidin in human trials [27, 28].

3.2. Gut microbiota was altered by CAF diet and hesperidin supplementation

3.2.1 Metagenomics approach: Microbial diversity analysis

To assess the impact of CAF diet and hesperidin consumption on gut microbiota composition, stool samples were collected to perform a metagenomics analysis.

The sequencing run produced a total of 13 156 718 reads that were reduced to 9 397 655 reads after quality filtering Those reads were then analyzed with QIIME, producing a total of 20°965 OTUs from the V3 and V4 regions of the 16S rRNA gene, which was used to summarize the relative abundance of the microbial clades at different taxonomic levels. The two phyla, Bacteroidetes and Firmicutes, dominate the community in all the samples with varying relative abundance, (25.7–61.1% and 29.1–71.7%, respectively), which is consistent with the published mammalian gut microbiome description [29].

The impact of diet in the microbial gut composition has already been reported [30]. In that way, a recent study showed an increase in the

relative abundance of Firmicutes and a decrease in abundance of Bacteroidetes in STD-fed rats when compared to CAF-fed rats [31]. However, in our study, the ratio of Bacteroidetes to Firmicutes (B/F) remained unaltered between the dietary groups (STD-VH 0.810; CAF-VH 0.814; p = 0.710). This lack of difference may be due to the short period of CAF diet intake but it must be noted that contradictory results have sometimes been found when relating the B/F ratio with pathological and dietary conditions [8, 32]. Therefore, the relevance of this ratio is debatable. This disparity can be caused by the significant variability between individuals, the different analytical methods employed to analyze the microbial composition (Real-Time PCR, Fluorescent in situ hybridization, 16S rRNA sequencing, among others), and/or by the different types of CAF-diet chow and the extent of CAF-diet study, among other factors.

In addition to the B/F ratio, the relative abundance of each phylum was also statistically compared between the dietary groups, but no significant differences were found. However, if the relative abundance was compared at the family level, some families were found to be differently distributed among the STD-VH and CAF-VH groups (Table 1). Most of these families came from Bacteroidetes (i.e., Prevotellaceae), which were downregulated in the STD-VH-fed group, whereas up- regulated families came from Firmicutes (Lachnospiraceae, among others). This result agrees with a recent study by our group [24], which found several families that were affected by the CAF diet in the same manner as in the present study. In addition to this, despite several works that have shown that obesity involves a reduction in the gut species richness [33], we did not find differences in the alpha-diversity values (chao1, Simpson and Shannon

values) between the STD-VH and CAF-VH groups, probably due to the short period of CAF-diet intake.

Table 1: Families significant either up or down abundant between groups. Metagenomics Analyses.

Family	CAF-VH (%)	STD-VH (%)	CAF-HESP (%)	STD-HESP (%)	Regulation	p-value
Aerococcaceae	0.050	0.008			up	0.020
Alcaligenaceae	2.577	0.160			up	0.000
Bifidobacteriaceae	0.159	0.010			up	0.015
Christensenellaceae	0.086	0.030			up	0.045
Enterobacteriaceae	0.780	0.148			up	0.007
Erysipelotrichaceae	0.587	0.021			up	<0.001
Lachnospiraceae	11.460	5.464			up	0.011
Micrococcaceae	0.027	0.002			up	0.001
Porphyromonadaceae	1.392	0.190			up	0.029
Prevotellaceae	3.320	8.787			down	0.041
Rikenellaceae	1.087	3.482			down	<0.001
Staphylococcaceae	0.013	0.001			up	0.011
Bacteroidaceae	13.288		22.983		up	0.031
RF16	0.002		0.001		down	0.031
Ruminococcaceae	13.603		5.758		down	0.029
Desulfovibrionaceae		0.148		0.051	down	0.030
Staphylococcaceae		0.001		0.002	up	0.032

When comparing the effect of hesperidin intake, both STD and CAF groups with and without hesperidin supple- mentation were analyzed. There was no significant change in the B/F ratio due to the effect of hesperidin in the STD groups (STD-VH 0.810; STD-HESP 1.184; p = 0.567) but a significant increase in the B/F ratio in the CAF group (CAF-VH 0.814; CAF-HESP 2.194; p = 0.021) was observed. According to this, an increase in the B/F ratio has already been reported to have beneficial effects on the reduction of SBP [34]. Therefore, our results suggest a possible role for hesperidin in rats fed a CAF diet, relying on the balance between the two major groups of intestinal bacteria. In fact, it has already been published that polyphenols also increase the B/F ratio [35]. This ratio has been implicated in the predisposition to

disease states [36]. Thus, dietary modulations with polyphenols may play a role in reshaping the gut microbial community to provide beneficial effects. However, as we have already mentioned, further studies are needed to confirm the role of the B/F ratio in maintaining a healthy, balanced state.

Apart from the B/F ratio, the relative abundance of each taxon was also compared. Both STD-diet groups did not show any differences in the microbiota diversity due to hesperidin supplementation at the phylum level and little significant differences were found at the family level, such as Desulfovibrionaceae from the phylum Proteobacteria, which is down-regulated in the no-hesperidin supplemented group. These results correlate with the lack of variation observed in SBP on both STD-fed rat groups, suggesting a minimum interplay between hesperidin action and microbiome composition in the normal blood pressure state. In that case, hesperidin intake did not promote either a decrease in SBP or a change in the microbial composition.

However, in the CAF-diet groups, significant changes in the Bacteroidaceae and Ruminococcaceae families were reported, supporting the increase in the B/F ratio when those rats were treated with hesperidin. The PCA in Fig. 1 shows the dissimilarity between the dietary groups based on the OTUs at the family. Groups are clearly separated due to significant differences between the relative abundance of several families. We show a decrease in the level of the Ruminococcaceae family accompanied by a decrease in SBP, but this result is not in accordance with a recent study with a different animal model that was reported to have a lower presence of the genus Ruminococcus in hypertensive mice compared with normal mice [37]. Otherwise, regarding what has been published in human models, we did not find significant differences in the Prevotella and Klebsiella

genus in the CAF-diet group before and after hesperidin treatment, although they have been found to be increased in hypertensive subjects [37]. In addition, although the gut microbial richness, diversity, and evenness had been reported to be decreased in hypertensive rats [38], we did not find differences in the alpha-diversity values (chao1, Simpson and Shannon values) among the CAF-diet groups.

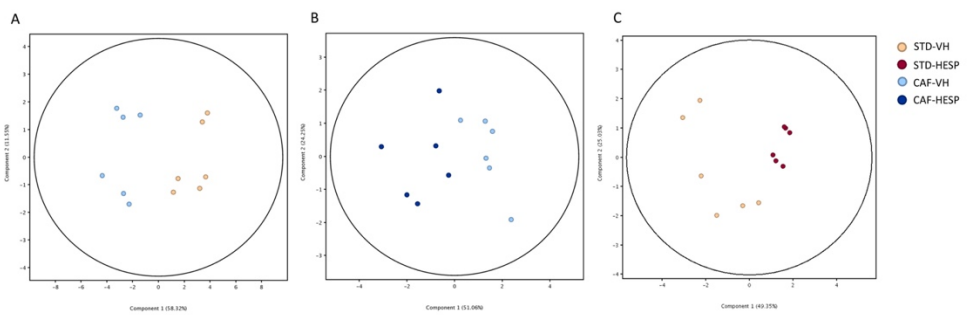

Figure 1. PCA on the family OTU abundance, showing the separation between the four dietary groups. The first two components areshown with the percent variance explained by them. The points correspond to the individual samples. (A) STD-VH versus CAF-VH, showing the effect of diet; (B) CAF-VH versus CAF-HESP, showing the effect of hesperidin supplementation in the CAF groups; (C) STD-VHversus STD-HESP showing the effect of hesperidin supplementation in the STD groups.

Our results suggest a complex relationship concerning the effect of hesperidin on the composition of the gut microbiota. In nonobese normal blood pressure animals, hesperidin did not promote a decrease in SBP nor a change in microbial diversity, whereas in obese, hypertensive rats, hesperidin promoted a reduction in SBP, reaching normal blood pressure values accompanied by changes in the microbiome composition. The decrease of SBP on obese rats treated with hesperidin could be the result of several factors, some related to microbiome composition and function and others to the described transport to cell nucleus of hesperidin/hesperetin fermentation metabolites [39] by its interaction with lipoproteins [40, 41].

Therefore, understanding nutrient–host–microbiome connections will offer new disease prevention and therapeutic strategies.

3.2.2. Metaproteomics reveal changes in the functional gut microbiota

To corroborate the hypothesis that the gut microbiota functionality is disturbed by diet and hesperidin, a metaproteomic approach was performed.

After 96 fractions were analyzed (corresponding to 24 samples), a total of 2824 bacterial proteins were identified. From them, 594 were selected as more confident by filtering the proteins present in a minimum of three samples in at least one of the four experimental conditions.

To understand the function of the whole gut microbiome, GO functions were attributed to each protein and the fold change of the 25 most abundant activities were represented as shown in Fig. 2D. As seen, mostly energy metabolic functions, such as the glycolytic process and glycogen biosynthesis, were altered when STD-VH and CAF-VH was compared.

To evaluate the differences caused by diet, the protein levels of the STD- and CAF-diet groups were compared. This resulted in 47 proteins with significantly different abundances, 17 of which were upregulated and 30 of which were decreased, represented in Fig. 2A and Table 2. Of these proteins, 40% were up or downregulated and were from the Lachnospiraceae family in the order Clostridiales, which was found to be increased in the metagenomics analysis.

Table 2: Proteins significantly altered between each pair of conditions. Metaproteomics analysis.

Compound	Function	Family	Regulation	p-value	
Phosphoserine aminotransferase n=1	AA metabolism	Clostridiaceae	down	0.050	a
Glycine/sarcosine/betaine reductase complex component C subunit beta n=2	AA metabolism	Lachnospiraceae	down	0.029	a
Glutamate dehydrogenase n=3	AA metabolism	Lachnospiraceae Clostridiaceae	down	0.049	a
sn-glycerol-3-phosphate import ATP-binding protein UgpC n=1	ATP/Energy metabolism	Acetobacteraceae	down	0.046	a
ATP-dependent 6-phosphofructokinase n=1	ATP/Energy metabolism	Eubacteriaceae	down	0.007	a
Glucose-1-phosphate adenylyltransferase. GlgD subunit n=1	ATP/Energy metabolism	Eubacteriaceae	down	0.036	a
Glucose-1-phosphate adenylyltransferase n=1	ATP/Energy metabolism	Lachnospiraceae	down	0.015	a
Glucose-1-phosphate adenylyltransferase subunit GlgD	ATP/Energy metabolism	Lachnospiraceae	up	0.029	a
Glucose-1-phosphate adenylyltransferase n=1	ATP/Energy metabolism	Ruminococcaceae	up	0.015	a
V-type ATP synthase alpha chain n=1	ATP/Energy metabolism	Ruminococcaceae	up	0.008	a
V-type H+-transporting ATPase subunit K n=2	ATP/Energy metabolism	Ruminococcaceae	down	0.026	a
ABC transporter. ATP-binding protein n=3	ATP/Energy metabolism	Ruminococcaceae Clostridiaceae	down	0.025	a
Flagellar motor protein MotA n=6	Bacteria	Bacteroidaceae	up	0.012	a
Flagellin n=1	Bacteria	Lachnospiraceae	down	0.043	a
Propanediol utilization protein PduA n=5	Bacteria	Lachnospiraceae Ruminococcaceae	up	0.043	a

Microcompartments protein n=1	Bacteria	Ruminococcaceae	up	0.010	a
Maltose/maltodextrin transport system ATP-binding protein n=1	CARB metabolism	Eubacteriaceae	down	0.028	a
Multiple sugar transport system ATP-binding protein n=1	CARB metabolism	Lachnospiraceae	down	0.018	a
Sugar transporter n=1	CARB metabolism	Rhodobacteraceae	up	0.044	a
Elongation factor G n=1	Celular division	Lachnospiraceae	down	0.049	a
Elongation factor Tu n=10	Celular division	Lactobacillaceae	up	0.023	a
Elongation factor Tu n=6	Celular division	Lactobacillaceae	up	0.031	a
Elongation factor Tu n=2	Celular division	Ruminococcaceae Clostridiaceae	down	0.021	a
Ethanolamine utilization protein eutM n=1	Phospholipids	Coriobacteriaceae	down	0.040	a
Glyceraldehyde-3-phosphate dehydrogenase n=1	Glycolysis	Lachnospiraceae	down	0.024	a
Glyceraldehyde-3-phosphate dehydrogenase. type I n=1	Glycolysis	Lactobacillaceae	up	0.036	a
Fructose-1 6-bisphosphate aldolase class II n=2	Glycolysis	Ruminococcaceae	down	0.017	a
Fructose-1 6-bisphosphate aldolase class II n=2	Glycolysis	Lachnospiraceae	down	0.007	a
Uncharacterized protein n=1	NON	Lachnospiraceae	down	0.009	a
Uncharacterized protein n=1	NON	Lachnospiraceae	up	0.008	a
Uncharacterized protein n=1	NON	Lachnospiraceae	down	0.018	a
Uncharacterized protein n=3	NON	Lachnospiraceae	down	0.035	a
Uncharacterized protein n=1	NON	Ruminococcaceae	up	0.028	a

Putative L-ribulose-5-phosphate 4-epimerase n=1	Pentose phospate pathway	Lachnospiraceae	up	0.017	a
FtsH protease regulator HflK n=4	Proteases	Lachnopiraceae Clostridiaceae	down	0.040	a
30S ribosomal protein S12 n=1	Ribosomal/Translation	Acetobacteraceae	up	0.044	a
30S ribosomal protein S11 n=1	Ribosomal/Translation	Bacteroidaceae	up	0.022	a
30S ribosomal protein S12 n=3	Ribosomal/Translation	Clostridiaceae	down	0.009	a
30S ribosomal protein S8 n=2	Ribosomal/Translation	Clostridiaceae	down	0.015	a
30S ribosomal protein S8 n=1	Ribosomal/Translation	Lachnospiraceae	down	0.014	a
Ribosomal protein S1 n=2	Ribosomal/Translation	Lachnospiraceae Ruminococcaceae	up	0.018	a
Tyrosine--tRNA ligase n=1	RNA	Clostridiaceae	down	0.021	a
Phosphoenolpyruvate carboxykinase (ATP) n=1	TCA	Bacteroidaceae	up	0.001	a
Pyruvate-flavodoxin oxidoreductase n=1	TCA	Eubacteriaceae	down	0.043	a
Formate acetyltransferase n=1	TCA	Lachnospiraceae	down	0.021	a
Pyrophosphate--fructose 6-phosphate 1-phosphotransferase n=1	TCA	Prevotellaceae	down	0.041	a
Pyruvate. phosphate dikinase n=1	TCA	Rhodobacteraceae	down	0.034	a
Enolase n=1	Glycolysis	Prevotellaceae	up	0.033	b
Enolase n=1	Glycolysis	Spirochaetaceae	up	0.036	b
Ribosomal protein L4/L1 family protein n=1	Ribosomal/Translation	Prevotellaceae	up	0.029	b
Uncharacterized protein n=1	NON	Clostridiaceae	down	0.010	b
Uncharacterized protein n=1	NON	Lachnospiraceae	down	0.030	b
Cell division protein FtsZ n=1	Celular division	Lachnospiraceae	down	0.048	c

Glucose-1-phosphate adenylyltransferase n=1	ATP/Energy metabolism	Lachnospiraceae	down	0.046	c
Glyceraldehyde-3-phosphate dehydrogenase n=1	Glycolysis	Clostridiaceae	up	0.042	c
NADH dehydrogenase subunit I n=1	ATP/Energy metabolism	Clostridiaceae	down	0.034	c

a) STD-VH vs CAF-VH;
b) CAF-VH vs CAF-HESP;
c) STD-VH vs STD-HESP.

