**UNIVERSITY OF SÃO PAULO**
**FACULTY OF PHARMACEUTICAL SCIENCE**
**GRADUATE PROGRAM IN FOOD SCIENCE**
**AREA OF FOOD SCIENCE**

# THE ROLE OF JABOTICABA (*Plinia jaboticaba* (VELL.) BERG) PHENOLIC COMPOUNDS IN OBESITY-ASSOCIATED INTESTINAL INFLAMMATION

**LARISSA RODRIGUES**

**SÃO PAULO**
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Advisor: Prof. Dr. Maria Inés Genovese Rodriguez

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To the Lord, who deserves everything, and to all the amazing people He has put on may path. You inspire me to be better every day.

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“The known is finite, the unknown infinite; intellectually we stand on an islet in the midst of an illimitable ocean of inexplicability. Our business in every generation is to reclaim a little more land, to add something to the extent and the solidity of our possession”.

Thomas Huxley

## ABSTRACT

RODRIGUES, L. **The role of jaboticaba (*Plinia jaboticaba* (VELL.) Berg) phenolic compounds in obesity-associated intestinal inflammation.** FACULTY OF PHARMACEUTICAL SCIENCES – UNIVERSITY OF SÃO PAULO, SÃO PAULO, 2021.

Jaboticaba (*Plinia jaboticaba* (Vell.) Berg) is a Brazilian native fruit belonging to the Myrtaceae family. Previously it was demonstrated that phenolic-rich extracts from jaboticaba (PEJ) possess health-beneficial properties in diet-induced obesity; however, whether PEJ modulates the obesity-associated intestinal inflammatory status remains unclear. The objective of the present study was to evaluate the effect of PEJ on intestinal inflammation associated with obesity induced by a high-fat-sucrose (HFS) diet. Thus, forty male C57BL/6J mice were distributed into two groups: negative control (CH, 10 animals), fed standard diet AIN96M and water *ad libitum*; and positive control (HFS, 30 animals), fed HFS diet and water *ad libitum* induced to obesity for an initial period of 14 weeks. After this period, the HFS group was redistributed in three groups of 10 animals each, and continuously fed HFS diet for another 14 weeks: HFS group received daily gavages of water, PEJ1 group received PEJ at the dose of 50 mg of gallic acid equivalent (GAE)/kg body weight (BW and PEJ2 group received PEJ at the dose of 100 mg GAE/kg BW. Feed intake and body mass were monitored weekly, and fasting glucose biweekly. The initial period of obesity-induction demonstrated that the HFS diet was efficient to promote a significant body weight gain and fasting hyperglycemia when compared to the negative control group (CH). At the end of the experiment the animals were euthanized under anesthesia and their organs and tissues were collected. The major classes of phenolic compounds found in PEJ were ellagitannins, anthocyanins including cyanidin and delphinidin glycosides, proanthocyanidins, and free ellagic acid. PEJ-treated animals presented a reduced body weight gain, adiposity and demonstrated significant reversion of insulin resistance and dyslipidemia. In addition, the inflammatory profile of colon demonstrated that PEJ prevented metabolic endotoxemia linked to an attenuation of the HFS diet-induced intestinal inflammation via down-regulation of pro-inflammatory mediators such as tumor necrosis factor (TNF-α), membrane transporter toll-like receptor-4 (TLR-4) and nuclear factor-κB (NF-κB) in the colon. These anti-inflammatory effects appear to be involved, at least in part, with an inhibition of the colonic inflammasome pathway of obese mice. Collectively, our data reveals that PEJ exerts a direct anti-inflammatory effect in obesity-associated intestinal inflammation and this outcome is linked to an amelioration of metabolic endotoxemia in obese mice.



**Keywords:** Jaboticaba, Myrtaceae, Inflammasome, Intestinal inflammation, Polyphenols.

## RESUMO

RODRIGUES, L. **O papel dos compostos fenólicos da jabuticaba (*Plinia jaboticaba* (VELL.) Berg) na inflamação intestinal associada à obesidade.** Faculdade de Ciências Farmacêuticas – Universidade de São Paulo, São Paulo, 2021.

A jabuticaba (Plinia jaboticaba (Vell.) Berg) é uma fruta nativa brasileira pertencente à família Myrtaceae. Anteriormente, foi demonstrado que extratos ricos em fenólicos de jabuticaba (PEJ) possuem propriedades benéficas à saúde na obesidade induzida por dieta; no entanto, se o PEJ modula o estado inflamatório intestinal associado à obesidade ainda não está claro. O objetivo do presente estudo foi avaliar o efeito do PEJ na inflamação intestinal associada à obesidade induzida por uma dieta rica em sacarose (HFS). Assim, quarenta camundongos C57BL / 6J machos foram distribuídos em dois grupos: controle negativo (CH, 10 animais), alimentados com dieta padrão AIN96M e água ad libitum; e controle positivo (HFS, 30 animais), alimentado com dieta HFS e água ad libitum induzida à obesidade por um período inicial de 14 semanas. Após este período, o grupo HFS foi redistribuído em três grupos de 10 animais cada, e continuamente alimentado com dieta HFS por mais 14 semanas: o grupo HFS recebeu gavagens diárias de água, o grupo PEJ1 recebeu PEJ na dose de 50 mg de ácido gálico equivalente (GAE) / kg de peso corporal (pc) e o grupo PEJ2 recebeu PEJ na dose de 100 mg GAE / kg pc. O consumo de ração e a massa corporal foram monitorados semanalmente e a glicemia de jejum quinzenal. O período inicial de indução da obesidade demonstrou que a dieta HFS foi eficiente em promover significativo ganho de peso corporal e hiperglicemia de jejum quando comparada ao grupo controle negativo (HC). Ao final do experimento os animais foram submetidos à eutanásia sob anestesia e seus órgãos e tecidos coletados. As principais classes de compostos fenólicos encontrados em PEJ foram elagitaninos, antocianinas incluindo cianidina e delfinidina glicosiladas, proantocianidinas e ácido elágico livre. Os animais tratados com PEJ apresentaram redução do ganho de peso corporal, adiposidade e reversão significativa da resistência à insulina e dislipidemia. Além disso, o perfil inflamatório do cólon demonstrou que o PEJ evitou a endotoxemia metabólica ligada a uma atenuação da inflamação intestinal induzida pela dieta de HFS por meio da regulação negativa de mediadores pró-inflamatórios, como o fator de necrose tumoral (TNF-α), transportador de membrana toll- como o receptor 4 (TLR-4) e o fator nuclear κB (NF-κB) no cólon. Esses efeitos anti-inflamatórios parecem estar envolvidos, pelo menos em parte, com uma inibição da via do inflamassoma colônico de camundongos obesos. Coletivamente, nossos dados revelam que o PEJ exerce um efeito anti-inflamatório direto na inflamação intestinal associada à obesidade e esse resultado está relacionado com uma melhora da endotoxemia metabólica em camundongos obesos.



**Palavras-chave:** Jabuticaba, Myrtaceae, Inflamassoma, Inflamação intestinal, Polifenóis.

## FIGURE LIST

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## SYMBOLS AND ABBREVIATIONS

°C – Celsius degrees
AMPK – AMP-activated protein kinase
AT – Adipose Tissue
AUC – Area under curve
BW – body weight
C18 – Octadecylsilane resin
CD – Crohn disease
CEAGESP – Companhia geral de entrepostos e armazens de São Paulo
CH – Chow (negative control group)
COX-2 – Cyclo-oxygenase-2
CXCL-IP10 – Interferon indutory protein
DAD – Diode Array Detector
DMAC - dimethylaminocinnamaldehyde
DSS – Dextran sulfate sodium
EDTA – Ethylenediaminetetraacetic Acid Tetrasodium Salt
EP – Epidydimal
ESI – Electrospray ionization
FBC – Food bioactive compounds
Fe2SO4 – Iron II sulfate
FEA – Free ellagic acid
g – grams
GI – Gastrointestinal
GAE – Gallic acid equivalent
GAPDH – glyceraldehyde 3-phosphate dehydrogenase
h – hour
H2O – Water
HCl – Chloride acid
HE – Hematoxylin/Eosin
HFS – High-fat-sugar diet
HPLC – High-performance liquid chromatography
IBD – Inflammatory bowel disease
IL – Interleukin

IFN – Interferon

iNOS – Inducible NO synthase

IG – Inguinal

JNK – c-jun-N-terminal protein kinase

Kcal – Kilocalories

kV – Kilovolts

LC-MS – Liquid chromatography/mass spectrometry

PEJ1 – Low dose

LEP – Leptin hormone

LEPR – Leptin receptor

LPS – Lipopolysaccharide

M – mol/L

Min – minute

m/z – mass-to-charge

MCP-1 – Monocyte Chemoattractant Protein-1

mg – Miligrams

mL – Mililiters

µL – microliters

µm – Milimeters

MLC – Myosin light chain

MLCK – Myosin light chain kinase

MS – Mass spectometry

N2 – Nitrogen

NaOH – Sodium hydroxide

NEFA – Non-esterified fat acids

NCDs – Noncommunicable chronic disease

NF-kB – Necrosis factor

nm – nanometers

oGTT – Oral glucose tolerance test

PAS – Periodic acid Shiff

PB2 – Procyanidin B2

PBS – Phosphate buffered saline solution

PCR – Polymerase chain reaction

PEJ – Phenolic extract from jaboticaba

PMSF – Phenylmethylsulfonyl fluoride

PTFE – Teflon

PVDF – polyvinylidene difluoride membrane

RT – Retention time

RT – Retoperitoneal

RT-PCR – Real time PCR

SC – Subcultaneous

SD – Standard deviation

SPE – Solid phase extraction

T2DM – Type 2 diabetes mellitus

TAG – Triacylglycerols

TJ – Tight junctions

TLR – Toll like receptor

TNF – Tumor necrosis factor

tWAT – Total white adipose tissue

UC - Ulcerative Colitis

UV – Ultraviolet

v/v – volume/ volume

WAT – White adipose tissue

WHO – World health organization

ZO – Zonula occludens

**SUMMARY**

# 1. INTRODUCTION

## 1.1. BIOACTIVE COMPOUNDS

Populations around the world have different incidences of chronic noncommunicable diseases (NCDs), such as cardiovascular diseases, diabetes, stroke, and cancer, and this fact has been associated with different diet characteristics. Mediterranean people, for example, have a lower incidence of NCDs than Western people, and this has been attributed to a wide variety of fruits and vegetables in their diets, very different from the hypercaloric Western diets (BASTOS; ROGERO; ARÊAS, 2009). In the last few decades, an increased interest has been given to natural compounds of plants, which may contribute to health. These phytochemicals are plant secondary metabolites and the regular consumption has been associated with reduced risk of some NCDs and health benefits (CROZIER; JAGANATH; CLIFFORD, 2009; DEL RIO et al., 2013).

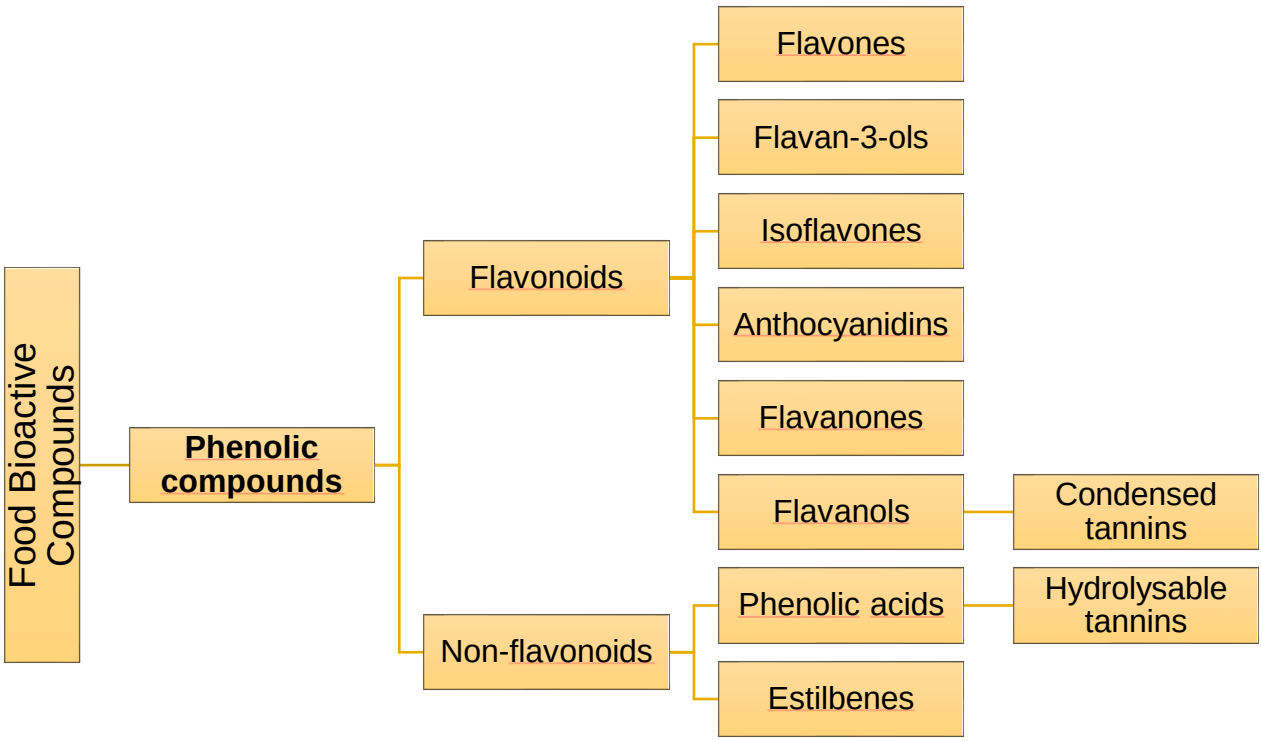
The protective effects of the diet could be attributed, in part, to a specific class of compounds, the polyphenols, a large class of plant-derived substances. They act protecting plants from herbivores, microbial infection, UV light, etc. The basic structure of these compounds presents is composed by one or more aromatic rings containing at least one hydroxyl group attached. Until 2009, more than 8000 phenolic chemical structures had been described, dispersed among the variety of plants, but many of them can be also found in plant foods (CROZIER; JAGANATH; CLIFFORD, 2009).

Phenolic compounds can be classified by the number and arrangement of their attached radicals and are commonly found as sugars and/or organic acids conjugates. Is possible to classify them into two groups, flavonoids, and non-flavonoids (**Figure 1**). Flavonoids are most numerous and are found throughout the plant kingdom. Among them, there are six major subclasses: flavonols, flavones, flavanones, isoflavones, anthocyanidins, and flavan-3-ols (CROZIER; JAGANATH; CLIFFORD, 2009; CROZIER; DEL RIO; CLIFFORD, 2010; DEL RIO et al., 2013).

Among the flavonols, the most common compounds are quercetin, myricetin, and kaempferol, which are mainly found in the glycosylated form. One of the main sources of quercetin and its derivatives is onion (*Allium cepa*), a food

commonly consumed worldwide. Flavones and flavanones such as hesperidin and naringenin are commonly found in citrus fruits, for example, sour orange (*Citrus aurantium*) and grapefruit (*Citrus paradise*). Legumes are almost exclusively sources of isoflavones which, due to their structural similarity to estrogen, are classified as phytoestrogens. Soy (*Glycine max*) is rich in daidzein and genistein, two isoflavones widely consumed in both Eastern and Western cultures. Fruits as raspberry (*Rubus idaeus L.*), strawberry (*Fragaria L.*), blackberry (*Rubus L.*), and blueberry (*Vaccinium L.*), are diversified sources of anthocyanins and tannins, such as ellagitannins and proanthocyanidins. In relation to the non-flavonoids, coffee is a significant source of chlorogenic acid and, due to its popularity and high consumption, this phenolic type is part of the daily diet of people around the world. In addition to these examples, red wine is also widely appreciated, rich in resveratrol and derivatives (CROZIER; JAGANATH; CLIFFORD, 2009; DEL RIO et al., 2013).

**Figure 1.** Classification of phenolic compounds.




Source: the author (2019).

The non-flavonoids are distributed into phenolic acids and stilbenes. Is possible to classify the phenolic acids, as hydroxybenzoic and hydroxycinnamic

acids and their derivatives. Gallic and ellagic acids are the main hydroxybenzoic acids and they are precursors of gallic and ellagic tannins, also known as hydrolysable tannins. Hydroxycinnamic acids occur conjugated to other compounds and are collectively referred as chlorogenic acid (DEL RIO et al., 2013).

## 1.2 OBESITY AND INTESTINAL INFLAMMATION

Overweight and obesity are characterized by abnormal or excessive body fat accumulation, outcoming from a chronic imbalance between energy intake and expenditure that affects health (XIE; WATERS; SCHIRRA, 2012). According to the World Health Organization (WHO), can be considered overweight individuals with Body Mass Index (BMI) between 25.0-29.9 Kg/m², and obese individuals with BMI over 30 Kg/m². This condition causes a chronic and low-grade inflammation, becoming a serious risk factor to develop other chronic associated diseases, like type 2 diabetes mellitus (T2DM), cardiomyopathies, cancers, insulin resistance, osteoarthritis, and decreases the reproductive performance (ITOH et al., 2011; WHO, 2018).

Nowadays obesity and its related diseases have become a subject of major importance, since the percentage of overweight and obese individuals has increased in both developed and developing countries, over the past few decades. According to the WHO, since 1975 it has almost tripled. In 2016, 39% of the total population older than 18 years, which represents more than 1.9 billion adults in this age category, were overweight, and over 13% were obese. Some decades ago, underweight was the main concern between most of the countries across the world; recently, overweight and obesity kill more people than underweight (WHO, 2018). Obesity is affecting population independently of age and ethnic groups, which means that is no longer restricted to one range of age or one specific group of people (LOPOMO; BURGIO; MIGLIORE, 2014).

In Brazil, according to the Ministry of Health in 2016, one on every five people was overweight. The prevalence of obesity is close to 19% and has almost doubled in a decade. With obesity, the prevalence of diabetes and cardiovascular diseases are increasing each year (BRASIL, 2017).

Many modern lifestyle factors as nutrition and limited physical activity, also contribute to the development of obesity and insulin resistance (DE WIT et al., 2008). Looking through the diet perspective, Western diets are rich in simple sugars, saturated and unsaturated fats, and low in fiber (MARTINEZ et al., 2017). High fat (HF) diet is suggested to induce low-grade inflammation since it increases body weight, adiposity, insulin resistance, plasmatic LPS levels, oxidative stress, and visceral adipose tissue inflammation, as demonstrated in several *in vivo* studies (CANI et al., 2008; LIU et al., 2016; ARAÚJO et al., 2017; MOURA et al., 2018).

Besides that, epigenetics factors might also contribute for the development of obesity onset. So far, some causal genes such as the leptin and leptin receptor genes were identified in monogenic forms of obesity, in studies with families and twins. Mutations in some of these genes are associated with early obesity and may contribute more than 10% of severely obese children (LOPOMO; BURGIO; MIGLIORE, 2014).

Another observed impact caused by the HF diet is microbiota modulation, which can happen very quickly, occurring within 24 and 48 hours after the HF diet consumption, and sustained if dietary habits persist. HF diet changes gut microbiota depending on the amount and the type of fat (saturated or unsaturated), as well as shift gut microbial function (MARTINEZ et al., 2017; DE LA SERRE et al., 2020).

The excessive lipids and carbohydrates intake in obesity acts as a potential trigger to uncontrolled inflammation. Thus, the excessive consumption of nutrients is considered as a major cause of the systemic inflammation. This state is sustained by specialized metabolic cells (such as hepatocytes and adipocytes) which mediate the interface between metabolic input and inflammatory output, what means that inflammatory response and damaged metabolic homeostasis are started by metabolic signals outcoming from metabolic cells (GREGOR; HOTAMISLIGIL, 2011).

