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FMUP FACULDADE DE MEDICINA
UNIVERSIDADE DO PORTO

UNRAVELING THE EFFECTS OF ANTHOCYANINS IN METABOLIC HEALTH AND DISEASE: INSIGHTS ON BIOAVAILABILITY AND GUT MICROBIOTA MODULATION

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CONTRIBUTO PESSOAL NOS TRABALHOS DE INVESTIGAÇÃO APRESENTADOS NESTA DISSERTAÇÃO

A candidata declara que teve uma contribuição determinante na realização do trabalho experimental (programação e execução do trabalho experimental), bem como na interpretação e discussão dos resultados apresentados em todos os artigos desta dissertação. A candidata contribuiu ainda ativamente na redação dos trabalhos apresentados.

Abbreviations

Adenosine monophosphate-activated protein kinase	**AMPK**
Body mass index	**BMI**
Brain-derived neutrophic factor	**BDNF**
Carbohydrate response element binding protein	**ChREBP**
Central nervous system	**CNS**
Endocannabinoid	**eCB**
Farnesoid-X-receptor	**FXR**
Fasting-induced adipose factor	**FIAF**
Gamma-aminobutyric acid	**GABA**
Glucagon-like peptide-1	**GLP-1**
Glucagon-like peptide-2	**GLP-2**
High-fat	**HF**
Interleukin-1β	**IL-1β**
Interleukin-6	**IL-6**
Lipopolyssacharide	**LPS**
Lipoprotein lipase	**LPL**
Non-alcoholic fatty liver disease	**NAFLD**
Peptide YY	**PYY**
Short chain fatty acids	**SCFA**
Sterol response element binding protein-1	**SREBP-1**
Toll-like receptor	**TLR**
Tumor necrosis factor-α	**TNF-α**
Vitamin D receptor	**VDR**
World health organization	**WHO**

Contents

ABSTRACT/RESUMO

Abstract

The microbes that collectively inhabit the gut - the gut microbiota - constitute the largest and most diverse community in the body. The gut microbiota is primarily responsible for the maintenance of the intestinal wall integrity and the protection against pathogens. These functions have long been attributed to the gut microbiota but the interest of the scientific community upon this collection of microorganisms began to emerge when the first studies demonstrating its envelopment in obesity were published.

Besides having an important role in the regulation of host energy metabolism, the gut microbiota can also influence neurodevelopment, modulate behavior and contribute to the development of neurological disorders. Thus, the gut microbiota might constitute a potential target for the treatment of obesity and the associated neuropsychiatry disorders.

Anthocyanins are a particular class of flavonoids that can be found mainly in red wine and red fruits. Anthocyanins bioavailability is considered to be low but it remains to be properly addressed. In addition, when they reach the colon they can be metabolized by the gut microbiota and may modulate bacterial growth. Whether gut microbiota modifications are responsible for the neuropreotective effects of anthocyanins remains unknown.

Using a Rat model of diet-induced obesity, the effects of blackberry anthocyanins upon gut microbiota composition were evaluated in the present thesis. Results from this work strongly suggest that dietary manipulation of the gut microbiota by anthocyanins can attenuate the neurologic complications of obesity, expanding the classification of psychobiotics to anthocyanins.

Regarding their bioavailability, the clinical trial conducted in the current thesis indicate that anthocyanins are extensively metabolized after absorption. Furthermore, it showed, for the first time, that ethanol enhances cyanidin metabolism, potentiating its conversion into methylated derivatives, especially in overweight and obese individuals.

These results should prompt the attention of the scientific community to the fact that the kinetic of these compounds is influenced by body composition and deserve special considerations since obese individuals might be the ones who would benefit the most from anthocyanins intervention.

This thesis brought new insights on anthocyanins bioavailability and have clarified the mechanisms by which anthocyanins participate in the bilateral communication between gut and brain. Future clinical interventional studies are warranted to validate these findings.

Resumo

Os microrganismos que habitam coletivamente o intestino - o microbiota intestinal - constituem a comunidade mais densa e mais diversa do corpo humano. O microbiota intestinal é principalmente responsável pela manutenção da integridade da barreira intestinal e pela proteção contra agentes patogénicos. Há muito que estas funções do microbiota intestinal são conhecidas, mas o interesse da comunidade científica sobre esta coleção de microrganismos começou a emergir quando foram publicados os primeiros estudos que demonstram seu envolvimento na obesidade.

Além de ter um papel importante na regulação do metabolismo energético do hospedeiro, o microbiota intestinal pode também influenciar o neurodesenvolvimento, modular o comportamento e contribuir para o desenvolvimento de perturbações neurológicas. Assim, o microbiota intestinal poderá constituir um potencial alvo para o tratamento da obesidade e das perturbações neuropsiquiátricas associadas à obesidade.

As antocianinas são uma classe particular de flavonoides, encontrando-se principalmente no vinho tinto e nos frutos vermelhos. A biodisponibilidade das antocianinas é considerada baixa, porém esta nunca foi devidamente avaliada. Além disso, quando atingem o colon, podem ser metabolizadas pelo microbiota intestinal e modular o crescimento bacteriano. No entanto, permanece por esclarecer se as modificações do microbiota intestinal são responsáveis pelos efeitos neuroprotetores das antocianinas.

Usando um modelo animal de obesidade induzida pela dieta, os efeitos das antocianinas presentes na amora sobre a composição do microbiota intestinal, foram avaliados nesta tese. Os resultados deste trabalho sugeriram fortemente que a manipulação do microbiota intestinal com antocianinas pode atenuar as complicações neurológicas da obesidade, alargando a classificação de psicobióticos às antocianinas.

Em relação à sua biodisponibilidade, o ensaio clínico conduzido nesta tese, revelou que as antocianinas, depois de absorvidas, são extensamente metabolizadas. Para além disso, revelou, pela primeira vez, que o etanol estimula o metabolismo da cianidina, potenciando a sua conversão em derivados metilados, especialmente em indivíduos com excesso de peso ou obesidade.

Estes resultados devem chamar a atenção da comunidade científica para o facto da cinética desses compostos ser influenciada pela composição corporal e merecem considerações

particulares uma vez que os indivíduos pré-obesos ou obesos são aqueles que mais beneficiarão da intervenção com antocianinas.

Esta tese trouxe novas considerações sobre a biodisponibilidade das antocianinas e clarificou os mecanismos pelos quais as antocianinas participam na comunicação bilateral entre o intestino e o cérebro. Estudos clínicos de intervenção são necessarios para a validação destes resultados

CHAPTER I

INTRODUCTION

AIMS

The human body harbors a collection of trillions of microorganisms. Bacteria, archaea, virus fungi and other eukaryotes live inside different organs establishing a symbiotic relationship with the host [1-3]. The microbes that collectively inhabit the gut - the gut microbiota - constitute the largest and most diverse community in the body [4]. Most of them are bacteria belonging to the Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria or Verrucomicrobia phylum (Table 1) [5].

Table 1 - Phylogenetic classification of the most abundant bacteria in the gut.

Phylum	Class	Order	Family	Genus
Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Lachnoclostridium
				Roseburia
				Acetatifactor
			Ruminococcaceae	Ruminococcus
				Ruminiclostridium
				Sporobacter
			Unclassified	Intestinimonas
				Flavonifractor
				Pseudoflavonifractor
			Eubacteriaceae	Eubacterium
			Clostridiaceae	Hungatella
				Clostridium
			Oscillospiraceae	Oscillibacter
	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus
			Enterococcaceae	Enterococcus
Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides
			Prevotellaceae	Prevotella
Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	Bifidobacterium
Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Desulfovibrio
Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Akkermansiaceae	Akkermansia

The gut microbiota is primarily responsible for the maintenance of the intestinal wall integrity and the protection against pathogens. The bacterial fermentation of non-digestible carbohydrates leads to the formation of short chain fatty acids (SCFA) which reduces luminal pH and inhibits the growth of pathogens [6, 7]. On the other hand, butyrate, one of the most abundant SCFA in the gut, regulates epithelial cell growth and differentiation, thus contributing to the intestinal barrier reinforcement [8-10]. Besides, the gut microbiota can synthesize certain vitamins, such as vitamin K and B group vitamins including biotin, cobalamin, folates, nicotinic acid, pantothenic acid, pyridoxine, riboflavin and thiamine [11]. Furthermore, it is involved in the bile acids transformation as some bacteria have the ability to hydrolyze the amide bond between the steroid nucleus and the conjugated amino acid (taurine or glycine) [12]. In addition to bile acid deconjugation, some bacteria can then convert the primary bile acids into secondary bile acids through 7α-dehydroxylation or 7α-dehydrogenation, thus increasing the diversity of the bile acid pool [12].

These structural, protective and metabolic functions have long been attributed to the gut microbiota but the interest of the scientific community upon this collection of microorganisms began to emerge when the first studies demonstrating its envelopment in obesity were published [13, 14].

The gut microbiota in the etiology of obesity

Obesity is defined by the World Health Organization (WHO) as an excessive fat accumulation that presents a risk to health [15]. The etiology of obesity is multifactorial but, in ultimate analysis, it results from an energy imbalance in favor of body energy input. Current estimates are that 70.7% of US adults (above 20 years old) are either overweight or obese, with approximately 38% obese [16]. In Portugal, 36.5% of the adult population (18-64 years old) reveals pre-obesity while 21.6% are obese [17]. However, the main cause of concern is not obesity itself but rather the comorbidities associated with it. Overweight (30 > body mass index (BMI) ≥ 25 kg/m^2) and obese people (BMI ≥ 30 kg/m^2) are at increased risk for many chronic diseases such as type 2 diabetes, cardiovascular diseases (the major cause of global mortality) and certain types of cancer [18-20]. Besides, obesity undeniably increases the risk of mental illness such as clinical depression, anxiety and other mental disorders [21, 22].

As genetic and environmental factors failed to solely explain the magnitude of obesity epidemic, new hypotheses emerged to explain the etiopathogenesis of this condition [23].

In 2004, little attention was being paid to the gut microbiota when it appeared to be a factor affecting predisposition towards obesity [13]. Using germ-free mice (mice raised without any

exposure to microorganisms), Backhed *et al* found that mice lacking microbiota had about 40% less total body fat than conventionally raised mice [13]. In addition, the authors found that colonizing germ-free animals with gut microbiota from conventionally raised mice resulted in 60% increase of body fat mass, in only two weeks [13].

In 2006, Turnbaugh *et al* demonstrated that the gut microbiota of genetically obese mice differed from their lean littermates in the relative abundance of Firmicutes and Bacteroidetes [14]. Similar results were obtained when the authors compared the gut microbiota of lean and obese human volunteers [24]. Moreover, Turnbaugh *et al* revealed that obesity could be transmitted to germ-free mice if these animals were colonized with an 'obese' microbiota [25].

These pioneering findings have established a causal relationship between gut microbiota and obesity and have encouraged subsequent research. Thenceforward, due to the development of culture-independent techniques and omics-based approaches (metagenomics, metatranscriptomics and metabolomics), which facilitated not only the identification and classification of the microorganisms existing in the gut but also the assessment of their encoded genes (microbiome) and gene products; the number of publications regarding the gut microbiota has increased exponentially: while ten years ago a search for "gut microbiome" in PubMed would retrieve 9 results, now more than 2 000 papers are published per year.

Mechanisms linking gut microbiota to obesity

The gut microbiota plays an important role in the regulation of host energy metabolism [26-32]. The initial studies, conducted in germ-free and conventionally raised mice, demonstrated that the gut microbiota can influence both sides of the energy balance equation (energy input and energy expenditure) by increasing energy harvest from components of the diet and by affecting host genes that regulate how energy is expended and stored (Figure 1) [25, 33].

In a nutshell, dietary polysaccharides that escape digestion in the upper part of the gastrointestinal tract are utilized by the gut microbiota when they reach the colon. Gut bacteria provide glycoside hydrolases and polysaccharide lyases required to cleave glycosidic linkages in plant glycans [34, 35]. The resulting monosaccharides are either absorbed (the gut microbiota increases monosaccharide uptake from the gut [13] and facilitates their delivery to the portal circulation [36]) or metabolized into SCFA (mainly acetate, propionate and butyrate). Both are substrates for *de novo* lipogenesis in the liver [37]. Accordingly, the gut microbiota increases the expression of key transcriptional factors in the liver, carbohydrate response element binding protein (ChREBP) and sterol response element binding protein 1 (SREBP-1), that enhance *de novo* lipogenesis (Figure 1) [13].

The newly synthesized lipids are then deposited in adipocytes through a process that involves, in part, microbial suppression of the intestinal production of fasting-induced adipose factor (FIAF), a secreted lipoprotein lipase (LPL) inhibitor [13, 38]. Thus, increased hepatic lipogenesis is coordinated with increased LPL activity, to promote storage of the additional energy harvested in adipocytes (Figure 1).

On the other side of the balance equation, the gut microbiota suppresses adenosine monophosphate-activated protein kinase (AMPK) activity, the fuel gauge of eukaryotic cells. Inhibition of AMPK results in reduced muscle and liver fatty acid oxidation leading to the accumulation of the excess fatty acids in these tissues (Figure 1) [33].

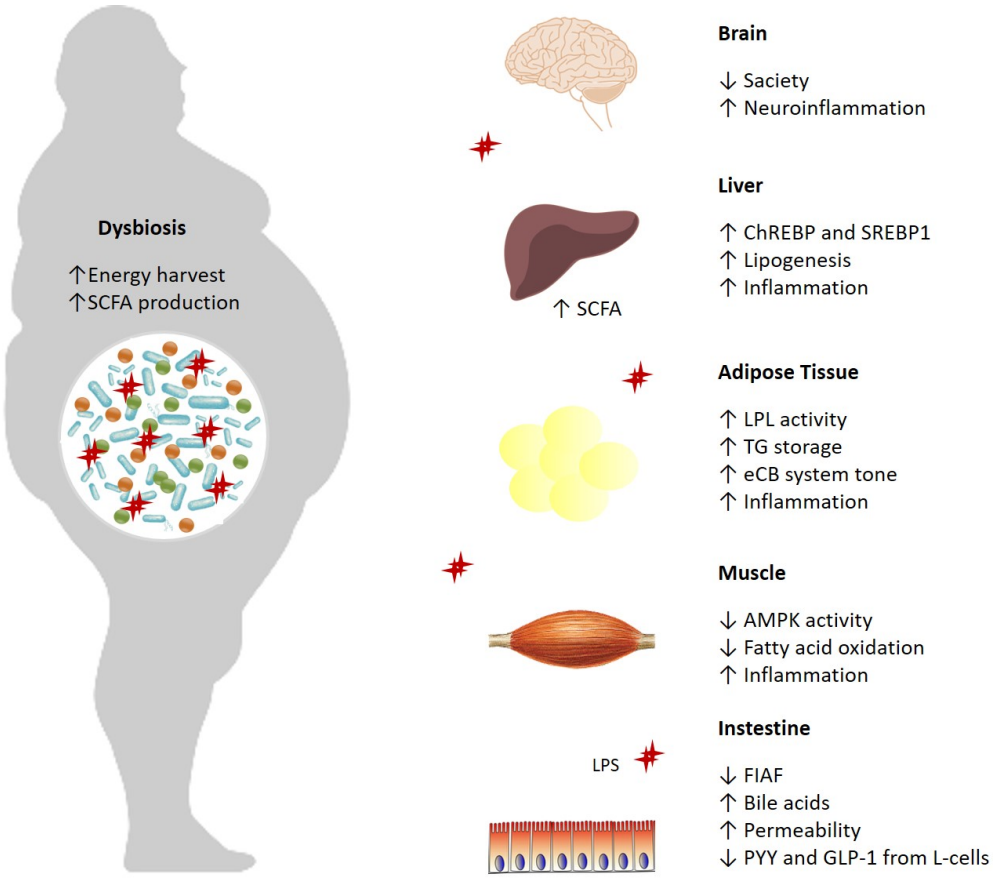


Figure 1 - Functional alterations of the gut microbiota in obesity (on the left). Proposed mechanisms by which gut microbiota regulates host energy metabolism and influences metabolic processes in peripheral organs (on the right). Adapted from Tremaroli *et al* [39]. AMPK, adenosine monophosphate-activated protein kinase; ChREBP, carbohydrate response element binding protein; eCB, endocannabinoid; FIAF, fasting-induced adipose factor; GLP-1, glucagon-like peptide-1; LPS, lipopolysaccharide; LPL, lipoprotein lipase; PYY, peptide YY; SCFA, short-chain fatty acids; SREBP-1, sterol response element binding protein 1; TG, triacylglicerides.

Genetically obese mice and obese individuals have an increased capacity to extract energy from food as their microbiome exhibit an overexpression of genes encoding enzymes able to digest complex plant-derived polysaccharides [25, 40]. As a result, they also present higher amounts of fecal SCFA [25, 41, 42]. Nevertheless, contrasting evidence has shown that SCFA interventions [43-48] as well as dietary interventions with nondigestable carbohydrates may have beneficial effects in the prevention of obesity [49-52]. SCFA can directly regulate host energy metabolism in peripheral tissues by binding to G-protein-coupled receptors (GPR41, also known as FFAR3 and GPR43, also known as FFAR2) found in colonic tissue, adipose tissue, muscle, liver and brain [53-58]. E.g. SCFA increase energy expenditure, stimulate the production of gut-derived satiety hormones PYY and GLP-1 by L-cells and enhance central appetite regulation [46, 47, 59-62]. Thus, despite the production of SCFA could be altered in obesity (including SCFA profile), the relevance of energy harvest from nondigestable carbohydrates is still debated. In the meantime, other mechanisms linking gut microbiota to obesity have been proposed.

In 2007, Cani *et al* presented lipopolysaccharide (LPS) as a triggering factor of obesity and metabolic diseases [63]. LPS is a component of gram-negative bacteria (prevailing in our gut) that can activate an extensive inflammatory cell signaling pathway when it binds to toll-like receptor 4 (TLR4) [64]. Crossing the intestinal barrier via a paracellular or a transcellular route [65], LPS may reach the systemic circulation at concentrations five times lower than those observed in sepsis, inducing a state denominated as 'metabolic endotoxemia' [63, 66]. In their work, the authors were successful in demonstrating that LPS infusions in chow-fed mice induce a metabolic response similar to high-fat (HF) diet feeding (increased body weight gain and adiposity) [63].

On the other hand, both *ob/ob* and diet-induced obesity mice display higher circulating levels of LPS than respective control mice, which is consistent with the increased gut permeability observed in these animals [66, 67]. Similarly, obese individuals [68], with type 2 diabetes [69-72] or non-alcoholic fatty liver disease (NAFLD) [73] also reveal elevated LPS levels.

LPS may be transported from the intestine towards target tissues through the newly synthesized chylomicrons in response to fat feeding [74-76]. Once in circulation, LPS may activate CD14/TLR4 signaling in adipose tissue [69], muscle [77], liver [78] and brain [79, 80]. These data support the hypothesis that LPS is involved in the onset of the low-grade inflammatory status that characterizes obesity and insulin resistance [81].

Among the putative mechanisms linking the gut microbiota to obesity, a role for the endocannabinoid system has also been proposed, probably involving LPS and its influence on

cannabinoid receptors signaling [82-85]. Activation of the endocannabinoid (eCB) system in the intestine (e.g. by the gut microbiota) increases gut permeability, which enhances LPS levels and, consequently, eCB system tone in peripheral tissues [85]. Increased eCB system tone in adipose tissue stimulates adipogenesis and promotes adipose tissue expansion [86]. Therefore, the crosstalk between gut microbiota and adipose tissue may be mediated by the eCB system.

During the last decade, bile acids have emerged as novel metabolic modulators. The major function of bile acids is to facilitate the emulsification of dietary fats and to aid the intestinal absorption of lipids and lipophilic vitamins [12]. Nevertheless, recent evidence have shown that bile acids represent signaling molecules in the host with the capacity to regulate cellular and metabolic activities by activating the farnesoid-X-receptor (FXR), the vitamin D receptor (VDR) or the G protein-coupled bile acid receptor TGR5 [87-89]. E.g. TGR5 signaling in enteroendocrine L-cells induces secretion of GLP-1, thereby enhancing glucose tolerance [88]. Since different receptors have different affinities for individual bile acids, subtle variations in the bile acid signature may alter the signaling properties of the bile acid pool with a subsequent impact on host physiology [12]. These variations are likely to occur whenever gut microbiota is perturbed. In diet-induced obesity models, higher amounts of bile acids are released into the small intestine in response to the fat content of the diet [90]. Given their amphipathic properties, bile acids may damage bacterial cell membranes as well as host cell membranes by interacting with membrane phospholipids [91]. Therefore, in addition to their role in lipid and glucose metabolism, bile acids may contribute to explain the gut microbiota alterations and the increased gut permeability associated to obesity [92-94].

In summary, the gut microbiota is an important organ involved in the maintenance of host energy homeostasis by means of a molecular crosstalk. Thus, the gut microbiota as well as gut microbial metabolites constitute potential targets for the treatment of obesity and metabolic disorders such as, type 2 diabetes, cardiovascular diseases and NAFLD. In this regard, the identification of robust biomarkers, on the basis of gut microbiota, for the early diagnosis of obesity related metabolic diseases is warranted.

The microbiota-gut-brain axis

Besides having an important role in the regulation of host energy metabolism, the gut microbiota can also influence neurodevelopment, modulate behavior and contribute to the development of neurological disorders [95-100]. Resembling obesity, behavioral phenotypes can be transmitted to germ-free mice after gut microbiota transplantation [101].

The microbiota-gut-brain axis encompasses the strong bidirectional communication between the gut microbiota and the central nervous system (CNS) [102]. Multiple mechanisms may be involved in this bilateral communication, including immune (cytokines), endocrine (cortisol) and neural (vagus and enteric nervous system) pathways (Figure 2). However, while the effects of the autonomic nervous system on gut physiology have long been understood, the effects of gut microbiota on CNS physiology are still being unraveled.

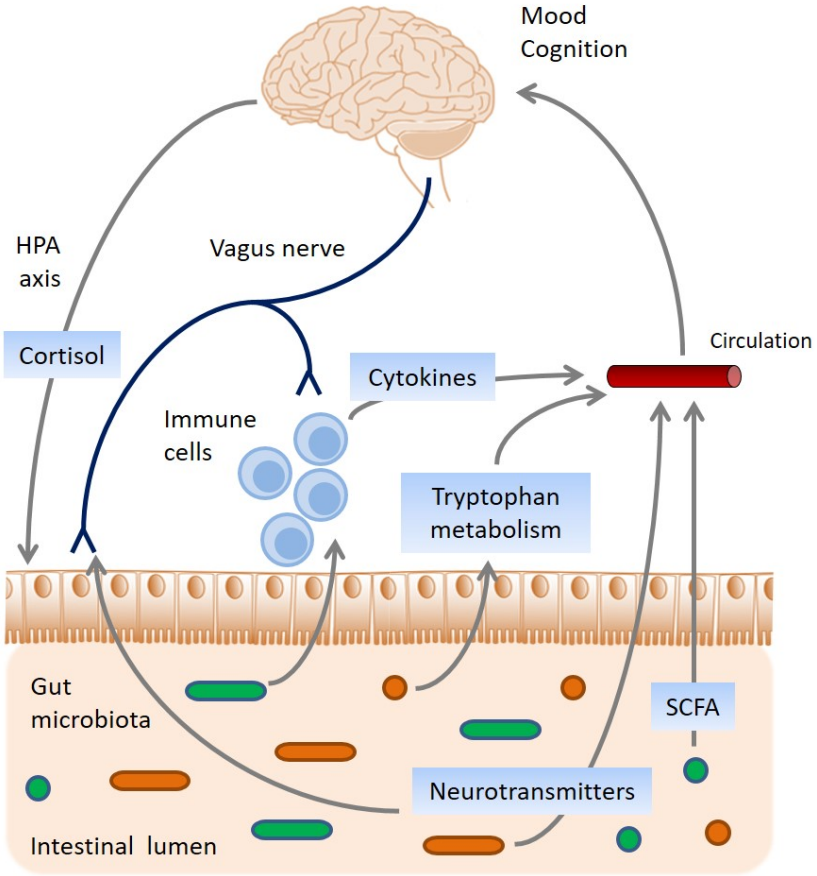


Figure 2 - Pathways involved in the bidirectional communication between gut microbiota and brain; the microbiota-gut-brain axis. Adapted from Cryan et al [103]. HPA, hypothalamic-pituitary-adrenal; SCFA, short-chain fatty acids.

Gut microbial metabolites such as SCFA are signaling molecules in the host, as described in the previous section. Nonetheless, SCFA have not only a significant role in the regulation of satiety, but also exhibit different neuroactive properties [99, 104, 105]. Besides, gut bacteria have the capacity to produce many neurotransmitters and neuromodulators such as gamma-aminobutyric acid (GABA), noradrenaline, serotonin and acetylcholine (Figure 2) [106, 107]. In addition, it has the ability to control host tryptophan metabolism, regulating the fraction of tryptophan available for serotonin synthesis and, on the other hand, the production of

neuroactive and neurotoxic metabolites [108-110]. Lastly, the gut microbiota can induce the secretion of neurotrophic factors by intestinal smooth muscle cells such as the brain-derived neurotrophic factor (BDNF), an important plasticity-related protein that promotes neuronal growth, development and survival [111]. Therefore, the gut microbiota can communicate with the brain by several mechanisms and may be implicated in the development of many neurologic conditions.

One of the key players in the development of neuropsychiatric comorbidities in obesity is the inflammatory process [112]. Inflammation can be originated in the gut, since gut microbiota alterations in obesity may increase intestinal permeability and, consequently, LPS circulating levels, leading to the development of chronic low-grade endotoxemia. The increased production of proinflammatory cytokines (e.g. interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α)) by immune cells may, ultimately, activate microglia within CNS. Nevertheless, while microglia activation normally exerts a protective action on the CNS, its unregulated and chronic activation may, in contrast, become deleterious, causing significant alterations in CNS functions (e.g. changes in neuroendocrine function, neurocircuitry, enzymatic pathways, and neurotransmitter metabolism/function) [113]. Thus, the gut microbiota can be viewed as a key triggering factor of inflammation-driven neuropsychiatric comorbidities. For that reason, modulation of the gut microbiota might be a tractable strategy to the development of novel therapeutics for complex CNS disorders, including those associated with obesity.

Modulation of gut microbiota by dietary factors

Diet is an important factor that can rapidly induce changes in gut microbiota composition [114-117]. Gut microbiota modifications in response to dietary changes may occur within 24-48 h [115]. These fast and diet-induced dynamics are supported by studies in which participants switched between plant- and meat-based diets. Animal-based diets increased the abundance of bile-tolerant bacteria (Alistipes, Bilophila and Bacteroides) while plant-based diets increased the levels of bacteria belonging to the Firmicutes phylum that have the ability to metabolize plant polysaccharides (Roseburia, *Eubacterium rectale* and *Ruminococcus bromii*) [115]. These dietary shifts alter the nutrients available for gut bacteria, favoring the growth of specific species, thus resulting in modifications in gut microbiota composition. Nevertheless, long-term dietary habits are a dominant force in determining the gut microbiota composition of an individual [118]. Despite the gut microbiota composition can detectably change within 24 h, enterotype identities may remain stable even after 10-day dietary interventions [5, 119].

On the other hand, this 'hidden' metabolic organ, as it is commonly referred, has also an important role in arbitrating the impact of dietary interventions on host metabolism. I.e., the effects of dietary interventions on host metabolic parameters may rely on the gut microbiota composition. For instance, dietary interventions with complex carbohydrates may increase microbiota diversity and the metabolic output (SCFA), but only if individuals have a diverse microbiota at baseline [120]. When the diversity of the gut microbiota is low, residual bacteria will not be able to handle all the complex carbohydrates available from the diet and the diversity of the ecosystem will probably remain low [118].

The gut microbiota plays therefore an important role at the intersection of diet and health. The resistance to ecological stress and the ability to recover from a stress-related perturbation (resilience) are the characteristics of a healthy microbiota that empowers its maintenance throughout life [121]. When the microbial ecosystem is perturbed to an extent that exceeds its resistance and resilience capabilities, alterations in its composition and function may occur (dysbiosis) [122].

