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THE ROLE OF GUT BRAIN AXIS IN NEURODEGENERATIVE DISORDERS

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ABSTRACT

Age-related cognitive decline and neurodegenerations represent a heavy burden on modern society due to the increasing aging population. Neurodegenerations are due to neuronal loss and disruption in neuronal structure and function that cause the impairment of brain function, cognition, memory retention, learning abilities, emotions, and motor functions. It is accepted that gene–environment interaction determines the risk of developing neurodegenerative disorders (NDDs). The gut-brain axis (GBA) represents the bidirectional communication between the gut microbiota and the brain and there is an interactive relationship between NDDs and gut dysbiosis. Dietary intervention targeting gut microbiota could be a promising strategy for treating symptoms and slowing down degenerative processes in NDDs.

In this PhD thesis, the effects of probiotic formulation SLAB51 on NDDs-related metabolic changes and sleep deprivation was explored. Furthermore, the effect of polyphenol microbial metabolites on aberrant proteolysis, which is considered as a hallmark of NDDs was analyzed. In addition, a functional prebiotic-based cookie enriched with probiotics, more suitable for elderly people, counteracting cognitive decline was designed.

Specifically, upon probiotics administration to Alzheimer's disease (AD) mice, cholesterol biosynthesis was inhibited with a process involving sterol regulatory element binding protein 1c and liver X receptors mediated pathways. Decreased plasma and brain concentration of 27-hydroxycholesterol and increased brain expression of cholesterol 24S-hydroxylase indicated that alternative pathways of bile acid synthesis are influenced. The plasmatic increase of arachidonic acid in treated AD mice reflects dynamic interactions among several actors of a complex inflammatory response, in which polyunsaturated fatty acids can compete each other and simultaneously cooperate in the resolution of inflammation. In addition, chronic sleep deprivation (CSR) is another condition characterized by increased oxidative stress and inflammation and altered gut-brain axis hormones. Probiotics oral administration boosted the antioxidant capacity of the brain, thus limiting the oxidative damage provoked by loss of sleep. Moreover, it positively regulated gut-brain axis hormones and reduced peripheral and brain inflammation induced by CSR.

In vitro and *in silico* studies demonstrated that the flavan-3-ol microbial metabolite phenyl-γ-valerolactones modulated cellular proteolysis via proteasome inhibition and compensatory autophagy upregulation, and inhibits cathepsin B activity, ultimately reducing the amount of intracellular and extracellular amyloid-beta (Aβ) (1-42) peptides in SH-SY5Y neuroblastoma cells stably transfected with the 717 valine-to glycine amyloid precursor protein mutated gene.

Driven by global dramatic demographic and lifestyle changes and based on the growing interest on on-the-go healthy snacks, a prebiotic-based cookie prototype enriched with probiotics with proved neuroprotective properties have been designed and developed. The functional cookie contains the optimal combination of selected prebiotics and probiotics resulting in a natural, safe, appealing and sustainable

people-centred food counteracting cognitive decline. Chronic consumption of the functional cookie improved glucose and lipid profiles, restored neuroprotective gut hormones plasma levels in 3xTg-AD mice, ultimately reducing Aβ load and improving cognitive performance, thereby delaying AD progression.

Collectively, our study makes an important contribution to the development of new therapeutic options for NDDs targeting the gut-brain axis. We have demonstrated that diet, prebiotics, probiotics and synbiotics can modulate the gut microbiome and effectively alleviate the hallmarks of NDDs through the gut-brain axis including pathological protein aggregation, neuroinflammation, oxidative stress, metabolic disorders, and neuronal death. Therapeutic strategies targeting the gut-brain axis are a promising approach to prevent and treat NDDs.

CHAPTER 1

GENERAL INTRODUCTION

1.1 Neurodegenerative disorders

1.1.1 A brief overview of neurodegenerative disorders

Age-related cognitive decline and neurodegenerations represent a heavy burden on modern society due to the increasing aging population. An urgent priority is to increase healthy life expectancy, which is an important index of population health, as it indicates whether any gains in life expectancy are lived in good health or with some health issues and disabilities.

Neurodegenerations are due to neuronal loss and disruption in neuronal structure and function that cause the impairment of brain function, cell signaling, cognition, memory retention, learning abilities, emotions, and motor functions. In fact, terminally differentiated cells of the nervous system possess limited capacity of self-renewal. Interestingly, it is accepted that gene–environment interaction determines the risk of developing neurodegenerative disorders (NDDs), such as Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD) [1].

1.1.2 Alzheimer's disease: the most prevalent neurodegenerative disorder

AD is a chronic, progressive NDD mostly affecting the elderly individual and is the most prevalent dementia, accounting for 60-70% of cases. It causes memory loss and behavioral abnormalities. Dr. Alois Alzheimer first described AD in 1906, by identifying extracellular amyloid beta (Aβ) plaques and intracellular neurofibrillary tangles (NFT). Aβ peptide is produced by cleavage of amyloid precursor protein (APP). Abnormal Aβ tends to aggregate to form toxic insoluble plaques that inhibit neuron function. Hyperphosphorylated tau protein aggregate to form NFTs. Tau stabilizes neuronal microtubules, but when hyperphosphorylated, it creates tangles that alter the neuron structure and cause dysfunction [2], [3]. Amyloid plaques and NFT cause neuronal death, synaptic dysfunction, brain shrinkage, and cognitive impairment.

AD progression can be simply divided into early, middle, and late stages. Early symptoms include minor memory loss and complex task difficulty. Memory loss, confusion, and disorientation may worsen in the middle stage. Late stages patients may lose communication and need assistance with daily tasks. AD has a multifactorial pathogenesis. Familial AD, which only accounts for a tiny number of AD patients, is mainly linked to APP and presenilin gene mutations. Most AD occurrences are sporadic, and environmental factors such as inadequate nutrition, lifestyle, sleep quality, and education are considered as risk factors [4].

1.1.3 Hallmarks of neurodegenerative disorders

Although each NDD displays unique clinical manifestations and pathological mechanisms, they share common hallmarks [5] (see Fig. 1). In this section, the major hallmarks of NDDs, which contribute to a

better understanding and prevention of NDDs will be discussed.

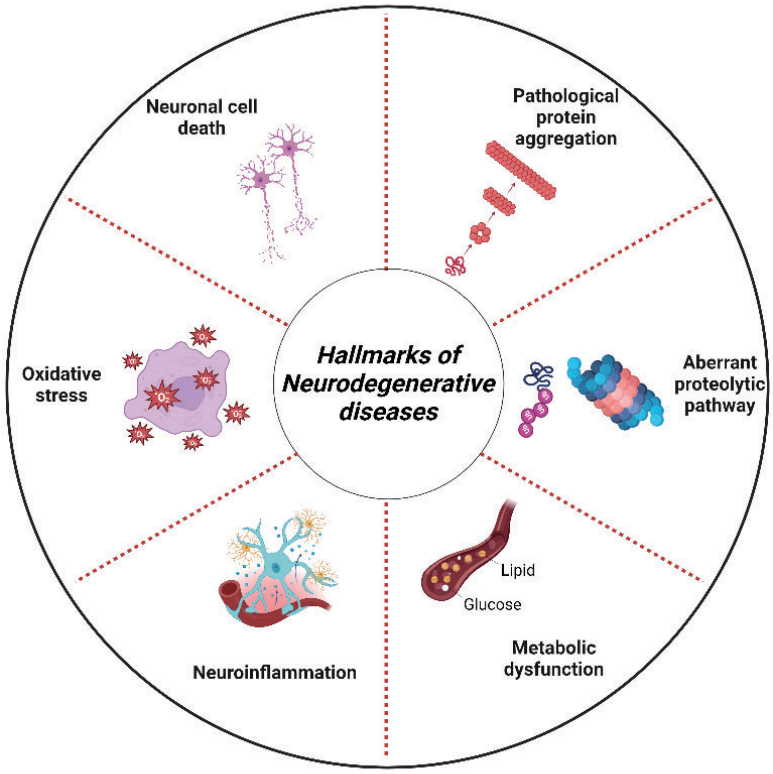


Figure 1. *Hallmarks of neurodegenerative disorders*

1.1.3.1 Pathological protein aggregation

The accumulation of protein inclusions has been observed in many NDDs that typically develop in older individuals, due to a combination of genetic and environmental factors. For example, Aβ and tau proteins aggregate to form amyloid plaques and NFTs respectively in AD. α-synuclein aggregates to form Lewy bodies in PD. Intracellular aggregates of mutant huntingtin are characteristic of HD [6]. The presence of protein aggregates in the majority of NDDs suggests a strong association between protein aggregation and the pathogenesis of these diseases.

The phenomenon of protein aggregation and sequestration within a specific cellular compartment can result in the depletion of its presence in other regions, consequently leading to the impairment of its physiological functionality. The consequences of pathological protein aggregation in NDDs are impaired functions of the nervous system, with aggregated proteins acquiring toxic functions and/or losing their normal functions [7]. For instance, Aβ aggregation in AD induces gain of toxic function, while NFTs lead to loss of normal tau function.

Prion-like propagation has been proposed as a mechanism for the propagation of protein aggregates in NDDs. Prion-like propagation involves misfolding of proteins and rapid dissemination of aggregated

proteins. Specific proteins misfold and aggregate into seeds, causing them to aggregate and form pathogenic assemblies ranging from small oligomers to massive amyloids. Misfolded proteins that can oligomerize and form insoluble aggregates associate with molecular chaperones and other elements of the proteolytic machineries that maintain cell homeostasis by promoting protein clearance and preventing aggregation.

1.1.3.2 Aberrant proteolytic pathway

Protein homeostasis is mainly maintained through two cellular mechanisms, the ubiquitin-proteasome system (UPS) (Fig. 2) and the autophagy-lysosome pathway (ALP) (Fig. 3). The UPS is a tightly regulated and selective protein degradation pathway that involves the conjugation of ubiquitin (Ub) molecules to protein substrates, leading to their degradation by the proteasome, primarily short-lived and damaged proteins [8]. This process is ATP-dependent and begins with the activation of Ub by forming a thioester bond with the Ub-activating enzyme E1. Ub is then transferred to the Ub-conjugating enzyme E2 and ultimately to the Ub ligase E3, which binds to the target protein and interacts with E2 to covalently attach Ub to the target protein [9]. Deubiquitinating enzymes are responsible for removing Ub moieties from protein substrates. They can edit elongating chains or completely remove/recycle Ub chains from substrates [10].

Ubiquitination generates a chain of four or more Ub, termed polyubiquitin, targeting the substrates to the 26S proteasome [11]. The 26S proteasome consists of a proteolytic 20S core particle capped at both ends by a 19S regulatory particle. The 19S particle binds to and unfolds the polyubiquitinated protein substrate, feeding the unfolded polypeptide chain into the chamber of the 20S particle [12]. The 20S particle contains specific peptidase activities that cleave the substrates into small peptides. During substrate feeding, the 19S particle also deubiquitinates the polyubiquitinated substrates to recycle Ub. In detail, the substrates are cleaved by the β5, β2, and β1 subunits of the 20S particle, which exert chymotrypsin-like, trypsin-like, and caspase-like or peptidylglutamyl-peptide hydrolyzing (PGPH) peptidase activities, respectively (see Fig. 2).

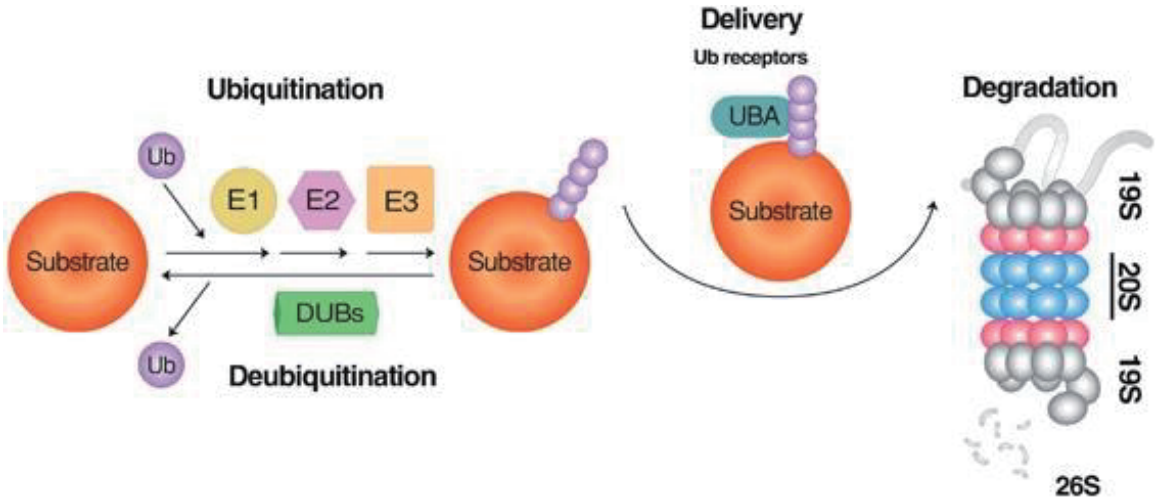


Figure 2. *Ubiquitin-proteasome system* [13]

The downregulation of the UPS is associated with the pathogenesis of various NDDs, including AD, PD, and HD [14]. Aging is a major risk factor for reduced UPS activities in degenerating brains. Proteasomal activities gradually decrease with age, leading to a reduced capacity to degrade misfolded proteins and contributing to formation of pathological protein aggregates. Another risk factor is the presence of aggregated proteins that inhibit UPS components, including the proteasome. For example, aggregated β-sheet-rich prion protein (PrP) blocks the opening of the 20S proteasome particle, resulting in reduced proteasomal activity [15]. Similarly, ubiquitinated and aggregated tau in AD can block the gate of the 19S catalytic particle by binding to its recognition site, resulting in a traffic jam and impaired proteasomal degradation.