The relationship between development of NCDs and excessive caloric intake is commonly focused in the main metabolic tissues, underlying their physiology and the molecular mechanisms, such as liver, adipose tissue, and skeletal muscle. Accumulation of adipose tissue (AT) mass is a major characteristic of obesity, but for a long time, the AT was not considered an

endocrine organ, seen only as a simple storage compartment for energy and excess nutrients. However, recently AT has been recognized as an endocrine organ and highly metabolic active, responsible for regulating immune and inflammatory processes during physiologic and pathologic conditions (AGRAWAL; KERN; NIKOLAJCZYK, 2017).

An immune cell accumulation, such as macrophages and lymphoid cells, occurs during the development of obesity, due to changes in AT, characterized by adipocyte hyperplasia and hypertrophy. The result of this process is an increased production of cytokines that characterizes the inflammatory response (ITOH et al., 2011; AGRAWAL; KERN; NIKOLAJCZYK, 2017). Many pathological conditions such as diabetes and cardiovascular diseases are developed with these contributions of AT changes (ITOH et al., 2011; KUWABARA et al., 2018).

There is emerging evidence showing that gastrointestinal tract (GI) has a pivotal role in the development of obesity and its related diseases (DE WIT et al., 2008; WINER et al., 2016). Besides intestine is an important organ, responsible for both digestion and absorption of nutrients, its contributions in metabolic disorders needs to be more investigated. Moreover, an intestinal inflammatory state caused by perturbed immune homeostasis, has also been reported to be related with prolonged exposure to obesogenic diets (PROGATZKY et al., 2014). Obesity-related traits, as altered immunity and gut microbial dysbiosis are common in obese individuals, they act in concert to produce compartmentalized responses that dictate alterations in metabolic profile of the host (ANHÊ et al., 2020).

The intestinal epithelial barrier is a primary interaction site between diet, host, and gut microbiota, this microenvironment represents the first line of defense protecting the host from invading enteric pathogens and encroaching bacteria (MARTENS; NEUMANN; DESAI, 2018). The highly specialized cells that composes the epithelium are responsible for regulating the physical barrier function, blocking pathogenic and non-pathogenic microorganisms entrance into the intestinal mucosa and bloodstream (NAGLER-ANDERSON, 2001). Metabolic disorders, such as obesity and insulin resistance, can induce intestinal barrier disruption, leading to exacerbated immune response and triggering intestinal inflammation (CHELAKKOT; GHIM; RYU, 2018). Consumption of diets rich in fat and simple carbohydrates, like the Western diet, are been investigated to

increase intestinal permeability, a major factor related with leakage of gut bacteria-derived toxins as LPS, which reaches the bloodstream and causes metabolic endotoxemia (GIL-CARDOSO et al., 2016; CHELAKKOT; GHIM; RYU, 2018). Additionally, long term intake of cholesterol and saturated fats are suggested to trigger intestinal inflammatory response, through inflammasome activation, and also cause chronic and systemic inflammatory response (PROGATZKY et al., 2014).

One first finding which has established the link between obesity, diabetes, and chronic inflammation was the tumor necrosis factor-α (TNF-α) overexpression in white adipose tissue of obese mice. After TNF-α, several other cytokines and inflammatory mediators were also seen to be overexpressed in AT and other tissues, both *in vivo* models and human trials. These cytokines work in networks and the real contribution of each inflammatory mediator itself on metabolic function depends on their place in the network; those more proximal, such as TNF-α, exhibits greater effects (HOTAMISLIGIL, 2006; GREGOR; HOTAMISLIGIL, 2011).

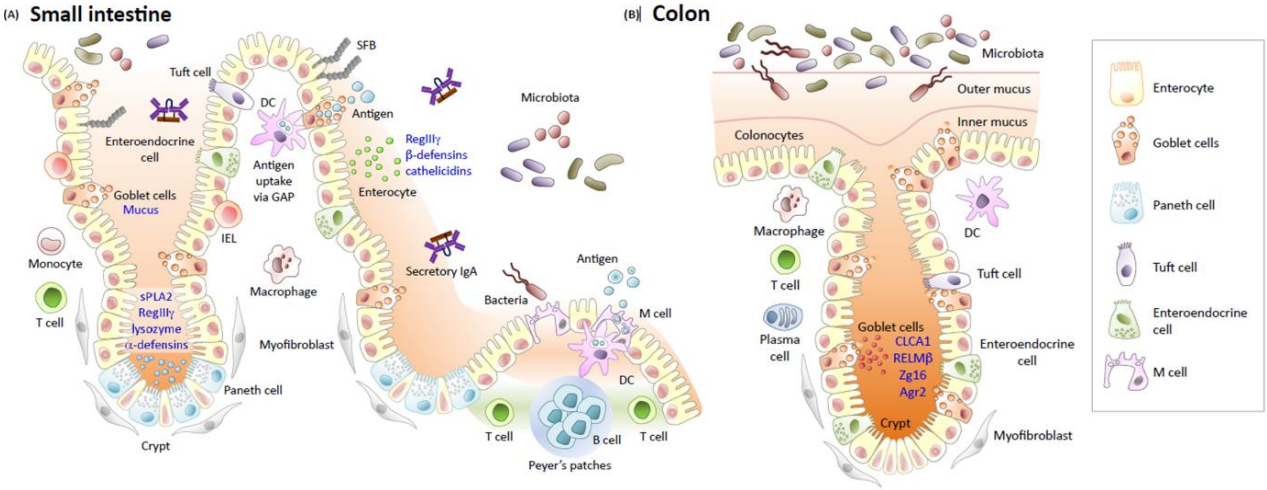
The intestinal epithelial barrier defines the physical interphase between body and diet, and serves as a gatekeeper. Energetic unbalances in the diet are strongly associated with changes in the gut microbiota composition, and may cause disturbance in the gastrointestinal tract, and consequently a systematic inflammatory response through different mechanisms (DE WIT et al., 2008).

A single layer of cells forms the intestinal epithelium, and differences in both cellular composition and architectural structure are found between the small intestine and the colon. The small intestine epithelium presents structures called villi, that project into the lumen, and allows an increase in the mucosal surface area and the absorption of nutrients. In the colon these villi are absent, presenting a relatively flat mucous surface, since its absorptive function is less expressive. Under homeostatic conditions, the intestinal crypts are replaced every 4-5 days, undergoing constantly cycles of renewal, (ALLAIRE et al., 2018).

In the intestinal epithelium are found several types of cells and each of them performs unique and specialized functions. As in structure, the variety of cell types are differently distributed between small intestine and colon, but in general, the majority are found in both intestinal segments. The enterocytes are responsible for water and nutrient absorption, and represent the most prominent

cell type in the intestinal epithelium. Several secretory cells such as enteroendocrine cells that secrete hormones, and goblet cells that secrete mucins are also present in the epithelium, the variety of cell type are represented at **Figure 2**. Some types of cells are characteristics of the small intestine (**Figure 2.A**), like Paneth cells, responsible for releasing antimicrobial factors and located at the bottom of crypts, where they intercalate with stem cells (ALLAIRE et al., 2018).

**Figure 2.** Anatomy of the Intestinal Mucosa. (A) Representative small intestinal epithelium and (B) Representative colonic epithelium.



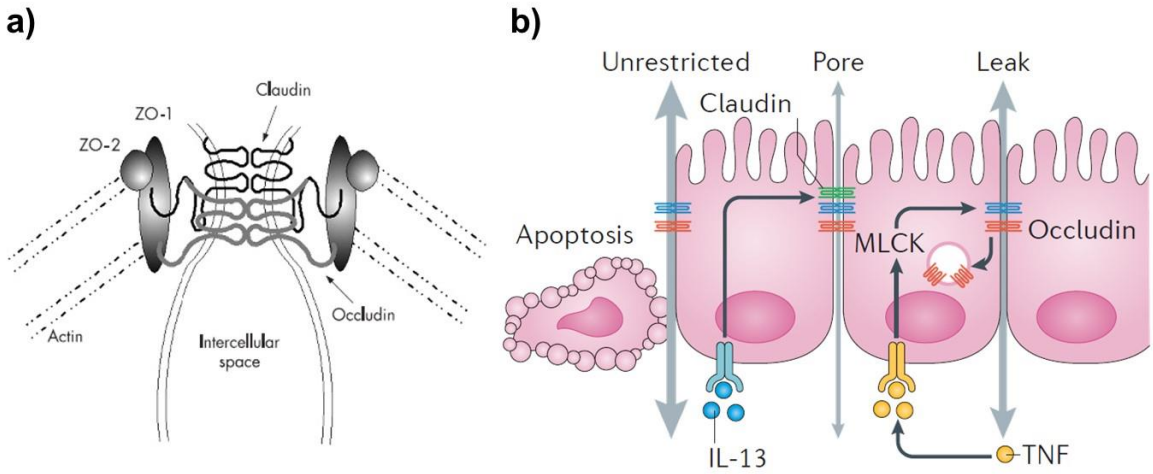

Source: Adapted from (ALLAIRE et al., 2018).

Some evidence suggests that the impairment of the intestinal barrier function could be associated with alterations in the localization and structure of tight junctions (TJ). The TJ is composed basically of occludin, claudin family members, and junctional adhesion molecules, which are linked to the actin cytoskeleton by cytoplasmic rafts formed by catenins (like β-catenin) and zonula occludens (ZO) proteins (**Figure 3a**). This complex of junctional proteins is responsible for the selective regulation of the paracellular transport of solutes, peptides, and ions from the lumen to the bloodstream (BRUN et al., 2007).

Gut microbiota is a major source of bacteria-derived endotoxins, and high circulating levels of those toxins are also related to insulin resistance and obesity, which also contributes to increased intestinal permeability (TEIXEIRA et al., 2012). One of the most important consequences of the gut barrier dysfunction is the entrance of endotoxins from the intestinal lumen. A highly discussed

endotoxin, is the lipopolysaccharide (LPS), a structural component of the cell walls of gram-negative bacteria. Once LPS reaches circulation, it can affect multiple organs and tissues, activating and modulating inflammatory pathways, leading to metabolic disturbances (GIL-CARDOSO et al., 2016). High circulating levels of endotoxins act as a trigger for local inflammation and even induce a systemic inflammation through cytokine release, which contributes to a sustained inflammatory state (AZUMA et al., 2013; KUWABARA et al., 2018).

**Figure 3.** Tight junction structure (a) and paracellular epithelial permeability pathway (b).




Source: a) (ARRIETA; BISTRITZ; MEDDINGS, 2006). Representative association of claudin and occluding (other proteins have not been described). b) (ODENWALD; TURNER, 2017).

The LPS binds to its membrane receptor and promotes the Toll-like receptor-4 (TLR4) activation, which induces the signaling that leads to necrosis factor-κB (NF-kB) translocation, a transcriptional factor that results in gene expression of several cytokines like adhesion molecules, tumor necrosis factor-α (TNF-α), interleukins, and inducible enzymes (inducible NO synthase (iNOS) and cyclo-oxygenase-2 (COX-2)) among others. The activation of pro-inflammatory signaling initiates an immune cell recruiting, such as macrophages and lymphocytes. The immune cell infiltration at the intestinal lumen reinforces the inflammation state and the production of proinflammatory mediators (**Figure 3b**) (DING et al., 2010; GIL-CARDOSO et al., 2016). The network of immune pathways is connected, which means that the overproduction of cytokines caused by the LPS-TLR-4 activation also affects the epithelial permeability by changing TJ. The IL-13 and TNF-α production may cause increased permeability across

the leak and pore pathways. TNF-α enhances the permeability by increasing myosin light chain kinase (MLCK) transcription, once activated, this kinase protein increases the leak pathway and causes occludin endocytosis at the TJ (ODENWALD; TURNER, 2017).

In summary, obesity is a complex of disturbances and interactions, which involves a series of factors, such as fat accumulation at AT, insulin resistance, and systemic inflammation. There are divergent opinions about the development of obesity and its related diseases, some authors defend the AT changes as the beginning of the systematic inflammation, others suggest that a gut barrier leakage could be a first signal indicating the effects of a harmful diet. Besides its complexity, the elucidation of the mechanisms involved allows the understanding of new points of intervention and regulation that might be explored to treat and prevent obesity and its associated diseases.

## 1.3 INTESTINAL INFLAMMATION, OBESITY, AND BIOACTIVE COMPOUNDS

Keeping the previous scenario, there is growing evidence showing a positive effect of some food bioactive compounds, particularly the flavonoids, in obesity and associated diseases through their effects on inflammatory pathways and mediators (TERRA et al., 2009). The link between intestinal inflammation and obesity as a possible cause-consequence state is quite recent. However, there is both *in vitro* and *in vivo* studies relating potential effects of phenolic compounds in the treatment and prevention of this inflammatory process, since they are already proven to be effective against obesity and related diseases in animal models (ALEZANDRO; GRANATO; GENOVESE, 2013; DONADO-PESTANA; BELCHIOR; GENOVESE, 2015; DONADO-PESTANA et al., 2018, 2021; MOURA et al., 2018; RODRIGUES et al., 2021).

Prior studies have demonstrated that certain phenolic compounds have health protective effects, and can prevent low-grade inflammation *in vivo*, by adjusting adipose tissue cytokine imbalance, diminishing proinflammatory and enhancing the anti-inflammatory molecules (TERRA et al., 2009, 2011; MOURA et al., 2021).

Polyphenol compounds have shown important anti-obesogenic, anticholesterolemic, and anti-inflammatory effects in animal models. Donado-

Pestana; Belchior; and Genovese (2015) demonstrated these effects in diet-induced obese animals and supplemented with phenolic extract of cagaita (*Eugenia dysenterica* DC.), a Brazilian native fruit.

Another study observed mostly the same effects in animals that received an extract of grape-seed, rich in procyanidins, which suggested a beneficial effect against low-grade inflammatory diseases (TERRA et al., 2009, 2011). This may be related with the inhibition of some proinflammatory molecules expression and enhanced production of anti-inflammatory cytokines. According to Terra et al. (2009), these findings may also provide suggestions for regulatory points to reduce obesity-related adipokine dysregulation and manage cardiovascular and metabolic risk factors.

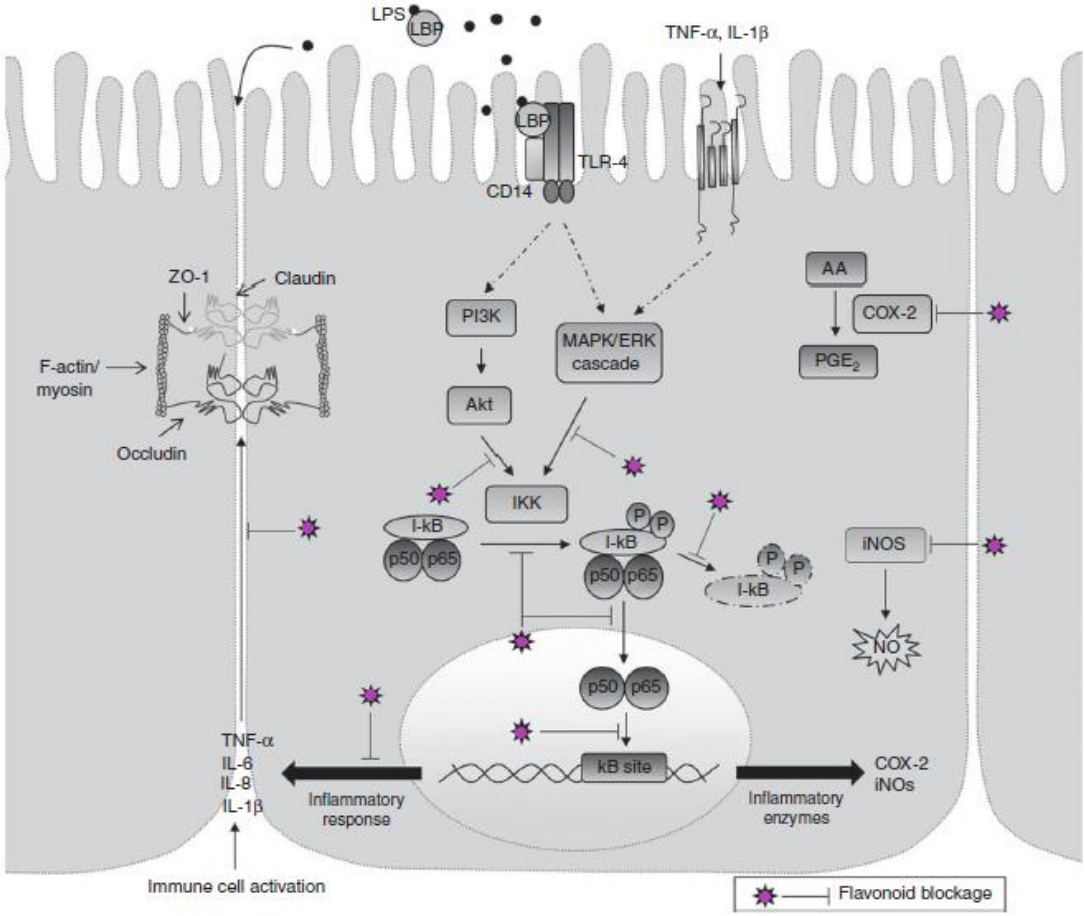
Furthermore, a significant decrease in inflammatory mediator levels, such as iNOS, COX2, MCP-1, ICAM-1, TNF-α, and lL-6, was observed after oral administration of naringenin for 10 days in animals submitted to chemical induction of intestinal colitis using DSS (dextran sulfate sodium). This indicates a protective effect of polyphenols from citric fruits, in DSS-induced colitis, by the suppression of the NF-kB signaling, a classical inflammatory pathway (DOU et al., 2013).

*In vitro* studies with luteolin, a flavone present in some vegetables and spices, demonstrated a positive effect through its administration in epithelial cells, observing the inhibition of the NF-κB pathway. A proposed mechanism that may lead to the inhibition of NF-κB signaling and gene expression in intestinal epithelial cells, is that some flavonoids are capable of to stopping the NF-κB translocation to the nucleus, which prevents the gene transcription of another pro-inflammatory mediators. Some authors suggest that it might related to a protective effect of some flavonoids over the IκB degradation, an intermediate of the NF-κB signaling (KIM; JOBIN, 2005; CHEN et al., 2007; GIL-CARDOSO et al., 2016).

Another *in vitro* study has reported a decreased inflammation through blocking Iκ-B proteins degradation and consequently inhibiting the translocation of NF-κB to the nucleus, and the expression of proinflammatory cytokines, after the administration of a phenolic extract from *Opuntia ficus*, a typical Portuguese cactus, in Caco-2 cells, a model used to analyze intestinal permeability (MATIAS et al., 2014).

There are many possible sites of flavonoids interactions with metabolism during homeostasis and disease pathogenesis of the epithelial barrier (**Figure 4**). According to Gil-Cardoso et al. (2016), a main mechanism is the reduction of the intestinal inflammatory processes characterized by active NF-κB through the down-regulation of the TLR-4/NF-κB pathway and inhibiting cytokine expression and synthesis. Further, once elucidated in human trials, these observations can contribute to recommendations about the consumption of flavonoid-rich foods or the use of flavonoid supplements as potential therapeutic and preventive adjuvants in the management of obesity and its associated disorders, as intestinal inflammation.

**Figure 4.** Regulation points of flavonols in the intestinal epithelium




Source: (GIL-CARDOSO et al., 2016).

The gut microbiota is also an important and emerging interaction point between metabolism, host, and diet, where phenolic compounds are proven to be beneficial. Barrier integrity and gut microbiota composition effects are also

being investigated, and some studies have indicated that the microbiota composition could be altered by polyphenols consumption, favoring a healthier profile (ANHÊ et al., 2015; ETXEBERRIA et al., 2015). Also, most parts of the dietary phenolic compounds present low bioavailability, and are not completely absorbed in the GI-tract, thus they reach the colon and are metabolized by the gut microbiota, generating metabolites. Therefore, their interaction with the gut microbiota represents an important point to relate potential beneficial effects of phenolic compounds with metabolic disorders (GIL-CARDOSO et al., 2016).

