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HIGH-FAT DIET-INDUCED BRAIN DAMAGES: NEW NUTRACEUTICAL APPROACHES

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Acknowledgments

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Glossary

The following is a glossary of common abbreviations or acronyms used in this thesis.

AD	Alzheimer's disease
AFA	Aphanizomenon flos aquae
AGEs	Advanced glycation end products
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
BECs	Microvascular endothelial cells
CAT	Catalase
CNS	Central nervous system
CREB	cAMP response element-binding protein
CRP	C-reactive protein
EGCG	Epigallocatechin-3-gallate
eNOS	Endothelial nitric oxide synthase
ETC	Electron transport chain
FFAs	Free fatty acids
GBA	Gut-brain axis
GFAP	Glial fibrillary acid protein
GSH	Glutathione
GSK-3β	Glycogen synthase kinase-3 β
HFD	High-fat diet
ICAM-1	Intercellular adhesion molecule-1
IKK	IκB kinase
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IR	Insulin receptor
IRS	Insulin receptor substrates
JNK	c-Jun N-terminal kinase
LPS	Lipopolysaccharides
LTP	Long-term potentiation
MAPK	Mitogen-activated protein kinases
MCP-1	Monocyte chemoattractant protein-1
MMP	Matrix metalloproteinases
mTOR	Mammalian target of rapamycin
NF-kB	Nuclear factor-κB
NO	Nitric oxide
PARP-1	Poly (ADP-Ribose) polymerase 1
PD	Parkinson's disease
PKB	Phosphatidylinositol 3-kinase/protein kinase B
PKC	Protein kinases C
PUFA	Polyunsaturated fatty acids
RAGE	Receptor for advanced glycation endproducts
ROS	Reactive oxygen species
SAT	Subcutaneous adipose tissue
SCFAs	Short-chain fatty acids
SOD	Superoxide dismutase
STD	Standard diet
TJ	Thight junction
TLR-4	Toll-like receptor 4

TNF-α	Tumor necrosis factor-α
VAT	Visceral adipose tissue
VCAM-1	Vascular cell adhesion molecule-1
WAT	White adipose tissue

PRELIMINARY CONSIDERATIONS

The modern nutritional transition towards a high fat, low-fiber diet (commonly known as the Western diet) negatively impacts human health and it significantly contributes to numerous non-communicable diseases and metabolic disorders, with obesity being foremost among them [Kopp, 2019].

Obesity develops because of a lack of balance between food intake and energy consumption. It represents a chronic complex condition characterized by excessive and abnormal body fat accumulation that can compromise human health. It increases the risk of developing type 2 diabetes and cardiovascular diseases, it affects bone and reproductive health, it alters the balance of the intestinal microbiota, and it increases the risk of certain types of cancer and neurodegenerative diseases. Additionally, obesity can affect the quality of life, including sleep and mobility [O'Brien et al., 2017; Patra et al., 2023].

The obesity comorbidities are numerous and can vary among individuals due to various genetic and environmental factors. However, at the core of obesity, as well as its associated comorbidities, lie two common pathophysiological events: oxidative stress and inflammation. Oxidative stress is the result of an imbalance between the production of free radicals and the body's ability to neutralize them. This imbalance may cause damage to cells and tissues and lead to a wide variety of chronic diseases. Inflammation, which is often chronic in obese individuals, contributes to cellular damage and it is crucial to the development of comorbidities such as insulin resistance, type 2 diabetes, cardiovascular diseases, cancer and neurodegenerative disorders [García-García et al., 2022; Feitosa et al., 2024].

The discovery that obesity can affect brain physiology enough to cause neurodegenerative disease development is relatively recent [Kueck et al., 2023]. Substantial evidence underscores the harmful impact of diets rich in saturated fats on brain health. In rodents, diet-induced obesity can potentially lead to memory disorders [Cordner & Tamashiro, 2015; Alzoubi et al., 2018] as well as depressive and anxiety-like behaviors [Kurhe et al., 2014; Zemdegs et al., 2016]. Concerning humans, some studies report that obesity is associated with reduced cognitive performance [Beydoun et al., 2008; Edwards et al., 2020], decreased brain volumes [Gunstad et al., 2008; García-García et al., 2022], increased probability of developing cognitive decline, including Alzheimer's disease (AD) [Cohen et al., 2010; Gunstad et al., 2010; Morys et al., 2023].

A broad range of strategies are recommended to reduce the increasing prevalence of obesity and its related co-morbidities, first of all a healthy lifestyle. This includes regular physical activity and healthful meals including intake of fruits and vegetables. Indeed, some biologically active natural compounds possess antioxidative and anti-inflammatory properties, helpful to contrast obesity and its related pathologies. To date, numerous phytochemicals, nutraceuticals and/or functional foods are considered protective and/or therapeutic against metabolic dysfunctions and related neurodegeneration [Singh et al., 2021; Piccialli et al., 2022]. For example, foods rich in antioxidants, micronutrients, phytochemicals, essential oils, probiotics and even algae have been found to be helpful in maintaining body weight and reducing the incidence of neurodegenerative diseases [Edwards et al., 2020; Onaolapo et al., 2022; Klose et al., 2022].

The research presented here deals with the effects of different foods or phytochemicals on obesity-related brain damage in the animal model. In particular, we used C57BL/6J male mice, that, when allowed *ad libitum* access to high-fat diet (HFD), progressively develop a pathology similar to human metabolic syndrome, including obesity, hyperglycaemia, insulin resistance, hepatic steatosis, systemic inflammation [Zhang et al., 2020], but also neuroinflammation and neurodegeneration [Nuzzo et al., 2015; Nuzzo et al., 2018; Nuzzo et al., 2020].

The animals were divided into separated groups, that were fed differently for a period, as it follows: A. Lean group (negative control group) fed with a standard diet (STD) (4RF25, Mucedola, Milan, Italy) consisting of 70% of energy as carbohydrates, 20% proteins and 10% fat. B. High-Fat Diet group (HFD) (positive control group) fed with HFD (PF4051/D, Mucedola, Milan, Italy) that supplied 60% of energy as fat, 20% proteins and 20% carbohydrates; C. Group to be examined, that were mice fed with an HFD supplemented with functional/novel food or phytochemicals (Diet composition details are given in Table1). The supplemented diets were custom-designed and prepared by Mucedola S.r.l., in such a way to be isocaloric to HFD. At the end of the experimental protocol stated, the animals were sacrificed, and the brains were rapidly explanted and properly stored for subsequent comparative molecular and histological analyses.

The results are presented in relation to the original published papers; therefore, methods are described within each chapter.

Table 1. Composition and energy densities of STD and HFD.

Ingredient (g/kg)	STD	HFD
Acid Casein 741	200	265
L-Cystine	2.8	4
Maltodextrine-0032	33.2	160
Sucrose	300	90
Cellulose (Arbocel)	50	65.5
Soybean Oil	25	30
Lard	19	220
Vitamin mix AIN-93-VX-PF2439	10	21
Mineral mix AIN-93G-MX-PF2348	45	48
Choline Bitartrate	1.9	3
Calcium Phosphate Dibasic	13	3.4
Total Energy, Kcal/g	3.5	6
Protein, %	20	20
Carbohydrate, %	70	20
Fat, %	10	60
Fatty Acids compositions (g/kg)		
total saturated	7.02	92.5
total monounsaturated	7.95	117.5
total polyunsaturated	20.41	40

Specifically, the first article examines whether long-term intake of honey and/or D-limonene, ingested separately or in combination, can counteract the HFD-induced neurodegeneration. Specifically, we analyzed the brain neurodegeneration, inflammation, oxidative stress, and the gene expression of AD markers.

The second article focuses on the neuroprotective potential of an extract of *Aphanizomenon flos-aquae* (AFA), a cyanobacterium considered a suitable supplement for its nutritional profile and beneficial properties. The extract is commercialized as KlamExtra®, including the two AFA extracts Klamin® and AphaMax®. Metabolic parameters, brain insulin resistance, expression of apoptosis biomarkers, modulation of astrocytes and microglia activation markers, and Aβ deposition were analyzed and compared among different groups of mice.

The third article concerns the impact of Indicaxanthin (Ind), a betalain abundant in *Opuntia ficus-indica* yellow fruit, with anti-oxidative and anti-inflammatory properties, on neuronal damage and gut microbiota dysbiosis induced by HFD. Fecal samples were used for DNA extraction and hypervariable V3-V4 regions of the 16S rRNA gene amplification.

All the described studies were performed in collaboration with the National Research Council of Italy (CNR), Institute for Biomedical Research and Innovation (IRIB), Palermo.

The introduction provides an overview of the effects of an HFD on brain health and the potential benefits of supplements in mitigating these effects.

PUBLICATIONS

The following publications are due to work performed during my PhD candidature and they represent the basis of this thesis:

Journal Articles

- Terzo S, **Calvi P**, Nuzzo D, Picone P, Allegra M, Mulè F, Amato A. Long-Term Ingestion of Sicilian Black Bee Chestnut Honey and/or D-Limonene Counteracts Brain Damage Induced by High Fat-Diet in Obese Mice. *International journal of molecular sciences.* 2023; 24(4):3467.
- Galizzi G, Deidda I, Amato A, **Calvi P**, Terzo S, Caruana L, Scoglio S, Mulè F, Di Carlo M. *Aphanizomenon flos-aquae* (AFA) Extract Prevents Neurodegeneration in the HFD Mouse Model by Modulating Astrocytes and Microglia Activation. *International journal of molecular sciences*. 2023; 24(5):4731.
- Terzo S, Amato A, **Calvi P**, Giardina M, Nuzzo D, Picone P, Palumbo-Piccionello A, Amata S, Giardina IC, Massaro A, Restivo I, Attanzio A, Tesoriere L, Allegra M, Mulè F. Positive impact of indicaxanthin from Opuntia ficus-indica fruit on high-fat diet-induced neuronal damage and gut microbiota dysbiosis. *Neural regeneration research*. 2024. doi: 10.4103/NRR.NRR-D-23-02039. Online ahead of print.

INTRODUCTION

It is well known that the brain needs a high amount of energy to work adequately. However, because of the energy minimal reserves, the brain heavily depends on the continuous supply of nutrients from the blood via the blood-brain barrier (BBB). Regularly, the brain uses glucose as a main fuel [Zhang et al., 2023b], but it can also utilize some alternative energy substrates such as ketone bodies, triglycerides and lactate during development or when the glucose availability is scarce [Bordone et al., 2019]. Recent evidence suggests that adequate dietary intake and nutritional status can affect markedly the neuronal function, neuronal signaling, cognitive capabilities and synaptic plasticity [Melzer et al., 2021]. Indeed, both clinical and preclinical studies have demostrated that consumption of saturated fats causes impaired cognitive performance [Fadó et al., 2022] and even short-term dietary intake of HFD is associated with impaired attention and visual memory in adult human subjects [González Olmo et al., 2023]. Notably, intake of a saturated fat-rich diet is widely considered as a driving factor for cognitive impairment and AD development [Amelianchik et al., 2022; Valentin-Escalera et al., 2024]. In contrast, diets rich in polyunsaturated fatty acids have been reported to promote cognitive health [Thangaleela et al., 2022]. It is therefore conceivable that the composition, abundance or lack of certain nutrients in the diet may differentially affect brain function.

1. The effects of HFD on the brain health

Traditionally, the consumption of saturated fat-rich diet has been linked to the development of metabolic and cardiovascular diseases, but increasing evidence indicates that patients with metabolic syndrome (including diabetes, obesity and hypertension) have an increased relative risk for cognitive decline, pathological brain aging and even AD [Biessels & Despa, 2018; Sun et al., 2020; Sharma, 2021].

The availability of suitable animal models is very critical to study the mechanisms linking metabolic syndrome to brain neurodegeneration. Considering the polygenic nature of metabolic diseases such as diabetes and obesity, the HFD feeding animal model seems helpful to study neuronal damage and neurodegeneration conditions associated with a wide range of disturbances such as obesity, insulin resistance, type 2 diabetes and dyslipidemia.

Numerous studies suggest that HFD not only causes neuronal mild disorders such as memory alterations, mood changes, anxiety, and depression [Fadò et al., 2022], but also it

contributes to neurodegenerative diseases like AD and Parkinson's disease (PD) [Boulos et al., 2019; Sánchez-Alegría & Arias, 2023].

Remarkably, in mice, HFD intake, just for one day, is enough to cause a rapid decline in episodic memory performance, suggesting that the negative impact of HFD on the brain can occur very quickly, impairing the ability to recall specific events and information [McLean et al., 2018]. However, the memory deficits can be rapidly reversed when mice switch from HFD to a normocaloric diet, suggesting that irreversible damage requires prolonged exposure.

Several evidence suggest that hypothalamic inflammation induced by HFD represents the initial stage of central nervous system (CNS) dysfunction in the context of HFD-induced cognitive impairment [Feng et al., 2017]. This phenomenon is initiated by peripheral inflammatory signals that, due to compromised BBB, reach the hypothalamus. Here, they induce gliosis, thereby triggering a detrimental process of inflammation and oxidative stress. Furthermore, saturated fatty acids can directly induce an inflammatory response in hypothalamic cells, by binding with the Toll-like receptor 4 (TLR4) receptor and activation of the Nuclear factor-κB (NF-kB) signaling cascade, which in turn induces the expression of inflammatory cytokines [Milanski et al., 2009]. Therefore, the TLR4 receptor is involved in detecting danger signals, including those from saturated fatty acids that are present in high concentrations during HFD [Chen & Trapp, 2016]. Microgliosis resulting from this leads to the activation of NF-κB and the production of increased amounts of inflammatory cytokines such as interleukin (IL)-1β, interleukin (IL)-6, and tumor necrosis factor-α (TNF-α) [Chen et al., 2018].

Astrocytes are activated by inflammatory mediators, and in turn contribute to the exacerbation of inflammation through the production of cytokines in the hypothalamus [Sewaybricker et al., 2023]. In addition, activated astrocytes secrete high levels of chemokines that attract both peripheral immune cells to the inflamed nervous system (e.g. T cells, monocytes) and resident CNS cells (microglia) to the injury sites. For example, the chemokine Monocyte Chemoattractant Protein-1 (MCP-1) also known as Chemokine (CC-motif) ligand 2 (CCL2) is upregulated by astrocytes at lesion sites, leading to the recruitment of inflamed monocytes into the CNS [Gutiérrez-Cuevas et al., 2024].

There has been shown that central inflammation extends beyond the hypothalamus following HFD feeding, subsequently affecting brain areas associated with cognition [Dionysopoulou et al., 2021]. In fact, monocytic infiltration involves other brain regions

such as the amygdala, prefrontal cortex, and hippocampus, expanding neuronal damage [Schneider et al., 2019; Gómez-Apo et al., 2021].

1.1 Potential mechanisms of HFD-induced neuronal dysfunction and cognitive deficits

The mechanisms and the signaling pathways affected by HFD are not completely known. There is evidence for the involvement of changes in energy metabolism leading to oxidative stress, reduced insulin sensitivity, increased ceramide production, neuroinflammation and reduced neuronal viability [Sánchez-Alegría & Arias, 2023].

It is largely accepted that white adipose tissue (WAT) is one of the main players responsible for the pathophysiological events associated with obesity, including neurodegenerative diseases. The WAT includes subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT), surrounding abdominal organs and is strictly associated with cardiometabolic risk [Reyes-Farias et al., 2021]. Indeed, WAT is not a simple inert tissue with a storage function [Sakers et al., 2022], but it is a dynamic tissue with complex endocrine functions, orchestrating energy metabolism and inflammatory processes [Longo et al., 2019]. It can synthesize and release cytokines and adipokines that exert profound effects on metabolism, homeostasis, and inflammation [Hotamisligil, 2017].

Adipokines play multifaceted roles in metabolic and physiological pathways, influencing insulin signaling, glucose uptake, fatty acid oxidation, and other energy-related processes [Pestel et al., 2023]. On the other hand, cytokines regulate the inflammatory response and play a key role in both the initiation and resolution of inflammation, as well as in adaptive and reparative angiogenesis [Hotamisligil, 2017]. The dysregulation of adipose tissue function and secretome, often observed in obesity, disrupts the delicate balance of energy regulation and immune response modulation. Chronic expansion of WAT triggers a cascade of events, including hypoxia-induced stress and the release of damage-associated molecular patterns, which recruit immune cells and perpetuate a pro-inflammatory environment [Zatterale et al., 2020]. Therefore, obesity induces metabolic dysfunctions and chronic low-grade systemic inflammation, affecting the entire body including the CNS [Więckowska-Gacek et al., 2021].

Additionally, other HFD-related consequences, such as insulin and leptin resistance, mitochondrial dysfunction, and oxidative stress, may accelerate the aging process (Figure 1). All these factors highlight the multifaceted impact of HFD on brain health, increasing susceptibility to neurological disorders and cognitive decline.

Recently, numerous investigations have suggested that cognitive decline induced by HFD feeding is associated with alteration of abundance, diversity, and composition of gut

microbiota [Shi et al., 2020; Qu et al., 2023; Wang et al., 2023], that, in turn has been found to be associated with neuro-inflammation impairment [Shi et al., 2020a; Shabbir et al., 2021]. Thus, it is likely that HFD feeding could be responsible for neuronal dysfunction and cognitive decline through various mechanisms.

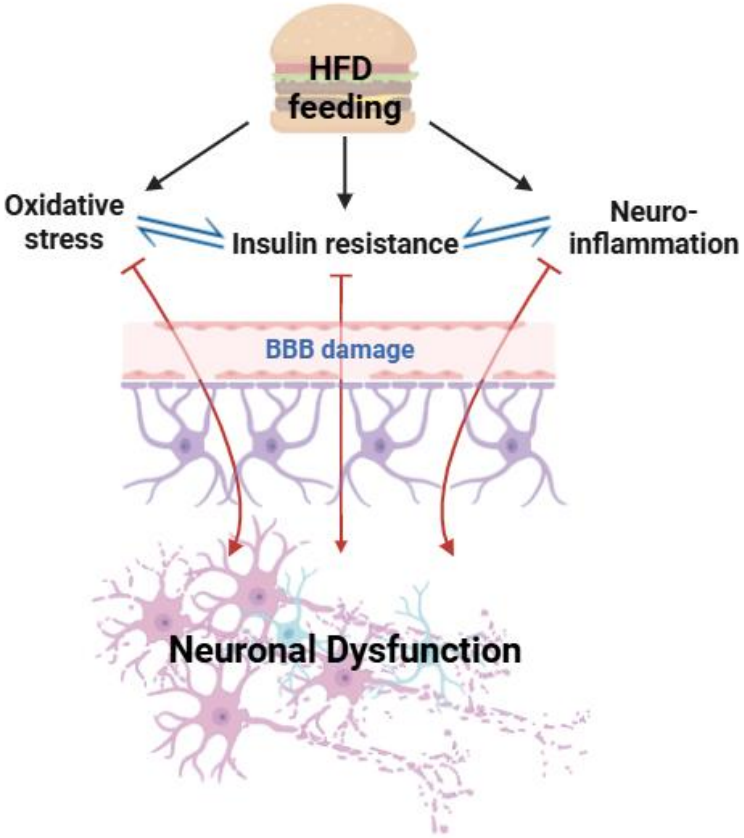

Figure 1 *High-Fat Diet mechanisms causing neurodegeneration.*

1.1.1 Systemic inflammation and neuroinflammation

The metabolic inflammation related to HFD, and obesity differs from the typical immune response, presenting as a persistent and relatively mild condition that fails to resolve, termed "chronic low-grade inflammation" [Khanna et al., 2022].

Studies on both animal models and humans have confirmed the association between increased adiposity and adipose tissue inflammation, caused by excessive caloric intake [Cavaliere et al., 2018; Hammarstedt et al., 2018]. Chronic fat intake leads to an expansion of adipose tissue, characterized by hyperplasia and hypertrophy of adipocytes. While hyperplasia seems to be tolerated well by the tissue, hypertrophy induces cellular stress, resulting in numerous molecular changes causing cellular dysfunction and ultimately leading to apoptosis [Cotillard et al., 2014]. Dysfunctional hypertrophic adipocytes produce an abnormal amount of adipokines stimulating chemotaxis to recruit local or circulating

macrophages [Zatterale et al., 2020]. The recruited macrophages then phagocytize apoptotic or dead adipocytes, forming "crown-like structures" (CLS), which are characteristic of inflamed adipose tissue [Zatterale et al., 2020].

Adipose tissue macrophages can be divided into two main phenotypes: M1 macrophages and M2 macrophages. The former produce pro-inflammatory cytokines and molecules such as IL-1 β, IL-6, TNFα, inducible nitric oxide synthase (iNOS), MCP-1 and inhibitor of NF-kB kinase beta (IKK β) [Artemniak-Wojtowicz et al., 2020]. On the contrary, the latter are involved in the maintenance of tissue homeostasis, and they are typical in the adipose tissue of lean individuals. Various studies have shown that the macrophages are more abundant in VAT compared to SAT in an HFD-induced obesity condition [Camastra & Ferrannini, 2022]. As a result, VAT shows increased secretion of inflammatory markers such as TNFα, IL-1 β, IL-6, and C-reactive protein (CRP), as well as increased macrophage and T-lymphocyte infiltration. In addition, visceral fat cells have a higher propensity for lipolysis, leading to elevated systemic levels of free fatty acids (FFAs), which further exacerbate inflammation. They are also more susceptible to macrophage infiltration, resulting in the release of pro-inflammatory cytokines [Reyes-Farias et al., 2021].

Furthermore, elevated levels of dietary saturated fatty acids and disturbances in lipid metabolism associated with dysfunctional adipose tissue have been shown to increase the expression of pro-inflammatory factors such as IL-6 and TNFα through the toll-like receptor (TLR) [Jiao et al., 2009]. This increased exposure of adipocytes to stressors, including oxidative stress, inflammatory cytokines, and lipid imbalance, triggers the upregulation of pro-inflammatory genes via the activation of transcription factors such as activator protein-1 (AP-1) and NF-kB [Balan et al., 2024]. These processes are mediated through various cellular kinase pathways, including mitogen-activated protein kinases (MAPK), IKK β, mammalian target of rapamycin (mTOR), and several protein kinases C (PKC). The persistent inflammatory state induced by HFD can extend to the CNS, contributing significantly to neurodegeneration and cognitive impairment [Margină et al., 2020].

Physiologically, the internal environment of the CNS is immunosuppressive, preventing excessive leukocyte entry into neural spaces. This property is largely facilitated by the function of the BBB in cooperation with the immune cells resident in the CNS, microglia cells (Figure 2) [O'Brown et al., 2018].

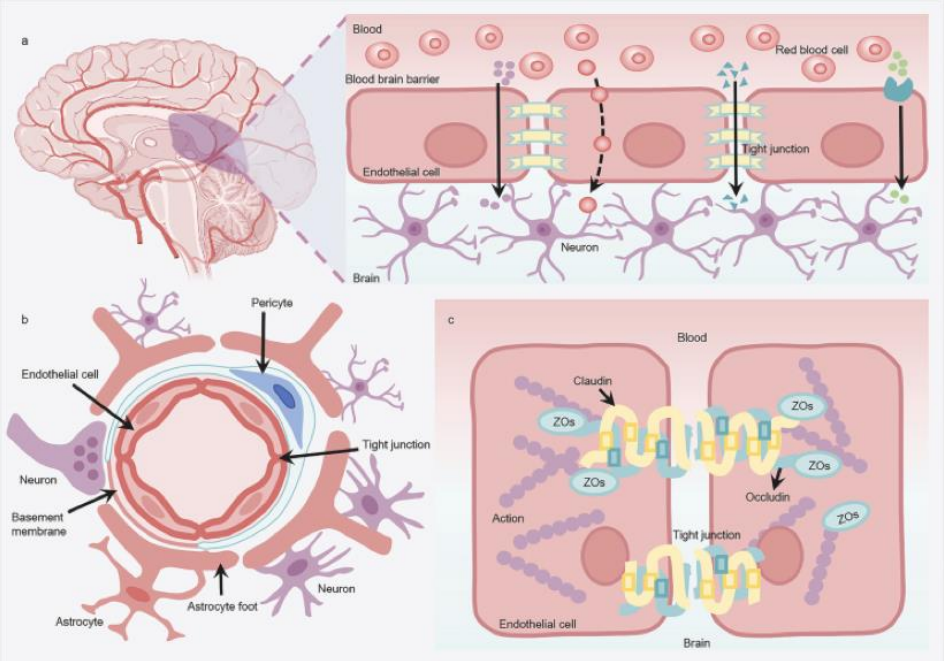

Figure 2 *BBB physiological structure.*

The chronic low-grade inflammation, present in obesity conditions, increases the permeability of the BBB, leading to leukocyte extravasation, solute diffusion, and entry of pathogens and toxins into the CNS. This further stimulates inflammatory responses in a vicious cycle. Therefore, impairment of BBB can represent a mechanism involved in the neurodegeneration caused by HFD [de Paula et al., 2021]. The extravasation of leukocytes across the vascular endothelium is a process that involves interaction between adhesion molecules on the leukocyte and endothelial cells. The leukocyte adhesion molecules expressed by endothelial cells include P-selectin, E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) [Rutledge & Muller, 2020]. The inflammation upregulates VCAM-1 and ICAM-1 [Mussbacher et al., 2022], as well as P- and E-selectin [Perkins et al., 2019], responsible for increased leukocyte extravasation. Migration of the activated leukocyte into the perivascular space occurs through a paracellular pathway and less commonly through a transcellular pathway [Rutledge & Muller, 2020]. The penetration of leukocytes across the basement membrane is facilitated by matrix metalloproteinases (MMP), such as MMP-9, which degrade the membrane filaments [Rashid & Bardaweel, 2023].

Inflammation activates many molecular pathways and changes gene expression in different ways. For example, NF-kB is a transcription factor that is activated by elevated levels of pro-inflammatory cytokines such as TNF-α and IL-1β [Msweli et al., 2024].

BBB integrity is also affected by inflammation-induced gene dysregulation. Tight junction (TJ) proteins, including claudin-5, ZO-1, and occludin, are down-regulated [Varatharaj & Galea, 2017; Huang et al., 2021].

In addition, the astrocytes, cells contributing to BBB, undergo structural changes including disruption of the end feet, that affect the BBB integrity. Astrocyte gene expression is shifted towards a pro-inflammatory and cytotoxic profile, resulting in increased production of IL-1β, IL-6, TNF-α, and prostaglandins [Ahadiat & Hosseinian, 2023]. TNF-α and IL-1β up-regulate CXCL1 and CCL2, chemokines involved in recruiting immune cells.

It is noteworthy that the microglia is activated by NOD-like receptor pyrin domain-containing protein 3 (NLRP3), a protein of the inflammasome secreted by dysregulated visceral adipose tissue [Guo et al., 2020].

Leptin is an adipokine, primarily produced by adipocytes, that is known to play an important role in brain inflammation associated with HFD and obesity. However, there is still much to be learned about its exact role and impact on neuroinflammatory processes and related disease. Leptin is a hormone whose levels are directly proportional to the amount of adipose tissue, and which acts primarily on the hypothalamus to increase satiety. Nevertheless, in obese individuals, the plasma hormone concentration is elevated and persistent, leading to leptin resistance due to receptor saturation and subsequent disruption of the satiety signal [Obradovic et al., 2021].

There are conflicting results in the scientific literature regarding the effect of leptin on the neurodegenerative process. *In vitro*, astrocytes and microglia have been shown to express the receptor for leptin and their activation causes pro-inflammatory responses [Tang et al., 2007; Shinjyo & Kita, 2021]. In contrast, recent studies have shown that treatment with leptin can reduce inflammation in the brain in models of neurodegenerative diseases. In AD mice, leptin reduced the expression of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α and decreased the number of Iba1-positive microglial cells in the hippocampus [Ma et al., 2023]. Other studies have shown that leptin promotes neuroprotective microglial phenotypes and reduces inflammatory responses. It also reduced apoptosis and local inflammation in a brain ischaemia-reperfusion injury model [Ma et al., 2023].
Furthermore, in myeloid cell-specific leptin receptor (LepR)-deficient mice, microglia showed less branching and reduced phagocytic capacity. This suggests that leptin may play a direct role in maintaining the homeostatic function of microglia, which is essential for the health of the CNS [Davis et al., 2017].

1.1.2 HFD, oxidative stress and neuronal damage

Oxidative stress is a condition characterized by an imbalance between the production of reactive oxygen species (ROS) and the body's ability to detoxify the reactive intermediates or to repair the resulting damages. The imbalance can lead to cellular and tissue damage, and it is implicated in the aging process and various diseases, including cancer, cardiovascular diseases, and neurodegenerative disorders. The presence of ROS excess can damage DNA, proteins, and lipids, disrupting normal cellular function and integrity [Korovesis et al., 2023] (Figure 3).

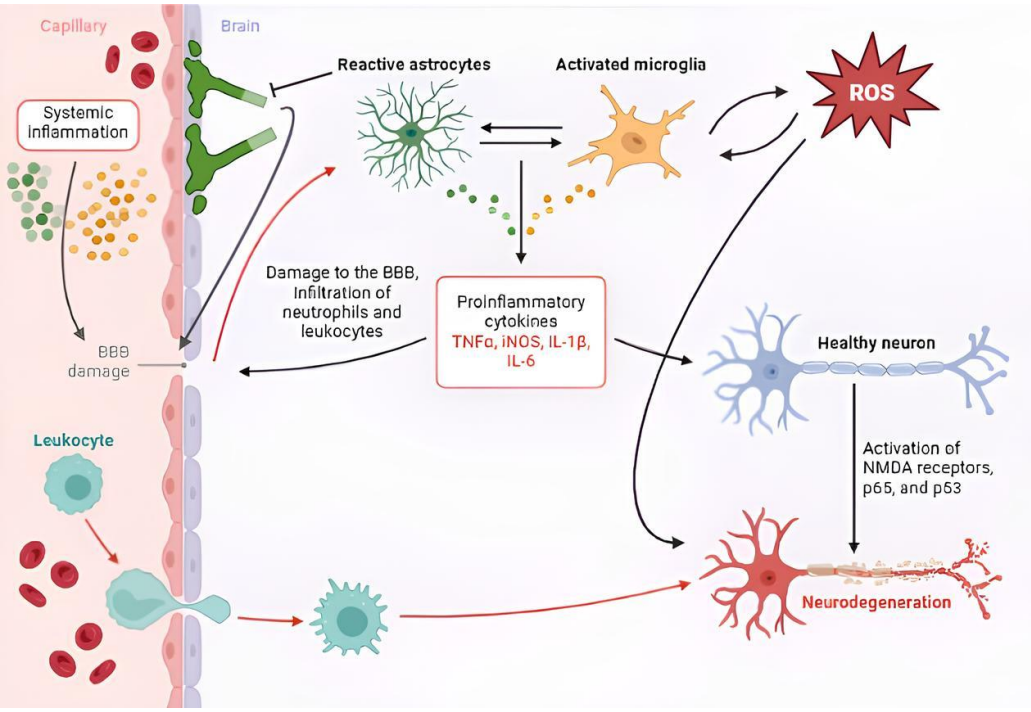

Figure 3 *Oxidative Stress in the CNS leading to different neurodegenerative pathologies.*

HFD leads to oxidative stress through several interconnected mechanisms.