Some of these changed proteins are involved in energy metabolism pathways, such as the tricarboxylic acid (TCA) cycle or ATP-binding pathways, while others participate in cell division. Similar results were found in studies performed with high-fat-diet-fed mice, but these results did not correlate these proteins with microbiota families and instead associated them with host alterations caused by the gut microbiota [42]. Another study showed that Firmicutes seems to be involved in coenzyme metabolism and transport in high-fat-diet-fed rats [43]. Since the majority of the significant proteins found belong to the Firmicutes phylum and they are downregulated in the CAF-VH group, this could indicate that the aforementioned biological processes are more active, which explains our findings in the context of these pathways.

Moreover, to contrast this information, Firmicutes appears to be most active in carbohydrate metabolism, whereas Bacteroidetes are more altered in several other activities, but it is important to comment that the functionality of Bacteroidetes looks to be underrepresented in databases because less is known about this phylum.

It is challenging to corroborate our metaproteomics findings with published works, as there are few studies assessing the microbiota

proteome in relation to the host. Nonetheless, a recent study was performed on the gut microbiota from obese children, which identified some differences in the NAD-dependent enzyme levels. This showed that the metaproteomes of the nonobese and obese group differed in a statistically significant way and demonstrated that there are considerable differences in the functionalities and supporting that the microbiota is active in energy metabolism in that specific obesity model [44]. This could support our results, but deeper knowledge and information is still needed.

Regarding the effect of hesperidin supplementation on SBP, only four (STD-VH vs. STD-HESP) and five (CAF-VH vs. CAF-HESP) proteins were significantly changed. The PCA in Fig. 2B,C shows the differences between groups based on altered proteins. In the standard groups, the altered proteins belong to the Lachnospiraceae and Clostridiaceae families, both from the Clostridiales order, but none of these families were found to have differences in terms of their abundance in the metagenomics approach. This could be explained by the fact that to not every family has a completed proteome in the databases. In addition to that, the microbiota function may be similar because of their proximity at the taxonomic level. However, the metaproteomic results obtained in the CAF groups correlate well with the metagenomic results, as the proteins altered belong to Lachnospiraceae, Clostridiaceae and Prevotellaceae. All of them are families from the Clostridiales and Bacteroidetes orders and have been identified in the metagenomics study.

Focusing on protein function, the four proteins identified in the STD groups are involved in metabolic pathways such as the TCA cycle or cell division, whereas the five proteins found in the CAF group are involved in glycolysis. It is remarkable that the protein that increased in

the STD-HESP group is 1-enolase, which is a glycolytic protein involved in several conserved functions in bacteria. 1-Enolase is also considered a moonlight protein, meaning it is a surface-localized bacterial protein that is related to the adhesion and modulation of the host immune response [45, 46]. This upregulated protein from the Clostridiales and Bacteroidetes orders could be responsible for the decrease in the SBP due to hesperidin action in the obese rat model.

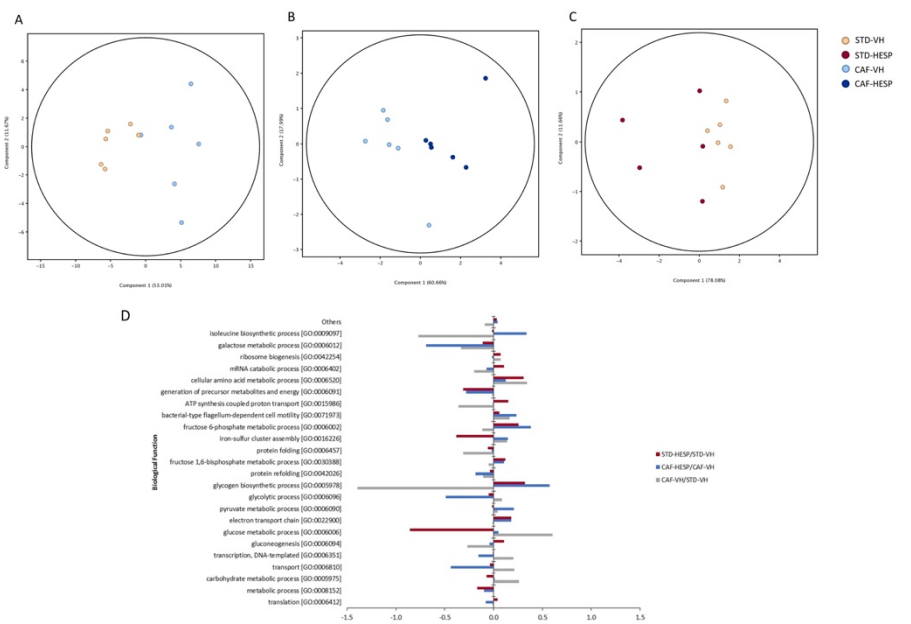

Figure 2. PCA on up- and downregulated proteins showing the separation between the four dietary groups. The first two components are shown with the percentage of variance that they explain. Points correspond to individual samples. (A) STD-VH versus CAF-VH, showing the effect of diet; (B) CAF-VH versus CAF-HESP, showing the effect of hesperidin supplementation in the CAF groups; (C) STD-VH versus STD-HESP showing the effect of hesperidin supplementation in the STD groups. (D) Fold changes of the protein content related to biological function (STD-HESP/STD-VH, CAF-HESP/CAF-VH, CAF-VH/STD-VH).

4. Concluding remarks

It is well known that the gut microbiota plays a significant role in obesity, and that its biodiversity is influenced by diet. Classic studies on phenotype profiling are restricted to the identification of microbial

diversity, where the molecular functionality of this community and its interaction with the host biology has gone unnoticed.

To overcome this lack of information, metaproteomics has emerged as a complementary approach to metagenomics. The advances in molecular separation methods coupled with mass spectrometry, as well as the development of proteome databases and bioinformatics tools, have all been crucial for novel, large-scale metaproteomic studies.

Here, we report the modulation of the microbiota in obesity by a diet-induced model where the CAF diet alters the gut microbiota composition and function, playing an important role in the final overweight phenotype. In addition, we report that hesperidin acts as a modulator of hypertension in obese rats, altering the gut microbiota balance that was reflected at both the metagenomic and metaproteomic levels.

It is worth mentioning that, although the modification of the gut microbiota composition by polyphenols has been reported before, there are remarkably few studies investigating their influence on the microbiota activity and to date, no metaproteomic approach has been performed deciphering the effects on functionality.

The current study sheds some light on the mechanism of action of polyphenols and represents significant progress in understanding polyphenol–microbiota interactions. However, although mechanistic insights are being generated, further detailed studies are still required to achieve a better understanding. In that sense, the combination of more than one omics science looks to be a promising strategy for studying the complex relations between the host, the gut microbiota, and diet.

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The authors have declared no conflict of interest.

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Manuscript 5

Protective effects of hesperidin against the Metabolic Syndrome induced by a cafeteria diet in rats

Maria Guirro, Andreu Gual-Grau, Albert Gibert-Ramos, Juan Maria Alcaide-Hidalgo, Núria Canela, Lluís Arola and Jordi Mayneris-Perxachs.

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Maria Guirro[1,2], Andreu Gual-Grau[2], Albert Gibert-Ramos[2], Juan Maria Alcaide-Hidalgo[3], Núria Canela[1], Lluís Arola[1,2,3], Jordi Mayneris-Perxachs[1,3,4,5]

[1] Eurecat, Centre Tecnològic de Catalunya, Technological Unit of Omics Sciences, Reus, Spain.

[2] Nutrigenomics Research Group, Department of Biochemistry and Biotechnology. Universitat Rovira i Virgili. Marcel.lí Domingo, 1. 43007. Tarragona, Spain.

[3] Eurecat, Centre Tecnològic de Catalunya, Technological Unit of Nutrition and Health, Reus, Spain.

[4] Department of Diabetes, Endocrinology and Nutrition. Girona Biomedical Research Institute (IdIBGi), Dr. Josep Trueta University Hospital, Girona, Spain.

[5] Center for Physiopathology of Obesity and Nutrition (CIBERobn), Instituto de Salud Carlos III, Madrid, Spain.

Abstract

Scope: Metabolic syndrome (MetS) has become a major public health problem and polyphenols such as hesperidin have been considered as potential candidates to MetS treatment ameliorating some of its major components. Our objective was to assess the impact of hesperidin supplementation in the components of diet-induced MetS in rats.

Methods and Results: Animals were fed either with a standard (STD) diet or a cafeteria (CAF) diet for 17 weeks and they were supplemented with vehicle (V) or two different doses of hesperidin (H1 or H2) for the final 8 weeks of the study. Urine and plasma metabolomics were performed and some biochemical and physiological parameters were assessed. CAF-fed animals developed MetS, characterized by obesity, insulin resistance, hypertension and dyslipidemia. Hesperidin supplementation in CAF-fed rats ameliorated blood pressure, insulin sensitivity and lipid metabolism. Metabolomics analyses also revealed an improvement of lipidomic profile and a reduction of oxidative- and inflammation-related metabolites and a decreased of serum amino acids were found.

Conclusion: Overall, our results suggest that hesperidin is a promising prebiotic to ameliorate MetS features.

1. Introduction

Metabolic syndrome (MetS) is defined as a clustering of interrelated cardio-metabolic risk factors, including abdominal obesity, insulin resistance (IR), hypertension, and dyslipidemia, which increases the risk of developing both cardiovascular disease (CVD) and type 2 diabetes (T2D) [1–3]. The MetS is highly prevalent worldwide (about 20-30% of all adults) and is rising in relation to the increasing incidence of obesity and T2D. Therefore, it has become a major public health concern and the seek for effective treatments is crucial [4]. Current guidelines point out therapeutic lifestyle modification, including diet and physical activity, as the first step in the management of MetS [1,5]. Despite a general agreement in reducing body weight and increasing physical activity, a consensus is lacking regarding the optimal diet [6]. However, epidemiological evidence highlights the importance of the consumption of fruits and vegetables. In particular, plant polyphenols have emerged as potential effective nutritional strategies to improve the health of patients with MetS due to their antioxidant, antiinflammatory, hipolipidemic, antiobesity, antidiabetic and antihypertensive properties [7–9]. Among the wide variety of poyphenols present in the diet, hesperidin, a flavanone glycoside found abundantly in citrus fruits, has shown a promising role against CVD due to their antihypertensive, lipid-lowering, hypoglycemic, antioxidant and anti-inflammatory properties [10–13].

Despite this, the mechanism by which hesperidin induce these benefits are far from being fully understood. In fact, the few intervention studies performed to date with pure hesperidin indicate a possible role of hesperidin in improving endothelial function, whereas the antihypertensive, lipid-lowering, antioxidant, and anti-inflammatory effects are inconsistent [14–17]. However, most of these studies have

assessed hesperidin effects using classical single biomarkers. Taking into account that polyphenols multiple biochemical targets and physiological actions [18], and most chronic diseases are multifactorial, these markers provide a reductionist and incomplete picture. Conversely, metabolomics, which involves the comprehensive study of complete profiles of small molecules in response to stimuli, provides an holistic picture and holds a gread potential for tackle the complex relationship between nutrition and health.

Importantly, the vast majority of polyphenols reach the colon intact where they are extensively metabolized by the gut microbiota into several lower molecular weight catabolites which may be responsible for the beneficial health effects, rather than the original compounds [19,20]. Many of these catabolites are absorbed in the colon and appear in blood and urine, merging with endogenous metabolites, thereby altering the metabolome and influencing host health. In turn, polyphenols can modulate gut microbial composition or functionality, affecting the release of microbioal-derived metabolites [21]. Because of this, metabolomics, has an inherent potential to elucidate the host-microbiome interplay and contribute to a better understanding of the underlying mechanims involved in polyphenol-derived health effects [23,24].

A CAF diet in rodents has been shown to be a robust model of human MetS [25]. We have previously shown that hesperidin supplementation in CAF-fed rats altered microbial diversity and functionality at proteome level [22]. Here, we report for the first time a comprehensive untargeted metabolomic approach combined with extensive biochemical measurements to study the effects and underlying mechanisms of different doses of hesperidin on rats fed a CAF diet. To this end, we measured the metabolic profiles of blood and urines

samples by ^{1}H nuclear magnetic resonance (NMR) and several markers of glucose and lipid metabolism, inflammation, endothelial function, arterial stifness and blood pressure.

2. Materials and Methods

2.1. Hesperidin source

Hesperidin were generously provided by NUTRAFUR S.A. (Murcia, Spain). Hesperidin was extracted from the fruit peel of *Citrus sinensis* (sweet orange), and the purity was 93% (HPLC).