In summary, the downregulation of the UPS is a significant contributor to the pathogenesis of NDDs. Age-related reduction in proteasomal activity, as well as the presence of aggregated proteins that inhibit UPS components, can lead to impaired degradation of misfolded proteins and the formation of pathological protein aggregates. Understanding the mechanisms underlying UPS dysfunction in NDDs may help identifying new therapeutic targets to prevent or slow down the progression of these devastating disorders.

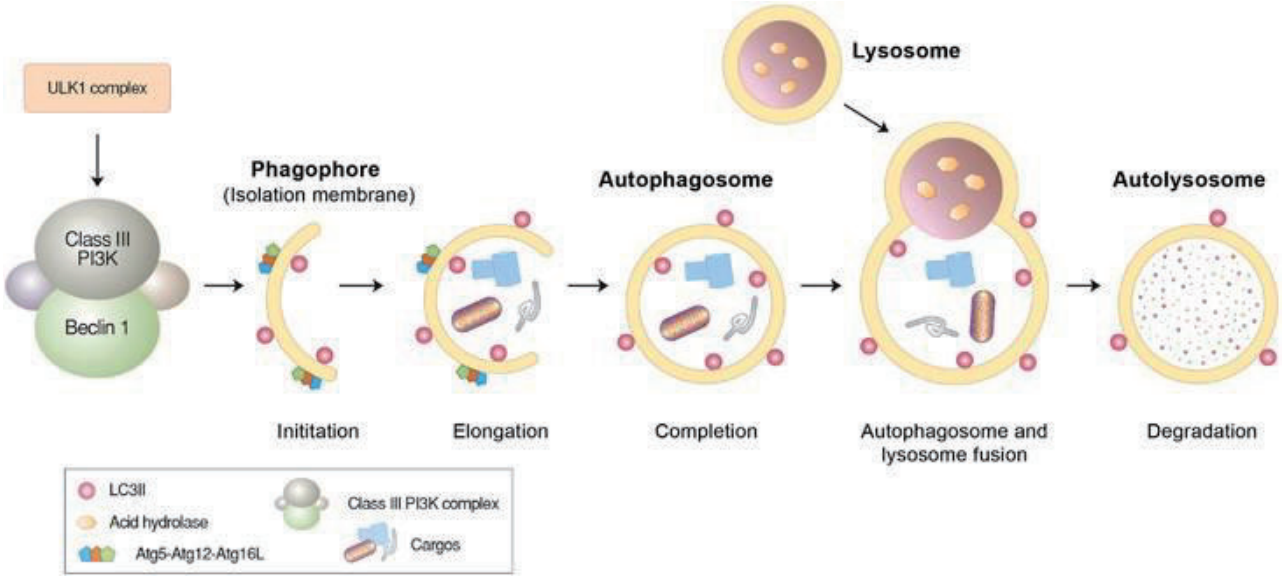


Figure 3. *Autophagy-lysosomal pathway* [13]

ALP is another proteolytic process in response to cellular stress, degrading obsolete cellular components and eliminating damaged long-lived protein aggregates and organelles [16]. The process consists of 5 major steps, 1) initiation and phagophore formation; 2) elongation; 3) autophagosome completion; 4) autophagosome lysosome fusion (autolysosome); and 5) degradation. According to the specific physiological role, cargo delivery mode and pathway, autophagy is usually divided into three groups, namely macroautophagy, chaperone-mediated autophagy (CMA), and microautophagy. Macroautophagy primarily degrades damaged or old organelles, long-lived or protein aggregates that are too large to be processed by the proteasome. Microautophagy represents the direct engulfment of cytoplasmic cargo by

lysosomes. CMA is involved in the specific degradation of protein substrates with the pentapeptide KFERQ motif.

Neurons heavily rely on the efficient removal of misfolded proteins by autophagy for their functions and survival since they cannot dilute cytotoxic proteins through cell division. However, autophagy presents challenges in neurons due to their unique cellular structure characterized by the expansion of dendrites and axons. In neurons, lysosomes are enriched in the perinuclear center, while misfolded proteins generated in axons and nerve terminals and packaged into autophagosomes, need to go through a long retrograde journey to reach the cell body and fuse with lysosomes; this process needs to consume more energy, time, and has a lower efficiency [13].

Defects in some autophagy-related genes (ATGs) known to be critical for autophagy regulation may contribute to neurodegeneration, such as mutations in p62 demonstrated to be related to the pathogenesis of NDDs. Additionally, transcription factor EB (TFEB), successfully ameliorated the disease in several mouse models of NDDs by coordinating autophagy induction with lysosomal biogenesis [17].

1.1.3.3 Metabolic dysfunction

Metabolic disturbances in macronutrients such as lipids, proteins, and carbohydrates have been linked to aging and have also been associated with NDDs. Lipid metabolism disorder induced obesity, has a double risk of developing AD compared with normal individuals [18].

Adipokines such as leptin affect hypothalamic appetite/satiety set points, causing dyslipidemia, hypertension, and insulin resistance. Insulin resistance and metabolic impairment have been associated with AD, and growing evidence links brain insulin signaling and glucose metabolism failure to AD. Insulin enhances transcription of Aβ-degrading proteases, insulin-degrading enzymes, and α-secretase and lowers the transcription of pro-amyloid proteins including APP and β-secretase. The hippocampus and the temporal lobe have many insulin receptors, however, AD patients have diminished insulin receptor expression and function. Aβ competes with insulin for receptor binding, disrupting insulin signaling pathway and causing insulin resistance. A large number of AD patients have type 2 diabetes mellitus (T2DM) or impaired fasting glucose, indicating that AD and T2DM share pathophysiology [19].

Additionally, the brain is the most lipid-rich organ and hypercholesterolemia can disrupt the blood-brain barrier (BBB), leading to infiltration of peripheral macrophages or substances, active microglia and astrocyte, aggravating neuroinflammation [20]. Furthermore, *in vitro* functional cell biology studies support cellular and membrane-bound cholesterol modulating β-secretase and γ-secretase activity and promoting Aβ formation. Dyslipidemia-induced elevated triglycerides influence leptin transport across the BBB, and leptin possesses a positive impact on hippocampus synaptic plasticity, memory, and cognition [21].

1.1.3.4 Neuroinflammation

In the central nervous system (CNS), nervous tissue is composed of neurons and glial cells (microglia, astrocytes, oligodendrocytes). Neurons are responsible for computation and communication. Neurons communicate between each other and with target cells through electric and chemical signals. Glial cells play a structural role in CNS, maintaining the extracellular environment around neurons, improving signal conduction in neurons and normally contribute to brain health by sensing and responding to pathogens or injury. However, in the context of NDDs, they become continually activated by danger signals like protein aggregates and misfolded proteins. Upon microglia activation, pro-inflammatory cytokines are secreted, reactive oxygen and nitrogen species (ROS and RNS) are produced, with consequent synaptic dysfunction and neuronal cell death. Astrocytes possess specific morphological and functional properties that differ within specific areas of the brain. These cells support neurons activities, modulate the BBB integrity and permeability, regulate synapses, energy metabolism, detoxification, and extracellular balance of ions, fluid, and transmitters. Neuroinflammatory reactive astrocytes can be observed during aging, injury, or neuroinflammation, upon microglia signaling [22].

In a recent study, scientists elucidated the underlying mechanism between age-related neurodegeneration and chronic inflammation. Specifically, aging or neurodegenerative conditions exhibit increased accumulation of mitochondrial DNA in the cytoplasm of microglial cells, triggers cGAS-STING cascade (cyclic GMP-AMP Synthase (cGAS) - Stimulator of Interferon Genes (STING)), an important immune signaling pathway that detects and responds to intracellular DNA damage and infection, which significantly promotes an inflammatory phenotype, and also activates astrocytes and oligodendrocytes involved in propagating neurotoxic signals, ultimately culminating in neuron loss and impaired memory capacity. Blocking this pathway has helped aged mice in suppressing chronic inflammation, leading to improvements in both cognitive and motor performance [23].

1.1.3.5 Oxidative stress

Oxidative stress is due to an imbalance between production and elimination of ROS and RNS, resulting in damage to macromolecules such as lipids, proteins, and nucleic acids. This imbalance can occur as a result of increased free radicals or a decrease in antioxidant defense. An altered oxidative status is often observed in the brain of aging subjects and in NDDs. The brain is very vulnerable to oxidative stress because of the high metabolic activity and oxygen consumption, and high polyunsaturated fatty acids content in membranes. For example, lipid peroxidation can damage the cell membrane structure, fluidity and cause membrane leakage; DNA peroxidation disrupts gene transcription and causes gene mutation, which in turn causes pathological aggregation of proteins; protein oxidation changes the structure of protein and loss of its normal physiological function. Oxidation of lipids, proteins, and DNA/RNA ultimately results in necrosis and apoptosis mediated cell death. AD manifests as an irreversible progressive deterioration of the nervous system, during which oxidative stress contributes to the pathophysiology by causing mitochondrial dysfunction, oxidation of macromolecules, generation of ROS

by the binding of metal ions to Aβ plaques and the upregulation of p-tau and Aβ synthesis [24], [25]. Antioxidant treatments have demonstrated that AD is associated with oxidative stress, being a multifactorial pathology.

1.1.3.6 Neuronal cell death

Neurons are highly susceptible to cell death in NDDs due to several factors such as their inability to replenish and replicate, high energy demands, and reliance on support from glial cells, as well as their special structure. NDDs hallmarks, such as protein aggregation, oxidative stress, and mitochondrial dysfunction, can individually and collectively contribute to neuronal loss through different mechanisms of cell death, including apoptosis, necrosis, and autophagy, ultimately resulting in a reduction in brain volume, and this a common phenomenon among AD patients [5].

1.1.4 Sleep deprivation as a risk factor for NDDs

Sleep is essential for human wellness. Widespread sleep disturbance or deprivation in modern society can cause many health problems, including NDDs. Chronic sleep restriction (CSR) is a risk factor for NDDs and may worsen cognitive and motor symptoms. The link between sleep loss and NDDs is complex and multifactorial. Sleep loss can impair brain health by affecting toxic protein clearance, oxidative stress, and inflammatory response, damage BBB, and neuronal function restoration [26]–[28].

Specifically, sleep deprivation can lead to brain and systemic inflammation, which activate brain immune cells microglia and astrocytes. Microglia shift morphologically and functionally to improve immune surveillance and clearance after activation. Prolonged microglial activation and production of pro-inflammatory cytokines such as IL-1β and TNF-α can cause neuroinflammation and neurodegeneration, accelerating toxic protein aggregates, such as Aβ and tau, to accumulate and harm neurons [29].

A reciprocal relationship between ROS and sleep quality exists. Sleep loss affects the body's oxidation and antioxidation equilibrium, increasing ROS generation. ROS causes lipid peroxidation, protein oxidation, and DNA damage, further enhancing neuronal malfunction, inflammation, and neurodegeneration [30]. Sleep loss, oxidative stress, and NDDs may be linked by circadian rhythm disruption. The circadian rhythm controls sleep-wake cycles and antioxidant synthesis. Shift work and jet lag can change antioxidant status and enhance oxidative stress. Disrupting the circadian cycle can potentially cause brain ROS excess and oxidative stress, and vice versa, and circadian cycle disturbance is a common phenomenon in NDDs.

A large number of studies have proved that lack of sleep can lead to gut microbial dysbiosis [31], and the interaction between gut microbes and NDDs has also been deeply studied, but the clear relationship between sleep deprivation, disturbances of the gut-brain axis and NDDs needs to be further clarified.

1.2 Gut-Brain Axis

The gut-brain axis (GBA) represents the bidirectional communication between gut microbiota and brain. The coordinated interaction of the nervous system, endocrine system, and immune system (Fig.4) is crucial in regulating whole physical and mental health and maintaining the homeostasis of the GI tract, CNS, and microbial systems, which exert direct or indirect effects by stimulating the release of chemical transmitters such as microbial hormones and metabolites [32]–[34]. In the following paragraphs various aspects of GBA in terms of its impact on NDDs and possible potential therapeutic effects are described.