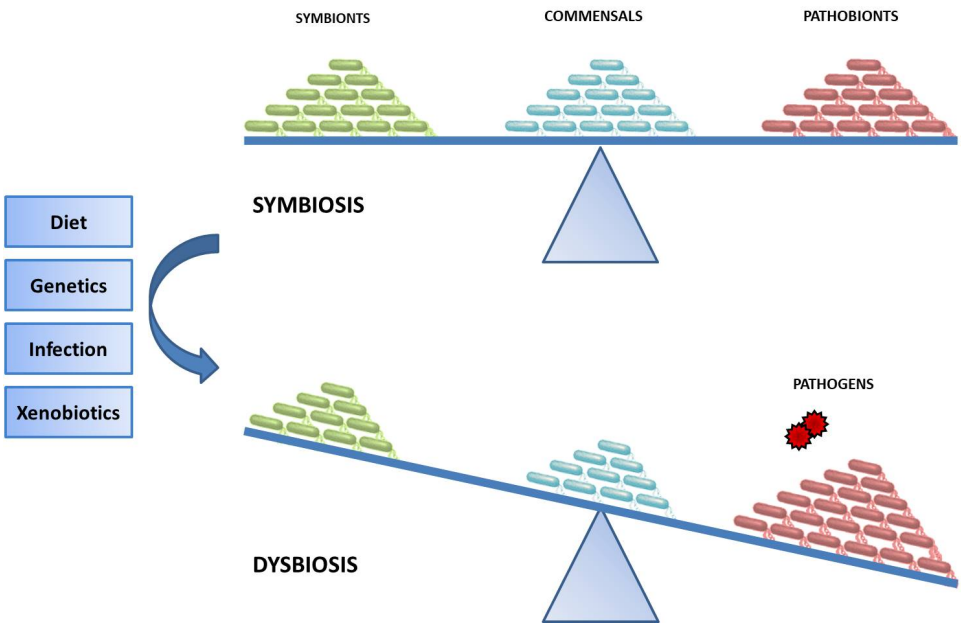


Figure 3 - Adapted from Round et al. [123] Dysbiosis typically features one or more of the following non-mutually exclusive characteristics: (i) loss of beneficial microbial organisms, (ii) expansion of pathobionts or potentially harmful microorganisms and (iii) loss of overall microbial diversity (both in terms of richness and evenness). Beyond diet, other factors may influence the composition of the intestinal microbial community. Xenobiotics such as antibiotics have the potential to dramatically alter the homeostatic commensal colonization [124-126]. Infection and

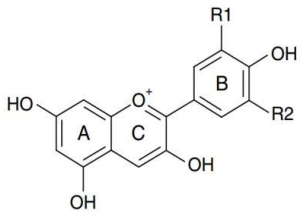
inflammation can also compromise the microbiota's ability to provide colonization resistance against invading microorganisms [127, 128]. In addition, genetic background is a stronger determinant of the gut microbiota composition [129, 130].

The expansion of pathobionts (commensal microorganisms that can cause pathology under uncontrolled proliferation), the loss of beneficial bacteria and the loss of diversity (microbial richness) are common characteristics of a dysbiotic state (Figure 3) [122, 131]. These features have detrimental consequences for the host (may initiate obesity and metabolic diseases as well as neurologic disorders) and can be caused by environmental factors such as dietary modifications that encompass high contents of fat. Other factors have been suggested as potential instigators of dysbiosis such as non-caloric artificial sweeteners [132, 133] and dietary emulsifiers [134, 135].

Anthocyanidins, a particular class of flavonoids

Polyphenols are phytochemicals that result from the secondary metabolism of plants. These chemicals are not essential for the immediate survival of plants but increase their chances to survival in hostile environments. They are important to plant defense mechanisms, propagation and development and contribute to their colors, aroma, flavor, bitterness and astringency [136].

Structurally, flavonoids are polyphenols characterized by a flavan nucleus which consists in a C6-C3-C6 structure [137]. Flavonoids are divided into 14 different classes. The level of oxidation and the pattern of substitution at the C ring dictate the class to which flavonoids belong [137, 138].



R1=H; R2=H: pelargonidin
R1=OH; R2=H: cyanidin
R1=OH; R2=OH: delphinidin
R1=OMe; R2=OH: petunidin
R1=OMe; R2=OMe: malvidin

Figure 4 - Representation of the general structure of anthocyanidins (flavylium form). These compounds differ in the methoxyl and hydroxyl substitution pattern of the aromatic B ring.

Anthocyanidins are a particular class of flavonoids. Attending to their chemical nature, anthocyanins naturally occur as glycosides of flavylium (2-phenylbenzopyrylium) salts and are commonly based on six anthocyanidins: pelargonidin, cyanidin, delphinidin, petunidin and malvidin (Figure 4). The sugar moieties vary but are usually glucose, rhamnose, galactose or

arabinose. The most widespread anthocyanins are glycosylated in the 3-OH position (3-O-monoglycosides) [139].

These compounds are naturally part of our diet as they can be found mainly in red wine and red fruits such as cherries, strawberries, plums, blackberries, raspberries, grapes, red currants and black currants but also in some cereals and root vegetables (e.g. aubergines, beans, cabbage, radishes and onions) [140].

The bioavailability of anthocyanins, i.e. the percentage of anthocyanins that reach the bloodstream after an administered dose, is considered to be low. Nevertheless, this might be attributed to several major drawbacks or limitations of the current studies [141]. In fact, the bioavailability of anthocyanins is the most difficult to assess amongst all flavonoids as a result of their occurrence under different structures depending on pH [142].

The analysis of anthocyanins and their metabolites in biological samples (plasma, urine and feces) requires important steps such as sample preparation and purification, which are decisive for an accurate estimation of anthocyanins bioavailability. Nonetheless, the analytical detection of anthocyanins and anthocyanin metabolites in biological samples is not an easy task since it requires the appropriate analytic standards that are not commercially available and have to be chemically synthetized. For that reason, the majority of studies have only looked for the parent structure of anthocyanins, underestimating the total anthocyanins content that might actually reach the target organs [143]. Besides, anthocyanins bioavailability might also be strongly influenced by the food matrix. In red wine, anthocyanins are consumed concomitantly with ethanol but it remains unclear whether ethanol enhances anthocyanins bioavailability.

Despite all these issues, anthocyanins are particularly interesting since those that are not absorbed in the small intestine might be metabolized by the gut microbiota when reaching the colon [144]. Microbial metabolism of anthocyanins may alter the bioactivity of these compounds but, on the other hand, may modulate bacterial growth. Only a few studies have analyzed the effect of anthocyanins on gut microbiota modulation and it remains unclear whether they can be used as prebiotics [145-147].

Moreover, anthocyanins have emerged as anti-inflammatory agents and are promising candidates for the prevention of neuroinflammation, a common hallmark of obesity-associated neuropsychiatry disorders [148-151]. The mechanisms underlying these effects might be related to the interaction of anthocyanins with neuron and microglia biology. Recent studies suggest

that anthocyanins may protect neurons against neuroinflammatory injury by stimulating the production of proteins involved in synaptic plasticity [148, 152].

Anthocyanins and their metabolites are able to cross the blood-brain barrier [153]. Nonetheless, to be neuroprotective, anthocyanins do not necessarily need to reach the brain. By changing the gut microbiota and acting on the gut-brain axis, anthocyanins may exert a biological activity even without being absorbed. However, whether the anti-neuroinflammatory properties of anthocyanins are related to the gut microbiota changes that these compounds might bring about remains to be unraveled.

The general aim of this investigation was to unravel the effects of anthocyanins on gut microbiota, a metabolic organ that can communicate with the brain and might be implicated in obesity related neuropsychiatry disorders. Additionally, this study aimed to clarify the bioavailability of anthocyanins.

The specific aims, related to our different approaches, were the following:

Chapter II

- To compare the metabolic and gut microbiota alterations driven by HF diet, in two Rat models of diet-induced obesity;

- To track potential plasma markers of intestinal inflammation and permeability as well as gut microbiota dysbiosis, in the most suitable Rat model of HF diet-induced obesity;

- To highlight HF diet-induced dysbiosis as a likely cause of neuroinflammation;

- To investigate whether blackberry anthocyanins can counteract HF-diet induced dysbiosis and whether gut microbiota modulation is behind the neuroprotective effects of anthocyanins.

Chapter III

- To evaluate the bioavailability of anthocyanins after blackberries consumption and the effect of ethanol on the bioavailability of these compounds.

CHAPTER II

A. "HIGH-FAT DIET-INDUCED OBESITY RAT MODEL: A COMPARISON BETWEEN WISTAR AND SPRAGUE-DAWLEY RAT"

ADIPOCYTE, 2015. 5(1):11-21

DOI: 10.1080/21623945.2015.1061723

B. "THE ROLE OF I-FABP AS A BIOMARKER OF INTESTINAL BARRIER DYSFUNCTION DRIVEN BY GUT MICROBIOTA CHANGES IN OBESITY"

NUTRITION & METABOLISM, 2016. 13(31)

DOI: 10.1186/S12986-016-0089-7

C. "HIGH-FAT DIET-INDUCED DYSBIOSIS AS A CAUSE OF NEUROINFLAMMATION"

BIOLOGICAL PSYCHIATRY, 2016. 80(1):e3-4.

DOI: 10.1016/J.BIOPSYCH.2015.10.027

D. "TARGETING GUT-BRAIN AXIS WITH ANTHOCYANINS: A NEW CLASS OF PSYCHOBIOTICS"

SUBMITTED

ADIPOCYTE
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RESEARCH PAPER

High-fat diet-induced obesity Rat model: a comparison between Wistar and Sprague-Dawley Rat

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ABSTRACT

In the past decades, obesity and associated metabolic complications have reached epidemic proportions. For the study of these pathologies, a number of animal models have been developed. However, a direct comparison between Wistar and Sprague-Dawley (SD) Rat as models of high-fat (HF) diet-induced obesity has not been adequately evaluated so far. Wistar and SD rats were assigned for 2 experimental groups for 17 weeks: standard (St) and high-fat (HF) diet groups. To assess some of the features of the metabolic syndrome, oral glucose tolerance tests, systolic blood pressure measurements and blood biochemical analysis were performed throughout the study. The gut microbiota composition of the animals of each group was evaluated at the end of the study by real-time PCR. HF diet increased weight gain, body fat mass, mesenteric adipocyte's size, adiponectin and leptin plasma levels and decreased oral glucose tolerance in both Wistar and SD rats. However, the majority of these effects were more pronounced or earlier detected in Wistar rats. The gut microbiota of SD rats was less abundant in Bacteroides and Prevotella but richer in Bifidobacterium and Lactobacillus comparatively to the gut microbiota of Wistar rats. Nevertheless, the modulation of the gut microbiota by HF diet was similar in both strains, except for Clostridium leptum that was only reduced in Wistar rats fed with HF diet. In conclusion, both Wistar and SD Rat can be used as models of HF diet-induced obesity although the metabolic effects caused by HF diet seemed to be more pronounced in Wistar Rat. Differences in the gut microbial ecology may account for the worsened metabolic scenario observed in Wistar Rat.

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Introduction

Obesity (especially visceral obesity) is a key feature of the metabolic syndrome, a set of interrelated risk factors for cardiovascular disease and diabetes, that also includes dysglycemia, raised blood pressure, elevated triglyceride levels and low high density lipoprotein cholesterol levels.[1]

In the past decades, obesity and associated metabolic complications have reached epidemic proportions.[2] For a better understanding of these pathologies and to evaluate potential treatments for the metabolic syndrome, a number of experimental animal models have been developed.[3,4]

Despite the multifactorial etiology of obesity, the rate at its incidence is increasing suggests that environmental and behavioral factors (including dietary factors) have been the major contributors to the obesity epidemic rather than genetic changes.[5] For this reason, instead of monogenetic models, polygenetic animal models of diet-induced obesity have been preferentially used.

Several weeks into a regimen of a semi-purified diet with a fat content of more than 40% energy based on animal fats can lead to obesity, hyperglycemia, hypertriglyceridemia and hyperleptinemia in rodents, mimicking the pathophysiology of human obesity and metabolic syndrome.[6] Wistar and Sprague-Dawley (SD) Outbred Rat can be considered the standard rodents for this experiment type since they are susceptible to diet-induced obesity and insulin resistance with individual variations.[6] Furthermore, due to their larger size, the evaluation of some metabolic parameters such as blood pressure is facilitated over mice.

However, a direct comparison between Wistar and SD Rat as models of high-fat (HF) diet-induced obesity has not been adequately evaluated so far. There are studies reporting some metabolic changes caused by HF diet in Wistar but not in SD rats.[7,8] Nevertheless, it is difficult to

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Table 1. Primer sequences and real-time PCR conditions used for gut microbiota analysis

Target group	Primer sequence (5′-3′)	Genomic DNA Standard	PCR product Size (bp)	AT	Reference
Firmicutes	ATG TGG TTT AAT TCG AAG CA AGC TGA CGA CAA CCA TGC AC	*Lactobacillus gasseri* ATCC 33323	126	60°C	[38]
Bacteroidetes	CAT GTG GTT TAA TTC GAT GAT AGC TGA CGA CAA CCA TGC AG	*Bacteroides vulgatus* ATCC 8482	126	60°C	[38]
Lactobacillus	GAG GCA GCA GTA GGG AAT CTT C GGC CAG TTA CTA CCT CTA TCC TTC TTC	*Lactobacillus gasseri* ATCC 33323	126	60°C	[39]
Enterococcus	CCC TTA TTG TTA GTT GCC ATC ATT ACT CGT TGT ACT TCC CT TGT	*Enterococcus gilvus* ATCC BAA-350	144	61°C	[40]
Clostridium leptum	GCA CAA GCA GTG GAG T CTT CCT CCG TTT TGT CAA	*Clostridium leptum* ATCC 29065	239	60°C	[41]
Bacteroides	ATA GCC TTT CGA AAG RAA GAT CCA GTA TCA ACT GCA ATT TTA	*Bacteroides vulgatus* ATCC 8482	495	60°C	[42]
Prevotella	CAC RGT AAA CGA TGG ATG CC GGT CGG GTT GCA GAC C	*Prevotella nigrescens* ATCC 33563	513	55°C	[42]
Bifidobacterium	CGC GTC YGG TGT GAA AG CCC CAC ATC CAG CAT CCA	*Bifidobacterium longum subsp. Infantis* ATCC 15697	244	60°C	[39]

AT, annealing temperature; bp, base pairs.

attribute those variations to the strain of the animal used since there are other variables like the age of animals, the duration of the study and the composition of the HF diet used that can also be behind those divergences. Therefore, in the present study, Wistar and SD rats were studied in parallel to evaluate the metabolic effects of an HF diet in comparison to a standard chow, in both strains. To assess some of the features of the metabolic syndrome, oral glucose tolerance tests, systolic blood pressure measurement, and blood biochemical analysis were performed. Given the growing body of evidence demonstrating the prominent role of gut microbiota in energy balance and metabolism, the gut microbiota composition and its modulation by HF diet were also evaluated in both strains.

Results

Energy ingestion, weight gain and body fat composition

HF diet increased energy ingestion, weight gain and fat mass and reduced water consumption in both Wistar and Sprague-Dawley rats ($P < 0.001$) (**Fig. 1** and **Table 2**).

Energy ingestion was higher in HF diet groups of both strains since the beginning of the study ($P < 0.05$) (**Fig. 1A**). However, the average of the energy ingested per day during the entirely study was significantly higher in Wistar rats fed with HF diet than in SD rats fed with the same diet (74.7 ± 1.4 vs 66.6 ± 1.3 Kcal/day, interaction $P = 0.001$) (**Table 2**).

Wistar rats on HF diet became heavier than their St counterparts from the 4th week of the study while SD rats became heavier only from the 7th week of the study ($P < 0.05$) (**Fig. 1B**). Nevertheless, HF diet caused a significant increase in total weight gain in both strains ($P < 0.001$) (**Table 2**). However, while the increase observed in Wistar rats was 66.9 ± 13.4% (from 190.0 ± 9.1 to 317.2 ± 25.5 g), in SD rats was only 32.2 ± 6.9% (from 208.8 ± 11.5 to 276.2 ± 14.3 g) ($P < 0.05$).

Through bioelectrical impedance it was possible to estimate the total fat mass of the animals, at the end of the study. HF diet lead to an increase of total fat mass in

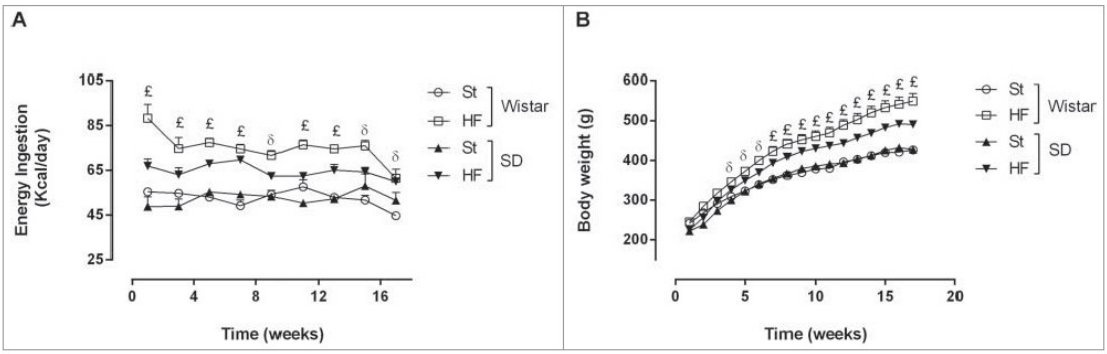

Figure 1. Energy ingestion (A) and body weight (B) of Wistar and Sprague-Dawley (SD) rats fed either with standard (St) or high-fat (HF) diet along the 17 weeks of the study. Data are presented as mean ± SEM (n = 6 rats per group). $^{£}P < 0.05$ between St and HF diet groups of both strains and $^{\delta}P < 0.05$ between St and HF diet groups of Wistar rats.

Table 2. Energy intake, weight gain and body fat mass of Wistar and Sprague-Dawley (SD) rats fed either with standard (St) or high-fat (HF) diet during 17 weeks

	Wistar Rat		SD Rat		Two-way ANOVA *P* values		
	St	HF	St	HF	Diet	Strain	Interaction
Energy Ingested (Kcal/day)	53.0 ± 1.2	74.7 ± 1.4*	53.7 ± 0.6	66.6 ± 1.3*,†	<0.001	0.005	0.001
Drink Ingested (mL)	31.9 ± 1.9	24.1 ± 1.1	29.6 ± 0.7	24.0 ± 1.4	<0.001	0.371	0.437
Weight Gain (g)	190.0 ± 9.1	317.2 ± 25.5	208.8 ± 11.5	276.2 ± 14.3	<0.001	0.506	0.082
Fat Mass (g)	181.3 ± 6.8	259.2 ± 16.8	179.3 ± 6.1	218.1 ± 8.5	<0.001	0.053	0.076

Data are presented as mean ± SEM (n = 6 rats per group).
*$P < 0.05$ vs respective St diet group and †$P < 0.05$ between HF diet groups.

both strains ($P < 0.001$). The increase observed was 43.0 ± 9.3% (from 181.3 ± 6.8 to 259.2 ±16.8 g) and 21.6 ± 4.7% (from 179.3 ± 6.1 to 218.1 ± 8.5 g) in Wistar and SD rats, respectively. However, the difference between these results did not reach statistical significance ($P = 0.068$).

Glycaemic response

OGTTs were performed in the middle (**Fig. 2**) and at the end of the study (**Fig. 3**) to evaluate the effects of HF diet in glycaemic response of both strains. During OGTTs, blood glucose was affected by time ($P < 0.001$) (**Figs. 2A and 3A**). Total area under the curve (AUC) of the glycaemic response was calculated for each experimental group (**Figs. 2B and 3B**). While at the end of the study, HF diet increased the AUC independently of the strain ($P < 0.05$), at the 9th week of the study this effect was only visible in Wistar rats (interaction $P = 0.003$).

Fasting insulin levels were doubled in Wistar rats fed with HF diet compared to those in the St diet group right after 9 weeks of HF feeding (interaction $P = 0.017$)

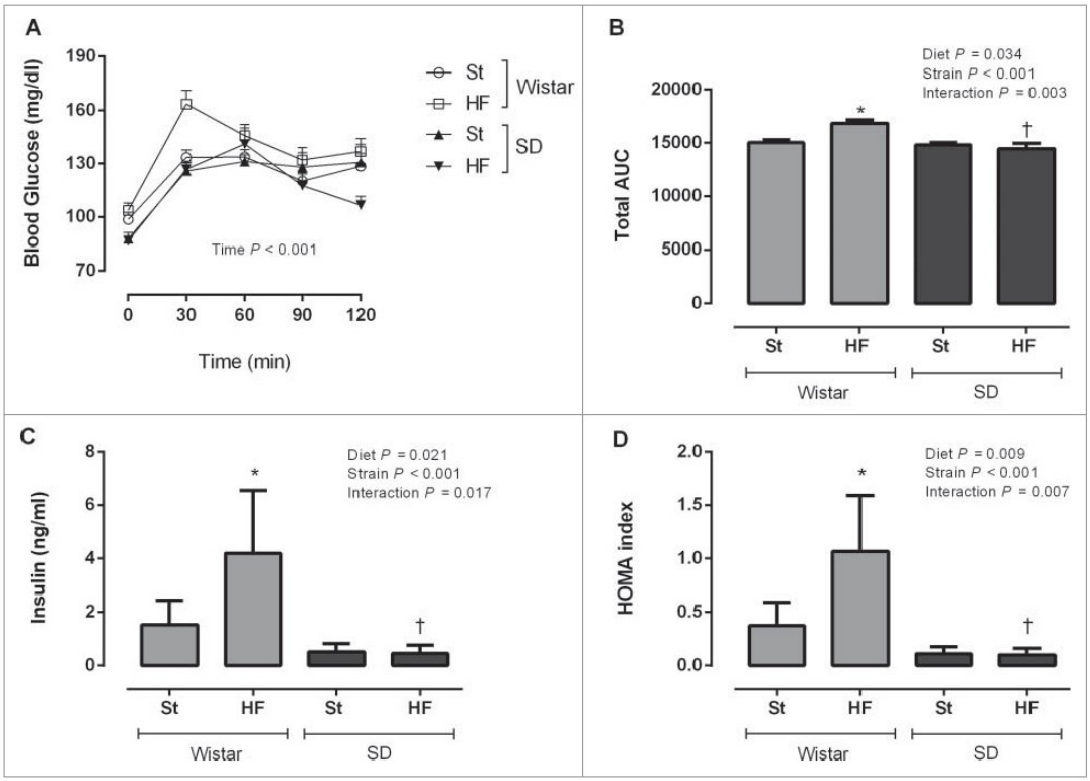

Figure 2. Glycaemic response during oral glucose tolerance test (A), total area under the curve (AUC) (B), fasting insulin plasma levels (C) and homeostasis model assessment (HOMA) (D) of Wistar and Sprague-Dawley (SD) rats after 9 weeks of feeding either with standard (St) or high-fat (HF) diet. HOMA was calculated using the formula: fasting glucose (mg/dl) × fasting insulin (ng/ml)/405. Data are presented as mean ± SEM (n = 5–6 rats per group). *$P < 0.05$ vs respective St diet group and †$P < 0.05$ between HF diet groups.

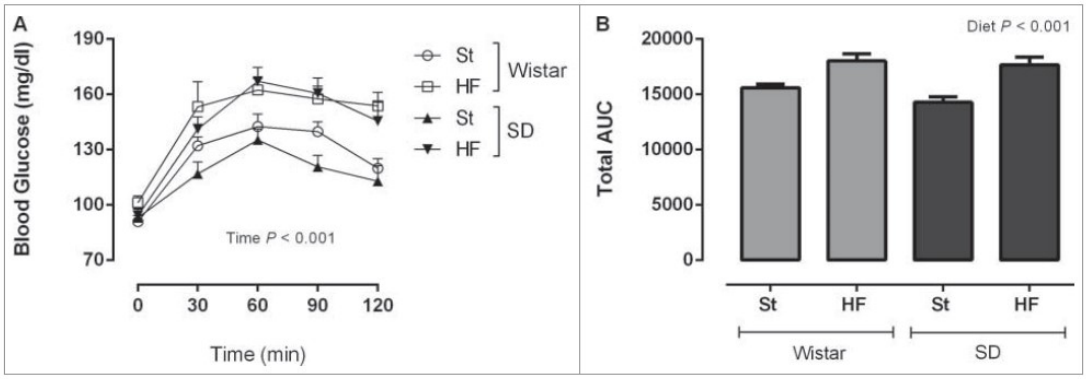

Figure 3. Glycaemic response during oral glucose tolerance test (A) and total area under the curve (AUC) (B) of Wistar and Sprague-Dawley (SD) rats after 16 weeks of feeding either with standard (St) or high-fat (HF) diet. Data are presented as mean ± SEM (n = 6 rats per group).

(Fig. 2C). Similarly, homeostasis model assessment (HOMA) of insulin resistance indicated that these animals were fold2- less insulin sensitive (interaction $P = 0.007$) (Fig. 2D). Insulin sensitivity remained unchanged in SD rats fed with HF diet in comparison with their St counterparts (Fig. 2C and 2D) which is in accordance to the results obtained for this strain in OGTT at the 9th week of the study.

Systolic blood pressure

After 15 weeks, HF diet was not sufficient to significantly cause an elevation of SBP in rats of both strains (Fig. 4A). SBP values did not differ either between strains.

Blood and urine biochemical profile

Values for the different biochemical parameters evaluated in serum and urine of Wistar and SD rats, fed either with St or HF diet, are displayed in Table 3. In general, HF diet did not change the values of the majority of biochemical markers analyzed, independently of the strain of Rat. However, HF diet decreased serum albumin ($P = 0.048$) and increased urinary urea ($P = 0.020$). Nevertheless, HF diet also caused a noticeable increase in serum urea (from 30.2 ± 0.7 to 36.6 ± 2.1 mg/dL, $P < 0.05$) and alkaline phosphatase (from 79.7 ± 5.2 to 132.0 ± 13.6 U/L, $P < 0.05$) but only in Wistar rats (interaction $P < 0.05$). A trend to increase serum creatinine and triglycerides was also observed in Wistar rats (interaction $P = 0.062$ and $P = 0.064$, respectively).

Serum creatinine, triglycerides, albumin and urinary creatinine values were significantly different between strains ($P < 0.05$). Serum creatinine values were higher in SD rats as well as urinary creatinine values ($P < 0.05$) while the serum values of triglycerides and albumin were more elevated in Wistar rats ($P < 0.05$).

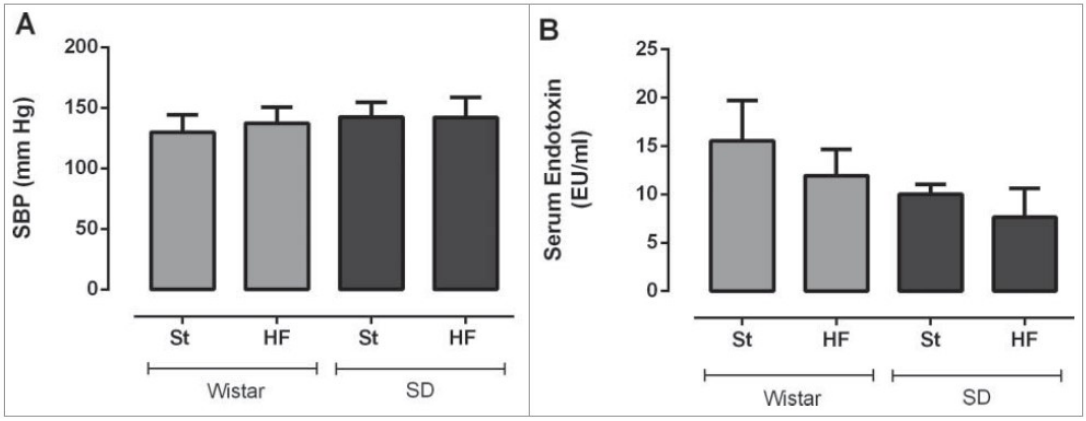

Figure 4. Systolic blood pressure (A) and serum endotoxin levels (B) of Wistar and Sprague-Dawley (SD) rats fed either with standard (St) or high-fat (HF) diet during 17 weeks. SBP measurements were recorded at 15th week of the study. Data are presented as mean ± SEM (n = 5–6 rats per group).