2.2. Animals, diets and treatments

The Animal Ethics Committee of the University Rovira i Virgili (Tarragona, Spain) and the *Generalitat de Catalunya* approved all of the procedures (DAAM 4840). The experimental protocol followed the 'Principles of laboratory animal care', and was carried out in accordance to the European Communities Council Directive (86/609/EEC). Forty-eight 8-week-old male Sprague-Dawley rats (Charles River Laboratories, Barcelona, Spain) weighting 251 ± 2.5 g were housed individually at 22 °C under a light/dark cycle of 12 h (lights on at 09:00 am) and were given free access to food and water. After quarantine (1 week) rats were randomly distributed into two dietary groups (n = 24) and fed *ab libitum* with either a standard (STD) chow (Teklad global 18% protein, Envigo) or a cafeteria (CAF) diet for 10 weeks. The CAF diet included the following components: standard chow, bacon (8-12g), biscuits with pâté (12-15g), biscuits with cream cheese (10-12g), carrot (6-9g), muffins (4-5g), and 100 mL of milk with sugar (220g/L). The STD diet (3.1 kcal/g) contained 24% calories from protein, 18% from fat, and 58% from carbohydrates, whereas the

caloric distribution of the CAF diet was 10% protein, 41% fat and 49% carbohydrates.

At the beginning of 10th week, to determine whether the CAF-fed animals had developed MetS features, blood pressure and body composition analyses were carried out, and blood samples were obtained under fasting conditions (8h of diurnal fasting) by saphenous vein puncture. Afterwards, both STD and CAF rats were further divided in three groups (n=8) depending on the treatment received over the remaining 8 weeks: vehicle (V), low dose hesperidin (H1), or high dose hesperidin (H2). Therefore, two groups in each dietary regime were supplemented every day (at 9:00 h) with hesperidin dissolved in low-fat condensed milk diluted 1:1 at a dose of 40 mg/kg (STD-H1 and CAF-H1) or 100 mg/kg (STD-H2 and CAF-H2). The other groups received the same volume of low-fat condensed milk as the vehicle (STD-V or CAF-V). All treatments were administrated orally with a syringe of 1 mL. To ensure voluntary consumption, four days before the beginning of the treatments, rats were trained to lick low-fat condensed milk (0.2 mL).

At the beginning of the 18th week, blood samples were obtained by saphenous vein puncture as described above. Rats were also placed in in individual metabolic cages so as to collect 24-h urinary samples. Urine samples collected in a solution of 1% (wt/vol) sodium azide were filtered and kept at -80°C until analysis. One week later, rats were sacrificed under anaesthesia (pentobarbital sodium, 80 mg/kg body weight) after 8 of diurnal fasting. Blood was collected by cardiac puncture, and serum was obtained by centrifugation and stored at -20 °C until analysis. Liver, kidney, heart, brain, soleus and gastrocnemius muscles, cecum, and white adipose tissue depots including mesenteric (MWAT), retroperitoneal (RWAT), inguinal (IWAT) and epididymal

(EWAT), were collected, weighed and immediately frozen in liquid nitrogen. All the samples were stored at -80°C until further analyses.

2.3. Blood pressure measurement

Systolic blood pressure (SBP) was measured weekly in the rats from the 10th to the 18th week of the study by the tail-cuff method [27]. Before each analysis, the animals were kept at 32 °C for 15 min to allow dilatation of the blood vessels, making the pulsations of the tail artery detectable and also to calm the rats. Measurements were performed in a peaceful environment using the LE 5001 non-invasive blood pressure meter (Panlab; Harvard Apparatus, Barcelona, Spain), were carried out at the same time of day (between 03:00 pm and 05:00 pm) to avoid the influence of the circadian cycle, and, for each animal, were taken by the same technician throughout the study. The SBP values were determined as the average of at least 5 measurements. To acclimatize the animals to the procedure and to ensure the accuracy of the results, the animals were subjected to a training period of 2 weeks prior to testing (the 8th and 9th weeks of the study) in which the SBP measurements were performed every day.

2.4. Body composition analyses

Lean and fat mass measurements were performed without anaesthesia at the beginning of weeks 10 and 18 using an EchoMRI-700™ device (Echo Medical Systems, L.L.C., Houston, USA). The measurements were performed in triplicate and in *ad libitum* conditions at 8.00 am. Data are expressed in relative values as a percentage of body weight.

2.5. Serum analyses

Enzymatic colorimetric kits were used to determine the levels of triacylglycerols, glucose and total cholesterol (QCA, Barcelona, Spain),

HDL-cholesterol and LDL/VLDL-cholesterol (Bioassay systems, California, USA) and non-esterified free fatty acids (FFAs) (WAKO, Neuss, Germany) in the serum obtained by saphenous vein puncture at the beginning of weeks 10 and 18. Circulating insulin levels were measured using a rat/mouse ELISA kit (Millipore, Barcelona, Spain) at the same time points.

At the end point, serum monocyte chemoattractant protein-1 (MCP-1), vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) were determined with rat ELISA kits (Thermo Scientific, Illinois, USA). Neuraminidase (NA) enzymatic activity in serum was analyzed using the kit Amplex™ Red Neuraminidase (Sialidase) Assay Kit (Thermo Scientific, Illinois, USA).

2.6. HOMA-IR and R-QUICKI analyses

The homeostasis model assessment-estimated insulin resistance (HOMA-IR) was calculated following the formula: (Glucose *x* Insulin)/22.5. The insulin sensitivity was assessed using the revised quantitative insulin sensitivity check index (R-QUICKI) using the following formula: 1/[log insulin (μU/mL) + log glucose (mg/dL) + log FFA (mmol/l)].

2.7. ^{1}H nuclear magnetic resonance spectroscopy-based metabolic profiling.

Sample preparation. Urine and serum samples at week 18 were analyzed with the use of ^{1}H NMR spectroscopy. All urine samples were prepared by combining 400 μL urine with 200 μL phosphate buffer (pH 7.4; 100% D_2O) that contained 1 mM of the internal standard/L, 3-trimethylsilyl-1-[2,2,3,3-2H4] propionate (TSP), and 2mM sodium azide. All samples were mixed with the use of a vortex and centrifuged

(10,000 x *g*) for 10 min at room temperature before transfer to a 5 mm NMR tube.

For serum extraction, 200 µL of serum were lyophilized for 16h. The resulting pellet was resuspended by adding 1200 µL of methanol (CH_3OH), 400 µL of miliQ water, and 400 µL of chloroform ($CHCl_3$). The sample was vigorously vortexed for 1 min and sonicated for 20 min before adding 2 mL of $CHCl_3$ and 1 mL of miliQ water. The sample was vortexed again and centrifuged at 2400 x *g* for 20 min at 4°C. The upper layer (CH_3OH/water) containing the serum aqueous extract and the lower organic phase ($CHCl_3$/CH_3OH) containing the lipophilic extract were recovered separately and evaporated to dryness under N_2. The aqueous extract was reconstituted with 600 µL phosphate buffer (pH 7.4; 100% D_2O) that contained 1 mM TSP and 2mM sodium azide, whereas lipid extract was dissolved into 600 µL 0.01% tetramethylsilane (TMS) solution of 3:1 $CDCl_3$:CD_3OD.

NMR spectra were measured at a 600.20 MHz frequency using an Avance III-600 Bruker spectrometer equipped with a 5mm PABBO BB-1H/D Z-GRD probe. For urine samples, a standard one-dimensional (1D) NOESY presaturation pulse sequence (RD-90°-*t*1-90°-*t*m-90°-acquire, *noesypr1d*) was used with water suppression. A recycle delay (RD) of 5.0 s, a mixing time (tm) of 100 ms, and an acquisition time of 3.4 s, and a 90° pulse of 21.16 µs, were used for all samples. Four dummy scans were used to establish spin equilibrium, then 128 scans were collected into 64K data points with a spectral width of 16 ppm.

For serum aqueous extracts, a standard 1D NOESY presaturation pulse sequence (*noesypr1d*) was used. A RD of 5.0 s, a mixing time of 100 ms, an acquisition time of 3.4 s, and a 90° pulse of 10.02 µs, were used. For each aqueous extract, 4 dummy scans were followed by 256 scans and

collected in 64K data points with a spectral with of 16 ppm. In the case of lipophilic extracts, a 90° pulse with presaturation sequence (*zgpr*) was used. A RD of 5.0 s, a mixing time of 100 ms, an acquisition time of 2.94 s, and a 90° pulse of 9.92 µs, were used. After 4 dummy scans, a total of 256 scans were collected into 64K data points with a spectral with of 18.6 ppm.

NMR data processing of urine and plasma. NMR spectra were processed using the software TopSpin 3.5pl4 (Bruker Biospin, UK). An exponential line broadening of 0.3 Hz was applied before Fourier transform. Spectra were manually phased, baseline corrected and referenced to the chemical shift of TSP (0.0 ppm). ^{1}H NMR spectra (δ = -1.0-10.0) were digitized into consecutive integrated spectral regions with a resolution of 0.00034 ppm and 0.00025 ppm for urine and serum, respectively. In urine spectra, the water region between δ = 4.70 and 5.17 was removed to minimize baseline effects caused by imperfect water suppression. The urea region was also removed (δ = 5.60 - 6.0). In serum aqueous extracts, the regions between δ = 4.70 and 6.0 were removed, whereas in lipophilic extracts the water (δ = 4.44 – 5.20) region and the CH_3OH (δ = 3.26 – 3.44) and $CHCl_3$ (δ = 7.4 – 7.6) regions were removed. Urine NMR spectra were normalized using a probabilistic quotient approach [28] in order to compensate for differences in concentration between samples and all urine and serum spectra were aligned using a recursive segment-wise peak alignment (RSPA) method [29].

Metabolite identification. Metabolite identification was carried out using information from the literature and public databases (Chenomx NMR Suite, Human Metabolite DataBase, Biological Mangetic Resonance Data Bank). Two-dimensional (2D) NMR experiments

(COSY, TOCSY, HSQC) were acquired for a number of samples to assist or confirm metabolite identification.

2.8. Univariate statistical analysis

Univariate analyses were performed using IBM SPSS Statistics 25.0 (SPSS, IBM Corp. Armonk, New York, USA). Grubbs' test was used to detect outliers, which were discarded for subsequent analyses. The assumption of normality was determined using the Kolmogorov-Smirnov test, and the homoscedasticity among groups was assessed using Levene's test. When one or both of these conditions were not met, data were transformed to a base-10 logarithm to obtain a normal distribution and/or similar variances before statistical testing. Differences in the anthropometric and biochemical parameters between rats fed the STD and CAF diets after 9 weeks were detected using a Student's *t* test. Differences between groups at the end of the study (week 18) were assessed using a general linear mixed model, including diet (*D*) and treatment (*T*) and their interaction (*D*x*T*) as fixed factors, followed by the Tukey's post-hoc test. For parameters measured at multiple times, such as SBP, a linear mixed model followed by the Sidak post-hoc test was used. Fixed effects included diet (*D*), treatment (*T*), time (*t*), and their corresponding interactions. The subjects were included as a random factor and an unstructured covariance matrix was employed. When significant interactions were observed, slice tests were used to determine the effects within the interaction levels. Data are presented as the means ± SEM (n = 8). The level of statistical significance was set at bilateral 5%.

2.9. Multivariate statistical analysis

Multivariate modeling was performed in MATLAB with the use of in-house scripts. Initially, principal component analysis (PCA) of the NMR

spectra was performed using pareto scaling to visualize patterns and outliers within the data set. This was followed by orthogonal projection to latent structures discriminant analysis (OPLS-DA). This approach was used for pair-wise comparisons between the study groups to identify discriminatory metabolites between groups. Correlation coefficients plots were generated with the use of back scaling transformation to display the contribution of each metabolite to the sample classification. The color scale represents the significance of a correlation for each metabolite to the class membership with red indicating strong significance and blue indicating weak significance. The predictive performance (Q^2Y) of the models was calculated using a 7-fold cross-validation approach. The significance of the OPLS-DA Q^2Y values was assessed through permutation testing (1000 permutations).

Clustering analysis. Unsupervised hierarchical clustering analysis (HCA) was performed to identify general patterns of biochemical and microbial variations between samples. For metabolome data, unsupervised clustering for all samples was performed with the use of the normalized abundance of metabolites that were identified through the OPLS-DA models. Metabolites that were identified as contributing to the separation between groups through the OPLS-DA models were used for sample clustering. For comparison purposes, only metabolites that were present in all diets were used. For a comparative analysis across different metabolites data were standardized as Z scores across samples for each metabolite before clustering so that the mean was 0 and the SD was 1. This standardized matrix was subsequently used in unsupervised HCA for samples and metabolites with the use of correlation-based distances (Pearson correlation) from which hierarchical clusters were generated with the use of an average-linkage method by means of the pdist and linkage functions in the

MATLAB bioinformatics toolbox. Heat maps and dendrograms after the HCA were generated with MATLAB images and dendrogram functions, respectively. In the heat maps, a red-blue color scale was used whereby shades of red and blue represented higher and lower values, respectively, compared with the mean. Different diet groups were color coded and shown under the dendrogram for each sample.

3. Results

3.1. CAF intake triggered MetS-like alterations.

As expected, after 10 weeks of diet, CAF-fed animals had already developed a MetS phenotype (Table 1), characterized by increased body weight and fat mass, hypertriglyceridemia, hyperglycaemia, and elevated SBP. They also had higher levels of TC and FFA and lower relative lean mass (Table 1). These deleterious effects could be attributed to the increase energy intake and decreased cumulative protein intake observed in these animals compared to STD-fed rats. At the end of the study (week 18th), CAF-fed had the same alterations in the previous parameters (Figures 1-3). They also had a higher adiposity, elevated insulin concentrations, lower insulin sensitivity and insulin resistance (Figure 2). Finally, CAF feeding also resulted in increased levels in markers of endothelial dysfunction (ICAM-1), arterial stiffness (Neuraminidase), and inflammation (MCP-1) (Figure 3).

Table 1. Body composition and dietary and biochemical parameters of rats fed with a standard (STD) or a cafeteria (CAF) diet at the 10th week of the study.