## 1.4 JABOTICABA AND BIOACTIVE COMPOUNDS

Jaboticaba is a Brazilian native fruit belonging to the Myrtaceae family, characteristic of the Atlantic Forest biome. There are nine known species of jaboticaba; four of them are most popular and commonly consumed. The jaboticaba Paulista (*Plinia cauliflora* (DC.) Berg) and jaboticaba Sabará (*Plinia jaboticaba* (Vell.) Berg) (**Figure 5**) are typically cultivated in São Paulo and Minas Gerais states (CITADIN; DANNER; SASSO, 2010).

Figure 5. Jaboticaba Sabará in natura.

Font: EMBRAPA (2015).

The fruit could be considered a berry and has smooth skin usually dark violet in the ripe mature stage. Its pulp is sweet and slightly acid, containing

between one and four seeds. In general, jaboticaba can be consumed *in natura* or as jams, beverages, liquors, juice, ice cream, and other preparations (ALEZANDRO et al., 2013).

Furthermore, jaboticaba is rich in polyphenols, such as anthocyanins, proanthocyanidins, and ellagitannins (MOURA et al., 2018). Abe; Lajolo; and Genovese (2012) analyzed the total ellagic acid content of several fruits commonly consumed in Brazil and, among these fruits, jaboticaba demonstrated the higher content of total ellagic acid, representing a promising source of ellagic acid derivatives in the diet.

A comparative study between the two most common jaboticabas, Sabará and Paulista, demonstrated that according to the total phenolic content, proanthocyanidins, ellagic acid contents (both total and free), and *in vitro* antioxidant capacity, jaboticaba Sabará is a superior source than Paulista (ALEZANDRO et al., 2013). In addition, the ellagitannin content decreased with ripening meanwhile the anthocyanin increased significantly. These characteristics can also equate jaboticaba to classical berries, fruits with proven health beneficial effects (ABE; LAJOLO; GENOVESE, 2012; ALEZANDRO et al., 2013).

Alezandro, Granato, and Genovese (2013) have reported a potential effect of the administration of two doses: 50 and 100 mg gallic acid equivalents (GAE)/kg body weight (BW) in streptozotocin-induced diabetic rats. The results showed positive effects against oxidative stress and ameliorated both cholesterol and triacylglycerol levels in plasma. According to the authors, these results suggest that the daily ingestion of jaboticaba may represent a dietary strategy for controlling oxidative stress in pathological conditions. A most recent study with phenolics at the same doses has indicated beneficial properties preventing obesity in C57BL/6J mice. The daily administration of a tannin-rich extract from jaboticaba was capable of attenuating hyperinsulinemia and preventing high fasting blood glucose concentrations. Moreover, they prevented high total cholesterol levels in mice with diet-induced obesity (MOURA et al., 2018).

In summary, jaboticaba phenolic compounds demonstrated a potential positive effect in obesity and diabetes models.

# 2. OBJECTIVES

The main objective was to evaluate the effect of polyphenols from jaboticaba in the intestinal inflammation associated with obesity induced by a high-fat-sucrose (HFS) diet in C57BL/6J mice.

## 2.1 SPECIFIC OBJECTIVES

- To characterize phenolic-rich extracts from jaboticaba (PEJ) in relation to total phenolic, proanthocyanidins, flavonoids, and ellagitannins profile/contents.
- To evaluate the effect of daily administration of 50 and 100 mg gallic acid equivalents (GAE)/kg body weight (BW) of the PEJ on body weight gain, energy intake, adiposity, glucose homeostasis, and lipid profile of HFS-fed obese mice.
- To determine pro-inflammatory cytokine production in intestinal segments.
- To investigate the effects of PEJ in the activity and expression of key proteins of intestinal TJ, inferring whether PEJ may affect intestinal permeability.

# 3. MATERIAL AND METHODS

## 3.1 PLANT MATERIAL

Jaboticaba Sabará (*Plinia jaboticaba* (Vell.) Berg fruits were obtained from a local supplier (Produtora Unidos Ltda.) located at the São Paulo Central Market (CEAGESP – Companhia de Entrepostos e Armazéns Gerais de São Paulo, São Paulo, Brazil). The fruits were hygienized with a solution of chlorinated water, frozen in liquid nitrogen, lyophilized, grounded in an analytical mill, and stored at -20 °C.

## 3.2 EXTRACTION OF PHENOLIC COMPOUNDS

The PEJ was obtained by hydromethanolic extraction of the dried jaboticaba powder, according to the procedure previously described by Moura et al. (2018) and Rodrigues et al. (2021). For this, a methanol/water/acetic acid solution (70:30:0.5 v/v/v) was used for extraction at 1:25 (m/v) for 2 hours under shaking, at 4 °C. The extract was vacuum-filtered through a Whatman #1 filter paper, and the residue re-extracted twice for 30 min each, under the same conditions previously described. Next, the extract was concentrated on a rotatory evaporator (Rotavapor R-210; Büchi, Switzerland) at a temperature of ≤ 37 °C and the volume completed to 500 mL with distilled water. The extract was purified by solid-phase extraction (SPE) with octadecylsilane (C18) column (Supelclean™ LC-18, Supelco), thereby eliminating other soluble constituents with potential functional and/or nutritional (i.e. vitamins, fiber, proteins, carbohydrates, etc). C18 column was preconditioned with 20 mL of methanol and 60 mL of distilled water per gram of resin. After the passage of the PEJ in the ratio of 3% GAE (gallic acid equivalents) of C18, columns were washed with water and the phenolic compounds eluted with methanol, at the rate of 50 mL per gram of resin. Finally, the extract was concentrated until methanol elimination and resuspended in water, thereby obtaining the PEJ.

## 3.3 CHEMICAL CHARACTERIZATION OF THE PEJ

### 3.3.1 Total phenolic compounds content

The total phenolics compounds content was determined through the reducing ability of the Folin-Ciocalteu reagent according to Singleton, et al. (1998). In a 96-well microplate, 50 µL of the standard curve of gallic acid or the diluted samples were pipetted. After the addition of 50 µL of the Folin-Ciocalteu reagent, the mixture was shaken for 5 min at room temperature. Then, 100 µL of NaOH (0.35 mol/L) was added to the mixture and shaken for 5 min. Absorbance was read at 760 nm in a spectrophotometer (BioTek Instruments, Winooski, VT). The results were expressed as gallic acid equivalents (GAE) per mL of extract.

### 3.3.2 Proanthocyanidins

Two methodologies were used to determine the content of proanthocyanidins including the acidified butanol method, and the DMAC method. The first analysis was performed according to Porter; Hrstich e Chan (1985), with some modifications. Briefly, an n-butanol solution: HCl (3:2) and iron II sulfate heptahydrate ($FeSO_4$ $7H_2O$) were added to 250 µL of extract, diluted when necessary. A standard of cyanidin-3-rutinoside (Extrasynthèse, Genay, France) was used. The tubes containing the standard curve and the samples were shaken in a bath at 95 °C for 15 min. Absorbance was read at 540 nm in a spectrophotometer (Ultrospec 2000, Pharmacia Biotech, NJ, USA). The results were expressed as mg equivalent of cyanidin-3-rutinoside (C3R) per mL extract.

The DMAC (4-dimethylaminocinnamaldehyde) method was performed according to Prior et al. (2010), with some modifications. An aliquot of 210 µL of the DMAC solution 0,1% (in EtOH 91%:HCl:$H_2O$ (75:12.5:12.5) was added to 70 µL control solution (80% aqueous ethanol), standard and extracts (diluted when necessary) in a 96-well microplate. After incubation at 25 °C for 25 min, the absorbance was read at 640 nm using a spectrophotometer (Synergy hybrid H1 reader, Biotech, IL, USA). A standard procyanidin B2 curve (Extrasynthèse, Genay, France) was used to calculate the content of proanthocyanidins. The

results were expressed as mg equivalent of procyanidin B2 (PB2) per mL of extract.

### 3.3.3 Identification and quantification of flavonoids and phenolic acids by HPLC-DAD-MS

As described by Rodrigues et al. (2021), the phenolic compounds profile of PEJ was performed according to Donado-Pestana et al. (2021). A high-performance-liquid-chromatography (HPLC) system (LC-20ADX Preminence, Shimadzu) was used, equipped with a DAD detector and a reverse phase column LiChroCART C-18 (250 mm x 4 mm, 4.5 µm, Merck, Darmstadt, Germany), coupled to a mass spectrometer (Amazon Speed, Bruker, Massachusetts, USA) (MS), with an ion-trap analyzer ($MS^n$) and an electrospray ionization (ESI) source. PEJ samples were injected (5 µL) in triplicates. The established chromatography conditions were: a) mobile phase of 0.1% formic acid/acetonitrile in a gradient elution starting with 90:10 during 3 min and reaching 80:20 in 22 min, 65:35 in 42 min, and finally 92:8 after a total of 65 min; a flow rate of 0.5 ml/min; b) 40°C of column temperature; c) ESI voltage set to 3.5 kV with 230°C of temperature, and depletion gas flow ($N_2$) of 6 L/min; d) operating in negative mode; e) *m/z* range of 100-1000. The HyStar software was used to control these parameters. The identification of main phenolic compounds was based in the elution order, UV-Vis spectra, retention time and MS/MS fragmentation patterns.

Phenolic compounds including delphinidin 3-O-glucoside (#0904S, Extrasynthese, Genay Cedex, France), cyanidin 3-O-glucoside (#79457, Sigma-Aldrich, St. Louis, MO, USA), ellagic acid (#E-2250, Sigma-Aldrich, St. Louis, MO, USA), myricetin 3-O-rhamnoside (#M-6760, Sigma-Aldrich, St. Louis, MO, USA) and quercetin 3-O-rhamnoside (#Q-3001, Sigma-Aldrich, St. Louis, MO, USA) were identified and quantified according to its respective commercial standard. Ellagic acid derivatives such as casuariin, pedunculagin, strictnin, casuarinin and tellimagrandin I, were identified according to literature data (PLAZA et al., 2016; MOURA et al., 2018), and quantified using calibration curve of ellagic acid.

## 3.3 BIOLOGICAL EXPERIMENT

### 3.4.1 Animals and experimental design

All experiments were approved by the Animal Ethics Committee of the Faculty of Pharmaceutical Sciences of the University of São Paulo (FCF/USP/CEUA/522), and carried out in strict compliance with Brazilian legislation.

Forty male C57BL/6J mice (8 weeks aged) were purchased from IQ/FCF bioterium and housed in a controlled animal facility (12/12 (light/dark), 20 °C), fed with mice standard feed (NUVILAB CR-1, Nuvital Nutrientes S/A, PR, Brazil) and water *ad libitum*, for 2 weeks before the beginning of the experiment.

The commercial standard diet used during the experiment followed the AIN 93 M protocol providing 3.8 kcal/g, composed by 81% of carbohydrates, 18% of lipids, and 14% of protein. A high lipid and sucrose (HFS) diet for induction of obesity was prepared manually according to Lemieux et al. (2003) and Moura et al. (2021), yielding 4.6 kcal/g, 20% of which is from protein, 39% from lipids and 41% from carbohydrates (simple sugars).

At the first phase of the protocol, the animals were randomly distributed into two dietary groups for 14 weeks, a negative control group (CH) (n = 10) fed a standard diet and water ad libitum; and a positive control group (HFS) (n = 30) fed a HFS diet and water ad libitum.

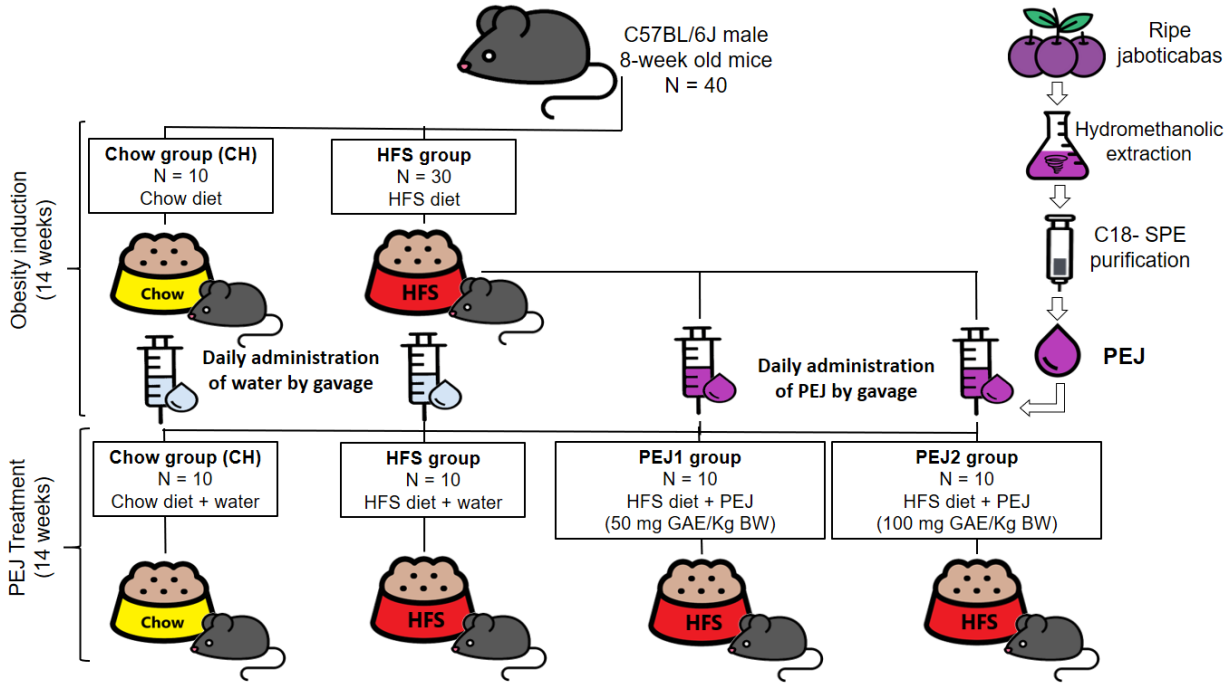
After these 14 weeks of obesity induction, the animals of the HFS group were redistributed into three different groups following the experimental protocol:

- *HFS Group: 10 animals fed HFS diet and daily gavage of water.*
- *PEJ1 Group: 10 animals fed HFS diet and daily gavage of PEJ at a dose of 50 mg EAG / kg of BW.*
- *PEJ2 Group: 10 animals fed HFS diet and daily gavage of PEJ at a dose of 100 mg EAG / kg of BW.*

Animals in the Ch group were continuously fed with the standard diet and daily gavage of water.

**Figure 6** presents the schematization of the experimental design. After 28 weeks, animals were euthanized by exsanguination under anesthesia. Plasma was obtained by centrifugation at 3000 *g* at 4 °C for 20 min. The organs and tissues of the gastrointestinal tract, including glandular stomach, six segments of the small intestine (proximal and distal duodenum, proximal and distal jejunum, proximal and distal ileum), cecum and two segments of the colon (proximal and distal) were removed, washed in phosphate-buffered saline solution (PBS), weighed and stored at -80 °C for further analysis.

**Figure 6**. Summary of the experimental design.




HFS – high-fat-sucrose diet; PEJ – Phenolic-rich extract from jaboticaba; GAE – Gallic acid equivalent; BW – body weight. SPE – solid-phase extraction; C18 – Octadecylsilane resin.
Font: The author (2019).

### 3.4.1.1 Fasting glycemia and oral glucose tolerance test (oGTT)

Glycemia was measured biweekly in animals fasted for 4 h from blood drawn of the caudal vein, using an Accu-Chek Performa glucometer (Roche, Mannheim, Germany). At the 26$^{th}$ week the oral glucose tolerance test (oGTT) was performed after 4 h fast. The animals received a glucose solution (1.0 g/kg BW) by gavage. Initial glycemia was determined prior to gavage (time 0) and in intervals of 15 min for 90 min. Blood samples (~50 µL) collected at 0, 30, and 90

min, and centrifuged (3000 g for 20 min at 4 °C) to obtain serum were analyzed for insulin determination using a Rat/Mouse Insulin ELISA Kit (Millipore, Missouri, USA).

#### 3.4.1.2 Plasma biochemical profile

Commercial kits were utilized to assess the plasma biochemical profile. Total cholesterol, HDL-cholesterol, LDL-cholesterol, triacylglycerols (TAG) from LABTEST (Lagoa Santa, MG, Brazil), non-esterified fat acids (NEFA) from FUJIFILM Wako Chemicals (Neuss, Germany), and lipopolysaccharide (LPS) from Abbexa (Cambridge, UK). All analysis was performed in quadruplicate and following the manufacturer's instructions.

### 3.4.2 Protein expression in gastrointestinal tissues

As detailed by Rodrigues et al. (2021), the immunoblotting was performed as previously described (Donado-Pestana et al., (2021). Proximal section of colon (30-50 mg) was homogenized (T10, Ultra-Turrax®) in lysis buffer (300 µL) containing 50 mmol/L HEPES, 40 mmol/L NaCl, 50 mmol/L NaF, 2 mmol/L EDTA, 10 mmol/L sodium pyrophosphate, 10 mmol/L sodium glycerophosphate, 1.5 mmol/L sodium orthovanadate, 10 mmol/L sodium β-glycerophosphate, and a protease inhibitor cocktail (cOmplete Mini, Roche, Germany). The obtained homogenate was centrifuged at 14,000 *g* for 40 min at 4 °C, and the supernatants collected. Protein concentration was performed using Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, USA). Before the loading of the samples, they were heated for denaturation in Laemmli buffer, and then added to 12% SDS-PAGE for separation. After separated and transferred to PVDF membranes (Merck Millipore, Massachusetts, USA), the membranes were incubated primary antibodies overnight. The primary antibodies used were: toll-like receptor-4 (TLR-4, #48-2300), claudin-1 (#71-7800) (Invitrogen, Thermo Scientific, Rockford, USA), c-Jun N-terminal kinase (phospho-SAPK/JNK (Thr183/Tyr185), #9251 and SAPK/JNK, #9252), nuclear factor-kB (phospho-NF-κB p65, #3033 and NF-κB p65, #3987), and β-actin (#3700) (Cell Signaling

Technology, Beverly, USA). Then membranes were washed in TBS-Tween 1% solution, and incubated overnight with peroxidase-conjugated secondary antibody. Finally, membranes were revealed using an enhanced chemiluminescence (ECL) substrate solution (Immobilon, Merck Millipore, Massachusetts, USA). The ImageJ program (National Institute of Health, USA) was used to determine the densitometry of the bands.

### 3.4.3 Gene expression by RT-PCR

The extraction of RNAs from gastrointestinal sections was performed using an RNA purification kit (GE Healthcare, USA), following the manufacturer's recommendations. The RNA content was determined on a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, USA). For the cDNA synthesis a 2 µg sample of RNA was subjected to the reverse transcription reaction with random primers. A mixture containing 1 µL of the sample cDNA, 0.5 µL of each primer sequence, 8 µL of ultrapure water and 10 µL of the SYBR Green PCR Master Mix (Sigma Aldrich, San Luis, EUA) was subjected to the following cycles: first cycle (single) at 95 ° C for 2 min, followed by 40 cycles with the denaturation phases at 95 ° C for 15 s and annealing/extension for 60 s and hold at 4 ° C (Rotor-Gene, Qiagen, Hilden, Germany). Specific primers for inflammation pathway genes are listed in **Table 1.** Quantification of the expression of the genes of interest was performed using the ΔCt method and the qBase analysis program, with the expression of GAPDH as an internal standard.

## 3.5 CYTOKINES QUANTIFICATION

The cytokine quantification of colon homogenates (as described at 3.4.2), was performed by ELISA method, using commercial kits for the following cytokines: IL-6, TNF-α, and MCP-1 (DuoSet ELISA®, R&D Systems), and according to manufacturer instructions. The results were expressed in ρg/mg of protein according to standard curves.