Table 3. Biochemical markers evaluated in serum and urine of Wistar and Sprague-Dawley (SD) rats fed either with standard (St) or high-fat (HF) diet during 17 weeks

	Wistar Rat		SD Rat		Two-way ANOVA *P* values		
	St	HF	St	HF	Diet	Strain	Interaction
Serum							
Creatinine (mg/dL)	0.40 ± 0.04	0.52 ± 0.02	0.67 ± 0.04	0.67 ± 0.03	0.098	**<0.001**	0.062
Urea (mg/dL)	30.2 ± 0.7	36.6 ± 2.3	35.0 ± 2.4	34.0 ± 0.7	0.121	0.513	**0.039**
Uric Acid (mg/dL)	0.83 ± 0.08	0.92 ± 0.12	1.00 ± 0.27	0.85 ± 0.12	0.773	0.699	0.425
Total Cholesterol (mg/dL)	103.8 ± 7.2	105.6 ± 8.6	101.8 ± 8.2	80.0 ± 4.0	0.177	0.069	0.115
Triglycerides (mg/dL)	150.2 ± 21.3	185.4 ± 14.9	62.7 ± 12.5	40.7 ± 3.1	0.654	**<0.001**	0.064
Albumin (g/dL)	3.7 ± 0.1	3.5 ± 0.1	3.3 ± 0.2	2.9 ± 0.1	**0.048**	**0.003**	0.296
Alkaline Phosphatase (U/L)	79.7 ± 5.2	132.0 ± 14.9	141.0 ± 14.2	118.0 ± 9.7	0.214	0.052	**0.004**
AST (U/L)	191.3 ± 39.0	168.6 ± 20.8	130.7 ± 28.4	135.8 ± 26.1	0.774	0.138	0.649
ALT (U/L)	47.2 ± 4.6	41.4 ± 4.1	51.5 ± 3.9	59.2 ± 9.3	0.883	0.095	0.296
Urine							
Creatinine (g/day)	0.010 ± 0.000	0.012 ± 0.002	0.016 ± 0.004	0.017 ± 0.002	0.606	**0.023**	0.825
Urea (g/day)	0.17 ± 0.02	0.24 ± 0.01	0.17 ± 0.01	0.21 ± 0.03	**0.020**	0.476	0.709

Data are presented as mean ± SEM (n = 5–6 rats per group).
$^{*}P < 0.05$ vs respective St diet group and $^{\ddagger}P < 0.05$ between St diet groups.
ALT, alanine transaminase; AST, aspartate transaminase.

Contrarily to what was expected, HF feeding did not increase endotoxin serum values, in both strains (**Fig. 4B**).

Adiponectin and leptin plasma levels (**Fig. 5A and 5B**) were higher in Wistar rats ($P < 0.05$) and when animals of both strains were fed with HF diet ($P < 0.05$). However, the increase in leptin plasma levels caused by HF diet was more pronounced in Wistar than in SD rats (interaction $P = 0.001$).

Adipocyte area

HF diet increased the area of adipocytes from mesenteric adipose tissue ($P < 0.05$, **Fig. 6A and 6B**). The effects of HF diet on adipocyte area did not differ between strains.

Gut microbiota

HF diet decreased Firmicutes, Bacteroidetes, Lactobacillus and Prevotella ($P < 0.05$) and increased Firmicutes to Bacteroidetes ratio and Bacteroides ($P < 0.05$), as displayed in **Table 4**.

A significant interaction between strain and diet was found for Firmicutes, Prevotella and Lactobacillus ($P < 0.05$). Accordingly, although HF diet decreased these bacterial groups in both strains, the effects on Firmicutes and Prevotella were more evident in Wistar rats while the effects on Lactobacillus were more noticeable in SD rats.

Clostridium leptum was reduced (a reduction of almost fold3-) only in Wistar rats fed with an HF diet (interaction $P = 0.010$).

The composition of the gut microbiota differ between the 2 strains of Rat, namely in Bacteroidetes, Firmicutes to Bacteroidetes ratio, Lactobacillus, Bacteroides, Prevotella and Bifidobacterium. The gut microbiota of SD rats was less abundant in Bacteroides and Prevotella. Since

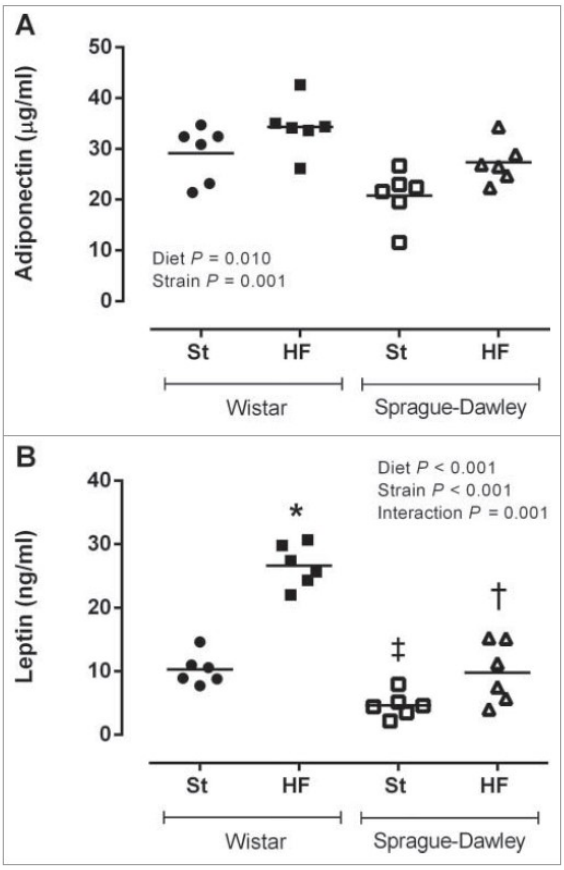

Figure 5. Adiponectin (A) and leptin plasma levels (B) of Wistar and Sprague-Dawley (SD) rats fed either with standard (St) or high-fat (HF) diet during 17 weeks. Data are presented as mean ± SEM (n = 6 rats per group). $^{*}P < 0.05$ vs respective St diet group, $^{\ddagger}P < 0.05$ between St diet groups and $^{\dagger}P < 0.05$ between HF diet groups.

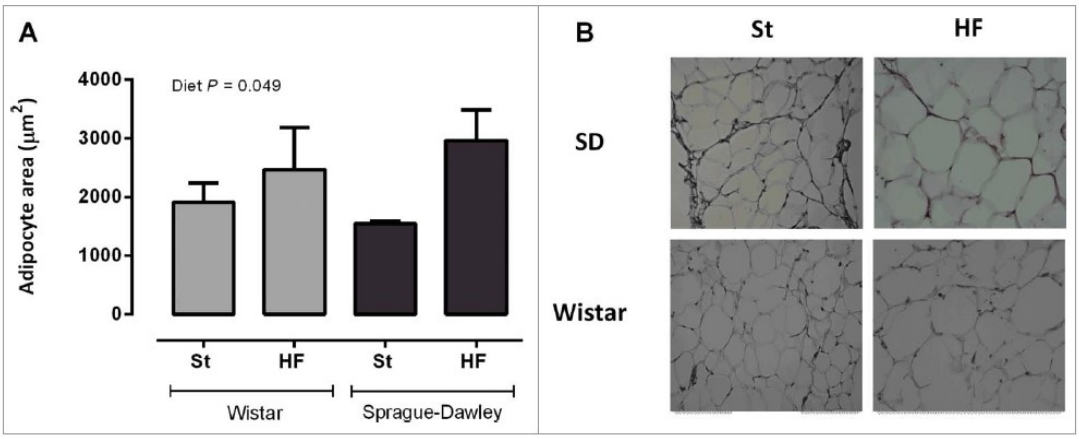

Figure 6. Mesenteric adipocyte's area (A) of Wistar and Sprague-Dawley (SD) rats fed either with standard (St) or high-fat (HF) diet during 17 weeks. Data are presented as mean ± SEM (n = 6 rats per group). (B) Representative images of hematoxylin and eosin stained-adipose tissue sections for each experimental group.

these 2 bacterial groups belong to Bacteroidetes phylum, the number of copies of Bacteroidetes was lower in SD rats. As a result, the Firmicutes to Bacteroidetes ratio was more elevated in this strain of Rat. On the other hand, the gut microbiota of SD rats was more abundant in Bifidobacterium and Lactobacillus comparatively to the gut microbiota of Wistar rats.

Discussion

The aim of the present study was to compare Wistar and SD Rat as models of HF diet-induced obesity. The results obtained showed that HF diet increased energy ingestion, weight gain, body fat mass, mesenteric adipocyte's size, adiponectin and leptin plasma levels and decreased oral glucose tolerance in both Wistar and SD Rat. Nonetheless, the majority of these effects were more evident or earlier detected in Wistar Rat.

Wistar rats fed with HF diet consumed higher amounts of food (data not shown) and, therefore, higher amounts of energy throughout the study when compared to SD rats fed with the same diet. Consequently, weight gain was larger in these animals and was mainly due to an expansion of adipose tissue mass.

Leptin is an adipocyte-derived hormone that controls food intake and energy expenditure.[9] Plasma leptin concentration increases in proportion to body fat mass.[10] As a result, Wistar rats fed with HF diet displayed higher leptin plasma levels than SD rats in the same diet regimen. In addition, the amount of leptin released by each gram of body fat mass (plasma leptin to body fat mass ratio) was also more elevated in Wistar than in SD rats (data not shown). This

Table 4. Quantification of gut microbiota phyla, genera and species in different experimental groups

	Wistar Rat		SD Rat		Two-way ANOVA *P* values		
	St	HF	St	HF	Diet	Strain	Interaction
Firmicutes	6.71 ± 0.06	6.28 ± 0.06	6.65 ± 0.03	6.55 ± 0.09	**0.001**	0.118	**0.017**
Bacteroidetes	6.16 ± 0.11	5.40 ± 0.05	5.55 ± 0.18	5.21 ± 0.12	**<0.001**	**0.004**	0.114
Firmicutes/Bacteroidetes	1.09 ± 0.01	1.16 ± 0.01	1.20 ± 0.04	1.26 ± 0.03	**0.026**	**0.001**	0.825
Lactobacillus	4.13 ± 0.31	3.56 ± 0.19	5.87 ± 0.12	2.84 ± 0.29	**<0.001**	**0.047**	**<0.001**
Enterococcus	2.65 ± 0.11	2.89 ± 0.08	2.89 ± 0.13	2.87 ± 0.17	0.403	0.410	0.314
Clostridium leptum	5.53 ± 0.07	5.09 ± 0.12	5.40 ± 0.06	5.52 ± 0.12	0.111	0.126	**0.010**
Bacteroides	4.07 ± 0.36	4.32 ± 0.12	3.21 ± 0.13	4.05 ± 0.17	**0.020**	**0.017**	0.192
Prevotella	4.09 ± 0.32	2.31 ± 0.14	2.80 ± 0.25	2.40 ± 0.14	**<0.001**	**0.015**	**0.006**
Bifidobacterium	2.13 ± 0.11	2.60 ± 0.27	4.33 ± 0.60	3.82 ± 0.37	0.949	**<0.001**	0.219

Values are presented as mean ± SEM and expressed as $\log_{10}$ 16S rRNA gene copies/20ng of DNA (n = 6 rats per group).
$^{*}P < 0.05$ vs respective St diet group, $^{\ddagger}P < 0.05$ between St diet groups and $^{\dagger}P < 0.05$ between HF diet groups.
HF, high-fat diet group; SD, Sprague-Dawley; St, standard diet group.

might be considered as an attempt to overcome the resistance to the leptin action which can aggravate, in turn, hyperphagia and obesity.[11,12]

Despite presenting higher adiponectin plasma levels, oral glucose tolerance was decreased, at the end of the study, in animals of both strains fed with HF diet. This is in accordance with other studies reporting an increase in adiponectin plasma levels and a glucose tolerance impairment in SD rats after 24 and 32 weeks of treatment with the same HF diet.[7,13] Adiponectin is recognized by its insulin sensitizing action, however, it has been proposed that obesity may induce a malfunction on adiponectin signaling (adiponectin resistance).[14]

Albumin is considered a negative acute phase protein, and might be decreased during inflammatory conditions, such as obesity.[15,16] Accordingly, in the present study, serum albumin was decreased after HF feeding in both strains.

Serum creatinine, urea and liver enzymes activity are biochemical parameters used to evaluate the function and damage of kidney and liver. The results from this study showed that, in Wistar rats, HF diet increased some of these metabolic markers. Despite the increase into values that did not differ from those of SD healthy animals fed with St diet, serum urea and alkaline phosphatase activity values in Wistar rats fed with HF diet might be, for this strain, already indicative of some renal and liver function impairment as a consequence of obesity.[17-19]

Hypertriglyceridemia is one of the criteria for diagnosis of the metabolic syndrome and seems to be present in Wistar rats fed with HF diet. The increase in free fatty acids flux to the liver (from an expanded adipose tissue mass) can lead to the overproduction of triglyceride-rich very low-density lipoproteins (VLDL) which results, in turn, in high circulating levels of triglycerides.[20] Hypertriglyceridemia is also a reflection of the insulin resistant condition.[20] In accordance, a glucose tolerance impairment, which is related to the inability of insulin to promote glucose uptake and metabolism by insulin-sensitive tissues, was clearly visible in Wistar rats fed with HF diet right from the 9th week of the study. On the other hand, SD rats which glucose tolerance was only impaired at the end of the study, did not develop hypertriglyceridemia as reported by previous studies using the same HF diet.[7,13]

High blood pressure is another component of the metabolic syndrome and represents a major risk factor for cardiovascular diseases. The development of hypertension in a Rat model of diet-induced obesity is described in the literature.[21] However, the low salt content of the HF diet used in this study (0.3%) in contrast with those used by other authors (0.8, 2 and 4%) may explain its lack of efficiency in increasing SBP of both Wistar and SD rats.[22]

The ability of gut microbiota to modulate host signaling pathways that can influence energy balance and metabolism has raised the interest of the scientific community in this subject. Several studies have already demonstrated the link between the gut microbiota and obesity.[23,24]

Here, a comparison between the gut microbiota of Wistar and SD Rat was performed for the first time. This analysis was conducted as an attempt to explain the differential metabolic effects caused by HF feeding in these 2 models of diet-induced obesity. According to Li et al, differences in gut microbiota may account for the differential metabolic response of the animals to a dietary intervention and, consequently, predispose to different pathological outcomes such as obesity and diabetes.[25] The results obtained showed that HF diet profoundly reduced the gut microbial community in both strains by decreasing its 2 dominant phyla (Bacteroidetes and Firmicutes). Furthermore, it also decreased Lactobacillus, an important bacterial genus recognized for its health promoting properties.[26,27] On the other hand, it increased the number of copies of Bacteroides and increased the Firmicutes to Bacteroidetes ratio that has been associated to obesity.[28-30] Most of these effects were more pronounced in Wistar rats, except for Lactobacillus. The gut microbiota composition of SD rats was richer in Lactobacillus what may justify the harshest effect of HF diet in the reduction of this genus, in this strain.

The analysis of the interrelationship between gut microbiota and host metabolic parameters (**Fig. 7**) showed that *Clostridium leptum* was significantly negatively correlated with insulineamia, leptin plasma levels, HOMA and with AUC of the glycaemic response at 9th week of the study. Previous studies had also shown that *Clostridium leptum* is negatively correlated with fat mass, fasting glycaemia, insulinaemia and HOMA.[27,31] In the present study, *Clostridium leptum* was only reduced in Wistar rats fed with HF diet and these were the animals presenting a worsened metabolic scenario.

The gut microbiota-derived LPS is one of the elements linking the gut microbiota to the low-grade inflammation observed in obesity.[32] Increased LPS plasma levels are observed after HF feeding, since the fat content of the food modulates LPS absorption.[33] However, in the present study, LPS was not elevated in the animals fed with HF diet, despite ingesting more amount of fat than the animals in St diet groups. LPS was only measured when animals were fasted and not in the postprandial state which could explain the absence of increased endotoxin levels in HF diet groups.[34]

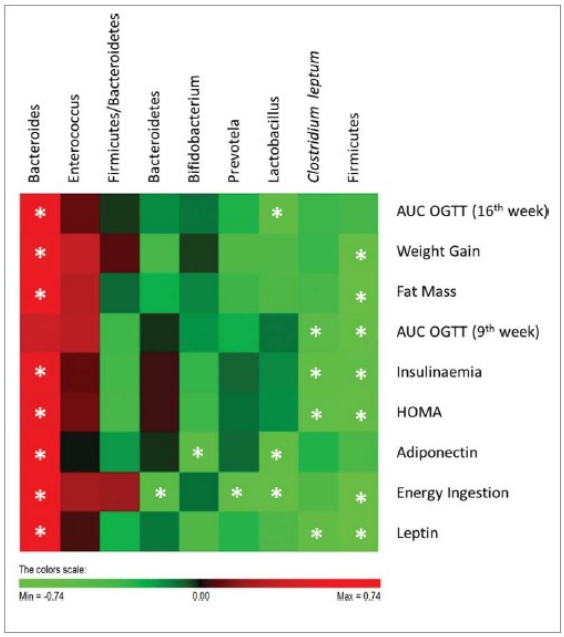

Figure 7. Correlations between gut microbiota and host metabolic parameters. Data of all experimental groups were gathered and analyzed by SPSS software (20.0 version) using 2-tailed Pearson's correlation test. The scores of Pearson's correlation were figured by PermutMatrix software (Version 1.9.3 EN) using heatmap plots. As shown by the colors scale, green color indicates a negative correlation while red color indicates a positive correlation. The symbol (*) indicates a statistical significant correlation ($P < 0.05$).

In summary, both Wistar and SD Rat can be used as models of HF diet-induced obesity, although most of the metabolic effects caused by HF diet were more pronounced, or earlier detected, in Wistar Rat. In addition, despite the differences in the gut microbiota composition of these 2 strains, the modulation caused by HF diet was similar in both groups, except for *Clostridium leptum*. Differences in the gut microbial ecology may account for the different responses to HF diet and to the development of a worsened metabolic and inflammatory status.

Materials and Methods

Animals and housing

Twelve male Wistar rats and 12 male SD rats were purchased from Harlan Laboratories (Santiga, Spain) and kept under controlled environmental conditions (22−24°C and 12 h light/dark cycles), for at least 1 week before starting the experiments. Animals from the 2 different strains (7 weeks of age) were randomly divided into 2 groups of 6 animals each: standard (St) and high-fat (HF) diet group. The diets were respectively "St" (Teklad 2014, Harlan Laboratories, Santiga, Spain) and "HF" with 45% of energy from lipids and 17% of energy from sucrose (D12451 Research Diets, New Brunswick, NJ, USA). Animals were subjected to different experimental conditions for a total of 17 weeks. The water and chow were supplied *ad libitum*. Food and beverage consumption and body weight were monitored weekly, to carefully characterize energy ingestion and weight gain.

At the end of the 17 weeks, food was removed 4−6 h before sacrifice and the animals were anesthetized with a mixture of ketamine (50 mg/kg) and medetomidine (1 mg/kg) and maintained with isoflurane. Meanwhile, using a Quantum /S bioelectrical impedance analyzer (RJL Systems, Akern SRL, Florence, Italy), the body composition of each rat was determined by bioelectrical impedance, according to the procedure already described in the literature.[35] Before perfusion of the vascular compartment with a saline solution (NaCl 0.9%, w/v), blood was drawn from the left ventricle into tubes with or without heparin to obtain plasma and serum, respectively. Aliquots were frozen at −80°C until further analysis. Fresh fecal samples were collected directly from the colon of all animals, snap-frozen in liquid nitrogen and stored at −80°C until further analysis.

Animal handling and housing protocols followed European Union guidelines (Directive 2010/63/EU) for the use of experimental animals in scientific research. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Faculty of Medicine of University of Porto.

Oral glucose tolerance tests (OGTTs)

In the middle (9th week of treatment) and at the end of the study (16th week of treatment), rats were fasted over 5h and a baseline blood draw from the lateral tail vein was collected for plasma fasting glucose and insulin measurements. Animals were gavaged with a glucose solution of 2 g/kg body weight and blood droplets from the tail vein were collected to measure glycaemia thereafter at 30, 60, 90 and 120 min. Glucose levels were measured with Precision Xtra Plus test strips and an Optium Xceed device (Abbott Diabetes Care, Ltd., Maidenhead, UK). Plasma insulin levels were measured using a Rat/Mouse Insulin ELISA kit (Merck Milipore, Madrid, Spain). The homeostasis model assessment (HOMA) was used to calculate approximate insulin resistance using the formula: glucose (mg/dL) × insulin (ng/ml)/405.[36]

Systolic blood pressure measurement

One week prior to the systolic blood pressure (SBP) measurement, animals were acclimated daily to the procedure room and handling. During the 15th week of treatment, indirect measurement of SBP in awake restrained rats was carried out by the non-invasive tail-cuff method, using LE 5000 (Letica Corporation, Rochester Hills, MI, USA).[37] Before the measurements, rats were kept at 37°C during 10 min to make the pulsations of the tail artery detectable. After obtaining a stable pulse (≈300 pulses per minute), 3 to 5 consecutive measurements of SBP were taken and the average of them was considered to analysis.

Blood and urine biochemical analysis

Biochemical evaluation of serum and urine was performed at the end of the study. For urine collection, rats were placed on metabolic cages, after being acclimated. Analysis of routine biochemical markers was performed in a certified Clinical Analysis Laboratory (Guimarães, Portugal).

Adiponectin and leptin were measured in plasma collected at the end of the study, using Rat Adiponectin ELISA Kit (Life Technologies Ltd, Paisley, UK) and Rat Leptin ELISA Kit (Merck Milipore, Madrid, Spain), respectively.

Quantification of bacterial endotoxin was performed using the Chromo-*Limulus* Amebocyte Lysate (Chromo-LAL) reagent (Associates of Cape Cod, Inc.., Falmouth, MA, USA). Briefly, serum samples were diluted 1:4 in ultrapure water (Merck Milipore, Billerica, MA, USA) and heated for 2 min at 100°C. Samples and Chromo-LAL were incubated at 37°C for 40 min and absorbance was read every 10 seconds at 405 nm.

Morphometric analysis of adipose tissue

A small portion of mesenteric adipose tissue was collected from all animals, at the end of the study. Adipose tissue was fixed at 4°C in 10% buffered formaldehyde for at least 48 h and then dehydrated and embedded in paraffin. Three to 5 μm-thick sections were obtained with a Leica Microtome (RM2125RT, Lisbon, Portugal) and stained with hematoxylin and eosin to assess morphology. Digital images were acquired, under specimen identity occultation, with a microscope (Nikon Eclipse 50i, Melville, NY, USA) at a magnification of 200×. The adipocyte area was calculated using ImageJ software (National Institute of Health, Bethesda, MD, USA) with the average of values obtained from 100 adipocytes per animal.

DNA extraction from stool

Genomic DNA was extracted and purified from stool samples using NZY Tissue gDNA Isolation Kit (nzytech, Lisbon, Portugal) with some modifications. Briefly, faeces (170−200 mg) were homogenized in TE buffer (10 mM Tris/HCl; 1 mM EDTA, pH 8.0) and centrifuged at 4000 × *g* for 15 min. The supernatant was discarded and the pellet was resuspended in 350 μL of buffer NT1. After an incubation step at 95°C for 10 min, samples were centrifuged at 11000 × *g* for 1 min. Then, 25 μL of proteinase K were added to 200 μL of the supernatant for incubation at 70°C for 10 min. The remaining steps followed manufacturer's instructions. DNA purity and quantification were assessed with a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Microbial analysis of Rat stool by real-time PCR

Real-time PCR was performed in sealed 96-well microplates using a LightCycler FastStart DNA Master SYBR Green kit and a LightCycler instrument (Roche Applied Science, Indianapolis, ID, USA). PCR reactions mixtures (total of 10 μL) contained 5 μL of 2× Faststart SYBR Green (Roche Diagnostics Ltd), 0.2 μl of each primer (final concentration of 0.2 μM), 3.6 μL of water and 1 μL of DNA (equilibrated to 20 ng). Primer sequences (Sigma-Aldrich, St. Louis, MO, USA) used to target the 16S rRNA gene of the bacteria and the conditions for PCR amplification reactions are reported in **Table 1**. To verify the specificity of the amplicon, a melting curve analysis was performed via monitoring SYBR Green fluorescence in the temperature ramp from 60 to 97°C. Data were processed and analyzed using the LightCycler software (Roche Applied Science). Standard curves were constructed using serial tenfold dilutions of bacterial genomic DNA, according to the following webpage http://cels.uri.edu/gsc/cndna.html. Bacterial genomic DNA used as a standard (**Table 1**) was obtained from DSMZ (Braunschweig, Germany). Genome size and the copy number of the 16S rRNA gene for each bacterial strain used as a standard was obtained from NCBI Genome database (www.ncbi.nlm.nih.gov). Data are presented as the mean values of duplicate PCR analysis.

Statistical analysis

Values are expressed as the arithmetic mean ± standard error of the mean (SEM). Two-way ANOVA was used to determine the main effects of diet (St vs HF diet), strain (Wistar vs SD Rat) and their interaction. Tukey's multiple comparison test was used to determine differences between all experimental groups, whenever a significant

interaction was identified. Two-way ANOVA repeated measures followed by Tukey's multiple comparison test was used to evaluate the differences between experimental conditions throughout time. To analyze the differences between 2 groups, a *t* test was used. The differences were considered statistically significant when $P < 0.05$. All statistical analyses were performed using GraphPad Prism 6 statistical software (GraphPad Software Inc., La Jolla, CA, USA).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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The role of I-FABP as a biomarker of intestinal barrier dysfunction driven by gut microbiota changes in obesity

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Abstract

Background: Intestinal fatty-acid binding protein (I-FABP) is expressed in epithelial cells of the mucosal layer of the small intestine tissue. When intestinal mucosal damage occurs, I-FABP is released into the circulation and its plasma concentration increases. In the context of obesity, the gut barrier integrity can be disrupted by dietary fat while intestinal permeability increases.

Objective: To investigate whether intestinal fatty acid binding protein (I-FABP) is a suitable plasma marker of intestinal injury and inflammation in obesity.

Methods: Twelve male Wistar rats were randomly divided into two groups of six animals each: standard (St) and high-fat (HF) diet fed groups for 12 weeks.

Results: HF fed animals developed obesity, insulin resistance and seemed to present increased plasma levels of proinflammatory cytokines (MCP-1 and IL1β). The gut microbiota composition of these animals was also altered, with lower number of copies of Bacteroidetes, Prevotella spp. and Lactobacillus spp., in comparison with those from St diet group. Fecal lipopolysaccharide (LPS) concentrations tended to be increased in HF fed animals. Intestinal expression of TLR4 seemed to be also increased in HF fed animals suggesting that HF diet-induced dysbiosis may be behind the systemic inflammation observed. However, in contrast to other intestinal inflammatory diseases, plasma I-FABP levels were decreased in HF fed rats whereas I-FABP expression in jejunum tended to be increased.

Conclusions: HF diet-induced obesity is characterized by dysbiosis, insulin resistance and systemic inflammation. In this context, plasmatic I-FABP should not be used as a marker of the intestinal barrier dysfunction and the low-grade chronic inflammatory status.

Keywords: Inflammation, Intestinal fatty acid binding protein, Intestinal permeability, Obesity, Gut microbiota

Background

The new concepts on the pathophysiology of obesity and insulin resistance highlight the role of intestinal microbiota and intestinal barrier in the development of these disorders [1, 2]. Microbiota seems to mediate obesity and associated metabolic disturbances through several mechanisms including energy storage and metabolic inflammation [1, 3]. On the other hand, intestinal mucosa plays not only an important part in the absorption of vital nutrients, but also in anatomical/barrier and immune functions, preventing bacterial translocation. High-fat diet changes gut microbiota composition and increases intestinal permeability, by a mechanism associated with a reduced expression of epithelial tight junction proteins [4]. The altered intestinal barrier and the subsequent translocation of bacteria or bacterial products, namely lipopolysaccharide (LPS) are now recognized as key

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mediators of the low-grade inflammation state, which characterize metabolic disorders.