As previously mentioned, the low-grade inflammatory state induced by chronic ingestion of HFD increases oxidative stress. In fact, the activation of NF-κB stimulates immune cells such as macrophages and neutrophils to produce ROS as part of their defense mechanisms. In addition, also the pro-inflammatory cytokines (interleukins and TNF-α) play a significant role in promoting ROS production during chronic inflammation [Masenga et al., 2023]. The vulnerability to oxidative damage is particularly high in obese individuals because of the depletion of antioxidant reserves. In fact, the activity of enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) undergoes

a drastic reduction in obese subjects [Naomi et al., 2023]. Also, serum levels of vitamins A, E, and C, β-carotene, and glutathione decrease drastically [Pérez-Torres et al., 2021].

Continuous consumption of an HFD results in a notable rise in FFAs [Dalvi et al., 2017]. FFAs are transported into cells and utilized for energy production through β-oxidation within the mitochondria. During β-oxidation, FFAs are broken down into Acetyl-CoA molecules, generating byproducts like Nicotinamide Adenine Dinucleotide (NADH) and Flavin adenine dinucleotide (FADH2). These molecules carry extra electrons that need to be oxidized to sustain the β-oxidation process. The electrons are transferred along the electron transport chain (ETC) to oxygen molecules, resulting in the production of water and ATP. However, when there is an excess of nutrients or increased metabolic activity, more electrons enter the ETC than they can efficiently handle. This overload increases the likelihood of electrons "leaking" from the chain. These leaked electrons can interact with oxygen within the mitochondria, leading to the formation of ROS such as the superoxide radical (O2•−). As β-oxidation continues to increase and the ETC becomes overloaded, rapidly ROS production increases [Zhao et al., 2023].

The ROS can react with unsaturated lipids in mitochondrial and cellular membranes, causing lipid peroxidation. Lipid peroxidation is a harmful process that compromises the integrity of cellular and mitochondrial membranes, and macromolecules like DNA and proteins, affecting their functionality and contributing to a self-perpetuating cycle of oxidative stress and cellular damage resulting in cell death [Valgimigli, 2023].

The detrimental effects of oxidative stress affect every cell, and they impair the function of all organs, but the brain suffers particularly severe consequences for several reasons. The brain is a highly metabolic aerobic organ, consuming about 20% of the body's energy [Ardanaz et al., 2022]. The high level of oxygen consumption can potentially lead to an elevated risk of generating ROS. The brain cells are incapable of regeneration and contain high concentrations of omega-3 polyunsaturated fatty acids (PUFAs), which are particularly susceptible to peroxidation [Galecki et al., 2015; Aoyama, 2021].

Brain tissue contains elevated levels of redox-active iron and copper, which heighten its sensibility to oxidative stress. [Lee et al., 2020]. Moreover, the brain is particularly susceptible to disturbances in redox balance due to its comparatively weaker antioxidant defense system compared to other tissues [Lee et al., 2020].

The brain's susceptibility to oxidative damage is also influenced by the activity of microglia. During normal phagocytic activity, microglia generate superoxide (O_2^-) and other reactive species. The production of O_2^- is primarily mediated by the NADPH oxidase 2

(NOX-2), an enzyme of active microglia. Consequently, microglial activity is closely linked to oxygen availability. Reactive species such as hydrogen peroxide and nitric oxide (NO) are released, and they activate microglia, promoting local inflammation and progression of neurodegenerative processes. Thus, while essential for brain maintenance, excessive microglial activation and the resulting release of reactive species can lead to deleterious effects, including synaptic dysfunction and neurodegeneration [Kim et al., 2020].

Finally, poly-ADP-ribose polymerase (PARP-1), a DNA repair enzyme, works by using NAD^+ to attach ADP-ribose molecules to nuclear proteins, thereby helping to repair DNA damage. However, excessive activation of PARP-1 can led to the depletion of NAD^+, which is essential for energy production and cellular metabolism. This depletion not only hinders energy production but also triggers the opening of transient receptor potential melastatin 2 (TRPM2) Ca^{2+} channels, leading to neuronal cell death [Sriram et al., 2016].

Similarly, to chronic inflammation, oxidative stress damages cells and structures of the BBB, including brain microvascular endothelial cells (BECs), pericytes, astrocytes, and the basement membrane [Kim et al., 2022]. Indeed, oxidative stress leads to the release of numerous pro-inflammatory cytokines that contribute to the degradation of the extracellular matrix and direct breakdown of proteins involved in TJs. In fact, ROS can induce cytoskeletal reorganization, disrupting the proper positioning of TJ proteins [Banks & Rhea, 2021]. Moreover, increased oxidative stress influences the gene expression of these proteins and it modulates the function of ATP-binding cassette (ABC) transporters [Sita et al., 2017]. These transporters, predominantly located on BECs, play a crucial role in expelling toxic metabolites from the BBB [Grewal et al., 2017]. Dysfunction of ABC transporters lead to the accumulation of these metabolites, exacerbating BBB damage.

Finally, MMPs, particularly MMP-2 and MMP-9, become activated or upregulated in response to oxidative stress, significantly contributing to BBB degradation. MMPs are enzymes that degrade extracellular matrix proteins and cell surface receptors. MMP-2 and MMP-9 are specifically associated with increased barrier permeability following oxidative damage [Turner & Sharp, 2016].

BECs are particularly susceptible to oxidative damage because of several factors, and their impairment is closely linked to AD and PD [Parodi-Rullán et al., 2021]. BEC cells must transport high amounts of glucose into the brain to produce energy. Glucose is metabolized in the mitochondria, where it is used to generate energy while simultaneously producing oxygen-free radicals. In addition, BECs produce high levels of NO through the enzyme endothelial nitric oxide synthase (eNOS). eNOS is essential for intracellular signalling and

regulation of vascular tone [An et al., 2021]. Besides glucose, BECs must transport dietary lipids and fatty acids into the brain, serving as alternative sources of energy and signaling molecules. This process increases the risk of lipid peroxidation [Rizzo et al., 2014]. Lastly, BECs contain a higher density of mitochondria compared to peripheral endothelial cells, occupying between 8 and 11% of the cytoplasmic volume. These organelles constitute the primary source of ROS [Banks & Rhea, 2021].

Therefore, the oxidative stress affects the integrity of BBB, and it acts as a self-propagating phenomenon that results in a dangerous accumulation of ROS within the CNS. These ROS can cause significant and often irreversible damage to nerve cells.

1.1.3 HFD, Insulin Resistance and Cognitive Decline

Insulin resistance, a common complication of obesity that contributes to metabolic syndrome, can develop in response to chronic inflammation and oxidative stress induced by HFD. Once established, insulin resistance, oxidative stress, and inflammation can interact synergistically, amplifying their negative effects both systemically and within the CNS. This interaction can exacerbate the metabolic, neurological, and vascular conditions associated with metabolic syndrome, increasing the risk of cardiovascular disease, type 2 diabetes, and other chronic complications [Pugazhenthi et al., 2017].

Insulin is a peptide hormone secreted by the β-cells of the islets of Langerhans in the pancreas. For a long period after its discovery, insulin was considered just a peripheral hormone that regulates glucose metabolism, food intake, and body weight but it did not affect the brain. Subsequently, insulin has been considered also vital for brain health because it plays different roles ranging from enhancing hippocampal long-term potentiation [Barutçu et al., 2023], modulation of synaptic plasticity [Zhao et al., 2019], learning and memory [Kullmann et al., 2017]. In particular, it regulates structural plasticity in the brain, by inducing expression of PSD95, a scaffold protein required for the formation of the postsynaptic junctions [Bramham & Messaoudi, 2005; Lee et al., 2005]. Insulin can cross the BBB via BECs through a selective, saturable, and receptor-dependent mechanism [Banks et al., 1997]. Numerous studies have shown that this transport is influenced by obesity, diabetes as well HFD [Gray et al., 2017].

Thus, alterations in insulin signaling, especially during insulin resistance can severely impact neuronal activity and result in neurodegeneration.

In the brain, insulin binds to its receptor in the isoform A, known as insulin receptor A (IR-A) [Sędzikowska & Szablewski, 2021]. Most of the IR-A receptors are located on neurons, specifically at the axon terminals and the postsynaptic zones [Arnold et al.,

2018]. Receptor activation triggers several signaling cascades that play key roles in brain functions. For example, the PI3-K/AKT pathway (phosphatidylinositol 3-kinase/protein kinase B, PKB) is responsible for metabolism, and lipid and protein synthesis. Additionally, the MAPK pathway (mitogen-activated protein kinase) influences cell growth, survival, and gene expression [Kleinridders et al., 2014].

Studies in animal models have identified IR elevated density in specific brain regions such as the hippocampus, and frontal cortex, areas known for their involvement in learning and memory processes [Grillo et al., 2015], suggesting a role in synaptic plasticity and remodeling [Kullmann et al., 2017]. In addition, insulin administration to mice improves object memory. Also in humans, chronic stimulation of the brain by intranasal administration of insulin has been shown to improve memory [Benedict et al., 2004].

As mentioned earlier, IR-A receptors are highly expressed in neuronal presynaptic and postsynaptic zones. Insulin promotes neurogenesis and cell growth through the activation of AKT and MAPK signalling cascades [Cui et al., 2010]. Insulin plays an important role in the maintenance of excitatory synapses and in promoting dendritic spine formation [Zhao et al., 2019; Seyer et al., 2020]. Furthermore, through AKT pathway activation, insulin promotes cell survival by inhibiting apoptosis [Spielman et al., 2015].

IR are not confined to neurons but are also present in glial cells, specifically astrocytes. When activated, the AKT and MAPK signalling pathways within astrocytes, play a critical role in regulating the secretion of inflammatory cytokines in response to inflammatory stimuli, providing evidence for insulin impact of insulin on inflammatory responses mediated by glial cells in the brain [Sędzikowska & Szablewski, 2021].

High concentrations of ROS can impair insulin-triggered signaling cascades in target cells by activating stress-sensitive enzymes like IKK-β. This enzyme phosphorylates multiple targets including IR, insulin receptor substrates (IRS)-1, and IRS-2, resulting in downstream negative effects such as reduced PI3K activation [Yaribeygi et al., 2020]. Additionally, oxidative stress inhibits the insulin-degrading enzyme (IDE), biliverdin reductase-A (BVR-A), AKT, IRS-1, and GSK-3 enzymes [Balbaa et al., 2017; Barone et al., 2021].

In addition, the low-grade inflammatory state promotes an increase in systemic TNF-α levels, which in turn stimulates the activity of proteins, such as IKK, p38 MAPK, c-Jun N-terminal kinase (JNK), and PKC, that interfere with the functionality of the IR by directly targeting serine residues on the IRS. TNF-α induces dephosphorylation of phosphotyrosine residues on the IR and IRS proteins. Elevated levels of IL-6 activate the JAK-STAT

signaling pathways, resulting in increased expression of SOCS1 and SOCS3 proteins. These proteins then sterically inhibit the interaction between IR and IRS. In addition, IL-1β induces the activation of the protein p38 MAPK within cells, leading to the phosphorylation of serine residues on IRS1 and IRS2 proteins, which are crucial for insulin signal transmission. This type of phosphorylation disrupts or reduces the efficiency of the insulin signalling pathway, thereby contributing to insulin resistance [Yang et al., 2020].

From the insulin signaling standpoint, Glycogen synthase kinase-3 β (GSK-3β) is an important player in mediating HFD-induced complications linking insulin resistance and cognitive decline. Feeding HFD to experimental animals has been shown to increase the expression of GSK3β and its association with cognitive decline [Elahi et al., 2021]. Alternatively, several researchers have highlighted that GSK3β inhibitors could reverse cognitive decline induced by HFD feeding [Datusalia & Sharma, 2014; Sathiya Priya et al., 2019].

1.1.4 HFD and brain ceramide accumulation

Accumulation of ceramide in the brain is one of the mechanisms contributing to the development of central insulin resistance in HFD animal models. Ceramide is a lipid that can accumulate intracellularly in response to various stimuli, including high levels of free fatty acids derived from HFD. This accumulation occurs primarily in adipose tissues, skeletal muscle, and the brain [Pan et al., 2024].

Ceramide is a compound composed of the amino alcohol sphingosine and a long-chain saturated fatty acid (C:16-C:32). It belongs to a group of biologically active sphingolipids that form the cell membranes of neurons and glial. Moreover, in physiological conditions, this fatty acid plays a crucial role in the growth, differentiation, proliferation, and aging of the brain cells [Kagan et al., 2022]. Notably, hepatic synthesis of ceramide increases following HFD intake [Xiao et al., 2023]. Once ceramide accumulates in the brain, it can interfere with insulin signaling at multiple levels [Pan et al., 2024].

Brain insulin resistance has been shown to be associated with ceramide-mediated IRS-1 phosphorylation, which inhibits PI3K-Akt signalling. Ceramide activates serine-threonine kinases such as JNK and IKK, which phosphorylate serine on IRS-1, thereby blocking IR function. In addition, ceramide reduces Akt phosphorylation through induction of protein phosphatase 2A (PP2A) and activates interleukin-1β-converting enzyme (ICE)-like proteases. As a result, insulin signalling is disrupted and neuronal apoptosis is promoted, exacerbating conditions such as cognitive decline and neurodegenerative diseases [Maciejczyk et al., 2019].

The negative modulation of the insulin signaling pathways by ceramide can lead to reduced insulin responsiveness in the brain, thereby contributing to the development of insulin resistance. Furthermore, ceramide has been shown to impair the vitality of brain cells, energy metabolism, and mitochondrial activity, thereby exacerbating neurocognitive deficits in insulin-resistance patients [Liu et al., 2015]. Moreover, ceramide down-regulates the anti-apoptotic Bcl-2 and inhibits protein kinases B (PKB) and C (PKC-α) [Maciejczyk et al., 2019].

In summary, the accumulation of ceramide in the brain following HFD is a key mechanism that promotes central insulin resistance. It negatively influences insulin signaling transmission and compromises the metabolic response of brain cells to insulin effects, as well as cellular vitality.

1.1.5 Mitochondrial Dysfunction

Mitochondrial dysfunction plays a key role in the neuronal damage induced by HFD and central insulin resistance [Crispino et al., 2020]. In neurons, synapses-located mitochondria are crucial for the proper functioning of the synaptic process, supporting the exocytosis and reuptake of neurotransmitters, as well as the proper functioning of receptors and ion channels. They are also involved in synthesizing proteins required for synaptic plasticity [Todorova & Blokland, 2017; Rangaraju et al., 2019].

As before mentioned, oxidative stress leads to neurotoxic effects in the brain by generating large quantities of reactive species that irreversibly damage biological macromolecules [Korovesis et al., 2023]. Insulin resistance itself promotes oxidative stress through dysregulation of metabolism, hyperactivation of GSK-3β, impairment of anti-apoptotic signaling, and mitochondrial function [De la Monte & Tong, 2014]. Oxidative stress and hyperglycemia resulting from insulin resistance contribute significantly to mitochondrial dysfunction. In fact, the neurons are mitochondria-rich cells, because of their aerobic metabolism and high energy demands [Liyanagamage & Martinus, 2020]. Consequently, dysfunction of these organelles leads to increased levels of neurotoxic glutamate, mitochondrial phagocytosis, and subsequent neuronal death. Mitochondrial dysfunction also results in the release of damage-associated molecular patterns (DAMPs), which are recognized by microglial cells through pattern recognition receptors (PRRs). Activation of PRRs triggers the TLR/NF-κB inflammatory pathway and promotes the release of pro-inflammatory cytokines and chemokines. The neuroinflammatory process initiated by DAMPs, in turn, exacerbates mitochondrial dysfunction and increases ROS production, thereby exacerbating the neurodegenerative process [Mishra et al., 2021].

HFD-induced mitochondrial dysfunction and neuroinflammation are pathological events that are also characteristic of AD. In fact, the chronic inflammation induced by HFD can contribute to the pathogenesis of AD, accelerating the deposition of β-amyloid plaques and the formation of neurofibrillary tangles [Sah et al., 2017].

Hyperglycemia also contributes directly to neuronal damage. In fact, elevated glucose levels lead to the formation of advanced glycation end products (AGEs). These compounds bind to their receptor called RAGE, which are expressed in cerebral neurons, microglia, astrocytes and endothelial cells [Verdile et al., 2015]. RAGE receptor activation results in up-regulation of the NF-κB factor, thereby contributing to neuroinflammation, oxidative stress, and neuronal death [Haspula & Clark, 2018].

1.1.6 HFD, transcriptional dysregulation and synaptic plasticity

Brain-derived neurotrophic factor (BDNF) has been object of extensive investigation in relation to HFD feeding. BDNF is a neurotrophin that plays an important role in regulating the survival, growth and differentiation of central and peripheral nervous systems. It has also been shown to be fundamental to neuronal plasticity and it can induce long-term potentiation and thus it has been involved in learning and memory [Leal et al., 2015]. BDNF exerts its effects on synaptic plasticity through several mechanisms. First, BDNF activates synapsin I, a protein essential for synaptic vesicle function that facilitates neurotransmitter exocytosis and maintains synaptic contacts. In addition, BDNF modulates the activity of growth-associated protein 43 (GAP-43) and cAMP response element-binding protein (CREB), both of which are critical for neuronal growth and synaptic plasticity. Once activated, CREB is well-reported to regulate the expression of proteins associated with learning and memory. Furthermore, BDNF supports neurotransmitter release, promotes axon formation and growth, and it helps in maintaining presynaptic structure by phosphorylating synapsin 1 [Kowiański et al., 2018].

HFD negatively affects BDNF levels in the brain. BDNF-reduced expression, as found in AD brains, has been linked to cognitive decline [Gao et al., 2022]. Moreover, a reduction in circulating levels of BDNF has been also found in diabetes and obesity conditions [Liu et al., 2016b; Celik Guzel et al., 2014]. Various preclinical studies have highlighted that HFD intake is associated with decreased expression of BDNF in the brain [Azman & Zakaria, 2022; Yi et al., 2024], particularly in the hippocampus [Kanoski et al., 2007; Stranahan et al., 2008].

It is likely that the negative effects of HFD intake on learning and memory may also be mediated in part by alteration of BDNF-related synaptic plasticity [Cavaliere et al., 2019; Zhao et al., 2020]. Various studies have demonstrated that HFD diet intake is associated with reduced CREB activity in the brain [Jeong et al., 2019; Spinelli et al., 2020; Bayazid et al., 2022]. Thus, probably the reduction in BDNF level and cognitive deficits observed as a result of HFD feeding could in turn be a result of reduced CREB activity.

Recent evidence suggests that in addition to BDNF, HFD also affects the expression levels of several non-coding RNAs (ncRNAs) in mice [Yoon et al., 2019]. ncRNAs are widely expressed in the brain. Together with various mRNAs, they play a crucial role in regulating synaptic plasticity [Subhramanyam & Hu, 2017] and memory formation [Smalheiser, 2014]. In addition, transcriptomic analysis of the cerebral cortex of HFD-fed mice showed a profound change in gene expression of genes associated with synaptogenesis and neurotransmitter release [Yoon et al., 2019].

1.1.7 HFD and dysbiosis

The deleterious effects of HFD also extend to the composition of the gut microbiota. Indeed, the overall effects of HFD are detrimental to microbial abundance and promote increased intestinal permeability [Jia et al., 2023].

HFD just after two days can alter the microbial phylum composition in mice. A longer period of 12 days induces significant dysbiosis, characterized by an imbalance in the gut microbial community [Ke et al., 2021]. Specifically, HFD decreases *Bacteroides*, bacteria mediating generally beneficial host functions, and it increases *Firmicutes*, bacteria with detrimental functions. In line with these results, other studies in animal models and humans have shown an increase in the ratio of *Firmicutes* to *Bacteroides* after HFD feeding [Magne et al., 2020]. In particular, Jo and colleagues have reported that the HFD increased the genera *Blautia*, *Bilophila*, *Enterorhabdus*, *Erysipelatoclostridium*, and *Oscillibacter*, bacteria that are positively correlated with chronic diseases such as obesity [Jo et al., 2021]. Moreover, mice lacking gut microbiota (germ-free) fed HFD are less susceptible to the damage caused by HFD on glucose metabolism, suggesting that the gut microbiota may play a role in the onset and development of type 2 diabetes [Rabot et al., 2010].

Dysbiosis caused by HFD results also in the disruption of intestinal barrier [Vancamelbeke & Vermeire, 2017]. This effect appears to be mediated by a reduced abundance of bacteria that promote the health and integrity of the intestinal barrier, such as *Akkermansia muciniphila, Bifidobacterium spp., Bacteroidetes spp., Lactobacillus spp.* and

Clostridiales spp., and increasing those that have the opposite effect, such as *Oscillibacter spp* and *Desulfovibrio spp*. [Malesza et al., 2021]. Specifically, some bacteria affect the integrity of the gut barrier by regulating TJ proteins. For example, *Bifidobacterium spp*. and *Lactobacillus spp.*, which tend to decrease following HFD, are associated with stimulating gene expression TJ proteins in enterocytes, such as cingulin, occludin (OCLN), tight junction protein 1 (TJP1) and tight junction protein 2 (TJP2) [Malesza et al., 2021]. *Akkermansia muciniphila*, on the other hand, promotes TJP1 and Occludin gene expression and it counteracts HFD-induced thinning of the intestinal mucosa [Wu et al., 2017].

The existence of a strong link between dysbiosis and various pathological conditions in the host has been confirmed by modern research [Rutsch et al., 2020]. Among these, the most recent evidence shows that there is a strong correlation between the health of the CNS and the gut microbiota [Cryan et al., 2019]. This bidirectional connection between the gut and the brain is known as the gut-brain axis (GBA).

An increase in pro-inflammatory bacteria, such as *Escherichia* and *Shigella*, and a decrease in anti-inflammatory bacteria, such as *Eubacterium rectale*, have been observed in patients with cognitive impairment and cerebral amyloidosis. These changes are associated with increased levels of IL-1β, CXCL2, and NLRP3 [Cattaneo et al., 2017]. There are many other ways in which gut bacteria affect cognitive function. They can alter the levels of certain neurotransmitters and affect synaptic plasticity by interfering with the function of certain proteins and receptors, such as NMDA, BDNF, and serotonin receptors [Shabbir et al., 2021].

The vagus nerve represents a link between the gut and CNS, and it is involved in signalling pathways that regulate satiety, stress and mood. Vagal nerve pathways, located beneath the intestinal epithelium, can be activated by gut microbes or their metabolites by TLR2, TLR3, TLR 4, and TLR 7 on the nerve endings. Bacterial metabolites include short-chain fatty acids (SCFAs) and lipopolysaccharides (LPS), which stimulate vagal nerve endings, resulting in opposite end effects [Han et al., 2022].

Another pathway linking the microbiota to the CNS is the bloodstream. In fact, bacterial metabolites can move through the bloodstream, they cross the BBB, and they can act as neuroprotective or detrimental compounds in the CNS.

At the neuronal level, SCFAs, by-products of fiber fermentation, can stimulate the production of neurotrophic factors such as nerve growth factor (NGF), BDNF and glial cell line-derived neurotrophic factor (GDNF). They can also stimulate the synthesis of neurotransmitters such as glutamate, glutamine and GABA [Silva et al., 2020]. Therefore,

these metabolites can regulate the growth, survival, differentiation and excitability of neurons and synapses in the CNS with consequences for learning, memory, stress management and mood. In addition, SCFAs, particularly butyrate, have been shown to improve the integrity of the BBB through an increase in occludin expression [Li et al., 2016]. Butyrate and, to a lesser extent, other SCFAs can act as potent histone deacetylase (HDAC) inhibitors [Davie, 2003]. HDACs have been implicated in several neuropsychiatric disorders, including depression and schizophrenia, as they may affect biological processes critical for neuronal function and synaptic plasticity. Clinically, HDAC inhibitors may represent potential cognitive enhancers in emotional response and memory consolidation disorders. SCFAs can exert neuroprotective effects by stimulating microglia to produce anti-inflammatory cytokines such as IL-10 [Borsom et al., 2020]. Furthermore, SCFAs can influence the differentiation and activation of immune system cells, including cytokines, dendritic cells, macrophages and T cells [Correa-Oliveira et al., 2016].

2. Nutraceutical approaches in neurodegeneration prevention

Neurodegenerative diseases are a common and growing cause of death worldwide and they are mainly associated with aging. However, numerous modifiable risk factors such as diet and lifestyle can accelerate the onset of symptoms and the negative impact on human health. The high cost and sometimes ineffectiveness of traditional pharmacological therapies have led researchers to seek and find alternative solutions, such as the incorporation of specific natural compounds (functional food, phytochemicals or nutraceuticals) into the daily diet. Indeed, a plethora of these compounds can be used in the prevention and/or treatment of certain diseases, including neurodegenerative disorders, because of valuable biological activities (Figure 4).

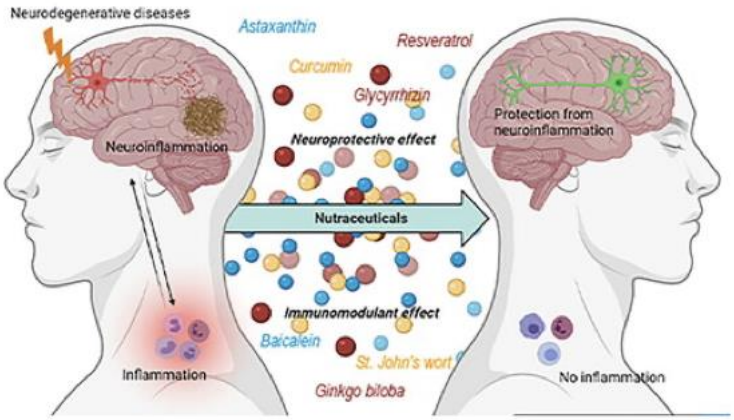

Figure 4 *Nutraceuticals and Neuroinflammation.*

2.1 Nutraceuticals and brain health

In the early 1980s, Dr. Stephen DeFelice coined the term "nutraceutical", a combination of the words "nutrition" and "pharmaceutical", to indicate a compound or food product that can exert a beneficial effect on health by preventing and/or treating disease. Since the definition of nutraceuticals has been established, research around the world has investigated a wide range of potential nutraceuticals and their effects on common diseases. It is important to emphasize that these substances are not drugs, and they are not intended to replace the treatment of disease. However, they can be a very useful tool to support traditional therapies and to help prevent certain secondary complications, particularly in chronic and multifactorial diseases such as obesity.

Nutraceuticals have been shown to be effective in counteracting many of the adverse effects of HFD and to offer a multifactorial approach to HFD-induced neurodegeneration.

These compounds act by reducing oxidative stress and inflammation, modulating the gut microbiota, protecting directly neuronal cells, and regulating lipid metabolism and insulin sensitivity.

2.1.1 Antioxidant properties

As previously mentioned, the CNS is extremely sensitive to oxidative damage. Therefore, nutraceuticals with antioxidant properties generate considerable interest for their application in neurodegenerative diseases [Gunawan & Boonkanokwong, 2024]. On the other hand, they show widespread availability, lack of toxicity, and minimal side effects. However, a limiting factor in their use in neurodegenerative disorders is BBB, which limits or prevents the brain uptake.

Indeed, numerous studies, both *in vitro* and *in vivo*, have shown that some nutraceuticals act as free radical scavengers, preventing free radicals from damaging lipids, proteins, and DNA. The most studied dietary antioxidants include vitamins (vitamins C and E), carotenoids (lycopene and β-carotene), flavonoids (quercetin and anthocyanin), polyphenols (curcumin and resveratrol), and phenolic acids (gallic and caffeic acids) [Feng et al., 2023].

Vitamin C is one of the most important water-soluble antioxidants, it is able to cross the BBB using GLUT1 in its oxidized form (dehydroascorbic acid) [Sage & Carruthers, 2014]. In the CNS, vitamin C has been shown to counteract effectively the formation and damage induced by superoxide and hydroxyl radicals [Kontoghiorghes et al., 2020]. Therefore, it has been hypothesized that vitamin C may play a protective role in certain neurodegenerative diseases.

Vitamin E is the generic name for a class of fat-soluble vitamins. The α-tocopherol isomer is the biologically most active form. Vitamin E can cross the BBB by passive diffusion. This mechanism allows vitamin E to reach higher concentrations within the CNS in comparison with vitamin C. Preclinical and clinical human studies have demonstrated that vitamin E possesses antioxidant capacity in the brain. In brain tissue, it increases glutathione (GSH) levels and the activity of antioxidant enzymes [Badgujar et al., 2016]. Experimental evidence suggests that vitamin E may offer significant benefits in the treatment and prevention of AD. In fact, a clinical trial showed that vitamin E supplementation can slow the onset of dementia in AD patients [Dysken et al., 2014]. Analyses in an AD mouse model showed that vitamin E reduces lipid peroxidation, a process that damages cell membranes, and it lowers the risk of developing the disease [Sung et al., 2004]. Vitamin E also prevents oxidative stress and the phosphorylation of the TAU protein by inhibiting the

activation of the p38 protein kinase [Giraldo et al., 2014]. Vitamin E may also slow the development of PD in humans by preventing lipid peroxidation [Etminan et al., 2005]. In animal models of PD, vitamin E has been o have shown significant neuroprotective effects. It counteracts damage caused by ROS, it increases GSH levels, and it reduces the negative effects of oxidative damage [Gaba et al., 2019].

Finally, vitamin E has been demonstrated to slow the progression of amyotrophic lateral sclerosis (ALS) by reducing lipid peroxidation. This antioxidant effect helps to protect neurons from oxidative damage, slowing the neuronal degeneration typical of ALS [Monteiro et al., 2024].

2.1.2 Anti-inflammatory effects

Various nutraceuticals, such as curcumin and green tea, have anti-inflammatory properties in addition to their antioxidant abilities.