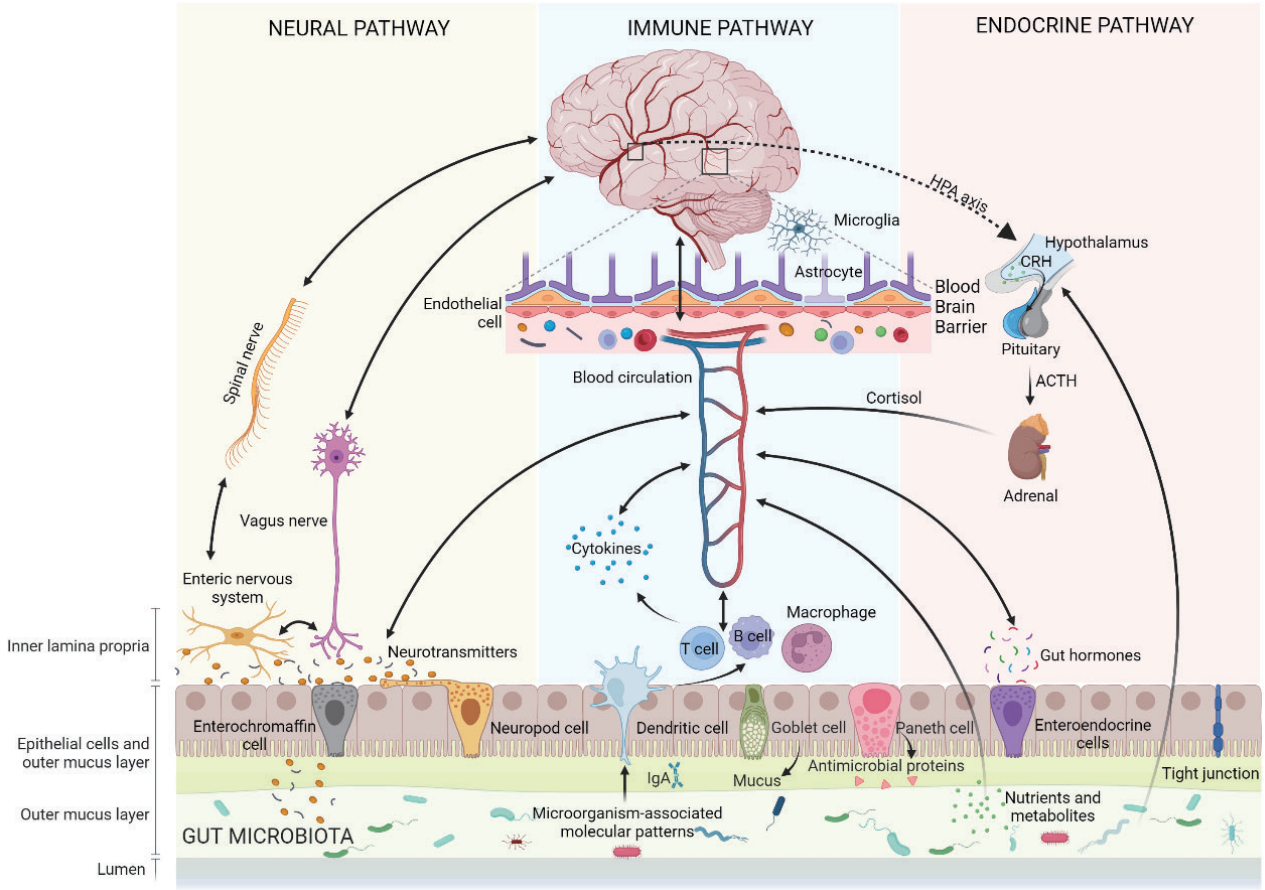


Figure 4. *Gut-brain axis*

1.2.1 Human gut microbiota

The gut microbiota refers to the collection of microorganisms that inhabit the GI tract of animals, including humans, constituting a dynamic community mainly composed of bacteria and to a lesser extent fungi, archaea, parasites, and viruses, which have co-evolved with their hosts over millennia, establishing an intricate and symbiotic association [35]. The collection of all gut microbial genes in an individual, referred to as the microbiome, represents a genetic repertoire containing a total of 3.3 million genes [35], more than an order of magnitude larger than the human genome [36]. Moreover, the number of bacteria in one human is nearly as high as the number of cells, greatly expanding the metabolic capabilities of humans, comparable to the liver [37].

The human gut microbiota consists of four major phyla (*Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria*) as well as two minor phyla (*Fusobacteria* and *Verrucomicrobia*), with *Firmicutes* and *Bacteroidetes* representing approximately 90% of gut microbiota [38], [39]. Across the GI tract, variations in microbial density and composition are influenced by chemicals, nutrition, and immunological factors. Specifically, the stomach exhibits an exceedingly acidic pH, while the small intestine also maintains a relatively elevated acidity and features a shorter transit duration, properties that limit microbiota growth and result in a few microbiota species. In contrast, the colon/large intestine, characterized by anaerobiosis, slower passage of food, water for absorption, and undigested food for fermentation, is densely colonized with microbiota [40]. This spatial diversity emphasizes the need to consider anatomical regions in gut microbiota analysis. Within an individual, gut microbiota communities are also dynamically changing entities that can alter their composition and activity in response to intrinsic host factors, such as genetics, age, and general health conditions, as well as extrinsic factors like diet, drugs, lifestyle, physical activity, infection, stress, and geographical location [37]. Variations also exist among individuals: each healthy human possesses a unique gut microbiota. The notion of a 'core microbiota' suggests a consistent set of abundant organisms across all individuals [41]. Nevertheless, greater similarity in the repertoire of microbial genes among individuals, as opposed to the taxonomic profile, indicates that defining the 'core microbiota' at a functional level might be more suitable [41].

The gut microbiota is closely related to human well-being, involving immune response, digestive function, metabolism as well as neurological signaling transmission. Studies suggested that ratio of *Firmicutes*/*Bacteroidetes* is an indicator of the gut microbiota health. This simple stratification scheme can help in the diagnosis, can determine risk or susceptibility to disease development, and provides a reference for possible therapeutic intervention [42]. Elevated levels of *Firmicutes* and reduced levels of *Bacteroidetes* have been linked to numerous pathological conditions, including T2DM, obesity, and dementia.

1.2.2 Autonomic nervous system

The autonomic nervous system (ANS) includes the sympathetic (SNS) and parasympathetic nervous systems (PNS). It is a neural network composed of neurons widely distributed in the central and peripheral region, regulating involuntary body functions such as heartbeat, breathing and digestion [43], [44]. The individual components of the GBA communicate bidirectionally with each other through this network. Considering the GI tract and the CNS, afferent signals from the lumen were transmitted to the CNS or efferent signals from the CNS to the GI tract, through both the SNS and the PNS (including enteric, spinal, and vagal pathways) [45]. In detail, the ANS controls the GI functions, such as intestinal motility and barrier integrity, luminal osmolarity, mucosal secretory and immune response. These changes in microbiota habitat further affect the relative abundance and diversity of specific microbial taxa. Conversely, gut autonomic nerves can carry sensory information directly to the brain when stimulated by gut microbial metabolites (metabolites interacting with gut ANS synapses) [46], including

serotonin, γ-aminobutyric acid (GABA), catecholamines, and precursors of tryptophan. These neurotransmitters can directly interact with the CNS. Incoming visceral information from the gut via the ANS is processed by the CNS, which then triggers an adaptive response with effects on peripheral organs. ANS serves as a conduit for immediate and precise neurological responses through innervation of the target organ.

1.2.3 Vagus nerve

The vagus nerve is a pivotal component of the PNS, deriving its name from the Latin word meaning "wandering". The vagus nerve consists of 80% afferent and 20% efferent neurons, which tonically transmit crucial information from visceral organs such as the GI, cardiovascular and respiratory systems to the CNS (bottom-up signaling) and furnish feedback to the viscera (top-down signaling). Substantial evidence supports the essential roles of vagal nerve pathways in the regulation of appetite, stress responses, inflammation, and cognitive reactions [47].

Vagal afferents establish three distinct categories of connections within the ENS: intraganglionic laminar terminals and intramuscular arrays, both end within the muscular layer, as well as terminal axon endings within the mucosal layer, and a connection with a subset of enteroendocrine cells, referred to as neuropods, which form synapses with vagal neurons. Due to their widespread distribution, types, and expression of a plethora of receptors, vagal afferents are considered multimodal, capable of detecting various molecules such as bacterial byproducts, intestinal hormones, or neurotransmitters. As a result, they are responsive to a range of mechanical, chemical, or hormonal signals [48].

Gut-related signals from vagal afferents travel to the nucleus tractus solitarius (NTS) in the brainstem [49]. This information then gets relayed to other nuclei within the brainstem and forebrain structures involved in regulating behavior, emotions, stress, and cognition [50]. Complex multisynaptic pathways originating from the NTS establish connections between visceral information and the entirety of the brain. For instance, projections to the bed nucleus of the stria terminalis and amygdala play roles in modulating emotions and behaviors, including anxiety, fear, and avoidance behaviors [51]. Similarly, projections to the nucleus accumbens and basolateral amygdala contribute to memory modulation after arousal. Additionally, projections to the lateral hypothalamus stimulate feeding behavior, the NTS also connects to the pituitary and ventral tegmental area, thereby influencing the hypothalamic-pituitary-adrenal (HPA) axis and cognition functions respectively. Further projections towards the arcuate nucleus merge endocrine and behavioral aspects, thereby regulating food intake and satiety. Through direct or multisynaptic projections, the NTS also affects neurotransmitters like norepinephrine, 5-HT. Essentially, the NTS efficiently coordinates the integration of gut-brain feedback via the vagus nerve, serving as a hub for GBA signaling [48].

The vagus nerve has a pivotal role in promoting neurogenic and neurotrophic signaling pathways. In fact, animal studies demonstrated that disruption of the vagus nerve reduced neurogenesis [52], activated

microglia in the hippocampus, consequently leading to aberrations in stress response and cognition, and causing anxiety- and fear-related behaviors [53]. Conversely, vagus nerve stimulation enhanced hippocampal neurogenesis, regulate the release of neurotransmitters [54], and increased hippocampal brain-derived neurotrophic factor (BDNF) expression, thereby improving synaptic plasticity, learning and memory [55].

1.2.4 Enteric nervous system

The Enteric Nervous System (ENS), a significant component of the ANS, is situated within the GI tract and constitutes a complex mesh of 200 to 600 million neurons facilitating control of gut functions, including motor activity, secretion, absorption and immune defense, playing a pivotal role in maintaining gut homeostasis and interacting with the microbiota and host systems [56]. The ENS is anatomically composed of two ganglionated plexuses, the myenteric and submucosal plexus, containing nitrergic and cholinergic neurons [57]. Intrinsic neurons of the ENS commonly communicate with the CNS through the PNS mainly the vagus nerve and the SNS such as prevertebral ganglia. These complicated intrinsic and afferent neural signals create avenues for factors originating from the gut lumen, potentially encompassing the microbiota, to influence not only intestinal functions but also the CNS. The structure and neurochemistry of the ENS resemble that of the CNS, that is why it often is referred to as the "second brain", and thereby any mechanisms implicated in CNS dysfunction may also result in ENS dysfunction or vice versa [58], [59].

The gut microbiota significantly influences ENS development and function through activation of pattern recognition receptors (PRRs) such as toll-like receptors, TLR2 and TLR4, which recognize microbial LPS, peptidoglycan, and viral RNA. Changes in ENS functions, including reduced stool output, water content, and gut motility were observed in TLRs deficient mice [60], [61]. Germ-free (GF) mice have been shown disrupted ENS structure, reduced enteric neurons, compromised gut motility, and impaired sensory signaling [56]. GF mice also exhibit abnormal neurochemistry and insufficient influx of enteric glial cells into the intestinal mucosa [62]. These observations are mirrored in mice with antibiotic-induced gut microbial dysbiosis [61]. The gut microbiota promotes serotonin biosynthesis by enterochromaffin cells, vital for mucosal and platelet function [63]. Gut microbiota also can produce neurotransmitters and metabolites like serotonin, GABA, histamine, catecholamines, acetylcholine and SCFA further shapes ENS activity. Conversely, the ENS seems capable of influencing the microbiota, an investigation involving a transgenic zebrafish model with deficient ENS function revealed a shift in the GI microbiota towards a pro-inflammatory microbial profile. Intriguingly, the introduction of ENS precursors through transplantation reversed the microbiota back to its normal state. These findings suggest a bidirectional interaction between the gut microbiota and the ENS [64]. Moreover, ENS has now been implicated in NDDs [65], including AD and PD, typically considered primary CNS conditions. This again underscores the vital role of ENS in intricate communication between the gut and the brain.