Intestinal fatty-acid binding protein (I-FABP) is an intracellular protein specifically and abundantly expressed in the epithelial cells of the mucosal layer of the small and large intestine tissue [5]. The location of I-FABP in the mature epithelium of villi facilitates its leakage into the circulation from enterocytes when intestinal mucosal damage occurs [5]. It has been shown that I-FABP is released into the circulation following small intestinal mucosal injury and its plasma concentration has been associated with small intestinal diseases - necrotizing enterocolitis and celiac disease [6–8]. Therefore, I-FABP has emerged as a possible non-invasive marker for evaluating gut wall integrity loss and inflammation. Defining new and early non-invasive markers of gut barrier dysfunction might be of great interest in order to manage a safe modulation of the intestinal microbiota before emergence of obesity and associated metabolic diseases.

The aim of this study was to investigate the role of I-FABP as a possible plasma marker of intestinal injury and inflammation and its relationship with microbiota dysbiosis in a high-fat diet-induced obesity Rat model.

Methods

Animals and housing

Twelve male Wistar rats were purchased from Charles River (Barcelona, Spain) and kept under controlled environmental conditions (22–24 °C and 12 h light/dark cycles), for at least 1 week before starting the experiments. Animals, 8 weeks of age, were randomly divided into two groups of six animals each: standard (St) and high-fat (HF) diet group. The diets were respectively "St" (Teklad 2014, Harlan Laboratories, Santiga, Spain) and "HF" with 45 % of energy from lipids and 17 % of energy from sucrose (D12451 Research Diets, New Brunswick, NJ, USA). Animals were subjected to different experimental conditions for a total of 12 weeks. The water and chow were supplied ad libitum. Food and beverage consumption and body weight were monitored weekly, to carefully characterize energy ingestion and weight gain.

At the end of the 12 weeks, food was removed 4–6 h before sacrifice and the animals were anesthetized with a mixture of ketamine (50 mg/kg) and medetomidine (1 mg/kg) and maintained with isoflurane. Meanwhile, using a Quantum/S bioelectrical impedance analyzer (RJL Systems, Akern SRL, Florence, Italy), the body composition of each rat was determined by bioelectrical impedance, according to the procedure already described in the literature [9]. Before perfusion of the vascular compartment with a saline solution (NaCl 0.9 %, w/v), blood was drawn from the left ventricle into tubes with or without heparin to obtain plasma and serum, respectively. Aliquots were frozen at −80 °C until further analysis. Colon and jejunum were dissected, pat dried and snap-frozen in liquid nitrogen. Fresh fecal samples were collected directly from the colon of all animals and snap-frozen in liquid nitrogen. Both tissues and fecal samples were stored at −80 °C until further analysis.

Animal handling and housing protocols followed European Union guidelines (Directive 2010/63/EU) for the use of experimental animals in scientific research. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Faculty of Medicine of University of Porto.

Oral glucose tolerance test (OGTT)

After 7 weeks of treatment, rats were fasted over 5 h and a baseline blood draw from the saphenous vein was collected for plasma fasting glucose and insulin measurements. Animals were gavaged with a glucose solution of 2 g/kg body weight and blood droplets from the saphenous vein were collected to measure glycaemia thereafter at 30, 60, 90 and 120 min. Glucose levels were measured with Precision Xtra Plus test strips and an Optium Xceed device (Abbott Diabetes Care, Ltd., Maidenhead, UK). Plasma insulin levels were measured using a Rat/Mouse Insulin ELISA kit (Merck Milipore, Madrid, Spain). The homeostasis model assessment (HOMA) was used to calculate approximate insulin resistance using the formula: glucose (mg/dL) × insulin (ng/mL)/405.

Blood and biochemical analysis

Biochemical evaluation of plasma was performed at the end of the study at São João Hospital Center Clinical Pathology Department. Routine biochemical parameters were measured using conventional methods with an Olympus AU5400® automated clinical chemistry analyzer (Beckman-Coulter, Izasa, Porto, Portugal).

Plasma content in leptin, adiponectin, monocyte chemoattractant protein-1 (MCP-1), I-FABP and glucagon-like peptide-2 (GLP-2) were determined using, respectively, Rat Leptin ELISA Kit (Merck, Milipore, Madrid, Spain), Human/Mouse/Rat Adiponectin Enzyme Immunoassay Kit (RayBiotech, Norcross, GA, USA), Rat MCP-1 ELISA Kit (RayBiotech, Norcross, GA, USA), Rat (FABP2) ELISA Kit (Shanghai Sunred Biological Technology Co., Ltd, Shangai) and GLP-2 ELISA Kit (Merck, Milipore, Madrid, Spain). Serum interleukin-1 *beta* (IL-1β) was determined by Luminex assay using custom Miliplex Rat Kits (Merck Milipore, Madrid, Spain), according to the manufacturer's protocols using the Luminex Xmap Multiplexing Technology platform.

Tissue RNA isolation and qRT-PCR

Total RNA from jejunum and colon was isolated using NZYol reagent (NZYTech, Portugal) according to

manufacturer's instructions. RNA samples were treated with DNase I (RQ1 RNase-free DNase; Promega, Portugal). cDNA was synthesized from 1 μg of treated mRNA with NZY First-Strand cDNA Synthesis Kit (NZYTech, Portugal). Quantitative real-time polymerase chain reaction (qRT-PCR) was run on Lightcycler96 (Roche Applied Science, Indianapolis, ID, USA). Cycling conditions were as follows: denaturation (95 °C for 10 min), amplification and quantification (95 °C for 10 s, annealing temperature for 10 s and 72 °C for 10 s, with a single fluorescence measurement at the end of the 72 ° C for a 10-s segment) repeated for 45 cycles and a final melting step with a temperature ramp from 60 to 97 °C. Rat-specific primer sequences (Sigma-Aldrich, St. Louis, MO, USA) used are described in Table 1. The Cq values obtained were transformed into relative quantification data using the formula $2^{-(\Delta Cq)}$.

DNA extraction from stool

Genomic DNA was extracted and purified from stool samples using NZY Tissue gDNA Isolation Kit (NZYtech, Lisbon, Portugal) with some modifications. Briefly, feces (170–200 mg) were homogenized in TE buffer (10 mM Tris/HCl; 1 mM EDTA, pH 8.0) and centrifuged at 4000 x *g* for 15 min. The supernatant was discarded and the pellet was resuspended in 350 μL of buffer NT1. After an incubation step at 95 °C for 10 min, samples were centrifuged at 11000 x *g* for 1 min. Then, 25 μL of proteinase K were added to 200 μL of the supernatant for incubation at 70 °C for 10 min. The remaining steps followed manufacturer's instructions. DNA purity and quantification were assessed with a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Microbial analysis of Rat stool by qRT-PCR

qRT-PCR was performed in sealed 96-well microplates using a LightCycler FastStart DNA Master SYBR Green kit and a LightCycler96 instrument (Roche Applied Science, Indianapolis, ID, USA). Primer sequences (Sigma-Aldrich, St. Louis, MO, USA) used to target the 16S rRNA gene of the bacteria and the conditions for PCR amplification reactions were previously described in Marques C et al. [10]. To verify the specificity of the amplicon, a melting curve analysis was performed via monitoring SYBR Green fluorescence in the temperature ramp from 60 to 97 °C. Data were processed and analyzed using the LightCycler software (Roche Applied Science, Indianapolis, ID, USA). Standard curves were constructed using serial tenfold dilutions of bacterial genomic DNA, according to the following webpage http://cels.uri.edu/gsc/cndna.html. Bacterial genomic DNA used as a standard was obtained from DSMZ (Braunschweig, Germany). Genome size and the copy number of the 16S rRNA gene for each bacterial strain used as a standard was obtained from NCBI Genome database (www.ncbi.nlm.nih.gov). Data are presented as the mean values of duplicate PCR analysis.

Table 1 Primer sequences and real-time PCR conditions used for gene expression analysis by qRT-PCR

Gene name	Primer Sequence (5'-3')	AT
I-FABP	ATGGAAAGGAGCTGATTGCT	59 °C
	TTGGCCTCCACTCCTTCATA	
TLR4	GATGCCTCTCTTGCATCTGG	60 °C
	TCATGAGGGATTTTGCTGAGA	
GAPDH	GGCATCGTGGAAGGGCTCATGAC	62 °C
	ATGCCAGTGAGCTTCCCGTTCAGC	

AT annealing temperature, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase (housekeeping gene), *I-FABP* intestinal fatty acid binding protein, *TLR4* Toll-like receptor 4

Fecal LPS quantification

Quantification of LPS was performed using the Chromo-Limulus Amebocyte Lysate (Chromo-LAL) reagent (Associates of Cape Cod, Inc., Falmouth, MA, USA). Briefly, 1 mL of sterile saline solution (NaCl 0.9 %) was added to 100 mg feces, vortexed and centrifuged (10 min, 10000 *g*, 4 °C) twice. Total supernatant (fecal water) was filtered with 0.45 μm filter and then with 0.22 μm filter. Fecal water and Chromo-LAL (1:1) were incubated at 37 °C for 20 min and absorbance was read every 10 s at 405 nm.

Statistical analysis

Values are expressed as the arithmetic mean ± standard error of the mean (SEM). Given the small sample size, a non-parametric test (Mann–Whitney test) was used to analyze the differences between St and HF groups. Correlation between variables was established using two-tailed Pearson's correlation test. The differences were considered statistically significant when $P < 0.05$. All statistical analyses were performed using GraphPad Prism 6 statistical software (GraphPad Software Inc., La Jolla, CA, USA).

Results

Energy and metabolic parameters

Energy intake was increased in the HF diet fed group when compared with St diet fed group (72.60 ± 0.15 vs. 57.9 ± 0.47 kcal/day, $P < 0.05$). Consequently, HF diet fed animals gained more weight during the 12 weeks of the study (205.5 ± 37.8 vs. 134.7 ± 22.5 g, $P < 0.05$) (Table 2). To assess whether these differences in weight gain were related to alterations in adiposity, we decided to evaluate the body composition of the animals of both groups. Our results showed that rats under HF diet had more body fat mass comparing to rats under St diet (206.3 ± 21.03 vs. 166.8 ± 21.26, $P < 0.05$).

Table 2 Energy ingestion, body composition and metabolic parameters of Wistar rats fed either with standard (St) or high-fat (HF) diet during 12 weeks

	St	HF	*P* value
Energy ingested (Kcal/day)	57.9 ± 0.5	72.60 ± 0.1	<0.05
Weight gain (g)	134.7 ± 9.2	205.5 ± 15.4	<0.05
Fat mass (g)	166.8 ± 8.7	206.3 ± 8.6	<0.05
Total cholesterol (mg/dL)	64.3 ± 3.1	69.8 ± 6.6	0.78
Triglycerides (mg/dL)	79.3 ± 11.5	79.2 ± 8.3	0.83
Leptin (ng/mL)	6.2 ± 1.2	19.1 ± 2.6	<0.05
GLP-2	7.0 ± 0.4	9.8 ± 0.8	<0.05

Values are presented as mean ± SEM ($n = 6$ rats per group). *GLP-2* glucagon-like peptide-2

To assess glycemic response, we performed an oral glucose tolerance test (OGTT). The total area under the curve (AUC) of the glycemic response was increased by HF diet feeding (Fig. 1a, b). In addition, HF diet fed rats had almost two fold less insulin sensitivity, as determined by homeostasis model assessment (HOMA) of insulin resistance (Fig. 1c).

We also examined plasma concentrations of leptin, which has postulated roles in obesity and insulin action (Table 2). HF diet fed group presented significantly higher leptin levels (19.1 ± 2.6 vs. 6.2 ± 1.2, P <0.05) than St diet fed group.

Total cholesterol and triglycerides did not differ between groups (Table 2).

Gut microbiota, LPS and inflammatory status

To study whether HF diet could induce modifications within the intestinal microbiota, we quantified some of the main bacterial groups presented in fecal samples. Analysis of the bacterial 16S rDNA revealed that, at the phylum level, animals from the HF diet group were characterized for having lower Bacteroidetes and higher Firmicutes to Bacteroidetes ratio (Table 3). Firmicutes/Bacteroidetes ratio was positively correlated with weight gain ($r = 0.829$, P <0.05) and AUC ($r = -0.723$, P <0.05) while Bacteroidetes were negatively correlated with weight gain ($r = -0.800$, P <0.05) and AUC ($r = -0.716$ P <0.05).

HF diet feeding also resulted in a decrease in the number of copies of Prevotella spp. and Lactobacillus spp. (Table 3). We further determined whether fecal LPS levels could be altered as a result of the changes in the gut microbiota. Our data showed that fecal LPS levels seemed to be more elevated in HF diet fed animals (Fig. 2a). In addition, LPS levels were positively correlated with Firmicutes/Bacteroidetes ratio ($r = 0.787$, P <0.05) and negatively correlated with Bacteroidetes ($r = -0.670$, P <0.05).

Afterwards, we evaluated the colonic expression of toll-like receptor 4 (TLR4) which is capable to recognize LPS. In agreement with our LPS findings, TLR4 expression tended to be increased in the colon of animals fed with HF diet (Fig. 2b). Next, we investigated whether HF diet and microbiota changes were associated with systemic inflammation. In accordance, the chemokines MCP-1 and Il-1β appeared to be more elevated in the plasma of HF diet fed rats (Fig. 2c, d). LPS was positively correlated with MCP-1 ($r = 0.726$, P <0.05).

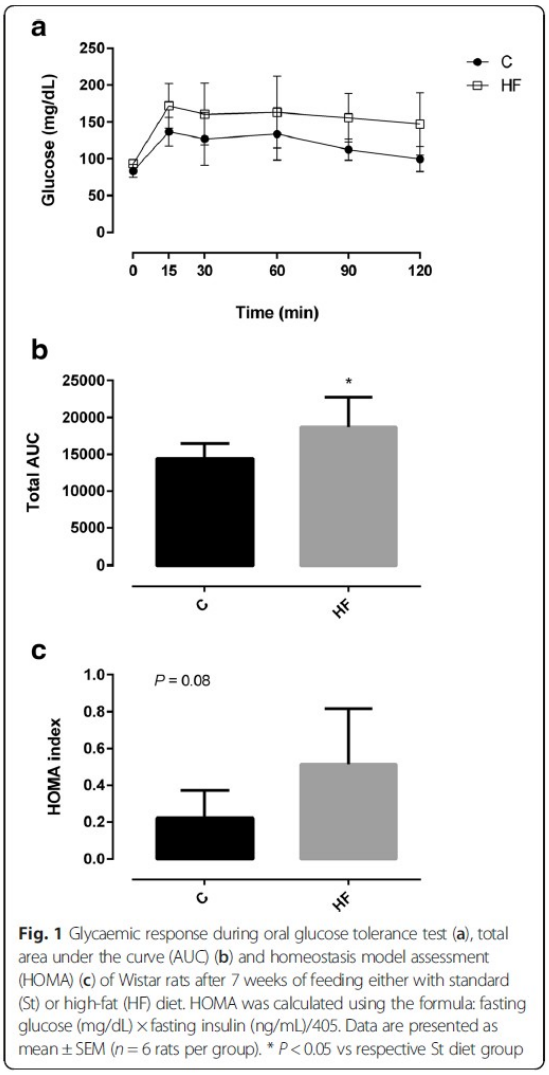

Fig. 1 Glycaemic response during oral glucose tolerance test (**a**), total area under the curve (AUC) (**b**) and homeostasis model assessment (HOMA) (**c**) of Wistar rats after 7 weeks of feeding either with standard (St) or high-fat (HF) diet. HOMA was calculated using the formula: fasting glucose (mg/dL) × fasting insulin (ng/mL)/405. Data are presented as mean ± SEM ($n = 6$ rats per group). * $P < 0.05$ vs respective St diet group

I-FABP and GLP-2 in high fat-diet induced obesity

To understand the relationship between I-FABP, HF diet feeding and systemic inflammation we quantified plasma I-FABP levels and its intestinal expression in both groups of rats. Surprisingly, plasma I-FABP levels were decreased after HF diet feeding (Fig. 3a). On the other

Table 3 Quantification of gut microbiota phyla, genera and species in different experimental groups

	St	HF	*P* value
Firmicutes/Bacteroidetes	1.03 ± 0.01	1.20 ± 0.03	<0.05
Firmicutes	6.61 ± 0.08	6.50 ± 0.09	0.35
Bacteroidetes	6.43 ± 0.10	5.45 ± 0.15	<0.05
Bacteroides spp.	4.36 ± 0.23	4.15 ± 0.50	0.18
Prevotella spp.	3.59 ± 0.29	2.21 ± 0.13	<0.05
Lactobacillus spp.	4.86 ± 0.27	3.77 ± 0.17	<0.05
Clostridium leptum	5.53 ± 0.04	5.43 ± 0.12	0.65
Bifidobacterium spp.	2.00 ± 0.24	2.12 ± 0.22	0.75

Values are presented as mean ± SEM and expressed as $\log_{10}$ 16S rRNA gene copies/20 ng of DNA ($n = 6$ rats per group). *HF* high-fat diet group, *St* standard diet group

hand, I-FABP relative expression in jejunum tended to be higher in HF diet fed rats (Fig. 3b) which could be considered an adaptive response to the increased dietary fat content of the diet. To determine intestinotrophic status of the animals we quantified GLP-2 plasma levels. GLP-2 plasma levels were significantly increased after HF diet feeding (Table 2). GLP-2 and intestinal expression of I-FABP seemed positively correlated with energy ingested ($r = 0.719$, $P < 0.05$ and $r = 0.770$, $P = 0.07$, respectively).

To determine whether I-FABP plasma levels could be used as a marker of the metabolic alterations and inflammatory status associated with obesity, we evaluated the correlation between plasma I-FABP and host metabolic and inflammatory parameters (Fig. 4). Plasma I-FABP levels were negatively correlated with fecal LPS ($r = -0.806$, $P < 0.05$) and IL-1B ($r = -0.623$, $P < 0.05$).

Discussion

Several studies have provided compelling evidence suggesting an association between gut microbiome dysbiosis, obesity and low-grade inflammatory state [11].

In consonance with previous reports, we found that the animals fed with HF diet had lower Bacteroidetes and higher Firmicutes to Bacteroidetes ratio [10]. This dysbiosis pattern might lead to an increased capacity of harvesting energy from food [12, 13]. HF diet also induced obesity and may have triggered intestinal inflammation since fecal levels of LPS and TLR4 expression tended to be increased in the animals fed with this diet. TLR4 is the LPS primary receptor that mediates its proinflammatory effects [14]. As a result, HF fed animals seemed to present higher plasma levels of proinflammatory cytokines and developed insulin resistance. As supported by other authors, it appears to be a causative role for the gut bacteria-induced proinflammatory state to the development of weight gain and insulin resistance in rats under HF diet [15, 16]. In this context, the gut barrier has an important role in the prevention of LPS leakage from the intestinal lumen to the portal blood. However, in animal models of diet-induced obesity, intestinal barrier function seems to be compromised [17].

In different intestinal diseases, I-FABP has emerged as a potential biomarker of intestinal barrier dysfunction

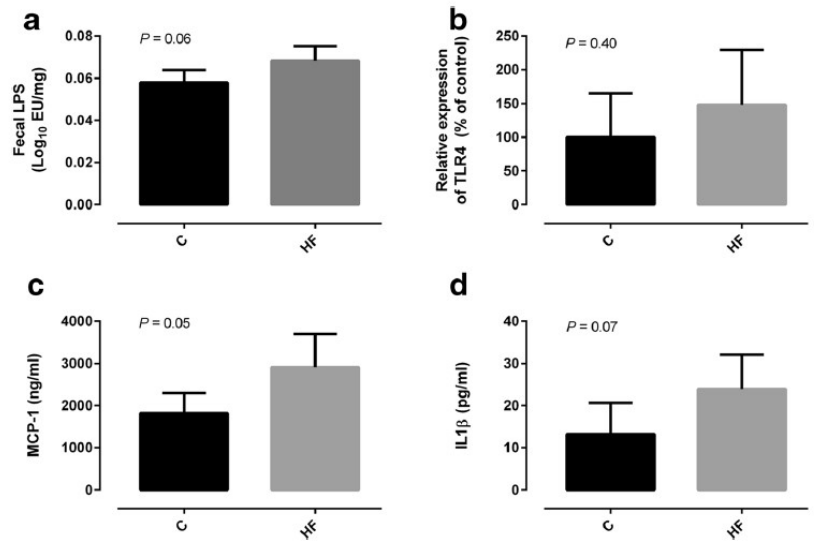

Fig. 2 Fecal lipopolysaccharide (LPS) (**a**), intestinal expression of toll-like receptor 4 (TLR4) (**b**), plasma monocyte chemoattractant protein-1 (MCP-1) (**c**) and serum interleukin-1 *beta* (IL-1β) (**d**) of Wistar rats fed either with standard (St) or high-fat (HF) diet during 12 weeks. Data are presented as mean ± SEM ($n = 3$–6 rats per group). * $P < 0.05$ vs respective St diet group

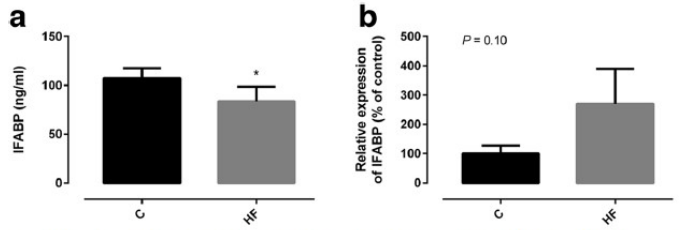

Fig. 3 Levels of plasma (**a**) and intestinal expression of intestinal fatty-acid binding protein (I-FABP) (**b**) of Wistar rats fed either with standard (St) or high-fat (HF) diet during 12 weeks. Data are presented as mean ± SEM (n = 3–6 rats per group). * $P < 0.05$ vs respective St diet group

[18, 19]. Basal I-FABP plasma levels may reflect the physiological turnover rate of enterocytes, whereas elevated levels might indicate intestinal epithelial cell damage [20]. Nevertheless, in the case of obesity-associated metabolic diseases, the existent data about I-FABP and intestinal barrier dysfunction is limited.

Verdam et al. have reported that chronically elevated glucose levels in obese individuals were associated with increased enterocyte loss, assumed by the increase on I-FABP levels [21]. It was therefore speculated that the increased enterocyte loss observed in subjects with chronic hyperglycemia might had contributed to the impaired intestinal barrier function, thereby promoting endotoxin-induced low-grade inflammation. However, increased I-FABP levels could also be a result of an increased production of I-FABP by enterocytes rather than enterocyte loss.

Interestingly, in our study we found that the relative expression of I-FABP tended to be increased in HF diet fed rats. As intestinal absorption capacity can be adapted to the dietary fat content, we hypothesize that HF diet may had up-regulated several genes known to play an important role in long-chain fatty acids uptake such as I-FABP [22]. The animals fed with HF diet also showed increased GLP-2 levels. GLP-2 is a 33 amino acid peptide associated with intestinal growth and adaptation in a variety of pathological conditions [23]. As suggested by other authors, GLP2/GLP2R system may be increased after HF diet to further promote fat absorption in the intestine [23]. On the other hand, the inflammatory state induced by microbiota changes after HF diet feeding might have increased GLP-2 production in order to improve the mucosal barrier integrity and, therefore, blunt the inflammatory stress [2].

Conclusions

To our best knowledge, this is the first study demonstrating that, inversely to what happens in other

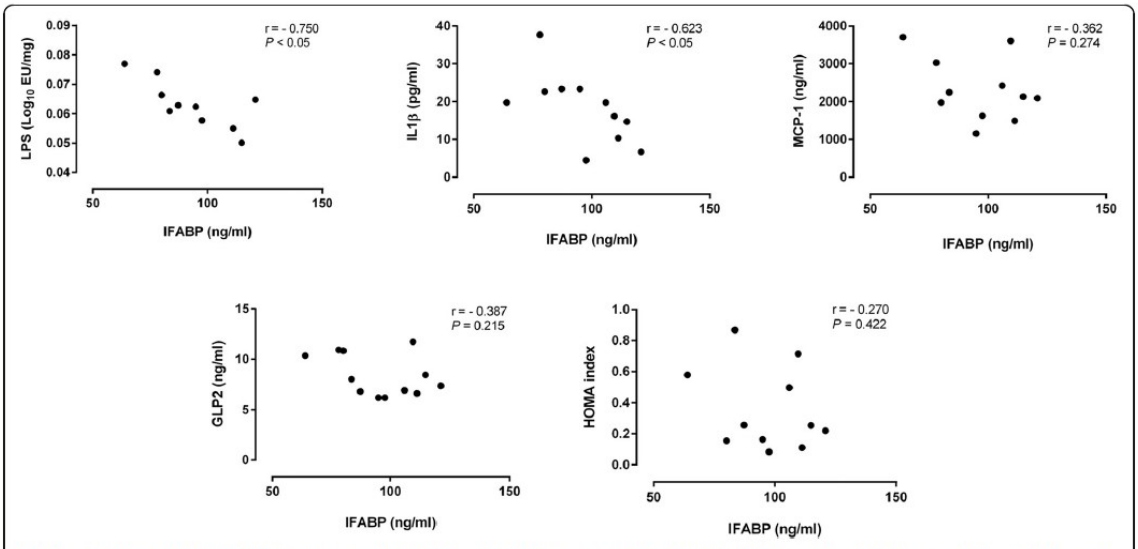

Fig. 4 Correlations between plasma levels of intestinal fatty-acid binding protein (I-FABP) and host metabolic and inflammatory parameters. Data of all experimental groups were gathered and analyzed using two-tailed Pearson's correlation test

intestinal inflammatory diseases, plasma I-FABP does not positively correlates with the inflammatory status presented in obesity. Instead, I-FABP is decreased in plasma but probably increased in jejunum in order to face dietary fat content. The search for noble biomarkers has to continue since it is extremely important to anticipate the progression of obesity-associated metabolic diseases and, thus, allowing the prevention or monitoring of the therapeutic strategies use.

Abbreviations
AUC: area under the curve; GLP-2: glucagon-like peptide-2; HF: high-fat; HOMA: homeostasis model assessment; I-FABP: intestinal fatty-acid binding protein; IL-1β: interleukin-1 beta; LPS: lipopolysaccharide; MCP-1: monocyte chemoattractant protein-1; OGTT: oral glucose tolerance test; qRT-PCR: quantitative real-time polymerase chain reaction; St: standard; TLR4: toll-like receptor 4.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
EL, CM, DC, PF and CC designed the study. EL, CM, DP and MS performed laboratory work. EL and CM analyzed and interpreted data and wrote the manuscript; DC, PF, CC critically revised the manuscript. All authors have read and approved the final manuscript.

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Biological Psychiatry

High-Fat Diet–Induced Dysbiosis as a Cause of Neuroinflammation

To the Editor:

In their outstanding article, Bruce-Keller *et al.* (1) demonstrated for the first time that gut microbiota altered by a high-fat (HF) diet decrease memory and increase anxiety and stereotypical behaviors in mice in the absence of obesity. Obesity and related metabolic comorbidities are characterized by a low-grade chronic inflammatory state accompanied by abnormal cytokine production (2). Gut microbiota dysbiosis may be implicated in the pathophysiology of these diseases through their impact on local and systemic inflammation (3).

Our group recently revealed that 17 weeks of HF feeding induces obesity and its associated metabolic complications in Wistar rats as well as modifications in the gut microbiota composition (4,5). These modifications included a significant reduction in *Clostridium leptum*, which belongs to the Ruminococcaceae order, and a severe reduction in *Akkermansia muciniphila* (4.5 ± 0.1 vs. 3.3 ± 0.2 $\log_{10}$ 16S ribosomal RNA gene copies in control and HF diet groups, respectively), similar to the results described by Bruce-Keller *et al.* (1).

In parallel, the animals fed the HF diet demonstrated an increased expression of several inflammatory markers in brain cortex (6). Furthermore, they exhibited decreased brain-derived neurotrophic factor (BDNF) levels in brain and plasma (4). Bruce-Keller *et al.* (1) did not find differences in BDNF levels between mice with control diet and HF diet microbiota. However, BDNF levels were assessed only in cortex, whereas a whole-brain homogenate was used in our study to quantify this neurotrophin.