Curcumin, or diferuloylmethane, is the polyphenolic component that gives turmeric its classic yellow color and it is mainly responsible for the beneficial effects attributed to the spice, from which it takes its name. This phytochemical has antioxidant and anti-inflammatory properties. It is also able to cross the BBB, and it exerts its effects directly on the CNS [Reddy et al., 2018]. In fact, the phytochemical has been reported to have significant benefits in neurodegenerative diseases, helping to protect neurons from oxidative and inflammatory damage [Maiti & Dunbar, 2018; Bagherniya et al., 2022]. The antioxidant properties of curcumin can protect the brain from oxidative damage, both directly by blocking the action of reactive species and indirectly by boosting the body's antioxidant defenses [Maiti & Dunbar, 2018]. Furthermore, curcumin supplementation significantly reduced serum levels of TNF-α, IL-6, transforming growth factor β (TGF-β), and MCP-1 in subjects with metabolic syndrome [Panahi et al., 2014]. In inflamed microglial cells, curcumin treatment led to a significant reduction in ROS, TNF-α, and MCP-1 levels [Guo et al., 2013]. In another study on mixed culture of neurons and glial cells, curcumin reduced the expression of several pro-inflammatory mediators, including CD11b (a marker of activated microglia), IL-1β and IL-6, by inhibiting NF-κB [Zhu et al., 2014]. Other studies report that curcumin reduces levels of NO, iNOS, TNF-α, COX-2 and PGE2 by inactivating MAPK signalling and inducing the expression of eme oxygenase-1 and nuclear factor erythroid-related factor 2 (Nrf2) [Jin et al., 2018; Yu et al., 2018].

The anti-inflammatory effects have been also confirmed *in vivo* in murine models of brain inflammation. Curcumin is able to reduce astrocyte activation and the subsequent

production of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) [Liu et al., 2016a; Kou et al., 2021].

Green tea has been shown to have clear neuroprotective effects and exerts multiple benefits on the CNS through a variety of mechanisms, including anti-inflammatory effects. The properties of the green tea are due to the substances it contains, such as epigallocatechin-3-gallate (EGCG), a flavonoid also found in red wine. This compound also has antioxidant activity and is able to cross the BBB [Wei et al., 2019]. *In vivo*, EGCG is able to reduce plasma levels of TNF-α, IL-1β and IL-6. It also inhibited NF-κB/p65 [Zhang et al., 2015].

At the neuronal level, EGCG has been shown to be involved directly in the beneficial and protective effects against certain neurodegenerative diseases. In fact, it inhibits the formation of Aβ fibrils, involved in AD pathogenesis [Gonçalves et al., 2021]. EGCG works by converting Aβ fibrils into smaller protein aggregates that are less toxic to mammalian cells. In addition, in cultured hippocampal neurons, EGCG protects against Aβ-induced neuronal apoptosis by scavenging reactive oxygen species [Choi et al., 2001].

2.1.3 Transcriptional effects of nutraceuticals

Some nutraceuticals are known to have a direct effect on nerve cells, protecting them from degeneration. They can promote neuronal survival, synaptic plasticity, and neurogenesis. One of the most famous examples is curcumin, which not only inhibits inflammation, but it also has effects on the survival of nerve cells.

In a rat model with brain inflammation, curcumin has been shown to reduce the glial fibrillary acid protein (GFAP) and ionized calcium-binding adaptor molecule 1 (Iba-1), two proteins expressed in astrocytes and microglia respectively, markers of activation of the glial cells [Khan et al., 2019a]. In addition, curcumin reduces apoptotic markers such as BAX, caspase-3, Cyt-c, and poly (ADP-ribose) polymerase-1, while at the same time increasing the levels of the anti-apoptotic protein Bcl-2 [Khan et al., 2019a].

Other foods or vegetable extracts, such as honey or Ginkgo biloba extracts, can counteract neurodegenerative disorders thanks to their abundance in polyphenols with anti-inflammatory and antioxidant properties. However, they can act directly on the cells, for example by modulating gene transcription in favor of neuronal survival [Hossen et al., 2017; Singh et al., 2019; Terzo et al., 2022]. For example, luteolin is a flavonoid found in honey that has been shown to enhance basal synaptic transmission and to facilitate the induction of long-term potentiation (LTP) in the rat hippocampal dentate gyrus by activating CREB. This mechanism contributes to the protection of synaptic function and the restoration of memory in neurodegenerative disorders [Xu et al., 2010]. Moreover, luteolin has neuroprotective

action against β-amyloid-induced toxicity in cultured mouse cortical neurons [Cheng et al., 2010].

Limonene and resveratrol are other examples of nutraceuticals that have been shown to have neuroprotective effects. Limonene is a monocyclic monoterpene that is found in large quantities in citrus fruits, especially in the peel. In addition to its antioxidant and anti-inflammatory properties, limonene has direct neuroprotective effects by inhibiting acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) [Szwajgier & Baranowska-Wójcik, 2019]. These enzymes are pathologically overactive in AD, leading to impaired cholinergic transmission and subsequent memory loss.

Resveratrol is a polyphenol with high biological activity. It is found in many vegetables, and it is particularly abundant in wine [Tian & Liu, 2020]. *In vitro* and *in vivo* studies have demonstrated its ability to cross the BBB and its consequent neuroprotective effects [Griñán-Ferré et al., 2021]. Red wine consumption reduces amyloid AD neuropathology and attenuates Aβ-associated memory loss in Tg2576 mice [Wang et al., 2006]. Resveratrol administration in diabetic rats improved cholinergic transmission, while in a hippocampal Sprague-Dawley rat model, it increases expression of BDNF [Schmatz et al., 2009; Rahvar et al., 2011]. Furthermore, intracerebroventricular (ICV) injection of resveratrol in a transgenic mouse model of AD, was effective in reducing hippocampal neurodegeneration by reducing levels of caspase-3 and GFAP [Kumar et al., 2007].

2.1.4 Effects of nutraceuticals on lipid and glucose metabolism

The healthy benefits of nutraceuticals in neurodegenerative diseases may result from the effects on lipid dysmetabolism and insulin resistance, important risk factors for cognitive decline.

Among nutraceuticals, omega-3 fatty acids (Ω-3), a category of PUFAs found in both animals (such as fish, krill, eggs and squid) and plants (such as algae, flaxseed, walnuts, edible seeds and clary sage) sources, are widely recommended internationally for the prevention of cardiovascular disease and dyslipidaemia [Cicero et al., 2021].

Omega-3 fatty acids exhibit anti-inflammatory, antioxidant and neuroprotective properties, making them promising agents for the treatment of complex conditions such as AD and PD [Kousparou et al., 2023]. Recent studies have linked dietary intake of Ω-3 with a reduced incidence of neurodegenerative diseases, highlighting the protective effects of DHA (a major component of Ω-3) against the key signs of Alzheimer's, such as cognitive impairment, accumulation of β-amyloid plaques and hyperphosphorylation of the tau protein. In addition, Ω-3 has been shown to improve β-amyloid phagocytosis by

macrophages in subjects with mild cognitive impairment (MCI), although similar benefits have not been observed in patients with advanced AD [Chitre et al., 2019].

Although clinical trials have demonstrated the safety and efficacy of Ω-3 prescribed for triglyceride reduction (FDA-approved), its neuroprotective mechanisms remain partially elucidated and study results can be variable [Hilleman et al., 2020]. Given the significant overlap between cardiovascular health and brain function, prescription Ω-3 may be a viable therapy for neurodegenerative diseases with both cardiovascular and neurological benefits. In light of the current evidence, the development of Ω-3 as a targeted treatment for neurodegenerative diseases is a plausible and scientifically justified path.

Quercetin is one of the most potent plant-derived antioxidants. It is a predominant flavonoid in many edible plants. Extensive research has demonstrated neuroprotective effects in several neurodegenerative conditions, including cognitive impairment, ischaemia, traumatic brain injury, PD and Huntington's disease (HD), in both *in vitro* and *in vivo* studies. Despite the limited ability to cross the BBB, quercetin effectively protects neurons from oxidative damage, particularly by reducing lipid peroxidation [Singh et al., 2022]. The neuroprotective properties of quercetin are largely attributed to the antioxidant effects. It inhibits Aβ fibril formation, prevents cell lysis, and disrupts inflammatory pathways associated with neurodegeneration. In addition, it has been shown to reduce neuroinflammation by decreasing nitric oxide production, iNOS gene expression in microglia, and inhibiting inflammatory cytokines including TNF-α, IFN-γ, IL-1β, IL-6, IL-12 [Khan et al., 2019b]. Moreover, quercetin may modulate glucose metabolism by improving insulin sensitivity. In fact, quercetin affects molecular pathways such as AMPK and PI3K/Akt signalling, which are essential for maintaining glucose and lipid homeostasis [Jiang et al., 2019]. By alleviating insulin resistance, quercetin not only optimises brain metabolism but also reduces inflammation and cellular stress, both of which are commonly associated with HFD-induced neurodegeneration.

In summary, quercetin efficacy in neuroprotection is due to both its direct antioxidant and anti-inflammatory properties and its ability to improve insulin signalling and metabolic balance.

2.2. Nutraceuticals and gut-brain axis

It is now widely accepted that altered microbiota composition is associated with several diseases including neurodegenerative disorders. Therefore, the diet may influence the composition and activity of the microbiota [Perler et al., 2023]. The use of nutraceuticals

to modulate the composition of the gut microbiota and to counteract the negative effects of gut dysbiosis represents a promising area of research and development.

AD has been the first neurodegenerative disease in which dietary interventions modulating gut microbiota have been tested for therapeutic or preventive purposes. These interventions are mainly based on several strategies, including probiotic supplementation, consumption of probiotic-enriched foods, fiber intake, and phytochemical supplementation [Lekchand Dasriya et al., 2022].

A clinical trial in older people showed that 12 weeks of probiotic treatment reduced stress scores and improved mental flexibility, increased plasma BDNF levels, and reduced the abundance of certain gut bacteria (*Eubacterium, Allisonella, Clostridiales, and Prevotellaceae*) [Kim et al., 2021]. Furthermore, there are evidence that the Mediterranean diet may have a neuroprotective effect, reducing the risk of AD and PD. This appears to be mediated, at least in part, by the promotion of a healthy gut microbiome [Solch et al., 2022].

Phytochemicals are the most extensively studied and tested nutraceutical compounds. Phytochemicals can exert probiotic effects by interacting with the gut flora. In general, they can alter the composition of the gut microbiota, because they act as preferential substrates for certain gut bacteria, helping them to grow. However, they may also negatively affect other bacteria, resulting in changes in the diversity and abundance of the microbiota.

For example, phenolic compounds found in tea, wine, olives and berries have been shown to have the ability to alter microbial growth in different ways [Merra et al., 2020; Santhiravel et al., 2022]. Catechins, found in tea, can also alter the amount of mucins in the ileum, and, in turn, bacterial adhesion and colonisation [Puupponen-Pimia et al., 2005]. Moreover, catechin promotes the growth of some bacterial groups such as *Clostridium coccoides, Eubacterium rectale* and *E. coli*, while inhibiting *Clostridium histolyticum*, promoting the growth of beneficial function-associated bacteria such *as Bifidobacterium spp*. and *Lactobacillus spp*. [Santhiravel et al., 2022]. Conversely, anthocyanin, a flavonoid, has been shown to have inhibitory properties against several pathogenic bacteria, including *Bacillus cereus, Helicobacter pylori, Salmonella spp.* and *Staphylococcus spp*. [Santhiravel et al., 2022].

AIMS OF THE RESEARCH

The present research was addressed to explore the possible beneficial impact of supplements and/or functional food on neuronal damage related to obesity, in an animal model with diet-induced obesity. To this aim, we used C57BL/6J male mouse, that, after 10 weeks on HFD, develops progressively obesity, hyperglycaemia, insulin resistance, hepatic steatosis, systemic inflammation [Zhang et al., 2020], neuroinflammation and neurodegeneration [Nuzzo et al., 2015; Nuzzo et al., 2020; Terzo et al., 2022].

In detail, we investigated the impact of:

1. Long-term ingestion of honey alone or in combination with D-limonene on obesity-related brain damage;
2. KlamExtra®, a commercialized extract from *Aphanizomenon flos aquae* (AFA), a blue-green microalga with excellent antioxidant and anti-inflammatory properties [Benedetti et al., 2010] on the molecular mechanisms involved in neurodegeneration induced by obesity. In particular, by using GFAP and soluble triggering receptors expressed on myeloid cells-2 (sTREM-2) as biomarkers, we explored the possibility that AFA can mitigate the central inflammatory process induced by HFD. Moreover, a possible protective response of AFA in reducing Aβ deposits was also investigated;
3. Indicaxanthin, a betalain extracted from *Opuntia ficus indica* yellow fruit, on the neurodegeneration induced by HFD and gut microbiota dysbiosis.

I° ARTICLE

LONG-TERM INGESTION OF SICILIAN BLACK BEE CHESTNUT HONEY AND/OR D-LIMONENE COUNTERACTS BRAIN DAMAGE INDUCED BY HIGH FAT-DIET IN OBESE MICE

The present study investigated the effects of long-term administration of honey and/or D-limonene, ingested separately or in combination, on neuronal damage induced by HFD. After 10 weeks on a high-fat diet, the mice were assigned to different treatment groups for further 10 weeks: one group continued on HFD alone (HFD), while the other groups received either HFD and honey (HFD-H), HFD and D-limonene (HFD-L) or HFD and a combination of honey and D-limonene (HFD-H + L). A separate group was maintained on a standard diet (STD) for the whole duration of the study. We focused on neurodegeneration, inflammation and oxidative stress to assess the effects of the treatments on brain health. In addition, we examined the gene expression of several markers that are associated with AD.

The consumption of honey and D-limonene counteracted HFD-induced upregulation of pro-apoptotic genes such as *Fas-L, Bim and P27*, and downregulation of anti-apoptotic factors *BDNF* and *BCL2* as well as the higher neuronal apoptosis, releaved bt Tunel assay. In addition, all treatments reduced the gene expression of the inflammatory cytokines IL-1β, IL-6 and TNF-α and decreased markers of oxidative stress such as COX-2, iNOS, ROS and nitrite. Notably, the combination of honey and D-limonene showed a stronger effect than either treatment alone. In addition, genes involved in amyloid plaque processing (APP and TAU), synaptic function (AChE) and hyperphosphorylation associated with AD were found to be upregulated in the brains of mice fed HFD. In contrast, these genes were significantly downregulated in the HFD-H, HFD-L and HFD-H + L treated groups.

In summary, long-term administration of honey and D-limonene, particularly in combination, effectively attenuated neuronal damage induced by a HFD, thereby demonstrating potential therapeutic benefits for brain health and AD.

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Long-Term Ingestion of Sicilian Black Bee Chestnut Honey and/or D-Limonene Counteracts Brain Damage Induced by High Fat-Diet in Obese Mice

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Abstract: Obesity is linked to neurodegeneration, which is mainly caused by inflammation and oxidative stress. We analyzed whether the long-term intake of honey and/or D-limonene, which are known for their antioxidant and anti-inflammatory actions, when ingested separately or in combination, can counteract the neurodegeneration occurring in high fat diet (HFD)-induced obesity. After 10 weeks of HFD, mice were divided into: HFD-, HFD + honey (HFD-H)-, HFD + D-limonene (HFD-L)-, HFD + honey + D-limonene (HFD-H + L)-fed groups, for another 10 weeks. Another group was fed a standard diet (STD). We analyzed the brain neurodegeneration, inflammation, oxidative stress, and gene expression of Alzheimer's disease (AD) markers. The HFD animals showed higher neuronal apoptosis, upregulation of pro-apoptotic genes Fas-L, Bim P27 and downregulation of anti-apoptotic factors BDNF and BCL2; increased gene expression of the pro-inflammatory IL-1β, IL-6 and TNF-α and elevated oxidative stress markers COX-2, iNOS, ROS and nitrite. The honey and D-limonene intake counteracted these alterations; however, they did so in a stronger manner when in combination. Genes involved in amyloid plaque processing (APP and TAU), synaptic function (Ache) and AD-related hyperphosphorylation were higher in HFD brains, and significantly downregulated in HFD-H, HFD-L and HFD-H + L. These results suggest that honey and limonene ingestion counteract obesity-related neurodegeneration and that joint consumption is more efficacious than a single administration.

Keywords: obesity; high-fat diet; neuronal damage; neurodegeneration; honey; D-limonene

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1. Introduction

Neurodegenerative diseases (NDs), including Alzheimer's disease (AD), are characterized by the progressive loss of neurons in areas of the brain, leading to cognitive and functional deterioration. NDs represent a serious problem because they affect about 50 million patients worldwide and this number is estimated to reach 115 million in 2050 [1]. Different factors contribute to the onset and progression of neurodegeneration such as aging, genetics, environment [2] and oxidative stress and inflammation [3,4]. Moreover, obesity and diabetes increase the risk of developing dementia and AD. In fact, the neurodegenerative process is exacerbated by obesity or diabetes, leading to the concept of metabolism-dependent neurodegeneration [5]. Indeed, insulin receptor down-regulation has been observed in the brains of patients with AD [6] confirming the theory that AD may be considered as "type 3 diabetes" [7]. Furthermore, studies on animal models pointed out that obesity affects learning and memory [8,9] and long-term ingestion of high-fat diet (HFD) in rodents is responsible for neuronal loss and synaptic plasticity damage [10–13].

NDs are as yet incurable and strongly debilitating for the patients. Nevertheless, current research is pushing towards effective therapies [14,15]. Numerous nutraceuticals and/or functional foods are considered protective and/or therapeutic against the metabolic dysfunctions and the related neurodegeneration [16–20]. For example, foods rich in antioxidants, micronutrients, phytochemicals, essential oils, and probiotics have been found to be helpful in maintaining body weight and reducing the incidence of neurodegenerative diseases [1,20].

In particular, honey might prove useful in the treatment of chronic diseases linked to oxidative stress and inflammation due to its high content in polyphenols [21]. Although the composition of honey is variable depending on various factors such as botanical origin, geographical region and climatic conditions, most of the polyphenols present in honey are flavonoids and phenolic acid derivatives that possess anti-inflammatory and neuroprotective properties [22].

More specifically, our recent investigation demonstrated the ability of honey consumption to prevent HFD-dependent neuronal injury. In particular, a 16-week-intake of Sicilian black bee chestnut honey, which is particularly rich in kaempferol and quercetin [22], prevented peripheral and central insulin resistance and neuroinflammation in mice fed with a hyperlipidic diet [23]. This neuroprotective effect proved to be mainly due to the positive modulation of brain genes involved in insulin signaling, neuroinflammation and apoptosis [23]. However, it remains to be investigated whether the long-term ingestion of honey is able to revert obesity-related metabolic dysfunctions and related neurodegeneration.

Recently, D-limonene (1-methyl-4-(1-methylethenyl) cyclohexane), a monocyclic monoterpene that is the major constituent of citrus essential oils, has also received notable scientific interest due to its ability to mitigate inflammation and oxidative stress and reduce apoptotic cell death [24]. In fact, it possesses antidiabetic, antioxidant, anti-inflammatory, antinociceptive and anticancer properties [25]. In animal models, D-limonene has been reported to alleviate obesity-related metabolic disorders [26,27]. However, although D-limonene has recently been shown in vitro to inhibit acetylcholinesterase [28] and to exert beneficial effects in the *Drosophila* AD model by reducing oxidative stress and neuroinflammation [29], data on neuroprotective actions against neuronal damage caused by HFD are lacking. Moreover, a recent study suggested that the usage of D-limonene together with other drugs, such as aminoguanidine, is more efficient in the prevention of secondary complications in diabetes in comparison to single treatment [30].

Therefore, the present research was undertaken with the purpose of exploring whether honey, administered alone or in combination with D-limonene, can represent a potential dietary supplement that can aid in ameliorating or reverting HFD-caused brain damage. In this view, we investigated the effects of the long-term ingestion of honey and D-limonene, separately or in combination, on brain damage in HFD mice when the pathological conditions were overt.

2. Results

2.1. Body Weight, Glycaemia and Serum Lipids

As shown in Figure 1A, at the end of the experimental protocol, HFD mice were significantly heavier than STD mice. The weight gain of HFD-L and HFD-H + L mice was significantly lower than that of HFD animals. The fasting blood glucose concentration of HFD mice was significantly higher than that of the STD group. HFD-H and HFD-H + L mice had similar fasting blood glucose concentrations to the HFD group. However, the D-limonene supplementation markedly reduced the fasting blood glucose levels induced by HFD (Figure 1B). The lipid profile of mice that were fed with the different diets is represented in Figure 1C. Total cholesterol and triglyceride levels, that were high in the plasma of the HFD mice compared to STD group, did not significantly differ in HFD-H, HFD-L and HFD-H + L mice, suggesting that honey and D-limonene, alone or in combination, did not impact on the lipid metabolism of obese mice.

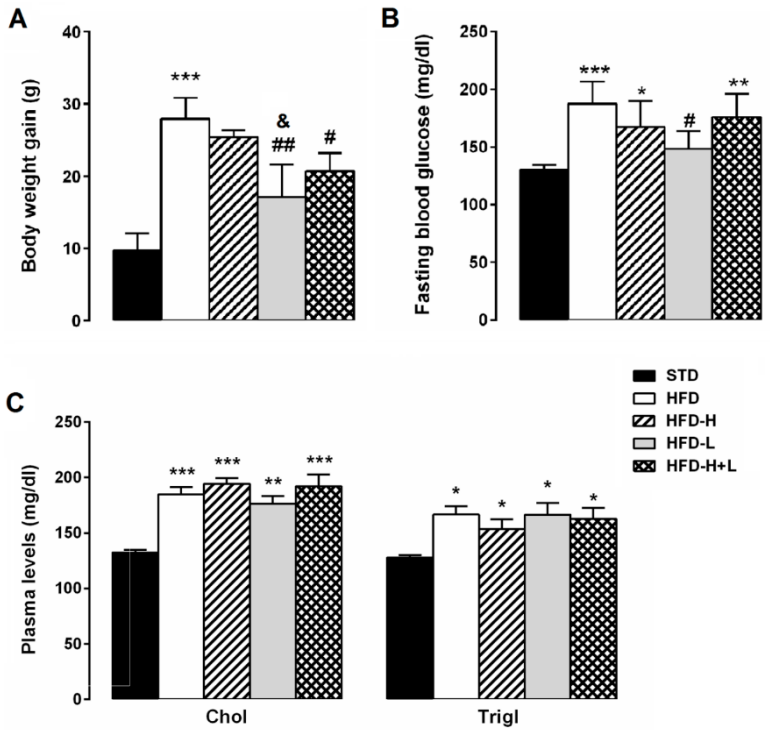

Figure 1. Metabolic parameters. (**A**) body weight gain, (**B**) fasting blood glucose concentrations and (**C**) plasma levels of cholesterol and triglycerides in obese mice. Data are mean values ± S.E.M. (n = 8/group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. STD mice; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$; $^{\&}$ $p < 0.05$ vs. HFD-H mice.

2.2. Neurodegeneration: TUNEL Assay

Neurodegeneration has been suggested to be associated with cell apoptosis. To identify whether apoptotic cells were present in the brain tissues of the different groups of mice, we used the TUNEL assay. A higher number of TUNEL-positive cells was observed in the cortex of HFD mice in comparison with STD mice. As shown in Figure 2, neuronal apoptosis resulting from a high-fat diet was significantly decreased in the cortex of HFD-H, HFD-L, and HFD-H + L mice, suggesting that both honey and D-limonene contributed to neuroprotective effects. Interestingly, the diet containing honey and D-limonene together was more efficacious than the single supplement.

2.3. Pro-Apoptosis and Anti-Apoptosis Genes Expression

In this work, the gene expression of the most important regulators of apoptosis. The pro-apoptotic factors *FAS-L*, *P27*, and *BIM* were significantly upregulated in mouse brain tissues from the HFD group compared to the STD group. A high-fat diet supplemented with honey, D-limonene or honey plus D-limonene significantly decreased the gene expression levels of all investigated factors, suggesting a reduced presence of neurons that undergo programmed cell death (Figure 3A,B). On the contrary, the brain gene expression of factors that help neuronal survival, such as BDNF and BCL2, was decreased in the HFD mice compared to the STD group. This down-regulation induced by HFD was counteracted by the simultaneous ingestion of honey or D-limonene. HFD-H + L proved to be the most efficacious diet to increase BCL2 and BDNF expression (Figure 3C,D).

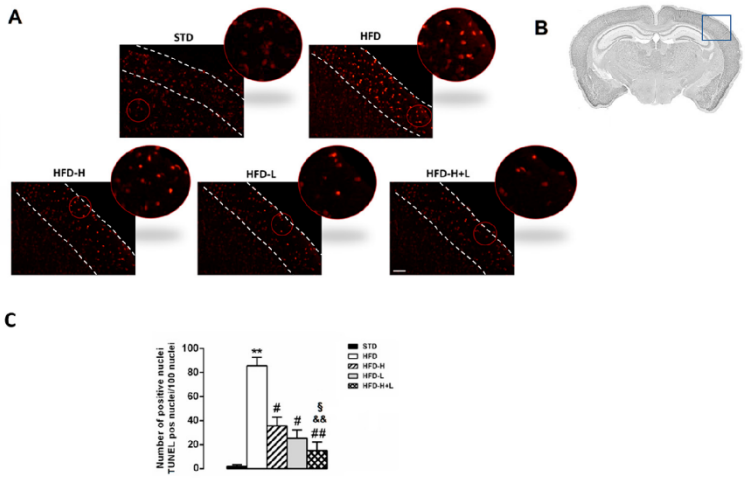

Figure 2. Neurodegeneration. (**A**) TUNEL assay; the outlined area is enlarged in the circle; white dotted lines represent the mouse brain cortex. (**B**) Schematic representation of cerebral cortex of positive areas. (**C**) Number of apoptotic nuclei in cerebral cortex of STD, HFD, HFD-H, HFD-L and HFD-H + L mice. Data are mean values ± S.E.M. (n = 8/group). ** $p < 0.01$ vs. STD mice; # $p < 0.05$, ## $p < 0.01$ vs. HFD mice; && $p < 0.01$ vs. HFD-H mice; § $p < 0.05$ vs. HFD-L mice. Microscope magnification 10×. Scale bar, 200 µm.

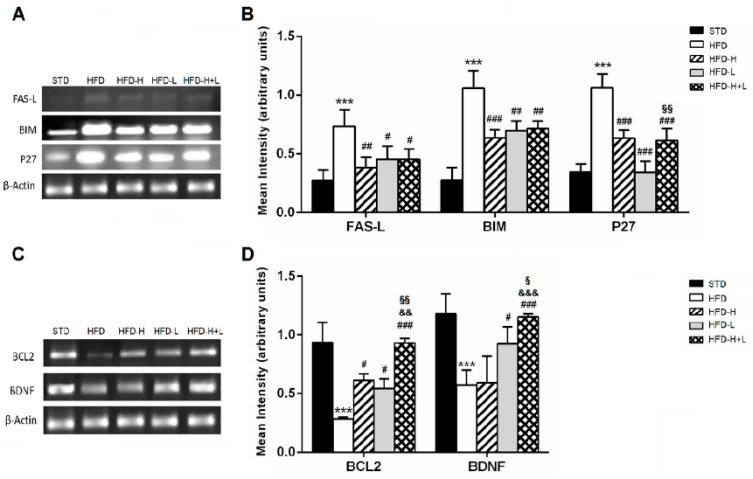

Figure 3. Apoptosis. (**A**) result of the RT-PCR and (**B**) mRNA levels of pro-apoptotic genes: *FAS-L*, *BIM* and *P27* in the mouse brain of different groups; (**C**) Representative image of the RT-PCR results and (**D**) mRNA levels of survival genes: *BCL2* and *BDNF* in the mouse brain of different groups. Data are mean values ± S.E.M. (n = 8/group). *** $p < 0.001$ vs. STD mice; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. HFD mice; && $p < 0.01$, &&& $p < 0.001$ vs. HFD-H mice; § $p < 0.05$, §§ $p < 0.01$ vs. HFD-L mice.

2.4. Brain Pro-Inflammatory Gene and Protein Expression

To determine whether honey and D-limonene, together or separately, reduced neuroinflammation, we examined the brain expression of some pro-inflammatory cytokines and other proteins, which are markers of inflammation. The IL-1β, IL-6 e TNF-α increased expression, found in HFD brains, was reduced by honey or D-limonene ingested separately, and it returned to control levels in the brain of HFD-H + L mice, suggesting that the combined administration of honey and D-limonene was more efficacious than single ad-

ministration (Figure 4A,B). Moreover, the elevated expression of COX-2 and iNOS induced by HFD, was mitigated by honey, D-limonene, and honey plus D-limonene (Figure 5A,B).

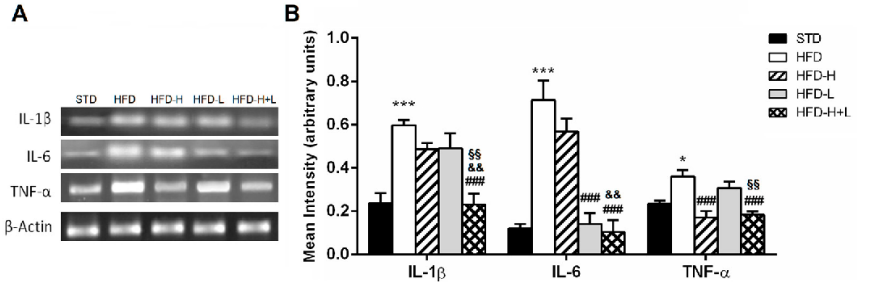

Figure 4. Brain inflammation. (**A**) Representative image of the RT-PCR results and (**B**) mRNA levels of IL-1β, TNF-α and IL-6 in the mouse brain of different groups. Data are mean values ± S.E.M. (n = 8/group). * $p < 0.05$, *** $p < 0.001$ vs. STD mice; ### $p < 0.001$ vs. HFD mice, && $p < 0.01$ vs. HFD-H mice; §§ $p < 0.01$ vs. HFD-L mice.

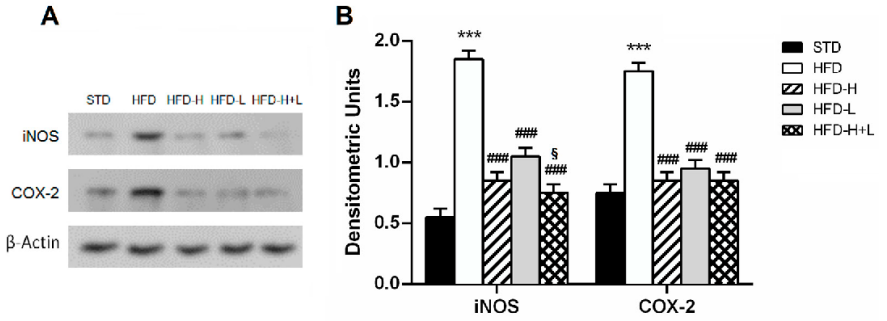

Figure 5. Brain stress. (**A**) protein expression levels of COX-2 and iNOS in the mouse brain of different groups; (**B**) densitometric analysis of iNOS and COX-2 protein levels normalized for β-actin levels. Data are mean values ± S.E.M. (n = 8/group). *** $p < 0.001$ vs. STD mice; ### $p < 0.001$ vs. HFD mice; § $p < 0.05$ vs. HFD-L mice.