1.2.5 Neuroendocrine hypothalamic-pituitary-adrenal axis

The HPA axis is considered an essential neuroendocrine pathway integral to GBA communication, orchestrating physiological adaptation to stress. During stress, the hypothalamus synthesizes and secretes corticotrophin-releasing hormone (CRH), which is the principal regulator of the HPA axis, CRH traverses to the anterior pituitary gland, binding to its corticotropin receptor and triggering the release of adrenocorticotropic hormone (ACTH) into the systemic circulation. ACTH stimulates the adrenal gland to synthesize and secrete glucocorticoids (cortisol in humans and corticosterone in rodents), serving as downstream effectors of the HPA axis. These glucocorticoids regulate physiological alterations through ubiquitously disseminated intracellular receptors to meet metabolic, physical, and psychological demands under stress. Nevertheless, both excessive or insufficient activation of the HPA axis leads to psychophysiological disturbances [66], [67].

Specifically, appropriate levels of glucocorticoids are essential for proper neurodevelopment, and cognitive processes such as learning and memory [68]. Experimental models investigating stress demonstrated a correlation between the HPA axis and alterations in microbiota composition as well as its metabolites [69]. Conversely, microbial modulation of the HPA axis also affects glucocorticoid concentrations [47]. Gut microbiota modulation through probiotics and prebiotics administration has been shown to ameliorate stress-dependent increase in corticosterone levels [70], [71]. Considering the widespread distribution of glucocorticoid receptors across multiple organs including the GI tract and the CNS, as well as on various cells such as neurons, epithelial cells, immune cells, and endocrine cells, glucocorticoids can affect both gut and brain functions through multiple pathways including neural, metabolic, immunological and endocrine pathways. Prolonged stress can induce the HPA axis dysregulation. In fact, it has been observed that increased cortisol is associated with cognitive decline and increased AD risk, with elevated cortisol in dementia patients involving interactions between inflammation, neurotransmitters, and oxidative stress [68].

1.2.6 Neurotransmitters

Neurotransmitters mediate intercellular signal transduction across diverse neuronal types and glial cells, influencing learning, memory, emotion, and movement. Neurotransmitters can be divided into excitatory neurotransmitters, such as glutamate, acetylcholine, norepinephrine, and dopamine, as well as inhibitory neurotransmitters, such as GABA, glycine, and serotonin. Their synthesis and regulation are orchestrated by neurons and glial cells through specific enzymes and their disruption is implicated in NDDs and psychiatric disorders, including AD, PD, depression, and anxiety [72].

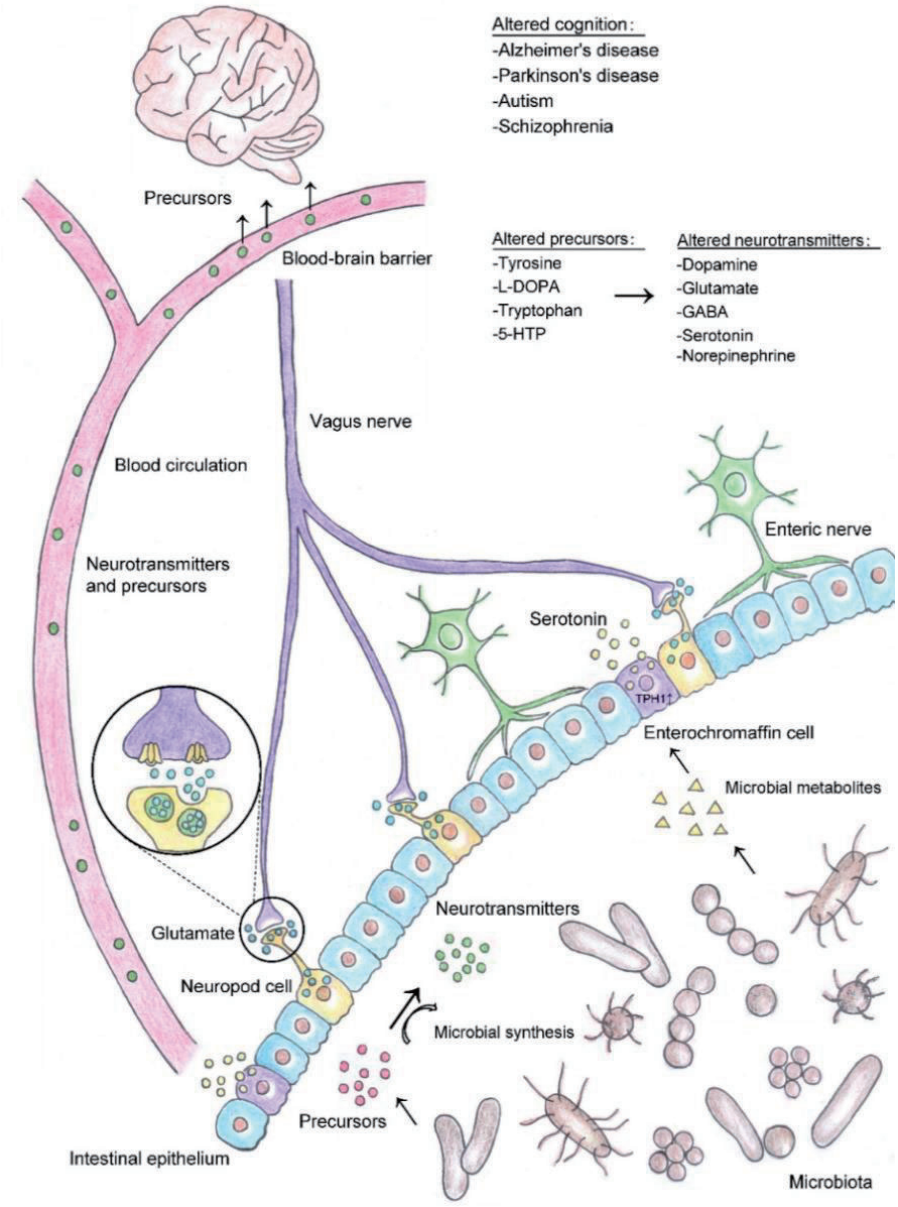


Figure 5. *Gut microbial-mediated neurotransmitter synthesis and its impacts on brain* [72]

Interestingly, emerging evidence underscores that certain microbiota also produce enzymes or metabolites fostering neurotransmitters or precursor synthesis, further influencing brain function [73]. This could introduce an additional communication pathway within the GBA (see Fig. 5). However, only a small number of neurotransmitters can directly pass the BBB and act on the CNS, and can indirectly modulate brain activity via local interaction with the ENS or rapid signaling to the brain via the vagus nerve [74]. Moreover, some neurotransmitter precursors can traverse the BBB through the carrier system present in capillary endothelial cells [75]. Subsequently, neurotransmitter-producing cells uptake these precursors, enabling their conversion into functional neurotransmitters through a series of intermediary steps facilitated by host enzymes. Specific strains respond to or produce catecholamines, the genus *Escherichia* is known to produce norepinephrine [76], and *Bacillus* can biosynthesize norepinephrine and

dopamine, both catecholamines that regulate emotion, cognition, and gut motility. *Staphylococcus* inhabited in the human gut also has been reported to express staphylococcal aromatic amino acid decarboxylase, able to convert precursor L-3,4-dihydroxy-phenylalanine (L-DOPA) into dopamine [73]. *Lactobacillus spp.* [77] and *Escherichia spp.* [78] have been reported to synthesize GABA, which regulates and coordinates neuronal signaling in the hippocampus, thereby influencing cognitive processes. Serotonin is involved in regulating cognition, GI secretion and motility, as well as circadian rhythm, and is almost 90% synthesized by enterochromaffin cells [79]. Interestingly, spore-forming bacteria, particularly *Clostridia*, have been linked to the promotion of serotonin biosynthesis by enhancing the gene expression of tryptophan hydroxylase 1, the rate-limiting enzyme for serotonin synthesis, and certain metabolites such as SCFA and bile acids can influence this synthesis process. Furthermore, *staphylococci* can use amino acid decarboxylase to decarboxylate the precursor 5-hydroxytryptophan into serotonin [73]. *E. coli* and *Morganella morganii* are some of the bacteria known to produce biogenic amines like histamine [80], which is responsible for regulating wakefulness as well as various immune functions, thereby potentially affecting the host immune system. Acetylcholine, a crucial cholinergic neurotransmitter, functions as a local modulator within both the central and peripheral nervous systems, facilitating excitatory signals between neurons [81]; its dysregulation is closely implicated in AD pathology [82], and can be synthesized by a variety of bacteria including *Bacillus subtilis*, *Lactobacillus plantarum*, *E. coli*, and *Staphylococcus aureus* [83], [84]. Research conducted on germ free animals and the utilization of antibiotics to deplete the gut microbiota have revealed significant alterations in neurotransmitters and their receptor levels in the brain, GI, and blood [85], [86]. Indeed, these collective findings underscore the complex interplay between the gut microbiome and host neurotransmitter levels. It is plausible that the gut environment may have direct and indirect effects on neuronal activity and cognitive function within the brain through such interconnected pathways.

1.2.7 Immune system pathway

The immune system plays a pivotal role in distinguishing between “harmful” and “harmless” signals and orchestrating appropriate responses, especially in the GI tract, where the immune system constantly interacts with microorganisms through innate and adaptive immunity. Enterocytes express innate immune receptors and can release cytokines and chemokines, while gut-associated lymphoid tissue (GALT) utilizes lymphocytes to generate a more specific immune response involving immunoglobulins. Notable immune receptors include PRRs like TLRs, which specifically identify microorganism-associated molecular patterns (MAMPs). Examples include LPS and polysaccharide A for gram-negative bacteria and peptidoglycan for gram-positive bacteria [87], [88]. Thereby enabling the immune system cells to detect and respond to microbial presence, recognize changes in bacterial balance, and maintain gut homeostasis. Specifically, activation of immune cells initiates a cascade of recruitment of inflammatory mediators, cytokines, and chemokines that act as chemical messengers to facilitate the immune system, gut microbiota, and CNS communication. Maintaining a balance between anti-inflammatory and pro-

inflammatory cytokines, and chemical messenger is a key determinant of an appropriate host defense against infection or tissue damage. Proper intestinal immune cytokine production maintains intestinal homeostasis, which in turn affects local microbial concentrations, while excessive cytokines may cross the BBB from the systemic circulation and directly affect brain function. Dysregulated microbiota can also compromise the integrity of both the intestinal barrier and the BBB, permitting the infiltration of microbes and their products into the CNS, triggering the activation of the brain's immune system such as resident immune cells like microglia, leading to a pro-inflammatory state. It's noteworthy that chronic inflammation is associated with cognitive impairment and behavioral alterations [89]. Gut microbiota depletion induced by antibiotic treatment or GF mice exhibit systemic and the CNS immune system responses [90], [91]. The microbiota can modulate neuroinflammation by affecting monocyte migration from the periphery to the CNS. Further analysis found that this monocyte migration is mediated by TNF-α, a cytokine produced by microglia [92], which can be reversed by the administration of probiotics [93]. A study on recombination activation gene 1 (Rag1) transgenic mice lacking lymphocytes revealed cognitive and anxiety-related behavioral changes, highlighting the crucial role of the immune system in the GBA, and this impairment of brain function was reversed by a probiotic combination—*L.rhamnosus* and *L.helveticus* [94]. Thus, probiotic treatment holds promise for alleviating adaptive immune damage and resulting behavioral changes.

1.2.8 Enteroendocrine signaling

Enteroendocrine cells (EECs) are widely distributed throughout the GI tract, comprising only 1% of the epithelial cell population within this milieu. Nonetheless, they collectively form the body's largest endocrine organ and wield pivotal regulatory effects on bidirectional communication between the gut and the brain (Fig. 6) [95], [96]. Currently, ten distinct EEC subtypes have been delineated [97], traditionally characterized based on their secretion of gut hormones, such as K-cells (glucose-dependent insulinotropic polypeptide (GIP)), I-cells (cholecystokinin (CCK)), and L-cells (glucagon-like peptide-1 (GLP-1)) [98]. However, recent indications suggest that EECs are more complicated multihormonal cells, largely influenced by their location and maturation within the gut [96]. EECs are predominantly open-type cells characterized by a bottleneck shape with an apical membrane in direct interaction with the lumen and a basolateral membrane close to blood vessels and innervating neurons [95]. EECs exhibit high expression of chemosensory components, including nutrient transporters and nutrient-specific G protein-coupled receptors (GPCRs). This unique configuration enables the EECs to sense changes in the gut lumen, including nutrients, as well as gut microbiota and their metabolites, acting as initial messengers to help the host maintain metabolisms such as energy and glucose homeostasis, as well as behavioral responses, such as food intake [95], [99]. Specifically, upon mechanical, chemical, or neural stimulation, the influx of intracellular calcium incites the release of gut peptides via the basolateral membrane into the extracellular milieu, where these gut peptides activate vagal afferent neurons that send signals to the NTS and then cascade to higher-order cerebral domains (see the vagal nerves system section for details). Vagal

afferent neurons can also be activated through the ENS via gut-derived neurotransmitters such as 5-HT. Additionally, gut peptides can enter the blood circulatory system and signal directly to the NTS [95].