	STD (n=24)	CAF (n=24)	*P*
Body composition			
Weight (kg)	422.2 ± 5.2	495.1 ± 9.4	<0.001
Weight change (kg)	170.0 ± 3.9	245.7 ± 10.3	<0.001
Fat (%)	6.3 ± 0.4	15.5 ± 1.1	<0.001
Lean (%)	88.5 ± 0.5	80.0 ± 1.1	<0.001
Dietary parameters			
Cumulative Energy intake (kcal)	681.8 ± 8.1	1411.3 ± 28.3	<0.001
Cumulative Protein (g)	40.9 ± 0.5	36.2 ± 0.8	<0.001
Cumulative Carbohydrates (g)	97.2 ± 1.2	209.6 ± 4.4	<0.001
Cumulative Fat (g)	13.6 ± 0.2	47.1 ± 1.5	<0.001
Cumulative Fibre (g)	7.7 ± 0.1	4.4 ± 0.2	<0.001
Cumulative SFA (g)	2.0 ± 0.0	18.9 ± 0.5	<0.001
Cumulative MUFA (g)	2.9 ± 0.0	18.1 ± 0.7	<0.001
Cumulative PUFA (g)	7.5 ± 0.1	10.2 ± 0.6	<0.001
Cholesterol (g)	-	0.22 ± 0.05	<0.001
Biochemical parameters			
TC (mg/dL)	87.9 ± 2.1	98.0 ± 3.3	0.013
TG (mg/dL)	102.6 ± 9.6	277.2 ± 17.8	<0.001
NEFA (mmol/L)	0.61 ± 0.02	0.82 ± 0.04	<0.001
Glucose (mmol/L)	7.75 ± 0.17	9.10 ± 0.20	<0.001
SBP (mmHg)	128.7 ± 1.1	143.6 ± 1.9	<0.001

3.2. Hesperidin supplementation had no effect on body weight or body composition

Hesperidin supplementation could not prevent weight gain in CAF-fed rats. Therefore, no significant changes in body weight, fat and lean mass percentages and adiposity index were observed after hesperidin supplementation in CAF-fed rats compared to those supplemented

with the vehicle (Figure 1). Similar results were observed in STD-fed rats (Figure 1).

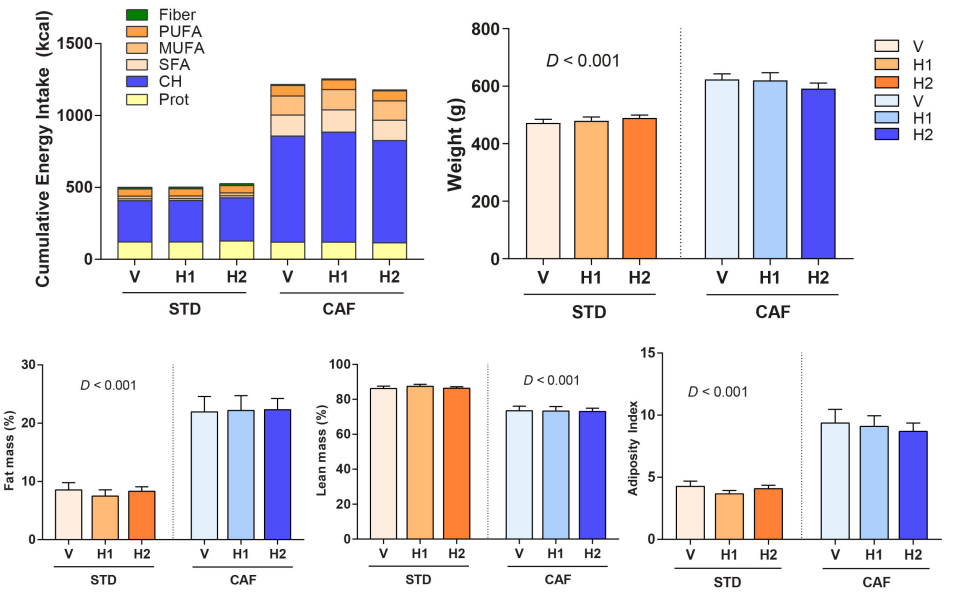

Figure 1. End point (week 18) cumulative energy intake, weight, fat mass, lean mass, and adiposity index of rats that were fed with a standard (STD) or a cafeteria (CAF) diet and received a daily oral dose of vehicle (V), hesperidin at 40 mg/kg (H1), or hesperidin at 100 mg/kg (H2), for the last 8 weeks. Data are given as means ± SEM. *D*: Diet effect.

3.3. Hesperidin supplementation improved the lipid profile in both STD and CAF-fed rats

Hesperidin supplementation in CAF-fed rats resulted in healthier lipid profiles in a dose-dependent manner. Therefore, CAF-H1 and CAF-H2 rats had lower TC and LDL-C levels compared to CAF-V rats, although no differences were observed in the HDL-C concentrations (Figure 2). The same trend was observed in the LDL-C levels of STD-fed rats. Hence, hesperidin supplementation in both dietary regimens improved the lipid profile. In addition, hesperidin supplementation decreased the circulating levels of FFA in CAF-fed rats, but not in STD-fed rats. No changes in the TG concentrations were observed after hesperidin

supplementation in either STD- or CAF-fed animals compared to their respective controls.

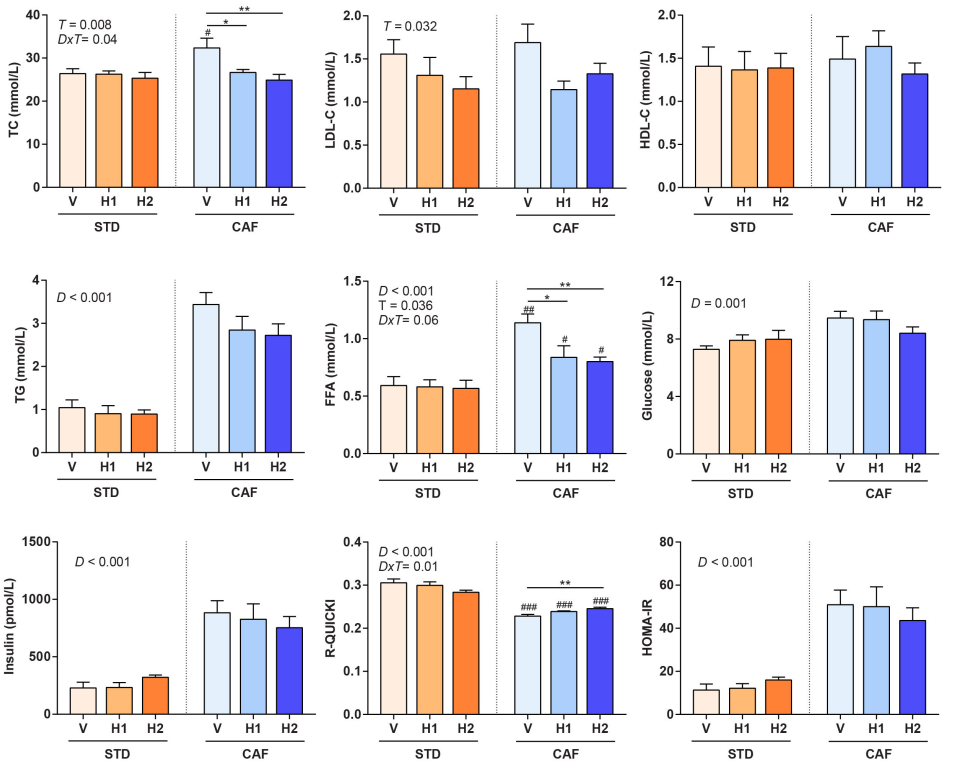

Figure 2. End point (week 18) lipid and glucose metabolism biochemical parameters of rats that were fed with a standard (STD) or a cafeteria (CAF) diet and received a daily oral dose of vehicle (V), hesperidin at 40 mg/kg (H1), or hesperidin at 100 mg/kg (H2), for the last 8 weeks. Data are given as means ± SEM. *D*: Diet effect; *DxT*: diet-treatment interaction; *T:* treatment effect. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ *vs* the corresponding supplementation group in the STD diet model. * $P < 0.05$, ** $P < 0.01$ vs the V group in the same dietary model. FFA, free fatty acids; HOMA-IR, Homeostatic model assessment of insulin resistance; R-QUICKI, revised quantitative insulin sensitivity check index; TC, total cholesterol.

3.4. Hesperidin supplementation increased insulin sensitivity in CAF-fed rats

Hesperidin supplementation had no effect on any of the glucose metabolism parameters (glucose, insulin, R-QUICKI, HOMA-IR) in STD-

fed rats (Figure 2). No significant changes were also observed in glucose, insulin levels or HOMA-IR in CAF-fed rats supplemented with hesperidin compared to their respective controls. However, CAF-H2 showed higher insulin sensitivity, as measured by the R-QUICKI index, compared to CAF-V rats, which is in line with the decrease in FFA observed in these animals due to their relationship with insulin resistance.

3.5. Hesperidin supplementation decreased systolic blood pressure in CAF-fed rats

A linear mixed model analysis showed a significant *time* (p<0.001) and *diet* effects (p<0.001) and significant *time x diet* (p=0.004) and *time x treatment x diet* (p=0.05) interactions (Figure 3). Therefore, slice tests were conducted at each time and diet. They showed that in the highest dose of hesperidin (H2) in CAF reduced SBP compared to CAF-V rats after six (130.3 ± 2.3 vs 140.7 ± 2.4, p = 0.010), seven (129.1 ± 2.7 vs 140.1 ± 2.9, p = 0.026), and eight (126.3 ± 2.6 vs 140.7 ± 2.7, p=0.001) weeks of supplementation. Although, the lower dose of hesperidin (H1) did no show significant differences compared the vehicle, there was a clear trend towards reducing SBP and it is likely that a longer supplementation period could have achieved significant results.

3.6. Effect of hesperidin supplementation on vascular function in CAF-fed rats.

After eight weeks of supplementation, no significant differences were observed in the circulating concentrations of markers of endothelial dysfunction (ICAM-1 and VCAM-1) among groups in any dietary regime (Figure 3). However, hesperidin supplementation considerably reduced arterial stiffness, as measured by the circulating Neuraminidase levels, in a dose-dependent manner. In addition, CAF-H2 rats had lower levels

of MCP-1, an inflammation marker, compared to CAF-H1 and CAF-V rats.

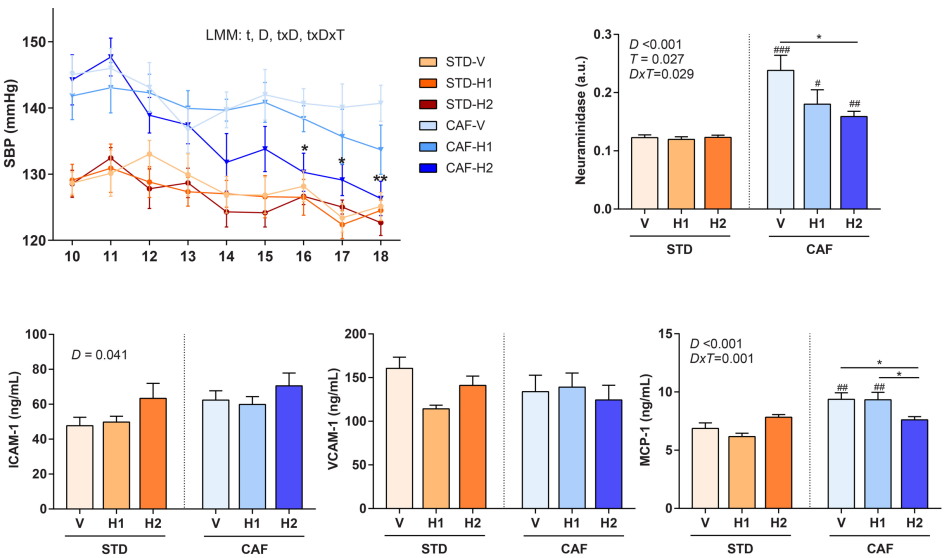

Figure 3. Systolic blood pressure (SBP) and end point (week 18) endothelial function parameters of rats that were fed with a standard (STD) or a cafeteria (CAF) diet and received a daily oral dose of vehicle (V), hesperidin at 40 mg/kg (H1), or hesperidin at 100 mg/kg (H2), for the last 8 weeks. Data are given as means ± SEM. LMM: linear mixed model; *D*: Diet effect; *DxT*: diet-treatment interaction; *T:* treatment effect, *t*: time effect. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ *vs* the corresponding supplementation group in the STD diet model. * $P < 0.05$, ** $P < 0.01$ *vs* the V group in the same dietary model.

3.7. Hesperidin supplementation improved the urinary metabolic profiles of CAF-fed rats in a dose dependent manner

Pairwise OPLS-DA models were built to compare metabolic profiles of rats fed either a STD diet or a CAF diet and supplemented with the vehicle. Significant models with good predictive abilities were obtained for the comparison of the urinary metabolic profiles (Q^2Y = 0.81, P <0.001), the serum aqueous metabolic profiles (Q^2Y = 0.58, P <0.001), and the serum lipid metabolic profiles (Q^2Y = 0.81, P <0.001). The metabolic alterations induced by a CAF diet compared with a STD

diet are shown in the OPLS coefficients plot for this model (Supplementary figure 1) and are summarized in Figure 4. Following a CAF-V diet, the urinary excretions of citrate, glycerophosphocholine (GPC), sucrose, *N*-methyl-4-pyridone-3-carboxamide (4-PY), and metabolites related to inflammation and oxidative stress (*N*-acetylglycoproteins [NAG], 2'-deoxycitidine) were increased compared to a STD-V diet. However, gut microbial-host co-metabolites (Hippurate, phenylacetylglycine [PAG], 2-phenylacetamide), branched chain amino acid (BCAA) catabolism intermediates (2-oxoisocaproate [2-OIC]), taurine and *N*-methylnicotinic acid (NMNA) were excreted in lower amounts. This lower urinary excretion of BCAA catabolites was in agreement with the higher plasma levels of BCAA (valine, leucine, isoleucine) and branched chain keto acids (BCKA) (3-methyl-2-oxovalerate [2-MOV]) observed in CAF-V compared to STD-V rats. Plasma levels of other amino acids (proline, threonine), TCA cycle intermediates (citrate, succinate), as well as acetate, creatine, carnitine, GPC, glyceraldehyde-3-phosphate (G3P), and glucose, were also in higher in CAF-V, but they had lower lactate levels. As expected, the most significant differences were found in the levels of lipids, which were increased in CAF-V as compared to STD-V rats. The former rats had higher levels of TC, free cholesterol (FC), esterified cholesterol (EC), FFA, diglycerides (DG), triglycerides (TG), phospholipids (PL), phosphocholines (PC), MUFA, PUFA, linoleic acid (LA), alpha linolenic acid (ALA), EPA, and DHA.