2.5. Brain Oxidative Stress

Increasingly, studies have demonstrated that oxidative stress is critical for neuronal injury. Therefore, we determined the effect of the different supplemented diets on ROS generation and nitrite content in the brain of the different groups of animals. After a 20-week HFD administration, ROS generation assessed with H_2DCF-DA was significantly increased in HFD brain compared not only to STD, but also to HFD-H, HFD-L, and HFD-H + L (Figure 6A). Moreover, we found a significant increase in nitrite levels in the brains of HFD obese animals in comparison with STD animals. HFD-H, HFD-L, and HFD-H + L mice showed nitrite values that were significantly lower than those of HFD mice (Figure 6B).

2.6. Expression of Genes Involved in AD

Using a Mouse Alzheimer's Disease RT^2 Profiler PCR Array we analyzed expression changes of genes involved in the onset, development and progression of Alzheimer's disease in the different groups of animals. Among them, there are genes that contribute to amyloid beta-peptide (Aβ) generation, clearance and degradation but also genes related to neuronal toxicity. The list of genes is shown in Table S1. We focused on the gene expression levels that were affected more than two-fold among the analyzed groups. The results showed that in the HFD brains, various genes involved in the processing of Amiloid β Precusor (APP) and TAU (*Aplp1*, *Aplp2*, *App*, *Apba3*, *Apbb2*, *Apoe*, *Ckk5*, *Clu*, *Ctsl*, *Mapt*, *Prkca*, *Prkce* and *Hsd17b10*), in synaptic function (*Ache*), in AD-related iperphosphorylation

(*Gsk3α*, *GCdk5* and *Prkca*), and in inflammation (*MPO* and *Il-1α*) (Table 1) were upregulated in comparison with lean brains. These abnormal expressions were significantly ameliorated in the brain of obese animals fed with honey, D-limonene and honey plus D-limonene with a major improvement in the HFD-H group (Table 1).

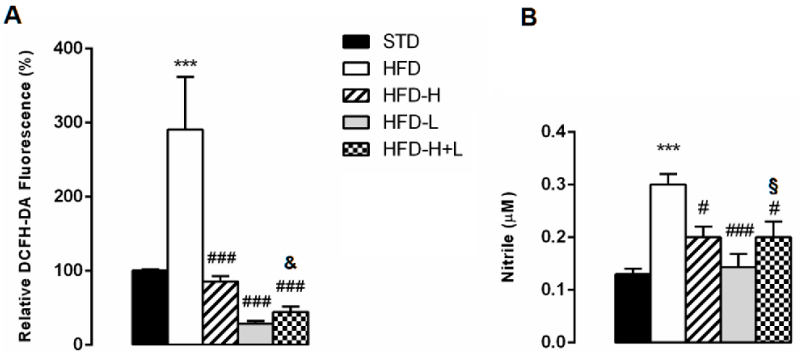

Figure 6. Brain oxidative stress. (**A**) Levels of ROS in the brain of STD, HFD, HFD-H, HFD-L and HFD-H + L mice. (**B**) Nitrite concentration in the brain of STD, HFD, HFD-H, HFD-L and HFD-H + L mice. Data are mean values ± S.E.M. (n = 8/group). *** $p < 0.001$ vs. STD mice; # $p < 0.05$, ### $p < 0.001$ vs. HFD mice; & $p < 0.05$ vs. HFD-H mice; § $p < 0.05$ vs. HFD-L mice.

Table 1. Gene expression profiles involved in AD in HFD/Lean, HFD-H/HFD, HFD-L/HFD and HFD-H + L/HFD, which were significantly downregulated by 2-fold.

Gene Name	Protein	HFD/Lean	HFD-H/HFD	HFD-L/HFD	HFD-H + L/HFD
Ache	Acetylcholinesterase	2.10	−30.84	−12.01	−16.31
Apba3	Amyloid beta (A4) precursor protein-binding, family A, member 3	44.94	−59.17	−30.02	−29.88
Apbb2	Amyloid beta (A4) precursor protein-binding, family B, member 2	2.60	−18.46	−10.71	−17.39
Aplp1	Amyloid beta (A4) precursor-like protein 1	5.17	−8.79	−8.53	−5.00
Aplp2	Amyloid beta (A4) precursor-like protein 2	15.97	−45.55	−15.49	−17.72
Apoe	Apolipoprotein E	5.16	−26.20	−9.36	−9.77
App	Amyloid beta (A4) precursor protein	6.05	−15.85	−9.94	−10.12
Bdnf	Brain derived neurotrophic factor	−17.99	−5.49	−3.44	−2.67
Cdk5	Cyclin-dependent kinase 5	2.92	−36.93	−17.68	−13.99
Clu	Clusterin	26.50	−4.04	−3.76	−2.19
Ctsl	Cathepsin L	5.08	−13.34	−4.65	−4.90
Gsk3a	Glycogen synthase kinase 3 α	7.20	−22.22	−14.11	−15.35
Hsd17b10	Hydroxy steroid deydrogenase10	5.87	−15.93	−9.38	−11.82
Il1a	Interleukin 1 α	4.31	−90.55	−49.60	−98.59
Mapt	Microtubule-associated protein tau	44.58	−23.78	−9.87	−8.28
Mpo	Myeloperoxidase	14.87	−33.10	−14.25	−28.87
Prkca	Protein kinase C, alpha	6.17	−16.16	−15.98	−10.33
Prkce	Protein kinase C, epsilon	5.89	−16.58	−12.56	−16.61
Psen1	Presenilin 1	4.68	−225.85	−152.13	−193.57

3. Discussion

The results of the present study suggest that long-term intake of Sicilian black bee chestnut honey and/or D-limonene, ingested separately or in combination, can protect central neurons against HFD-induced cerebral damage by reducing oxidative stress and neuroinflammation. To our knowledge, our study is the first report on the neuroprotective effects of D-limonene against the damage induced by HFD.

Epidemiological human studies pointed out that a high-calorie diet is associated with worse performance on cognitive tasks [31]. It increases the risk of dementia because high lipid content causes oxidative stress and neuronal dysfunctions [32]. Indeed, high stress oxidative triggers the up-regulation of pro-inflammatory factors leading to neuroinflammation [33]. However, different biological mechanisms including insulin resistance, developmental disturbances, altered membrane functioning, and altered vascularization have been involved in HFD-induced neuronal damage and cognitive decline [5,32].

In our experiments we used mice which, following chronic consumption of HFD, developed obesity accompanied by hyperglycemia, dyslipidaemia, insulin resistance [34–36], activation of amyloidogenic pathways, neuroinflammation and neurodegeneration [10,18,37–39]; consequently, they are suitable for verifying the potential effects of functional food/phytochemicals on neuronal survival. First of all, we analyzed the presence of apoptosis in the cerebral cortex and the gene expression of pro- and anti-apoptotic factors in the brains of the different animal groups. It is well known that apoptosis plays a key role in the pathogenesis of neurodegenerative diseases [40], involving mainly the BCL-2 protein family. This family includes proteins that control the mithocondrion membrane permeability such as Bax, Bim (pro-apoptotic proteins) and BCL-2, Bcl-xL, Bcl-w (anti-apoptotic proteins). Additionally, FAS ligand (FAS-L) has been involved in neuronal death [41] and P-27, an inhibitor of cyclin-dependent kinase, has been reported to promote neuronal apoptosis induced by the neurotoxic αβ42 peptide [42]. According to our previous reports [10,17,18], our results confirmed the presence of neurodegeneration caused by HFD as suggested by the increase of apoptotic neurons in the brain cortex of obese mice in comparison with STD mice. In HFD-H or HFD-L cerebral cortexes, the level of apoptotic neurons was significantly reduced suggesting that the daily ingestion of honey or D-limonene inhibits programmed cellular death. Moreover, honey and D-limonene ingested in combination further decreased the apoptotic neuron number, suggesting a synergistic neuroprotective action. The results from molecular analysis also supported our hypothesis on the neuroprotective effect of honey and D-limonene. In fact, the pro-apoptotic gene up-regulation and the anti-apoptotic gene down-regulation that was found in the HFD brain was attenuated in HFD-H, HFD-L and HFD-H + L animal groups. We also found a down-regulation of BDNF in the HFD brain, which was in accordance with previous studies that reported reductions in levels of BDNF in the hippocampus of obese rodents [11,43] as a consequence of increased oxidative stress [44]. However, honey and D-limonene when separately ingested increased the BDNF gene expression; even more so when ingested in combination, suggesting that an increase of survival factors can also be responsible for the observed beneficial effects.

Neurodegeneration can be triggered by various pro-inflammatory and neurotoxic mediators, such as IL-1β, IL-6, and TNF-α, and neuroinflammation is strictly associated with oxidative stress [45]. Indeed, several studies demonstrated that neuroinflammation is linked to high levels of ROS and high expression of AD biomarkers in the brains of HFD mice [10,46,47]. Because both honey and D-limonene have been reported to possess anti-inflammatory and antioxidant properties, leading to the assumption that they could be used as a supplement in anti-inflammatory therapies [21,48,49] we examined and compared the expression of pro-inflammatory factors, the levels of oxidative stress and nitrite in the brains of the different animal groups. The results suggested that HFD increases the gene expression of inflammatory cytokines (IL-1β, IL-6, TNF-α) and other proteins, markers of inflammation (i-NOS and COX-2) and ROS and nitrite levels in the brain as previously shown [10,18,32,50,51]. Interestingly, long-term ingestion of honey or D-limonene, and even more so, the combined ingestion of honey and D-limonene reduced the inflammatory and

oxidative stress markers suggesting once more a beneficial action against damage induced by HFD in the brain. We can only speculate about the honey compounds responsible for the observed beneficial effects, which generally have been attributed to polyphenols [21]. However, it is noteworthy that we used Sicilian black bee chestnut honey, whose kaempferol and quercetin levels corresponded to 69% of the total content [22]. Quercetin as well as kaempferol can cross the blood–brain barrier [52]. Quercetin has been reported to protect neurons from oxidative stress and inflammation and to have beneficial properties against mechanisms involved in AD in different in vitro and in vivo models [53], and kaempferol can act positively in various models of neurodegenerative diseases [54,55].

Although recent research using a Drosophila AD model suggested that D-limonene has a neuroprotective action against $A\beta_{42}$-induced toxicity associated with its antioxidant and anti-inflammatory properties [29], the effects of D-limonene on AD have not been well-studied yet. Therefore, by using a mouse Alzheimer's disease microarray, we have analyzed and compared the expression of genes involved in amyloid beta-peptide (Aβ) generation and processing and/or genes related to neuronal toxicity in the brains of different mouse groups. The results clearly suggest that long-term HFD feeding promotes the expression of genes associated with AD, including *Ache*, *App*, *Apba3*, *Apbb2*, *Aplp1*, *Aplp2*, *Apoe*, *CdK5*, *Clu*, *Ctls*, *GSK3α*, *Hsd17b10*, *Mapt*, *Psen1*, *Prkca*,*Prkcb* and genes linked to inflammation such as *Mpo and Il1α* [56,57]. However, these deleterious changes in gene expression were counteracted in the brains of HFD-H, HFD-L and HFD-H + L, suggesting that the increased neurotoxicity induced by HFD may be mitigated by long-term ingestion of honey and D-limonene, both separately and in combination. In particular, the down regulation of *App*, *Apba3*, *Apbb2*, *Aplp1*, *Aplp2*, *Apoe* and *Psen1* could suggest that the eventual endogenous APP generation and processing were reduced after the long-term ingestion of honey and D-limonene [58]. Moreover, *Cdk5*, a promoter of neuronal death [59] and *Clu*, encoding clusterin, a protein involved in several processes such as suppression of the complement system, lipid transport, and neuronal cell death and cell-survival mechanisms, whose levels are increased in AD [60], were mitigated by the intake of honey and D-limonene either alone or in combination.

4. Materials and Methods

4.1. Animals and Diets

Male C57BL/6 mice, purchased from Envigo (S.Pietro al Natisone, Udine, Italy) were maintained in the ATeN center animal house according to the European guide lines. The animals (4-weeks old) were housed (2 mice/cage) in a temperature- (23 ± 1 °C) and relative humidity (55% ± 5%)-controlled facility, under a 12-h light–dark cycle, according to the Italian legislative decree n. 26/2014 and were approved by the Ministry of Health (Rome, Italy; Authorization n. 891/2018-PR).

After two weeks of acclimatization, 8 mice were fed a standard diet (STD) (negative control) containing protein 20.0%, fat 10.0%, carbohydrate 70.0%, *w*/*w*, and water (code 4RF25, Mucedola, Milan, Italy), and 32 mice were fed a HFD, containing protein 20.0%, fat 60.0%, carbohydrate 20.0%, *w*/*w* (PF4215, Mucedola, Milan, Italy) for 10 weeks to induce obesity. Subsequently, HFD mice were divided randomly into four groups Then, four groups (n = 8/group) were created from the HFD mice: one group received HFD, the second group received HFD supplemented with honey (45 mg per day/mouse) (HFD-H), the third received HFD supplemented with D-limonene (0.5% *w*/*w*) (HFD-L) and the last one received HFD supplemented with honey and D-limonene in combination at the same doses (HFD-H + L), for another 10 weeks. The doses of D-limonene (Sigma—St. Louis, MO, USA) and honey (Prezzemolo and Vitale Supermarket, Palermo, Italy) were taken from the literature [23,27,61] and added to the HFD cow in a percentage amount that was useful so as not to change the HFD caloric value. Body weight and food intake were monitored every week.

At the end of the experimental protocol (20th week), biochemical analyses were performed on blood collected from the tail vein and then the animals were sacrificed. The

aorta was perfused with a buffer solution of Dulbecco and the right atrium was incised to allow outflow. Brains were rapidly explanted, weighed and coronally cut into two halves. One part was fixed in 4% formalin was utilized for histological investigation; the other half was ice-covered and used for molecular analysis.

4.2. Biochemical Analyses

Glucose concentration was measured using a glucometer (GlucoMen LX meter, Menarini, Florence, Italy) in overnight fasting mice. Plasma total cholesterol and triglyceride concentrations were determined using the ILAB 600 Analyzer (Instrumentation Laboratory, Bedford, MA, USA).

4.3. Apoptosis Investigation

The Tunel assay was used to determine the level of apoptosis (Promega, Madison, WI, USA) in the cerebral cortex sections, following the manufacturer's instructions. The values of the damaged nuclei were counted by two blind investigators and the ratio of apoptotic nuclei in respect of normal nuclei was calculated.

4.4. Reactive Oxygen Species Analysis

To determine the reactive oxygen species (ROS), 5 mg of brain tissue was homogenized with 1 mL of cold PBS1X and 10 µL of protease inhibitors (Amersham Life Science, Munich, Germany). The preparate brain homogenates were incubated with 1 mM dichlorofluorescein diacetate (DCFH-DA) at room temperature in the dark for 15 min, then the fluorescence was measured by fluorimeter (GloMax® Plate Reader, Promega, Milano, Italy) with an excitation filter set at 485 nm and an emission filter set at 530 nm. ROS levels were expressed as a percentage of the fluorescence emitted by STD cerebral samples.

4.5. Determination of Nitric Oxide (NO) Levels

The level of nitric oxide (NO) in the brains was evaluated by using Griess reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA). Briefly, 5 mg of brain tissue was homogenized with 1 mL of PBS1X and centrifuged at 14,000 rpm, for 30 min at 4 °C. 100 µL of supernatant was incubated with equal volumes of Griess reagent (1% sulphanilamide in 5% phosphoric acid and 0.1% N-(1-naphthyl)-ethylenediamine), the absorbance was immediately read at 520 nm in a microplate reader (GloMax® Plate Reader, Promega).

4.6. Molecular Analyses

Whole brain was used to extract RNA by using a RNeasy plus Mini Kit (Qiagen, Valencia, CA, USA). Subsequently, by using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA). cDNA was prepared by 2 ng of total RNA. Then the expression of target genes was performed by using Reverse Transcription Polymerase Chain Reaction (RT-PCR) with the subsequent primers: β-actin *For* 5′-CGGGATCCCCGCCCTAGGCACCAGGGT-3′; *Rev* 5′-GGAATTCGGCTGGGGTGTTGAAG GTCTCAAA-3′; for pro-inflammatory factors: IL-1β *For* 5′-CATGGGATGATGATAACCTGCT-3′; *Rev* 5′-CCCATACTTTAGGAAGACACGATT-3′; IL-6 *For* 5′-CTGGTGACAACCACGGCC TTCCCT-3′; *Rev* 5′-ATGCTTAGGCATAACGCACTAGGT-3′; TNF-α *For* 5′-AGCCCACGTC GTAGCAAACCA-3′; *Rev* 5′-GCAGGGGCTCTTGACGGCAG-3′; for pro-apoptotic factors: FAS-L *For* 5′-CAAGTCCAACTCAAGGTCCATGCC-3′; *Rev* 5′-AGAGAGAGCTCAGATAC GTTTGAC-3′; BIM *For* 5′-AACCTTCTGATGTAAGTTCT-3′; *Rev* 5′-GTGATTGCCTTCAGG ATTAC-3′; p27 *For* 5′-TGCGAGTGTCTAACGGGAG-3′; *Rev* 5′-GTTTGACGTCTTCTGAGG CC-3′; for anti-apoptosis factors: BCL-2 *For* 5′-ATGTGTGTGGAGAGCGTCAA-3′; *Rev* 5′-AGAGACAGCCAGGAGAAATCA-3′; BDNF *For* 5′-GGCTGACACTTTTGAGCACGTC-3′; *Rev* 5′-CTCCAAAGGCACTTGACTGCTG-3′. The amplification cycles comprised denaturation (45 s at 95 °C), annealing (45 s at 52 °C) and elongation (45 s at 72 °C), for 40 cycles. The amplification products were visualized by ultraviolet light using E-Gel GelCapture (Thermo Fisher Scientific, Monza, Italy) after separation on agarose gel. The quantification

of gene expression was obtained by using E-Gel GelQuant Express Analysis Software (version 1.14.6.0 (Dongle)) (Thermo Fisher Scientific, Monza, Italy). The signal intensity of the products was normalized to its respective β-actin signal intensity.

Protein expression. Brains dissected from the experimental animals were homogenized in ice-cold solubilization buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM DDT, 1% Triton X-100, 24 mM sodium deoxycholate, 0.01% SDS, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM sodium orthovanadate, 1.5 µM aprotinin, 1 mM phenylmethanesulfonylfluoride and 2.1 µM leupeptin) and centrifuged at 12,000× *g* at 4 °C for 30 min. Then, the supernatants were used for protein determination, as previously described [62]. Samples containing 50 µg protein were resolved by SDS-PAGE electrophoresis on 12% acrylamide gels and transferred to nitrocellulose membranes. After blocking for 2 h in 5% (*w*/*v*) skimmed dry milk, the membranes were incubated in the presence of primary antibodies overnight at 4 °C (Santa Cruz, Milan, Italy, 1:1000 dilution): anti-COX-2 (sc-376861), anti-iNOS (sc-7271). Subsequently, the samples were incubated with the secondary for 90 min. HRP-conjugated antibodies (Dako, Milan, Italy, 1:10,000 dilution) and chemiluminescent bands were detected by a C-Digit Blot Scanner (LI-COR, Lincoln, NE, USA) and densitometric analysis was used to analyze band intensities, by using LI-COR Image Studio 4.0.

4.7. RT² Profiler PCR Array

Mouse Alzheimer's disease array (Alzheimer's Disease RT² Profiler PCR Array, QIAGEN, Monza, Italy) was used in order to analyze factors in HFD brains that are involved in the onset, development and progression of AD. The 96 genes reported in the plate are listed in the Supplementary data (Table S1).

RNA from whole brains was utilized. A High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Bedford, MA, USA) was used to synthetize cDNA from 2 ng of RNA. The array was executed by using a StepOne Real-Time instrument (Applied Biosystem) and the results were obtained through the relative quantification method ($2^{-\Delta\Delta CT}$).

We chose to highlight only the genes showing changes in the expression levels that were more than two-fold among the different groups analyzed (HFD vs. Lean; HFD-H vs. HFD; HFD-L vs. HFD; HFD-H + L vs. HFD).

4.8. Statistical Analysis

The results are presented as mean values ± the standard error of the mean SEM. The number of animals is indicated with the letter 'n'. The comparison between the groups was performed by ANOVA, and then a Bonferroni post hoc test was used. All the analyses were obtained using Prism 6.0, GraphPad software (San Diego, CA, USA). Results with a p-value ≤ 0.05 were considered statistically significant.

5. Conclusions

In conclusion, our results confirm that HFD causes detrimental effects on AD-related neuropathological and neuroinflammatory pathways leading to neurodegeneration. However, the long-term ingestion of honey and D-limonene, either separately or in combination, is able to counteract and to ameliorate the cerebral stressing conditions related to HFD-induced metabolic disorders.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms24043467/s1.

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II° ARTICLE

APHANIZOMENON FLOS-AQUAE (AFA) EXTRACT PREVENTS NEURODEGENERATION IN THE HFD MOUSE MODEL BY MODULATING ASTROCYTES AND MICROGLIA ACTIVATION

The aim of the present study was to investigate the potential preventive effects of an extract of *Aphanizomenon flos-aquae* (AFA) on brain damage induced by HFD.
C57BL/6 mice were fed a standard diet (Lean control), HFD, or HFD+AFA for 28 weeks. Molecular, histopathological, and immunohistochemical analyses were conducted on the brains of animals to investigate the impact of AFA supplementation on the expression of apoptosis biomarkers, cerebral insulin resistance, oxidative stress, neuroinflammation, and its potential beneficial role in the deposition of β-amyloid plaques.
The supplementation of AFA to the HFD resulted in a significant attenuation of neurodegeneration, which was achieved by reducing insulin resistance and neuronal loss. AFA supplementation also enhanced the expression of synaptic proteins (PSD95 and synaptophysin), it mitigated the HFD-induced activation of astrocytes and microglia (evidenced by increased TREM2 and reduced GFAP expression) and it reduced the accumulation of beta-amyloid (Aβ) plaques.

The findings suggest that regular consumption of AFA extract may counteract the metabolic and neuronal dysfunction caused by HFD, reducing neuroinflammation and promoting Aβ plaque clearance.

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Article

Aphanizomenon flos-aquae (AFA) Extract Prevents Neurodegeneration in the HFD Mouse Model by Modulating Astrocytes and Microglia Activation

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Abstract: Obesity and related metabolic dysfunctions are associated with neurodegenerative diseases, such as Alzheimer's disease. *Aphanizomenon flos-aquae* (AFA) is a cyanobacterium considered a suitable supplement for its nutritional profile and beneficial properties. The potential neuroprotective effect of an AFA extract, commercialized as KlamExtra®, including the two AFA extracts Klamin® and AphaMax®, in High-Fat Diet (HFD)-fed mice was explored. Three groups of mice were provided with a standard diet (Lean), HFD or HFD supplemented with AFA extract (HFD + AFA) for 28 weeks. Metabolic parameters, brain insulin resistance, expression of apoptosis biomarkers, modulation of astrocytes and microglia activation markers, and Aβ deposition were analyzed and compared in the brains of different groups. AFA extract treatment attenuated HFD-induced neurodegeneration by reducing insulin resistance and loss of neurons. AFA supplementation improved the expression of synaptic proteins and reduced the HFD-induced astrocytes and microglia activation, and Aβ plaques accumulation. Together, these outcomes indicate that regular intake of AFA extract could benefit the metabolic and neuronal dysfunction caused by HFD, decreasing neuroinflammation and promoting Aβ plaques clearance.

Keywords: High-Fat Diet; neurodegeneration; AFA extract; supplementation; inflammation; astrocytes; microglia; amyloid beta

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1. Introduction

The increase in lifespan leads to a growth in the incidence of age-related diseases, including neurodegenerative diseases. These disorders significantly impact the quality of life of patients and their caregivers and relatives, becoming a social and economic burden. However, aging is not the only risk factor for neurodegenerative diseases onset. Increasing evidence in humans and animals has shown a robust correlation between obesity and the development of neurodegenerative diseases, such as Alzheimer's disease (AD) [1,2]. This pathology is characterized by progressive cognitive and memory loss that ultimately ends in dementia [3]. At the root of this condition is the widespread loss of neurons and their synapses in the particular brain area known as the hippocampus and entorhinal cortex. AD histopathological hallmarks are the so-called senile plaques, and neurofibrillary tangles obtained, respectively, by deposition of the aggregated β amyloid peptide (Aβ) and hyperphosphorylated Tau protein [4].

Metabolic changes caused by overweight and unhealthy lifestyle habits are associated with central nervous system (CNS) dysfunction, leading to neuronal death and alteration

of synaptic plasticity that impairs memory ability. Intake of foods rich in fats and sugars and poor in vitamins and minerals are major risk factors for obesity and associated neurodegeneration [5,6].

The standard biological and molecular mechanisms involved in obesity and neurodegenerative diseases include insulin resistance, inflammatory cytokines activation, oxidative stress generation, mitochondrial dysfunction, and cell death. Brain insulin resistance is when brain cells fail to respond to insulin [5,7]. In AD and related disorders, insulin resistance is due to impaired insulin signaling, so AD is denominated as brain diabetes or "Type 3 Diabetes" [8,9]. In addition, central insulin resistance induces mitochondrial alterations, such as mitophagy, mitochondrial quality control, mitochondrial dysfunction, and apoptosis [10]. In agreement with these outcomes, High-Fat Diet (HFD) consumption causes cognitive impairments, reduces synaptic plasticity, induces neuroinflammation, and changes mitochondrial function and astrocytes activation [11,12]. However, the involved mechanisms are not well clarified, and several studies seek discoveries regarding their pathophysiology and prevention.

Since an unhealthy diet is the cause of metabolic dysfunctions and the development and progression of associated comorbidity, nutrition rich in antioxidant and anti-inflammatory compounds could help to reduce the risk of metabolic diseases, such as obesity or Type 2 Diabetes (T2D), and protect from related neurodegenerative disorders. The beneficial effect of antioxidant phytochemicals, as components of functional foods or used in supplementation, on obesity, neurodegeneration, and related comorbidity has been demonstrated in in vitro and in vivo model systems [13–18].

Recent data showed that the daily consumption of pistachios and honey could increase obesity-related dysmetabolic conditions, such as T2D, adiposity, and neurodegeneration in an animal model of diet-induced obesity [19–26].

Among supplements with potent antioxidant and anti-inflammatory effects, the extracts of blue-green algae play a relevant role. Spirulina, for example, contains numerous bioactive molecules, including beta-carotene, phycocyanin, tocopherols, micronutrients, fatty acids, and phenolic compounds [27]. It possesses antioxidant and anti-inflammatory properties and lipid-lowering ability. Further, its benefits on obesity and neurodegeneration can be extended to antiviral, anticancer, antidiabetic, hepatoprotective, and cardioprotective properties [27].

Recently, a growing interest has existed in the *Aphanizomenon flos-aquae* (AFA), another blue-green alga. AFA is a cyanobacterial unicellular organism endowed with several health-enhancing properties and spontaneously grows in Upper Klamath Lake (southern Oregon, USA). AFA contains all the vitamins; it is the only living food with 72 minerals; and it has the most comprehensive spectrum of carotenes, such as beta-carotene, xanthophyll, and an unusually high concentration of chlorophyll [28,29]. Among its bioactive molecules, particularly relevant are phenylethylamine, an important neuromodulator, and a particular type of AFA phycocyanin, also composed of phycoerythrocyanin, with very high antioxidant, anti-inflammatory and antiproliferative properties, that are reinforced by the further presence of mycosporine-like amino acids (MAAs) and various polyphenols [30–33].

Further, it has been reported that the AFA extract Klamin® which concentrates phenylethylamine, can influence mood, reduce anxiety, and enhance attention and learning, suggesting that it could have a role in clinical brain areas [34,35]. An in vitro study on neuronal cells stimulated with Aβ demonstrated that the AFA extract Klamin® plays a protective role in neurodegenerative processes, such as oxidative stress generation, inflammation, and formation of amyloid plaques [36].

Further, the possibility of using the AFA extract to develop functional food for the health and wellness market has been evaluated. Another study considered employing the AFA extract as an additive in biscuit dough, demonstrating that AFA antioxidant properties are also maintained after exposure to high temperatures [37].

Moreover, cellular molecules and mechanism that joins dysmetabolism and obesity-related neurodegeneration have yet to be thoroughly explored. Neuroinflammation is

closely related to the pathogenesis of AD and obesity/T2D [38]. In the brain, astrocytes and microglia cells maintain homeostasis and support many functions of neurons. Moreover, they play an essential role in the inflammatory process of neurodegenerative diseases [39].

Recently a new AFA product reached the market, KlamExtra®, which combines the Klamin® extract (EU patent n° 2046354), which has more specific neuromodulatory and immunomodulatory properties, with the AphaMax® extract (EU patent n° 2032122), which concentrates the AFA-phycocyanins and, so, has increased antioxidant and anti-inflammatory properties.

Here, we aimed to study the effect of KlamExtra® on the molecular mechanisms involved in neurodegeneration induced by obesity. Further, by using glial fibrillary acid protein (GFAP) and soluble triggering receptors expressed on myeloid cells-2 (sTREM-2) as biomarkers, we explored the possibility that AFA can mitigate the central inflammatory process induced by the HFD diet by modulating astrocytes and microglia activation. A possible protective response of AFA in reducing Aβ deposits was also explored. From now on, we shall define KlamExtra® as AFA.

2. Results

2.1. AFA and Metabolic Parameters

The effects of the AFA assumption on animal body weight, food intake, and circulating lipids are illustrated in Figure 1. HFD animals gradually and more rapidly enhanced throughout the twenty-eighth week compared with the Lean group. In AFA-fed mice, the body weight gain was lower than in HFD mice (Figure 1A). At the end of the experimental protocol, the body weight mean values were 34.74 ± 2.14 g for Lean mice, 50.85 ± 1.00 g for HFD animals, and 46.00 ± 2.20 g for the HFD supplemented with AFA groups (Figure 1B). Food intake was approximately similar between the HFD and HFD + AFA groups, but it was significantly different from the Lean group (Figure 1C). Plasma Triglycerides (TG) and Cholesterol (Chol) levels were higher in HFD mice compared with the Lean group or the HFD + AFA group (Figure 1D).