Our results revealed that on one hand, a HF diet alters the gut microbiota composition, and on the other hand, it causes neuroinflammation. The hypothesis that HF diet–induced gut microbiota dysbiosis is capable of inducing by itself neurologic dysfunction, as demonstrated by Bruce-Keller *et al.* (1), brought new insights for a better understanding of these HF feeding outcomes. We believe that changes in the gut microbiota may have occurred first and triggered an inflammatory pathway from the gut to the brain.

Gut microbiota can synthesize a range of neurotransmitters, induce the secretion of neurotrophic factors such as BDNF by intestinal smooth muscle cells, and mediate local and systemic inflammation (7-9). As shown by Bruce-Keller *et al.* (1), gut microbiota can also modify the intestinal permeability, allowing the entry of a potent proinflammatory endotoxin (lipopolysaccharide [LPS]) into the bloodstream. Unpublished results from our group showed that after 12 weeks of HF feeding, changes in the gut microbiota are already detected, and fecal LPS levels are increased. Leakage of LPS from the intestine might trigger peripheral inflammatory responses that can lead to de novo production of cytokines in certain regions of the brain (10). Chronic low-grade inflammation may result in changes in brain structure and synaptic plasticity and consequently contribute to the development of neurologic dysfunction and numerous psychopathologies (11). There is also evidence from human studies that intestinal permeability is increased in depression. Patients with depression display higher levels of LPS binding protein and 16S rDNA, both markers of bacterial translocation (12,13).

Inflammation underlies many risk factors for depression, such as stress and disturbed sleep, that, along with obesity, are also characterized by an altered gut microbiota (14,15). As a result, in addition to anti-inflammatory drugs and supplements (nonsteroidal anti-inflammatory drugs or ω-3 fatty acids), the use of psychobiotics or prebiotics that enhance the growth of beneficial bacteria for patients with psychiatric illness emerges as a therapeutic strategy for the treatment of neurophysiatric disorders. Clinical trials are warranted to validate the efficacy of these compounds on the restoration of the gut microbiota balance and in the treatment of anxiety, depression, and other mood disorders.

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TARGETING GUT-BRAIN AXIS WITH ANTHOCYANINS: A NEW CLASS OF PSYCHOBIOTICS

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Background Anthocyanins, a particular class of flavonoids found in berries, are able to control neuroinflammation in high-fat (HF)-diet induced obesity models. On the other hand, anthocyanins are subjected to gut microbiota metabolism and may modulate bacterial growth. The goal of this work was to test the hypotheses: 1- Anthocyanins can modulate gut microbiota composition and counteract HF-diet induced dysbiosis; 2- Modifications in gut microbial environment may be involved in the anti-neuroinflammatory properties of anthocyanins.

Methods Wistar rats were randomly divided into 4 groups (n=6 per group): (C) standard diet; (C+BE) standard diet + blackberry anthocyanin rich extract; (HF) high-fat diet; (HF+BE) high-fat diet + blackberry anthocyanin rich extract. BE was supplied daily in food (25 mg/kg body weight). After 17 weeks, the animals' gut microbiota composition was evaluated by sequencing 16S rRNA gene. Fecal and urine metabolome was analyzed by HPLC/Orbitrap. These outcomes were then correlated with the neuroinflammatory markers previously measured in the hippocampus of these animals.

Results BE counteracted some of the features of HF-diet induced dysbiosis. P*seudoflavonifractor* and *Sporobacter* (bacterial genus increased by BE in C and HF diets, respectively) were negatively correlated with thymus chemokine-1 (TCK-1), a potent chemoattractant which expression is decreased in the hippocampus of animals supplemented with BE. In addition, BE altered host tryptophan metabolism increasing the production of the neuroprotective metabolite kynurenic acid, possible responsible for the effects of BE on neuroinflammation.

Conclusions Our results demonstrate that anthocyanins may counteract the HF diet-induced neuroinflammation through gut microbiota modulation, thereby acting on the bilateral communication between gut and brain.

Keywords: Anthocyanins; Gut-brain axis; Microbiota; Neuroinflammation; Obesity; Tryptophan

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INTRODUCTION

The incidence of neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases has been increasing as global population gets older. The World Health Organization (WHO) predicts that by 2040, neurodegenerative diseases will overtake cancer to become the second leading cause of death after cardiovascular disease [141]. Accumulating evidence suggests that neurodegeneration occurs, in part, because neurons' environment is disturbed in a cascade of processes collectively called neuroinflammation [142]. Neuroinflammation is also central in other psychiatry disorders such as anxiety and depression which are often associated with obesity [143, 144]. Neuroinflammatory processes are profoundly modulated by peripheral inflammatory stimuli, especially those coming from the gut microbiota [145-147]. In this regard, both central nervous system (CNS) and gut microbiota offer legitimate targets for novel therapeutic strategies aiming to treat the rising burden of neuropsychiatry disorders.

Flavonoids are a class of polyphenolic compounds that have been pointed as key elements in the treatment of neurodegenerative diseases [148]. Undeniably, anthocyanins (often consumed in higher amounts than other flavonoids [149]) do interact with neurons and microglia biology, facilitating synaptic connectivity under both regular and pathologic conditions [150]. In addition, as recently demonstrated, anthocyanins are able to attenuate the negative impact of high-fat (HF) diets on neuroinflammation [151]. On the other hand, anthocyanins may also exert their effects through gut microbiota modulation [137].

The aim of the present study was to test the hypothesis that anthocyanins modulate gut microbiota composition and prevent HF diet-induced dysbiosis. After confirming these assumptions and given the bilateral connectivity that exists between gut and brain (the gut-brain axis) [152, 153], we went further to investigate whether gut microbiota modulation was behind the previously observed effects of anthocyanins on neuroinflammation.

METHODS AND MATERIALS

Animals

Twenty-four male Wistar rats were randomly divided into four groups (n=6 per group), as previously described: (C) standard diet; (C+BE) standard diet + blackberry anthocyanin rich extract; (HF) high-fat diet; (HF+BE) high-fat diet + blackberry anthocyanin rich extract [151, 154]. Animals were fed ad libitum with “standard” (Teklad 2014, Harlan Laboratories, Santiga, Spain) or “high-fat” diets (D1245 Research Diets, New Brunswick, USA) for 17 weeks. Blackberry anthocyanin rich extract (BE, 25 mg/kg body weight/day) was obtained as previously described [151]. BE was dissolved daily in sterile water and embedded in food pellets that animals had daily access to. Animal handling and housing protocols followed European Union guidelines (86/609/EEC) for the use of experimental animals. The study obtained ethical approval from the Ethical

Committee of the Faculty of Medicine of University of Porto.

DNA extraction from stool and 16S rRNA sequence analysis

Fresh fecal samples were collected directly from the colon of all animals, snap-frozen in liquid nitrogen and stored at -80 °C until further analysis. Genomic DNA was extracted and purified from stool samples using NZY Tissue gDNA Isolation Kit (NZYTech, Lisbon, Portugal) as previously described by Marques et al. [155]. Libraries were prepared following the 16S Metagenomic Sequencing Library Preparation protocol from illumina (illumina; San Diego, CA, USA). The region of interest was captured using the Klidnworth et al. set of primers that covered the hypervariable region V3-V4 of the bacterial 16s rRNA [156]. Samples were pooled and loaded into the illumina MiSeq System and, then, sequenced using a 300PE combination according to manufacturer's specifications.

Raw sequencing reads were merged with PEAR v0.9.6. Amplification primers were trimmed from the sequences obtained using the default program settings of cutadapt v1.9.1 [157]. Sequencing quality filtering was subsequently applied to isolate the sequences having more than 300 nts with a mean quality score ≥ 20. Sequences were excluded from all downstream analyses. Sequences were also inspected for PCR chimera constructs. The resulting sequence reads were clustered into operational taxonomic units (OTU) at 98% similarity, using cd-hit program. NCBI database was used to assign a taxonomic classification to each read in the representative set. Reads with no hits in the reference sequence collection were classified as "null". OTUs with a relative abundance < 1% in all samples were considered non-significant and are not presented. Shannon's richness index was calculated using the formula described in [158].

Neuroinflammation assessment

As previously described, neuroinflammatory markers were measured in the hippocampus of all animals using a predefined cytokine glass-based array (Quantibody Rat Cytokine Array; RayBiotech), according to manufacturer's instructions [151].

Fecal LPS quantification

Quantification of LPS was performed using the Chromo-Limulus Amebocyte Lysate (Chromo-LAL) reagent (Associates of Cape Cod, Inc., Falmouth, MA, USA). Briefly, 1 mL of sterile saline solution (NaCl 0.9 %) was added to 100 mg feces, vortexed and centrifuged (10 min, 10000 *g*, 4 °C) twice. Total supernatant (fecal water) was filtered with 0.45 µm filter and then with 0.22 µm filter. Fecal water and Chromo-LAL (1:1) were incubated at 37 °C for 20 min and absorbance was read every 10 s at 405 nm.

Fecal and urine metabolome analysis by HPLC/Orbitrap

The fecal water obtained for LPS quantification was also used for metabolomics experiments. Urine samples were prepared according to the procedure described by Marques et al. [159]. Samples were analyzed by HPLC/Orbitrap according to the method described by Fernandes et al. [160]. MS data was uploaded into XCMS Online and was processed as a multi-group experiment using the default

HPLC/Orbitrap parameters in negative mode (fecal samples) or positive mode (urine samples) [161]. Isotopes and adducts were annotated using CAMERA and arranged into feature groups. Metabolite features were selected to assess the differences between the fecal and urine samples of C, C+BE, HF and HF+BE groups. Tryptophan, kynurenine and kynurenic acid (MilliporeSigma, St. Louis, MO, USA) were used as standards.

Statistical analysis

Two-way ANOVA was used to determine the main effects of diet (CDe vs HF diet), BE supplementation (No BE vs BE) and their interaction. In XCMS online, one-way ANOVA followed by a post hoc multi comparison test was used in multi group experiment. To compare the differences between two groups, *t*-test was used. Correlation between variables was established using Spearman's correlation test. Statistical analyses were performed using SPSS Statistics 23 (IBM, USA) software. Differences were considered statistically significant when $p < 0.05$. Principal component analysis (PCoA) was performed in R 3.0.2 (The R Foundation, New Zealand) with the RStudio 0.97.310 package. Heatmaps were elaborated using CIMminer platform (https://discover.nci.nih.gov/cimminer/home.do).

RESULTS

Blackberry anthocyanins modulate gut-microbiota composition and counteract HF diet-induced dysbiosis

We compared the gut microbial community of Wistar rats fed either with standard (C) or high-fat (HF) diet and supplemented with blackberry anthocyanin extract (BE) by sequencing the V3-V4 regions of the 16S rRNA gene.

The gut microbiota of the animals was dominantly constituted by five phyla: Firmicutes, Bacteroidetes, Actinobacteria, Verrucomicrobia and Proteobacteria (Fig. 1A). Firmicutes to Bacteroidetes ratio was not significantly different among groups ($p>0.05$) (Fig. 1B). Despite no differences were observed at phylum level, at genus level, major differences were noted.

Results illustrated in Fig. 2A indicate the genus level distribution for the 4 diet groups. After 17 weeks of HF feeding, the gut microbiota of HF fed animals was largely altered comparatively to the gut microbiota of C animals. Notably, bacteria belonging to the Firmicutes phylum such as *Rumminococcus*, *Blautia, Erysipelatoclostridium, Streptococcus* and Parasporobacterium were more abundant in HF fed animals ($p<0.05$), whereas *Lachnoclostridium*, *Pseudoflavonifractor, Oscillibacter* and *Anaerostipes* (also Firmicutes) were more abundant in the animals of C group ($p<0.05$) (Fig. 2A). In addition, HF diet decreased the abundance of *Akkermansia*, *Prevotella* and *Paraeggerthella* ($p<0.05$), whereas increased the abundance of *Desulfovibrio*, *Rothia* and *Enterohabdus* ($p<0.05$) (Fig. 2A).

BE instigated several modifications in the gut microbiota composition of the animals. Specifically, BE increased *Pseudoflavonifractor* when the animals were fed with C diet whereas increased *Oscillobacter* independently of the

diet fat content (p<0.05) (Fig. 2A). Moreover, BE struggled to recover the gut microbiota's diversity when the animals were challenged with HF diet, as indicated by Shannon's richness index (Fig. 2C).

To reduce the number of variables, a principal coordinate analysis (PCoA) was performed. The gut microbiota communities from the four different groups (C, BE, HF and HFBE) were grouped into four different clusters (Fig. 2C). HF fed rats had a distinct gut microbiota that clustered separately from C and BE rats. Nevertheless, the gut microbiota communities of the HF+BE animals were also differentiated from those of HF rats (Fig. 2C). The reduction of *Rumminococcu*s and the prevalence of *Sporobacter* were the main features that justified the segregation of HF+BE from HF group (Fig. 2D).

In summary, our data indicate that HF feeding strongly affect the composition of the gut microbiota and that BE can counteract some of the features of HF-diet induced dysbiosis.

Changes in gut bacterial genera prompted by blackberry anthocyanins are correlated with some of their anti-neuroinflammatory properties

After characterizing the gut microbiota of the animals supplemented with BE, we sought to investigate if the protection against neuroinflammation, previously evaluated by our group, was associated with the changes observed in the gut bacterial genera of these animals.

In a previous work, we showed that BE consumption decreased TCK-1 expression in rat's hippocampus whereas fractalkine expression increased [151]. Here, we found that TCK-1 was negatively correlated with *Pseudoflavonifractor* and *Sporobacter* when the animals were fed with C and HF diet, respectively (p<0.05) (Fig. 3A and 3B). Interestingly, these bacterial genera were those increased by BE in C and HF diet groups, respectively (Fig. 2A). On the other hand, it was interesting to note that fractalkine, a chemokine extremely important in the crosstalk between neurons and microglia, was not correlated with any bacterial genus despite being upregulated in BE supplemented rat's hippocampus (Fig. 3A and 3B).

Blackberry anthocyanins modulate CNS inflammation via the microbial metabolites of tryptophan

As a component of the gram-negative bacteria and a trigger of the inflammatory response, lipopolysaccharide (LPS) was measured in the fecal samples of all animals. Although fecal LPS levels were not statistically different among groups (p> 0.05), a noticeable increase in LPS levels was observed in the fecal samples of the animals of HF diet group (Fig. 4). On the other hand, in HF+BE animals, fecal LPS seemed to return to control levels, probably as a reflection of the gut microbiota modifications that BE brought about in HF fed animals (Fig. 4).

The LC/MS analysis revealed the existence of additional compounds in the animals' fecal samples. Twenty-six compounds with a maximum intensity above 1 000 000 were significantly altered between samples (p< 0.01) (Fig. 5). Tryptophan (one of the identified metabolites) was decreased in the groups supplemented with BE (p< 0.05, Fig. 5).

Interestingly, fecal concentrations of tryptophan were positively correlated with TCK-1 expression in the hippocampus (r = 0.733, p< 0.05). Changes in the gut bacterial genera prompted by BE could have altered host tryptophan metabolism. To verify this assumption, tryptophan and tryptophan metabolites (kynurenine and kynurenic acid) were searched in the urine of all animals. Tryptophan and kynurenic acid (Figure 6) were increased in the urine of the animals fed with HF diet and supplemented with BE (p< 0.05), while kynurenine levels remained unchanged. On the contrary, this was not observed in the animals of C+BE group.

DISCUSSION

The present findings demonstrate for the first time that blackberry anthocyanins are able to counteract HF diet-induced dysbiosis. In addition, we demonstrate that anthocyanin-induced changes in the gut microbiota composition are related with their anti-neuroinflammatory properties. Finally, we propose that anthocyanins are able to counteract diet-induced neuroinflammation through the stimulation of tryptophan metabolism along the kynurenine pathway.

A number of studies have shown that mental illness may have origins in the gut [95, 162, 163]. Indeed, compelled by the study of Bruce-Keller et al., we have already proposed that HF diet-induced dysbiosis could be the trigger of neuroinflammation, a common hallmark of neuropsychiatry disorders [147].

Besides changing the gut microbiota composition abruptly (HF-diet induced dysbiosis), HF diet can also increase intestinal permeability, facilitating the passage of LPS into the circulation [61]. In addition, LPS transport from the intestine towards target tissues can also be enabled by the chylomicrons synthesized in response to fat feeding [57]. Once in circulation, LPS may activate CD14/TLR4 signaling in target tissues, including the brain. LPS can also disrupt the blood-brain barrier and induce neuroinflammation [164]. Nevertheless, LPS plasma levels of the animals fed with HF diet were not altered, probably because the animals were fasted before sacrifice [165]. However, the propensity for increased fecal LPS levels may reflect the alterations in the gut microbiota composition brought about by HF diet and might be indicative of higher amounts of LPS in plasma. On the other hand, increased fecal LPS levels may activate dendritic cells within the intestinal barrier, increasing the production of cytokines and, thus contributing to peripheral and systemic inflammation [166]. In the present study, we evaluated whether anthocyanins were able to counteract these effects of HF diet.

Anthocyanins bioavailability was initially considered to be low. This was due to some major drawbacks of the former studies in the field which, among other issues, have overlooked anthocyanin metabolites produced by the gut microbiota [167]. In fact, anthocyanins are extensively metabolized by the gut bacteria and these compounds can predominate during several days in the organism given the enterohepatic recirculation [137, 167]. On the other hand, at the same time they are utilized by these microorganisms,

anthocyanins can selectively stimulate the growth of some bacterial groups [137]. Hidalgo et al. analyzed the effects of anthocyanins upon gut microbiota composition by real-time PCR, using batch culture fermentations and they have shown that anthocyanins significantly enhanced the growth of *Lactobacillus-Enterococcus spp* and *Bifidobacterium spp* [138]. Here, we showed for the first time a complete picture of the modulation of gut microbial genera by blackberry anthocyanins, in an animal model. Anthocyanins were able to counteract some of the features of HF-diet induced dysbiosis, including fecal LPS levels and, in the context of a standard diet, anthocyanins increased *Pseudoflavonifractor*. *Pseudoflavonifractor* was found to be decreased in long-term proton pump inhibitor users which utilization is associated to an increased risk of *Clostridium difficile* infection [168]. Besides, in a different study, *Pseudoflavonifractor* increased the success of obese patients in losing weight consistently [169].

After confirming that anthocyanins are able to modulate gut microbiota composition and counteract HF-diet induced dysbiosis, we demonstrated that the anthocyanin-induced changes in the gut microbiota profile were correlated with their anti-neuroinflammatory properties previously assessed on the hippocampus of the same animals. This reinforces the idea that protection against neuroinflammation can be due to gut microbiota modifications.

Besides LPS, other pathways may be involved in the bilateral communication between the gut and the brain [170]. Since tryptophan was decreased in the fecal samples of the animals supplemented with BE, we decided to explore the metabolic pathway of tryptophan. As a precursor of serotonin, kynurenine and downstream metabolites of the kynurenine pathway, changes in the supply and availability of this essential amino acid has many implications for CNS functioning [104]. The dietary protein intake was the same in the groups supplemented with BE compared to the respective controls. Therefore, if tryptophan was decreased in the fecal samples of the animals supplemented with BE, it could be because BE was stimulating either the microbial catabolism of tryptophan or the host tryptophan metabolism. Around 90% of tryptophan is metabolized along the kynurenine pathway [102]. In this regard, we looked for kynurenine and kynurenic acid (tryptophan metabolites produced along this pathway) in the urine of all animals. Interestingly, we found that tryptophan and kynurenic acid were increased in the HF+BE group. This result suggests that tryptophan is being converted in kynurenine and, in turn, kynurenine is being converted into kynurenic acid. Kynurenic acid acts as an antagonist of the excitatory amino acid receptors and has been implicated in major psychiatric diseases [171]. Alterations in the gut microbial composition might result in changes in serum and urine kynurenic acid levels and could thus modify CNS excitation and behavior [170]. Some studies have also report the anti-inflammatory properties of kynurenic acid [172, 173].

The increased production in kynurenic acid could explain, at least in part, the anti-

neuroinflammatory properties of anthocyanins, especially in the context of HF diet.

Nonetheless, tryptophan could be also being used for the serotonin synthesis in the gut [174] or for indole production by gut bacteria which has been shown to increase intestinal barrier integrity [175].

However, the direct role of anthocyanins in the brain may not be ruled out. Anthocyanin metabolites can accumulate in the brain and exert their effects directly, including the stimulation of fractalkine secretion [150, 176].

In conclusion, anthocyanins alter host tryptophan metabolism, generating metabolites responsible for the control of CNS inflammation which may constitute another mechanism behind their anti-neuroinflammatory properties. Anthocyanins may act, therefore, as mediators of the microbiota-gut-brain axis, allowing the control of neuroinflammation by gut microbiota modulation.

These results strongly suggest that dietary manipulation of the gut microbiota by anthocyanins could attenuate the neurologic complications of obesity. Anthocyanins emerge, therefore, as a new class of psychobiotics.

Lastly, these preclinical studies have prompted interest in whether targeting the gut microbiota with anthocyanins might be a viable strategy to influence tryptophan availability for kynurenine metabolism and neuroinflammatory control within CNS.

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The authors declare no conflict of interests.

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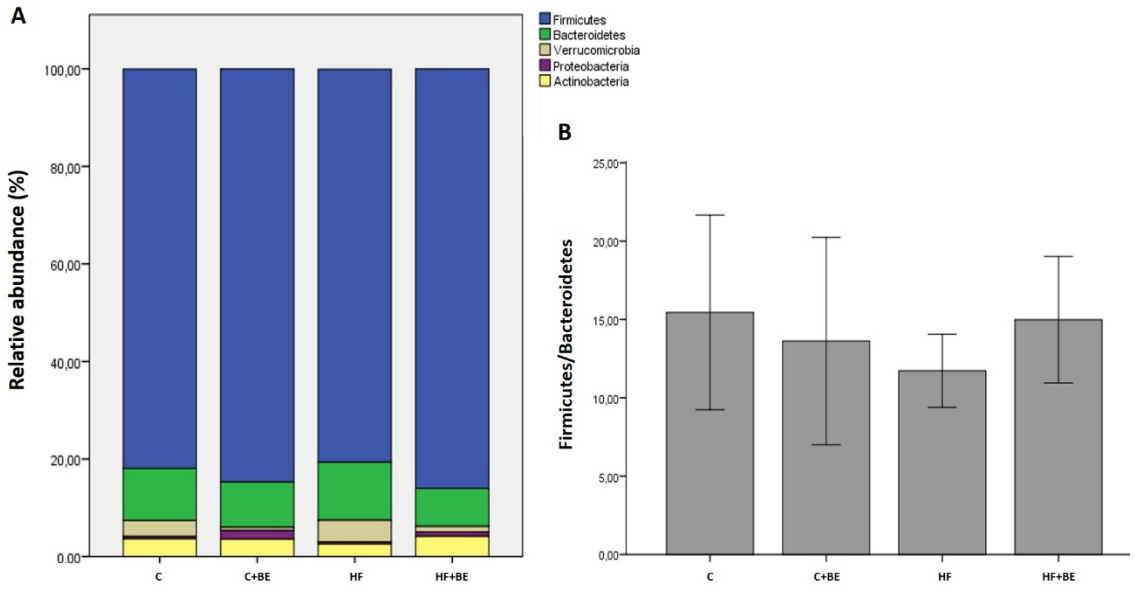

Figure 1 - (A) Major bacterial phyla in the gut microbiota of rats after 17 weeks of high-fat (HF) feeding and anthocyanins-rich blackberry extract (BE) supplementation. Bars represent the average of each phylum relative abundance in the 4 different diet groups. Each phylum is represented by a different color (n=5-6 rats per group). **(B)** Firmicutes to Bacteroidetes ratio among groups. This ratio was calculated by dividing the relative abundance of Firmicutes by the relative abundance of Bacteroidetes. Values are expressed as mean ± SEM (n=5-6 rats per group).

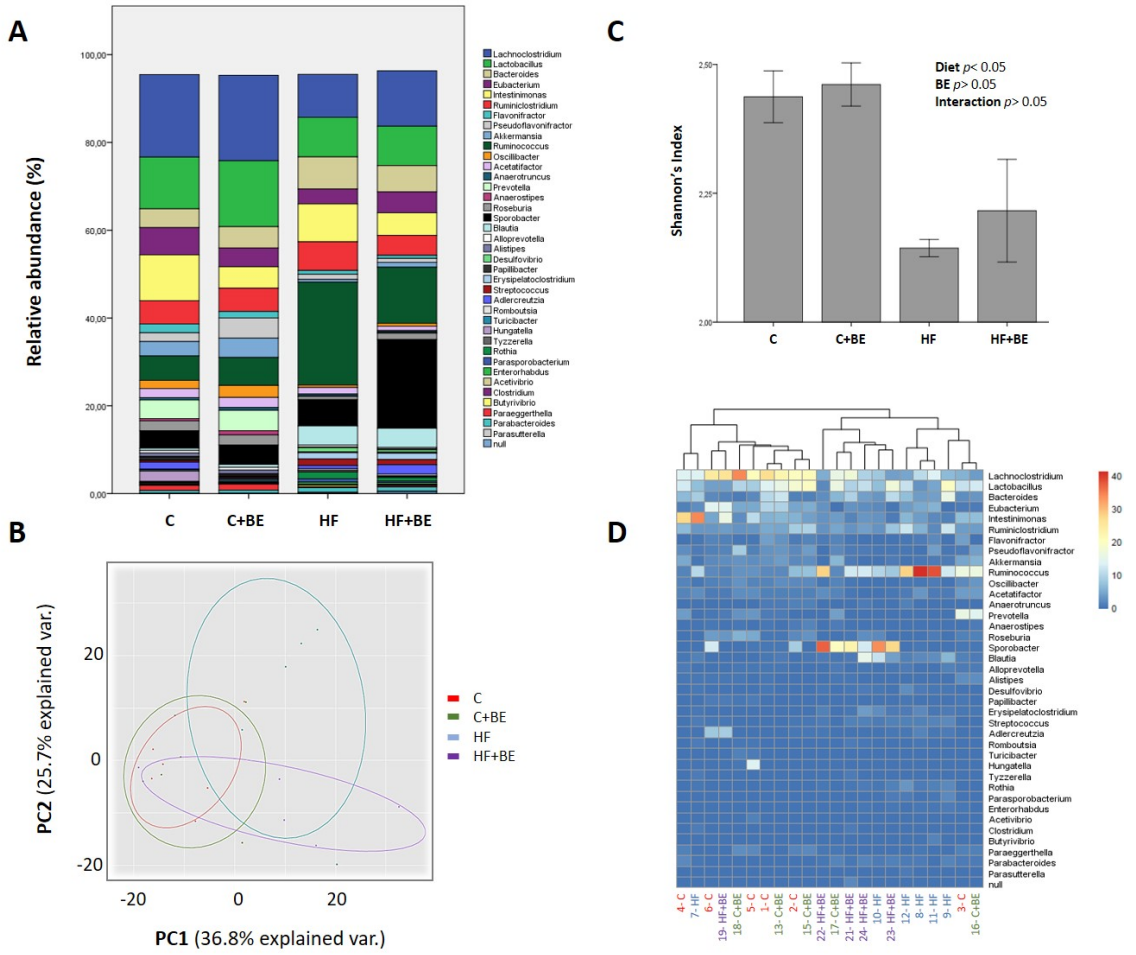

Figure 2 - Gut microbiota composition at the genus level among groups (n=5-6 per group). **(A)** Relative abundance of gut bacterial genera. Bars represent the average of each genus relative abundance in the 4 different diet groups. Each genus is represented by a different color. **(B)** Shannon's diversity index among groups. Values are expressed as mean ± SEM. **(C)** Gut bacterial genera were clustered using principal component analysis (PCoA). Results are plotted according to the first two principle components, which explain 36.8% (PC1) and 25.7% (PC2) of the variation in gut microbial composition (at genus level) between samples. Each point represents one sample and each diet group is denoted by a different color. Circles combine samples from the same diet group by their respective 95% confidence interval ellipse. **(D)** Heatmap and hierarchical clustering of the relative abundance of gut bacterial genera. Rows correspond to operational taxonomic units (OTUs) and columns represent the animals of the 4 different diet groups.