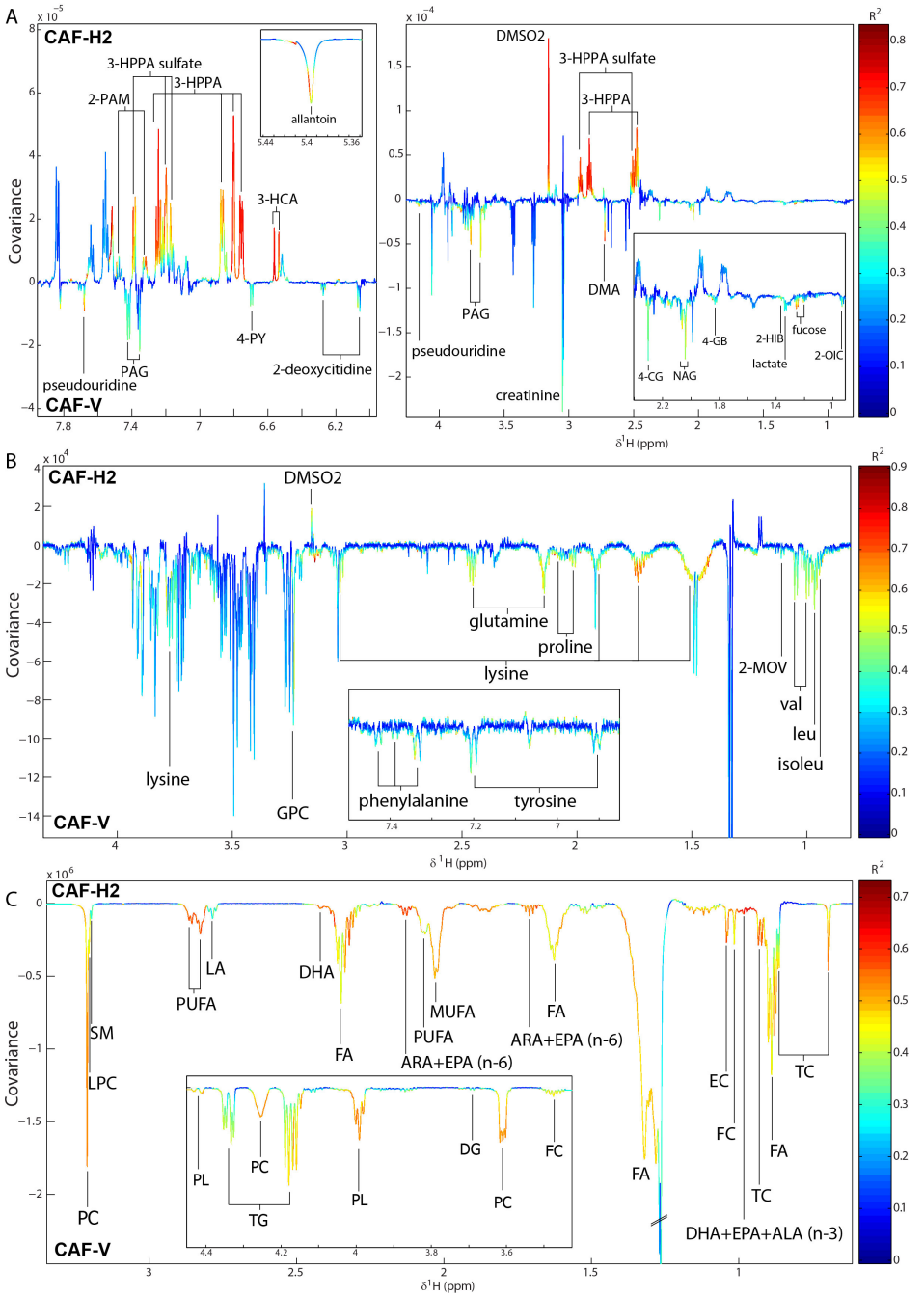

Figure 4. Orthogonal projection to latent structures-discriminant analysis (OPLS-DA) coefficients plot comparing the (A) urine metabolic profiles, (B) the serum aqueous metabolic profiles, and (C) the serum lipid metabolic profiles of CAF-fed rats supplemented with either the vehicle (V) or hesperidin at 100 mg/kg (H2). The colour scale represents the significance of the correlation for each metabolite to the class membership with red indicating strong significance and blue indicating weak

significance. 2-HIB, 2-hydroxyisobutyrate, 2-MOV; 3-methyl-2-oxovalerate; 2-OIC; 2-oxoisocaproate; 2-PAM, 2-phenylacetamide; 3-HCA, 3-hydroxycinnamic acid; 3-HPPA, 3-hydroxhyphenylpropionic acid; 4-CG, 4-cresol glucuronide; 4-GB, 4-guanidinobutanoate; ALA, α-linolenic acid; DG, diglycerides; ARA, arachidonic acid; DHA, docosahexaenoic acid; DMA, dimethylamine; DMSO2; dimethylsulfone; EC, esterified cholesterol; EPA, eicosapentaenoic acid; FA, fatty acids; FC; free cholesterol; GPC; glycerophosphocholine; LA, linoleic acid; LPC, lysophosphatidylcholines; MUFA, monounsaturated fatty acids; NAG, *N*-acetylglycoproteins; PAG, phenylacetylglycine; PC, phosphatidylcholines; PL, phospholipids; PUFA, polyunsaturated fatty acids; SM, sphingomyelins; TC, total cholesterol, TG, triglycerides.

Regarding to hesperidin supplementation in STD-fed rats, it only induced significant changes in the urinary metabolic profiles compared to the vehicle (Supplemental table 1, supplemental Figures 2 and 3, and Figure 4). The most significant difference was a much higher excretion of Hippurate and dimethyl sulfone (DMSO2) by H1 or H2 supplemented rats compared to the V. In addition, STD-H2 rats excreted lower amounts of inflammatory metabolites (NAG, fucose), acetamide, 2-OIC, creatinine, but higher amounts of 3-hydroxyphenylpropionic acid (3-HPPA) and 3-hydroxycinammic acid (3-HCA) compared to STD-V rats.

In the case CAF-fed rats, hesperidin supplementation induced significant alterations in both the urinary and plasma metabolic profiles (Supplementary table 1). Similar to STD-H1 rats, CAF-H1 rats excreted higher urinary amounts of Hippurate, DMSO2, 3-HPPA, and 3-HCA than CAF-V rats. They also excreted higher amounts of 3-HPPA sulfate, but lower amounts of dimethylamine (DMA), fucose, 2-oxoisovalerate (2-OIV), 2-hydroxyisobutyrate (2-HIB), and 4-guanidinobutaonic acid (4-GB) compared to CAF-V rats (Supplementary figure 4 and Figure 4). These metabolic alterations were enhanced after supplementation with H2. Hence, CAF-H2 rats

also excreted lower amounts of metabolites related to inflammation (fucose, NAG) and oxidative stress (2-deoxycitidine, pseudouridine, allantoin), uremic toxins (4-cresol glucuronide [4-CG], DMA), guanidino compounds (creatinine, 4-GB), PAG, and 4-PY (Figure 3 and 4). Supplementation with H2 also had a stronger influence on the serum metabolic profiles. While H1 supplementation only had significant effect on the serum lipid profile, H2 supplementation modulated both the lipid and aqueous serum profiles (supplementary table 1). H2 supplementation in CAF rats reverted some of the metabolic alterations observed between CAF and STD rats. Therefore, CAF-H2 rats had lower levels of BCAA, 2-MOV, proline, GPC, TC, FC, EC, FFA, DG, TG, PL, PC, MUFA and PUFA (Figure 5), all of them metabolites that were increased in CAF rats compared to STD rats. CAF-H2 rats also had lower levels of other amino acids such as lysine, glutamine, proline, phenylalanine, and tyrosine, as well as lysophosphocholines (LPC) and sphingomyelins (SM).

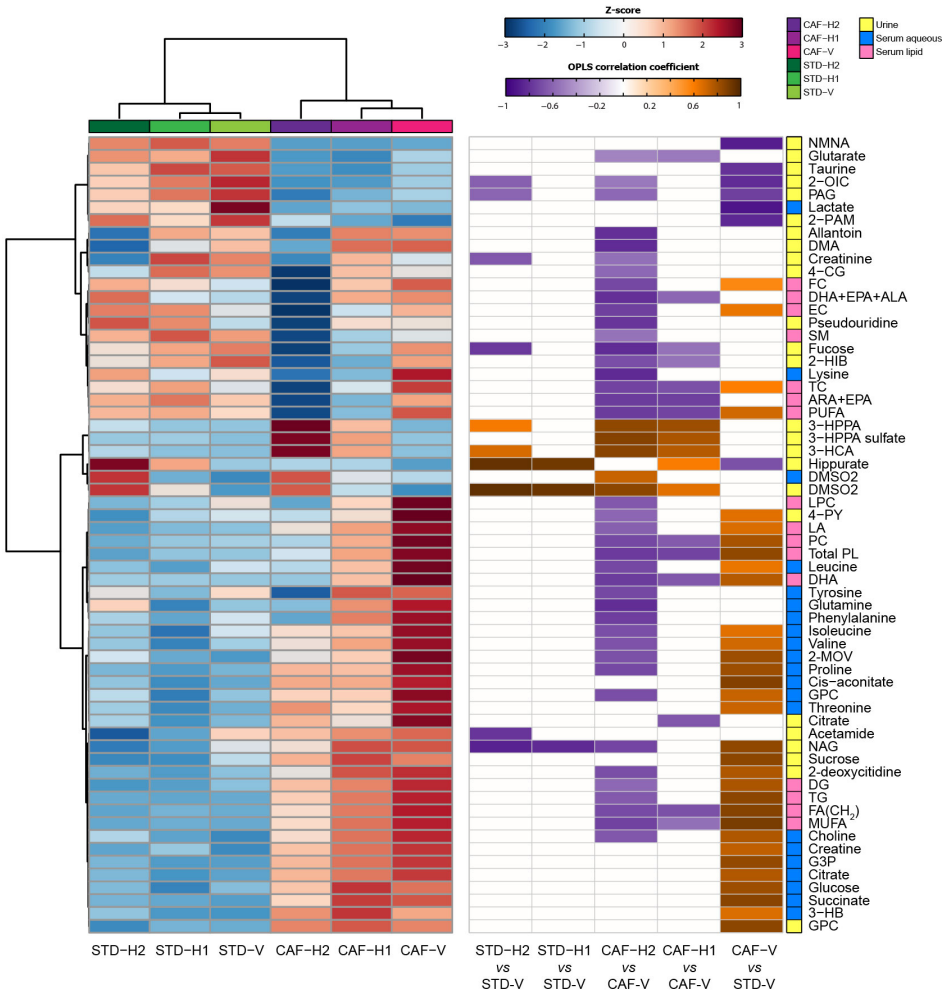

Figure 5. Summary of the diet- and hesperidin-induced metabolic alterations identified by the OPLS-DA models. (Left) Dendrogram and metabolite heatmap displaying the group averages. A metabolite z-transformation was performed on the intensity of each metabolite across samples. Metabolites and groups were clustered by Euclidean distance and Ward linkage hierarchical clustering. (Right) Significant OPLS-DA correlation coefficients associated with the indicated pairwise models. 2-HIB, 2-hydroxyisobutyrate, 2-MOV; 3-methyl-2-oxovalerate; 2-OIC; 2-oxoisocaproate; 2-PAM, 2-phenylacetamide; 3-HCA, 3-hydroxycinnamic acid; 3-HPPA, 3-hydroxhyphenylpropionic acid; 4-CG, 4-cresol glucuronide; 4-GB, 4-guanidinobutanoate; ALA, α-linolenic acid; DG, diglycerides; ARA, arachidonic acid; DHA, docosahexaenoic acid; DMA, dimethylamine; DMSO2; dimethylsulfone; EC, esterified cholesterol; EPA, eicosapentaenoic acid; FA, fatty acids; FC; free cholesterol; GPC; glycerophosphocholine; LA, linoleic acid; LPC,

lysophosphatidylcholines; MUFA, monounsaturated fatty acids; NAG, N-acetylglycoproteins; PAG, phenylacetylglycine; PC, phosphatidylcholines; PL, phospholipids; PUFA, polyunsaturated fatty acids; SM, sphingomyelins; TC, total cholesterol, TG, triglycerides.

4. Discussion

The MetS is highly prevalent in Western societies. However, due to globalization and the widespread of western diet, both MetS prevalence and incidence are rapidly increasing in developing countries [1]. Therefore, it has become a major global public health problem and a profound burden for national health care systems. Importantly, hesperidin has emerged a promising therapeutic agent for the treatment of the MetS due to its wide range of biological properties and constituting 90% of the flavanones oranges, the most important fruit tree crop in the world [10–12]. Here, we coupled exhaustive biochemical measurements and non-targeted comprehensive metabolomics with multivariate statistical analysis to explore underlying mechanims of the effects of hesperidin on rats fed a CAF diet, which a robust model of MetS. We showed that hesperidin supplementation in CAF-fed animals improved dyslipidemia, insulin sensitivity, hypertension, vascular function and inflammation. Metabolomics analyses revealed that these changes were accompanied by decreased levels of several serum amino acids, decreased excretion of oxidative stress- and inflammation-related metabolites and a significant improvement of the lipidomic profile.

One clinical features of MetS is central obesity, as it usually precedes the emergence of other MetS risk factors [2,3]. As expected, a CAF feeding resulted in increased body weight, fat mass and adiposity index compared to a STD diet. Obesity also plays a main role in the development of oxidative stress and low-grade inflammation that are

associated to the MetS [30,31]. Consistently, CAF-fed rats displayed higher levels of MCP-1, a molecule widely recognized as the major component of chronic inflammation, compared to STD-fed animals. In addition, metabolomics analyses revealed that the former animals also had higher levels of metabolites related to inflammation and oxidative stress. Interestingly, one of the main characteristics of polyphenols is their anti-inflammatory effect associated with their antioxidant capacity and enhancement of the levels of antioxidant enzymes [32]. Consequently, hesperidin supplementation improved the inflammatory and oxidative status of CAF-fed rats as evidenced by the decreased excretion of NAG, fucose, 2-deoxycitidine, pseudouridine, and allantoin. A protective role of fucose has been suggested in gut-centered and systemic inflammation [33], whereas NAG have recently emerged as useful biomarkers of systemic acute and chronic inflammation [34]. Pseudouridine and 2-deoxycitidine have been used as biomarkers of oxidative stress of DNA and RNA, respectively [35]. Allantoin, the end product of purine catabolism, has also been associated with inflammation and oxidative stress. Humans lack the enzyme uricase that converts uric acid to allantoin, so that uric acid is the final compound of purine catabolism [36]. Importantly, several studies have reported a tight relationship between uric acid and MetS [2].