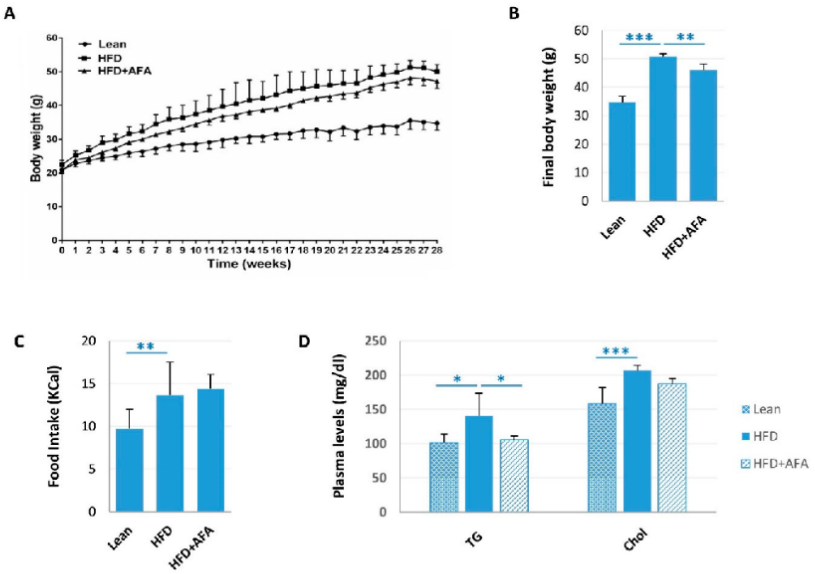

Figure 1. Effects of AFA chronic ingestion on body weight, food intake, and plasma lipids in HFD mice. (**A**) Body weight changes. (**B**) Final body weight. (**C**) Food intake. (**D**) Triglyceride (TG) and total cholesterol plasma (Chol) levels. Data are mean values ± S.E.M. (n = 8 mice/group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. STD mice and vs. HFD-fed mice.

To investigate the effects of AFA on glucose homeostasis, we measured the fasting plasma glucose and performed intraperitoneal glucose and insulin tolerance tests. Interestingly, in the AFA-supplemented HFD group, fasting blood glucose concentration (128.3 ± 6.23 mg/dL) was lower than in the HFD group (153 ± 11.78 mg/dL), and there was no statistically significant difference between Lean animals (122 ± 3 mg/dL) and the HFD + AFA group (Figure 2A).

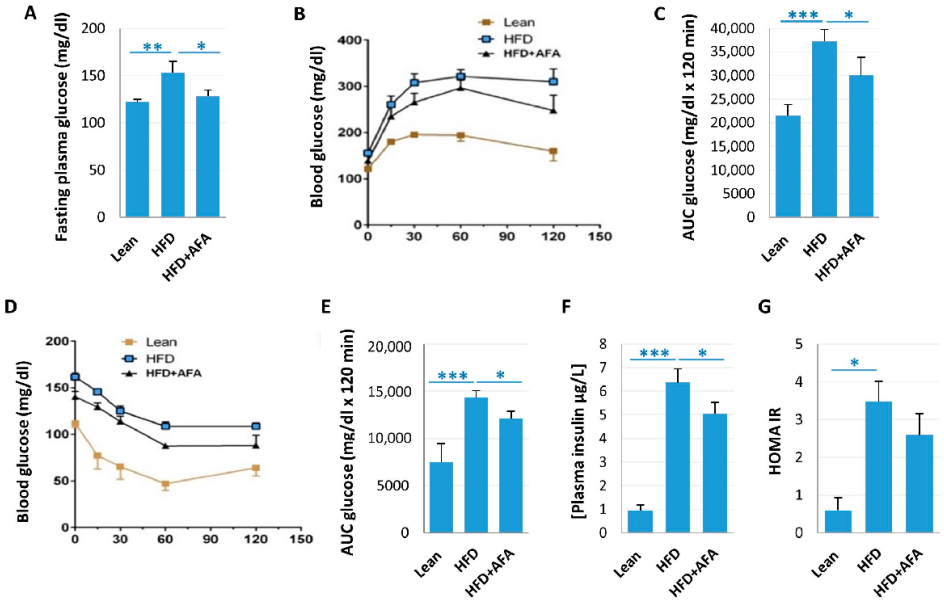

Figure 2. AFA assumption improves glucose dysmetabolism in HFD mice. Fasting glycaemia (**A**), glucose tolerance test (GTT) (**B**), area under the curve (AUC) for GTT (**C**), insulin tolerance test (ITT) (**D**), area under the curve for ITT (**E**), plasma insulin levels (**F**) and HOMA index (**G**). Results are shown as means ± SEM of 8 animals/group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. STD mice and vs. HFD-fed mice.

Figure 2B represents the blood glucose concentrations over 2h after i.p. glucose injection in different mice. The glucose tolerance curve and the related AUC in HFD were significantly higher compared with Lean mice (Figure 2B,C), indicating an impairment of glucose tolerance. The curve and AUC in HFD + AFA were considerably lower than in HFD, suggesting a beneficial effect on glucose homeostasis.

During the insulin tolerance test, HFD mice displayed higher blood glucose concentration than Lean mice (Figure 2D,E), suggesting impaired insulin sensitivity. In HFD + AFA mice, we found lower glycemic values and decreased AUC after insulin injection, suggesting an improved insulin sensitivity (Figure 2D,E).

Insulin concentrations were significantly higher in HFD in comparison with Lean and HFD + AFA mice (Figure 2F). In addition, HOMA-IR was slightly ameliorated in HFD + AFA mice (Figure 2G). These results indicate that AFA chronic ingestion improves insulin resistance and glucose intolerance in HFD mice.

2.2. *AFA Improves Brain Insulin Resistance in HFD Mice*

A reduction of insulin receptor expression and impairment of insulin signaling characterizes insulin resistance. In the brain, insulin resistance has been associated with neurodegenerative disorders [2]. In HFD-fed mice, phosphorylated brain insulin receptor (p-IR) protein expression was decreased compared with the control group, suggesting that

cerebral insulin resistance is diet-induced. In contrast, the HFD + AFA group showed a level of expression of p-IR similar to the Lean group (Figure 3A,B).

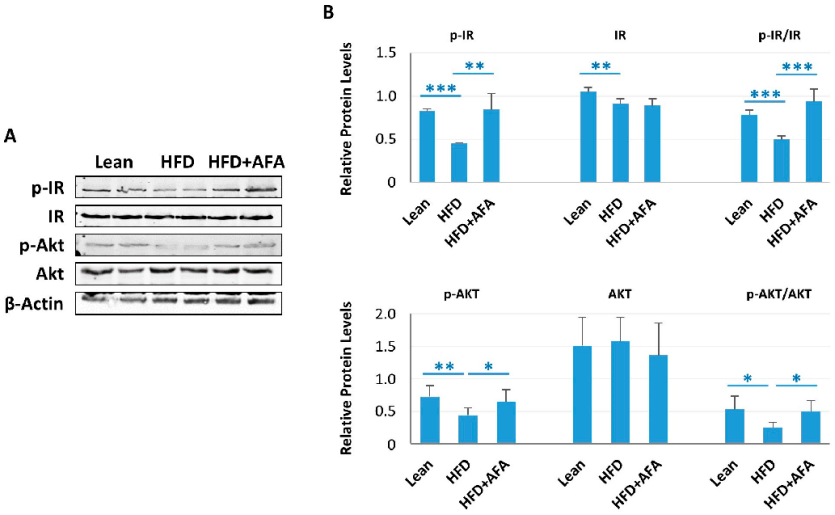

Figure 3. AFA chronic ingestion reduces insulin resistance. (**A**) Immunoblot of total lysate of Lean, HFD and HFD + AFA cortex incubated with anti-IR, anti-phospho-IR (p-IR), anti-Akt, anti-phospho-Akt (p-Akt) and anti-β-actin. (**B**) Quantification of immunoreactivity was performed by using densitometric analysis; uniformity of gel loading was confirmed with β-actin utilized as standard. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$ vs. indicated groups.

Furthermore, we analyzed the expression of proteins involved in insulin signaling in the brain. Decreased levels of posho-Akt were found in HFD mice compared with the Lean group (Figure 3A,B). In contrast, HFD + AFA-fed mice showed higher levels of phospho-AKT, suggesting that AFA extract ingestion can counteract HFD-induced brain insulin resistance (Figure 3A,B).

2.3. AFA Consumption Induces Neuroprotection

The effect of the different diets on brain morphology was analyzed by staining with Hematoxylin–Eosin. Histopathological analysis showed, in the cortex of the HFD group, damaged/disorganized neurons and the presence of numerous pyknotic cells. Further, a robust vacuolization in other brain cortical layers was observed. In contrast, in the HFD + AFA group, neuronal morphology was comparable to the Lean controls, except for certain pyknotic cells (Figure 4A). Further, a clear-cut result regarding the protective role of AFA extract on neurons was obtained by TUNEL assay. A significantly increased number of fragmented nuclei were detected in the cerebral cortex of the HFD group compared with the Lean and HFD + AFA mice, suggesting that AFA extract consumption can attenuate the degenerative neuronal process induced by the HFD diet (Figure 4B,C).

Furthermore, synaptic loss is present in obesity-related neurodegeneration [40]. The presynaptic protein synaptophysin and the postsynaptic protein PSD95 were downregulated in the HFD-fed mice compared with the Lean group. In contrast, HFD + AFA-fed mice exhibited a significant increase in synaptophysin. Although it does not reach significance, a trend towards an increase in PSD95 levels has been observed, suggesting a beneficial effect of AFA extract on synaptic transmission (Figure 4D,E).

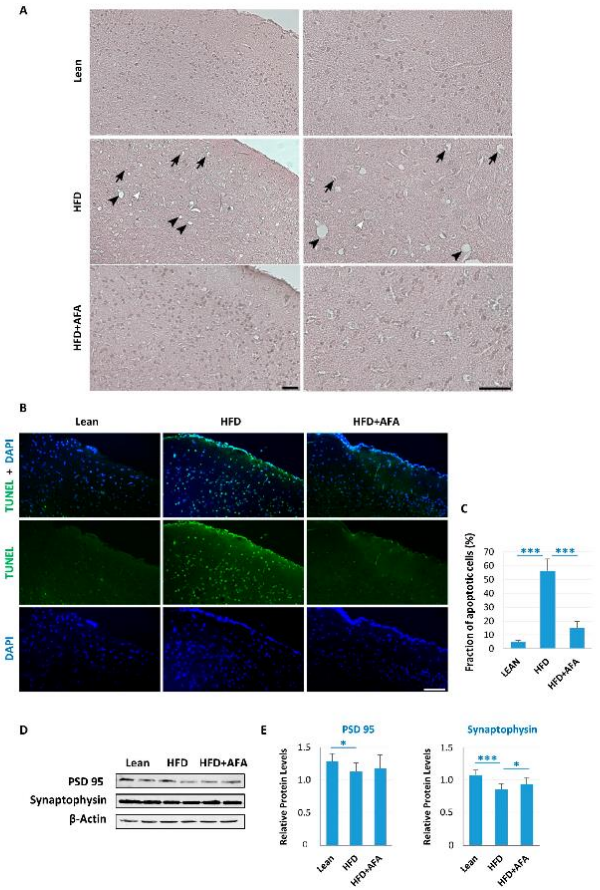

Figure 4. AFA ameliorates degeneration in the brain of diet-induced obese mice. (**A**) Morphology assessments on the cortex of Lean, HFD and HFD + AFA mice with hematoxylin and eosin stain. In the HFD group, there are numerous pycnotic (black arrows) and swollen (white arrows) cells, while strong vacuolation (arrowheads) was observed in all cortical layers. A sharp reduction in degenerative signs is observed in the sections of the AFA-treated group; (**B**) TUNEL assay on cerebral cortex sections; (**C**) Number of apoptotic nuclei in the cerebral cortex of Lean, HFD and HFD + AFA mice. Data are mean values ± S.E.M. (n = 6/group). *** $p < 0.001$. Scale bar, 50 µm; (**D**) Western blot of protein extracted from brain lysates of different mouse groups and incubated with anti-PSD95, anti-synaptophysin, and anti-β-actin antibodies; and (**E**) Quantification of immunoblot was performed by using densitometric analysis; uniformity of gel loading was confirmed with β-actin utilized as standard. Results are shown as means ± SEM of 6 animals/group. * $p < 0.05$, *** $p < 0.001$ vs. indicated groups.

2.4. AFA Reduces Aβ Accumulation

We also examined the levels of expression of BACE1 and PSN1, two enzymes involved in processing APP and Aβ production [41]. Although not significant, BACE1 expression shows an upward trend in the HFD compared with the HFD + AFA group (Figure 5A,B). Furthermore, in the HFD brain, PS1 expression levels showed an increase in both the whole protein (Holo) and the proteolytic fragments NTF and CTF. In the HFD + AFA group, the NTF fragment was reduced (Figure 5A,B).

Further, we investigated neuronal APP-Aβ presence in the brain of different animal groups. Aβ immunoreactivity was reduced in the Lean and HFD + AFA groups compared

with the HFD mice. In addition, in the Lean and HFD + AFA groups, we observed diffuse staining around the nuclei, indicating accumulation of intraneural Aβ. In contrast, in HFD mice, an APP punctate staining around the neurons was found, suggesting an increase in APP processing and Aβ aggregation (Figure 5C). Furthermore, this result was validated by staining with Thioflavin T (Figure 5D), a dye used to visualize the presence of β-sheet protein aggregates or amyloid plaques, whose existence was detected mainly in HFD mice.

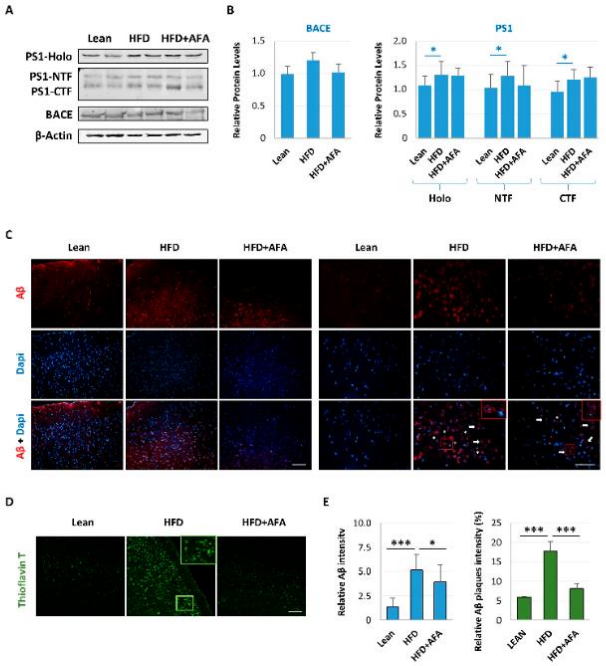

Figure 5. AFA treatment reduces Aβ deposition in HFD mouse brains. (**A**) Immunoblot of total lysate of Lean, HFD and HFD + AFA cortex incubated with anti-PS1, anti-BACE1 and anti-β-actin; (**B**) Quantification of immunoreactivity was performed using densitometric analysis; uniformity of gel loading was confirmed with β-actin utilized as standard. * $p < 0.05$, for Holo protein and PS1 fragments HFD vs. Lean; (**C**) Representative fluorescence images of the cortex of Lean, HFD and HFD + AFA mice. The Aβ fluorescence signal appears in the intracellular compartment of the cortex cells (arrows) in HFD + AFA mice and extracellular Aβ deposits are also present (asterisks) in HFD mice. Nuclei were stained with DAPI. Scale bars, 50 µm and 20 µm. (**D**) Thioflavin T staining of Aβ aggregates on cerebral cortex section of Lean, HFD and HFD + AFA mice. The outlined area in D is enlarged to the right to show the stained plaque. Thioflavin T-positive amyloid deposits are prominent in cortex areas of HFD mouse compared with those from Lean and HFD + AFA counterparts. Scale bar, 100 µm. The outlined areas in C and D are enlarged. (**E**) The number and intensity of Aβ plaques detected by Thioflavin T were quantified using Image J software. All images were taken using a fluorescence microscope. Asterisks indicate significant differences between HFD vs. Lean and HFD + AFA vs. HFD (* $p < 0.05$, *** $p < 0.001$).

2.5. *AFA Counteracts Neuroinflammation*

Peripheral inflammation triggered by obesity is associated with neuroinflammation [5]. Increased expression of TNF-α (Figure 6A) and decreased expression of IL-10 (Figure 6D) were detected in the brain of HFD-fed mice as compared with the Lean group, indicating activation of the inflammatory response. In contrast, in the HFD + AFA group, an expression level similar to that of the Lean mice was found for IL-10 (Figure 6D).

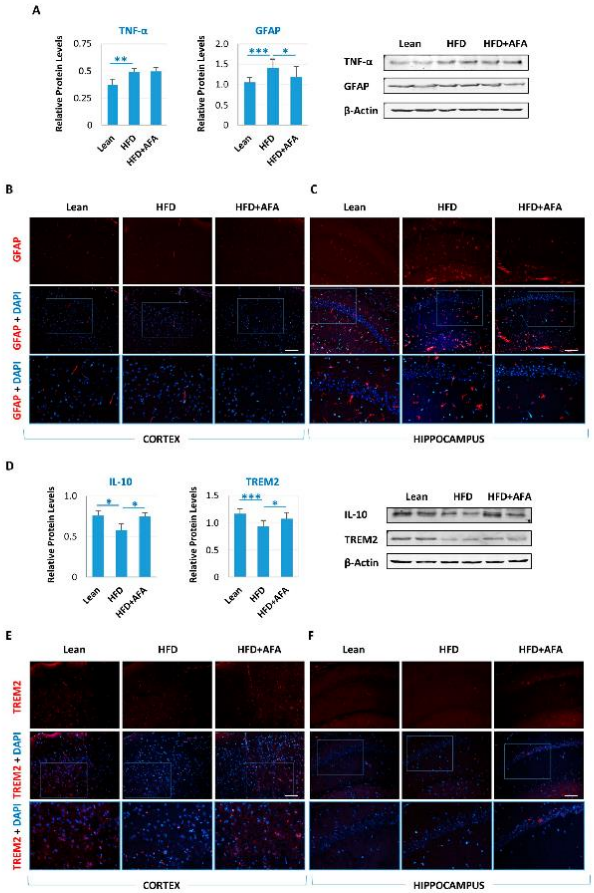

Figure 6. AFA improves inflammation induced by HFD in mouse brains. (**A**) Immunoblot of total lysate of Lean, HFD, and HFD + AFA cortex incubated with anti-TNFα, anti-GFAP, and anti-β-actin. Quantification of immunoreactivity was performed by using densitometric analysis; uniformity of gel loading was confirmed with β-actin utilized as standard. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$ HFD vs. Lean and HFD + AFA vs. HFD; Representative GFAP fluorescence images of the cortex (**B**) and hippocampus (**C**) of Lean, HFD, and HFD + AFA mice. (**D**) Immunoblot of total lysate of Lean, HFD, and HFD + AFA cortex incubated with anti-IL-10, anti-TREM2, and anti-β-actin. Quantification of immunoreactivity was performed by using densitometric analysis; uniformity of gel loading was confirmed with β-actin utilized as standard. * $p < 0.05$, *** $p < 0.001$ HFD vs. Lean and HFD + AFA vs. HFD; Representative TREM2 fluorescence images of the cortex (**E**) and hippocampus (**F**) of Lean, HFD, and HFD + AFA mice. Nuclei were stained with DAPI. Scale bars, 50 µm. The outlined areas in (**B**,**C**,**E**,**F**) are enlarged to show astrocytes and microglia.

Further, we analyzed the expression of GFAP, a biomarker for the activation of astrocytes [42]. The increase of GFAP expression observed in the HFD group with respect to the lean group was significantly counteracted by AFA consumption (Figure 6A). Accordingly, immunofluorescence analysis showed an increase in GFAP intensity mainly in the hippocampus (Figure 6C) of HFD-fed mice compared with the Lean group. In addition, a significant reduction of fluorescent intensity in the brain of HFD + AFA-fed mice indicate that the increase of astrocytes in response to HFD can be counteracted by adding an AFA supplement to the food (Figure 6A–C).

Further, we analyzed the expression levels of TREM2, a receptor expressed mainly on microglia and modulated in obesity-induced insulin resistance [43], a condition in which TREM2 exerts anti-inflammatory and neuroprotective effects. Consistent with the IL-10 result, TREM2 decreased in the brains of the HFD group. In contrast, higher levels of this protein were detected in HFD + AFA-fed mice brains (Figure 6D). We also observed higher TREM2 immunoreactivity especially in the cortex of the Lean and HFD + AFA mice than the HFD group, suggesting a protective effect on microglial cell viability (Figure 6E). All these results indicate that the bioactive molecules contained in AFA extract can have an impact on multiple mechanisms of the neuro-inflammation process and promote the healthy neuronal homeostasis.

2.6. *AFA Modulates Astrocytes and Microglia Activation and Aβ Deposition*

Hippocampus brain sections of Lean, HFD, and HFD + AFA-fed mice were used for GFAP and Aβ staining. Analysis of GFAP immunoreactivity astrocytes increase in response to the HFD diet and the presence of Aβ deposition that was attenuated in HFD + AFA-fed mice (Figure 7A,B). We also examined the presence of TREM2 and Aβ accumulation in the cortex of the different groups. TREM2 reduction observed in HFD-fed mice affected the clustering of microglia around the Aβ deposits, which was improved by AFA supplementation (Figure 7C,D).

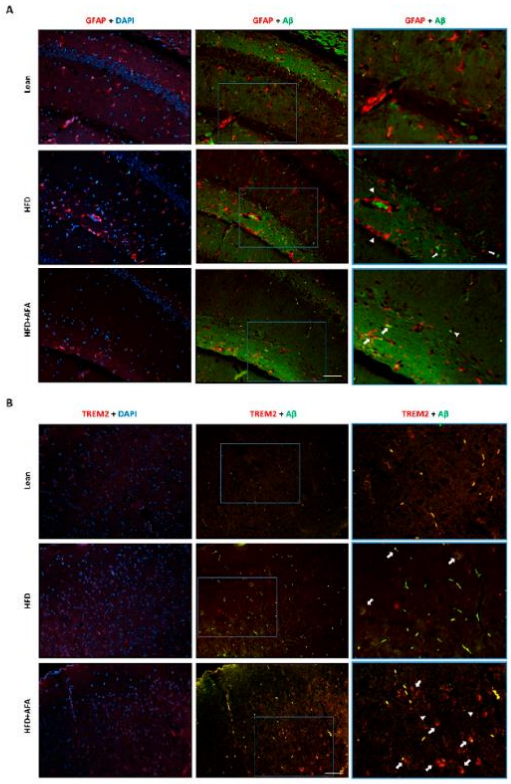

Figure 7. AFA modulates astrocytes and microglia activation induced by HFD in mouse brains. Representative fluorescence images of GFAP + Aβ (**A**) in the hippocampus and TREM2 + Aβ (**B**) in the cortex of Lean, HFD, and HFD + AFA mice. The outlined areas in A and B are enlarged to show the co-staining. Arrows indicate colocalization of astrocytes and microglia with Aβ plaques. Arrowheads indicate Aβ plaques not associated with astrocytes. Nuclei were stained with DAPI. Scale bars, 50 μm.

3. Discussion

Growing evidence indicates how unhealthy nutrition can be considered a potential cause of metabolic-related disease and disorders of the central nervous system (CNS). A correct lifestyle and constant physical exercise can prevent these pathologies, and nutritional supplements can help with this. Here, we explored the impact of the two AFA extracts (KlamExtra®) intake in the brain of obese mice. We applied an HFD feeding protocol to induce obesity and neurodegeneration in mice. We observed the preventive action of AFA extract as a dietary supplement in reducing brain metabolic and molecular impairment. AFA bioactive molecules have the chemical characteristics to cross the BBB, and its dietary consumption can be seen as a way to compensate for the loss of efficacy of the endogenous defenses [44].

A slight but significant reduction in body weight after regular food intake of KlamExtra® was observed in comparison with the obese control group. A considerable reduction of plasma triglyceride levels was also observed in the obese group supplemented with AFA. A high amount of dietary fiber and anti-obesity active compounds, including carotenoids, could account for the positive effect of these algae. Analysis of metabolic parameters showed that HFD fasting glycemia, insulin concentration, and HOMA index were more elevated than in Lean mice, indicating an impairment of glucose metabolism and insulin resistance condition, which was lightly improved by AFA consumption. Thus, the effects of regular AFA extract intake can be assigned to beneficial actions on glucose metabolism.

In line with previous results and in accordance with metabolic data, we demonstrated that brain dysfunction in long-term HFD-fed mice is associated with peripheral and central insulin resistance [2]. Brain insulin resistance in HFD-fed mice at molecular levels was confirmed by the reduced expression of insulin receptors and molecules involved in insulin signaling, such as Akt/p-Akt. In contrast, a supplement of AFA extract counteracted insulin sensitivity and insulin signaling impairment.

It has been widely reported that HFD consumption causes increased neuroinflammation and neuron loss [45]. AFA seems to reduce the neuroinflammatory profile modulating the expression of cytokines and activating astrocytes and microglia through the action of GFAP and TREM2 proteins. An increase of TREM2 and a decrease of GFAP expression in the AFA group suggest a protective reaction to the damage of brain homeostasis induced in the HFD model. In addition, our results could indicate that AFA protects from Aβ injury produced by the HFD diet by promoting microglial clearance.

AFA consumption mitigated degeneration and loss of neurons induced by the HFD diet, as demonstrated by histopathological analysis and TUNEL assay. Further, in the HFD group, loss of neurons was associated with loss of synapses, as suggested by the reduced expression of PSD95 and synaptophysin that was prevented by AFA addition, signifying a beneficial effect of the supplement on neurons' health and communication.

Consistent with this result, we found that specific proteins related to APP processing, including BACE1 and PSN1, were up-regulated in the brain of HFD-fed mice. In contrast, although not significant, their expression level was reduced in HFD + AFA mice. The increased presence of these proteins is associated mainly with the augmented production of Aβ. This complies with the accumulation of extracellular insoluble Aβ fibrillar aggregates and amyloid plaques found in the cerebral cortex of HFD brain sections and evidenced by ThT staining. In contrast, Aβ intracellular presence in the HFD + AFA group suggested that AFA could exert a neuroprotective role by interfering with APP processing and Aβ aggregation.

GFAP is an astrocyte protein overexpressed after a neurological insult known as astrogliosis [46,47]. The astroglial reaction has been observed in a different case in which memory performance is weakened [48]. We found an increase of GFAP and astrocytes in response to hypercaloric feeding suggesting a neuroinflammatory state and the occurrence of astrogliosis that was prevented by AFA supplementation. It has been reported that unhealthy nutrients and metabolites in an HFD diet can impact brain function by crossing the blood–brain barrier (BBB), interacting with neurons and triggering glia [49]. AFA

extract intake could reduce or eliminate metabolites peripherally generated by the HFD diet which, no longer being transported to the brain, elude astrogliosis. In addition, our results agree with the finding that growth factors and cytokines, such as IL-1, IL-6, TNF-α, and reactive oxygen species are among the main signaling molecules that can regulate astrogliosis [46,47].

Several studies have demonstrated that TREM2 protects against neurodegeneration by controlling neuroinflammation closely related to the pathogenesis of AD and obesity [50].

Overexpression of TREM2 was reported to upregulate synaptic proteins synaptophysin and PSD95, improving synaptic transmission in long-term HFD-fed mice, whose loss is related to memory and learning impairment [51]. Similarly, supplementation of AFA to HFD upregulates TREM2 and synaptic proteins, suggesting that it supports neuron metabolism and synapses.

While astrocytes have been assigned the role of filling tissue voids caused by degenerative events, microglia, the brain's immune cells, function as brain phagocytes responsible for removing debris from degenerating neurons and Aβ deposit that interferes with neuron communication [52].

Further, it was also demonstrated that TREM2 is essential for promoting microglial clustering around fibrillar Aβ plaques in AD mouse models and postmortem human brain sections [53]. Its deletion induces a reduction in plaque-associated microglia [53,54]. In addition, during AD development, homeostatic microglia respond to Aβ accumulation evolving into disease-associated microglia (DAM) [55].

This is consistent with the immunofluorescence results in which, in the AFA group, an increase of TREM2 is associated with a reduction of the formation of Aβ deposits. Further, the data examined here indicate that TREM-2 regulates microglia activation in response to dietary factors.

Recently, a study on patients affected by neurogenerative diseases has evidenced altered levels of GFAP and TREM-2 in CSF, suggesting that they could be used as biomarkers of central inflammation and have been proposed as prognostic tools of neurodegenerative progression [56].

However, we cannot exclude that the improved metabolic conditions and the restored homeostasis observed in the brain of the HFD + AFA group might have slowed down the APP processing and Aβ deposition, favoring the microglia response.

In conclusion, our results suggest that KlamExtra®, a natural product, works as a "functional food" to activate a compensatory mechanism mainly for mitigating HFD-induced systemic and central dysmetabolism.

AFA extract can alleviate central neuroinflammation HFD- induced by regulating astroglial and microglial activation and modulating anti-inflammatory cytokines. These outcomes associated with increased synaptic protein expression and Aβ plaques removal suggest that AFA extract could have promising protective activity on neurodegenerative diseases. However, additional investigations are necessary to confirm our findings.

4. Materials and Methods

4.1. Animals and Diets

All animals received care in compliance with the recommendations of the European Economic Community (2010/63/UE) and the guidelines for animal experimentation (Italian D.L. No. 26/2014 and subsequent variations). The Ministry of Health authorized the experimental protocol (Rome, Italy; Authorization Number 46/2020-PR, date of approval: 21 January 2020).

KlamExtra® includes the two AFA extracts Klamin® and AphaMax® whose composition was previously described in EU patents n° 2046354and 2032122 and by Nuzzo et al. [36].

Four-weeks-old male C57BL/6J mice were purchased from Harlan Laboratories (San Pietro al Natisone, Udine, Italy). As described in previous papers [22,23], after a 1-week habituation period, the animals were weighed and divided into separated three groups:

(A) Lean group (Lean, $n = 8$) fed a standard diet consisting of 70% of energy as carbohydrate, 20% proteins and 10% fat (code 4RF25, Mucedola, Milan, Italy); (B) High-Fat Diet group (HFD, $n = 8$) fed HFD (PF4215, Mucedola, Milan, Italy) that supplied 60% of energy as fat, 20% proteins and 20% carbohydrates; (C) mice fed a HFD supplemented with KlamExtra® (HFD-AFA, $n = 8$) for 28 weeks. HFD-AFA was custom designed and prepared by Mucedola S.r.l (4RF25), obtained by adding 8.3 g of KlamExtra/Kg HFD. All animals (two animals per cage) were maintained under a 12 h dark–light cycle at 23 ± 1 °C and 55% ± 5% humidity, with free access to food and water ad libitum.