**Table 1**. List of primers sequences used.

| Primer name | Forward sequence | Reverse sequence |
|---|---|---|
| IFN-γ | TGCTGCTGATGGGAGGAGATG | CACATTCGAGTGCTGTCTGGC |
| TNF-α | GGGCAGTTAGGCATGGGATG | TACCTACGACGTGGGCTACAG |
| NF-Kb | TCAGAACTCTGCAGGTGAGACC | CAGAACTCTGCAGGTGAGACC |
| JNK | TCAGAAGCAGAAGCCCCACC | ACGGCTGCCCTCTTATGACTC |
| IL-1β | GCCACCTTTTGACAGTGATGAG | TGATCTGCTGCTGCGAGATT |
| IL-6 | AGACGCATCTCAGCTGGTAAAG | TGGGGGAGGATGTTTGGATG |
| TLR-4 | CCAGTATTTCAGGCGGGAAGC | TGGAAGGGGTCAGAGCTAACAG |
| iNOS2 | TTCTCAGCCACCTTGGTGAAG | ACTCCGTGGAGTGAACAAGACC |
| Caspase-1 | TGCCGTGGAGAGAAACAAGG | GGGCCTTCTTAATGCATCATC |
| CXCL-IP10 | CCTCACCATATGCTCGGACACCA | GCTGTGCAGAGCCCTCGGAGC |
| NLRP3 | GCCAACAACAATGATCTTGGCGA | TTCACCCAACTGTAGGCTCT |
| ASC | GCCAGAACAGGACACTTTGTG | ACACTGCCATGCAAAGCATC |
| IL-18 | GATCAAAGTGCCAGTGAACCC | GGTCACAGCCAGTCCTCTTAC |
| Claudin-1 | TGCCCCAGTGGAAGATTTAC | CGAAGACTTTGCACTGCATC |
| Claudin-7 | GACAAAGCGAAGAAGGCCCG | ACCCTGCCCAGCCGATAAAG |
| Ocludin | CTTTCCTTAGGCGACAGCGG | ATAAGCGAACCTGCCGAGCC |
| ZO-1 | ATGTTTATGCGGACGGTGGC | ATCTTGTCTCTCTCCGCGCC |
| MLCK | GTGGTCACAGGATGGGAACTC | TAGAGGCGTCAGCTTGCACAC |
| GAPDH | AGCGGAACCGACAAAGGTTA | GTCTGAGTCATCTGGGTGCC |

IFN-γ, interferon-gamma, TNF-α, tumor necrosis factor-alpha, NF-κB, necrosis factor-kappa B, JNK, c-jun-N-terminal protein kinase, IL-1β, interleukin 1 beta, IL-6 interleukin 6, TLR-4, toll-like receptor 4, iNOS2, inducible nitric oxide synthase 2, IFN-γ, interferon γ, CXCL-IP10, interferon-inducible protein 10, COX-2, Ciclooxygense-2, ZO-1, zonula occludens-1, myosin light-chain kinase-3, MLCK-3, glyceraldehyde 3-phosphate dehydrogenase, GAPDH. (housekeeping/internal control).

## 3.6 HISTOLOGY

After the euthanasia, segments of colon, were collected and fixed in 10% formaldehyde, embedded in paraffin, and cut into 6 μm-thick non-serial sections. To analyze the morphology, sections were placed on slides and stained with hematoxylin and eosin (H&E). For the analysis of intestinal goblets cells in the colon, sections were placed on slides, passed by a histological reaction with Periodic Acid-Schiff (PAS) and counterstaing with hematoxylin. Cuts were washed under water, immersed in Schiff's Reagent for 30 min and washed again. Sections were stained with hematoxylin and mounted in Damar gum. The

quantification of PAS-positive marked cells was performed using a light microscope (Alphaphot-2 YS2, Nikon, Japan) by integrating eyepiece with a reticle (8X, type Kp12, Zeiss, Germany) and immersion objective (100X). Between 15 and 20 fields per animal were counted, and the result was expressed as a percentage of positive PAS cells per fields/animal. The capture of the images was performed using a light microscope coupled to a computer and connected to a photographic system (Olympus Bx51, Montreal, Canada). The measurements were performed using the image analysis software Image ProPlus v.5.2 (Media Cybernetics, Bethesda, USA).

## 3.8 STATISTICAL ANALYSIS

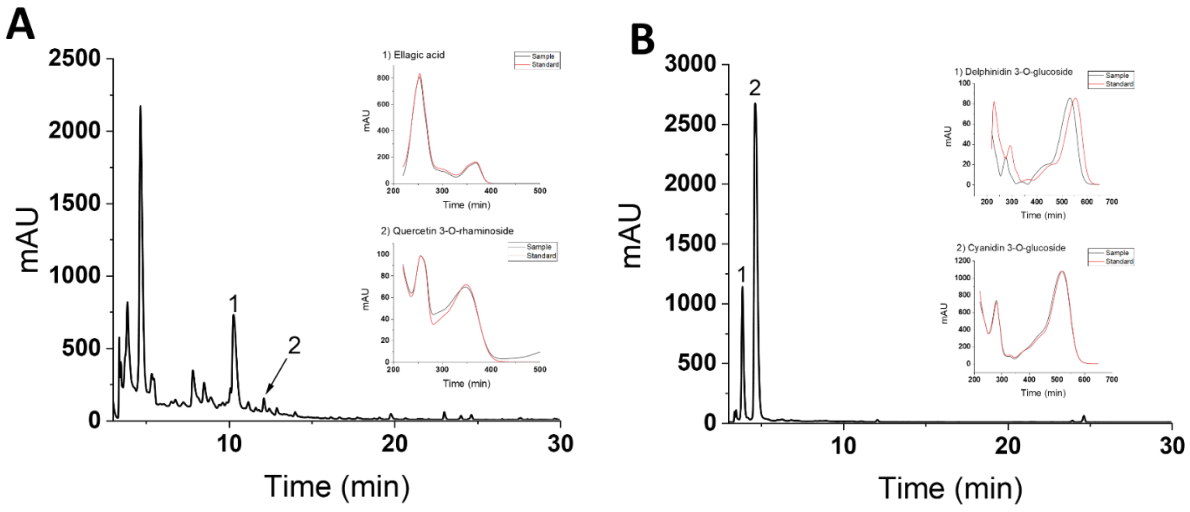
The results were expressed as mean ± standard deviation. First, the data were analyzed by the Shapiro-Wilk test to determine the nature of its distribution. For comparisons between two groups with parametric distribution it was used the t-test followed by Walch test, and t-test followed by Man-Whitney for non-parametric data. For three groups comparisons, it was used the analysis of variance (ANOVA) with Tukey test (normal distribution) or Kruskal-Wallis with Dunn test (non-parametric) were used to determine significant differences among the treatments. The analysis was performed using the GraphPad Prism software (GraphPad Software, 6.0 version, La Jolla, CA, USA).

# 4. RESULTS AND DISCUSSION

## 4.1 CHEMICAL CHARACTERIZATION OF THE PHENOLIC-RICH EXTRACT FROM JABOTICABA (PEJ)

Due to its physicochemical characteristics, the C18 resin allows the extraction of all the phenolic compounds present, meanwhile vitamins, sugars, and minerals are eliminated by the SPE technique. In this way, the PEJ is concentrated in both flavonoids and non-flavonoids, including phenolic acids and tannins. The reverse-phase liquid chromatographic profiles of PEJ are shown in **Figure 7**.

**Figure 7.** HPLC-chromatograms obtained at 270 (A) and 525 (B) nm and UV spectra (inserted) of flavonoid glycosides and phenolic acids from the phenolic-rich extract from jaboticaba (PEJ).




PEJ was characterized in relation to the total phenolics and proanthocyanidins contents. Four phenolic compounds were identified as anthocyanins (delphinidin and cyanidin derivatives), flavonols (quercetin derivatives) and phenolic acid (ellagic acid) glycosides. The main compounds found in the PEJ were the ellagitannins, which corresponded to approximately ~33% of the total phenolics, followed by anthocyanins (~9%), free ellagic acid (~3%), proanthocyanidins (~1%), and minor amounts of quercetin (**Table 2**).

Among ellagitannins, six molecules were identified (**Figure 8**) including casuariin, pedunculagin, vescalagin, strictinin, casuarinin, and tellimagrandin I.

**Table 2.** Phenolic characterization of the PEJ.

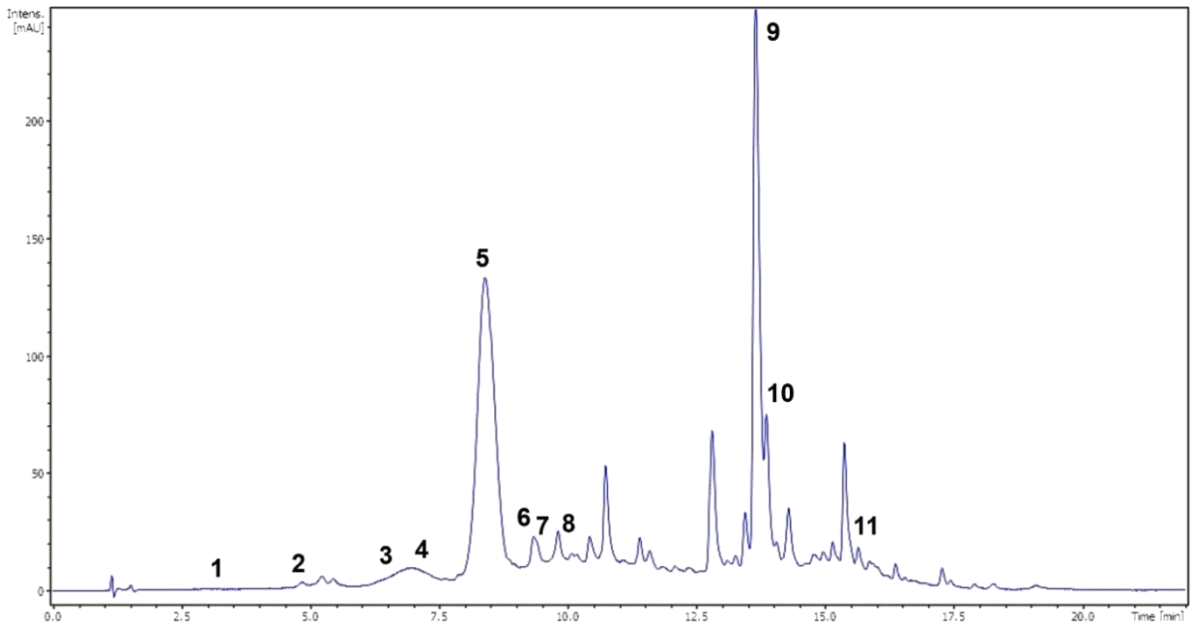
| | |
|---|---|
| Total phenolic compounds (µg GAE/mL)[1] | 12,500 ± 317 |
| Proanthocyanidins (µg Cya-chlor/mL)[2] | 119 ± 3 |

***Mass spectroscopy characteristics and quantification of identified phenolic compounds***

| ***Compounds*** | **RT (min)** | ***m/z*** | **MS/MS** | **PEJ (µg/mL)** |
|---|---|---|---|---|
| Casuariin* | 3.9 | **783** | 481, 301 | 3.16 ± 0.02 |
| Pedunculagin* | 6.5 | **783** | 481, 301 | 83.81 ± 1.00 |
| Delphinidin 3-O-glucoside | 7.0 | **463** | 300, 283 | 239.75 ± 0.48 |
| Cyanidin 3-O-glucoside | 8.1 | **447** | 285, 241 | 880.28 ± 23.41 |
| Strictinin* | 9.1 | **633** | 301, 275 | 48.00 ± 0.04 |
| Casuarinin* | 9.4 | **935** | 917, 633 | 96.98 ± 1.12 |
| Tellimagrandin I* | 10.1 | **785** | 483, 301 | 21.25 ± 0.29 |
| Ellagic acid | 13.8 | **301** | 257, 229 | 269.77 ± 5.50 |
| Myricetin 3-O-rhamnoside | 14.0 | **463** | 316, 271 | 14.59 ± 0.35 |
| Quercetin 3-O-rhamnoside | 15.6 | **447** | 301, 179 | 8.57 ± 0.37 |

Results are expressed as mean ± SD values (*n* = 3);
[1]GAE, gallic acid equivalent; [2]Cya-chlor, Cyanidin-3-O-rutinoside chloride; RT, retention time.
*Ellagic acid derivatives identified by comparison with data in the literature (PLAZA et al., 2016; MOURA et al., 2018), and expressed as ellagic acid equivalents.

**Figure 8.** HPLC-chromatogram (270 nm) of the phenolic-rich extract obtained from jaboticaba (PEJ).

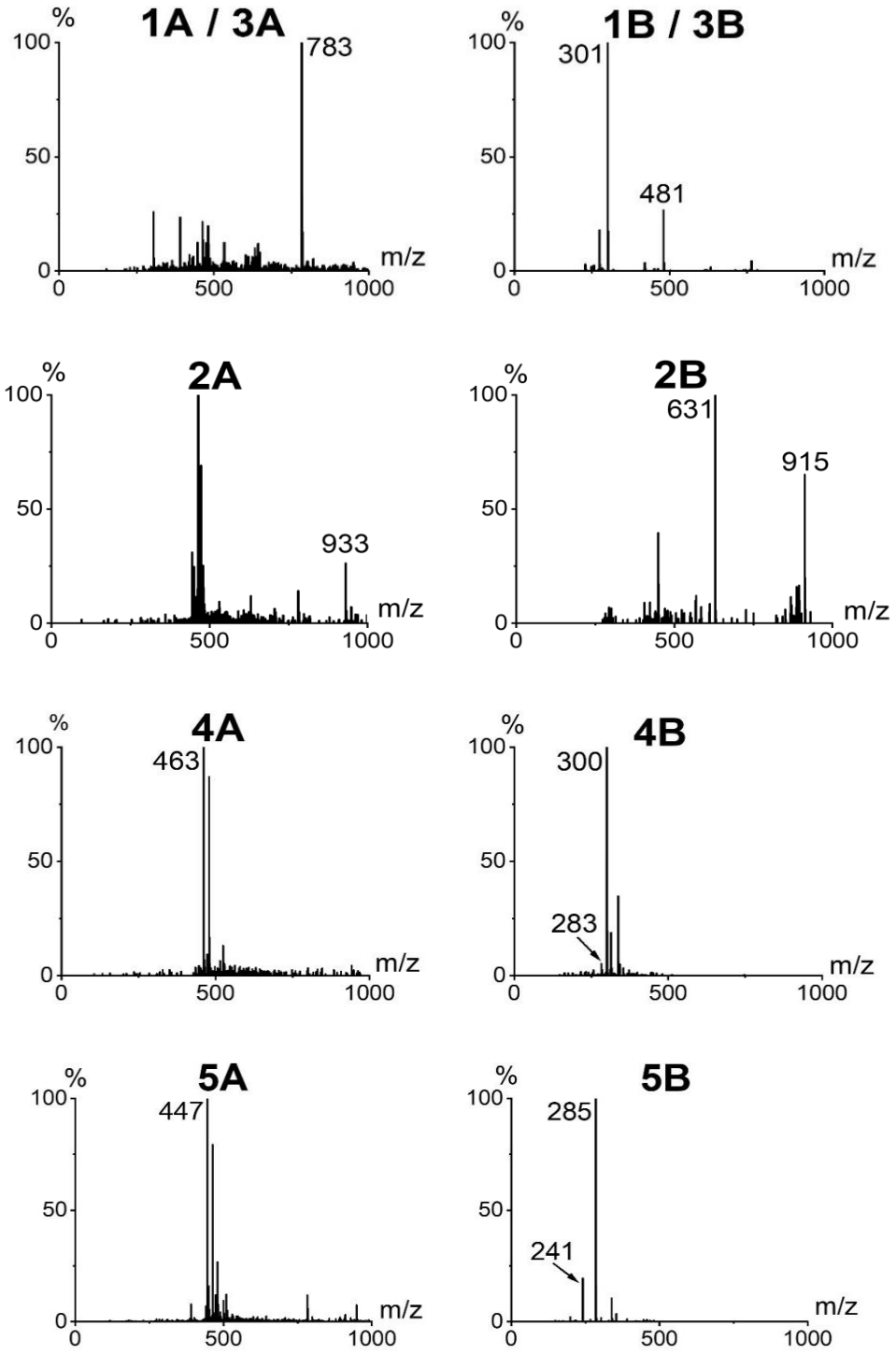



1: casuariin; 2: vescalagin; 3: pedunculagin; 4: delphinidin 3-*O*-glycoside (myrtilin); 5: cyanidin 3 *O*-glycoside (kuromanin); 6 strictinin; 7: casuarinin; 8: tellemagrandin I; 9: myricetin 3-*O*-rhamnoside (myricetrin); 10: ellagic acid; 11: quercetin 3-*O*-rhamnoside (quercetrin).

All the identified compounds were described by Moura et al. (2018) in the chemical characterization of a phenolic-rich extract from jaboticaba. The mass

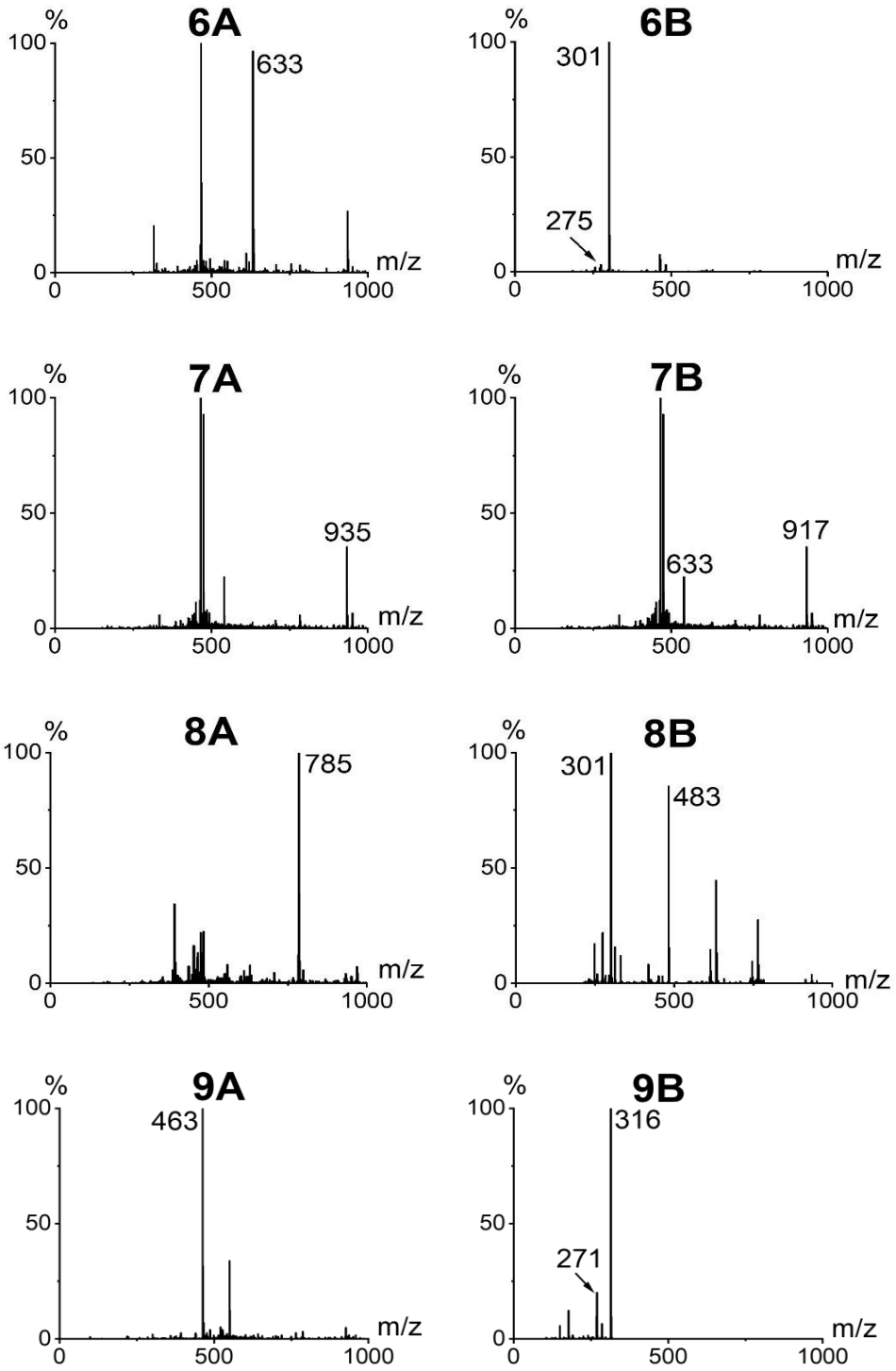
spectra and the fragmentation patterns of all identified compounds is presented at **Figure 9**.