Both obesity-related oxidative stress and low-grade inflammation seem to play a pivotal role in the pathogenesis of IR [37,38], which is the other central clinical feature of MetS and triggers its development. FFA have also shown to play a substantial role in the onset of IR [2]. In agreement with these findings, a CAF feeding lead to an impairment in glucose and insulin homeostasis and increased levels of FFA compared to a STD feeding, which resulted in the development of IR and

decreased insulin sensitivity. Therefore, it is likely that the improvement in oxidative and inflammatory status of CAF-H2 rats may have contributed partly to the significant decrease in serum FFA and an increase in insulin sensitivity compared to CAF-V rats. In line with our results, treatment of MetS patients with hesperidin caused a trend towards an improvement of IR [39].

Obesity and IR have also been recognized as the leading cause of MetS associated comorbidities, such as hypertension [40,41] or dyslipidemia [42]. Hypertension is by far the most prevalent individual MetS component, being present in about 85% of the MetS patients [3]. In agreement with our results, the antihypertensive effects of hesperidin have been previously described in spontaneously hypertensive rats [64,65] and recently in rats fed a high-fat, high-sucrose diet [66]. Diminished insulin sensitivity activates the sympathetic nervous and renin-angiotensin-aldosterone systems, which increases sodium reabsorption, eventually promoting hypertension [41]. FFA also seem to play a possible important role in hypertension development in patient with obesity and MetS [40]. Interestingly, SBP reduction in CAF-fed rats after hesperidin supplementation was accompanied by an increase in insulin sensitivity and a reduction in FFA. Accumulating evidence indicates that both IR and FFA may promote SBP through endothelial dysfunction and arterial stiffness [38,40,43–45]. However, hesperidin supplementation had no effect on serum levels of ICAM-1 and VCAM-1, both used as surrogate markers of endothelial dysfunction. Despite this lack of agreement, we must take into account that we did not find differences between STD- and CAF-fed rats in these parameters. In fact, these molecules are not specific of endothelial cells, but can be shed from other cells and thus reflect the activity of other biological processes. Nonetheless, CAF rats showed higher levels of

neuraminidase, an enzyme involved in elastogenesis that has recently been associated with arterial stiffness [46], which is now recognized as a major contributor to hypertension in elderly people [47].

Several studies cross-sectional and prospective cohorts studies revealed positive associations of circulating amino acids, especially BCAA, with visceral obesity, IR, hypertension, dyslipidemia, MetS and T2D [48]. Increased branched-chain keto acid dehydrogenase (BCKD) is the main cause of BCAA and BCKA accumulation, which may impair mitochondrial oxidation of fatty acids leading to IR [49]. In particular, leucine has recently shown to increase arterial pressure through activation of mTORC1signalling [50]. Accordingly, the serum metabolic profiles of CAF-fed rats were characterized by higher levels of BCAA and their catabolites (BCKA) compared to STD-fed rats. Notably, the reduction in SBP in CAF-H2 compared to CAF-V rats was accompanied by a decrease in the circulating levels of BCAA and 2-MOV, an isoleucine catabolite. Recently, 2-MOV has been identified as the strongest predictor of impaired fasting glucose in two independent human cohorts [51].

However, lysine was the amino acid that showed the strongest decrease after hesperidin supplementation in CAF-fed rats compared to the vehicle. Lysine competes with arginine for cellular uptake and enhances catabolism by activating kidney arginase, thereby decreasing its bioavailability. Importantly, arginine is the precursor of nitric oxide (NO), which is synthesized by endothelial NO synthase (eNOS) and plays a pivotal role in vascular homeostasis and vasodilation. In fact, diminished NO bioavailability is a hallmark of endothelial dysfunction [52]. Therefore, it is tempting to speculate that the reduction of lysine levels in CAF-H2 rats could improve NO bioavailability and partly explain the improvement in SBP observed in these animals compared

to the CAF-V group. In fact, supplementation with free amino acids or protein isolate with a high arginine/lysine ratio reduced SBP and angiotensin-I converting enzyme activity and increased plasma nitrate levels compared to vehicle-supplemented hypertensive rats [53]. Furthermore, we also found decreased levels of glutamine in CAF-H2, which has shown to be an important precursor for de novo synthesis of arginine [54].

Increased urinary lysine levels have also been associated with SBP in moderately hypertensive volunteers [55]. This occurred in parallel with increased cis-aconitate levels, which we found as one of the most discriminatory metabolites between CAF-V and STD-V rats. Similarly, patients with pulmonary arterial hypertension had increased cis-aconitate in the lung [56]. Cis-aconitate is formed from citrate by the enzyme aconitase, and iron dependent enzyme. Interestingly, we found that in CAF-fed rats, cis-aconitate had the strongest negative correlation with the *Bacteroidaceae* family (Supplementary figure 5). We have previously shown that CAF-H2 increased the relative abundance of *Bacteroidaceae* and it has been reported that *Bacteroides spp.* have shown a strong heme-dependence. The high iron demand associated with increased levels of this family in CAF-H2 rats can inhibit the activity of the iron-dependent aconitase and explain the reduced plasma cis-aconitate levels.

Most patients with MetS also exhibit dyslipidemia [2,3], including elevated TG, LDL-C, FFA and low HDL-C levels. Consistently, CAF-fed rats had higher circulating levels of TC, LDL-C, TG, and FFA compared to STD-fed animals. Lipidomic analyses corroborated these results and also revealed and increase in DG, PL and PC after a CAF diet. One of the most consistent effects of hesperidin is their ability to regulate lipid metabolism [13]. Hence, hesperidin supplementation has shown a

consistent lipid-lowering effect in animals models of diet-induced obesity, although the results in animal studies are inconsistent [10–13]. These are in agreement with our lipidomic findings showing a really significant reduction in all lipid classes in CAF-H2 compared to CAF-V rats. In addition, a reduction in LDL-C after hesperidin supplementation was the only effect that we found also found in STD-fed rats.

Finally, hesperidin supplementation resulted in higher urinary excretions of hippurate, 3-HPPA and 3-HCA in STD- and CAF-fed rats. These metabolites have been previous described as hesperidin catabolites [57]. In fact, the major portion of polyphenols are not absorbed (90-95%) in the small intestine, but they are extensively metabolized in the colon by the enzimatic activity of the gut microbiota to a relatively small number of metabolites that can be absorbed into the bloodstream [20]. Accordingly, the urinary levels of 3-HCA and 3-HPPA correlated positively with the *Bacteroidaceae* family, which we have previously shown to be increased after hesperidin supplementation [22]. Importantly, phenolic catabolites may be present at higher concentrations than the original compounds and/or have increased biological activity, thereby being potentially responsible for the systemic effects of polyphenols [19,20]. For example, 3-HPPA showed considerable higher *in vitro* vasodilatory effect than quercetin or related flavonoids [58]. Notable, it was the most potent among several phenolic catabolites and results were confirmed by decreased blood pressure after supplemenation in both normotensive and hypertensive rats. In addition, hippurate excretion was lower in SHR compared to normotensive Wistar Kyoto rats. As the diet was the same for both groups, athors attributed to strain differences in the gut microbiota [59].

Another metabolite found in higher levels in both serum and urine after hesperidin supplementation was DMSO2. Little is known about DMSO2, but a recent study showed an impressive increase in DMSO2 concentrations in obese subjects after several bariatric procedures [60], which have shown to produce significant improvements in obesity and hypertension partly due to changes in the microbiota composition [61,62]. Interestingly, DMSO2 may derive from microbial metabolism of methionine [63], thereby reflecting a change in the gut microbiome composition. Consistently, we found a positive correlation between DMSO2 and *Bacteroidaceae*, both increased after hesperidin supplementation.

5. Conclusions

Here, we demonstrated that hesperidin supplementation in rats fed a CAF diet has a positive effect to MetS components which are interconnected to CVD. Specifically, our results shown a decrease in blood pressure, an improvement of insulin sensitivity and an amelioration of lipid metabolism in those supplemented animals with H2. In fact, these results were accompanied by a reduction of oxidative stress and inflammation-related metabolites in plasma and urine. We also corroborate its relation with gut microbiota, due to the increased amount of hesperidin catabolites found that are metabolized by gut microbiota, especially in CAF-H2.

Therefore, these results support the hypothesis that hesperidin should be considered as a potential prebiotic to treat MetS. Further studies are needed addressing its effectiveness against some MetS components.

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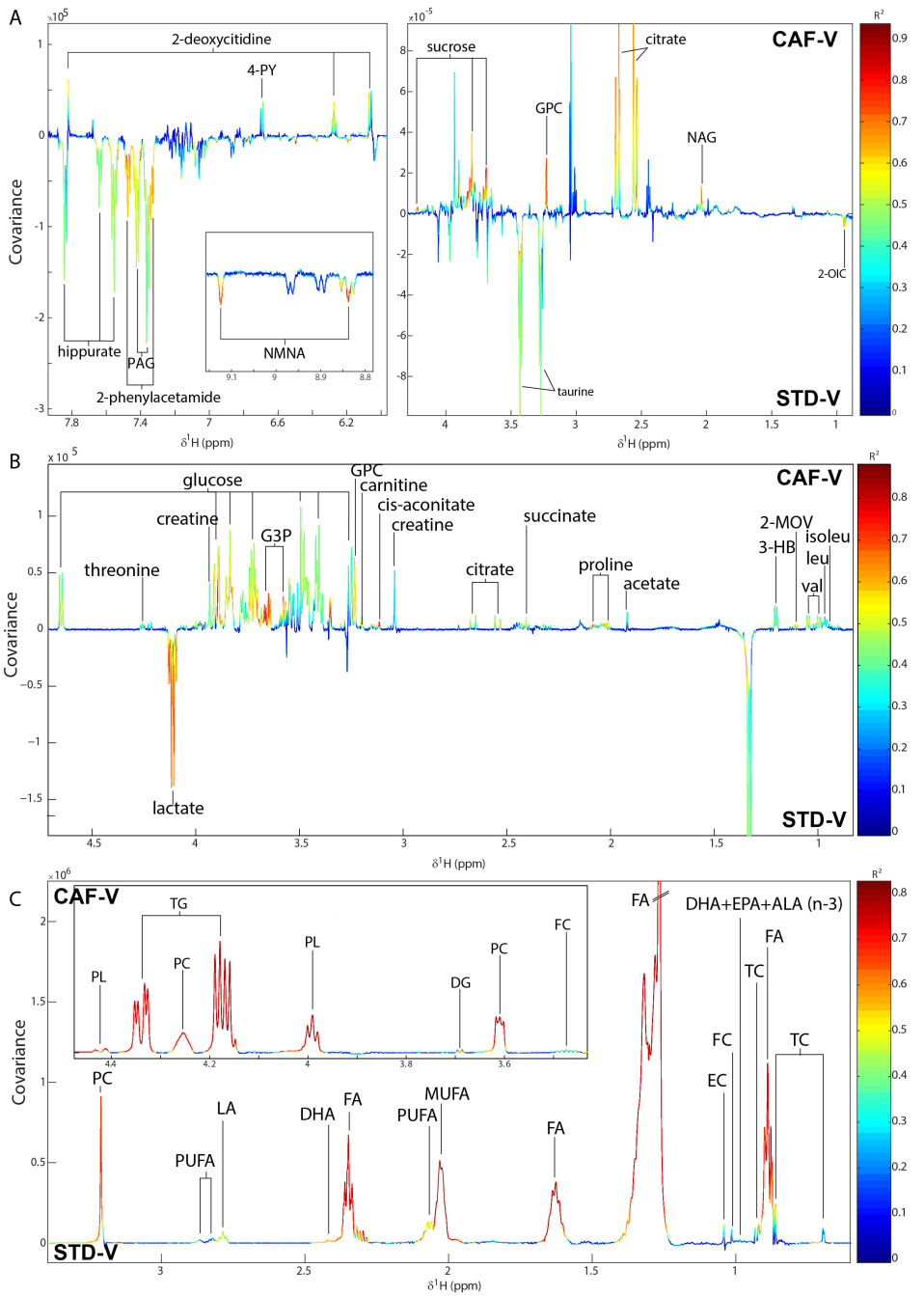
Supplementary Information

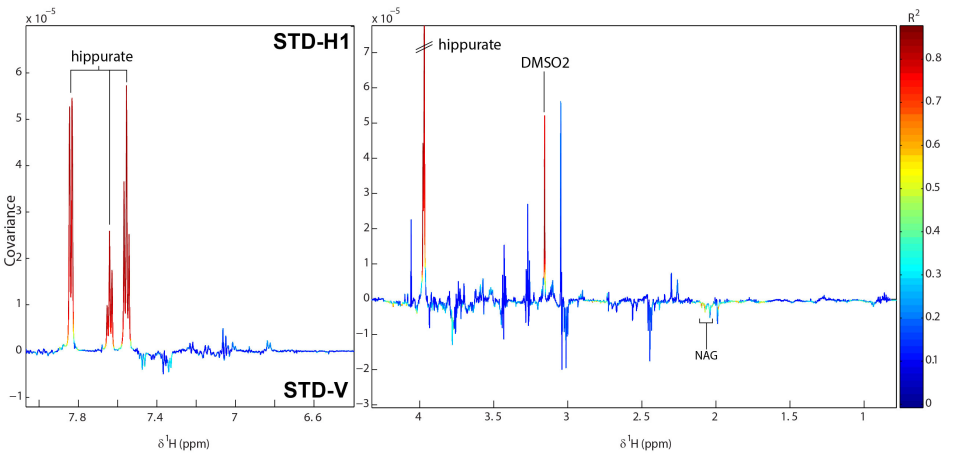
Supplementary Table 1. Models characteritics. Significant models are shown in bold.