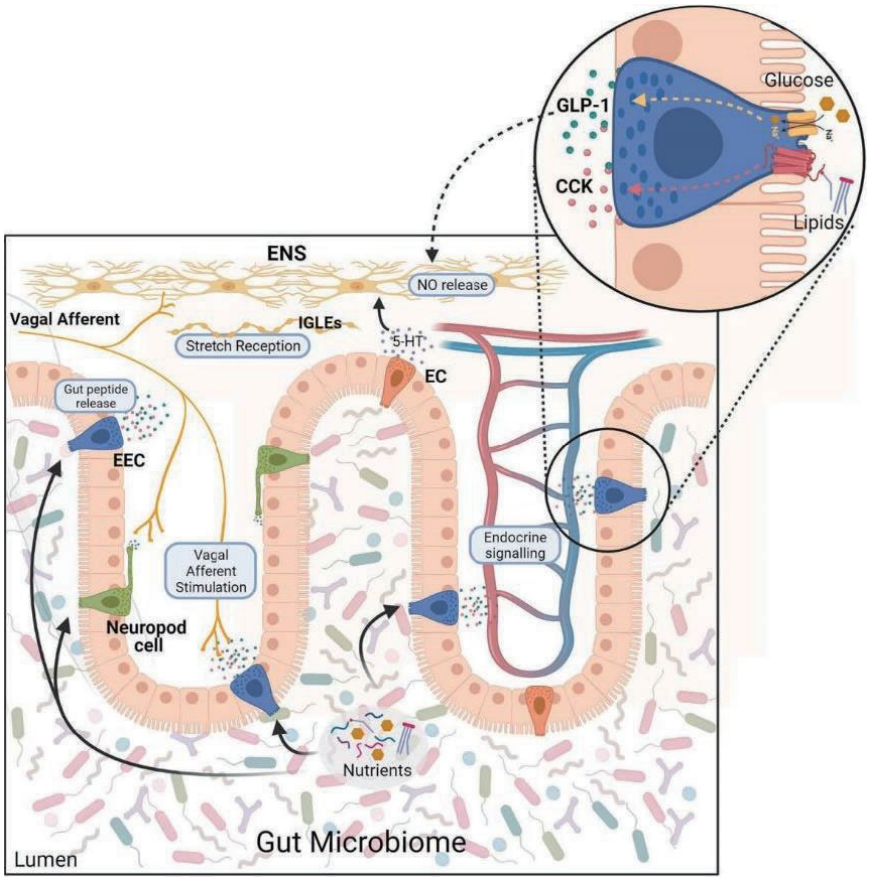


Figure 6. *Enteroendocrine signaling* [95]

While over 20 gut peptides have been identified within the GI tract, most of these assume a central role in modulating energy and glucose homeostasis. Notably, an array of gut hormones is implicated in pivotal facets of gut-brain crosstalk, such as ghrelin, peptide YY (PYY), GLP-1, GIP, and CCK, all of these hormones are released in response to food intake and mutually regulate with the brain [100], [101]. For instance, GLP-1 has been demonstrated to enhance hippocampal synaptic plasticity, improve learning, memory, and motor functions, as well as mitigate neuroglial cell activation, suggesting potential neuroprotective effects in NDDs such as AD and PD [102]. Ghrelin has been described as an important link connecting metabolism, aging, and NDD, affecting glucose and lipid metabolism, and having an impact on memory and learning consolidation [103].

1.2.9 Blood-brain barrier

The BBB appears to be a dynamic interface that separates the CNS from the systemic circulation, maintaining brain homeostasis. Functioning as a selective barrier, it allows essential nutrients, oxygen, and waste products to pass while preventing the entry of toxins, pathogens, and harmful molecules. Disruption of BBB integrity is implicated in the pathology and progression of NDDs. The BBB is

structurally organized by endothelial cells lining cerebral microvessels, along with tight junction proteins, pericytes, basement membranes, and glial cells (Fig. 7).

Endothelial cells of CNS possess unique properties with low rates of transcytosis, higher amounts of mitochondria, diminished expression of leukocyte adhesion molecules, and elevated expression of various transporters and enzymes [104]. Such features enable selective movement of solutes influx and efflux of the CNS parenchyma, maintaining a stable microenvironment for optimal neuronal function [105]. The integrity of the BBB is maintained by the presence of tight junction proteins, which consist of transmembrane proteins such as occludin, claudins, and junctional adhesion molecules (JAMs), also involving the recruitment of various membrane-associated cytoplasmic proteins such as zonula occludens (ZO) and actin cytoskeleton [106]. Accumulating evidence indicated a correlation between alteration of tight junction proteins, BBB dysfunction, and progression of NDDs like PD and AD. Pericytes, located on the abluminal surface of the endothelial cell wall, are involved in various physiological processes associated with BBB maintenance, such as angiogenesis, immune cell infiltration, modulation of extracellular matrix, healing of wounds, and regulation of blood flow. Pericytes also regulate BBB permeability through interactions with endothelial cells and secretion of factors that support BBB function. Astrocytes with their endfeet ensheathing blood vessels as a cellular link between the CNS and blood vessels, provide metabolic support to endothelial cells and also secrete factors that modulate tight junction integrity and transport activity, thus maintaining BBB integrity and functionality [104].

Postmortem and MRI imaging analysis confirmed the impairment of BBB in AD patients [107]. Interestingly, gut-derived signals, including microbial metabolites such as LPS, SCFAs, trimethylamines (TMAs), and vitamins, have been shown to modulate BBB permeability. Dysregulation of the gut microbiota observed in NDDs may contribute to BBB dysfunction and disease progression [108]. Systemic immune activation has been identified as a potential cause of BBB integrity damage, which was simulated by injecting LPS to animals, resulting in a significant 60% elevation in BBB permeability. Targeted manipulation of gut microbiota can increase the expression of claudin-5 and occludin, and decreasing BBB permeability. Conversely, germ-free mice exhibit a more permeable BBB compared with mice with a typical composition of gut microbiota, and implanting a normal microbiota into germ-free mice partially restored barrier function [109], [110].

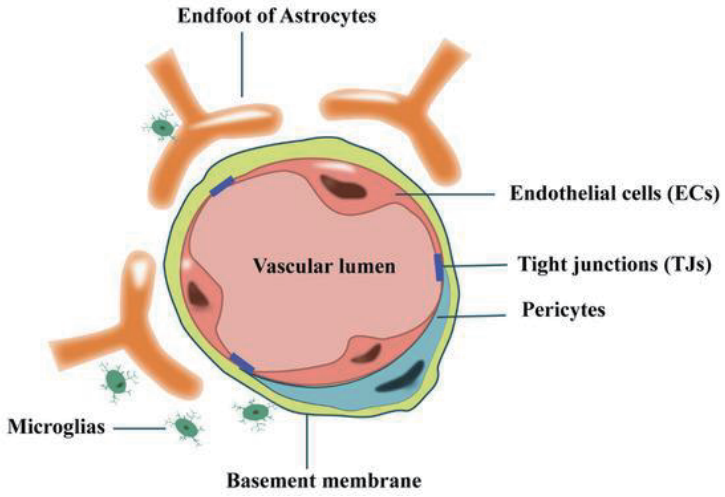


Figure 7. *Blood-brain barrier* [111]

1.2.10 Intestinal barrier

The intestinal barrier is a semipermeable surface responsible for the intricate selective functions of gut, which facilitates the absorption of essential nutrients and immune surveillance, while concurrently restricting the transport of pathogenic molecules and microorganisms. Both structural and molecular components of the barrier interplay in a dynamic manner to fulfill the complex task and maintain intestinal integrity and immune homeostasis [112].

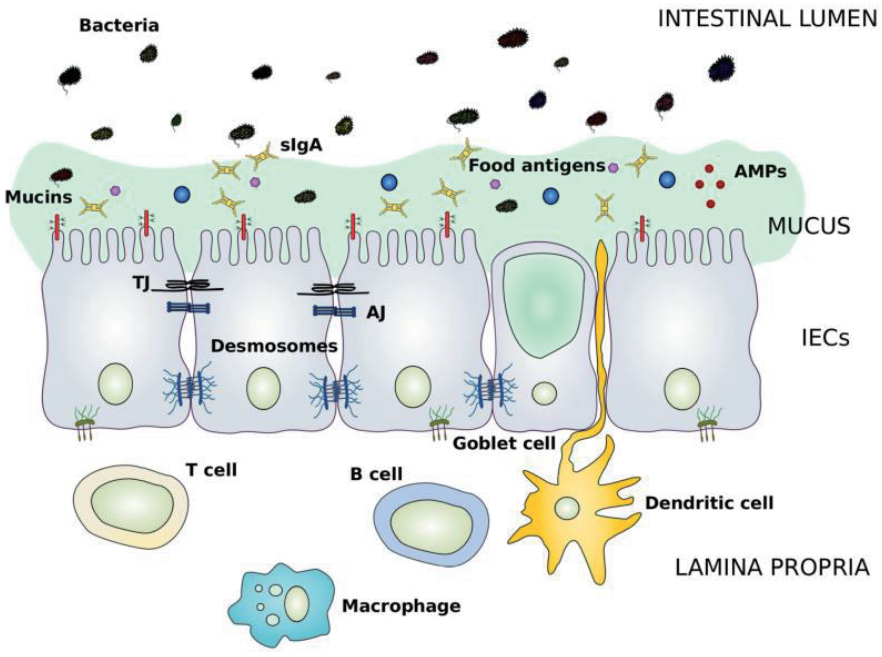


Figure 8. *Intestinal barrier* [112]

Structurally, the intestinal barrier is mainly composed of the outer mucus layer, a continuous monolayer formed by the epithelial cells, and the inner lamina propria carrying the adaptive and innate immune systems (Fig. 8) [113]. The outer mucus layer forms a gel-like sieve structure of highly glycosylated mucins such as mucin 2 that covers the epithelium and serves as the first line of physical defense against

direct contact of external molecules and bacteria with epithelial cells [114]. Immune regulators such as antimicrobial peptides (AMP) and secretory IgA molecules are also released and distributed in the mucus gel as immune sensing and regulatory proteins [115]. The outer mucus layer provides a habitat for the gut commensal microbiota and provides nutrients (glycans), the composition of the mucus layer can influence the microbiota, while the microbiota can shape the mucus gel. Beneath the mucus layer, the continuous and polarized monolayer of intestinal epithelial cells is constructed by five distinct cell types, including goblet cells, absorptive enterocytes, enteroendocrine cells, microfold cells, and Paneth cells, originated from a reservoir of pluripotent stem cells in the crypts [112]. They are tightly connected to each other through junctional complexes. Specifically, tight junctions are situated at the apical side of the cells, serve to seal the intercellular space, and regulate the transportation of small molecules and ions, it is composed of transmembrane proteins, such as claudins and occludins, as well as peripheral membrane proteins, such as zonula occludes (ZO)-1 and ZO-2, along with regulatory proteins. The adherent junctions (AJ), situated underneath the tight junctions, together with the desmosomes form strong cell-adhesion bonds to maintain the integrity of the intestinal barrier [116]. Inner lamina propria lies behind the epithelium and consists of cells of the adaptive and innate immune systems, including T cells, B cells, dendritic cells, and macrophages, involved in the immune defense mechanisms of the intestinal barrier.

Any damage to the structure and composition of the intestinal barrier may dramatically affect the functionality of the intestinal barrier and its dysfunction has been linked to a range of human diseases, GI tract diseases such as irritable bowel syndrome, as well as extraintestinal disorders including type 1 diabetes, obesity, AD, PD, and depression. It is widely postulated that the breakdowns of the intestinal barrier and the unregulated movement of antigens and pathogenic molecules across the intestinal epithelium may pose a challenge to the immune system of vulnerable individuals and disrupt the equilibrium between the host and microbial communities, as such potentially triggering inflammatory responses in the GI tract or even in distant organ systems [117].

1.3 Gut dysbiosis and neurodegenerative disorders

1.3.1 Gut dysbiosis

Gut dysbiosis refers to an imbalance in the gut microbiota, characterized by a decrease in microbial richness, abundance and the loss of beneficial bacteria such as *Bacteroides* and *Firmicutes*, and an increase in pathogenic bacteria such as *Prevotellaceae* and *Enterobacteriaceae* [118]. This imbalance can have negative effects on host's health, including metabolic disorders, endogenous intoxication, systemic inflammation, and reduced essential metabolites.

Dysbiosis can stem from a multitude of factors, encompassing an imbalanced dietary regimen predominantly composed of refined constituents, inadequate dietary fiber incorporation, elevated alcohol utilization, exposure to exogenous substances and contaminants, administration of pharmaceutical agents including antibiotics, persistent psychological stress, sleep deprivation, bacterial or viral infection, as

well as various medical conditions such as metabolic disorders notably type 2 diabetes, and NDDs [118].

Several potential biomarkers of dysbiosis have been applied in the diagnosis of diseases, and when combined with clinical and other biomarkers, microbiome-based biomarkers can improve the precision of disease classification [119]. Notably, urolithin (a class of metabolites derived from colonic microbial degradation of dietary fiber in the human gut) detection in urine offers a non-invasive method to identify gut dysbiosis and inflammation in PD, while reduced *Roseburia species* levels show potential as a PD marker [120]. Microbial metabolites like indoxyl sulfate could serve as diagnostic biomarkers for dysbiosis and neurological conditions, and *Enterobacteriaceae* abundance may predict post-stroke cognitive impairment [119].