**Figure 9**. Mass spectra and fragmentation patterns of compounds found in the phenolic-rich extract of jaboticaba (PEJ).



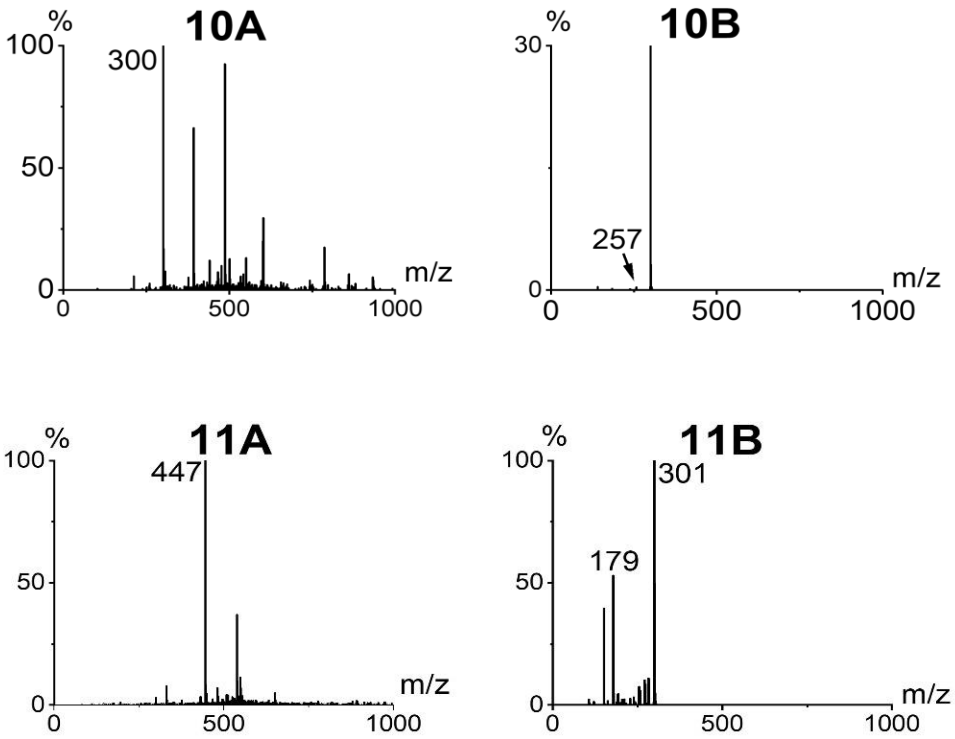

1- casuariin; 2- vescalagin; 3 – pedunculagin; 4- delphinidin 3-O-glycoside (myrtilin); 5 - cyanidin 3 O-glycoside (kuromanin); 6 – strictinin; 7 – casuarinin; 8- tellemagrandin I; 9: myricetin 3-O-rhamnoside; 10: ellagic acid; 11: quercetin 3-O-rhamnoside (quercetrin). A – molecular ion and B - mass fragments.

**Figure 9.** Mass spectra and fragmentation patterns of compounds found in the phenolic-rich extract of jaboticaba (PEJ). (Continuation).




1- casuariin; 2- vescalagin; 3 – pedunculagin; 4- delphinidin 3-O-glycoside (myrtilin); 5 - cyanidin 3 O-glycoside (kuromanin); 6 – strictinin; 7 – casuarinin; 8- tellemagrandin I; 9: myricetin 3-O-rhamnoside; 10: ellagic acid; 11: quercetin 3-O-rhamnoside (quercetrin). A – molecular ion and B - mass fragments.

**Figure 9.** Mass spectra and fragmentation patterns of compounds found in the phenolic-rich extract of jaboticaba (PEJ). (Continuation).



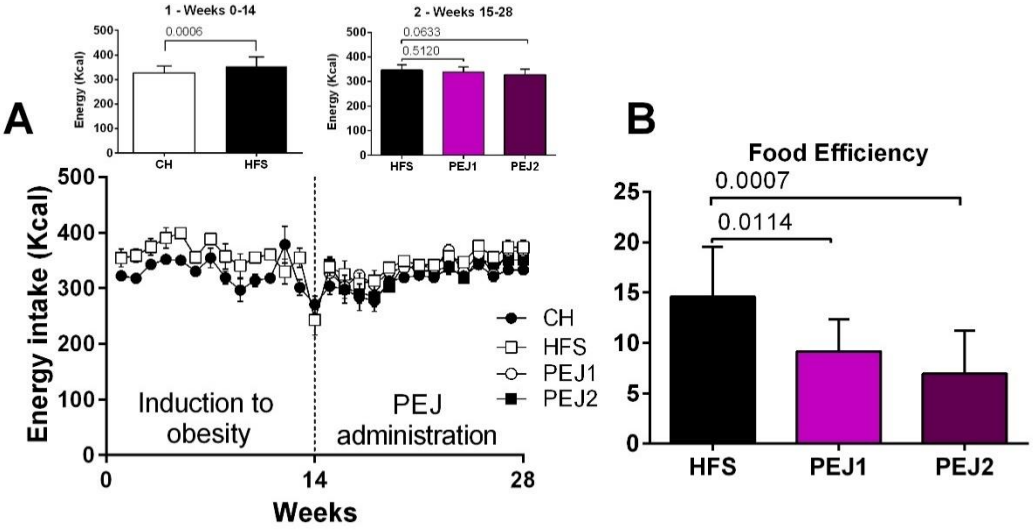

1- casuariin; 2- vescalagin; 3 – pedunculagin; 4- delphinidin 3-O-glycoside (myrtilin); 5 - cyanidin 3 O-glycoside (kuromanin); 6 – strictinin; 7 – casuarinin; 8- tellemagrandin I; 9: myricetin 3-O-rhamnoside; 10: ellagic acid; 11: quercetin 3-O-ramnoside (quercetrin). A molecular ion and B - mass fragments.

## 4.2 EFFECTS OF PEJ ON OBESITY AND INTESTINAL INFLAMMATION

### 4.2.1 Obesity

During the first period of induction to obesity, it was observed a statistical difference in the energy intake between the Ch group and the HFS group (0-14 weeks) (**Figure 10.A1**). After the redistribution of the HFS group, during the PEJ administration, such difference was not observed (15-18 weeks) (**Figure 10.A2**). On the other hand, as is shown in **Figure 10.B**, the feed efficiency was statistically lower for the PEJ-administrated groups, when compared to the HFS group, 32 and 38% lower for PEJ1 and PEJ2, respectively. Since the animals were fed with the same diet, the PEJ was proven to be effective in reducing feed efficiency.

**Figure 10.** Energy intake (A) and food efficiency (B) of mice fed with high-fat-sucrose (HFS) or standard diet and receiving water (Ch and HFS groups) or HFS and phenolic-rich extract from jaboticaba (PEJ) at two doses (PEJ1 and PEJ2 groups) by gavage.

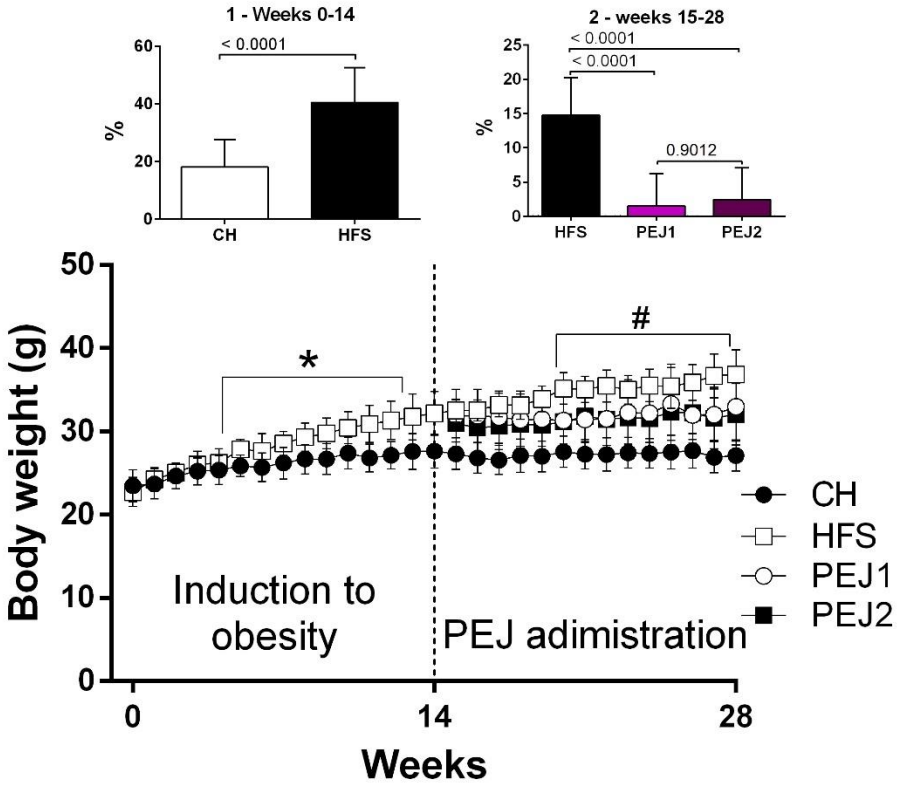



Values are mean ± SD. N = 8-12 animals/group.

During the obesity induction period (**Figure 11.1)**, the body weight of CH and HFS groups started to be statistically different from the fifth week. For the initial 14 weeks, the HFS group presented a body weight gain of 39% and the Ch group of 18% (**Figure 11.1**). This means that the HFS group had more than twice the body weight gain compared to the Ch group. According to other studies using animal models with diet-induced obesity, this proportion of body weight gain shows that induction of obesity was effective (CHU et al., 2017). After the beginning of the PEJ administration, on the 20$^{th}$ week, the body weight of animals was reduced ($p$<0.05) in PEJ1 and PEJ2 groups, when compared to the HFS group. On average, the body weight gain was 14% for the HFS group, while the supplemented groups were 1.5 and 2.4%, for PEJ1 and PEJ2, respectively (**Figure 11.2**).

The relative mass of white adipose tissue: retroperitoneal (RT), inguinal (IG), epididymal (EP), and total (tWAT) is presented in **Figure 12**. The HFS group presented, on average, 48 and 34% more WAT than the groups PEJ1 and PEJ2, respectively. The adiposity of the animals in the HFS group was significantly higher than that of animals receiving PEJ.

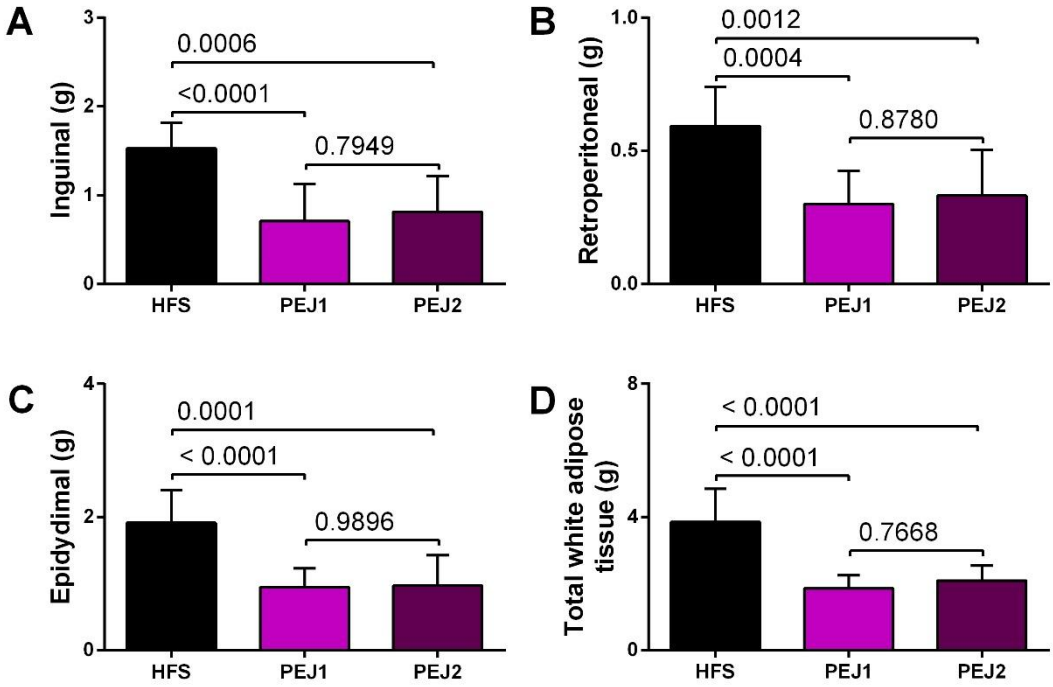
**Figure 11.** Body weight (1) and relative body weight gain (2), of mice fed with high-fat-sucrose (HFS) or standard diet and receiving water (Ch and HFS groups) or HFS and phenolic-rich extract from jaboticaba (PEJ) at two doses (PEJ1 and PEJ2 groups) by gavage.




*($p$<0.05) Ch *vs* HFS. # ($p$<0.05) HFS *vs* PEJ1 and PEJ2. Values are mean ± SD. N = 8-12 animals/group.

The absolute mass of adipose tissue of the HFS group, in comparison with the PEJ1 and PEJ2 groups, was 46 and 53% higher for IG, respectively (**Figure 12.A**), 41 and 56% for RT (Figure 12.B), 50 and 51% for EP (**Figure 12.C**), which represents 48 and 54% higher relative total WAT for HFS-fed animals than PEJ-administrated groups, PEJ1 and PEJ2 (**Figure 12.D**). Therefore, the PEJ administration was able to prevent the excessive WAT gain, which suggests an important effect of the phenolic compounds from jaboticaba in the management of adiposity.

**Figure 12**. Mass (g) of white adipose tissue inguinal (IG), retroperitoneal (RT), epididymal (EP) and total (WAT) of mice fed with high-fat-sucrose (HFS) or standard diet and receiving water (Ch and HFS groups) or HFS and phenolic-rich extract from jaboticaba (PEJ) at two doses (PEJ1 and PEJ2 groups) by gavage.




Values are mean ± SD. N=6-12 animals/group.

During the study we observed that HFS-obese animals developed excessive weight gain, corroborated by a considerable increase in fat accumulation. Besides that, PEJ administration was capable of decelerating the weight gain and adiposity, after 5 weeks. Previous studies with jaboticaba phenolic compounds have described similar results in HFS diet models (BATISTA et al., 2018; MOURA et al., 2018), such as decreased body weight gain independently of caloric intake, suggesting a protective effects of PEJ against diet-induced obesity. Thus, the exactly mechanisms by which PEJ protects against weight gain on already established obesity remain unclear; but, is presumed that it involves multiple pathways (RODRIGUES et al., 2021). Based on prior studies with phenolic compounds from native Myrtaceae fruits, including jaboticaba, conducted *in vitro* by our research group and others, it was attributed to phenolic compounds effects like enhanced *in vivo* fecal lipid excretion, lipolysis and fatty acid β-oxidation, increased energy expenditure, and inhibitory capacity

on lipid-hydrolyzing enzymes (mainly pancreatic lipase) (DONADO-PESTANA et al., 2015; BATISTA et al., 2018; MOURA et al., 2018; ANHÊ et al., 2020).

### 4.2.2 Glucose homeostasis

The decreased metabolic response to insulin in specific cells, or in the whole organism is defined as insulin resistance. It is considered a hallmark of obesity and a precursor of type 2 diabetes (T2DM). Moreover, a deregulated effect of circulating insulin is related to obesity and metabolic syndrome (CZECH, 2017).

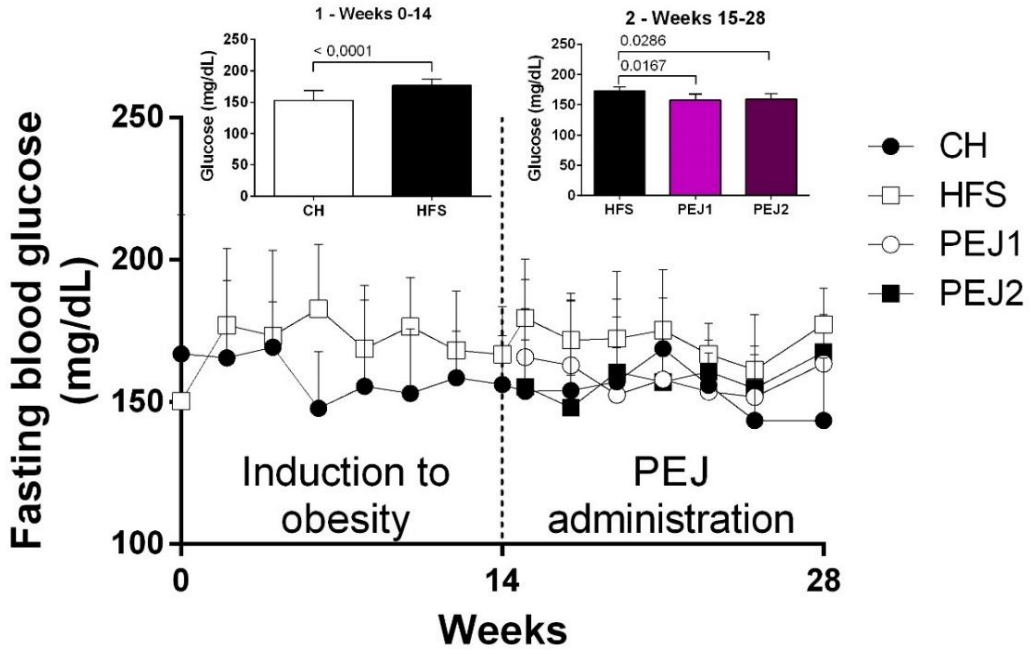
The insulin response and its mechanisms are complex and based on various biochemical reactions. There is evidence demonstrating beneficial effects of polyphenols on the management of blood glucose in insulin resistance and diabetes, by decreasing the digestion and intestinal absorption of dietary carbohydrates and regulating their metabolism, by improving glucose uptake in the muscle and adipose tissue, and also by improving β-cell function and insulin action (BAHADORAN; MIRMIRAN; AZIZI, 2013).

The fasting glycemia was monitored biweekly (**Figure 13**). In the first period it was observed a statistical difference between the HFS fed group (HFS) and the negative control group (CH). The average fasting glycemia was 16% higher for the HFS group at the initial 14 weeks (**Figure 13.A**). Then, with the PEJ administration, the mean glycemia was statistically lower for the supplemented groups (**Figure 13.B**). Since PEJ1 and PEJ2 groups presented the same average fasting glycemia, the relative difference among them and the HFS group was 8% lower for the PEJ-administrated groups.

In the 26$^{th}$ week, an oral glucose tolerance test (oGTT) was performed. In relation to the oGTT, it was observed a statistical difference, with AUC significative different ($p$<0.05), between the HFS group and the PEJ2 group (**Figure 14.A**). The relative percentage of difference was 15 and 21% lower for the PEJ1 and PEJ2 groups, respectively.