Bodyweight and food intake were detected weekly throughout the study. At the end of the study period, metabolic parameters were analyzed, then the animals were sacrificed by cervical dislocation. Blood was immediately drawn by cardiac puncture, and plasma was recovered after centrifugation at 3000 rpm at 4 °C for 15 min and stored at −80 °C until analysis. The aorta was cannulated and perfused with Dulbecco's buffered solution containing 2 mM EDTA, and the right atrial incision allowed blood outflow. Brains were explanted, weighed, and processed for subsequent analysis. AFA (0.9 mg/mouse) was administered daily for 28 weeks. The doses given to the Diet-Induced Obese (DIO) mice were extrapolated from the human dosage (1.6 g/day) and calculated on the basis of the average body weight (40 mg) [22].

4.2. Metabolic Parameters

Plasma triglyceride and total cholesterol were measured using the ILAB 600 Analyzer (Instrumentation Laboratory, Bedford, MA, USA). Fasting blood glucose concentrations were determined by a glucometer (GlucoMen LX meter, Menarini, Florence, Italy). Intraperitoneal glucose tolerance test (IPGTT) and insulin tolerance test (ITT) were carried out in overnight-fasting mice. For IPGTT, mice were injected intraperitoneally (i.p.) with glucose (2 g/kg b.w.) (D-glucose, Sigma-Aldrich, Milan, Italy) in 0.9% saline. For ITT, mice were injected i.p. with insulin (0.5 U/kg b.w.) (Insuman Rapid, Sanofi Aventis, Italy) in 0.9% saline. Tail-vein-measured glucose concentrations were taken at different time points (0, 15, 30, 60, and 120 min). Plasma insulin was quantified using a mouse ELISA kit (Alpco diagnostics, Salem, NH, USA) according to the manufacturer's instructions, and the HOMA-IR, index of insulin resistance, was calculated as the ratio of fasting insulin (ng/mL) and fasting glucose (mg/dL) divided by the constant 22.5.

4.3. Total Protein Extraction and Western Blot

Brain tissue from Lean, HFD and HFD + AFA mice was homogenized in RIPA buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM Na3VO4, 10 mM NaF, 1mM EDTA, 1 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, 1% Triton, 0.1% SDS, and 0.5% deoxycholate) with protease inhibitor (Amersham, Life Science, Les Ulis, France) and phosphatase inhibitor cocktail (Sigma-Aldrich, Poole, Dorset, UK). The samples were sonicated and centrifuged for 30 min at 14,000 rpm at 4 °C to remove insoluble material, and the supernatants were quantified by the Bradford method (Bio-Rad) and collected. Proteins (50 µg) were separated by 10% or 12% acrylamide gel and were transferred onto a nitrocellulose filter. The filter was incubated with the anti-insulin receptor (IR) (1:1000, Invitrogen, Waltham, MA, USA), anti-phospho insulin receptor (p-IR) (1:1000, Invitrogen, Waltham, MA, USA), anti-protein kinase B (Akt) (1:1000, Cell Signaling Technology, Danvers, MA, USA), anti-phospho protein kinase B (p-Akt) (1:1000, Cell Signaling Technology, Danvers, MA, USA), anti-presenilin1 (PSN1) (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-beta secretase enzyme 1 (BACE1) (1:500, Cell Signaling Technology, Danvers, MA, USA), anti-postsynaptic density protein 95 (PSD-95) (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-synaptophysin (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-tumor necrosis factor α (TNFα) (1:500, Thermo Fisher, Preprotech, Waltham, MA, USA), anti-glial fibrillary acidic protein (GFAP) (1:1000, Cell Signaling Technology Danvers, MA, USA), anti-interleukin 10 (IL-10) (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), an anti-triggering receptor expressed on myeloid cells 2 (TREM2) (1:1000,

Invitrogen, Waltham, MA, USA), and anti-β-actin (β-Actin; 1:10,000, Sigma-Aldrich, St. Luois, MO, USA). Primary antibodies were detected using the Odyssey® scanner (Li-cor), according to the manufacturer's instructions, using secondary antibodies (anti-mouse and anti-rabbit) labeled with IR790 and IR680 (1:10,000; Life Technology, Carlsbad, CA, USA). Band intensities were analyzed with ImageJ software, and expression was adjusted to β-Actin expression. The protein levels were expressed as intensity relative to the control.

4.4. Histopathology and Immunohistochemistry

For histopathological and immunohistochemical analyses, whole-brain specimens/samples were collected and fixed in 4% paraformaldehyde in 0.1 M PBS (pH 7.4) overnight at 4 °C and then transferred into dehydrated in graded ethanol and paraffin-embedded. Serial sagittal sections (5μ thick) were deparaffinized in xylene, rehydrated with a graded series of ethanol, and then processed for routine hematoxylin and eosin (HE) used according to the manufacturer's instructions. For immunofluorescence assays, sections were treated in 10 mM citrate buffer (pH 6.0) with microwaves for 6 min at 500 W for antigen retrieval. Then, cells were blocked with 10% normal goat (NGS) serum in PBS for 1 h at room temperature (RT) and were incubated at 4 °C overnight with anti-GFAP (1:200), anti-TREM2 (1:200), and anti-Aβ (1:100) primary antibodies (3% NGS in PBS and 0,3% Triton X-100, PBS-T). Immunopositive reactions were detected by incubation with the anti-rabbit Alexa 488 or anti-mouse Alexa 555 (1:500; Cell Signaling Technology) secondary antibodies (2% NGS in PBS) for 2 h at RT.

Finally, all sections were coverslipped using Sigma mounting medium, including 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories), and visualized with Axioskop-2 Zeiss or Leica DM4000 microscope. All photomicrographs were collected using the same magnification (10×, 20×, or 40× objectives), exposure time, and other parameters, and the representative images from five sections for each brain specimen were edited with Adobe Photoshop software. Negative controls were performed for every set of experiments by omitting the primary antibodies.

For quantitative analysis, the images were imported into the ImageJ software program (NIH, Bethesda, MD), converted to grayscale, and the total area of immunoreactivity was quantified by measuring the mean intensity of all stained areas of each micrograph, and the data were expressed as mean density.

4.5. Thioflavin T Staining

Following deparaffinization with xylene and ethanol, tissue sections were incubated in 70 μM of Thioflavin T (ThT, Sigma-Aldrich, St. Louis, MO, USA) for 10 min at RT. Sections were then rinsed in 70% ethanol. After washing in deionized water, the slides were mounted in aqueous mounting media. The images were visualized by a Leica DM4000 microscope at 10× magnification. The number and area of plaques detected by ThT were quantified using Image J software.

4.6. TUNEL Assay

In situ detection of DNA fragmentation was performed using the DeadEnd™ Fluorometric TUNEL Detection System kit (Promega, Madison, WI, USA), following the manufacturer's instructions. Pre-incubating sections performed positive controls with DNase I for 10 min at room temperature, and negative controls by omitting the TdT enzyme. Nuclei were stained with DAPI (Vector Lab, Burlingame, CA, USA), and the slides were analyzed using a fluorescent microscope (Leica Microsystems) at a magnification of 20X. TUNEL-positive cells were quantified and results were expressed as mean ± SEM values of three different experiments.

4.7. Statistical Analysis

The results are presented as the mean ± the standard error of the mean (SEM). The letter 'n' indicates the number of animals. Statistical analyses were performed using

the Prism Version 6.0 Software (Graph Pad Software, Inc., San Diego, CA, USA). The comparison between the groups was performed by one-way Analysis of Variance (ANOVA), followed by Bonferroni's post-test for significance analysis. Results with a p-value ≤ 0.05 were considered statistically significant.

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Institutional Review Board Statement: The study was conducted according to the Italian legislative decree n. 26/2014 and the European Directive 2010/63/UE and approved by the Ministry of Health (Rome, Italy; Authorization n. 46/2020-PR).

Data Availability Statement: The data presented in this study are available from the corresponding author on reasonable request.

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Conflicts of Interest: S.S. is the R&D Director of the company that owns KlamExtra®'s patent.

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III° ARTICLE

POSITIVE IMPACT OF INDICAXANTHIN FROM *OPUNTIA FICUS-INDICA* FRUIT ON HIGH-FAT DIET–INDUCED NEURONAL DAMAGE AND GUT MICROBIOTA DYSBIOSIS

The objective of the study was to assess the neuroprotective efficacy of indicaxanthin and its impact on gut dysbiosis in a murine model of HFD-induced obesity.

Four-week-old mice were fed a standard diet (STD) or a HFD for a period of 10 weeks. Thereafter, the HFD mice were divided into two groups: an obese control group (HFD) and an HFD + Ind group (subjected to indicaxanthin treatment) for the final 4 weeks. The potential neuroprotective effects were evaluated through analyses of brain apoptosis, redox status, and inflammation. Additionally, fecal samples were used to evaluate potential changes in microbiota composition.

Indicaxanthin treatment attenuated the neurodegeneration typically induced by HFD, as evidenced by a reduced number of apoptotic cells in the cerebral cortex and by the specific pro-apoptotic and anti-apoptotic gene expressions.

In terms of oxidative stress, indicaxanthin reduced malondialdehyde, RONS and NO levels, while upregulating SOD-2 expression and activating NRF-2, a key protein in cellular antioxidant defence. The treatment also attenuated neuroinflammatory responses, as evidenced by reduced expression of inflammation-related proteins and genes, and decreased GFAP immunoreactivity in the cortex.

Beyond neural effects, indicaxanthin positively affected gut microbiota, increasing beneficial function associated genera, known to produce SCFAs, such as *Lachnospiraceae*, *Alloprevotella* and *Lactobacillus*, while reducing bacteria associated with negative health outcomes, such as *Blautia*, *Faecalibaculum* and *Bilophila*.

Together, these findings suggest that indicaxanthin both protects against HFD-induced brain degeneration and supports a healthier gut microbiota, highlighting a potential interplay between neuroprotection and microbial composition

Research Article

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Positive impact of indicaxanthin from *Opuntia ficus-indica* fruit on high-fat diet–induced neuronal damage and gut microbiota dysbiosis

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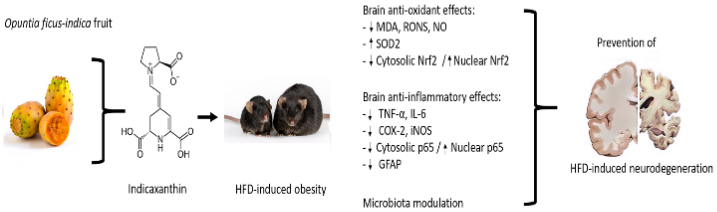
From the Contents

Graphical Abstract

Neuroprotective effects of indicaxanthin against neuronal damage induced by a high-fat diet (HFD)



Abstract

Indicaxanthin is a betalain that is abundant in *Opuntia ficus-indica* orange fruit and has antioxidative and anti-inflammatory effects. Nevertheless, very little is known about the neuroprotective potential of indicaxanthin. This study investigated the impact of indicaxanthin on neuronal damage and gut microbiota dysbiosis induced by a high-fat diet in mice. The mice were divided into three groups according to different diets: the negative control group was fed a standard diet; the high-fat diet group was fed a high-fat diet; and the high-fat diet + indicaxanthin group was fed a high-fat diet and received indicaxanthin orally (0.86 mg/kg per day) for 4 weeks. Brain apoptosis, redox status, inflammation, and the gut microbiota composition were compared among the different animal groups. The results demonstrated that indicaxanthin treatment reduced neuronal apoptosis by downregulating the expression of proapoptotic genes and increasing the expression of antiapoptotic genes. Indicaxanthin also markedly decreased the expression of neuroinflammatory proteins and genes and inhibited high-fat diet–induced neuronal oxidative stress by reducing reactive oxygen and nitrogen species, malondialdehyde, and nitric oxide levels. In addition, indicaxanthin treatment improved the microflora composition by increasing the abundance of healthy bacterial genera, known as producers of short-chain fatty acids (*Lachnospiraceae*, *Alloprovetella*, and *Lactobacillus*), and by reducing bacteria related to unhealthy profiles (*Blautia*, *Faecalibaculum*, *Romboutsia* and *Bilophila*). In conclusion, indicaxanthin has a positive effect on high-fat diet–induced neuronal damage and on the gut microbiota composition in obese mice.

Key Words: gut microbiota dysbiosis; high-fat diet; indicaxanthin; microflora; neuronal apoptosis; neurodegeneration; neuroinflammation; obesity; *Opuntia ficus-indica* fruit

Introduction

According to the World Health Organization (WHO), obesity is rapidly increasing worldwide and affects more than 1.12 billion people by 2030 (World Health Organization, 2021). Obesity is caused mainly by a high intake of sugars and fat associated with a sedentary lifestyle and can contribute to various health problems that reduce quality of life and life expectancy, leading to the development of insulin resistance, type 2 diabetes, dyslipidemia, nonalcoholic fatty liver disease and cardiovascular disease. Chronic low-grade inflammation and oxidative stress are linked to these various pathological conditions (Marseglia et al., 2015). Furthermore, obesity affects brain homeostasis, becoming a crucial cause of the development of dementia (O'Brien et al., 2017; Pugazhenthi et al., 2017). Consequently, chronic consumption of a high-fat diet (HFD) is considered a risk factor for cognitive impairment,

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early brain aging and even Alzheimer's disease (AD) (Nuzzo et al., 2015; Snowden et al., 2017; Chudoba et al., 2019; Galizzi et al., 2023). Cerebral damage induced by HFD has been widely characterized in animal models. This process involves neuroinflammation, extensive oxidative stress, alterations of the blood–brain barrier, synaptic loss, central insulin resistance and neuronal cell death (Dinel et al., 2011; Valladolid-Acebes et al., 2011; Nuzzo et al., 2015, 2020; Kim et al., 2016; Wanrooy et al., 2018; Galizzi et al., 2023; Terzo et al., 2023). Several mechanisms have been proposed to link obesity to central nervous system impairment (Picone et al., 2020), including changes in the gut microbiota (Zhang et al., 2019; Shi et al., 2020), but no therapies have been identified yet. Indeed, obese people consuming HFD exhibit gut microbiota dysbiosis associated with worse cognitive ability (Shi et al., 2020), and in rodents, HFD-induced microbiota alterations can impair learning and memory (Zhang et al., 2019). Interestingly, a recent study reported that many cases of dementia may be prevented by addressing diet and lifestyle (Livingston et al., 2020). In particular, a plethora of potential bioactive compounds or food components, such as monoterpene and triterpene polyphenolic compounds, have been reported to possess neuroprotective properties (Habtemariam, 2018; Noori et al., 2021; Wang et al., 2022).

Indicaxanthin (Ind), a phytochemical abundant in the yellow fruits of Opuntia ficus-indica, has been shown to act as an antioxidant in several *in vitro* and *in vivo* experiments (Rahimi et al., 2018; Allegra et al., 2019; Attanzio et al., 2019). Moreover, it exerts anti-inflammatory effects in various models of acute and chronic inflammation, prevents insulin resistance and improves obesity-related glucose dysmetabolism in HFD-fed mice (Allegra et al., 2014; Terzo et al., 2021). Ind is highly bioavailable (Tesoriere et al., 2004) and can cross the blood–brain barrier (Allegra et al., 2015). Nevertheless, very little is known about its neuroprotective potential (Campisi et al., 2021), despite its healthy effects.

In light of the biological activities described above, the purpose of the present study was to evaluate the neuroprotective potential of Ind against the neuronal damage and gut microbiota dysbiosis induced by HFD in animal models.

Methods

Extraction and purification of Ind

Ind was extracted from *Opuntia ficus-indica* fruits (yellow cultivar, San Cono, Sicily, Italy) as detailed in Italian Patent Application No. 102021000015167, filed on 10.06.2021. In brief, fruits were peeled and cut, the pulp was separated from the seeds, and 100 g was homogenized and centrifuged at 3000 × *g* for 10 minutes. The supernatant was recovered, and the pellet was extracted with 100 mL of distilled water and centrifuged as described above. The combined supernatants were subjected to cryodesiccation, and Ind in the resulting aqueous extract was separated by size exclusion chromatography on Sephadex G-25. Fractions containing the pigment were subjected to cryodesiccation, followed by solid-phase extraction on J.T. Baker, Bakerbond SPE C18 columns (VWR, Milan, Italy). The eluate was subjected to rotary evaporation to remove methanol, and the residue was dissolved in phosphate-buffered saline (PBS). The Ind concentration was evaluated via spectrophotometric revelation at 482 nm, with an extinction coefficient of 48/mM/cm. All the samples were aliquoted and stored at –80°C until further use.

Animals and diet

Four-week-old male C57BL/6J (B6) mice (*n* = 24; body weight 18–20 g), purchased from Envigo Laboratories (San Pietro al Natisone, Udine, Italy), were housed in the animal house of the Advanced Technologies Network (ATeN) center under specified environmental conditions (12-hour light/dark cycle, temperature 22–24°C, relative humidity [55 ± 5]%), with free access to water and food.

We chose to use only male mice to avoid hormonal changes linked to the reproductive cycle of the female animals, as previously reported (Terzo et al., 2021). The procedures for the care and use of laboratory animals conformed with European guidelines and were approved by the Ministry of Health (Rome, Italy; Authorization No. 37/2020-PR on 16 January 2020). After 1 week of acclimation, the mice were weighed and randomly divided into two groups, which were fed a standard diet (STD) (70% energy as carbohydrates, 20% protein, and 10% fat; 4RF25, Mucedola, Milan, Italy) (negative control group; *n* = 8) or HFD (*n* = 16) (60% energy as fat, 20% protein, and 20% carbohydrates; PF4051/D, Mucedola) to generate diet-induced obesity. After 10 weeks on HFD, the obese mice were further separated into two subgroups: one group was fed HFD (positive control group; *n* = 8), and the other was fed HFD while receiving Ind orally twice a day (0.4 mg/kg) for 4 weeks (HFD + Ind group, *n* = 8). The mice were assigned to the positive control (HFD) or HFD + Ind group on the basis of body weight to obtain two groups with similar initial conditions before treatment. Ind was administered in water (20 µL). Ind was tested at a nutritionally relevant dose that had previously been demonstrated to prevent glucose impairment and insulin resistance in obese mice (Terzo et al., 2021). At the end of the experimental protocol period, the animals were weighed and sacrificed, and blood was collected via cardiac puncture. The brains were rapidly removed from their skulls, weighed, and cut into two halves coronally. A portion was fixed in 4% formalin for histological assays; the other portion was stored at –80°C for biomolecular analyses. Blood samples were centrifuged at 5,7297 × *g* at 4°C for 15 minutes to obtain plasma, which was stored at –80°C (**Additional Figures 1** and **2**).

Metabolic parameters

The concentrations of triglycerides and cholesterol in blood were determined via a MultiCareIn biochemistry analyzer (Biochemical Systems International, Arezzo, Italy), whereas blood fasting glucose concentrations were determined via a commercial glucometer (GlucoMen LX meter, Menarini, Italy).

Brain tissue preparation

Explanted mouse brains from the STD, HFD, and HFD + Ind groups were coronally divided into two halves. One part was homogenized on ice via a manual Dounce homogenizer

(Omni International, Kennesaw, GA, USA), separated into aliquots (5 or 10 mg), immediately transferred to liquid nitrogen and stored at –80°C until analysis (gene expression, immunoblotting, enzyme-linked immunosorbent assay [ELISA], malondialdehyde [MDA] assays, reactive oxygen and nitrogen species [RONS] analyses and nitric oxide [NO] levels). The other part was used for immune-histological assays. Thus, the halves of each brain were fixed in 4% formalin for 24 hours, followed by incubation with graded ethanol (50%, 70%, 85%, and 96%) for 5 minutes each, embedded in paraffin overnight and subsequently sectioned (5 µm thick) via an automatic microtome (Leica Biosystems, Buffalo Grove, IL, USA) (terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling [TUNEL] assay and immunofluorescence).

Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling assay

Apoptosis was evaluated via the TUNEL assay via a cell death detection kit (Promega, Madison, WI, USA) as previously reported (Nuzzo et al., 2015, 2020; Shi et al., 2020). Briefly, brain samples were cut into 5 µm slices after being embedded in paraffin. Then, the deparaffinized slices were hydrated in a series of graded ethanol solutions (96%, 85%, 70%, and 50%) for 5 minutes each, washed in PBS and incubated with TUNEL working solution for 1 hour at 37°C. Diamidino-2-phenylindole (DAPI) (Invitrogen -Thermo Fisher Scientific, Waltham, MA, USA) solution was used to stain the nucleus. Slices were observed under a fluorescence microscope (Leica Microsystems, Heidelberg, Germany) at a magnification of 20×, and images were collected via the image analysis software Basic Research NIS Elements F 2.30 (Nikon, Florence, Italy). The number of apoptotic nuclei was manually counted by two researchers, who were blinded to the diet type, and the results are expressed in relation to normal nuclei observed in selected fields of the cerebral cortex.

Gene expression

RNA was extracted from the homogenized brain tissue via an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. A high-capacity cDNA reverse transcription kit (Applied Biosystems, Waltham, MA, USA) was subsequently used to reverse transcribe 2 ng of total RNA into cDNA. Only high-quality RNA samples were used for the experiments.

Semiquantitative polymerase chain reaction experiments

Semiquantitative polymerase chain reaction (PCR) experiments were performed to evaluate the expression of proapoptotic and antiapoptotic genes (*Fas-L*, *Bim*, *P27*, *Bcl-2*, and brain-derived neurotrophic factor [*BDNF*]). The primer sequences are listed in **Table 1**. Semiquantitative gene expression was evaluated by densitometry. The band intensities of the specific amplicons were obtained via ImageJ software (version 1.53f, Laboratory for Optical and Computational Instrumentation-LOCI, University of Wisconsin, USA) and normalized to the corresponding *β-actin* mRNA expression. The expression levels of the mRNAs are represented as the ratio of the mean optical density of the mRNA to that of *β-actin* and are shown as arbitrary units.

Table 1 | Semiquantitative polymerase chain reaction primer sequences for various genes

Gene	Forward primer (5'–3')	Reverse primer (5'–3')	Size (bp)
Fas-L	5'-AGC AGT CAG CGT CAG AGT TC-3'	5'-GTA CTG GGG TTG GCT CAC G-3'	442
Bim	5'-GGA GGA GGC GGA GGA TGA T-3'	5'-TCC TGT CTT GCG GTT CTG TC-3'	366
P27	5'-AGA TAC GAG TGG CAG GAG GT-3'	5'-TGT TTA CGT CTG GCG TCG AA-3'	381
Bcl-2	5'-ATG TGT GTG GAG AGC GTC AA-3'	5'- AGA GAC AGC CAG GAG AAA TCA-3'	182
BDNF	5'-GGC TGA CAC TTT TGA GCA CGT C-3'	5'-CTC CAA AGG CAC TTG ACT GCT G-3'	133
β-actin	5'-CGG GAT CCC CGC CCT AGG CAC CAG GGT-3'	5'-GGA ATT CGG CTG GGG TGT TGA AGG TCT CAA A-3'	301

Real-time PCR assays

Real-time PCR (qPCR) was performed to evaluate the expression of inducible nitric oxide synthase (*iNOS*), tumor necrosis factor-α (*TNF-α*) and interleukin (*IL-6*) genes in the brains of the mice. cDNA was amplified via RT2 SYBR Green/ROX qPCR Mastermix (Qiagen) and a StepOne Real-Time instrument (Applied Biosystems). The primer sequences are listed in **Table 2**. Real-time PCR experiments were performed in compliance with the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines (Bustin et al., 2009). The amplification efficiencies for all the genes analyzed ranged from 88% to 102%. Gene-specific amplification was confirmed by a single peak in the melting curve analysis and a single band of the expected size on a 2% agarose gel stained with SYBR™ Green nucleic acid gel stain. The average standard deviation of all the samples studied was 0.10 cycles. Relative mRNA expression was calculated via the $2^{-\Delta\Delta C_T}$ approximation method via SDS software (Applied Biosystems). The expression of the *β-actin* gene was also quantified in a similar way for normalization. The expression levels of mRNAs are indicated as the fold change.

Table 2 | Quantitative polymerase chain reaction primer sequences for various genes

Gene	Forward primer (5'–3')	Reverse primer (5'–3')	Size (bp)
iNOS	5'-GCA GAA TGT GAC CAT CAT GG-3'	5'-ACA ACC TTG GTG TTG AAG GC-3'	503
TNF-α	5'-GCC CAC GTC GTA GCA AAC CAC-3'	5'-GGC TGG CAC CAC TAG TTG GTT GT-3'	260
IL-6	5'-TCC AGT TGC CTT CTT GGG AC-3'	5'-GTG TAA TTA AGC CTC CGA CTT G-3'	600
β-actin	5'-CGG GAT CCC CGC CCT AGG CAC CAG GGT-3'	5'-GGA ATT CGG CTG GGG TGT TGA AGG TCT CAA A-3'	301

Western blot analysis

Protein quantification from homogenate brain tissue was performed via Bradford assays. Fifty micrograms of protein sample was resolved via SDS-PAGE (Sigma-Aldrich, St. Louis, MO, USA) on 8% acrylamide gels and blotted onto nitrocellulose membranes (Thermo Fisher Scientific, Monza, Italy). After blocking for 2 hours in 5% (w/v) skim milk, the membranes were incubated overnight at 4°C in the presence

of the primary antibodies shown in **Table 3** (1:1000 dilution, Santa Cruz Biotechnology, Milan, Italy). The membranes were then incubated for 90 minutes at room temperature with goat anti-mouse IgG and HRP-conjugated secondary antibodies (1:10,000 dilution; Merck, Milan, Italy; Cat# 12--349; RRID: AB_390192). Specific chemiluminescent bands were detected via a C-Digit Blot Scanner (LI-COR, Lincoln, NE, USA), and densitometry was performed via LI-COR Image Studio 4.0 (LI-COR). Protein expression levels were normalized to those of β-actin and laminin β.

Table 3 | **Primary antibodies used for Western blot analysis**

Protein	Cat#	Host organism	RRID	Clone	Molecular weight (kDa)
COX-2	sc-376861	Mouse	AB_2722522.	H-3	72/70
iNOS	sc-7271	Mouse	AB_627810	C-11	130
p65	sc-8008	Mouse	AB_628017	F-6	65
SOD-2	sc-137254	Mouse	AB_2191808	E-10	25
Nrf-2	sc-365949	Mouse	AB_10917561	A-10	61
Insulin Rβ	sc-57342	Mouse	AB_784102	CT-3	95
β-actin	sc-47778	Mouse	AB_626632	C-4	43
Lamin B	sc-365962	Mouse	AB_10848566	C-5	67

COX-2: Cyclooxygenase-2; iNOS: inducible nitric oxide synthase; SOD-2: superoxide dismutase-2.

Enzyme-linked immunosorbent assay

TNF-α and IL-1β levels in mouse plasma were measured via commercially available ELISA kits (Life Technologies, Frederick, MD, USA); plasma insulin levels were quantified via an ELISA kit (Mercodia, Uppsala, Sweden) and calculated via a plate reader system (Synergy HT microplate reader, Biotech, Winooski, VT, USA).

Immunofluorescence

Portions of the brains removed from the mice were fixed in 4% formalin for 24 hours and embedded in paraffin. Coronal sections (5 μm) were mounted on slides and deparaffinized using xylene solution. Next, the sections were incubated with a mouse primary antibody against glial fibrillary acidic protein (GFAP) (1:300, Cell Signaling Technology, Danvers, MA, USA, Cat# 3670, RRID: AB_10693476) at 4°C overnight and then with an anti-mouse IgG-Alexa Fluor 594 secondary antibody (goat, 1:300, Invitrogen, Cat# A-11005, RRID: AB_2534073) for 2 hours at room temperature. Nuclear staining was performed via Hoechst 33258, and the samples were visualized with a Leica DM4000 microscope (Leica Microsystems) at a magnification of 10×. Negative controls were generated by omitting the primary antibodies. GFAP marker expression was calculated as the percentage of cells that were doubly positive for Hoechst and GFAP compared with the total number of Hoechst-positive cells.

Malondialdehyde assay

MDA levels were assessed in homogenized tissue obtained from coronal portions of mouse brains according to a previously reported method (Allegra et al., 2015). Briefly, a mixture containing 0.2 mL of homogenate, 0.2 mL of 8.1% SDS, 1.5 mL of acetic acid solution at pH 3.5 with NaOH, and 1.5 mL of 1% TBA aqueous solution was prepared, increased to 4.0 mL with distilled water and heated at 95°C for 60 minutes. After the solution cooled, 1.0 mL of distilled water and 5.0 mL of an n-butanol/pyridine solution (15/1, v/v) were added. The mixture was shaken vigorously and then centrifuged at 14,668 × *g* for 10 minutes. The absorbance of the organic layer was evaluated at 532 nm, and the MDA levels were expressed as nmol MDA/g tissue, with 1,1,3,3-tetramethoxypropane used as an external standard. Measurements were performed via a microplate reader (LTek, INNO, Seongnam, South Korea).

Analysis of reactive oxygen and nitrogen species

To evaluate the RONS levels, 50 mg of brain tissue was homogenized with 500 μL of cold PBS and 10 μL of protease inhibitors (Amersham Life Science, Munich, Germany) and then centrifuged at 1123 × *g* for 5 minutes at 4°C. The supernatant was incubated with 10 μM dichlorofluorescein diacetate (DCFH-DA) (Sigma–Aldrich, Milan, Italy) in the dark at 37°C for 30 minutes, after which the fluorescence was evaluated with a fluorimeter (GloMax® Plate Reader, Promega) with an excitation filter at 490 nm and an emission filter at 540 nm.

Determination of nitric oxide levels

The levels of NO in brain tissue were assessed via the Griess reagent. Briefly, 50 mg of brain tissue was homogenized with 500 μl of cold PBS and centrifuged at 1123 × *g* for 5 minutes at 4°C. Afterward, 100 μL of the supernatant was mixed with 100 μL of Griess reagent (equal volumes of 1% sulphanilamide (w/v) in 5% phosphoric acid (v/v) and 0.1% naphthylenediamine-HCl (w/v)) and incubated at room temperature for 10 minutes. The absorbance was then evaluated at 550 nm via a microplate reader (LTek, INNO). Nitrite levels in the samples were evaluated by referring to a standard sodium nitrite serial dilution curve.