1.3.2 The role of gut dysbiosis on the pathophysiology of neurodegenerative disorders

Recent studies have shown that gut microbiota dysbiosis can lead to increased permeability of both the intestinal barrier and the BBB, along with alterations in intestinal mucus and the translocation of gut microbes and their metabolites. These changes contribute to the induction of a toxic inflammation state, causing a series of disturbances in physiological homeostasis, including oxidative stress, pathological protein aggregation, abnormal proteolysis, neuroinflammation, neuronal death and altered brain morphology. All of these gut dysbiosis-mediated dysfunction in the GBA signaling is linked to the progression of the neurodegeneration, which ultimately results in behavioral abnormalities and cognitive impairment.

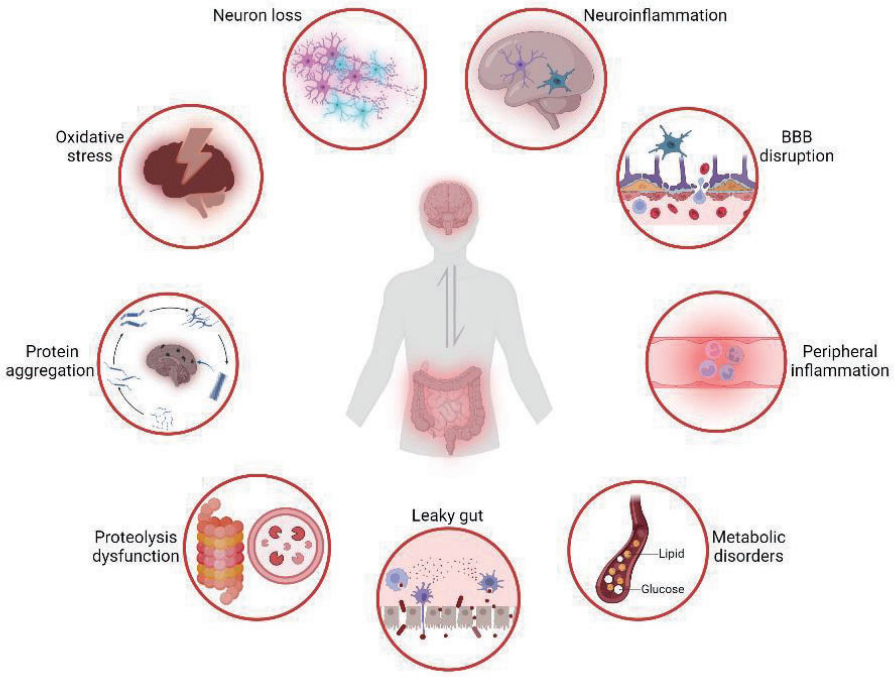


Figure 9. *The role of gut dysbiosis on the pathophysiology of neurodegenerative disorders*

The neurodegeneration process caused by gut dysbiosis is very complex and various physiological changes are interrelated (see Fig. 9). Specifically, the primary pathological mechanism triggered by gut

dysbiosis increases the permeability of the intestinal barrier, and the gut microbiota and their metabolites constantly interact with PRRs expressed in various host cells, including intestinal epithelial cells, immune cells in peripheral blood, as well as neurons and glial cells within the CNS. These PRRs such as TLRs, formyl peptide receptors, and the receptor for advanced glycation end products, can recognize pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs), which are highly conserved microbial structures including nucleotides, proteins, and LPS [121]. Consequently, active immune signaling pathways like the inflammasome, NF-κB, MyD88-dependent and type 1 interferon, lead to chronic inflammation. Chronic neuroinflammation fosters the aggregation of misfolded proteins around neurons, disrupting neuronal function, permeability, and synaptic integrity. These changes result in neuronal death and the release of misfolded neurotoxic aggregates, further exacerbating neuroinflammation.

Furthermore, decreased beneficial gut microbiota due to dysbiosis resulted in impaired microbial metabolism of neuroactive substances (such as tryptophan, SCFA, 5-HT levels). Conversely, increase in harmful microbiota elevated the production of toxic metabolites. AD, PD and amyotrophic lateral sclerosis (ALS) patients showed an increased abundance of cyanobacteria in the gut, which secretes higher levels of β-N-methylamino-L-alanine (BMAA), an excitotoxin that binds to metabotropic glutamate receptor 5 and depletes the major antioxidant glutathione. This results in excessive production of ROS and RNS in the brain, which in turn stimulates microglia and astrocytes, suggesting the direct link between gut dysbiosis and oxidative stress in NDDs [122].

Emerging research highlights a bidirectional link between gut microbiota and autophagy. Autophagy degrades invading pathogens, such as *Salmonella enterica* and *E.coli*, and plays a role in antigen presentation and lymphocyte development. Disruption of autophagy in the gut can worsen gut dysbiosis. In turn, gut dysbiosis can lead to chronic inflammation and oxidative stress, impairing autophagic clearance processes in both the gut and the brain.

Gut dysbiosis also contributes to fibril formation. The curli protein is one of the major components of the bacterial extracellular matrix, was shown to accelerate fibrilization by cross-seeding and aggregation of α-synuclein and β-amyloid [123]. Interestingly, numerous studies have revealed changes in the microbiome associated with specific NDDs. Studies have reported increased levels of pro-inflammatory bacteria like *Escherichia*/*Shigella* spp. and decreased levels of anti-inflammatory bacteria such as *Eubacterium rectale* spp. in the fecal microbiome of AD patients [124].

Collectively, gut dysbiosis can disrupt the intestinal and blood-brain barriers, alter the spectrum of microbial metabolites, induce intestinal, systemic, and neural inflammatory responses, trigger autophagic defects, and lead to increased oxidative stress and pathological protein aggregation. Gut dysbiosis disturbs these GBA communication pathways, involving neural, immune, and metabolic functional impairments, ultimately contributing to the pathophysiology of NDDs.

1.4 Therapeutic approaches targeting gut-brain axis

The link between gut dysbiosis and NDDs, suggests that dietary interventions targeting gut dysbiosis could be a promising strategy for treating symptoms and slowing down the neuroinflammatory and degenerative processes in NDDs.

To establish eubiosis and promote overall gut health, complementary nutritional interventions aimed at modulating gut microbiota composition and associated metabolites could complement existing therapeutic approaches. These interventions include various dietary plans, prebiotics, probiotics, synbiotics, and fecal microbiota transplantation (FMT) (Fig. 9), which have shown positive effects in reversing gut dysbiosis and promoting a healthy gut state [125].

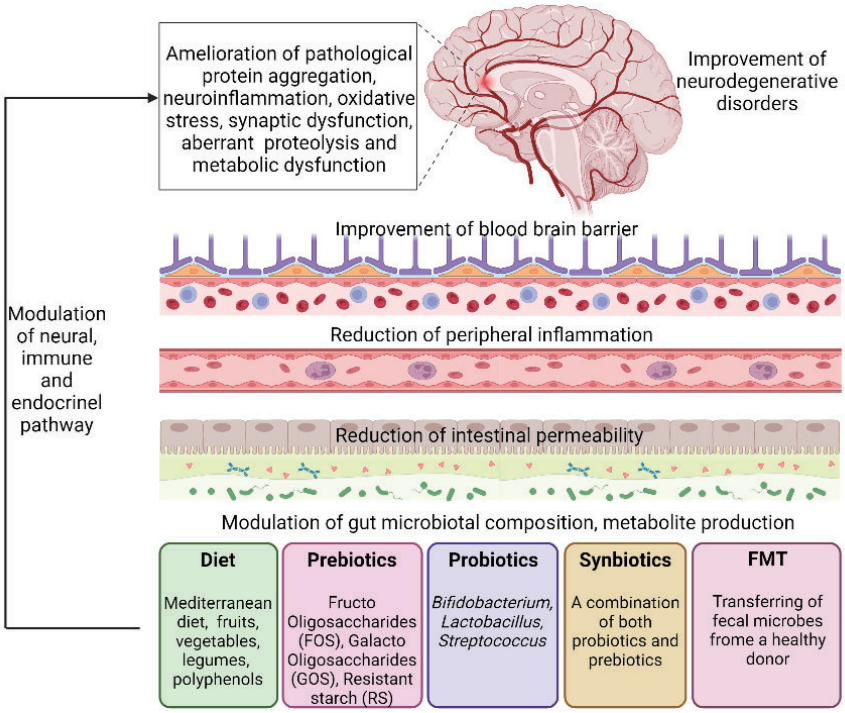


Figure 9. *Therapeutic approaches targeting gut-brain axis*

1.4.1 Diet

Diet can affect gut microbiota composition, which plays a critical role in maintaining host homeostasis. The Mediterranean diet (MD) emphasizes fruits, vegetables, legumes, and cereals, and is considered a healthy dietary receipt. One trial found that MD adherence slowed AD progression by 1.5 to 3.5 years, beneficial effects of MD may be mediated through gut microbiota changes and its anti-inflammatory characteristics.

High consumption of plant-based foods consistent with the MD diets modulates the gut flora, increasing SCFAs in feces and decreasing TMAO in urine [126]. A microbiota-accessible carbohydrate (dietary fiber) supplementation improved gut dysbiosis, intestinal barrier integrity, and systemic inflammation in mice,

further reducing neuroglial activation and synaptic dysfunction, but broad-spectrum antibiotic depletion reversed these effects [127]. Moreover, a healthy diet can avoid obesity and improve insulin resistance, which are considered potential risk factors for NDDs, such as AD.

Polyphenols, rich in fruits and vegetables, exhibit diverse physiological roles, they can regulate ROS to prevent oxidative stress, regulate autophagy pathway to reduce apoptosis, affect gut microbiota composition to improve the integrity of the intestinal barrier, and repair gut inflammation. In animal models of AD, resveratrol, a grape and wine polyphenol improved cognition and reduced neuroinflammation. It has also been observed potential to decrease pathological protein aggregation, including Aβ plaques and NFT. In a rat model of PD, resveratrol prevents dopaminergic neuron loss and reduces oxidative stress, lipid peroxidation, and protein carbonyl [128].

1.4.2 Prebiotics

Prebiotics are defined as indigestible dietary components in the upper part of human GI tract but selectively utilized by beneficial gut microbes in the large bowel, thereby promoting host health [129]. According to the structure of prebiotics, they can be divided into fructans including inulin and fructo-oligosaccharide or oligofructose (FOS); galacto-oligosaccharides (GOS); resistant starch (RS) known to resist digestion in the upper GI tract; glucose-derived oligosaccharides; and non-carbohydrate oligosaccharides. Prebiotics provide energy source to specific gut bacteria, altering their composition and activity. They can be fermented through a complex series of processes, producing byproducts that encourage cross-feeding between microorganisms. Prebiotics produce acidic fermentation products that reduce intestinal pH, affect gut microbial composition and abundance, mainly provide a favorable environment for beneficial bacteria, like *Lactobacilli* and *Bifidobacteria*, and inhibit pathogenic bacteria. The primary by-products produced starting from prebiotics are SCFAs. These small molecules can permeate gut enterocytes and enter the bloodstream, enabling prebiotics to have an impact not only on the GI tract but also on distant organs and systems, including brain [130].

RS has been shown to maintain bacterial abundance, improve lipid metabolism and intestinal function. Recently in a murine model of aging, RS from pinto beans, black-eyed peas, lentils, and chickpeas administration improved SCFA production while reducing bile acids and cholesterol, modulated gut metabolomic pool, therefore mitigating obesity-related metabolism disorders [131]. FOS as prebiotic ingredient, usually found in fruits and vegetables, promotes the growth of healthy gut microbiota and maintains microbial diversity and stability. FOS can also mitigate neuronal apoptosis, brain tissue swelling, and improve neurotransmitter synthesis and release. It has also been demonstrated to ameliorate cognitive damage and neurodegeneration in AD mice by regulating the gut microbiota and activating GLP-1 pathway [132]. In another study, FOS from *Morinda officinalis* administration successfully improved behavior like learning and memory abilities in a D-galactose and $A\beta_{1\text{-}42}$ induced AD deficient rats, reduced oxidative stress and inflammation and increased neurotransmitters, such as 5-HT, 5-hydroxy indole acetic acid, acetylcholine and dopamine, these beneficial effects were contributed by FOS

modulating gut and brain homeostasis [133].

1.4.3 Probiotics

Probiotics refer to live nonpathogenic microorganisms that confer health benefits when consumed in adequate amounts. Some of the most commonly used probiotics including *Bifidobacterium*, *Lactobacillus*, *Bacillus*, *Streptococcus*, and *Enterococcus*. Studies illustrated that probiotics exert their benefits through various mechanisms: they regulate gut microbial populations, reduce pathogens colonization and invasion; they increase epithelial cell proliferation and differentiation to reinforce intestinal barrier and reduce immunomodulation; probiotics also produce beneficial chemicals such as SCFAs with anti-inflammatory and neuroprotective effects that enter the circulatory system and cross the BBB, modulating CNS immune cell activity, inflammatory cytokines, BBB integrity, and neurogenesis, prompting brain health [134], [135]; they stimulate the synthesis and release of neurotransmitters, affecting BDNF levels, synaptic plasticity, neuronal function in NDDs [136].