The oGTT result was consistent with previous results (DONADO-PESTANA et al., 2015; MOURA et al., 2018), suggesting beneficial effects of phenolic compounds in glycemic homeostasis, also PEJ2 group demonstrated statistical difference in the insulin levels (**Figure 14.B**).

**Figure 13** – Weekly variation of fasting blood glucose (4 h) of mice fed with high-fat-sucrose (HFS) or standard diet and receiving water (Ch and HFS groups) or HFS and phenolic-rich extract from jaboticaba (PEJ) at two doses (PEJ1 and PEJ2 groups) by gavage.

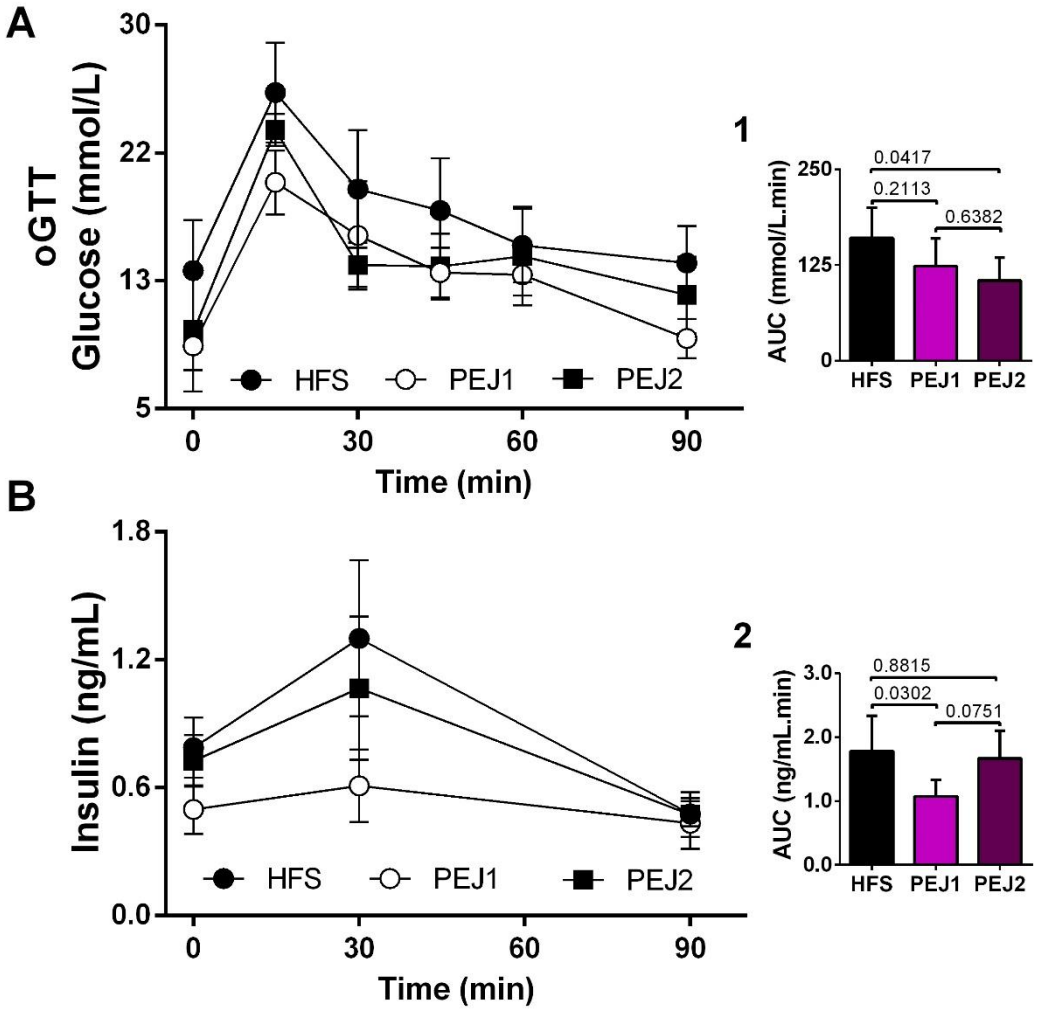



Values are mean ± SD. N = 8-12 animals/group.

Fruits and vegetables are recognized fonts of phenolics compounds and seem to play key role in glycemic homeostasis (SUN; MIAO, 2020). PEJ showed fasting glucose-lowering effects and ameliorated glucose tolerance. HFS-fed obese animals presented hyperinsulinemia, which was also decreased by PEJ in PEJ1 group, this result may be related to, at least in part, to decreased gluconeogenesis in liver or peripheral insulin sensitivity improvement (KANG et al., 2020). The higher dose did not differ significantly in relation to the HFS control group, although a lower tendency was observed. Disrupted glucose homeostasis and consequently insulin resistance is obesity-associated disorders (Zhang et al., 2016), considering that, to role of phenolic compounds form the diet has been investigated for their influence on glucose metabolism. The raised hypothesis is that phenolics can affect multiple mechanisms, such as nutrient absorption through reduction of intestinal carbohydrate digestion and glucose uptake, regulation of hepatic glucose output, and pancreatic β-cells protection, favoring the insulin-secretory function, and improving glucose uptake in insulin-sensitive

tissues such as muscle and adipose tissue (HANHINEVA et al., 2010; LERI et al., 2020).

**Figure 14** – Blood glucose (A) and the glucose area-under-the-curve (AUC; inset, 1) and plasma insulin (B) and the insulin AUC (inset, 2) during the glucose tolerance test of mice fed with high-fat-sucrose (HFS) or standard diet and receiving water (Ch and HFS groups) or HFS and phenolic-rich extract from jaboticaba (PEJ) at two doses (PEJ1 and PEJ2 groups) by gavage.




Data were analyzed by ANOVA and Tukey's multiple comparison test, and expressed as mean ± SD from each treatment (n = 5-6).

In fact, as discussed by Rodrigues et al. (2021), prior studies with fruits from Myrtaceae family, that includes jaboticaba reported that phenolic compounds present in these fruits have potential and beneficial properties that can mitigate insulin resistance in HFS-fed obese mice. One of the proposed mechanisms that might be associated with this effect is the improvement of signal transduction

through the insulin receptor/insulin receptor substrate-1 (IRS-1)/Akt/forkhead box protein pathway, or by mTORC2- and AMPK-mediated activation of Akt in liver, adipose tissue and skeletal muscle (Donado-Pestana et al., 2021; Dragano et al., 2013). Despite that, phenolic compounds form cagaita (*Eugenia dysenterica* DC.), another *Myrtaceae* Brazilian native fruit, in a HFS obesity-induced model showed regulatory effects over the hepatic gluconeogenesis through down-regulation of key genes involved, including pyruvate carboxylase, glucose-6-phosphatase (G6Pase), and PEPCK (Donado-Pestana et al., 2018). Further, Bao et al. (2020), has recently demonstrated gluconeogenesis suppressive effects of phenolic compounds from *Moringa oleifera Lam.,* also blocking the PEPCK and G6Pase activity, which stimulate glycolysis, and enhancing pyruvate kinase and hexokinase in liver of obese mice.

In summary, the PEJ administration at both doses was able to prevent the accumulation of excessive white adipose tissue, decreased the efficiency to convert the energy consumption in body weight, and ameliorated the glucose metabolism by lowering the fasting glycemia and the glucose intolerance in HFS-fed obese mice.

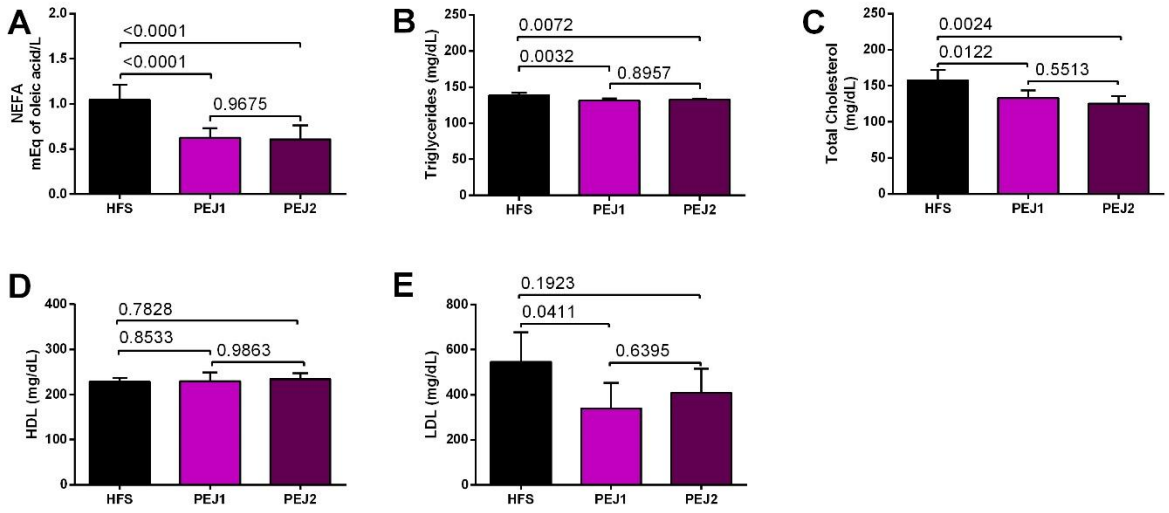
### 4.2.3 Plasma lipid profile

The lipid profile was assessed in relation to the cholesterol, triacylgyerols (TAG) and non-esterified fatty acids (NEFA) concentrations in plasma (**Figure 15**). Our results demonstrated that PEJ influenced on dyslipidemia of HFS-obese mice. The PEJ significantly reduced plasmatic NEFA levels ($p < 0.001$) in both doses (**Figure 15.A**). Furthermore, PEJ decreased triacylglycerols (TAG), total cholesterol, and LDL-cholesterol (only PEJ1) levels, but did not affected HDL-cholesterol (**Figure 15B-E**).

Several studies with polyphenol-supplemented animals demonstrated positive effects of polyphenols in the lipid metabolism. Mice lacking the LDL receptor (*Ldlr* $^{-/-}$*)* fed a HF diet and supplemented with 3% of naringenin, a flavanone present in citric fruits, for 4 weeks, showed decreased plasma lipids, decreased liver triglycerides and cholesterol, reduced overproduction of total triglycerides, inhibited the stimulated hepatic lipogenesis, and increased hepatic β-oxidation (MULVIHILL et al., 2009). Di Donna et al. (2014) reported decreased

concentrations of total cholesterol, VLDL, LDL and TAG, and increased HDL concentration, in diet-induced hypercholesterolaemic rats and supplemented with a enriched extract from bergamot fruit (*C. bergamia Risso*) (60 mg/kg BW/day), also, it was observed increased expression of important genes related to both cholesterol and TG metabolism. Further, the HMG enriched extract, has demonstrated similar results with a control group supplemented with a widely used hypocholesterolemic drug (simvastatin) in the treatment of cardiovascular diseases, that acts blocking the 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) in liver (DI DONNA et al., 2014).

**Figure 15**. Plasma lipid profile, plasmatic levels of non-esterified fat acids (NEFA) (A), triacylglycerols (TAG) (B), cholesterol (C), HDL-cholesterol (D), and LDL-cholesterol (E) of obese mice fed a high-fat-sucrose (HFS) diet receiving water (HFS group) or phenolic-rich extract from jaboticaba at two doses (PEJ1 and PEJ2 groups).




Data were analyzed by ANOVA and Tukey's multiple comparison test, and expressed as mean ± SD from each treatment (n = 5-6).

Numerous reports have also described that phenolic compounds affect the lipid metabolism by increasing bile acids (BA) excretion and reducing total and LDL cholesterol in animal and in-vitro studies. Rats supplemented with 0.4% of quercetin for 5 weeks presented lower levels of cholesterol and increased excretion of bile acids (ZHANG et al., 2016b). Furthermore, C57BL/6J mice fed with HF diet enriched with 0.25% of pomegranate extract for 4 weeks presented reduced hepatic levels of total cholesterol and TAG, and increased fecal excretion

of cholesterol and bile acids (YANG et al., 2018). Phenolics affect the bile acid metabolism by increasing the expression of cholesterol 7 α-hydroxylase (CYP7A1); a central enzyme of cholesterol metabolism and BA biosynthesis, reducing the expression of intestinal BA transporters; and altering the gut microbiota (CHAMBERS et al., 2019; PERINO et al., 2021).

Studies with phenolic compounds from green tea (*Camellia sinensis*) demonstrated significative effects against body weight gain, metabolic syndrome, diabetes and cardiovascular diseases in animal models and humans. A suggested mechanism for these actions is the activation of AMPK by tea phenolic compounds in the liver, skeletal muscle, and adipose tissues. Activated AMPK would act decreasing gluconeogenesis and fatty acid synthesis, and increasing catabolism, since its activation is associated with inhibition of cholesterol synthesis, stimulation of fatty acid oxidation, lipogenesis and triglyceride synthesis in liver (YANG et al., 2016). Thus, the active form of AMPK, can regulate other pivotal enzymes related to the lipid metabolism, such as peroxisome proliferator-activated receptor alpha (PPARα) and sterol regulatory element-binding protein (SREBP-1) (NELSON; COX, 2014).

Niazirin, a phenolic glycoside extracted from *Moringa oleifera* Lam. seeds, daily administered for 4 weeks to db/db mice, was efficient to reduce body weight gain, decreased levels of NEFA, TC, TAG, LDL, increased HDL levels, and ameliorated the glucose metabolism (BAO et al., 2020). NEFA plasmatic levels were also reduced in PEJ treated-animals. High circulating levels of NEFA are related with obesity-associated disorders, as insulin resistance and high levels of proinflammatory cytokines. They are produced by the hydrolysis od fatty acids and acts as substrates for their synthesis. High levels of circulating NEFA reduces hepatic glycogenesis, insulin secretion, and muscle glucose uptake. Therefore, insulin does not stimulate the uptake of NEFA in the cells, which exacerbates the lipotoxicity and hyperglycemia (NELSON; COX, 2014; USHIRODA et al., 2019; MOURA et al., 2021).

### 4.2.4 Intestinal inflammation

The anti-obesogenic effect of polyphenol-rich extracts was already previously described (MATIAS et al., 2014; DONADO-PESTANA et al., 2015,

2021; MOURA et al., 2018, 2021). The characterization of the obesity state was the first step in the present study. Next, the intestinal inflammation that is associated with obesity and the potential beneficial effects of the polyphenols from jaboticaba were studied.

A major evidence of systemic inflammation is the endotoxemia, evaluating the effects of PEJ in the LPS plasmatic levels in HFS-obese animals, it was observed that, in both doses, PEJ was capable of significantly reducing the LPS-circulating levels (PEJ1, $p < 0.05$; PEJ2, $p < 0.001$) (**Figure 16.A**). Considering that metabolic endotoxemia proceeds and is associated with intestinal inflammation, we investigated the role of PEJ administration in main inflammatory cytokines and proinflammatory mediators in the colon. As observed in **Figure 16.B**, when compared with HFS group, both PEJ groups demonstrated decreased contents of TNF-α, a key cytokine involved with proinflammatory signaling. We also found expressive decreased levels of TLR-4 protein content (**Figure 16.C**) and mRNA expression (**Figure 16.D**), in PEJ supplemented groups (PEJ1 and PEJ2) in relation to the HFS control group.

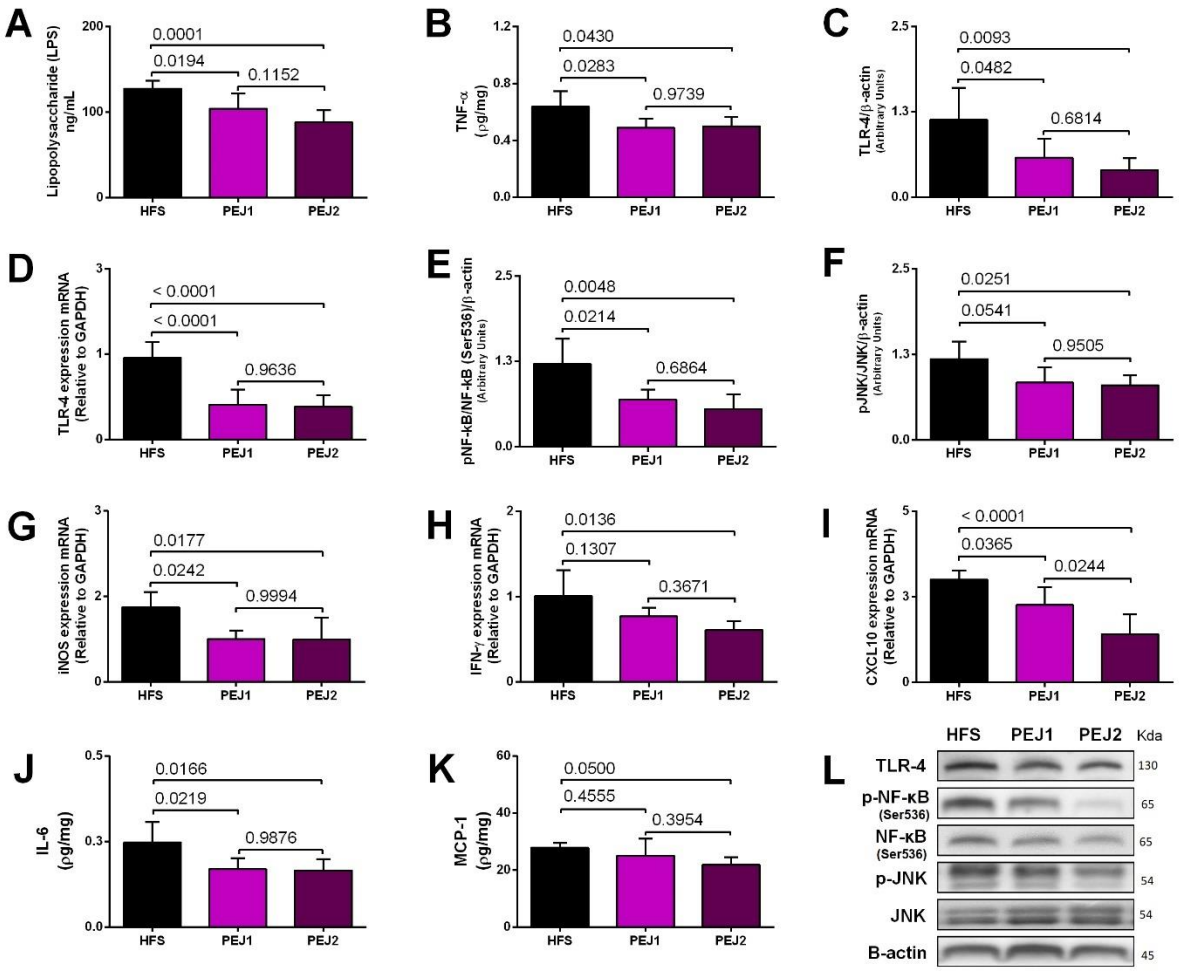
These inflammatory markers are associated with the NF-κB and JNK signaling pathways, which were also investigated, and PEJ groups presented significant reduction in both NF-κB and JNK protein contents (**Figure 16.E-F**) assessed by immunoblotting. Further, the gene expression of another pro-inflammatory mediators such as iNOS, IFN-γ (only to PEJ2) and CXCL10 (**Figures. 16.G-I**), were reduced in PEJ-administrated groups when compared with HFS-obese mice. In addition, the colonic contents of IL-6 and MCP-1 were also diminished by PEJ groups (**Figures. 16.J-K**).

Fruits and vegetables when regularly consumed has been proved to be beneficial for the health, with main effects associated with reduced risk of development of obesity-related NCDs. Besides that, the actual biological benefits and effects of these dietary components on lowering the NCDs risk are not completely elucidated yet. Active metabolic organs such as liver, pancreas, adipose tissue and skeletal muscle are the most focused organs in studies about obesity and its associated disorders, because their insulin-dependent-metabolism.