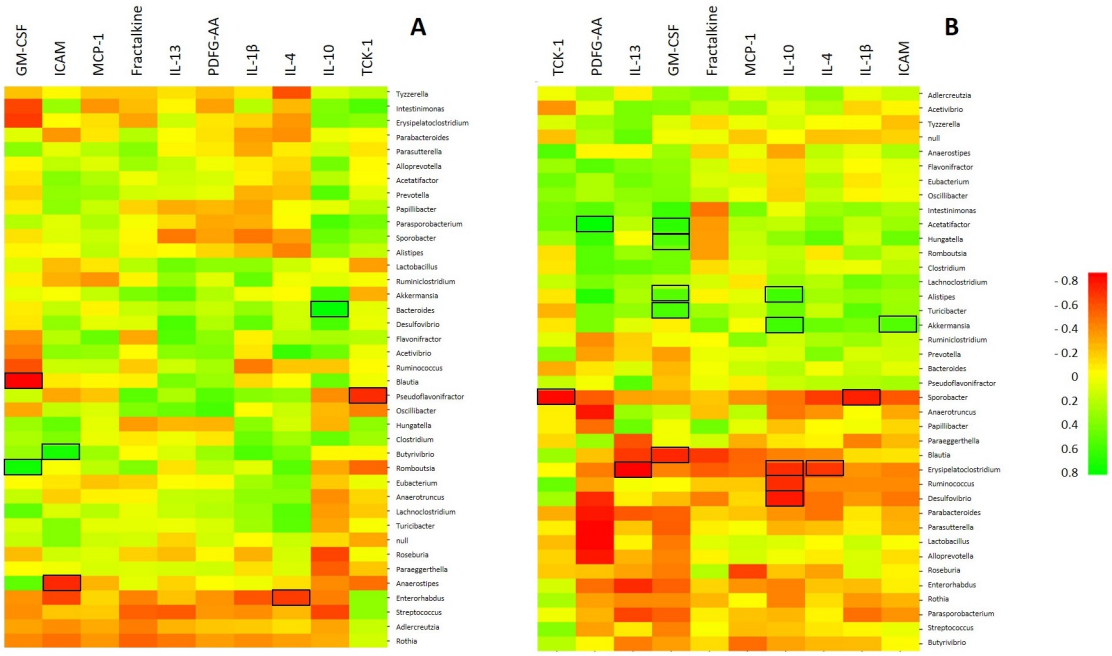

Figure 3 - Heatmap of Spearman's correlation test between gut bacterial genera and neuroinflammatory markers measured in hippocampus, **(A)** in the animals fed with standard diet (C and BE groups) and **(B)** in high-fat fed animals (HF and HFBE groups). Green color indicates a positive correlation while red color indicates a negative correlation. Squared cells represent correlations with statistical significance ($p< 0.05$).

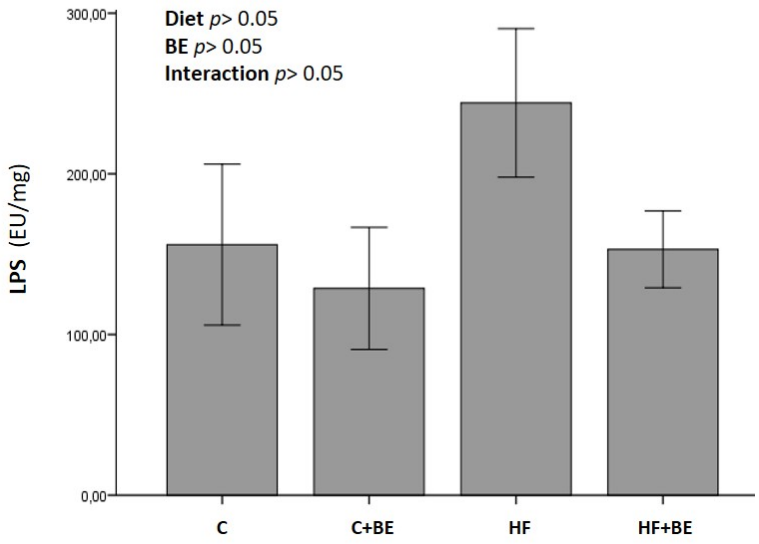

Figure 4 - Fecal LPS concentrations. Values are expressed as mean ± SEM (n=5-6 rats per group).

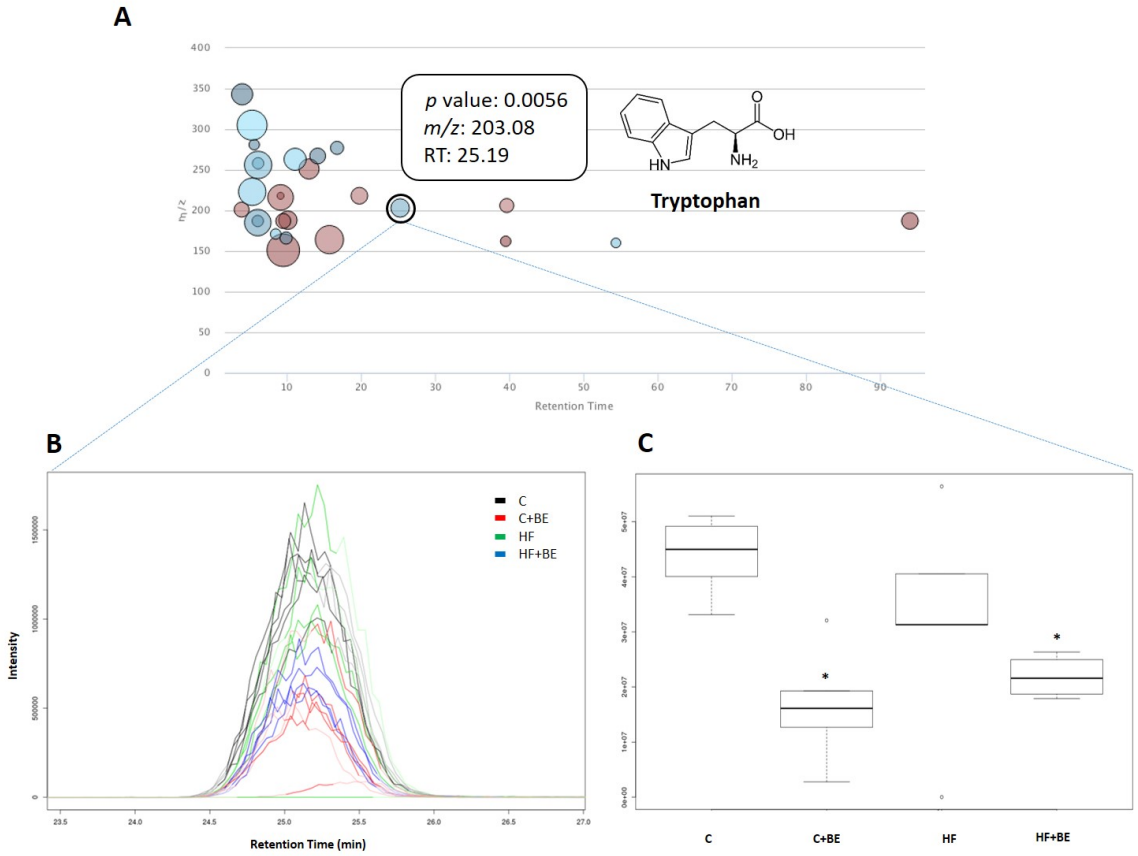

Figure 5 - (A) Metabolite features whose level varies significantly (p< 0.01) across groups are projected on the cloud plot depending on their retention time (x-axis) and m/z (y-axis). Statistical significance (p-value) is represented by the bubble's color intensity. The size of the bubble denotes feature intensity (only features with maximum intensity above 1 000 000 are displayed). Feature assignments (p-value, m/z, RT) are displayed in a pop-up window for the identified metabolite tryptophan. **(B)** Extracted ion chromatogram (EIC) and **(C)** Boxplot of tryptophan. *p<0.05 vs respective control.

A

Metabolites	m/z	Retention Time (min)
Kynurenin	189	15.63
Tryptophan	204	24.33
Kynurenic acid	208	43.57

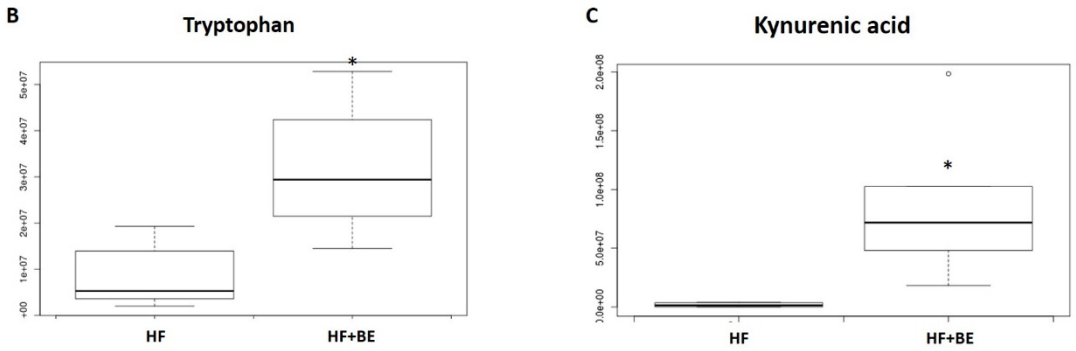

Figure 6 - (A) Tryptophan and tryptophan metabolites searched in the urine of the animals of all groups. **(B)** Boxplot of tryptophan and **(C)** Boxplot of kynurenic acid. *p<0.05 vs respective control.

CHAPTER III

"PHARMACOKINETICS OF BLACKBERRY ANTHOCYANINS CONSUMED WITH OR WITHOUT ETHANOL: A RANDOMIZED AND CROSSOVER TRIAL"

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RESEARCH ARTICLE

Pharmacokinetics of blackberry anthocyanins consumed with or without ethanol: A randomized and crossover trial

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Scope: This study was designed to evaluate the influence of ethanol on the bioavailability of blackberry anthocyanins.

Methods and results: A total of 18 participants were recruited to consume 250 mL of a blackberry puree (650 mg of anthocyanins) without (BBP) or with 12% ethanol (BBP 12%). Venous blood was collected from participants at baseline and at 15, 30, 60, and 120 min after puree ingestion. Urine samples were collected at baseline and at 120 min. Plasma and urine concentration of anthocyanins and anthocyanin conjugates were quantified by HPLC-DAD. Methyl-cyanidin-glucuronide (Me-Cy-Glucr) and 3′-methyl-cyanidin-3-glucoside (3′-Me-Cy3glc) were the main anthocyanin conjugates detected in all plasma and urine samples. Urinary concentration of these anthocyanin conjugates were positively correlated with their plasma concentration. Ethanol increased plasma C_{max} of Me-Cy-Glucr and 3′-Me-Cy3glc. Participants were then stratified according to their body mass index (BMI) and body fat mass. After BBP consumption, plasma C_{max} of Me-Cy-Glucr and 3′-Me-Cy3glc tended to be decreased in overweight/obese participants, in comparison to normal weight participants. The increase on plasma C_{max} of Me-Cy-Glucr and 3′-Me-Cy3glc induced by ethanol was more pronounced in the group of overweight/obese participants.

Conclusions: Ethanol seems to enhance Cy3glc metabolism that appears to be compromised in overweight and obese individuals.

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Anthocyanins / Bioavailability / Ethanol / Metabolites / Obesity

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1 Introduction

Anthocyanins are water-soluble pigments found in plants, flowers, and fruits, making them naturally part of the human diet [1]. Several epidemiologic studies have associated the consumption of flavonoid-rich foods with beneficial health

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Abbreviations: **AUC**, area under the curve; **BBP**, blackberry puree without ethanol; **BBP 12%**, blackberry puree with 12% ethanol; **BMI**, body mass index; **Cy**, cyanidin; **Cy3glc**, cyanidin-3-glucoside; **Cy3rut**, cyanidin-3-rutinoside; **Glucr**, glucuronide; **Me**, Methyl; **MRM**, multiple reaction monitoring; **Sulp**, sulfate

*These authors equally contributed to this work.
Colour Online: See the article online to view Figs. 1 and 4 in colour.

outcomes, highlighting the role of these compounds as active agents in health [2–4].

Although several reports have addressed the biological properties of anthocyanins in different biological systems in vitro, many of these studies have tested the compounds in their native forms and in concentrations far from the physiological range [5–7]. The study of anthocyanins bioavailability is, therefore, important to identify which compounds can reach the target organs and in which concentrations to further direct in vitro studies toward more accurate findings.

It has been widely accepted that anthocyanins are extensively metabolized in the organism and that their biological activity may not be attributed to the native forms but to the metabolized forms [8–10]. The identification and quantification of these forms has not been an easy task but the improvement of the analytical methods has allowed some advance in this area.

Anthocyanins bioavailability can be affected by different variables such as the motility and permeability of the gastrointestinal tract as well as gut microbiota [11]. The food matrix is also a very important factor since the availability of the compounds to be absorbed is dependent on the release of anthocyanins from the food matrix where they are inserted and on the lipophilicity of the environment that may influence the solubility of these compounds [12].

Due to their large presence in red wine, anthocyanins are often consumed concomitantly with ethanol. The effect of ethanol on anthocyanins bioavailability has not been properly addressed so far. Two previous studies have evaluated the effect of ethanol on anthocyanins bioavailability using red wine and dealcoholized red wine or red grape juice [13, 14]. However, apart from the considerable differences in phenolic concentrations of the beverages used, these studies have only searched for the parent anthocyanin structures in plasma and urine.

The aim of this study was to evaluate the effect of ethanol on the bioavailability of anthocyanins in healthy adults. Both anthocyanins and anthocyanin conjugates were analyzed in plasma and urine samples after blackberries consumption.

2 Materials and methods

2.1 Subjects

Eighteen healthy men and women were recruited among students and staff of the University of Porto. Exclusion criteria were as follows: age < 18 y; body mass index (BMI) < 18.5 kg/m^2; current or previous cardiovascular disease, diabetes or other severe chronic disease; women who were pregnant or lactating; participation in other clinical or food study in the preceding month; use of antibiotics in the last 3 months; subjects under prescription of any chronic medication that could interfere with anthocyanins bioavailability. All subjects signed their written informed consent after receiving oral and written information about the study.

2.2 Study design

To investigate whether ethanol modifies the bioavailability of blackberry anthocyanins, a randomized and crossover intervention study was conducted between March and June 2015. The study was designed to provide 80% power ($\alpha = 0.05$) to detect an ethanol induced change of 0.07 μmol/L in the plasma maximum concentration of cyanidin-3-glucoside (Cy3glc), on the basis of a previous study [15].

The study was carried out in the Center for Health Technology and Services Research (CINTESIS) according to the principles expressed in the Declaration of Helsinki. This trial was approved by the Ethics Committee for Health of the S. João Hospital Center (CES 180.13) and registered at clinicaltrials.gov as NCT02613715.

2.3 Intervention

Participants were instructed to maintain their usual lifestyle and to avoid the intake of specific anthocyanin-containing foods and beverages, including red wine and red fruits such as blackberries, raspberries, blueberries, strawberries, and grapes, 24 h prior intervention. After an overnight fasting period, participants were required to orally confirm that they had adhered to the aforementioned dietary restrictions. Afterwards, they were randomly assigned to drink a blackberry puree without ethanol (BBP) or with 12% ethanol (BBP 12%). Participants receiving BBP in visit 1 received BBP 12% in visit 2 and vice versa.

The blackberry puree was considered to be a suitable food matrix to study blackberry anthocyanins bioavailability since volunteers could promptly "drink" 250 g of blackberries. In addition, the effect of ethanol on anthocyanins bioavailability could be easily evaluated by mixing it either with water or ethanol instead of using, e.g. red wine, a much more complex food matrix.

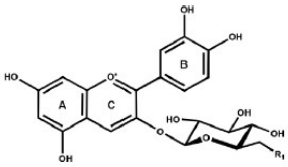
Blackberry purees (250 mL) were prepared with 250 g fresh blackberries, 80 mL water or alcoholic beverage (Cachaça 51, Brazil) and 17 g sugar, using Thermomix (TM31, Vorwerk, Germany). The final concentration of ethanol in the puree was 12%. This concentration was chosen to mimic the concentration present in red wine (another important source of anthocyanins). Total anthocyanins content in blackberry purees was measured after centrifugation (15 min at 2000 × *g*) by HPLC-DAD. Anthocyanins were naturally present in fresh blackberries and no anthocyanins were artificially added to the puree.

2.4 Study protocol

After a 15 min rest, blood pressure measurements were performed on the left upper arm using an automatic blood pressure monitor (BMG 4907, AEG, Germany). Height of subjects was measured to the nearest 0.5 cm with a stadiometer (Model 206, Seca, Germany). Body composition was determined by

Mol. Nutr. Food Res. 2016, *60*, 2319–2330

Table 1. Anthocyanins content of blackberry purees

	Anthocyanin (abbreviation)	R_1	M^+ / MS^2	Amount in purees (% total anthocyanins)
	Cyanidin-3-glucoside (Cy3glc)	OH	449/287	80.7
	Cyanidin-3-rutinoside (Cy3rut)	Rhamnosyl	595/449; 287	10.7
	Cyanidin-3-dioxaloyl-glucoside (Cy3dioxaglc)	Dioxaloyl	593/187	5.02
	Cyanidin-3-malonyl-glucoside (Cy3manglc)	Malonyl	535	3.56

conventional bioelectrical impedance analysis (TANBC545, Tanita, USA). Venous blood was collected from participants before and 15, 30, 60, and 120 min after puree ingestion. Blood was collected into vacuum tubes containing EDTA and centrifuged for 15 min at 2000 × *g*. Urine was collected before and 120 min after puree ingestion. After this protocol, a meal was available for all participants. Before the participants left the Research Center, alcohol levels were measured and they were only allowed to leave with a negative alcohol test. After approximately one month, participants returned for a second visit which followed identical procedures.

2.5 Biochemical analysis

Biochemical evaluation of plasma samples collected at baseline was performed at São João Hospital Center Clinical Pathology Department. Routine biochemical parameters were measured using conventional methods with an Olympus AU5400 automated clinical chemistry analyzer (Beckman-Coulter, Izasa, Portugal).

2.6 Determination of anthocyanins and metabolites in plasma and urine

Before HPLC quantification of anthocyanins in plasma and urine, isolation and concentration of anthocyanins were performed using solid phase extraction. Plus Sep-Pak cartridges (Waters, USA) were preconditioned with 4 mL methanol and then 4 mL of 1% HCOOH. After adding the sample, water-soluble compounds were eluted with 4 mL of 1% HCOOH, followed by elution of anthocyanins with 2 mL of 50% acetonitril /1% HCOOH. Acetonitril was evaporated in a vacuum concentrator centrifuge (UNIVAPO 100, UniEquip, Germany). Samples were then freeze-dried and reconstituted in 50 μL MeOH + 200 μL 1% HCOOH. All samples were centrifuged for 5 min at 1500 rpm (Centrifuge, Orto Alresa, DIGTOR, Madrid, Spain) before HPLC analysis and qTOF analysis (in Supporting Information).

2.7 Pharmacokinetic and data analysis

Pharmacokinetic modeling of metabolites in the serum was performed with PKSolver, an add-in program for Excel 2010 (Microsoft, USA), using non-compartmental analysis [16]. Area under the curve (AUC) was calculated using the trapezoidal rule.

2.8 Statistics

Values are expressed as the arithmetic mean ± SEM. To analyze the differences between two groups, a *t* test was used. Correlation between variables was established using two-tailed Pearson's correlation test. Subjects were stratified according to their BMI and body fat mass percentage [17]. Two-way ANOVA was used to determine the main effects of ethanol (BBP versus BBP 12%), BMI (normal weight versus overweight/obese) and their interaction. The differences were considered statistically significant when $p < 0.05$. All statistical analysis were performed using SPSS Statistics 23 (IBM, USA) software.

3 Results

3.1 Blackberry puree

The blackberry puree used in this human intervention study was comprised of four main anthocyanins (Table 1 and Fig. 1A). The LC–MS analysis allowed the identification of the four anthocyanins: Cy3glc, cyanidin-3-rutinoside (Cy3rut), cyanidin-3-dioxaloyl-glucoside (Cy3dioxaglc), and cyanidin-3-malonyl-glucoside (Cy3manglc). Anthocyanins concentration did not differ between BBP and BBP 12%. The total anthocyanin concentration of blackberry purees was 2.62 ± 0.08 mg/mL, which corresponds to approximately 650 mg of total anthocyanins ingested per volunteer.

3.2 Subjects

Alcohol consumption was moderate among the participants. Anthropometric and biochemical characteristics of the study participants are summarized in Supporting Information Table 1. Subjects were stratified according to their BMI and body fat mass percentage [17]. The mean BMI of normal weight participants was 23.2 ± 0.7 kg/m² while overweight/obese subjects presented a mean BMI of 30.3 ± 1.2 kg/m². BMI and body fat mass percentage

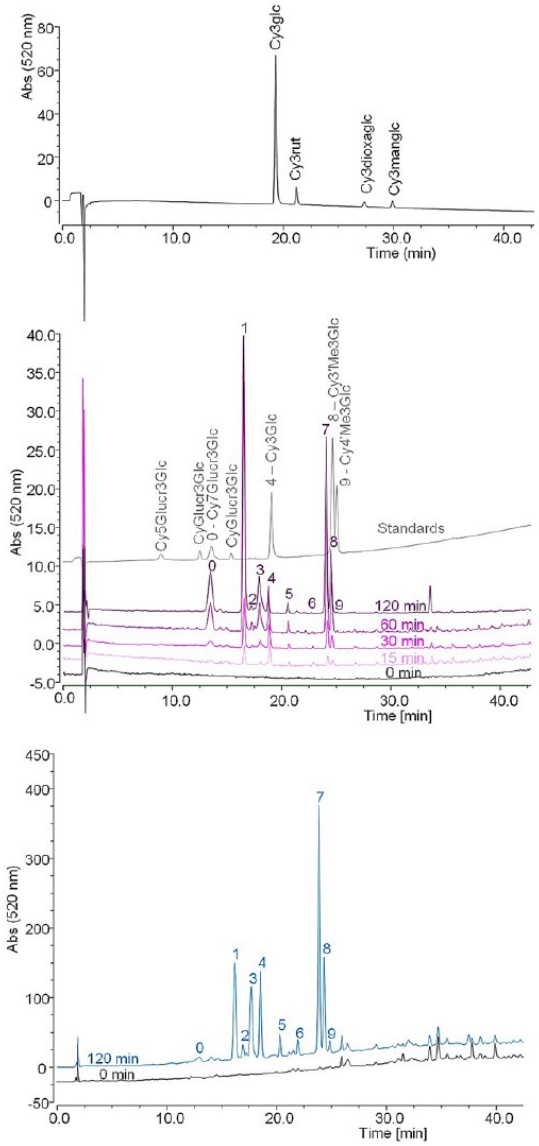


Figure 1. Representative HPLC chromatograms of (A) blackberry purees (B) plasma (0, 15, 30, 60, and 120 min) and (C) urine (0 and 120 min) samples. Peak 0, Cy3glc-7-Glucr; peak 1, Me-Cy3glc-Glucr; peak 2, Cy3glc-Sulp; peak 3, Cy-Glucr (a); peak 4, Cy3glc; peak 5, Cy3rut; peak 6, Cy-Glucr (b); peak 7, Me-Cy-Glucr; peak 8, 3′-Me-Cy3glc and peak 9, 4′-Me-Cy3glc.

were significantly different between normal weight and overweight/obese participants ($p < 0.05$). In addition, overweight/obese subjects presented higher levels of plasma triglycerides, total cholesterol, LDL, and C-reactive protein as well as increased systolic blood pressure.

3.3 MRM analysis of anthocyanin conjugates

The identification of anthocyanin conjugates in plasma and urine samples was performed by triple quadrupole MS analysis. Each sample was programmed to record multiple reaction monitoring (MRM) corresponding to the break up of the anthocyanins presented in the original puree (Cy3glc, Cy3rut, Cy3dioxaglc, and Cy3manglc) or conjugate of interest.

Eighteen possible anthocyanin conjugates were investigated including methylated, glucuronidated, and sulfated conjugates (Supporting Information Table 2). Among those, only three were previously detected in plasma samples: Cyanidin-glucuronide (Cy-Glucr) ($m/z = 463 > 287$; $C_{max} = 14.51$ nM) [18–20], methyl-Cy-Glucr (Me-Cy-Glucr) ($m/z = 477 > 301$; $C_{max} = 1.7–32.79$ nM) [18–21] and Me-Cy3glc ($m/z = 463 > 301$; $C_{max} = 0.46–0.80$ nM) [20, 21]. The detection of Me-Cy ($m/z = 301 > 287$) [20], Cy-Glucr ($m/z = 463 > 287$) [9, 18–20], Cy-Glucr-Glucr ($m/z = 639 > 463$) [19], Me-Cy-Glucr ($m/z = 477 > 301$) [9, 18–20, 22], Me-Cy3glc ($m/z = 463 > 301$) [9, 19–22], Me-Cy3glc-Glucr ($m/z = 639 > 477$) [9, 19, 21], Cy3glc-Glucr ($m/z = 625 > 463$) [22], and Cy3glc-disulfate (Cy3glc-diSulp; $m/z = 609 > 447$) [19] in urine samples was already described.

In both plasma and urine samples, only Cy3glc (peak 4 in Fig. 1B and C) and Cy3rut (peak 5 in Fig. 1B and C) from the original blackberry puree were detected (Fig. 1A). This result was not totally surprising since these were the two main anthocyanins present in the original puree. Considering anthocyanin conjugates, the HPLC profile between plasma and urine samples was quite similar (Fig. 1B and C). In the case of peak 9, only trace amounts were detected in plasma.

The identification of the position of insertion of glucuronyl group in peak 0 ($m/z = 625 > 463$) and methyl group in peaks 8 and 9 ($m/z = 463 > 301$) was only possible since these three standards had been previously synthetized. The chemical synthesis of the two Cy3glc ring A glucuronyl conjugates, cyanidin-7-*O*-glucuronyl-3-glucoside, and cyanidin-5-*O*-glucuronyl- 3-glucoside, was performed according to the procedure already described [23]. These standards allowed the identification of peak 0 as cyanidin-7-*O*-glucuronyl-3-glucoside (Fig. 1B and C). The identification of the position of the attached methyl group in Cy3glc (peak 8 and 9 in Fig. 1B and C) was anticipated from the comparison with the natural standard peonidin-3-*O*-β-D-glucoside [24] and the current available standard cyanidin-4′-*O*-methyl-3-glucoside, previously obtained by hemi-synthesis [25].

Peak 7 ($m/z = 477 > 301$) that corresponds to the conjugation of Cy with a methyl and glucuronyl group was the main conjugate detected (Fig. 1B and C). Although the position of conjugation is unknown, the most probable may be the insertion of methyl group on position C3 of ring B and the insertion of glucuronyl group in position C3 of ring C.