Gut microbiota composition

Fecal samples were collected from individual mice in autoclaved tubes and stored at –80°C. A DNA isolation kit (QIAamp DNA StoolHandbook Kit, Qiagen) was used to extract DNA (eight replicates in each dietary group) following the manufacturer's instructions. The extracted DNA was used for the metagenomic study carried out by the BMR Genomics Company (Padova, Italy). For NGS, PCR was performed with modified primers (Pro341F/Pro805R) (Takahashi et al., 2014) targeting the V3–V4 regions of microbial ribosomal RNA genes (small subunits), following the standard protocol of targeted amplicon sequencing. PCR amplification was performed in a final volume of 25 μL via Taq Platinum HiFi (Thermo Fisher Scientific) at 94°C for 1 minute, followed by 25 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 68°C for 45 seconds. After that, the PCR products were purified via Agencourt XP 1X magnetic beads (Thermo Fisher Scientific) and processed directly into the preparation of sequencing libraries via the Illumina MiSeq platform (San Diego, CA, USA). The UCLUST algorithm (Qime 2) was used for taxonomic annotations for seeds and unmatched nonseed sequences to obtain a minimum threshold of 97% against the Greengenes v13.8 database (Qime 2) (https://docs.qiime2.org/2022.2/

data-resources/). Operational taxonomic units (OTUs) were collected from the biom file and filtered at 0.005% abundance to eliminate spurious OTUs that were present at a low frequency.

Determination of short-chain fatty acids in plasma

Short-chain fatty acids (SCFAs) were evaluated in the plasma samples of the different animal groups via derivatization with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and 3-nitrophenylhydrazine (3-NPH) and determined via reversed-phase high-performance liquid chromatography coupled with mass spectrometry with electron spray ionization and a triple-quadrupole time-of-flight detector (HPLC/MS/ESI/Q-TOF) (Agilent Technologies Inc., Santa Clara, CA, USA), adapting a previously reported method (Jardou et al., 2021).

Statistical analysis

No statistical methods were used to predetermine sample sizes; however, our sample sizes are similar to those reported in previous publications (Terzo et al., 2021). The results are presented as the mean ± SEM, and the n values represent the number of samples. The data were analyzed via GraphPad Prism 6 software (Graph Pad software, San Diego, CA, USA). Statistical significance was evaluated by analysis of variance followed by the Bonferroni *post hoc* comparison test. Permutational multivariate analysis of variance (PERMANOVA) was used to analyze beta diversity among the different groups of animals. A P value < 0.05 was considered statistically significant.

Results

Metabolic parameters

Compared with those of STD mice, the body weights, plasma fasting glucose and insulin concentrations, HOMA indices, cholesterol and triglyceride levels, and liver and fat masses of HFD-fed mice were significantly greater (**Figure 1A–L**). These observations suggest that 14-week HFD leads to the development of obesity, with dysregulation of glucose and lipid metabolism and the onset of insulin resistance.

In accordance with previously reported results (Terzo et al., 2021), Ind supplementation significantly and selectively improved weight gain, fat mass and glucose dysmetabolism, counteracting peripheral insulin resistance (**Figure 1A–E** and **1I**). Moreover, Western blot analysis revealed that insulin receptor expression in HFD-fed brains was significantly lower (P < 0.001) than that in STD-fed brains, suggesting the presence of central insulin resistance. This downregulation was not evident in the HFD + Ind brains, as their insulin receptor expression was similar to that in the STD brain (**Figure 1F** and **G**), suggesting a positive impact of Ind on central insulin resistance.

Ind prevents neuronal apoptosis in the cerebral cortex of high-fat diet-fed mice

The presence of neuroapoptosis was evaluated in superficial cortex sections via a TUNEL assay, which can reveal fragmented DNA, indices of cell death, and comparative analyses of the gene expression of proapoptotic and antiapoptotic factors. Compared with STD animals, HFD-fed brains presented a significant increase in the number of apoptotic neurons; strong gene overexpression of the proapoptotic proteins *Fas-L*, *Bim*, and *P27*; and a significant reduction in the expression of the antiapoptotic proteins *Bcl-2* and *BDNF* (**Figure 2A–F**). Moreover, we observed significantly fewer apoptotic cells in Ind-treated mice than in HFD-fed mice (P < 0.001; **Figure 2A** and **B**). Furthermore, the HFD-induced gene upregulation of the proapoptotic proteins *Fas-L* (P < 0.01), *Bim* (P < 0.05), and *P27* (P < 0.01) was significantly attenuated by Ind treatment compared with that of the HFD-fed group (**Figure 2C** and **D**). Accordingly, the gene expression of the antiapoptotic proteins *Bcl-2* and *BDNF* was similar to that of STD mice in the HFD + Ind group (**Figure 2E** and **F**), suggesting that Ind was able to prevent HFD-induced neuroapoptosis.

Ind mitigates brain oxidative damage induced by a high-fat diet

Oxidative stress plays a crucial role in brain damage, including obesity-related neurodegenerative pathologies (Anjum et al., 2018). Consequently, we evaluated the impact of Ind on oxidative stress in the brain. As shown in **Figure 3A–C**, the MDA levels, lipid peroxidation indices, RONS and NO levels were significantly greater in HFD-fed brains than in STD-fed brains (P < 0.001) but were not elevated in the brains of Ind-treated mice. In addition, Western blot analysis revealed that the expression of the antioxidant enzyme superoxide dismutase (SOD-2) was significantly lower (P < 0.001) in HFD-fed brains than in STD-fed and HFD + Ind-fed brains (**Figure 3D** and **E**).

In light of the key role of Nrf-2 in the maintenance of intracellular redox homeostasis, we next evaluated Nrf-2 activation (assessed as its nuclear translocation) in the brains of the different groups of mice. We detected reduced nuclear accumulation of Nrf-2 in HFD-fed brains than in STD-fed brains (P < 0.001). In addition, HFD + Ind brains presented greater Nrf-2 nuclear accumulation that HFD-fed brains (P < 0.001), suggesting a key role for this transcription factor in the antioxidative activity of Ind (**Figure 3F** and **G**). These results suggest that Ind is able to ameliorate the oxidative stress induced by HFD in the brain, probably through the Nrf-2 pathway.

Ind ameliorates high-fat diet-induced brain inflammation

Oxidative stress and inflammation are mutually linked and are closely associated with neurodegeneration (Kempuraj et al., 2016). Consequently, we evaluated whether and how Ind treatment could alleviate HFD-induced neuroinflammation. Molecular analyses of proinflammatory parameters revealed that the gene upregulation of *iNOS*, *TNF-α*, and *IL-6* observed in HFD-fed brains was significantly decreased in HFD + Ind-fed brains (P < 0.001), as was the protein overexpression of iNOS and cyclooxygenase-2 (COX-2) (P < 0.001; **Figure 4A–C**). Moreover, the nuclear translocation of NF-kB, a key transcription factor involved in the elaboration of proinflammatory mediators, was increased in HFD-fed brains. Notably, these effects were mitigated by Ind treatment (**Figure 4D** and **E**).

Furthermore, to confirm the anti-inflammatory activity of Ind in the brain, the presence of GFAP, an index of astrocyte activation and gliosis in the brain, was evaluated in brain sections from different animal groups via

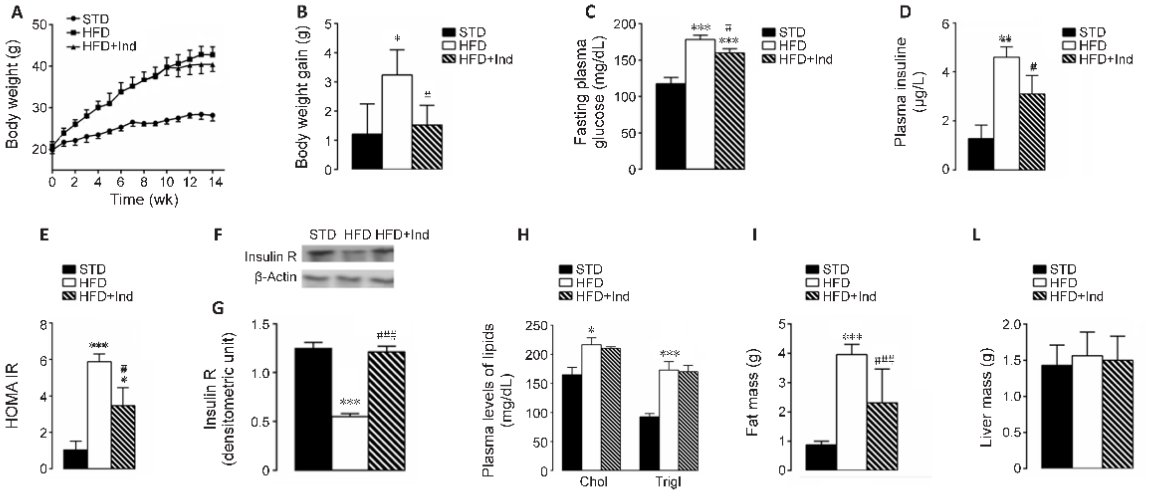

Figure 1 | **Metabolic parameters of the different groups of mice.**
(A) Body weight during the study period. (B) Final body weight gain. (C) Fasting blood glucose concentration. (D) Fasting insulin concentration. (E) The HOMA index was calculated as fasting glucose (mg/dL) × fasting insulin (ng/mL)/22.5. (F) Brain insulin receptor expression. (G) Densitometric analysis of insulin R protein levels normalized to β-actin levels in STD, HFD, and HFD + Ind mice. (H) Plasma levels of cholesterol and triglycerides. (I) Fat mass. (L) Liver mass. Data are presented as the mean ± SEM (n = 8/group). *P < 0.05, **P < 0.01, ***P < 0.001, *vs*. STD mice; #P < 0.05, ###P < 0.001, *vs*. HFD-fed mice. Chol: cholesterol; HFD: high-fat diet; Ind: indicaxanthin; STD: standard diet; Trigl: triglycerides.

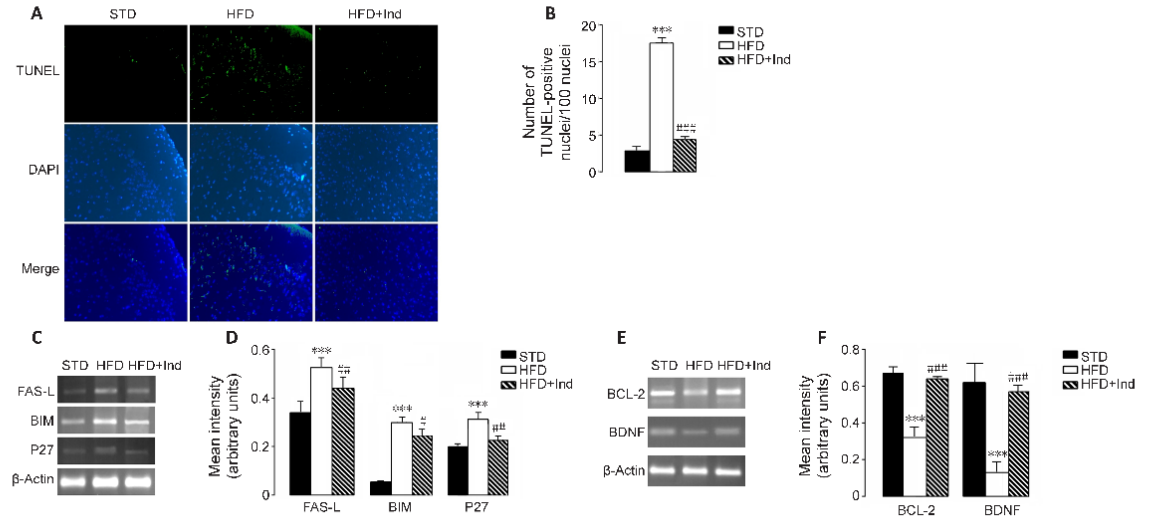

Figure 2 | **Indicaxanthin prevents HFD-induced neuronal apoptosis.**
(A) TUNEL assay in the superficial cerebral cortex (microscope magnification 20×), showing blue (DAPI-stained nuclei) and red (TUNEL-positive cells) staining. Scale bars: 200 µm. (B) Number of apoptotic nuclei in the superficial cerebral cortex. (C) Results of semiquantitative PCR and (D) mRNA levels of the proapoptotic genes Fas-L, Bim, and P27 in the mouse brain. (E, F) Representative image of the semiquantitative PCR results (E) and mRNA levels of the survival genes *Bcl-2* and *BDNF* (F) in the mouse brain. Data are presented as the mean ± SEM (n = 8/group). ***P < 0.001, *vs*. STD mice; #P < 0.05, ##P < 0.01; ###P < 0.001, *vs*. HFD-fed mice. BDNF: Brain-derived neurotrophic factor; HFD: high-fat diet; Ind: indicaxanthin; PCR: polymerase chain reaction; STD: standard diet; TUNEL: terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling.

immunofluorescence. The analysis revealed increased GFAP immunoreactivity in the superficial cerebral cortex of HFD-fed animals compared with that in STD-fed animals (P < 0.001). This increase was not observed in HFD + Ind mice. In fact, the number of GFAP-positive cells in the cerebral sections from HFD + Ind mice was similar to that in the STD-mice (**Figure 4F** and **G**). However, the plasma concentrations of the inflammatory cytokines TNF-α and IL-1β, which were significantly greater in HFD-fed mice than in STD-fed mice (P < 0.001), were not significantly modified by Ind treatment (**Figure 4H** and **I**). These results suggest that Ind is able to mitigate HFD-induced neuroinflammation.

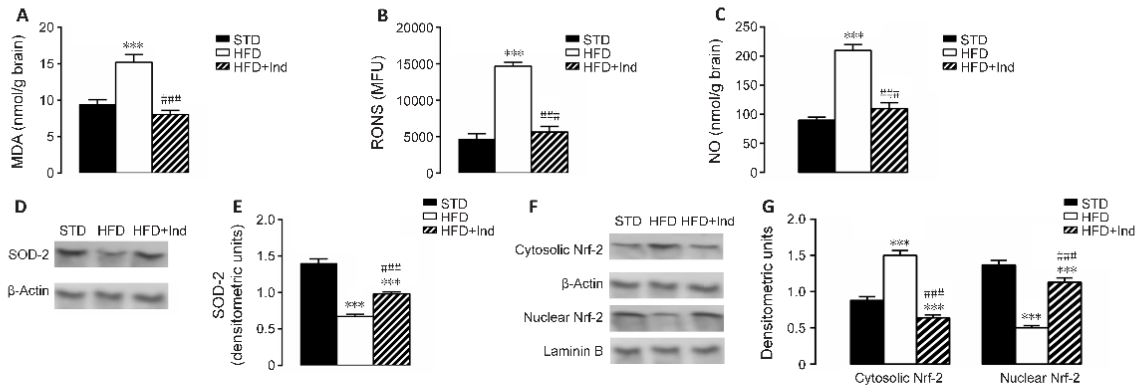

Figure 3 | **Indicaxanthin mitigates brain oxidative damage induced by HFD.**
(A–D) Levels of cerebral MDA (A), RONS (B), and NO(C), and SOD-2 protein expression (D). (E) Densitometric analysis of SOD-2 protein levels normalized to β-actin levels. (F) Brain expression of cytosolic and nuclear Nrf-2. (G) Densitometric analysis of cytosolic Nrf-2 protein levels normalized to β-actin levels and nuclear Nrf2 normalized to laminin B levels. Data are presented as the mean ± SEM (n = 8/group). ***P < 0.001, *vs.* STD mice; ###P < 0.001, *vs.* HFD-fed mice. HFD: High-fat diet; Ind: indicaxanthin; MDA: malondialdehyde; NO: nitric oxide; RONS: reactive oxygen and nitrogen species; SOD-2: enzyme superoxide dismutase-2; STD: standard diet; TUNEL: terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling.

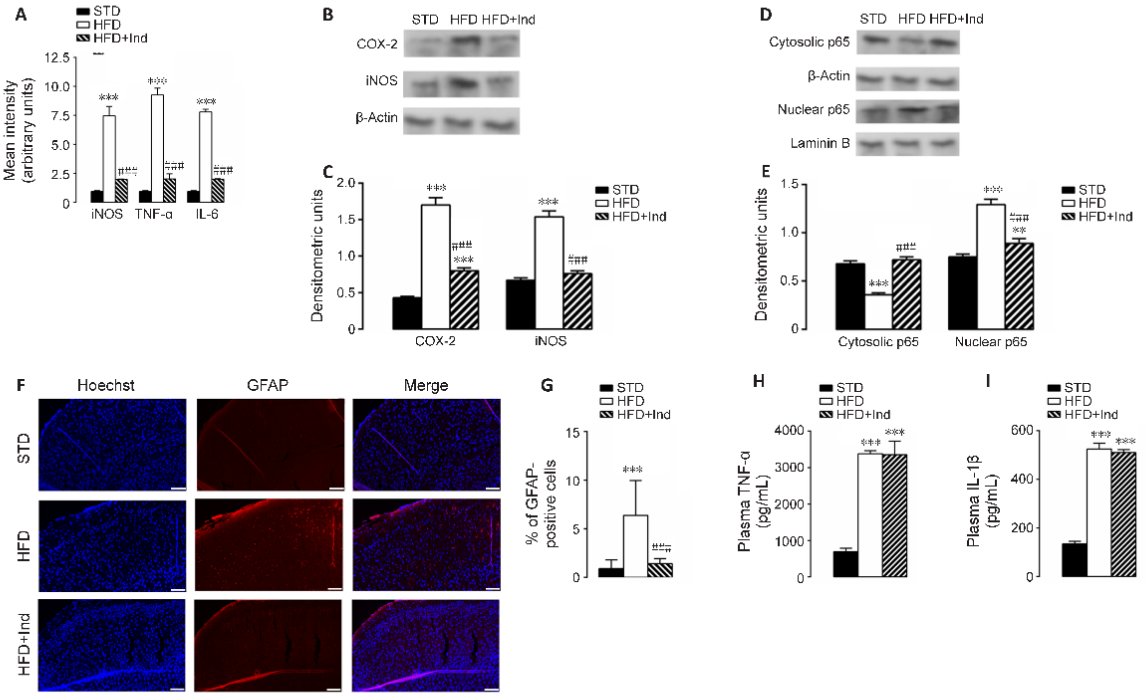

Figure 4 | **Indicaxanthin ameliorates brain inflammation induced by HFD.**
(A) Transcription levels of iNOS, TNF-α, and IL-6 determined by quantitative real-time PCR in the brain. (B) Brain expression of COX-2 and iNOS. (C) Densitometric analysis of COX-2 and iNOS protein levels normalized to β-actin levels. (D) Brain expression of cytosolic and nuclear p65. (E) Densitometric analysis of cytosolic p65 protein levels normalized to β-actin levels and nuclear p65 levels normalized to laminin B levels. (F) Representative images of GFAP-positive cells (red) on the surface. Hoechst staining was used to label the nuclei (blue) (microscope magnification 10×); scale bars: 100 μm. (G) Percentage of GFAP-positive cells; (H, I) Plasma circulating levels of TNF-α (H) and IL-1β (I). Data are presented as the mean ± SEM (n = 8/group). ***P < 0.001, *vs.* STD mice; ###P < 0.001, *vs.* HFD-fed mice. COX-2: Cyclooxygenase-2; GFAP: glial fibrillary acidic protein; HFD: high-fat diet; IL-6: interleukin-6; Ind: indicaxanthin; iNOS: inducible nitric oxide synthase; PCR: polymerase chain reaction; STD: standard diet; TNF-α: tumor necrosis factor-α.

Ind modulates the gut microbiota in high–fat diet-fed mice

Changes in the gut microbiota have been associated with neuroinflammatory, neurodegenerative and psychiatric disorders, leading to the hypothesis that modulation of the gut microbiota may prevent or improve the development and progression of CNS pathologies (Fung et al., 2017). Therefore, we examined whether Ind affects the diversity and composition of the gut microbiota in HFD-fed mice via next-generation sequencing (NGS) analysis. Compared with those of STD mice, the α diversity indices of HFD-fed mice (observed features, Shannon entropy and Pielou evenness) were significantly lower ($P < 0.05$), but no difference was observed in comparison with those of the HFD + Ind group (**Figure 5A–C**). β-Diversity analysis revealed that the HFD group was different from the STD group ($P < 0.001$) but similar to the HFD + Ind group (**Figure 5D** and **E**).

At the phylum level, HFD ($P < 0.01$) and HFD + Ind ($P < 0.05$) mice presented a significant reduction in the abundance of *Bacteroidetes* and an increase in *Firmicutes* ($P < 0.01$) and, consequently, in the *Firmicutes/Bacteroidetes* ratio in comparison to STD mice ($P < 0.001$; **Figure 6A** and **B**). In terms of the relative abundance of the other minor phyla, we found a significant reduction in *Desulfobacterota* ($P < 0.05$), *Proteocacteria* ($P < 0.001$) and *Deferribacterota* ($P < 0.001$) in the HFD + Ind group; however, these bacteria were increased in HFD-fed mice (**Figure 6C**).

At the family level, the HFD-fed group presented decreased *Lactobacillaceae* ($P < 0.05$) and *Prevotella* ($P < 0.001$) and increased *Erysipelotrichaceae* and *Deferribacteraceae* ($P < 0.01$) in comparison with the STD-fed group, whereas Ind supplementation mainly increased *Lactobacillaceae* ($P < 0.01$) and *Prevotellaceae* ($P < 0.01$) and significantly decreased *Erysipelotrichaceae* ($P < 0.001$) and *Deferrobacter* ($P < 0.001$) in comparison with the HFD-fed group (**Figure 7A**). Intriguingly, Ind treatment also increased *Lachnospiraceae_NK4A136*, *Lactobacillus*, and *Alloprevotella* but reduced *Blautia*, *Faecalibaculum*, *Mucispirillum*, *Bilophila*, and *Candiatus Saccharimonas* at the genus level, with increased *Bacteroides vulgatus* and *Lactobacillus johnsonii* and a decrease in *Clostridium* at the species level, suggesting that Ind could positively modulate HFD-induced changes in the gut microbiota (**Figure 7B** and **C**).

Finally, we analyzed SCFA levels, namely, acetate, propionate and butyrate, in the plasma of different mice using HPLC/MS. We found that HFD decreased acetate, whereas Ind supplementation increased plasma acetate (**Figure 7D**). The propionate and butyrate concentrations were under the limit of detection (0.5 μM) in all the cases.

Discussion

In the present study, we show, for the first time, a large range of beneficial effects of Ind, which is extracted from the yellow cultivar *Opuntia ficus-indica* fruit, on HFD-induced brain neuronal damage in mice. In particular, Ind treatment, at a nutritionally relevant dose, was able to prevent neurodegeneration, improve brain oxidative stress and neuroinflammation and positively modulate the composition and abundance of some bacterial phyla, families, genera and species in the gut.

Obesity is a key risk factor for the development of neurodegenerative diseases and cognitive impairments (Miller and Spencer, 2014; O'Brien et al., 2017). Epidemiological studies have demonstrated that obese people who consume a high proportion of saturated fats exhibit a dysmetabolism-related reduction in cognitive ability (Morris et al., 2004). Accordingly, in rodents, HFD induces neurodegeneration, impaired spatial learning and significant behavioral deficits (Dinel et al., 2011; Valladolid-Acebes et al., 2011).

Our results clearly show that chronic HFD intake leads to obesity, hyperglycemia, dyslipidemia and peripheral insulin resistance, in accordance with previous data (Kim et al., 2016; Nuzzo et al., 2020; Terzo et al., 2021, 2023), along with neuropathologies characterized by central insulin resistance, neuroapoptosis, oxidative stress and neuroinflammation. Treatment with Ind positively impacted metabolic and

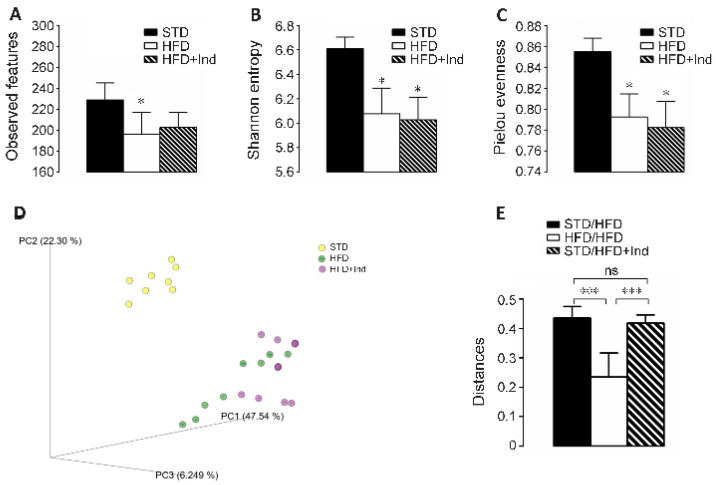

Figure 5 | Gut bacterial diversity in the different animal groups.、
(A–C) Alpha diversity of the gut microbiota on the basis of average observed features (A), average Shannon entropy (B), and average Pielou evenness (C). Beta diversity (unweighted UniFrac) of the gut microbiota in STD, HFD, and HFD + Ind animals: (D) principal coordinate (PC) analysis of each fecal sample bacterial profile analyzed in the different animal groups; (E) statistical comparison of beta diversity among the STD, HFD, and HFD + Ind group (permutational multivariate analysis of variance). Data are presented as the mean ± SEM (n = 8/group). *$P < 0.05$, *vs*. STD mice; ***$P < 0.001$, *vs*. HFD-Ind mice. HFD: High-fat diet; Ind: indicaxanthin; ns: not significant; STD: standard diet.

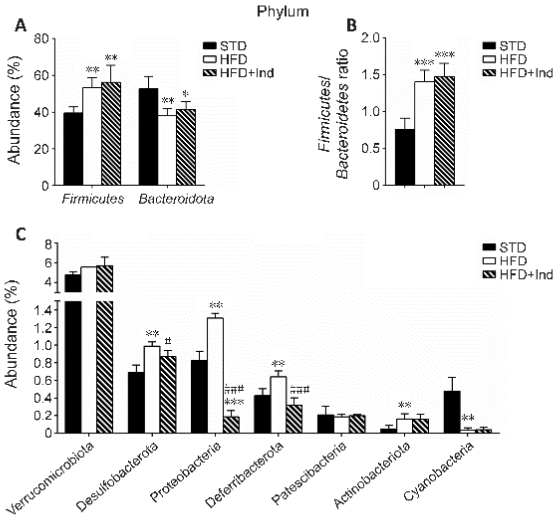

Figure 6 | Phylum-level taxonomic distributions of the gut microbial communities in different animal groups.
(A) Graphic representation of the relative abundance (%) of the *Firmicutes* and *Bacteroidota* phyla. (B) Ratios of *Firmicutes* to *Bacteroidetes* in the STD, HFD, and HFD + Ind groups. (C) Graph representing the mean relative abundance (%) of the minor phyla detected in the three animal groups. Data are presented as the mean ± SEM (n = 8/group). *P < 0.05, **P < 0.01, ***P < 0.001, *vs.* STD mice; #P < 0.05, ###P < 0.001, *vs.* HFD-fed mice. HFD: high-fat diet; Ind: indicaxanthin; STD: standard diet.

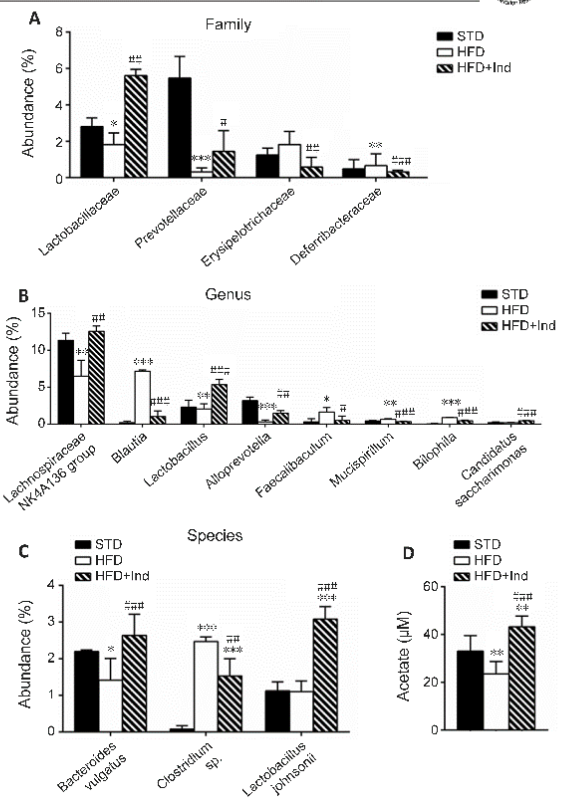

Figure 7 | Taxonomic distributions of the gut microbial communities in the different animal groups at the family, genus, and species levels.
(A–C) Family (A), genus (B), and species abundance (C) significantly modified by indicaxanthin treatment. (D) Analysis of the plasma acetate concentration in the STD, HFD, and HFD + Ind groups. Data are presented as the mean ± SEM (n = 8/group). *P < 0.05, **P < 0.01, ***P < 0.001, *vs.* STD mice; #P < 0.05, ##P < 0.01, ###P < 0.001, *vs.* HFD-fed mice. HFD: High-fat diet; Ind: indicaxanthin; STD: standard diet.

neurodegenerative parameters. In particular, it reestablished brain insulin receptor expression, suggesting that this phytochemical has the ability to ameliorate insulin functions in the brain.

Because dysregulated apoptosis has been implicated in the pathogenesis of neurodegenerative disorders (Jaiswal and Sharma, 2017), we examined the effects of Ind on programmed neuronal death. Compared with that in the STD cortex, the number of TUNEL-positive neurons, which was significantly greater in the HFD cortex, was lower in the cortex of HFD + Ind animals, demonstrating the ability of Ind to exert significant neuroprotective effects. Furthermore, we evaluated the expression of several pro- and antiapoptotic genes, including *Bim*, *Fas-L*, *P27*, *Bcl-2* and *BDNF*, in the different animal groups. Bim belongs to the BH3-only protein family and promotes apoptosis by activating Bax (Maes et al., 2017). Fas-L has previously been reported to mediate apoptosis in neurodegenerative disorders (Ethell and Buhler, 2003). *P27*, an inhibitor of cyclin-dependent kinases, injures neurons under neurotoxic conditions by promoting apoptosis (Jaiswal and Sharma, 2017). *Bcl-2*, an antiapoptotic factor, enhances cell survival in response to diverse apoptotic stimuli by regulating mitochondrial membrane permeability (Morris et al., 2021). BDNF plays a neuroprotective role because of its neurotrophic, antiapoptotic and antioxidative properties and is able to increase the expression of SODs (Chen et al., 2017; Pemberton et al., 2021; Camuso et al., 2022). In our experiments, Ind significantly reduced *Bim*, *Fas-L* and *P27* upregulation and increased *Bcl-2* and *BDNF* downregulation induced by HFD. Taken together, these results suggest that Ind treatment can prevent neuronal apoptosis by modulating the expression of key apoptosis-related genes.