A probiotic formulation of *Lactic acid bacteria* and *Bifidobacteria*, named SLAB51®, strategically modulated gut microbiota composition, increasing *Bifidobacterium spp.*, and decreasing *Campylobacterales*, with an increased production of SCFAs and neuroprotective gut peptide hormones, which contribute to reduce Aβ aggregates and prevente cognitive decline. Chronic supplementation with SLAB51® restored the functionality of neuronal proteolytic pathways, reduced cerebral oxidative stress via SIRT1-dependent pathways and improved glucose homeostasis in 3xTg-AD murine model of AD . Akbari et al. examined the cognitive effects of supplementing AD patients with *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium bifidum*, and *Lactobacillus fermentum*. Supplementation of these probiotics increased cognitive performance and metabolic status [137]. Long-term administration of probiotic *Lactobacillus paracasei* K71 prevented age-dependent cognitive impairment, the proposed mechanism was by upregulating BDNF levels in the hippocampus [138]. Probiotic-4, containing *Lactobacillus*, *Bifidobacterium*, *Typhimurium*, and *Eosinophilus*, was found to ameliorate age-related BBB and intestinal barrier disturbances. It also reduced plasma and brain LPS and proinflammatory markers such as IL-6, TNF-α, inhibited TLR4, and NF-kB inflammatory pathways in ALS mice model [139].

1.4.4 Synbiotics

Synbiotics are defined as specialized formulations of prebiotics and probiotics in which the prebiotics selectively favor the growth and metabolic activity of probiotics, enhance their viability and benefits, positively affect the microbiota composition of the host, increasing the abundance of beneficial microbes in the GI tract, resulting in potential health benefits for the host [140]. The composition employed in synbiotics should be appropriate so that the viability of probiotics in the GI tract is supported. Research findings have indicated that the utilization of synbiotics is more efficacious compared to the individual administration of probiotics or prebiotics.

A novel form of synbiotic, comprising three metabolically active probiotics—*Lactobacillus plantarum* NCIMB 8826, *Lactobacillus fermentum* NCIMB 5221, and *Bifidobacteria longum* spp. *infantis* NCIMB 702255—along with a polyphenol-rich prebiotic, acts through the gut-brain axis to heighten survivability and improve motility, reduces Aβ deposition and acetylcholinesterase activity, delays the onset of AD in *Drosophila melanogaster* model [141].

1.4.5 Fecal microbiota transplantation

FMT aims to re-establishing the healthy gut microbiome, increase gut microbiota diversity and function by transferring prescreened donor feces into patients' GI tract. Many studies have focused on the potential of FMT in the treatment of inflammatory bowel disease, metabolic disorders, and NDDs.

In APP/PS1 transgenic mice, Harach et al. found that 16S rRNA sequencing of conventionally raised APP/PS1 mice showed a significant gut microbiota alteration compared to wild-type mice. While GF APP transgenic mice had much lower Aβ (Aβ38, Aβ40, and Aβ42) than control mice. FMT from conventionally raised APP/PS1 exacerbated cerebral Aβ pathology in germ-free APP/PS1 mice, but FMT from wild-type mice did not [142].

Frequent transfer and transplantation of fecal microbiota from wild-type mice to $ADLP^{APT}$ mice have been found to reduce amyloid plaque and NFT formation, glial responses, and cognitive impairment. FMT has also been shown to reverse the abnormal expression of intestinal macrophage activity-related genes and the increase of circulating blood inflammatory monocytes in $ADLP^{APT}$ recipient mice [143]. In addition, in a study of PD patients, the researchers used FMT to treat 11 PD patients, reconstructed the gut microbiota, and improved the motor and non-motor symptoms of the patients [144].

The availability of well-organized stool banks and various routes of administration, such as capsules, enemas, or colonoscopies, provides opportunities to exploit FMT for the treatment of NDDs, making it a potentially convenient and efficacious therapy. However, FMT treatment poses significant unique and complex challenges for clinicians and regulators, including poorly defined mechanisms of action, stool availability, donor selection, adverse effects, and a relative lack of long-term follow-up data. Standardization of technical procedures, safety assessment, stool bank services and management, and other aspects are still in their infancy and require further study [145].

CHAPTER 2

GUT MICROBIOTA MODULATION IN ALZHEIMER'S DISEASE: FOCUS ON LIPID METABOLISM

Abstract

Background & aims: Unhealthy diet and lifestyle can unbalance the intestinal microbiota composition and, consequently energy metabolism, contributing to the pathogenesis of neurodegenerative disorders (NDDs) such as Alzheimer's disease (AD). Impairment of cerebral cholesterol metabolism occurs in both aging and AD, and lipid-lowering agents have been associated to a lower risk of NDDs, but the link between blood lipid profile and NDDs remains a matter of debate. Gut microbiota-host interactions have a key role in controlling glucose and lipid metabolism. Modulation of the gut microbiota composition by food-based therapy or by probiotic supplementation represents a promising preventive and therapeutic opportunity for NDDs. Understanding how lipid metabolism influences neurodegeneration and the regulatory effects of probiotics on lipid metabolism could provide valuable insights for the development of innovative therapies targeting abnormal lipid metabolism in NDDs.

Methods: This work focuses on the evaluation of the effects of SLAB51 chronic administration on lipid metabolism in 3xTg-AD mice and the respective wild-type counterpart. On this purpose, 8 weeks old mice were orally administered with SLAB51 for 4 and 12 months to analyze the plasma lipid profile (using lipidomic analyses and enzymatic colorimetric assays), along with the cerebral and hepatic expression levels of key regulators of cholesterol metabolism (through Western blotting and ELISA).

Results: Upon probiotics administration, cholesterol biosynthesis was inhibited in AD mice with a process involving sterol regulatory element binding protein 1c and liver X receptors mediated pathways. Decreased plasma and brain concentration of 27-hydroxycholesterol and increased brain expression of cholesterol 24S-hydroxylase indicated that alternative pathways of bile acid synthesis are influenced. The plasmatic increase of arachidonic acid in treated AD mice reflects dynamic interactions among several actors of a complex inflammatory response, in which polyunsaturated fatty acids can compete each other and simultaneously co-operate in the resolution of inflammation.

Conclusions: These evidence, together with the hypocholesterolemic effects, the ameliorated fatty acids profile and the decreased omega 6/omega 3 ratio successfully demonstrated that microbiota modulation through probiotics can positively change lipid composition in AD mice, with arachidonic acid representing one important hub metabolite in the interactions among probiotic-induced lipid profile changes, insulin sensitivity, and inflammation, all of which are recognized risk factors for NDDs.

Keywords: Neurodegenerative disorders; Alzheimer's disease; Microbiota modulation; Probiotics; Lipid metabolism; Cholesterol

2.1 Introduction

Neurodegenerative disorders (NDDs) encompass a range of debilitating conditions that afflicts millions of adults worldwide, with AD being a prominent example and the leading cause of late-life dementia. While cognitive dysfunction in NDDs is strongly linked to the accumulation of specific protein aggregates, such as amyloid-beta (Aβ) plaques and tau pathology [146], the precise molecular mechanisms underlying the development of these disorders are not fully understood. Among the modifiable risk factors associated with NDDs, diabetes, obesity, hypertension, and hypercholesterolemia can be effectively managed through healthy diet, regular physical activity and cognitive training. In particular, a diet rich in saturated fats and simple carbohydrates increases the risk of dementia and a suboptimal diet is associated with a more severe cognitive impairment in individuals affected by NDDs, such as AD [147], whereas Mediterranean diet correlates with better cognitive status in healthy people with reduced risk of AD [148]. Experimental and clinical evidences indicate that NDDs are often associated with metabolic dysfunction [149], which may worsen neurological symptoms [150]. Impairment of cerebral cholesterol metabolism occurs in both aging and AD [151], [152] and lipid-lowering agents have been associated to a lower risk of developing NDDs [153], but the link between blood lipid profile and NDDs such as AD continues to be a matter of debate [154].

In this context, gut microbiota-host interactions have a key role in controlling oxidative status, neuroinflammation, glucose and lipid metabolism. Modulation of the gut microbiota composition by food-based therapy or by probiotic supplementation represents a promising preventive and therapeutic opportunity for NDDs [155], [156]. Preclinical studies indicate that changes in the gut bacterial profile can reduce amyloid plaques in AD animal models, improving memory and lowering inflammation [157], which is a crucial pathogenic hallmark in neurological disorders [158]. Interestingly, polyunsaturated fatty acids (PUFAs) such as arachidonic acid (AA), α-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) can exert pro/anti-inflammatory effects and neuronal protective functions [159]. *Hjorth* et al. reported that DHA and EPA can be beneficial in AD by enhancing the removal of Aβ, increasing the production of growth factors like neurotrophin, and decreasing the secretion of pro-inflammatory cytokines [160]. Moreover, treatment with EPA and DHA markedly reduced the release of pro-inflammatory cytokines in peripheral blood mononuclear cells from AD patients [161].

Recently, new insights into the pathways controlling the levels of lipids, sterols and their metabolites indicated their important implications in AD, one of the primary NDDs. Specifically, cholesterol levels are sensed and regulated by two distinct transcription factor pathways, namely the sterol regulatory element-binding proteins (SREBPs) [162] and the liver X receptors α and β (LXRα and LXRβ) [163]. In detail, sterol response element binding protein 1c (SREBP-1c) can be modulated in different tissues by LXR α/β in response to altered sterol and oxysterols levels, consequently regulating the expression of genes that coordinate cholesterol and fatty acids metabolism [164].

Interestingly, SREBPs regulate 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR), which is the rate-limiting enzyme in cholesterol biosynthesis, but the exact mechanism of action of cholesterol lowering drugs and their implication in reducing susceptibility to NDDs are not exactly defined [165], [166]. Additionally, LXRs represent attractive therapeutic opportunities in AD because their activation controls the expression of genes involved in cholesterol uptake, efflux, transport, and excretion in multiple tissues, counteracts inflammation by modulating innate and adaptive immune responses, positively impacts glucose homeostasis, and decreases Aβ production by modulating amyloid precursor protein processing [167], [168].

Upon increased cellular sterol levels, LXRs can enhance the expression of the inducible degrader of LDL receptor (IDOL), an E3 ubiquitin ligase involved in the ubiquitin-proteasome mediated degradation of important substrates including LDL receptor [169] thus regulating the structural and functional plasticity of synapses and neural circuits [170], [171]. However, the contribution of LXR-IDOL pathway to feedback inhibition of cholesterol uptake and the complementary role of SREBP pathway in regulating lipid homeostasis in NDDs need to be further explored.

Differently from lipoprotein-bound cholesterol, oxidized cholesterol metabolites can cross the blood brain barrier (BBB) [172]. Increased levels of 27-hydroxycholesterol (27-OHCE) were observed in the brain of AD patients [173] due to hypercholesterolemia, increased BBB permeability and reduced neuronal metabolism of 27-OHCE by oxysterol 7 α-hydroxylase, a cytochrome p450 enzyme responsible for the first and rate limiting step in bile acid synthesis [174]. Abnormal levels of oxysterols in the brain can increase Aβ production [175] and worsen neuroinflammation [176]. In detail, oxysterols are endogenous activators of LXRs, which in turn play a role in the regulation of Aβ in the brain by mechanisms involving cholesterol transporters [177]. Moreover, cerebral cholesterol turnover is mainly performed by cholesterol 24-hydroxylase (CYP46A1), a cytochrome p450 enzyme that is mainly expressed in hippocampal and cortical neurons [178]. Several works demonstrated that increased CYP46A1 activity improved memory [179], reduced Aβ plaques and restored spatial memory performances in mice [180].

Recent studies have identified the modulation of gut-brain axis as a successful opportunity to counteract age-related decline and NDDs [155], [181]. It was previously demonstrated that the oral administration of SLAB51 probiotic formulation reduced Aβ aggregates and brain damages, and partially restored the impaired neuronal proteolysis in 3xTg-AD mice. The increased gut content of anti-inflammatory short-chain fatty acids and the increased plasma concentrations of neuroprotective peptide hormones partially explained the improved learning and memory and the reduced inflammation in treated AD mice [157]. In addition, SLAB51 mitigated cerebral oxidative stress through sirtuin-1-dependent mechanisms and ameliorated glucose uptake and metabolism in AD mice .