Peaks 1–3 and 6 (Fig. 1B and C) were identified as Me-Cy3glc-Glucr, Cy3glc-Sulp, and Cy-Glucr, respectively, based on the fragmentation patern ($m/z = 639 > 477$; $m/z = 529 > 287$, $m/z = 463 > 287$). Peaks 3 and 6 differ on the

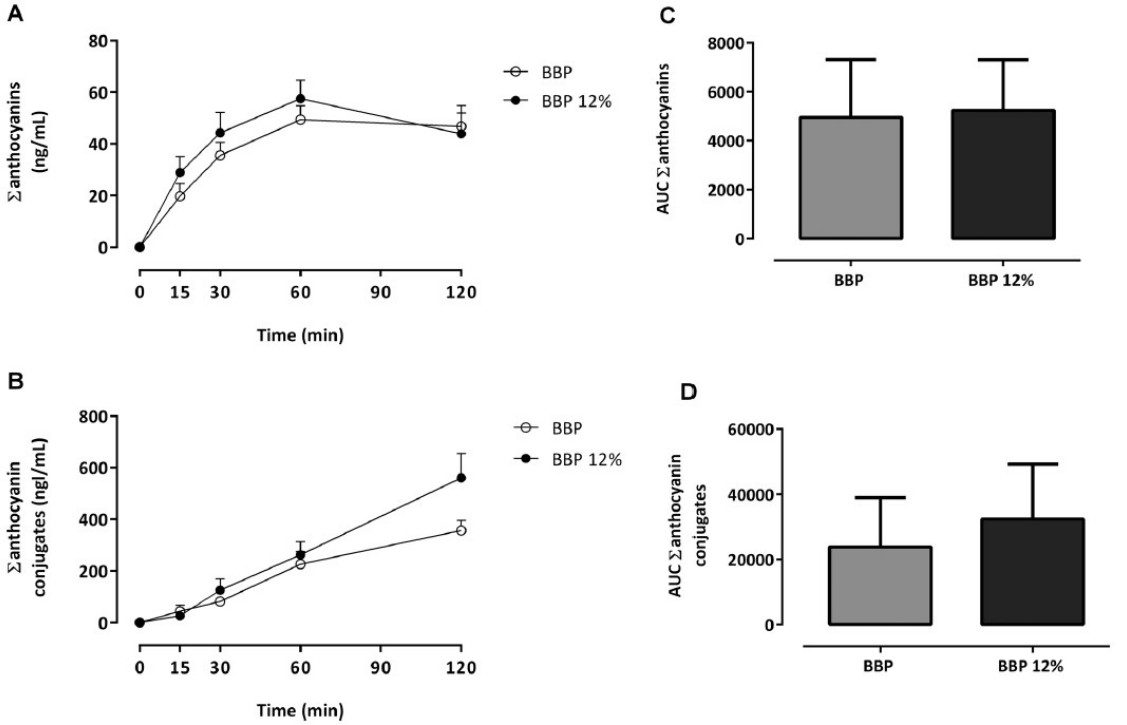


Figure 2. Plasma concentration of total anthocyanins (A and C) and total anthocyanins conjugates (B and D) over 120 min after consumption of 250 mL BBP and with BBP 12%. Σ Anthocyanins was calculated for each participant by adding the concentration of Cy3glc and Cy3rut in plasma at each time point. Σ Anthocyanin conjugates was calculated for each participant by adding the concentration of Cy3glc-Glucr, Me-Cy3glc-Glucr, Cy3glc-Sulp, Cy-Glucr (a), Cy-Glucr (b), Me-Cy-Glucr, and 3′-Me-Cy3glc in plasma at each time point. Values are presented as mean ± SEM (n = 14–18).

position of insertion of glucuronyl group that was not possible to determine given the absence of standards.

3.4 Anthocyanins and anthocyanin conjugates in plasma and urine of all volunteers

The analysis of the plasma collected after the consumption of blackberry purees revealed that blackberry anthocyanins were absorbed intact as native compounds and extensively metabolized into methylated, glucuronidated and sulfated derivatives (Fig. 1B). None of the anthocyanins or anthocyanin conjugates were detected in plasma collected before blackberry purees consumption, indicating that the 24 h period of an anthocyanin-free diet was adequate (Fig. 1B). Plasma concentration of total anthocyanins and total anthocyanin conjugates over 120 min, as well as the respective AUCs, after the intake of blackberry purees, are presented in Fig. 2A–D. All individuals (n = 18) were considered, independently of their BMI.

Ethanol did not alter the AUC of total anthocyanins but tended to increase the AUC of total anthocyanin conjugates (Fig. 2C and D). In particular, ethanol tended to increase the AUC of Me-Cy-Glucr (from 10 653 ± 1529 to 16 251 ± 2437 ng/mL, $p = 0.053$) and significantly increased the AUC of 3′-Me-Cy3glc (from 3908 ± 488 to 5965 ± 607 ng/mL, $p < 0.05$). Ethanol has also increased plasma C_{max} of Me-Cy-Glucr (from 187 ± 27 to 299 ± 34 ng/mL, $p < 0.05$) and plasma C_{max} of 3′-Me-Cy3glc (from 64 ± 8 to 101 ± 8 ng/mL, $p < 0.05$). Plasma concentrations of Me-Cy-Glucr and 3′-Me-Cy3glc at 120 min were positively correlated to their urinary concentrations (r = 0.573 and $r = 0.346$, respectively, $p < 0.05$).

3.5 Anthocyanins and anthocyanin conjugates in plasma of normal weight and overweight/obese participants

Plasma concentration of anthocyanins and anthocyanin conjugates after the intake of blackberry purees in both groups of participants (normal weight and overweight/obese) is reported in detail in Supporting Information Table 3. Anthocyanins (Cy3glc and Cy3rut) were detected in the plasma right after 15 min of the consumption of both blackberry purees and their plasma concentration remained relatively stable after 60 min (Fig. 3A). Nevertheless, plasma anthocyanins

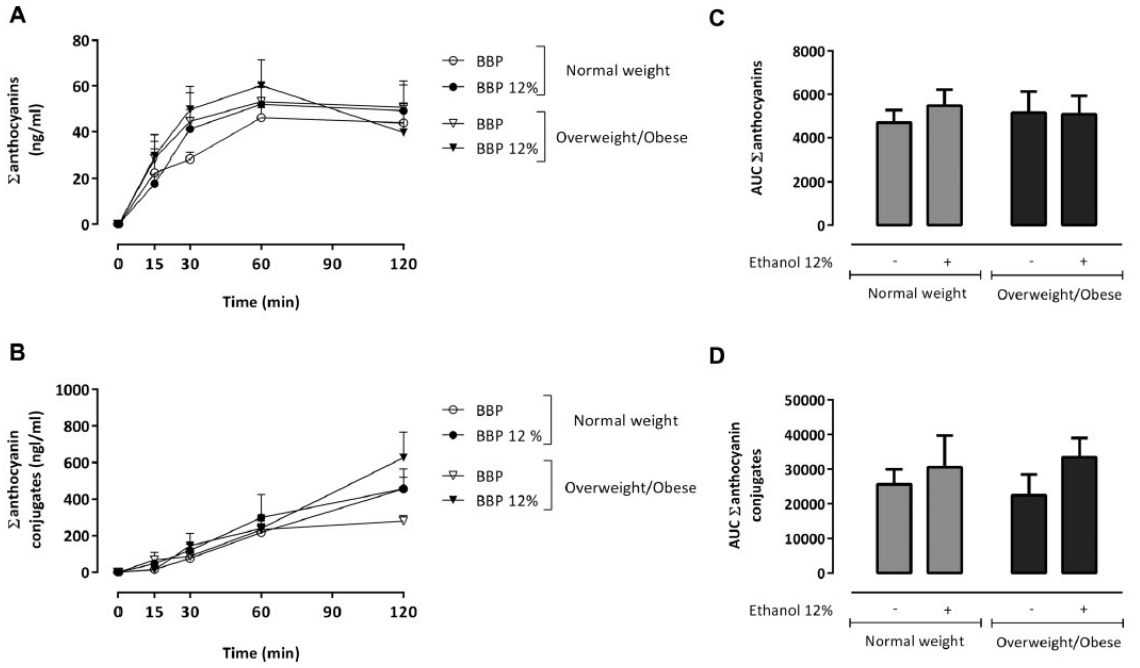


Figure 3. Plasma concentration of total anthocyanins (A and C) and total anthocyanins conjugates (B and D) over 120 min in normal weight and overweight/obese participants, after consumption of 250 mL of blackberry puree without (BBP) and with 12% ethanol (BBP 12%). Σ Anthocyanins was calculated for each participant by adding the concentration of Cy3glc and Cy3rut in plasma at each time point. Σ Anthocyanin conjugates was calculated for each participant by adding the concentration of Cy3glc-Glucr, Me-Cy3glc-Glucr, Cy3glc-Sulp, Cy-Glucr (a), Cy-Glucr (b), Me-Cy-Glucr, and 3′-Me-Cy3glc in plasma at each time point. Values are presented as mean ± SEM (n = 5–9).

concentration tumbled down after 60 min in the group of overweight/obese participants when they had ingested BBP 12% (Fig. 3A). In parallel, plasma anthocyanin conjugates concentration increased promptly after that time, in the same group of participants (Fig. 3B). However, total anthocyanins concentration over 120 min was not significantly different between groups (Fig. 3C). Me-Cy-Glucr and 3′-Me-Cy3glc were the two main anthocyanin conjugates originated after blackberry purees consumption and were detected in all plasma samples analyzed (Table 2).

The plasma C_{max} of Me-Cy-Glucr and 3′-Me-Cy3glc tended to decrease in overweight/obese subjects (Me-Cy-Glucr, P_{BMI} = 0.069; 3′-Me-Cy3glc, P_{BMI} = 0.081) but was significantly increased by ethanol ($P_{ethanol}$ < 0.05) (Table 2). The C_{max} of Me-Cy-Glucr increased from 272 ± 39 to 298 ± 71 ng/mL and from 121 ± 14 to 301 ± 38 ng/mL, in normal weight and overweight/obese individuals, respectively, while C_{max} of 3′-Me-Cy3glc increased from 88 ± 11 to 98 ± 16 ng/mL and from 45 ± 6 to 103 ± 10 ng/mL (Table 2). The effect of ethanol in the C_{max} of these anthocyanin conjugates was considerably more pronounced in overweight/obese individuals (Me-Cy-Glucr, $P_{interaction}$ = 0.059; 3′-Me-Cy3glc, $P_{interaction}$ < 0.05) (Table 2). In the same line, the AUC calculated for Me-Cy-Glucr and 3′-Me-Cy3glc was increased when participants ingested BBP 12% (Me-Cy-Glucr, $P_{ethanol}$ = 0.064; 3′-Me-Cy3glc, $P_{ethanol}$ < 0.05), especially in overweight/obese individuals, although the interaction between BMI and ethanol did not reach statistical significance (Table 2).

Cy3glc-Glucr, Me-Cy3glc-Glucr, Cy3glc-Sulp, Cy-Glucr (a), and Cy-Glucr (b) were the additional anthocyanin conjugates detected in plasma samples (Table 2). Given the wide interindividual variations in the plasma concentration of these compounds, no differences were found between groups. Total metabolites concentration over 120 min tended to be increased by ethanol (Fig. 3D), a result that is probably due to the effect of ethanol on Me-Cy-Glucr and 3′-Me-Cy3glc.

3.6 Urinary excretion of anthocyanins and anthocyanin conjugates in normal weight and overweight/obese participants

All anthocyanins and anthocyanin conjugates identified in plasma were detected in the urine collected after blackberry purees consumption (Table 3). The detection of 4′-Me-Cy3glc in urine may indicate that this compound was also present in plasma samples but its concentration was probably too low to be detected.

The main urinary anthocyanin conjugates detected were also Me-Cy-Glucr and 3′-Me-Cy3glc. Similarly to plasma

Table 2. Pharmacokinetic characteristics of plasma anthocyanins and anthocyanin conjugates over 120 min, in normal weight and overweight/obese participants, after consumption of 250 mL of blackberry puree without (BBP) and with 12% ethanol (BBP 12%)

	Normal weight		Overweight/obese	
	BBP	BBP 12%	BBP	BBP 12%
Cy3glc	(100%)	(100%)	(100%)	(100%)
AUC $_{0–120}$	3876 ± 728	4945 ± 632	4512 ± 903	4474 ± 801
C_{max} (ng/mL)	47 ± 8	55 ± 6	54 ± 11	68 ± 11
t_{max} (min)	66 ± 15	72 ± 20	80 ± 13	66 ± 13
Cy3rut	(71%)	(100%)	(89%)	(100%)
AUC $_{0–120}$	888 ± 163	616 ± 82	817 ± 103	741 ± 94
C_{max} (ng/mL)	11 ± 3	7 ± 1	10 ± 1	11 ± 1
t_{max} (min)	108 ± 12	84 ± 15	86 ± 13	94 ± 13
Cy3glc-7-Glucr	(29%)	(40%)	(56%)	(25%)
AUC $_{0–120}$	1990 ± 377	2236 ± 281	8268 ± 6763	5190 ± 3219
C_{max} (ng/mL)	33 ± 2	37 ± 4	85 ± 68	116 ± 79
t_{max} (min)	120 ± 0	120 ± 0	87 ± 21	120 ± 0
Me-Cy3glc-Glucr	(43%)	(0%)	(56%)	(38%)
AUC $_{0–120}$	4657 ± 1261		2486 ± 847	11432 ± 8678
C_{max} (ng/mL)	78 ± 21		34 ± 11	250 ± 197
t_{max} (min)	100 ± 20		102 ± 18	120 ± 0
Cy3glc-Sulp	(71%)	(100%)	(100%)	(88%)
AUC $_{0–120}$	2516 ± 951	3527 ± 1055	4135 ± 1944	2777 ± 796
C_{max} (ng/mL)	34 ± 13	46 ± 17	62 ± 29	54 ± 18
t_{max} (min)	108 ± 12	108 ± 12	77 ± 11	111 ± 9
Cy-Glucr (a)	(71%)	(100%)	(67%)	(88%)
AUC $_{0–120}$	2700 ± 408	2772 ± 563	2980 ± 978	2603 ± 739
C_{max} (ng/mL)	42 ± 4	52 ± 12	35 ± 11	53 ± 17
t_{max} (min)	120 ± 0	84 ± 15	110 ± 10	111 ± 9
Cy-Glucr (b)	(14%)	(80%)	(89%)	(25%)
AUC $_{0–120}$	516 ± 0	513 ± 209	1422 ± 800	494 ± 236
C_{max} (ng/mL)	6 ± 0	6 ± 2	26 ± 18	5 ± 2
t_{max} (min)	30 ± 0	60 ± 21	71 ± 16	75 ± 45
Me-Cy-Glucr	(100%)	(100%)	(100%)	(100%)
AUC $_{0–120}$	14 704 ± 2591	16 921 ± 6152	7502 ± 1019	15 832 ± 1720
C_{max} (ng/mL)	272 ± 39	298 ± 71	121 ± 14	301 ± 38[1]
t_{max} (min)	120 ± 0	108 ± 12	107 ± 9	113 ± 8
3′-Me-Cy3glc	(100%)	(100%)	(100%)	(100%)
AUC $_{0–120}$	5081 ± 791	6412 ± 1461	2995 ± 435	5686 ± 483[*]
C_{max} (ng/mL)	88 ± 11	98 ± 16	45 ± 6	103 ± 10[*,£]
t_{max} (min)	120 ± 0	96 ± 15	100 ± 10	120 ± 0[3]

Values are presented as mean ± SEM (n = 5–9). Percentage of the detection of compounds in the analyzed samples is represented in brackets. Plasma concentrations of anthocyanins and anthocyanin conjugates were quantified by HPLC-DAD.
$AUC_{0–120}$, area under the curve (0–120 min); Cy, cyanidin; Cy3glc, cyanidin-3-glucoside; Glucr, glucuronide; C_{max}, maximum plasma concentration; Me, methyl; t_{max}, time to reach maximum plasma concentration.
*$p_{ethanol} < 0.05$ and £ $p_{interaction} < 0.05$.

samples, the concentration of these compounds in urine was decreased in overweight/obese individuals ($P_{BMI} < 0.05$) and tended to be increased by ethanol (Me-Cy-Glucr, $p = 0.053$ and 3′-Me-Cy3glc, $p = 0.093$).

4 Discussion

Several health benefits have been attributed to anthocyanins. However, since the bioactive forms in vivo are not necessarily those occurring in nature, but rather conjugates or metabolites originated in the human body, the identification of these compounds assumes a special relevance [18].

Blackberries were chosen for the present study because of their content in anthocyanins. The use of a fruit containing glycosides of only one anthocyanidin (cyanidin) containing one *orto*-catechol group was essential to establish the origin of plasma methylated compounds. Furthermore, the elevated concentration of cyanidin-3-glycosydes in blackberries allowed the detection of plasma cyanidin phase II conjugates

Table 3. Anthocyanins and anthocyanin conjugates presented in the urine of normal weight and overweight/obese participants, 120 min after consumption of 250 mL of blackberry puree without (BBP) and with 12% ethanol (BBP 12%)

	Normal weight		Overweight/Obese	
	BBP	BBP 12%	BBP	BBP 12%
Cy3glc	1.8 ± 0.4 (100%)	2.3 ± 0.5 (100%)	1.2 ± 0.3 (100%)	1.7 ± 0.4 (100%)
Cy3rut	0.2 ± 0.0 (88%)	0.5 ± 0.2 (100%)	0.2 ± 0.0 (90%)	0.2 ± 0.1 (100%)
Σ anthocyanins	2.0 ± 0.4 (100%)	2.8 ± 0.6 (100%)	1.4 ± 0.3 (100%)	2.0 ± 0.5 (100%)
Cy3glc-7-Glucr	0.1 ± 0.0 (38%)	0.3 ± 0.2 (57%)	0.1 ± 0.0 (70%)	0.2 ± 0.1 (33%)
Me-Cy3glc-Glucr	0.3 ± 0.0 (50%)	0.4 ± 0.0 (14%)	0.2 ± 0.1 (60%)	0.5 ± 0.2 (67%)
Cy3glc-Sulp	0.4 ± 0.2 (88%)	0.6 ± 0.3 (86%)	0.3 ± 0.1 (90%)	0.3 ± 0.1 (89%)
Cy-Glucr (a)	0.9 ± 0.2 (75%)	1.3 ± 0.5 (100%)	0.5 ± 0.1 (100%)	0.6 ± 0.1 (100%)
Cy-Glucr (b)	0.2 ± 0.1 (75%)	0.2 ± 0.1 (57%)	0.1 ± 0.0 (60%)	0.1 ± 0.0 (44%)
Me-Cy-Glucr	3.0 ± 0.8 (100%)	4.8 ± 1.6 (100%)	1.2 ± 0.3 (100%)	2.7 ± 0.4$^{\#}$ (100%)
3′-Me-Cy3glc	1.2 ± 0.3 (100%)	1.7 ± 0.5 (100%)	0.7 ± 0.1 (100%)	1.1 ± 0.2$^{\#}$ (100%)
4′-Me-Cy3glc	0.2 ± 0.0 (100%)	0.2 ± 0.0 (100%)	0.1 ± 0.0 (100%)	0.2 ± 0.1 (100%)
Σ anthocyanin conjugates	5.8 ± 1.4 (100%)	8.9 ± 2.9 (100%)	3.1 ± 0.6 (100%)	5.4 ± 0.8$^{\#}$ (100%)

Σ anthocyanins was calculated for each participant by adding the concentration of Cy3glc and Cy3rut in urine at 120 min. Σ Anthocyanin conjugates was calculated for each participant by adding the concentration of Cy3glc-7-Glucr, Me-Cy3glc-Glucr, Cy3glc-Sulp, Cy-Glucr (a), Cy-Glucr (b), Me-Cy-Glucr, 3′-Me-Cy3glc, and 4′-Me-Cy3glc in urine at 120 min. Values are expressed as mean ± SEM (n = 7–10). Values were corrected to urinary creatinine values and expressed as μg/mg creatinine. Percentage of the detection of compounds in the analyzed samples is represented in brackets. Urinary concentration of anthocyanins and anthocyanin conjugates were quantified by HPLC-DAD. Cy, cyanidin; Cy3glc, cyanidin-3-glucoside; Cy3rut, cyanidin-3-rutinoside; Glucr, glucuronide; Me, methyl; Sulp, sulphate.
$^{\#}p_{BMI} < 0.05$.

that usually appear at such low concentrations (below the detection limit of the analytical methods) that end up not being reported.

The occurrence of Cy3glc and Cy3rut in plasma right after 15 min of blackberry purees consumption suggested that blackberry anthocyanins may be quickly absorbed in the stomach in their intact form. Given the viscosity of the blackberry puree, gastric emptying may have been delayed. Previous reports from our group have shown that anthocyanins can cross MKN-28 cell monolayers, a human gastric barrier model, probably through glucose transporters [26, 27]. Nevertheless, the absorption of anthocyanins also occurs throughout the gastrointestinal tract, as suggested by other in vitro studies using intestinal epithelial cells [28, 29].

Cytosolic β-glucosidase and lactase-phlorizin hydrolase are enzymes responsible for the hydrolysis of Cy3glc into Cy. The removal of the glucose moiety from Cy3glc to form Cy is essential so that Cy-Glucr can be formed, but only if the insertion of the glucuronyl group occurs in the position C3 of ring C. The absence of Cy in plasma and urine sample may be related to the instability of this molecule in physiological conditions. Given the elevated hydrophobicity of this molecule, Cy could be bound to circulating plasma proteins, similarly to other polyphenols and, therefore, not be detected by the current methodology [30]. On the other hand, the absence of the aglycone moiety can be a result of the spontaneous degradation of Cy into protocatechuic acid [31]. However, in the present investigation, phenolic acids and their derivatives were not determined since the occurrence of these anthocyanin metabolites is well documented in the literature [9, 19].

On the basis of the rapid appearance of anthocyanin conjugates (most of them were also detected right after 15 min of blackberry purees consumption), anthocyanins may have been metabolized in the upper gastrointestinal tract before postabsorption phase II metabolism in the liver. The contribution of the gastric mucosa to the metabolism of anthocyanins should not be ruled out since the stomach also possesses conjugative enzymes (UDP-glucuronosyltransferase, sulfotransferase, and catechol-O-methyl transferase) [26]. However, the majority of these compounds may be originated in the liver since the plasma concentration of total anthocyanin conjugates continued to increase after 60 min while the plasma concentration of total anthocyanins remained relatively stable or started to decrease (Fig. 2A and B; Fig. 3A and B).

After 120 min of blackberry purees consumption, the concentration of total anthocyanin conjugates in plasma was about ten times higher than the total parent anthocyanins concentration. This result corroborated the assumption that anthocyanins are extensively metabolized and that their putative health effects may be mediated by the originated metabolites instead of the parent anthocyanins. Methylation and glucuronidation were the major metabolic pathways observed for anthocyanin metabolism (Fig. 4). In normal weight individuals, after 120 min of BBP consumption, methylated compounds represented 74.1 and 55.4% of the total compounds detected in the plasma and urine, respectively, while glucuronides represented 71.6 and 53.2%. Kay et al. described glucuronidation as the major metabolic pathway followed by methylation [18]. Nevertheless, the authors have only identified Cy-Glucr and 3′-Me-Cy-Glucr as anthocyanin conjugates.

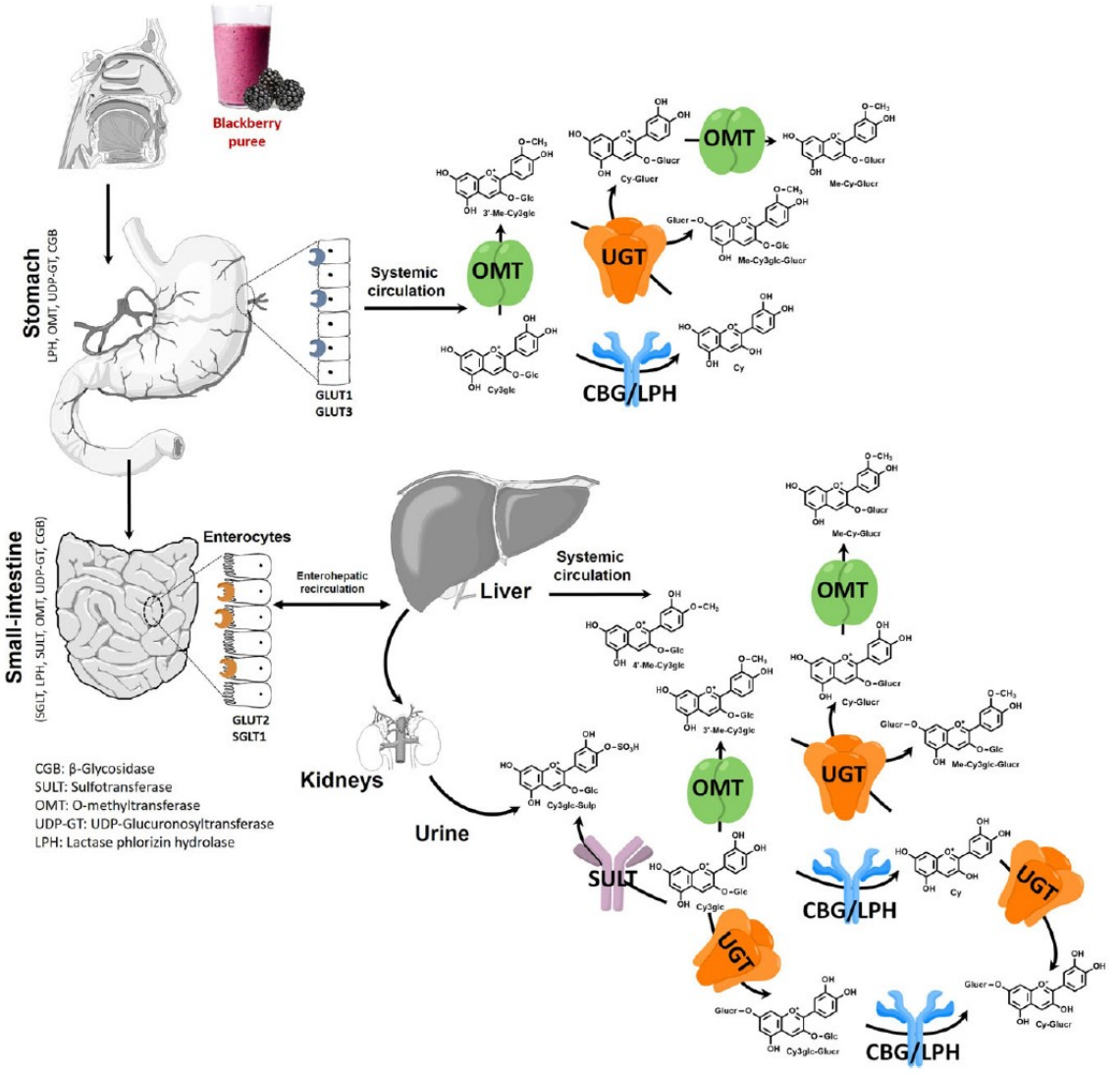


Figure 4. Proposed pathway for the metabolism of cyanidin-3-glucoside in humans. OMT, *O*-methyl transferases; Cy, cyanidin; Cy3glc, cyanidin-3-glucoside; CBG, cytosolic β-glucosidase; GLUT1, facilitative glucose transporter type 1; GLUT2, facilitative glucose transporter type 2; GLUT3, facilitative glucose transporter type 3; LPH, lactase-phlorizin hydrolase; Me, methyl; SGLT1, sodium-dependent glucose transporter; SULT, sulfotransferease; UGT, UDP-glucuronosyltransferase.

The present study has demonstrated that a wide array of anthocyanin phase II conjugates can be detected in plasma and urine after anthocyanins consumption. On the other hand, it has also revealed that anthocyanins may undergo different metabolic pathways and, therefore, an expanded interindividual variability for some of these compounds should be expected.

In the present study, BBP 12% did not increase the rate of appearance of Cy3glc or Cy3rut in plasma. This result suggests that ethanol may not interfere with anthocyanins absorption that is in accordance to other in vitro experiments performed by our group [27]. Conversely, anthocyanins absorption was higher after grape juice ingestion in comparison with red wine [13]. Nevertheless, this may not be related to the absence of ethanol but rather with additional components of red wine matrix that may interfere with anthocyanins absorption.

On the other hand, ethanol increased the plasma concentration of two methylated compounds (Me-Cy-Glucr and 3′-Me-Cy3glc). Bell et al. had already proposed that ethanol could accelerate the conversion of red wine flavonoids into methylated derivatives to explain the rapid elimination of (+)-catechin from the plasma compartment [32]. The AUC for Me-Cy-Glucr and 3′-Me-Cy3glc were also increased after BBP 12% consumption. To our knowledge, this is the first clinical trial showing that ethanol enhanced Cy3glc metabolism potentiating its conversion into methylated derivatives. The bioactivity of Me-Cy-Glucr and 3′-Me-Cy3glc remain to be clarified but previous studies performed by our group have shown that 3′-Me-Cy3glc exert some biological effects. The Cy3glc methylated metabolites were found to retain significant radical scavenging activity and reducing activity, suggesting that they could act as potential antioxidants in vivo. They also exert antiproliferative effects against gastric, colon, and breast cancer cells [33]. In addition, these metabolites can cross the blood–brain barrier and may be involved in the modulation of neuroinflammatory processes [34, 35]. Anthocyanin glucuronides might also be responsible for the biological effects of anthocyanins since, after red wine anthocyanins extract consumption, these compounds appeared in higher concentrations in plasma whereas circulating levels of MCP-1 decreased [36].