The relationships among neuronal apoptosis, oxidative damage, neuroinflammation and gut microbiota dysbiosis in the development of neural pathologies are well documented. Oxidative stress is one of the most important risk factors for neuronal apoptosis and can initiate neuroinflammatory responses mediated by microglia and astrocytes. Conversely, neuroinflammation can amplify oxidative damage and neuronal apoptosis, perpetuating a cycle of neurodegeneration (Shandilya et al., 2021). Moreover, dysbiosis of the gut microbiota can exacerbate neuroinflammation through various means, including increasing intestinal permeability and/or beneficial metabolite production, affecting neuronal health (Solanki et al., 2023).

Consequently, we considered the impact of Ind on the brain redox state and neuroinflammation triggered by HFD. In

agreement with previous studies (Moroz et al., 2008; Nuzzo et al., 2015, 2020; Terzo et al., 2023), we found that HFD increased malondialdehyde, RONS and NO levels, likely by downregulating the expression of the antioxidant enzyme SOD-2. Conversely, Ind supplementation prevented HFD-induced increases in oxidative stress markers and increased SOD-2 expression. These results, which are consistent with the antioxidative properties of the pigment betalain (Allegra et al., 2019; Attanzio et al., 2022; Terzo et al., 2021), provide evidence of the beneficial effects of Ind at the cerebral level in HFD-fed mice. We also verified the involvement of Nrf-2 in the protection of Ind against oxidative stress. Nrf-2 is a transcription factor that plays a significant role in the regulation of the cellular redox balance in neurons via genes encoding detoxifying and antioxidant enzymes (Hayes and Dinkova-Kostova, 2014). The observation that Nrf-2 nuclear accumulation was increased in the HFD + Ind brain suggests that Ind may facilitate Nrf-2 activation, leading to SOD-2 overexpression, which, in turn, reduces RONS and MDA. Therefore, activation of the Nrf-2/SOD-2 axis may be one of the mechanisms underlying the neuroprotective effects of Ind.

We found that Ind prevented the HFD-induced increase in the expression of some proinflammatory genes (*TNF-α* and *IL-6*) and proteins (iNOS and COX-2) in the brain. The expression of these proinflammatory mediators is tightly controlled by the activation of the redox-dependent transcription factor NF-B (Guo et al., 2024).

Interestingly, HFD induced NF-B activation. Therefore, it is possible to hypothesize that Ind is able to counteract HFD-induced neuroinflammation via the reduced activation of transcription factors, leading to decreases in downstream proinflammatory enzymes and gene expression.

To confirm the anti-inflammatory activity of Ind in the brain, we also evaluated the expression of GFAP, an astrocyte protein that is overexpressed in astrogliosis and inflammation (Jurga et al., 2021). The increase in HFD-induced gliosis and glial activation was markedly improved by Ind in superficial cortical sections. Moreover, since serum TNF-α, IL-1β and IL-6 can induce astrogliosis by crossing the blood–brain barrier, we evaluated the impact of Ind on plasma cytokine concentrations. Ind failed to affect the HFD-induced increase in TNF-α and IL-1β levels, suggesting that the beneficial effect on neuroinflammation is not mediated by an indirect action at the systemic level but rather by direct action in the brain. Indeed, unlike the majority of phytochemicals, Ind is able to cross the blood–brain barrier (Allegra et al., 2015).

Numerous studies have noted that the composition and metabolome of the gut microbiota are altered in various brain disorders, leading to the hypothesis that modulation of the gut microbiota may prevent or improve the development and progression of central nervous system pathologies (Rekha et al., 2024; Fung et al., 2017). In particular, evidence suggests that changes in the gut microbiota are involved in the neuroinflammation and cognitive impairment associated with obesity (Agustì et al., 2018; Zhang et al., 2019). Consequently, we analyzed the effects of Ind on the diversity and composition of the gut microbiota. Consistent with previous studies (Lee et al., 2019; Fang et al., 2021; Ye et al., 2021), HFD-fed mice presented decreased microbial diversity and formed a cluster that was distant from that of STD-fed mice. However, the microbial communities of Ind-treated mice clustered closely to those of HFD-fed mice, and the abundance of Ind-treated mice was similarly lower than that of STD-fed mice, suggesting that Ind failed to reverse HFD-induced gut dysbiosis. In addition, the *Firmicutes/Bacteroidetes* ratio, which is significantly increased in HFD-fed mice and is an indicator of obesity-linked microbial imbalance (Turnbaugh et al., 2006), was not modified by Ind supplementation. Interestingly, we detected decreases in the abundance of several minor phyla in HFD + ind mice: *Desulfobacterota*, *Proteobacteria* and *Deferribacterota*. In particular, the decrease in the abundance of *Proteobacteria*, the major source of LPS (Lin et al., 2020), could be beneficial because this phylum is associated with metabolic diseases, obesity and the development of AD (Hung et al., 2022).

Moreover, increases in *Desulfobacterota* and *Deferribacterota* have been associated with inflammatory damage, leading to alterations in energy metabolism (Gryaznova et al., 2022). Intriguingly, at the family level, Ind supplementation increased the abundance of *Lactobacillaceae* (*Lactobacillus* at the genus level and *Lactobacillus jhosonii* at the species level) and *Prevotellaceae* (*Alloprevotella* at the genus level), suggesting a shift toward a healthier microbial composition. In fact, *Lactobacillus* and *Alloprevotella* are considered probiotics with protective effects against dysfunctions of the intestinal barrier, inflammation and cognitive deficits (Wu et al., 2023; Zhang et al., 2023). In addition, these bacteria, as well as *Lachnospiraceae* and *Candidatus Saccharimonas*, which were increased in the HFD + Ind group, are known producers of SCFAs, primarily acetate, propionate and butyrate (De Filippo et al., 2010; Kong et al., 2019; Liu et al., 2019). SCFAs exert various beneficial effects because they improve the function of the intestinal barrier, preventing the translocation of harmful bacteria and toxins into the blood and promoting gut health (Saad et al., 2016). Additionally, SCFAs produced by gut bacteria play important roles in gut–brain communication (Silva et al., 2020). Once transported into the brain, they can modulate neuronal development and function (Rekha et al., 2024) and play anti-inflammatory and neuroprotective roles (Wang et al., 2018). Consequently, we determined SCFA levels in the plasma of the different animal groups. Surprisingly, in our experiments, we detected only acetate, whereas the concentrations of propionate and butyrate were under the revealing threshold. In any case, the levels of acetate, which were greatly decreased by HFD, were markedly increased by Ind, suggesting that acetate could mediate the beneficial impact of Ind in counteracting HFD-induced neuronal damage. Consistent with our hypothesis, a recent study demonstrated that acetate improves cognitive impairment and suppresses neuroinflammation in an AD mouse model by inhibiting the phosphorylation of NF-kβ and decreasing COX-2 and IL-1 expression (Liu et al., 2020).

Furthermore, Ind decreased the abundance of *Erysipelotrichaceae* and *Deferribacteraceae*, which are families that have the potential to induce inflammation (Liu et al., 2019) and are negatively correlated with SCFA-producing

bacteria (Do et al., 2023). Another positive modulator of the HFD microbiota by Ind consists of a reduction in the abundances of *Faecalibaculum Mucispirillum* and *Bilophila*, which are inflammation indicators associated with metabolic impairment (So et al., 2021; Yu et al., 2022). Finally, we found a significant reduction in *Blautia* abundance in HFD + Ind mice. Because *Blautia* has been linked to the development of glucose dysmetabolism, inflammation and diabetes (Kashtanova et al., 2018) as well as neurodegeneration (Keshavarzian et al., 2015; Voght et al., 2017), our observation fits well with the improvements induced by Ind in HFD-fed brains.

This study has several limitations that should be noted. Our results allow us to clarify whether the improvement in dysbiosis is the key mechanism by which Ind intake ameliorates neuronal damage. That changes in the gut microbiota are the main players responsible for the beneficial effects of Ind, we should examine the Ind impact in the absence of microbiota by using germ-free mice or animals treated with a cocktail of broad-spectrum antibiotics.

In conclusion, the present study shows, for the first time, the ability of Ind to combat HFD-induced neurodegeneration, brain oxidative stress and neuroinflammation. Modulation of redox-dependent NF-κB/Nrf2 activation and its downstream signaling axis appears to be a key mechanism underlying Ind-mediated neuroprotective effects. Importantly, phytochemical administration also positively modulates the composition and abundance of selected bacterial phyla, families, genera and species in the gut. Further experiments are necessary to clarify whether and how gut microbiota modulation is responsible for the neuroprotective effects of Ind.

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Author contributions: *Conceptualization, TL; data curation: AA and MF; formal analysis: TS and AA; investigation: TS; methodology: CP, GM, ND, PP, PPA, AS, ICG, MA, RI, and AA; project administration: AM; resources: TL; supervision: AA and MF; writing – original draft: MF; writing – review & editing: TL and AM. All authors approved the final version of the paper.*

Conflicts of interest: *The authors declare that they have no conflicts of interest.*

Data availability statement: *All relevant data are within the paper and its Additional files.*

Additional files:

Additional Figure 1: *Flowchart of the study.*

Additional Figure 2: *Schematic diagram representing the timeline of the experimental protocol.*

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GENERAL DISCUSSION

The results presented in this thesis have been the object of detailed analysis in their respective sections. The following general discussion explores the broader implications, significance, and potential overall impact of this work on human health. This approach will facilitate the integration of the results into a broader framework and enable an assessment of the relevance of this research in the context of public health and future research.

High dietary intake of saturated fatty acids, frequently referred to as the "Western diet", has become a dominant dietary pattern in industrialized countries and it is rapidly spreading to numerous less developed regions, giving rise to significant public health concerns [Kopp, 2019]. In fact, high consumption of fat along with a sedentary lifestyle have been associated with the development of obesity, insulin resistance and type 2 diabetes, cardiovascular diseases, different types of cancer and even it represents a risk factor for neurodegenerative diseases, including AD. Consequently, there is a strong need to implement the knowledge of the common underlying molecular mechanisms, identifying new agents able to slow down or block the course of the diseases.

Indeed, in our research, we used an animal model, the HFD mouse, in which the diet highly enriched in saturated fat causes both metabolic impairments and neuronal defects. HFD contained 60% of fats relative to the total caloric intake, mimicking as closely as possible a human diet enriched in fats. Typically, a human HFD contains about 50-60% of fats relative to total caloric intake [Speakman, 2019]. It should be noted that in order to standardise our model and reduce confounding variables, we chose to exclude female mice to avoid the potential influence of hormonal fluctuations typical of female physiology. In addition, we focused exclusively on investigating the effects of our compounds in the context of a pathological condition induced by the HFD, rather than the overall potential beneficial effects of the treatments on general health. As a result, we did not evaluate the effects of treatments on healthy mice, which are used in our studies only as a negative control.

Research on animal models has advantages and disadvantages. Studies on animals offer the opportunity to evaluate the impact of potential functional food/nutraceutical/phytochemical by a mechanistic point of view. They allow full control over dietary composition in a shorter timeframe, to an extent that it is not possible in humans. Furthermore, they avoid ethical concerns of clinical trials. For example, inducing obesity by HFD to determine neurodegeneration in humans would be unethical. However, preclinical results cannot always to be translated into clinical outcomes. In fact, the results obtained *in*

vitro and *in vivo* frequently fail to reflect those observed in clinical applications. A discrepancy in efficacy may be attributed to various factors that influence the effectiveness of functional food/nutraceuticals within human applications.

The main questions of our studies are: Is the HFD mouse a good model to investigate the mechanisms responsible for an unhealthy brain and the possible dietary strategies to prevent and to improve neuronal damage? Are the results translatable to humans?

Concerning the first question, the results reported in this thesis provide clear and consistent evidence for the presence of neuroinflammation, increased oxidative stress, and neuroapoptosis in the mouse brain after HFD chronic ingestion, confirming that high dietary intake of saturated fatty acids can induce neuronal damage in the brain and, consequently, neurodegeneration. On the other hand, previous studies [Evans et al., 2024; Kim et al., 2019; Nuzzo et al., 2018a; Nuzzo et al., 2019; Nuzzo et al., 2020; Sharma, 2021; Terzo et al., 2022] suggest that HFD mouse can be useful to analyse molecular mechanisms linking metabolic disease with cognitive deficits and to evaluate the impact of diets affecting disease progression. In the studies here reported, the duration of exposure to high dietary intake of fat ranges from 14 to 28 weeks. This depends on the endpoints assessed. The first study investigated the effects of the long-term ingestion of honey and D-limonene, separately or in combination, on brain damage when the pathological conditions were overt. Therefore, it aimed to explore ameliorating or reverting impact on HFD-caused brain impairment. The second and third studies examined the preventive impact of *Aphanizomenon flos-aquae* or indicaxanthin against the development of neurodegeneration in a long or short time, respectively. A longer period on HFD diet along with age allows us to explore an eventual impact on AD markers. Indeed, AD-like pathology does not spontaneously develop in mice and, because of short lifespans, mouse model cannot mimic fully AD diseases [Valentin-Escalera et al., 2024]. However, in the first and second papers, where the studies adopted a long-period protocol, molecular analyses pointed out, in HFD brains, overexpression of genes involved in the processing of Amiloid Precursor Protein (*APP*) and *TAU*, in AD-related hyperphosphorylation (*Gsk3a, GCdk5* and *Prkca*), in inflammation (*MPO* and *Il-1a*) and loss of proteins related to synaptic function (synaptophysin and PSD95). In addition, in cortex areas of HFD mice, thioflavin T-positive amyloid deposits were prominent compared to lean animals, suggesting that long-term exposure causes the progressive build-up of molecular hallmarks of AD. In these cases, the animal model is used to evaluate how the experimental diet impacts the classical features of AD.

In our studies, neuroinflammation is evident even after a less long period of exposure to HFD (14 weeks). HFD leads to the activation of glial cells in the brain, primarily astrocytes and microglia, as shown by increased expression of the GFAP protein, an index of astrocyte activation. A notable increase in inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, and over-expression of pro-inflammatory enzymes such as COX-2 and iNOS, was also observed in HFD brains. Additionally, HFD enhances the levels of NF-κB, a transcription factor promoting the expression of multiple pro-inflammatory genes. On the other hand, pro-inflammatory cytokines, such as TNF-α and IL-1β have been reported to be increased in mouse brains after 2 months of HFD [Kim et al., 2016] while activation of astroglia and microglia has been observed as early as 3 days after the start of HFD [Thaler et al., 2012; Kim et al., 2019].

Furthermore, HFD increases the production of ROS and other free radicals, which exceed the brain's antioxidant capacity and generate a redox imbalance. In HFD brains, the level of nitrites and other indicators of oxidative stress were significantly higher than negative controls, confirming that the HFD creates a pro-oxidant environment that impairs neuronal function. HFD reduces the levels of the antioxidant enzyme, SOD-2, and it inhibits the activation of the transcription factor Nrf-2, which is essential for maintaining intracellular redox homeostasis. The Nrf-2 factor is a key regulator of the cellular antioxidant response, controlling genes essential for redox balance, including those for antioxidant enzymes and detoxification proteins. It plays a critical role in neuronal homeostasis by preventing oxidative damage [Kensler et al., 2007]. However, stressors like HFD or aging can significantly reduce Nrf-2 activity, weakening cellular defences against oxidative stress [Sah et al., 2017]. The decreased expression of SOD-2 and reduced Nrf-2 activity observed in the brains of HFD-fed mice suggest that this diet not only increases free radical production but also impairs endogenous antioxidant defences, thereby further aggravating oxidative damage.

In summary, the data indicate that chronic inflammation and oxidative stress, induced by HFD, act as key factors in damage to the CNS and they create an environmental condition leading to neuronal apoptosis and neurodegeneration.

Several biological mechanisms, including insulin resistance, impaired BBB integrity, and gut dysbiosis, have been reported to be involved in HFD-induced neurodegeneration [de Paula et al., 2021; Huang et al., 2021]. Our findings align with those of previous research, suggesting that HFD promotes cerebral insulin resistance, which in turn contributes to neuronal injury and neurodegeneration [Gray et al., 2017; Pugazhenthi et al., 2017]. In our

experiments, a significant reduction in the expression of the insulin receptor in the HFD brain was observed already at the 14th week on HFD diet, which is indicative of central insulin resistance, and it could contribute to cognitive decline. However, we did not perform any analysis about cognitive performance and behavioural reactivity.

A substantial body of evidence has demonstrated the neuroprotective potential of nutraceuticals as complementary medicine for neurodegenerative diseases. Nutraceuticals exert their beneficial effects on neuronal health through the modulation of several key mechanisms, including the regulation of energy metabolism, the reduction of oxidative stress within the nervous system, and the modulation of neuroinflammation [Maccioni et al., 2022; Goyal et al., 2024]. Furthermore, nutraceuticals have been shown to promote neurogenesis by stimulating neural progenitor cell proliferation and supporting the production of growth factors and neurotrophins [Chiu et al., 2020].

Preclinical studies frequently utilize elevated doses of nutraceuticals/phytochemicals or experimental conditions that are challenging to replicate in humans, limiting the translation of positive findings into clinical practice [Reza-Zaldívar & Jacobo-Velázquez, 2023]. A common approach to avoid high doses is the combination of different bioactive substances [Watanabe et al., 2020; Puri et al., 2022; Jayatunga et al., 2022]. The combination approach has the potential to yield superior therapeutic outcomes, enabling the reduction of the high doses typically employed in preclinical studies while enhancing the overall efficacy of treatments. This is achieved without the necessity to resort to dosages that may be impractical or unsafe for human use [Puri et al., 2022].

The first study, reported in this thesis, focused on the synergistic interaction between a complex food (honey) and a single phytochemical (limonene). The combined treatment of honey and D-limonene, had superior neuroprotective efficacy compared to each compound administered separately, suggesting that the synergistic interaction between these agents may confer additional benefits and enhance the overall efficacy of the therapeutic regimen. The treatments administered to the animals included doses of honey and D-limonene that were realistically transferable to human use, thus adding value to the results. In particular, the dosage of honey employed corresponded approximately to 10 grams per day for human subjects, while D-limonene was administered at a dose corresponding to approximately 0.6 grams per kilogram of body weight, for 10 consecutive weeks. The utilisation of realistic dosages confers particular significance upon the results, rendering them highly pertinent for future clinical applications.

It is noteworthy that, in humans, the ingestion of honey has been linked to elevated serum levels of antioxidants, including vitamin C, β-carotene, uric acid, glutathione reductase, elevated phenolic content, responsible for antioxidant activity [Palma-Morales et al., 2023], but no clinical evidence is currently available about neural and behavioural effects. Although the number of clinical studies supporting the use of D-limonene is considerably smaller than that supporting the use of honey, recent evidence suggests that it may have potential benefits for humans, particularly in the behavioural field. Indeed, one study demonstrated that vaporised D-limonene can attenuate acute delta-9-tetrahydrocannabinol (THC)-induced anxiety [Spindle et al., 2024], suggesting that D-limonene may exert a beneficial influence on neural circuits.

However, our study explored the therapeutic effects of honey, alone or in combination, in counteracting HFD-induced neuronal damage, and it did not investigate the neuroprotective preventive impact of honey and D-limonene. A preventive approach would have required the introduction of honey and D-limonene from the beginning of the HFD diet, rather than after a period of exposure to the diet itself. This limitation do not allow us to state whether honey and limonene are able to prevent HFD-induced damage entirely, even if it is likely. Moreover, the minimum effective dose for combination treatments remains to be explored. Although the observed efficacy is notable, it remains unclear whether lower doses of honey and D-limonene in combination can maintain significant neuroprotective effects.

The combination of honey and D-limonene could represent an example of "novel food", an innovative food with emerging potential for improving human health. In fact, the novel foods encompass not only new foods derived from non-traditional sources but also advanced nutraceuticals [Quintieri et al., 2023].

Biologically active compounds derived from microalgal biomass also fall into this category [Galasso et al., 2019], and a further example of novel food is represented by the alga *Aphanizomenon flos-aquae*, which has been the subject of the second study in this thesis. AFA, a blue-green microalga, has been identified as a notable source of valuable nutrients. Elements contained in AFA act in a synergistic manner to reduce oxidative stress and neurodegeneration, as evidenced by *in vitro* studies [Nuzzo et al., 2018b]. In particular, AFA extracts are able to counteract neuronal toxicity caused by oxidants and Aβ peptide aggregation, preventing mitochondrial dysfunction and lowering the levels of inflammatory cytokines such as IL-6 and IL-1β [Nuzzo et al., 2018b]. This helps to limit neuronal damage associated with neurodegenerative diseases.

Evidence gathered from the experimental work discussed in this thesis demonstrated that an AFA extract also exerts a neuroprotective action *in vivo*, thus further supporting the potential of this alga as a therapeutic agent for the treatment of HFD-related neurodegenerative diseases. The AFA dose was calculated from a human dose of 1.6 g daily, which was then adjusted to the average weight of the animals, resulting in a dose of approximately 40 mg. The use of a dose adapted to human consumption facilitates the transferability and relevance of the results for future nutritional and preventive developments. To facilitate administration and minimise stress for the animals, the AFA powder was directly incorporated into the food. A 28-week experimental protocol was designed to evaluate the preventive effect of AFA. The animals were randomly assigned to one of three experimental groups at the beginning of the experimental protocol (time zero). The first group was fed a standard diet, the second group was fed a HFD, and the third group was fed an AFA-enriched HFD diet. This approach enabled a comparison of the effects of the HFD diet and an evaluation of the preventive influence of AFA supplementation on neuronal damage. The concept of prevention is a central element in the field of functional foods [Bocanegra et al., 2021; Essa et al., 2023]. Functional foods are designed not only to nourish the body but also to provide additional health benefits, thereby reducing the risk of disease and maintaining general well-being [Essa et al., 2023]. The protocol designed for the AFA study enables observation of the impact of alga at an early stage and highlights its potential in mitigating the adverse effects of a high-fat diet.

Indeed, the protocol long period, adopted in the AFA study, allows us to investigate the preventive impact of the micro-alga extracts on the brain accumulation of Aβ-peptide induced by HFD. It is well known that senile plaques, due to the deposition of aggregated Aβ-peptide in the extracellular spaces, and intracellular neurofibrillary tangles, represent the hallmarks of AD and there is a vicious circle among oxidative stress, neuroinflammation, mitochondrial dysfunction, presence of Aβ-plaques and neuronal apoptosis [Selkoe, 2000].

The results clearly support a beneficial role, not only on stress oxidative and neuroinflammation markers but also on the clearance of the Aβ-peptide and expression of synaptic proteins, supporting the hypothesis that AFA supplementation could prevent or delay AD progression. However, clinical trials are necessary to verify this suggestive and promising hypothesis.

The third study presented in this thesis offers a significant advantage bypassing one of the main limitations in the use of nutraceuticals and phytochemicals, particularly those with neuroprotective potential: bioavailability [Goyal et al., 2024]. The limited

bioavailability of nutraceutical compounds represents a significant challenge that limits their concentration in target tissues [Dima et al., 2020], including the brain [Crespo-Bujosa et al., 2019]. Lower bioavailability, in turn, reduces the capacity to exert therapeutic effects.

The betalain pigment, indicaxanthin, which is present in high concentrations in the yellow fruit of *Opuntia ficus indica*, overcomes the bioavailability that is typical of other similar compounds. It has been demonstrated that this compound is capable of effectively crossing the BBB and accumulating in the brain [Allegra et al., 2015]. Tests on human subjects who consumed 500 grams of prickly pear pulp revealed elevated plasma levels of the pigment [Tesoriere et al., 2004]. Pharmacokinetic studies on rats demonstrated a prolonged half-life and minimal excretion (21%) following a single oral dose. Additionally, indicaxanthin enters the bloodstream in its native form, without undergoing metabolic transformation [Gómez-Maqueo et al., 2020], preserving its bioactive properties and facilitating its action on target tissues, thereby increasing its therapeutic potential in comparison to other compounds. The high bioavailability of indicaxanthin, its capacity to cross the BBB, and its efficacy in counteracting glucose dysmetabolism induced by a HFD [Terzo et al., 2021], provided the basis for the study presented in this thesis.

The indicaxanthin dosage (0.8 mg/kg/day for four weeks) was selected based on our previous research, that indicated the dose as efficacious and safe for achieving significant benefits on glucose metabolism [Terzo et al., 2021]. It is a dose nutritionally relevant, and it corresponds to the ingestion of four fruits in humans. Moreover, indicaxanthin is derived from a food source that is readily accessible and consumed by a significant proportion of the population, making its utilisation particularly appealing for nutritional and therapeutic applications. Prickly pear fruit is rich in a wide range of nutrients and bioactive compounds, including dietary fiber, vitamins, minerals and other phytocompounds. It should be interesting to investigate indicaxanthin efficacy when ingested with the fruit because it could be influenced by interactions with other substances. These could modulate the absorption, metabolism or biological activity of indicaxanthin, thereby enhancing or limiting the neuroprotective effects.

A challenge to the clinical application of nutraceuticals is the existence of significant inter-individual variability, that is influenced by a range of factors including genetics, metabolic processes and environmental conditions [Vazquez-Vidal et al., 2019]. Among these factors, the gut microbiota, which comprises communities of microorganisms such as viruses, bacteria, fungi and eukaryotes that colonize the human digestive tract, emerges as one of the most influential [Cuevas-Sierra et al., 2019; Delzenne & Rodriguez, 2022]. The

microbiota of everyone is distinct, contributing to inter-individual variability by modulating disease manifestation and therapeutic response [Businaro et al., 2021]. The information stored in the microbiota may facilitate early diagnosis and prognostic assessment in at-risk populations, and its modulation may represent a personalized, effective and safe therapeutic option [Delzenne & Rodriguez, 2022; Ratiner et al., 2024].

It is noteworthy that both metabolic dysfunction and neurodegenerative processes have been linked to gut dysbiosis, which refers to an imbalance in the resident gut microbiota [Kwon et al., 2023]. Furthermore, an increasing body of evidence points to a correlation between gut dysbiosis and AD [Varesi et al., 2022]. *In vivo* research indicates that the primary mechanism through which an altered microbiota contributes to the pathogenesis of neurodegenerative diseases is the induction of neuroinflammation through the modulation of glial function [Więckowska-Gacek et al., 2021; Abdolmaleky et al., 2023].

Since HFD chronic ingestion results in gut dysbiosis [Ke et al., 2021; Jia et al., 2023] we evaluated the impact of indicaxanthin on gut microbiota in our animal model. The analysis of the microbiota provided evidence of the positive effects of indicaxanthin on the bacterial composition. In fact, we found in obese animals receiving indicaxanthin, a significant reduction of the *Desulfobacterota, Proteobacteria,* and *Deferribacterota* abundance, compared to HFD. These phyla have been associated to metabolic diseases, obesity, inflammation, and the development of AD [Gryaznova et al., 2022; Hung et al., 2022]. Moreover, indicaxanthin significantly increased the abundance of families, genera and species linked to a healthier profile such as *Lactobacilacee* and *Alloprevotella* and it inhibited the growth of bacteria associated with dysmetabolism and inflammation such as *Clostridium*. Positive modulation of gut microbiota by indicaxanthin could represent one of the mechanisms through which the phytochemical exerts neuroprotective effects. In fact, our results do allow us to clarify if dysbiosis improvement is the key mechanism by which indicaxanthin ameliorates HFD-induced neuronal damages. In order to conclude that the gut microbiota change is the main player responsible for the beneficial effects of indicaxanthin on brain health we should examine the betalain impact in animals treated with a cocktail of broad-spectrum antibiotics to eliminate microbiota. Moreover, although the observed alterations in the gut microbiota may offer valuable indications, the translability of these effects to humans remains uncertain.

During my training stage at the Institute of Biomedical Research of Girona (IDIBGI) in Girona (Spain), I acquired new skills in the analysis of big data obtained from sequencing analyses and I carried out association tests to identify bacteria that may be correlated with

certain metabolic and neural variables in the samples studied. This allowed me to evaluate more in-depth the effects of indicaxanthin on the gut dysbiosis induced by the HFD. Correlation analyses demonstrated that the abundance of bacterial genera, including *Romboutsia*, *Roseburia*, and *Blautia*, increased in response to HFD. This increase was positively correlated with the expression of pro-apoptotic genes, specifically *BIM*, *P-27*, and *FAS-L*. Conversely, a negative correlation was observed between these bacterial genera and the anti-apoptotic genes *BCL-2* and *BDNF*. In contrast, other bacterial genera, such as *Lachnospiraceae* and *Alloprevotella*, exhibited a positive correlation with the expression of anti-apoptotic genes and a negative correlation with the expression of pro-apoptotic genes. Moreover, the abundance of these two bacterial genera was augmented by indicaxanthin administered to the obese mice. Additionally, a negative correlation was observed between the abundance of *Alloprevotella* and the expression levels of IL-6 and TNF-α in the brain. The genera *Alloprevotella* and *Lachnospiraceae* are recognised as beneficial function-associated bacteria, mainly due to SCFAs production [Liu et al., 2019; Zhang et al., 2023a; Wang et al., 2024]. SCFAs, such as acetate, propionate, and butyrate, affect GBA mainly through inflammatory and neurochemical modulation mechanisms. These compounds act as energy sources for intestinal cells, they strengthen the intestinal barrier, and they can cross the BBB. In the brain they exert anti-inflammatory and neuroprotective effects. In addition, SCFA stimulates neurotransmitter production and influences immune cell activity, helping to maintain a balance between the gut and the CNS [Wang et al., 2018].

Overall, the results of the studies demonstrate that neuroprotection against HFD-induced damage occurs through multiple levels and mechanisms of action, resulting in a holistic effect. In fact, each treatment demonstrates the ability to target different biological processes, including reduction of oxidative stress and inflammatory response, and modulation of the expression of crucial genes involved in neurodegeneration. These actions work in parallel to contribute to neuronal protection and the prevention of neurodegenerative damage.

In light of the reported results in this thesis, obtained using doses compatible with human use, we hypothesize that the neuroprotective effects of the nutraceuticals examined are potentially transferable to humans. Further investigation is required to confirm these findings and to obtain evidence for concrete applications, offering new perspectives for the prevention and treatment of neurodegenerative diseases associated to hyperlipidic diets.

Taken together, the results of this thesis show that alimentation can influence negatively or positively the brain health and that neuroprotective opportune

supplementations could be advantageous for preventing or reversing brain neuronal damages. However, clinical studies are necessary to verify the human translability, because the bioavailability in the human brain, thereby the ability to cross BBB, should be established.

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