Recently, the intestine has been recognized as a crucial organ in a metabolic perspective, and seen as an essential effector, and initiator for the direct

and anticipatory response in the metabolic regulation, and no longer, as a restricted nutrient provider (DE WIT et al., 2008; FÄNDRIKS, 2017).

**Figure 16.** Plasmatic levels of lipopolysaccharide (LPS) (A), intestinal TNF-α level (B), intestinal TLR-4 protein content (C) and gene expression (D), intestinal protein contents of phospho-NF-κB p65 (Ser536) (E) and phospho-JNK (Thr183/Tyr185) (F), intestinal gene expression of iNOS (G), IFN-γ (H), CXCL10 (I), intestinal levels of IL-6 (J) and MCP-1 (K), and representative immunoblots (L) of obese mice fed a high-fat-sucrose (HFS) diet receiving water (HFS group) or phenolic-rich extract from jaboticaba at two doses (PEJ1 and PEJ2 groups).




Data were analyzed by ANOVA and Tukey's multiple comparison test, and expressed as mean ± SD from each treatment (n = 5-6).

Prolonged and excessive fat intake can drive to intestinal inflammation through the inflammasome activation (PROGATZKY et al., 2014). The NLRP3 inflammasome activation requires two signals, the first is provided by TLR ligands, such as bacterial toxins like LPS, urate crystals and silica, or by cytokines, like TNF-α, that promotes the NLRP3 activation via NF-kB induction. The second

signal triggers directly the caspase-1 activation, and can also be promoted by microbial toxins (CHEN; NÚÑEZ, 2011; WEN et al., 2011).The product of inflammasome activation is the release of pro inflammatory cytokines IL-1β and IL-18 in their active form. PEJ also demonstrated effects on some specific intermediate targets of inflammasome. The gene expression of NLRP3 was only slightly reduced (non-significant) in both PEJ groups (**Figure 17.A**). As well as the ASC gene expression, another main component of inflammasome pathway, which was also slightly reduced in PEJ1, but not in the higher dose group (PEJ2), that demonstrated an unexpected increase comparing with the control HFS-group (**Figure 17.B**)

Despite that, the gene expression of caspase-1, a key NLRP3 -activated enzyme, and IL-1β, a main product of inflammasome pathway, were decreased in both PEJ groups (PEJ1 and PEJ2) (**Figure 17.C-D**). The IL-18 gene expression was also decreased, but only in PEJ2 group (**Figure 17.E**).

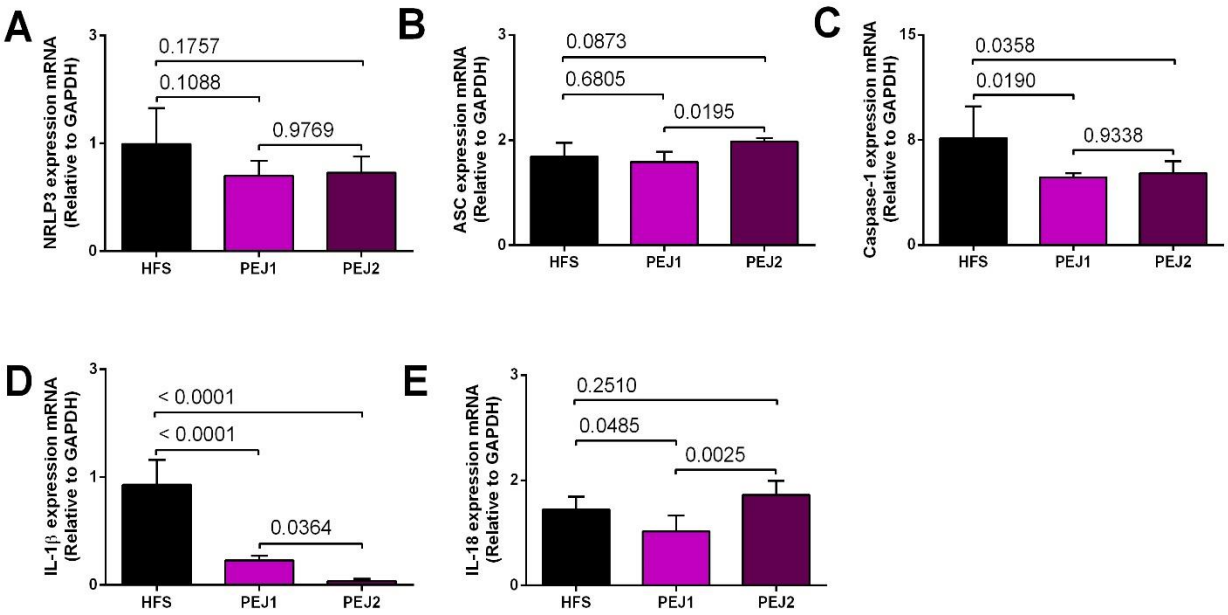
The demonstrated anti-inflammatory effects of PEJ, is proposed to be, at least in part, related to the inhibition of inflammasome activation in the colon of the HFS-obese animals, which may uncover a potential novel mechanism to explain the observed effects and the biological properties of thee phenolic compounds from jaboticaba in the intestine and its associated disorders.

According to the chemical characterization, PEJ is majorly composed by ellagitannins and ellagic acids derivates (**4.1**), when metabolized by gut microbiota, these ellagitannins are converted to urolithins (INADA et al., 2019). Recently, was demonstrated that urolithins can mitigate colitis in both preventive and therapeutic ways, since they has shown to enhance the gut barrier function and presented protective effects against the intestinal inflammation (SINGH et al., 2019). Similarly, a study with DSS-induced colitis and phenolic-rich extracts from apple demonstrated an important inhibition of inflammatory cytokines production, and consequently diminished inflammatory activation, as well as a protective effect over the intestinal mucosal integrity and reduced shortening of colon (LIU et al., 2020). Considering this study, our observations about the slight, non-significant increased colon weight in PEJ-administrated groups (**Supplementary figure 1**) compared to the HFS control group, may be, at least in part, explained.

There are two mechanisms by which urolithins can act: a) protecting the immune cells and preventing LPS-induced inflammation; and b) through its

antioxidant properties, and direct affecting the aryl hydrocarbon receptor (AhR) - nuclear factor erythroid 2-related factor 2(Nrf2) dependent pathways, that, in turn, upregulates the epithelial tight junction (TJ) proteins. Furthermore, another important effect of urolithins is the potential mitochondrial dysfunction regulation, a main contributor to the pathophysiology of the inflammatory bowel disease (IBD) (SINGH et al., 2019).

**Figure 17.** Intestinal gene expression of NLRP3 (A), caspase-1 (B), IL-1β (C), IL-18 (D) and ASC (E) of obese mice fed a high-fat-sucrose (HFS) diet receiving water (HFS group) or phenolic-rich extract from jaboticaba at two doses (PEJ1 and PEJ2 groups).




Data were analyzed by ANOVA and Tukey's multiple comparison test, and expressed as mean ± SD from each treatment (n = 5 – 6).

Several animal studies supported that increased intestinal permeability is closely related with an excessive fat intake, since it contributes to increase the entrance of toxins derived from the gut microbiota (i.e. LPS) (ROHR et al., 2020). A remarkable finding in the present study was the significant decreasing of plasmatic LPS in animals that received PEJ in both doses, as discussed LPS circulating levels is major factor in the development of intestinal and systemic inflammation, causing disruption of the intestinal barrier and leading to metabolic endotoxemia and chronic low-grade inflammation. These mechanism, mainly through TLR-4 activation is reported to be related with the pathogenesis of other obesity-associated metabolic disorders, such as non-alcoholic fatty liver disease

(NAFLD), cardiovascular disease, insulin resistance and type 2 diabetes mellitus (T2DM) (KIM et al., 2012; MOREIRA et al., 2012). These results provide evidence, demonstrating significant impacts of PEJ in the glucose homeostasis, improving glucose tolerance and lowering the fasting glycemia in HFS-obese animals.

The elevated endotoxemia, with higher levels of circulating LPS, induces inflammatory activation, and a principal characteristics of this state is the enhanced expression of pro-inflammatory cytokines, such as iNOS, TLR4,and the NF-kB pathway in colon of HF-fed mice (KIM et al., 2012). PEJ administration was capable of ameliorating significant inflammatory markers in the intestine of the animals. These observations are compatible with previous studies with phenolic compounds from different sources, whereby they act by inhibiting the TLR-4 pathway, and down-regulating the contents and expression of pro-inflammatory markers, such as TNF-α, MCP-1, iNOS, and IL-6, as demonstrated in the colon of animals with colitis induced by DSS (DOU et al., 2013).

Similarly, a study with phenolic compounds form green tea has also demonstrated anti-inflammatory effects, by unstimulating the TLR-4 signaling pathway and mitigating the over expression of pro-inflammatory mediators, in HF diet-induced mice (LI et al., 2020). Another previous study has shown a different mechanism by which phenolic compounds can also act, the upregulation of the gene expression of Toll interacting protein, that acts as a negative regulator of TLR4 signaling through 67LR via cell surface receptors such as 67-kDa laminin receptor (67LR), and which plays a central role mediating the anti-inflammatory action (BYUN et al., 2010). Moreover, phenolic compounds may also interfere in the TLR-4 oligomerization, since this factor is determinant for the activation of the receptor upon ligand biding, that is another plausible mechanism by through phenolic compounds affect the LPS-mediated inflammatory response (CAPIRALLA et al., 2012).

In addition, our results indicate that PEJ-administrated groups presented lower expression of Thr183/Tyr185-phosphorilated JNK in colon. Some authors suggest that activated JNK can drive the TJ integrity, and consequently, intestinal permeability, intermediated by IL-6 and targeting claudin-2 mRNA expression, affecting both transcription and synthesis of this specific member of claudin family, a major class of TJ components (AL-SADI et al., 2014). Further, PEJ, in the higher dose, also demonstrated decreased levels of IFN-γ and CXCL10, a IFN-γ-induced

protein, and according to Wang et al. (2005) IFN-γ is a key modulator of gut permeability. A study using a colitis DSS-induced model, has previous described a suppressive effect on IFN-γ expression with naringenin administration, a common flavanone characteristic of citric fruits, thus protecting from mucosal epithelial barrier impairment and ameliorating endotoxemia (AZUMA et al., 2013).

The inflammasome is assembled by NLRP3, is a cytosolic platform protein, ASC, an adapter protein, and procaspase-1, and the interactions between these proteins are responsible for regulating the inflammasome function (ZAKI; LAMKANFI; KANNEGANTI, 2011; SHAO et al., 2015). Although we did not observed decreased expression of NLRP3 and ASC, PEJ reduced the caspase-1 expression in both treated groups, since caspase-1 plays a crucial role in the regulation of NLRP3-inflammasome machinery, we considered is a potential site of action for phenolics in this specific inflammatory pathway (SWANSON; DENG; TING, 2019).

Curiously, the ASC expression was slightly, non-significant increase in higher dose group (PEJ2), when compared to HFS control group (*p*-value = 0.09). ASC is a key mediator of gut homeostasis regulation, this effect observed may derive from a compensatory mechanism stimulated by PEJ (ZAKI; LAMKANFI; KANNEGANTI, 2011). In fact, a previous study with ASC $^{-/-}$ mice in a DSS-induced colitis model, showed gastrointestinal disease, severe epithelial barrier impairment, and dysplasia, which suggests a protective role of ASC (ALLEN et al., 2010).

Pro-inflammatory cytokines as IL-1β and IL-18 have a critical role in inflammasome and inflammatory processes (ZAKI; LAMKANFI; KANNEGANTI, 2011). The PEJ groups presented reduced expression of IL-1β in both groups PEJ1 e PEJ2) and IL-18 only in the lower dose (PEJ1). Accordingly, Kim et al. (2012), has demonstrated that HF diet causes increased expression of IL-1β, intestinal inflammation and shortening of the colon, despite that, phenolic compounds from pomegranate was capable of reducing both level and expression of IL-1β, mitigating the negative effects of the hypercaloric diet (ZHAO et al., 2019).

In together, our findings demonstrate the potential anti-inflammatory effects of PEJ in intestinal inflammation, by lowering endotoxemia, decreasing

cytokine levels and inhibiting the expression of main pro-inflammatory mediators (RODRIGUES et al., 2021).

### 4.2.5 Intestinal permeability

Evidence from animal models suggests a link between gut microbiota, increased intestinal permeability, endotoxemia, and obesity. The intestinal permeability evaluation can be used to address the intestinal barrier integrity. In this sense we intended to investigate whether PEJ can affect the intestinal permeability in a HFS-obesity model, which was already established and well characterized.

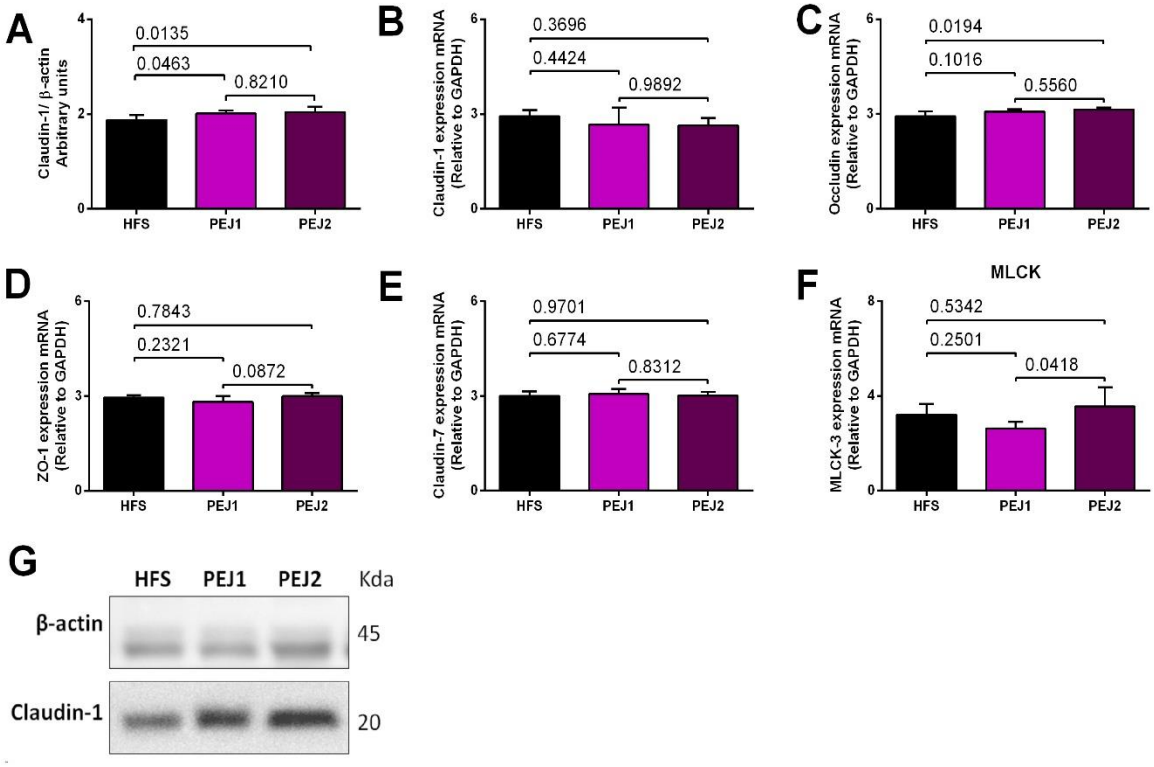
The TJ regulates the paracellular pathway, and is constituted majorly by zonulins, occludin, claudins, and junction adhesion molecules (JAMs) proteins. Our data demonstrated that PEJ, in both doses, increased the protein content of claudin-1 (**Figure 18.A**), but did not affect its gene expression (**Figure 18.B**), and significantly improved the gene expression of occludin in PEJ2 group (**Figure 18.C**). Besides, PEJ did not impact the gene expression of ZO-1 (**Figure 18.D**), claudin-7 (**Figure 18.E**) and myosin light-chain kinase-3 (MLCK-3) (**Figure 18.F**).

A major mechanism involved with the paracellular permeability is the direct interaction between the transmembrane claudin-1 and occludin with cytosolic protein zonula occludens-1 (ZO-1) which binds to the actin cytoskeleton to control paracellular permeability, preventing the penetration of proinflammatory agents such as antigens, microorganisms, toxins, and LPS present in the intestinal lumen. ZO-1 proteins play a crucial role as key molecules in the paracellular contact, maintaining the TJ structure and the epithelial barrier function (CARRASCO-POZO; MORALES; GOTTELAND, 2013).

As described at item 4.2.4, the HFS-fed animals demonstrated an elevated level of circulating LPS, which suggests an increased entrance of these toxins trough the paracellular pathway. Our findings also showed an elevated expression of inflammatory mediators, as TLR-4, TNF-α, IFN-γ and NF-κB (**Figure 16**), which may be involved in the regulation of the intestinal permeability. According to Liu et al. (2017), studies with intestine, liver, and brain demonstrated that increased permeability through the TJ proteins regulation, such as occludin levels in the colon are highly related to increased TLR4 levels. Also, the

overproduction of proinflammatory cytokines such as IFN-g, alone or in combination with other cytokines as TNF-α, increases intestinal permeability through changes in expression and localization of TJ proteins as well as rearrangement of the cytoskeleton (BRUEWER et al., 2020). High levels of TNF-α are also related to increased permeability by enhancing the myosin light chain kinase (MLCK) transcription and activity at the tight junction and causing occludin endocytosis (ODENWALD; TURNER, 2017).

**Figure 18**. Intestinal protein content of claudin-1 (A), gene expression of claudin-1 (B), occludin-1 (C), ZO-1 (D), claudin-7 (E), MLCK-3 (F), and representative immunoblots (G) of obese mice fed a high-fat-sucrose (HFS) diet receiving water (HFS group) or phenolic-rich extract from jaboticaba at two doses (PEJ1 and PEJ2 groups).




Data were analyzed by ANOVA and Tukey's multiple comparison test, and expressed as mean ± SD from each treatment (n = 5 – 6).

An *in vitro* study with phenolic compounds including quercetin, epigallocatechingallate (EGCG), rutin, and resveratrol demonstrated a protective effect of these compounds in a model of indomethacin (INDO)-induced gastrointestinal damage, through mitochondrial dysfunction, oxidative stress, and apoptosis. It was observed an important effect of these phenolic compounds (mainly quercetin) in the protection of the mucosal integrity improving the ZO-1

and occludin expression, that was affected by INDO, an effect attributed to mitochondrial protecting property (CARRASCO-POZO; MORALES; GOTTELAND, 2013).

Several *in vitro* studies using cytokine stimuli have demonstrated the protective effect of phenolic compounds, mostly evidenced by improving the trans epithelial electrical resistance (TEER) and increasing the expression of ZO-1, occludin, and claudins involved in the functioning of TJs (NODA; TANABE; SUZUKI, 2013; CONTRERAS et al., 2015; VALENZANO et al., 2015; CREMONINI et al., 2017, 2018; VAZQUEZ-OLIVO et al., 2019). Animal studies with diet-induced obesity also confirmed this hypothesis, as observed by Gil-Cardoso et al. (2018), that used a model of cafeteria diet-induced obesity in Wistar rats supplemented with grape-seed proanthocyanidins extract (GSPE). The co-administration of these phenolic compounds contributed to improve the TEER, decrease the endotoxemia and inflammatory markers, as well as increase TJ proteins expression such as ZO-1 and occluding.

PEJ has demonstrated to be effective in increasing claudin-1 protein content and occludin mRNA expression, what suggests a positive effect on intestinal permeability. However, further investigations of whether PEJ may affect intestinal barrier function are necessary.

### 4.2.6 Histology

The intestinal mucus layer present in the lumen is the first physical and biochemical barrier that prevents contact of bacteria and other microbes with the epithelium. The main composition of the mucus layer are mucins (the most abundant being MUC2), mucin interacting-peptides, and antimicrobial peptides, such as lysozyme and defensins (ARAÚJO et al., 2017).