Another interesting finding from the present investigation was that anthocyanins metabolism may be compromised in overweight and obese individuals. Plasma and urinary concentrations of Me-Cy-Glucr and 3′-Me-Cy3glc after blackberry purees consumption were positively correlated and partly decreased in this group of participants. It is well established that the gut microbiota, which composition differs between lean and obese adults [37], has an important role in anthocyanins metabolism [38]. However, the analyzed compounds are not resulting from the colonic metabolism of anthocyanins and other mechanisms may possibly be behind the differences observed. Catechol-O-methyl transferase (COMT) is an enzyme responsible for xenobiotics methylation and its hepatic expression is decreased in rats with diet-induced obesity [39]. Alterations in COMT expression or activity in obese individuals may justify the decreased formation of methylated compounds in this group of participants.

Lastly, finding that the effects of ethanol were more pronounced in the overweight/obese group have also raised important considerations. In the overweight/obese group, the C_{max} of Me-Cy-Glucr and 3′-Me-Cy3glc was increased by ethanol toward levels similar to those presented by normal weight participants after drinking BBP12% (Table 2). Despite increasing the levels of Me-Cy-Glucr and 3′-Me-Cy3glc also in normal weight individuals, the effects of ethanol were more evidenced in the overweight/obese group. The mechanisms behind this effect need further investigation but it seems that ethanol is more effective in promoting Cy3glc metabolism when its metabolism is impaired. In addition, the bioactivity of these methylated compounds should be clarified in future studies in order to understand whether overweight and obese individuals can actually benefit from the consumption of foods containing simultaneously anthocyanins and ethanol, such as red wine.

In summary, the effects of ethanol on the pharmacokinetics of anthocyanins and anthocyanin conjugates were first and properly evaluated in the current study, by ensuring the same food matrix and the same amount of anthocyanins in both groups (BBP and BBP12%). The current study indicated for the first time that ethanol enhances Cy3glc metabolism potentiating its conversion into methylated derivatives (Me-Cy-Glucr and 3′-Me-Cy3glc). This effect was more pronounced in overweight and obese individuals, in whom Cy3glc metabolism appeared to be compromised. These results should prompt the attention of the scientific community to the fact that the kinetic of these compounds is influenced by ethanol and the body composition.

C.C., N.M., A.F., I.F., and C.M. designed the study; C.M., A.F., S.N., C.S., D.T., and C.C. conducted the clinical trial; C.M., I.F., and A.F. prepared all the biological samples for analysis; I.F. and V.F. performed the HPLC–MS/MS analysis, pharmacokinetic modeling, and compiled raw data; C.M. analyzed data and performed statistical analysis; C.M., I.F., and A.F. contributed to the development of the manuscript; C.C. and N.M. contributed to the critical review of the manuscript. All authors read and approved the final manuscript.

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The authors have declared no conflict of interest.

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SUPORTING INFORMATION

Methods

uHPLC analysis

uHPLC analysis of anthocyanins and conjugates was performed on Dionex Ultimate 3000 (Thermo Scientific; USA) equipped with a BDS Hypersil 150 x 4.6 mm i.d. (particle size 3 µm) reversed phase C18 column (Thermo Scientific; USA). Detection was carried out at 520 nm using a diode array detector (DAD). The solvents were (A) H_2O/HCOOH (9:1) and (B) H_2O/HCOOH/CH_3CN (6:1:3). The gradient consisted of 100-64% A for 40 min, followed by 10 min for cleaning with 100% solvent B and 10 min with initial conditions, at a flow rate of 1 mL/min.

qTOF analysis

The equipment was comprised of two main instruments: Waters ACQUITY UPLC™ System with ACQUITY UPLC® Photodiode Array Detector and Waters Quattro Premier™ XE benchtop tandem quadrupole mass spectrometer. The source temperature was set at 120 °C and the desolvation temperature was 350°C. The gas flow was 600 L/h for desolvation and 50 L/h for the cone. The autosampler temperature was maintained at 4°C. The mobile phase was comprised of (A) H_2O/HCOOH (9.9:0.1) and (B) H_2O/HCOOH/CH_3CN (6.9:0.1:3). The conditions established for the gradient elution program were 80–15% A for 70 min, followed by 10 min for cleaning with 100% solvent B and 10 min with initial conditions, at a flow rate of 0.4 mL/min. The injection volume was 10 µL for each sample injected.

Data acquisition, data handling and instrument control were performed by MassLynx software (Waters; USA).

Metabolite identification was performed by multiple reaction monitoring (MRM), optimized for the detection of pure standards with m/z of the parent and daughter fragments. Metabolites were confirmed on the basis of retention time (using authentic and synthesized standards whenever possible).

Table 1 - Characteristics of the study participants.

	All participants (n = 18)	Normal weight (n =8)	Overweight/Obese (n = 10)
Sex M/F (*n*)	10/8	5/3	5/5
Age (y)	27.8 ± 0.9	27.0 ± 3.6	28.5 ± 3.7
BMI (kg/m^2)	27.1 ± 1.1	23.2 ± 0.7	30.3 ± 1.2 *
Fat mass (%)	27.4 ± 2.4	20.0 ± 2.5	33.2 ± 2.6 *
SBP (mmHg)	129.1 ± 2.6	122.8 ± 2.4	134.2 ± 3.7 *
DBP (mmHg)	79.5 ± 2.0	77.5 ± 2.2	81.1 ± 3.2
HR (beats/min)	73.7 ± 2.1	73.5 ± 3.1	73.9 ± 2.9
Plasma			
Albumin (g/l)	44.5 ± 0.4	44.2 ± 0.6	44.8 ± 0.6
Glucose (mg/dl)	89.2 ± 1.3	91.1 ± 2.2	87.7 ± 1.5
Triglycerides (mg/dl)	81.8 ± 12.9	53.8 ± 2.7	104.2 ± 20.9*
Cholesterol (mg/dl)	169.2 ± 7.7	148.5 ± 6.5	185.8 ± 10.2*
HDL (mg/dl)	53.0 ± 3.6	53.6 ± 2.7	52.5 ± 6.3
LDL (mg/dl)	99.8 ± 7.4	97.3 ± 7.7	131.0 ± 10.1*
CRP (mg/l)	3.1 ± 0.7	1.1 ± 0.3	4.7 ± 1.0*
Uric Acid (mg/dl)	5.0 ± 0.3	4.6 ± 0.4	5.3 ± 0.4
Urea (g/l)	29.7 ± 1.3	29.2 ± 2.0	30.1 ± 1.7
Creatinine (mg/l)	0.8 ± 0.0	0.8 ± 0.1	0.8 ± 0.0
Urine			
Urea (g/l)	25.2 ± 1.4	24.0 ± 1.8	26.2 ± 2.1
Creatinine (mg/l)	1989.6 ± 131.3	1930.5 ± 214.9	2036.8 ± 171.3

Values are expressed as mean ± SEM.* $P < 0.05$ vs normal weight participants. CRP, C-reactive protein; DBP, diastolic blood pressure; HR, heart rate; SBP, systolic blood pressure.

Table 2 - HPLC-MS/MS parameters (MRM) for the identification of anthocyanins and anthocyanin conjugates in human samples.

	M^+	MS^2	Previously described in plasma	Previously described in urine
Cy	287	137 (-150)	X	X
Me-Cy	301	287 (-14)	X	[1]
Cy-Glucr	463	287 (-176)	[1-3]	[1-4]
Cy-Glucr-Glucr	639	463 (-176)	X	[3]
Cy-Sulp	367	287 (-80)	X	X
Me-Cy-Glucr	477	301 (-176)	[1-3, 5]	[1-4, 6]
Cy3glc	449	287 (-162)	[1, 3-5, 7, 8]	[1, 3-7, 9-12]
Me-Cy3glc	463	301 (-162)	[1, 5]	[1, 3-6]
Cy3glc-Glucr	625	463 (-162)	X	[6]
Cy3glc-Glucr-Glucr	801	639 (-162)	X	X
Me-Cy3glc-Glucr	639	477 (-162)	X	[3-5]
Me-Cy3glc-Glucr-Glucr	815	653 (-162)	X	X
Cy3glc-Sulp	529	449 (-80)	X	X
Cy3glc-diSulp	609	447 (-162)	X	[3]
Cy3rut	595	449 (-146)	X	X
Cy3rut	595	287 (-308)	X	X
Me-Cy3rut	609	301 (-308)	X	X
Cy3rut-Glucr	771	463 (-308)	X	X
Me-Cy3rut-Glucr	785	477 (-308)	X	X
Cy3rut-Sulp	675	367 (-308)	X	X
Cy3rut-diSulp	755	607 (-308)	X	X
Cy3manglc	535	287 (-162, -86)	X	X
Cy3dioxaglc	593	287 (-144, -162)	X	X

Cy, cyanidin; Cy3dioxaglc, cyanidin-3-dioxaloyl-glucoside; Cy3glc, cyanidin-3-glucoside; Cy3manglc, cyanidin-3-malonyl-glucoside; Cy3rut, cyanidin-3-rutinoside; diSulp, disulphate; Glucr, glucuronide; Me, methyl; Sulp, sulphate.

Table 3 - Anthocyanins and anthocyanin conjugates presented in plasma samples of normal weight and overweight/obese participants at 15, 30, 60 and 120 min after consumption of 250 ml blackberry puree without ethanol (BBP) and with 12% ethanol (BBP 12%).

	Normal weight								Overweight/Obese							
	BBP				BBP 12%				BBP				BBP 12%			
	15	30	60	120	15	30	60	120	15	30	60	120	15	30	60	120
Cy3glc	26 ± 11	27 ± 3	39 ± 4	34 ± 9	26 ± 6	39 ± 13	46 ± 6	45 ± 9	19 ± 4	37 ± 8	44 ± 9	42 ± 12	29 ± 9	45 ± 10	53 ± 11	31 ± 10
Cy3rut	0 ± 0	4 ± 1	8 ± 2	11 ± 3	3 ± 1	6 ± 1	7 ± 1	7 ± 2	3 ± 1	6 ± 1	8 ± 1	8 ± 1	2 ± 0	5 ± 1	7 ± 1	11 ± 2
Cy3glc-Glucr	0 ± 0	8 ± 0	14 ± 7	33 ± 2	14 ± 0	5 ± 1	17 ± 8	37 ± 4	356 ± 0	58 ± 50	79 ± 67	45 ± 28	4 ± 0	192 ± 183	31 ± 17	116 ± 79
Me-Cy3glc-Glucr	4 ± 0	16 ± 6	40 ± 13	75 ± 23	0 ± 0	0 ± 0	0 ± 0	0 ± 0	7 ± 3	18 ± 7	22 ± 8	43 ± 9	5 ± 3	26 ± 24	74 ± 52	265 ± 190
Cy3glc-Sulp	3 ± 0	11 ± 10	21 ± 6	33 ± 13	12 ± 1	13 ± 2	33 ± 13	44 ± 17	5 ± 2	12 ± 5	64 ± 33	45 ± 21	4 ± 0	8 ± 2	21 ± 6	47 ± 17
Cy-Glucr (a)	0 ± 0	10 ± 4	24 ± 5	36 ± 7	9 ± 2	23 ± 13	34 ± 13	43 ± 15	8 ± 4	16 ± 8	22 ± 5	29 ± 8	6 ± 4	8 ± 3	20 ± 4	53 ± 17
Cy-Glucr (b)	5 ± 0	6 ± 0	6 ± 3	4 ± 0	5 ± 2	6 ± 2	5 ± 2	4 ± 1	7 ± 3	4 ± 1	6 ± 1	26 ± 21	4 ± 2	4 ± 2	4 ± 2	4 ± 1
Me-Cy-Glucr	11 ± 4	42 ± 10	118 ± 31	272 ± 39	22 ± 11	62 ± 26	154 ± 88	278 ± 61	11 ± 2	26 ± 5	75 ± 15	110 ± 15	5 ± 2	38 ± 5	124 ± 22	300 ± 38
3'-Me-Cy3glc	3 ± 1	13 ± 4	39 ± 9	88 ± 11	9 ± 3	19 ± 8	67 ± 24	82 ± 18	4 ± 2	12 ± 2	31 ± 7	40 ± 4	3 ± 1	14 ± 4	46 ± 7	103 ± 10

Values are expressed as mean ± SEM in ng/ml (n=5-9). Plasma concentrations of anthocyanins and anthocyanin conjugates were quantified by HPLC-DAD. Cy, cyanidin; Cy3glc, cyanidin-3-glucoside; Cy3rut, cyanidin-3-rutinoside; Glucr, glucuronide; Me, methyl; Sulp, sulphate

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CHAPTER IV

CONCLUDING REMARKS

FUTURE PERSPECTIVES

Concluding Remarks

When it comes to our microbes, we are never alone. The human gut households trillions of microorganisms that have been evolving with us for millions of years [154]. On account of the technological advances, the scientific community have been exploring the dynamics of such complex ecosystem. The role these microorganisms play in our health is still a mystery but emerging evidence suggest that gut microbiota dysbiosis is implicated in a number of diseases ranging from localized gastrointestinal disorders to autoimmune, hepatic, respiratory, cardiovascular, oncologic, metabolic, neurologic and psychiatric diseases [155].

The incidence of these diseases, specially obesity and obesity associated metabolic disorders has substantially increased during the past two centuries. This period encompasses only a limited number of human generations, which makes obesity epidemic unlikely to be explained by genetic factors alone. On the other hand, the environmental and behavioral changes (including dietary modifications and exposure to xenobiotics), largely adopted by post-industrial revolution societies, are probably related to the increasing incidence of these inflammatory and metabolic diseases [122, 156]. Nonetheless, both human and microbial genomes have been subject to these rapid environmental pressures. Therefore, another gene pool - the gut microbiome - has to be considered when evaluating the impact of such environmental factors on human health.

Humans and their microbiome form a composite organism, the so-called “holobiont” [157, 158]. The hologenome theory of evolution proposes that natural selection acts not on the individual organism but on the “holobiont” [159]. Thus, when a “holobiont” is challenged by dramatic changes, such as altered diet, reduced physical activity or drugs, it employs adaptive mechanisms in the form of reshuffling/balancing its microbiome [159]. The significantly shorter generation time of microorganisms, make the microbiome responsive to rapid evolutionary changes on a much shorter timescale than the host genome [122]. The human microbiome is highly mutable and its functional capacity is dynamic [160]. Nevertheless, every time it changes to adapt to new environmental conditions, some functions might get lost, which can be detrimental for human health.

The compilation of studies unveiled in this thesis intended to explore the effects of a particular class of dietary compounds (anthocyanins) on the gut microbiota composition. The bioavailability of anthocyanins is considered to be low, therefore, it is expected that high amounts of these compounds reach the colon after consumption of anthocyanins-rich foods.

The main aim of this work was to verify whether they could prevent the dysbiosis associated with obesity and the consequent metabolic and neurologic disorders that can emerge from such a disrupted microbiome. To obtain an animal model of obesity and its associated metabolic disorders, in which the effects of anthocyanins could be studied, our primary approach was to use an high-fat and high-sucrose diet (45 % and 17 % of energy, respectively) to induce obesity in rats (**Chapter II-A, Chapter II-B, Chapter II-D**). A genetic animal model of obesity was not used since, as discussed above, genetics are probably not the major driving force of obesity epidemics.

The high-fat and high-sucrose diet (HF diet) was carefully chosen to adequately mimic the modern dietary pattern (Western diet) adopted by post-industrial revolution societies [161]. This type of diet is able to induce obesity and metabolic disorders in rodents resembling human metabolic syndrome [161, 162]. Moreover, HF diet abruptly change the composition of the gut microbiota and these changes seem to be associated with the development of obesity and its related metabolic complications [163-168].

Gut microbiota is probably indispensable for obesity development, as germ-free animals are resistant to HF diet-induced obesity [33]. On the other hand, it is well established that a disrupted microbiome, either from HF diet-induced obesity rodents, obese individuals (that usually have a diet rich in fat, sugar and food additives) or from *ob/ob* animals (that continuously have a sense of starvation and exhibit hyperphagia), initiates obesity after transplantation to germ-free animals [25, 169, 170]. Therefore, it is important to characterize these diet-induced changes on the gut microbiota composition since they might be responsible for the onset of obesity and metabolic disorders.

Wistar and Sprague-Dawley are the main Rat strains used as HF diet-induced obesity models [171-174]. Nevertheless, a suitable comparison between these two Rat strains as HF-diet induced obesity models was not available in the literature. Furthermore, the effects of HF diet upon the gut microbiota of these animals were not previously characterized. Therefore, in **Chapter II-A**, Wistar and Sprague-Dawley rats were studied in parallel to determine the most appropriate model where the effect of anthocyanins could be investigated (**Chapter II-D**).

As discussed in **Chapter II-A,** both strains can be used as models of HF diet-induced obesity, although Wistar rats seemed to be particularly predisposed to HF diet-induced obesity and metabolic disorders [175]. The gut microbiota composition of the two Rat strains differed in some of the bacterial genera analyzed which can explain different metabolic responses to HF

diet [175]. As previously reported, host genetics may influence the gut microbiota composition which may predict, in turn, the effects of dietary interventions on host metabolic parameters [176-178].

In **Chapter II-B**, Wistar rats with high-fat diet induced obesity were further characterized concerning intestinal injury and inflammation [179].

The intestinal epithelium is a highly regulated physical barrier that secretes several compounds such as mucus and antimicrobial peptides which, together, act as front lines of defense protecting the host against bacterial translocation [180]. Recently, it has been hypothesized that gut barrier dysfunction in obesity leads to the passage of microbial components into circulation, which drives systemic inflammation [181]. Moreover, HF diets can compromise gut mucosal integrity and, therefore, contribute to metabolic endotoxemia [182-186]. Herein (**Chapter II-B**), intestinal fatty-acid binding protein (I-FABP) and glucagon-like peptide-2 (GLP-2) were evaluated as putative biomarkers of intestinal permeability in the context of HF-diet induced obesity [179]. Although I-FABP ended up not being a good biomarker of intestinal permeability, GLP-2 was surprisingly increased in the plasma of HF diet fed animals and were positively correlated with systemic inflammatory markers [94].

As discussed in that chapter**,** the inflammatory state driven by gut microbiota modifications after HF-diet feeding might have increased GLP-2 production in order to improve the mucosal barrier integrity and, therefore, blunt the inflammatory stress [179]. Accordingly, a very recent report has shown that the L cell is as a key mucosal sensor of gut injury, which responds to mucosal damage by secretion of glucagon-like peptides which, in turn, promote restoration of mucosal integrity and attenuation of inflammation [187]. Therefore, GLP-2 may be used as a biomarker of intestinal permeability to anticipate the progression of obesity-associated metabolic disorders.

Apart from being implicated in the onset and development of metabolic diseases, the HF-diet disrupted microbiota may also be implicated in several neurologic conditions involving inflammation. The idea that HF diet-induced dysbiosis is a rational cause of obesity-related neuroinflammation was significantly reinforced in **Chapter II-C**, after the publication of Bruce Keller *et al* [188, 189]. These authors have demonstrated that mice receiving an obese-type microbiota exhibited increased neuroinflammation together with significant disruptions in exploratory, cognitive and stereotypical behaviors (increased anxiety and decreased memory) [188].

Given the immunomodulatory properties of the gut microbiota, immune cell pathways have been highlighted as important mechanisms mediating microbial modulation of brain function and behavior [190]. Intestinal microbes modulate the maturation and function of microglia and astrocytes within the CNS while they also influence the activation of peripheral immune cells that regulate responses to neuroinflammation, brain injury and neurogenesis [190]. Consequently, germ-free mice which are raised under sterile conditions exhibit substantial alterations in behavior (reduced anxiety-like behavior, reduced social behavior, hyperactivity) and display learning and memory deficits compared to conventional raised mice [191-194] .

The activation of these immune pathways in the host result from the recognition of microbial derived products via TLRs. TLRs are the most well characterized family of pattern recognition receptors and are expressed not only on innate immune cells but also on CNS cell populations, including neurons and glial cells [195]. Thus, TLR ligands derived from the intestinal microbiota (e.g. LPS) may be capable to directly trigger innate immune pathways to affect CNS function [196, 197].

In HF-diet induced obesity mice, fecal LPS levels are increased (as a result of HF-diet induced dysbiosis) along with systemic LPS levels (LPS is absorbed together with fat) [63]. Moreover, the increased intestinal permeability observed in these animals intensifies the passage of TLR ligands from the gut microbiota to the systemic circulation [66]. Thenceforward, TLR ligands may activate microglia in the brain and, consequently, induce neuroinflammation [198, 199]. Accordingly, results from **Chapter II-A**, **II-B** and **II-C** show that Wistar rats with HF-diet induced obesity exhibited gut microbiota dysbiosis, increased fecal LPS concentrations, increased intestinal permeability (assessed by GLP-2) and neuroinflammation [148, 175, 179, 189]. Altogether, these data highlight the importance of targeting HF-diet induced dysbiosis in the treatment of neuropsychiatry disorders.

Besides being tangled in anxiety and depression (neuropsychiatry disorders whose risk is tremendously increased by obesity), neuroinflammation plays a prominent role in the progression of neurodegenerative diseases such as Alzheimer's and Parkinson's disease [200, 201]. As population gets older, the incidence of neurodegenerative diseases, become increasingly more prevalent and new therapeutic strategies are warranted [202]. Recent evidence have shown that neurodegenerative alterations in Parkinson's disease are accompanied by gastrointestinal symptoms that may precede or follow CNS impairment [100]. Consequently, gut microbiota modifications may also constitute a promising therapeutic option for neurodegenerative diseases.

Targeting the gut microbiota with prebiotics might be a good strategy to nurture a beneficial microbiome and prevent dysbiosis-associated diseases [95, 203].

The most studied prebiotics are non-digestible carbohydrates to fulfill the criteria of previous prebiotic definition [204]. However, the definition of prebiotics has been modified to "a substrate that is selectively utilized by host microorganisms conferring a health benefit" [205]. Other substances, beyond carbohydrates, might fit the updated definition if convincing evidence demonstrates their health benefits. Compounds such as polyphenols, namely anthocyanins, may be considered prebiotics, according to this new definition.

Anthocyanins turn out to be particularly relevant since their bioavailability is considered to be low. Therefore, after consumption of anthocyanin-rich foods, it is expected that high amounts of anthocyanins reach the intestine to modulate bacterial growth at the same time they are metabolized by the existing bacteria.

In **Chapter II-D**, the effects of anthocyanins on gut microbiota were evaluated using the HF-diet induced obesity model characterized in **Chapters II-A, II-B and II-C**. The modifications in the gut microbiota composition brought about by anthocyanins were not sufficient to prevent the onset and development of obesity and metabolic diseases but, somehow, attenuated the detrimental effects of neuroinflammation in a high-fat challenged brain [148, 206].

Moreover, in the same chapter, an attempt to unravel the mechanisms behind the neuroprotective effects of anthocyanins was conducted. Despite some modifications in the gut microbiota composition, the fecal LPS concentrations were not significantly decreased by anthocyanins nor GLP-2 plasma levels (data not shown), which indicate that anthocyanins did not preclude gut barrier dysfunction. Nevertheless, anthocyanins may interfere in other mechanisms by which the gut can communicate with the brain.

One of these mechanisms is related to the ability of intestinal bacteria to modulate the host metabolome. Circulating metabolites can enter the CNS and directly affect neuroactivity [207]. Conversely, particular metabolites can regulate the function of peripheral immune cells which can then influence brain function [208].

Results from this chapter revealed that the alterations in gut microbial composition brought about by anthocyanins resulted in changes in the levels of tryptophan and kynurenic acid which has been implicated in CNS inflammation, excitation and behavior **(Figure 5)**.

Psychobiotics were previously defined as live bacteria which, when ingested, confer mental health benefits through interactions with commensal gut bacteria [209]. Recently, this definition was expanded to encompass prebiotics [210]. Therefore, according to the results present in this thesis, anthocyanins might constitute a new class of psychobiotics.

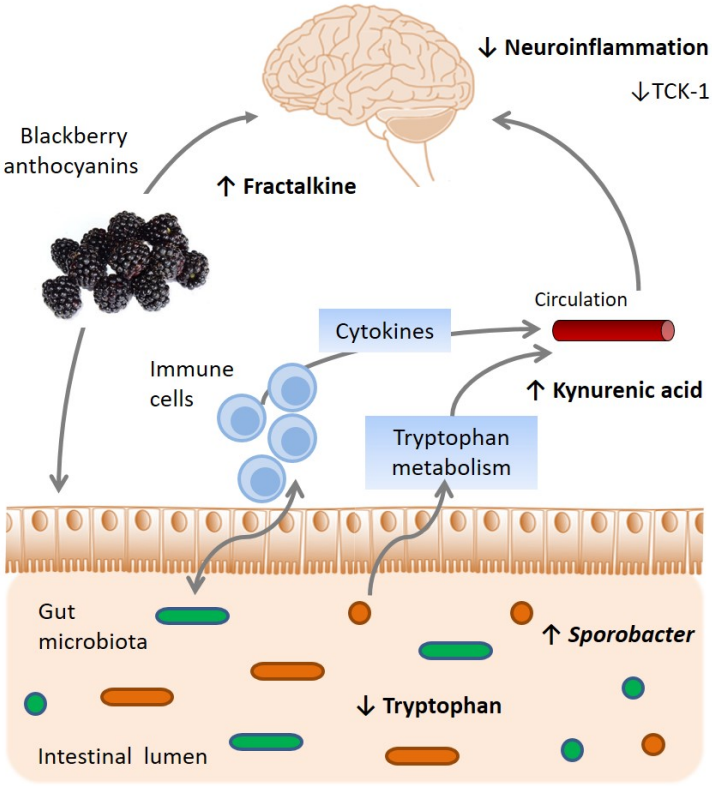

Figure 5 - Mechanisms behind the neuroprotective effects of anthocyanins, in the context of HF-diet induced obesity. Anthocyanins act directly in the brain increasing the expression of fractalkine, a chemokine extremely important in the crosstalk between neurons and microglia during synaptic plasticity [148]. On the other hand, anthocyanins modulate the gut microbiota composition, increasing the bacterial genus *Sporobacter* and alter host tryptophan metabolism. The amount of tryptophan available is decreased by anthocyanins to undergo the kynurenine pathway and originate kynurenic acid, a metabolite whose neuroprotective actions were recently identified [211, 212]. Through these routes, anthocyanins counteract the HF-diet induced neuroinflammation and may attenuate the neurological complications of obesity as well as neurodegenerative diseases. Anthocyanins might constitute, therefore, a new class of psychobiotics. TCK-1, thymus chemokine-1.

The preclinical studies presented in **Chapter II** require further validation in humans. Clinical trials are necessary to confirm the effects of anthocyanins on the gut microbiota and whether they can be a useful tool in the management of neuropsychiatry and neurodegenerative disorders.

The first thing to address in humans is the bioavailability of these compounds. The bioavailability of anthocyanins is considered to be low but in fact, anthocyanins are rather extensively metabolized. In **Chapter III**, the analysis of plasma and urine samples of healthy volunteers after the ingestion of a blackberry puree revealed that the plasma concentration of anthocyanins' parent structure (the one that naturally occurs in fruits and is actually consumed) is almost ten times lower than the newly formed metabolites [213]. Nonetheless, this discrepancy might be even bigger if the metabolites that are produced by the gut microbiota were considered. Thus, the bioavailability of anthocyanins might not be that low. Given the enterohepatic recirculation, these compounds may prevail in the human body for several days [214]. Moreover, they can accumulate in several organs where they can exert their effects [215].

Another interesting point addressed in **Chapter III** was the difference between normal weight and obese individuals regarding the metabolism of anthocyanins [213]. The disrupted gut microbiota as well as the generalized metabolic dysfunction unveiled by obese individuals may be behind the variability observed between these two groups. Obesity may comprise the metabolism of these compounds which deserves special consideration since obese individuals might be the ones who would benefit the most from anthocyanins intervention.

The first steps in the human studies were already undertaken in **Chapter III** of this thesis. However, quoting George Bernard Shaw, "science never solves a problem without creating ten more".

Which metabolites are originated from the microbial metabolism of anthocyanins? Are they more bioactive than the parent compounds? How they interfere with host tryptophan metabolism? Do they contribute to the prevention of neuroinflammation? These are questions that still need to be unraveled and will inspire future clinical trials.

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