Model	Ortho components	R^2Y	Q^2Y	*P*
Urine				
STD-V vs CAF-V	1	1.0	0.81	<0.001
STD-V vs STD-H1	7	1.0	0.84	<0.001
STD-V vs STD-H2	1	0.98	0.87	<0.001
STD-H1 vs STD-H2	8	1.0	0.71	0.30
CAF-V vs CAF-H1	2	1.0	0.63	0.006
CAF-V vs CAF-H2	7	1.0	0.93	<0.001
CAF-H1 vs CAF-H2	3	1.0	0.40	0.15
Serum AQ				
STD-V vs CAF-V	2	1.0	0.58	<0.001
STD-V vs STD-H1	4	1.0	0.54	0.17
STD-V vs STD-H2	4	1.0	0.58	0.21
STD-H1 vs STD-H2	4	1.0	0.49	0.36
CAF-V vs CAF-H1	3	1.0	0.36	0.60
CAF-V vs CAF-H2	2	1.0	0.62	0.017
CAF-H1 vs CAF-H2	4	1.0	0.82	0.03
Serum LIP				
STD-V vs CAF-V	5	1.0	0.81	<0.001
STD-V vs STD-H1	0	0.48	-0.34	-
STD-V vs STD-H2	0	0.44	-0.06	-
STD-H1 vs STD-H2	0	0.36	-0.13	-
CAF-V vs CAF-H1	0	0.48	0.30	0.034
CAF-V vs CAF-H2	0	0.60	0.51	0.005
CAF-H1 vs CAF-H2	0	0.49	-0.35	-

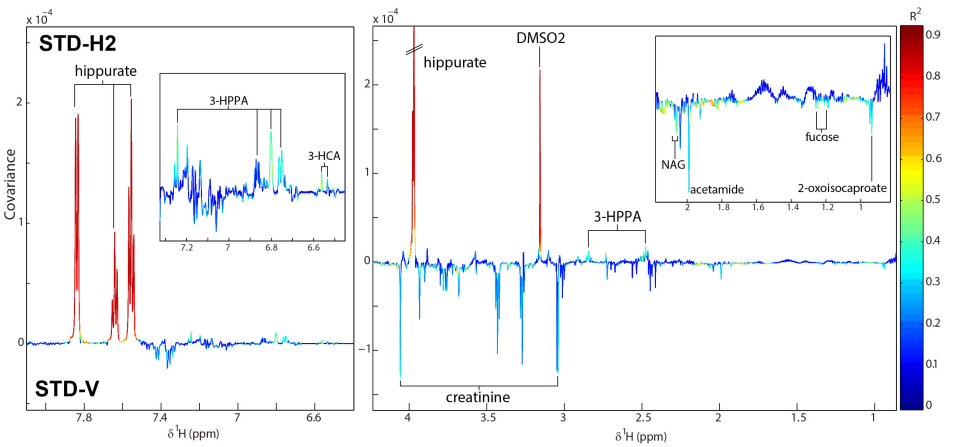
Supplementary Figure 1. OPLS-DA models comparing the metabolic profiles of rats fed a STD or a CAF diet and supplemented with the vehicle (V). A) urine metabolic profiles, B) serum aqueous metabolic profile, C) serum lipid metabolic profile.



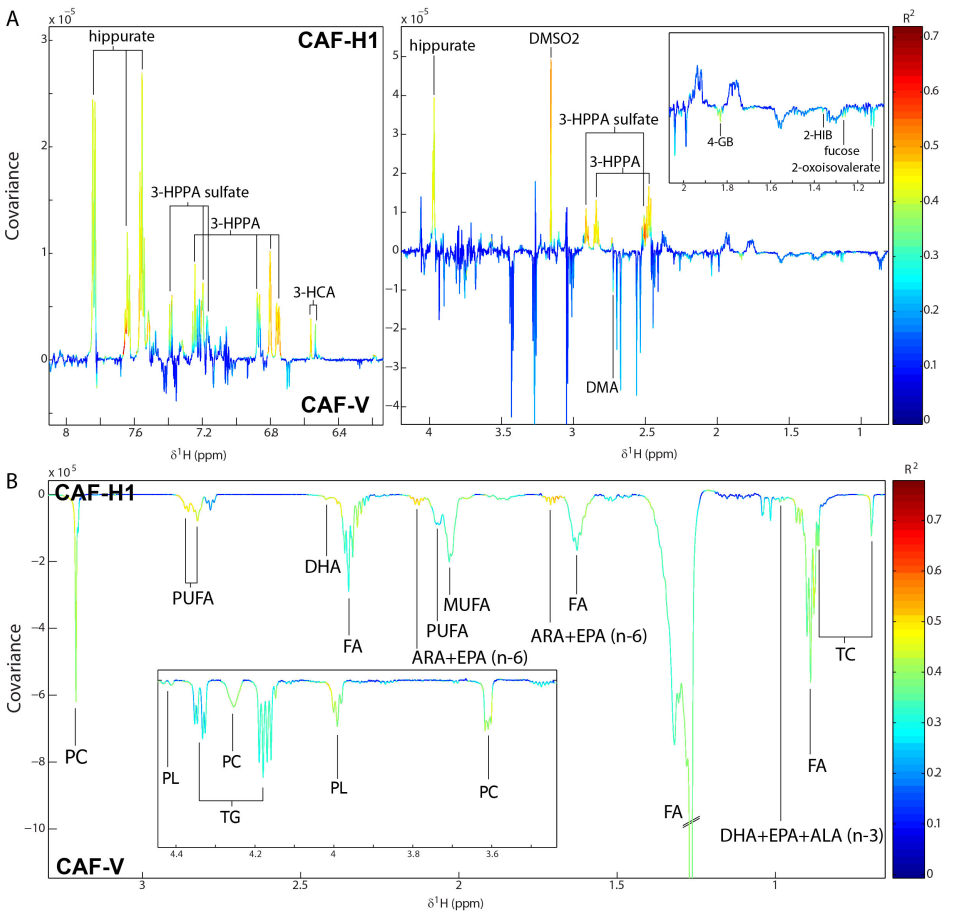
Supplementary Figure 2. OPLS-DA models comparing the urine metabolic profiles of STD-fed rats supplemented with either the vehicle (V) or hesperidin at dose1 (H1).



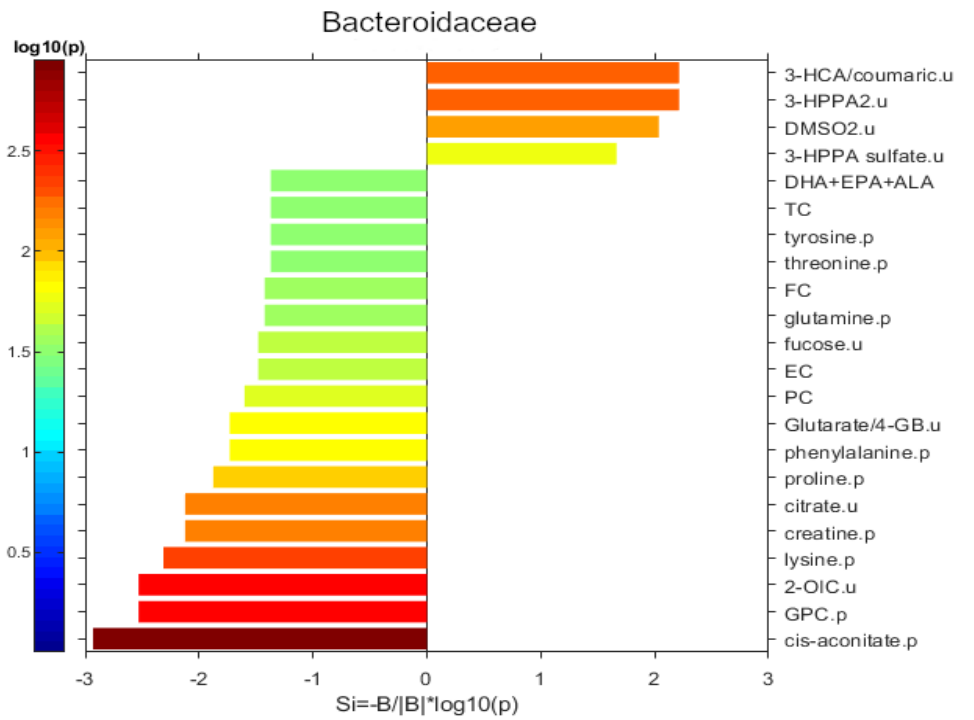
Supplementary Figure 3. OPLS-DA models comparing the urine metabolic profiles of STD-fed rats supplemented with either the vehicle (V) or hesperidin at dose2 (H2).

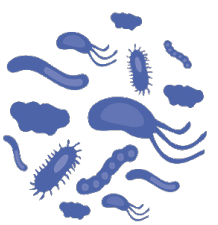


Supplementary Figure 4. OPLS-DA models comparing the metabolic profiles of CAF-fed supplemented with either the vehicle (V) or hesperidin at dose1 (H1). A) urine metabolic profile, B) serum lipid metabolic profile.



Supplementary Figure 5. Correlation between Bacteroidaceae family and metabolites from urine and plasma between CAF-V and CAF-H2.





GENERAL DISCUSSION

GENERAL DISCUSSION

The link between gut microbiota and diet has been extensively demonstrated, and it is well known that intestinal microbiota can be modulated by diet and subsequently play a key role in disorders such as MetS or obesity, where diet is strictly related to its development [1,2]. Several studies have shown the involvement of dietary patterns in gut bacteria balance between beneficial and harmful microbes. Normally, intestinal microbiota diversity decreases when a hypercaloric diet is consumed, and currently, this loss of richness has been proposed as a hallmark of Western societies [3]. Taken together, evidence shows patterns associated with gut microbiota composition and an obese phenotype.

Therefore, the first objective was to assess the effect of CAF diet on microbiota biodiversity and its implications in host physiology (**Manuscript 1**). To our knowledge, this was the first time that gut microbiota activity was related to CAF diet, being a more realistic animal model for comparison with human studies. Thus, gut microbiota characterization demonstrated remarkable changes associated with physiological and biochemical alterations characteristic of CAF-induced obesity previously described [4–7]. Due to the lack of consensus on how the CAF diet affects the pattern of phyla variation, the most controversial part was to corroborate in which direction these alterations lead to gut microbiota modulation. Several studies agree with our results, affirming that the Firmicutes phylum seems to be responsible for the decrease in alpha-diversity and that consequently, an increase in Bacteroidetes occurs [8,9], but previously, other authors demonstrated the opposite [10,11]. Additionally, an increase in the

Actinobacteria phylum proportion was also observed in CAF animals and was associated with obesity [12]. In fact, the *Propionibacterium* genus was positively associated with plasma concentrations of insulin and glucose [13]. All these changes together have also been demonstrated in both preclinical models and human subjects [14,15]

In this study, we also suggested a strong correlation between diet ingredients and gut microbiota. It is known that fiber, carbohydrate, and fat content shape the gut microbiota population [3,16]. The reduction in the populations of positive fermenters, such as Firmicutes, could mislead the breakdown of non-digestible carbohydrates and its products, such as SCFAs, which are beneficial to health [17] and cannot be used as an energy source and as precursors for fatty acids [18]. Thus, it is plausible to hypothesize that the low fiber intake in CAF-fed animals is tightly related to a reduction of metabolites (mainly SCFAs) produced by fermentative bacteria, the populations of which are also reduced. If this reduction in SCFA levels was also found in blood, it could contribute to suboptimal activation of catabolic pathways and could increase fat deposition in adipose tissues and liver. Therefore, these metabolites suggest that microbiota are interconnected with adipose tissue remodeling and liver health to maintain the homeostasis of lipid metabolism, highlighting the relevance of microbiota in energy metabolism [19,20].

Despite studying the relationship between the microbiota population and host physiology, the microbes that compose the whole gut microbiota exercise specific functions [21]. Hence, there is a need to have some technical tools to fully comprehend the role of the gut microbiota in maintaining the health-disease balance. The application of omic technologies in microbiota studies has allowed the creation of a new subfield, ‘metaomic sciences’, which have been considered to

be the pioneering techniques to characterize gut microbiota functions in the host, providing the information required to describe the phenotype [22]. To date, metagenomics has been widely used to study the 16S rRNA gene of gut bacteria, which identifies microbes, although the specific functions that they have are still unclear. For this reason, metaproteomics is a 'new' promising methodology that will enhance knowledge on gut microbiota functions and has the ability to directly measure the functional activity [23]. Classical proteomic workflows must be adapted to microbiota complexity, and to achieve that objective, some research is required to find new methodologies adapted to this kind of sample (**Manuscript 2**).

Therefore, five different methodologies were tested to determine the optimum protocol to elucidate microbiota functions. Three critical steps were evaluated: cell lysis, tryptic digestion and sample fractionation. In the literature, some authors attempted to reach this goal before, but there are no articles where these key steps were combined, and in the end, a comparison of protein function between the different methods was tested.

The type of cell lysis is important to achieve a broad vision of the proteome of the microbiota population. Bacterial walls are difficult to break, and some aggressive buffers are needed. After comparing both SDS-based buffer and non-detergent, the former was the most effective. Some authors [24,25] have confirmed this statement by comparing several lysis buffers. Moreover, the combination of lysis and tryptic digestion was another step to optimize. FASP is a widely used method for digestion, but some controversial results were found due to its poor technical reproducibility [26–28]; in our study, when FASP was compared to an in-gel digestion, similar results were obtained, although in-gel digestion was more reproducible and more

confident [29]. However, the innovative part of this new methodology was the incorporation of a fractionation step to expand protein coverage and obtain a better protein characterization. In fact, few studies have applied a fractionation methodology [28,30,31], but none has compared more than one protocol. Our results correlated with the expectations that fractionation increased the number of proteins identified and their coverage, and in particular, off-gel fractionation on the peptide level was chosen for its great performance.

Finally, the impact of the data analysis workflow on identification and prediction of the metaproteome functions was also compared. In this sense, particular studies have assessed the functions of its own results, but none have compared more than one workflow [32]. The reference database could also be considered a critical step to reach an accurate peptide/protein taxonomic assignment and, consequently, a high-quality annotation to each function. Generally, the quantity of peptides shared by multiple species is considerably high, and only some unique peptides can be assigned to a specific species. This issue makes the identification of peptides/proteins by downstream taxonomic and functional data analysis more challenging. For this reason, an in-house gut microbiome database is by far the better option to identify the peptides/proteins in a metaproteome after comparing other database options. In this respect, the choice of metagenomics data from the same sample collection to create a metaproteomics database improves the results; for example, a dramatic increase in terms of general identifications and assignments can be observed in some studies in mice [33]. The search against different databases types can significantly be dependent on taxa annotation and functional characterization of the gut metaproteome. For this reason, several studies support the idea to couple experimental metagenomic

sequencing to metaproteomic analysis for database construction [34], and it is recommended to avoid comparing results with multiple databases to prevent the conclusion from being unreliable and erroneous [35].