Mucus properties are likely influenced by the bacteria that are in close proximity to the epithelium, an altered microbial community composition can promote differences in metabolite production, including short-chain fatty acids (SCFAs), which have been described to modulate mucus expression. It is related, for example, to the preference of butyrate as energy source by the goblet cells, and accordingly changes in the SCFA profile may have serious effects on physiology (SCHROEDER et al., 2020).

Considering the importance of mucus in the maintenance of the intestinal barrier integrity, we further investigated, qualitatively, the mucus activity in the colon of HFS-obese mice and PEJ supplemented groups. The intestinal goblet cells are responsible for synthesize and secret mucins in the intestine lumen, and these mucins are basically composed by glycoproteins, easily colored with the Periodic acid-reactive Schiff reaction (WANG et al., 2019). The relative proportion of PAS positive marked cells demonstrated a decreasing tendency in mucins secretion in PEJ2 groups, that received the higher dose ($p$=0.3007) (**Figure 19.A** and representative **Figure 19**).

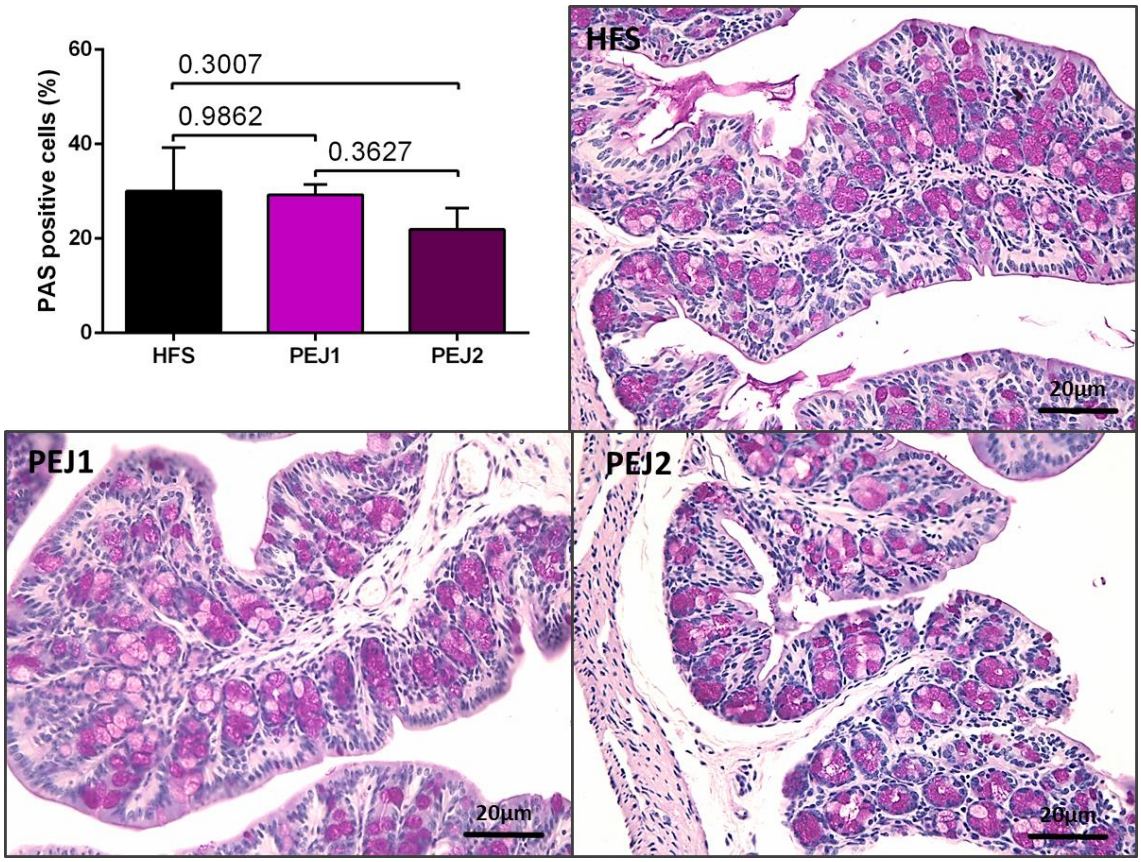
Besides some studies suggest a positive effect of increased levels of mucins content, it is known that mucus is secreted to protect the intestinal barrier. HF diet has been reported to induce GI changes by modifying the gut microbiota composition, such as the levels of gram-positive and gram-negative bacteria, the Firmicutes/Bacteroidetes ratio, and increasing LPS levels in intestinal epithelia. These alterations are suggested to be associated with decrease in mucus layer thickness and an increase in pro-inflammatory cytokines secretion, what is strongly associated with systemic inflammation, insulin resistance, obesity, and type 2 diabetes.

Araújo et al. (2017) discuss four major altered mechanisms caused by HF diets in the small intestine: decreased gene expression of antimicrobial peptides, disrupted mucus layer, impaired electrolytes secretion, and increased epithelial permeability. Considering the mucus layer disruption, the authors point that a MUC2 accumulation in goblet cells may be involved with these negative effects. Thus, the higher percentage of PAS-positive cells for HFS group (**Figure 19**) demonstrates an accumulation of glycoproteins, and the qualitative observation of decreasing proportion of PAS marked cells in PEJ groups might indicate a positive effect of PEJ administration against the mucus layer disruption. However, a more complete analysis could address better the effects of PEJ over this mechanism.

In summary, we intended to investigate whether PEJ may affect multiple aspects of intestine associated disorders in HFS-induced obese mice, and we found important anti-obesogenic, anti-inflammatory, and promising effects in maintaining intestinal permeability. **Figure 20**shows a graphical abstract of our main findings, demonstrating the inflammatory state of the HFS-fed obese mice

(**Figure 20.A**), compared to animals treated with PEJ (**Figure 20.B**), pointing the principal observation in relation to inflammatory pathway, inflammasome and intestinal permeability.

**Figure 19.** PAS positive cells ratio (A) and representative PAS stained of obese mice fed a high-fat-sucrose (HFS) diet receiving water (HFS group) or phenolic-rich extract from jaboticaba at two doses (PEJ1 and PEJ2 groups).



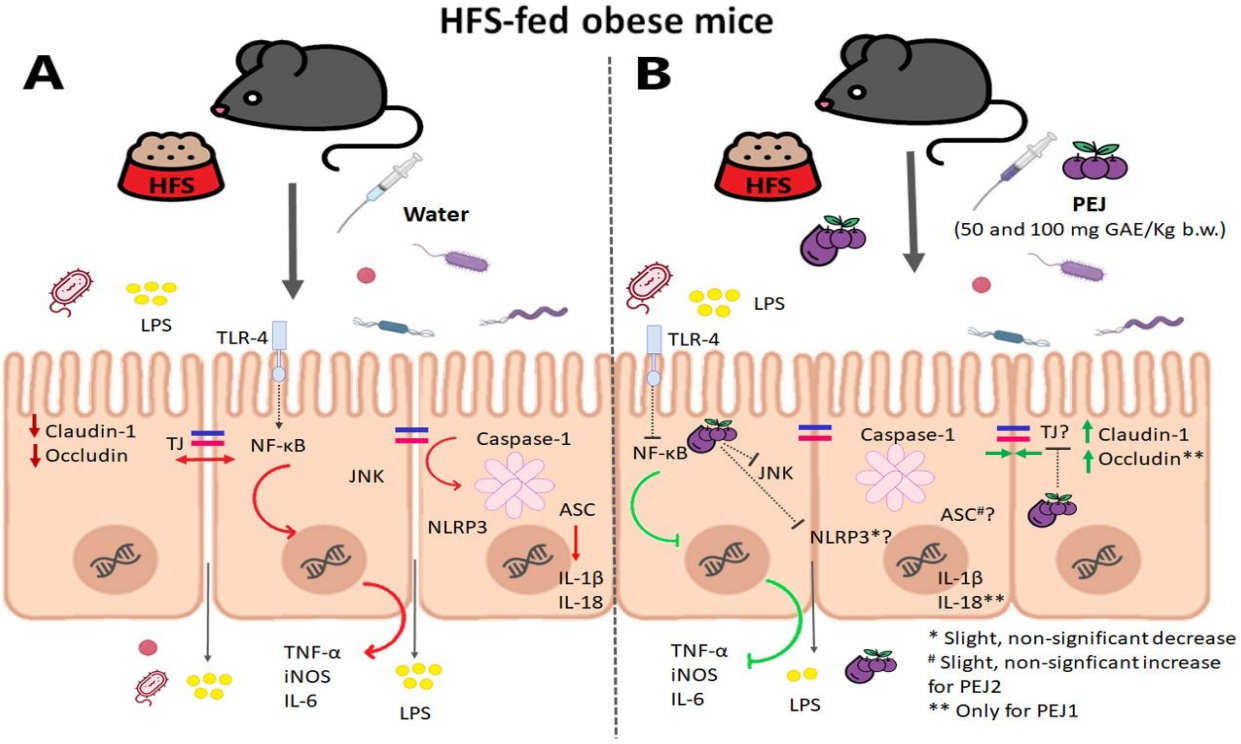

Data were analyzed by ANOVA and Tukey's multiple comparison test, and expressed as mean ± SD from each treatment (n = 3).

Original magnification X 400.

An increased risk of gastrointestinal disease is commonly associated with obesity, which is suggested to be caused by a gut health impairment. The exact definition of gut health has been discussed and remains undefined, but, as proposed by Bischoff (2011), is possible to ascertain gut health according to five major criteria and signs of gastrointestinal disorders. These criteria include effective digestion and absorption of food, absence of gastrointestinal illness, normal and stable intestinal microbiota, and effective immune status. Based on these criteria is possible to classify several gastrointestinal disorders according to

signs and factors, since both obese individuals and animal models have demonstrated alterations in these parameters (TEIXEIRA et al., 2012).

**Figure 20**. Graphical schematization of collected data and representation of the main proposed effects of PEJ on the intestine of HFS-fed obese mice, compared to the HFS control group.




Source: addapted from (RODRIGUES et al., 2021)

Relating these gut health criterions to our major findings about the anti-inflammatory effects of PEJ, as decreased activity and expression of main cytokines of inflammatory pathways, such as TLR-4, NF-κB, TNF-α, and IL-1β (discussed at **4.2.4**), and increased claudin-1 and occludin levels, suggesting potential benefits of PEJ on maintaining the intestinal permeability, we propose that, collectively, these observations might provide supporting data to recommend the use of phenolic compounds from jaboticaba as an adjuvant for the treatment and prevention of obesity and its associated disease, such as intestinal inflammation.

## 5. CONCLUSION

The major classes of phenolic compounds found in PEJ are represented by ellagitannins, anthocyanins including cyanidin and delphinidin, proanthocyanidins, and free ellagic acid. The phenolic compounds from jaboticaba, demonstrated anti-obesogenic effects, preventing the body weight gain and improved the glucose homeostasis of animals fed on a HFS diet. Further, PEJ administration improved the glucose homeostasis by lowering the fasting glycemia and the glucose intolerance, and prevented the accumulation of excessive white adipose tissue, decreased the efficiency to convert the energy consumption in body weight, and enhanced the lipid profile of HFS-fed obese animals.

Summarily, our data clearly reveals the directly anti-inflammatory effects of PEJ in obesity-associated intestinal inflammation. Our findings suggests that this outcome was directly linked with a reduction of metabolic endotoxemia in the HFS-obese mice. Additionally, PEJ inhibited classical inflammatory intermediates and partially modulated the inflammasome pathway in the colon of obese mice. PEJ has also demonstrated to be effective in increasing activity and expression of key proteins of intestinal TJ, what suggests a protective effect against increased intestinal permeability caused by HFS diet. Further studies should address the effects of PEJ in intestinal integrity and elucidate this specific mechanism, as well as the PEJ interactions and effects on the commensal microbiota, which plays an important role in maintaining intestinal homeostasis and in orchestrating mucosal immunity.

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**ANEXO I**

# UNIVERSIDADE DE SÃO PAULO

**FACULDADE DE CIÊNCIAS FARMACÊUTICAS**

**Comissão de Ética no Uso de Animais - CEUA**

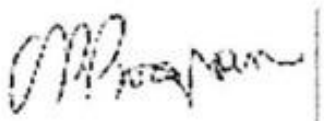
Ofício CEUA/FCF **006.2021**

São Paulo, 15 de janeiro de 2021.

Prezado (a) Senhor (a),

A Comissão de Ética no Uso de Animais da Faculdade da FCF/USP, em reunião realizada em 15 de janeiro de 2021, **APROVOU** o relatório final, relativo ao projeto de pesquisa "Compostos fenólicos da jabuticaba-sabará (*Plinia jaboticaba* (Vell.) Berg) na redução dos riscos à saúde causados pela obesidade e pelo diabetes mellitus tipo 2" do pesquisador Márcio Hercules Caldas Moura (Protocolo CEUA nº**522**).

Cordialmente,



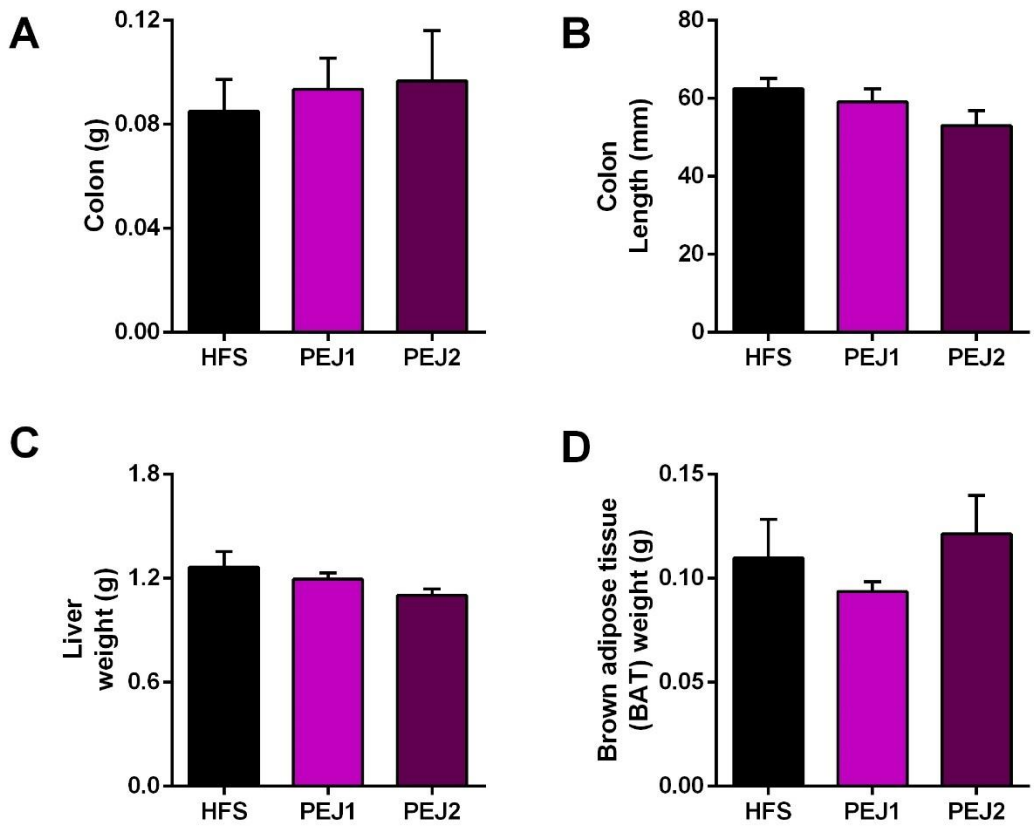
**Profa. Dra. Cristina Stewart Bittencourt Bogsan**

Coordenadora do CEUA/FCF/USP

Ilmo Sr. Márcio Hercules Caldas Moura
Orientadora: Profa. Dra. Maria Inés Genovese
FBA/FCF/USP

## ANEXO II

**Supplementary figure 1.** (A) Colon weight (g), (B) colon length (mm), (C) liver weight (g), and (D) brown adipose tissue weight (g).




Data were analyzed by ANOVA and Tukey's multiple comparison test, and expressed as mean ± SD from each treatment (n = 5-6).

# ANEXO III

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## Phenolic compounds from jaboticaba (*Plinia jaboticaba* (Vell.) Berg) ameliorate intestinal inflammation and associated endotoxemia in obesity

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ABSTRACT

Jaboticaba (*Plinia jaboticaba* (Vell.) Berg) is a Brazilian native fruit belonging to the Myrtaceae family. Previously it was demonstrated that phenolic-rich extracts from jaboticaba (PEJ) possess health-beneficial properties in diet-induced obesity; however, whether PEJ modulates the obesity-associated intestinal inflammatory status remains unclear. Thus, male C57BL/6J obese mice were fed a high-fat-sugar (HFS) diet and received PEJ at two doses, 50 mg gallic acid equivalent (GAE)/kg body weight (BW) (PEJ1 group), and 100 mg GAE/kg BW (PEJ2 group), or water (HFS group) by oral gavage for 14 weeks. PEJ groups presented a reduced body weight gain and adiposity and were protected against insulin resistance and dyslipidemia. In addition, PEJ prevented metabolic endotoxemia linked to an attenuation of the HFS diet-induced intestinal inflammation via down-regulation of pro-inflammatory mediators such as tumor necrosis factor (TNF-α), membrane transporter toll-like receptor-4 (TLR-4) and nuclear factor-κB (NF-κB) in the colon. These anti-inflammatory effects appear to be involved, at least in part, with an inhibition of the colonic inflammasome pathway of obese mice.

### 1. Introduction

Obesity is one of the most serious public health problems in modern society. This condition is regularly associated to a prolonged and excessive consumption of Western-type diets rich in fat and sugar, which results in an increased risk for the development of many prevalent non-communicable diseases (NCDs) including type 2 diabetes mellitus, non-alcoholic fatty liver disease, cardiovascular disease, as well as certain cancers (i.e., colon, breast cancers) (Christ, Lauterbach, & Latz, 2019). Obesity and related NCDs are commonly studied and focused on the physiology and underlying molecular mechanisms in metabolic tissues such as adipose tissue, liver, and skeletal muscle. However, there is growing evidence that also the intestine can play a role in the etiology of obesity and associated complications (de Wit et al., 2008; Winer, Luck, Tsai, & Winer, 2016). In fact, evidence has shown that prolonged exposure to high-fat diet and obesity can indirectly induce intestinal inflammation by perturbing immune homeostasis, and gut microbial dysbiosis, thereby dictating metabolic alterations in the host (Anhê et al., 2020; Progatzky et al., 2014).

Although the intestine is an essential organ for the digestion and absorption of nutrients, its role in metabolic diseases has been poorly investigated. The intestinal epithelium and its specialized cells acts as a highly regulated physical barrier being a first line of host defense against both encroaching commensal bacteria and invading enteric pathogens (Martens, Neumann, & Desai, 2018; Nagler-Anderson, 2001). The disruption in the intestinal barrier structure can induce an uncontrollable immune reaction in the intestinal microenvironment, which leads to intestinal inflammation and metabolic disorders such as diabetes and obesity (Chelakkot, Ghim, & Ryu, 2018). It has been suggested that the consumption of high-fat diet increases intestinal permeability, which in turn causes the leakage of gut microbiota-derived lipopolysaccharide (LPS) and other toxins into the bloodstream, leading to metabolic endotoxemia (Chelakkot et al., 2018; Gil-Cardoso et al., 2016). Furthermore, prolonged intake of saturated fats and cholesterol can induce chronic and systemic inflammatory responses through inflammasome activation in the intestinal epithelium, leading to inflammation in the intestine (Progatzky et al., 2014).

Praga, Croft, Kennedy, and Tomás-Barberán (2019) reported an association between the intake of phenolic compounds and decreased risk of obesity-related NCDs. In mice, previous studies have shown that

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