Despite the improvement in methodological sample preparation and data analysis, some other topics related to the assessment of intestinal microbiota functions in obesity have been explored in animal models. The resulting methodology of manuscript 2 was tested in an animal study with rats fed either a STD or CAF diet. We previously observed that the CAF diet caused gut microbiota dysbiosis, and we could corroborate our previous findings on metagenomics data. The addition of the metaproteomics analysis gathered the required information to correlate the functions modulated by gut microbiota disruption, as we expected.

To continue studying the gut microbiota effect on the host, a second aim was proposed to verify the impact of antibiotics and corroborate the role of diet by the implication of FMT (**Manuscript 3**).

Antibiotics are known to cause a depletion in the gut microbiota, resulting in a dysbiosis state [36], which alters the gut microbiota balance within the host. In addition, FMT is a straightforward therapy that manipulates the microbial ecosystem and can reverse antibiotic effects. Nonetheless, the FMT effect on the host regarding microbiota function has not been fully studied, so the combination of both metaomics (metagenomics and metaproteomics) allows to corroborate the impact of diet on microbiota functions and the effects of antibiotics and FMT in obese rats. Regarding diet, our results agree with what has been described before, and some authors conclude the implication of diet in important metabolic functions [37]. The novelty

relies on the corroboration that FMT was successfully achieved and gut microbiota function was redirected. First, gut microbiota richness and evenness were reestablished after antibiotic treatment as expected, and the composition was more similar to animals fed a diet with low amounts of fat [38,39]. Similarly, gut microbiota function was also restored after FMT, and although no studies compared its functionality before and after FMT, important metabolic functions such as ATP/energy metabolism or glycolysis were equally regulated in LFD and FMT animal groups.

Moreover, metagenomics and metaproteomics results, interestingly, correlated and among all the proteins, glyceraldehyde-3-phosphate dehydrogenase was highlighted. It is known that this protein controls NAD-dependent glycolytic activity in *Clostridium* species, which are fermenters directly related to butyric acid fermentation, considering butyric acid a SCFA product from microbiota activity [40]. These results could confirm the beneficial effect of SCFAs produced by gut microbiota and elucidate their important implication in host homeostasis.

In addition to FMT, some prebiotics, probiotics and other compounds can exert beneficial effects in the host by the action of gut microbiota. Specifically, some polyphenols must be metabolized by gut microbiota to exercise their benefit in host physiology. Hesperidin, a flavanone hydrolyzed by gut microbiota, and its bioactive compound are being proven as an effective treatment to reduce hypertension, one of the major comorbidities of MetS. Therefore, the last objective was to evaluate the effect of hesperidin in hypertension through the modulation of microbiota diversity and its function (**Manuscript 4**) by combining both metaomic methodologies.

First, we corroborated the hypotensive effect of hesperidin in a diet-induced hypertension animal model, and the evidence suggested that hesperidin was acting through gut microbiota modulation, elucidating the complex relationship between the alteration of gut microbiota composition and the effect of hesperidin supplementation; additionally, the decrease in SBP in obese rats could be due to changes in microbiome composition and function [41,42]. The major changes were found between obese animals and lean animals, but slight differences were found in obese hesperidin-supplemented animals and untreated animals. Some bacterial families differed, producing alterations in gut microbiota functions, increasing the expression of Enolase-1, a glycolytic protein involved in several bacterial functions known as a moonlighting protein [43,44].

Regardless, the mechanism by which hesperidin induces these benefits is far from being fully understood. According to the literature, the possible role of hesperidin in improving endothelial function seems to be clear, whereas the antihypertensive, lipid-lowering, antioxidant, and anti-inflammatory effects are inconsistent [45–48]. Thus, we explored the hesperidin effect regarding biochemical and physiological parameters and also combined with urine metabolomics analysis to decipher the role of gut microbiota (**Manuscript 5**).

In general, hesperidin supplementation did not completely affect all the biochemical parameters, as expected. In addition to the increase in insulin sensitivity, in the literature, diminished insulin sensitivity was reported to activate the RAAS system, promoting hypertension, which correlated with our results [49]. Similar results were found with the reduction in FFAs, which appears to play an important role in hypertension development in patients with obesity and MetS [50].

Finally, hesperidin supplementation resulted in higher urinary excretions of hippurate, 3-HPPA and 3-HCA, catabolites from hesperidin that are produced by gut microbiota [51–53], specifically 3-HPPA. Some authors attributed this result to the vasodilatory effect [54], and they were considered a modulator in the decrease in BP after supplementation in both normotensive and hypertensive rats, with its effect attributed to the differences in the gut microbiota [55].

Considering all the results of this thesis, it was confirmed that gut microbiota plays a leading role in host physiology. As expected, diet is a major modulator of microbiota composition, and consequently, its function related to the host is modified. However, the necessity to study gut microbiota has led researchers to find new strategies to comprehend the complex system. Metagenomics and metaproteomics have emerged as the perfect tools to combine to fully understand it.

Moreover, the gut microbiota is modulated by other factors, such as antibiotics and prebiotics/probiotics, which can disrupt its balance, producing dysbiosis. Here, we demonstrated the effect that antibiotics can have on the health-disease balance and how this balance can be restored by FMT, a new strategy that has started to be used to treat metabolic disorders. Nevertheless, another strategy to consider should be dietary intervention with the supplementation of polyphenols, which can act as prebiotics that exert several benefits through gut microbiota functions.

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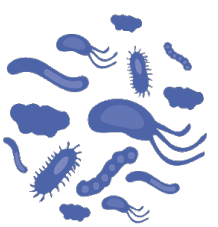

CONCLUSIONS

CONCLUSIONS

1. In the CAF-fed rat (a useful model for studying diet-induced dysbiosis in obesity) the balance between fiber, simple carbohydrates and fats determines the abundance of different phyla with a significant decrease in gut bacterial diversity.

2. In the CAF-fed rat model, the reduction in Firmicutes and the increase in Actinobacteria, Bacteroidetes and Proteobacteria abundances were responsible for the elevated endotoxemia and scarce SCFAs.

3. In the CAF-fed rat model, clear associations can be drawn between dysbiosis and increased adiposity and altered fat metabolism, in addition to hepatic lipid accretion.

4. The finest strategy, to improve gut microbiota metaproteome characterization by enlarging protein coverage, includes the use of SDS-buffer lysis, in-gel digestion, and peptide off-gel fractionation.

5. Metaproteomics must be preceded by metagenomics. The creation of the metaproteomics database constructed from each metagenomic result is the key step in obtaining the most accurate protein identification. Afterwards, the integration of metagenomics and metaproteomics provides results that are more reliable.

6. A multi-omics approach is the most accurate method to elucidate the gut microbiota activity. The metagenomics and metaproteomics combination has confirmed the biochemical alteration in gut microbiota composition caused by diet and the disruption of gut microbiota ecosystem by antibiotics.

7. FMT is an effective therapy to recolonize the gut microbiota after the use of antibiotics, and it can be considered as a treatment for dysbiosis induced by hypercaloric diets.

8. Hesperidin supplemented chronically (100 mg/kg) in diet-induced hypertensive rats acts as a modulator of gut microbiota, in turn producing catabolites with vasodilatory properties that can regulate blood pressure system.

9. Hesperidin ameliorates dyslipidemia, insulin sensitivity, hypertension, vascular function and inflammation caused by diet-induced MetS features.

CONCLUSIONS

1. En rates alimentades amb dieta CAF (un bon model per a estudiar la disbiosi induïda per la dieta en obesitat) el balanç entre fibra, carbohidrats simples i greixos determina l'abundància dels diferents fílums, amb un augment significatiu de la diversitat bacteriana intestinal.

2. En el model de rates alimentades amb dieta CAF, la reducció de Firmicutes i l'augment d'Actinobacteria, Bacteroidetes i Protoebacteria són els responsables de l'endotoxemia augmentada i l'escassetat de SCFA.

3. En el model de rates alimentades amb dieta CAF, es poden trobar associacions clares entre la disbiosi i l'augment de l'adiposita i l'alteració del metabolisme dels greixos, malgrat l'acumulació de lípids hepàtics.

4. La millor estratègia per a millorar la caracterització del metaproteoma de la microbiota intestinal, inclou l'ús d'un tampó de lisi amb SDS, una digestió en gel i un fraccionament *Off-gel* a nivell de pèptids.

5. La metaproteòmica ha d'anar precedida per la metagenòmica. La creació de la base de dades de metaproteòmica construïda amb els resultats de metagenòmica és la clau per a obtenir una identificació de proteïnes més acurada. Així, la integració de metagenòmica i metaproteòmica dóna resultats fiables.

6. Una aproximació multi-òmica és la manera més acurada d'aclarir l'activitat de la microbiota intestinal. La combinació de metagenòmica i metaproteòmica ha confirmat l'alteració bioquímica de la composició de la microbiota intestinal causada per la dieta i la disrupció d'aquest ecosistema causada per antibiòtics.

7. El FMT és una teràpia efectiva per a recolonitzar la microbiota intestinal després de l'ús d'antibiòtics i es pot considerar com un tractament per a la disbiosi induïda per dietes hipercalòriques.

8. L'hesperidina suplementada crònicament (100mg/Kg) en rates amb hipertensió induïda per la dieta, que produeix catabòlits amb propietats vasodilatadores pot regular el sistema de pressió arterial.

9. L'hesperidina millora la dislipèmia, la sensibilitat a la insulina, la hipertensió, la funció vascular i la inflamació causades pels components de MetS induïts per la dieta.

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LIST OF PUBLICATIONS

Papers included in the thesis

A) Published papers

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. Electrophoresis. 2018;39: 1692–1701. doi:10.1002/elps.201700476. Impact factor: 2.569. SI Journal Citation Reports © Ranking: 35/ 78 (Q2), (Biochemical Research Methods)

Del Bas JM, **Guirro M**, Boque N, Cereto A, Ras R, Crescenti A, Caimari A, Canela N, Arola L. Alterations in gut microbiota associated with a cafeteria diet and the physiological consequences in the host. Int J Obes (Lond). England; 2018;42: 746–754. doi:10.1038/ijo.2017.284. Impact factor: 5.159. SI Journal Citation Reports © Ranking: 10/ 83 (Q1) (Nutrition and Dietetics)

B) Submitted papers

Guirro M, Herrero P, Costa A**,** Gual-Grau A, Ceretó Massagué A, Hernández A, Torrell H, Arola L, Canela N. Deciphering the functions of gut microbiota in an animal model of obesity using an optimized metaproteomics workflow. [Submitted to Journal of Proteomics].

Guirro M, Costa A**,** Gual-Grau A, Herrero P, Torrell H, Canela N, Arola L. Obesity-induced gut microbiota dysbiosis can be ameliorated by fecal microbiota transplantation: a multiomics approach. [Submitted to PLoS ONE].

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].

Other papers

A) Published papers

Hernández-Aguilera A, Sepúlveda J, Rodríguez-Gallego E, **Guirro M**, García-Heredia A, Cabré N, et al. Immunohistochemical analysis of paraoxonases and chemokines in arteries of patients with peripheral artery disease. Int J Mol Sci. 2015;16: 11323–38. doi:10.3390/ijms160511323

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B) Submitted papers

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. [Submitted to The Journal of Nutritional Biochemistry].

LIST OF CONFERENCE PAPERS

XVII Congreso de la Sociedad Española de Nutrición. 2018. Spain. Diet-induced gut microbiota dysbiosis can be ameliorated by faecal microbiota transplant. **Guirro M**, Torrell H, Costa A, Herrero P, Canela N, Arola L. Poster Presentation.

V Reunión de Jóvenes Investigadores. Sociedad Española de Nutrición. 2018. Spain. Diet-induced gut microbiota dysbiosis can be ameliorated by faecal microbiota transplant. **Guirro M**, Torrell H, Costa A, Herrero P, Canela N, Arola L. Oral Presentation.

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.

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.

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IX Seminario Sobre Alimentación y Estilos de Vida Saludables. 2017. Spain. Metaproteómica: comparativa de metodologías para el estudio de la microbiota. **Guirro M**, Costa A, Herrero P, Arola L, Canela N. Poster 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. A comprehensive study of microbiota by omic sciences. **Guirro M**, Herrero P, Arola L, Canela N. 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.

IV Bioinformatics and Genomics Symposium. 2016. Spain. In depth evaluation of the Fecal Microbiota: A Flexible and Optimized Ion Torrent 16S rRNA Gene-Based Analysis workflow. Cereto-Massagué A, Torrell H, **Guirro M**, García L, Hernàndez A, Canela N. Poster Presentation.

Gut microbiota has become a key player in the pathophysiology of metabolic syndrome and its associated comorbidities, such as hypertension. Treatment of metabolic syndrome is complicated because it is a cluster of disorders; thus, polyphenols can exert beneficial effects to ameliorate metabolic syndrome, especially hesperidin, decreasing the increased levels of blood pressure.

Moreover, some factors such as diet or antibiotics can disrupt the gut microbiota-host equilibrium and trigger a set of detrimental events. To fully comprehend the role of gut microbiota in metabolic syndrome, metaomic sciences have been proposed as the most accurate strategy. A multi-omics strategy combining metagenomics, which provides the taxonomic profile, and metaproteomics, which helps to understand the function in host, is the most adequate approach to study the role of the gut microbiota.

In this regard, this thesis aims to evaluate the role of gut microbiota through a multi-omics strategy in a metabolic syndrome situation. We proposed a cafeteria diet as an accurate model for studying gut microbiota dysbiosis produced by diet. The gut microbiota composition was altered, and its function in the host was modulated; thus, metaproteomics should be preceded by metagenomics to better characterize gut microbiota activity. Moreover, the effectivity of fecal microbiota transplantation was corroborated after a short period of antibiotic depletion, making it an accurate therapy to restore gut microbiota disruption caused by a hypercaloric diet.

Additionally, hesperidin, a polyphenol metabolized by gut microbiota, alters the biodiversity and the functions of the intestinal microbiota, which are responsible for the amelioration of blood pressure caused by cafeteria diet, and regulates insulin sensitivity and dyslipidemia after chronic administration.

The results of this thesis elucidate the importance of using a multi-omics strategy to explain gut microbiota activity and confirm the significance of the gut microbiota in the maintenance of host homeostasis and energy metabolism.