Several studies described the cholesterol-lowering effects of probiotics in both animals and humans [182]. Considering the key role of impaired cerebral lipid metabolism in NDDs and histopathological alterations,

the amelioration of lipid metabolism represents a promising approach in NDDs therapy and deserves more attention than it has received until now. In this perspective, the present work has focused on exploring the effects of SLAB51 oral administration on lipid metabolism in 3xTg-AD mice, a model of NDDs and their wild-type (wt) counterpart. For this purpose, 8-week-old mice were orally administered with SLAB51 for 4 and 12 months to investigate the hypocholesterolemic effects and the ability to qualitatively ameliorate plasma lipid profile. The brain expression level of LXRs, SREBPs, IDOL, and the liver expression level of HMGCR, LXRs and SREBPs, were evaluated to demonstrate that cholesterol biosynthesis is influenced, following the activation LXRs and SREBPs dependent pathways. Moreover, the plasma and cerebral amounts of the oxidative metabolite 27-OHCE and the brain expression of CYP46A1 enzyme were monitored to explore the ability of the probiotics to influence alternative bile acids biosynthetic pathways, maintain cholesterol turnover and homeostasis and support neuronal function in the context of NDDs, with the aim of elucidating the mechanisms through which the probiotic formulation counteracts the progression of NDDs using AD mice as the research model.

2.2 Material and methods

2.2.1 Reagents and chemicals

SLAB51 probiotic formulation was provided by Ormendes SA (Jouxtens-Mézery, Switzerland, https://agimixx.net). SLAB51 contains eight different live bacterial strains: *Streptococcus thermophilus* DSM 32245, Bifidobacterium lactis DSM 32246, Bifidobacterium lactis DSM 32247, *Lactobacillus acidophilus* DSM 32241, *Lactobacillus helveticus* DSM 32242, *Lactobacillus paracasei* DSM 32243, *Lactobacillus plantarum* DSM 32244, *Lactobacillus brevis* DSM 27961.

Enzymatic colorimetric kits for total cholesterol, high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) determination were kindly provided by Chema Diagnostica (Monsano, AN, Italy). HMGCR, HMG-CoA, NADPH, $NADP^+$, CoA, potassium phosphate, sodium phosphate, magnesium sulfate, phenyl-methanesulfonyl-fluoride, ethylenediaminetetraacetic acid (EDTA), tosylphenyl-alanyl-chloromethyl-ketone, dithiothreitol (DTT), and dimethyl sulfoxide (DMSO) were all purchased from Sigma-Aldrich. HPLC grade methanol was obtained from JT Baker. All solvents and reagents were of the highest purity available.

The reverse phase Luna C18 column (5 µm particle size, 250 × 4.6 mm, equipped with a 5 mm guard column) was purchased from Phenomenex S.r.l. (Bologna, Italy).

Membranes and reagents for western blotting analyses were purchased from Merck KGaA, (Darmstadt, Germany). The rabbit polyclonal anti IDOL (ab-74562) and the rabbit monoclonal anti-HMGCR [EPR1685(N)] (ab174830) antibodies were from AbCam (Milano, Italy). The mouse monoclonal antibodies anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), anti CYP461A antibody, anti-LXRα/β antibody and anti-SREBP-1c were from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany).

2.2.2 Animal model

AD triple-transgenic mice, B6; 129-Psen1^{tm1Mpm} Tg (APPSwe, tauP301L)1Lfa/J (named 3xTg-AD) and the wild type B6129SF2 mice, were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). 3xTg-AD mice contain three mutations associated with frontotemporal dementia or familial AD (amyloid precursor protein [APP]Swe, tau MAPT P301L, and presenilin-1 M146V). This reliable model of human AD displays both plaque and tangle pathology, with Aβ intracellular immunoreactivity detectable at three months of age and hyperphosphorylation of tau protein occurring by 12-15 months of age [183]. Consequently, in 12-15 months it is possible to reliably reproduce traits similar to those observed in the entire life of Alzheimer's disease patients.

Experiments complied with the ARRIVE guidelines, in accordance with the EU Directive 2010/63/EU for animal experiments and with a protocol approved by the Italian Ministry of Health (518/2018-PR). Mice were housed in plastic cages (Makrolon, Covestro A.G., Filago, Italy) in a temperature-controlled room (21 ± 5 °C) and 60% humidity on 12-h light/dark reversed cycle (light was switched on at 8:00 p.m.)

and maintained on standard laboratory diet (Mucedola, Italy) and water ad libitum. Appropriate measures minimized pain and discomfort in experimental animals.

2.2.3 Experimental design

Eight-week-old AD male mice (n = 48) were organized in two groups and were treated with SLAB51 dissolved in water (n = 24) or with water (control group, n = 24). At the same time, 48 age-matched wild type (wt) mice were organized into wt control (n = 24) and wt-treated (n = 24) groups. Based on the body surface area principle the dosage of SLAB51 2×10^{11} bacteria/kg/day was calculated. The body weight was monitored during the treatment to ensure single-housed animals received the proper intake of the probiotic. Preliminary studies were performed to evaluate both viability and stability of the probiotic formulation as previously described and probiotic drinking solution was freshly prepared every day. With the aim to start treatment before the deposition of protein aggregates, and based on preliminary experiments, 8, 24 and 56 weeks of age were selected as timepoints, in order to evaluate the preventative and therapeutic properties of chronic supplementation with probiotics. Eight mice per group were euthanized by CO_2 overdose at 8, 24 and 56 weeks of age, and the tissues were properly collected for biochemical analyses. In detail, murine brains and livers were quickly removed and placed on an ice-cold glass plate. Plasma samples and tissue homogenates were promptly supplemented with protease inhibitors (1 mM tosyl phenylalanyl chloromethyl ketone (TPCK) and Pefabloc).

2.2.4 Plasma lipid analysis

Plasma total cholesterol, high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) were determined enzymatically, using commercially available colorimetric kits (Chema Diagnostica, Italy) according to the manufacturer's instructions. Data are expressed as mg/dL ± SE.

2.2.5 Preparation of brain and liver homogenates

Upon sacrifice, tissues were homogenized in 50 mM Tris buffer, 150 mM KCl, 2 mM EDTA, pH 7.5 (1:5 weight/volume of buffer). Homogenates were immediately centrifuged at 13,000 × g for 20 min at 4 °C and the supernatants were used. Protein concentration was measured with the Bradford protein assay [184].

2.2.6 Liver microsomes preparation

Liver microsomes were prepared as previously reported [185]. In detail, liver tissue samples (0.1 g) were added to 1 mL of cold homogenization buffer (50 mM Tris-HCl buffer, 0.3 M sucrose, 10 mM EDTA, 10 mM DTT and 50 mM NaCl at pH 7.4 in the presence of protease inhibitors and homogenized using a bench-top Ultra-Turrax TP 18/10 homogenizer (Janke and Kunkel; Staufen, Germany). The homogenate was centrifuged at 20,000 × g for 15 min at 4 °C. Supernatant was collected and (ultra)centrifuged at

100,000 × g for 60 min at 4 °C. Microsomal pellet was finally resuspended in the activity buffer (100 mM phosphate buffer, 10 mM DTT, 1 mM EDTA, 2% DMSO at pH 6.8). Total protein concentration was determined with the Bradford assay.

2.2.7 High performance liquid chromatography (HPLC) analysis

The Amersham Biosciences AKTA basic HPLC system equipped with a UV/VIS detector was used for the analysis. Reaction mixture consisting of ultracentrifuged liver homogenates, NADPH and HMG-CoA was incubated at 37 °C (aliquots were withdrawn at indicated times and separated by HPLC). Each species was injected and separated with Phenomenex Luna C18 reverse-phase-HPLC column (5 μm particle size, 250 × 4.6 mm) with the following linear gradient of 100 mM potassium phosphate (solvent A) and methanol (solvent B): 10%-30% B in 3 column volumes, 30%-30% B in 1 column volume and 30%-10% B in 1 column volume at flow rate of 0.6 mL/min, UV/VIS detector set at 260 nm. After each chromatographic elution, column was regenerated with two column volumes of 60% methanol. HMG-CoA, NADPH, and $NADP^+$ were directly monitored, whereas mevalonate was determined by monitoring CoA production (mevalonate/CoA 1:1 stoichiometric ratio)

Liver microsomes dissolved in the activity buffer (100 mM sodium phosphate buffer containing 1 mM EDTA, 10 mM DTT, 2% DMSO, and 1 mM magnesium sulfate, pH 6.8) were incubated with substrate and NADPH for 60 min at 37 °C. The resulting mixture (10 μL) was separated with the above-described Luna C18 column thermostatted at 26 ± 0.1 °C.

2.2.8 Western blotting

The expression levels of liver microsomal HMGCR were analyzed through Western blot assay with the aim to verify a possible change upon probiotics treatment. In detail, for each time point (8-week-old, 24-week-old and 56-week-old) microsomes (20 μg of total proteins) were resolved on a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) then transferred on polyvinylidene fluoride (PVDF) membranes. Successively, upon incubation with specific antibodies, the immunoblot detection was carried out with an enhanced chemiluminescence (ECL) Western Blotting ChemiDoc™ System (Biorad, Milano, Italy). Molecular weight markers were included in the gel. With the same approach LXRs (α/β) and SREBP1c expression levels were measured.

GAPDH was used to check equal protein loading and to normalize Western blot data. The densitometric analysis was conducted as previously described [186]. Briefly, ChemiDoc acquired images or scanned autoradiographs (16-bit gray scale) were processed through Image J (NIH) to calculate the background mean value and its standard deviation. The background-free image was then obtained subtracting the background intensity mean value from the original digital data. The integrated densitometric value associated with each band was then calculated as the sum of the density values over all the pixels belonging to the considered band having a density value higher than the background standard deviation.

The band densitometric value was then normalized to the relative GAPDH signal intensity. The ratios of band intensities were calculated within the same Western blot. All the calculations were carried out using the Matlab environment (The MathWorks Inc., Natick, MA, USA).

2.2.9 Plasma fatty acid profile

The determination of the fatty acid composition of plasma glycerophospholipids was obtained using a previously described method [187], with the aim to verify the ability of the treatment to shift the lipid metabolic pathway exerting a neuroprotective effect. Briefly, 100 µL of plasma and 0.6 mL methanol (precooled at 5 °C) were combined in glass tubes and shaken for 30 s. The precipitated proteins were separated from the methanolic phase by centrifugation at 900 × g for 5 min. The methanolic supernatant was transferred into another glass tube. 25 µL of sodium methoxide solution were added to the supernatant, then the tubes were shaken at room temperature during selective synthesis of methyl esters. The reaction was stopped after 3 min with 75 µL of methanolic HCl. Fatty acid methyl esters (FAME) were extracted by adding 300 µL hexane and shaking the tubes for 30 s. The upper hexane phase was transferred into a 2 mL vial. The extraction was repeated, and combined extracts were dried under nitrogen flow at RT. The dry residue was taken up in 50 mL hexane (containing 2 g/L tert-buthyl-hydroxy-toluene) for GC analysis.

FAME were quantified using gas chromatography by a standard procedure using a capillary column ZB-FAME, 30 m × 0.25 mm, film thickness 0.20 µm (Phenomenex, USA) in a 7820 A GC System (Agilent Technologies, Santa Clara, California, EUA). The FAME was evaluated by calculating each FA as a percentage over the total FA cluster (relative %). GC peaks were identified as 97% of the total peaks present in the GC analysis by comparison with commercially available standards. Results were interpreted using the post hoc Tukey's test and are expressed as percent abundance.

2.2.10 ELISA determination of 27-hydroxycholesterol

27-Hydroxycholesterol (27-OHCE) was measured in the plasma and the brain of control and treated 56-week-old AD mice using the mouse 27-hydroxycholesterol solid-phase ELISA kit (My BioSource, San Diego, California, USA) following the manufacturer's instructions. Data are expressed as ng/mL ± SE.

2.2.11 Statistical analysis

Biochemical data are expressed as mean values ± SE. Statistical analysis was performed with one-way ANOVA, followed by the Bonferroni and Tukey test using Sigma-Stat 3.1 software (SPSS, Chicago, IL, USA). Statistical significance of treated mice compared to untreated 8-week-old mice of the same genotype is indicated with asterisks ($^{*}p < 0.05$). To describe the effect of SLAB51 treatment, statistical significance of treated mice compared to age-matched untreated mice of the same genotype is indicated with hashtags ($^{\#}p < 0.05$).

2.3 Results

2.3.1 Probiotic administration ameliorates blood lipid profile in AD

Cholesterol is involved in APP processing and high levels of cholesterol correlate with increased risk of AD [188]. Considering that cholesterol lowering therapies improve cognitive performance in AD subjects, the ability of SLAB51 to ameliorate blood lipid profile was investigated. As expected, cholesterol plasma concentration in 3xTg-AD mice was significantly higher than that of age-matched wt mice (Fig. 1). Interestingly, upon probiotic administration, a significant decrease of total cholesterol concentration was detected in the plasma of 56-week-old AD mice (Fig. 1). Conversely, no significant variations were observed in wt animals.

Moreover, old AD mice showed decreased levels of high-density lipoprotein cholesterol (HDL-C) and increased levels of low-density lipoprotein cholesterol (LDL-C) with respect to age matched wt mice. Probiotics oral administration induced a significant increase of HDL-C and a significant decrease of LDL-C plasma concentrations, indicating a positive effect on blood lipid profile and suggesting an amelioration of AD pathology.

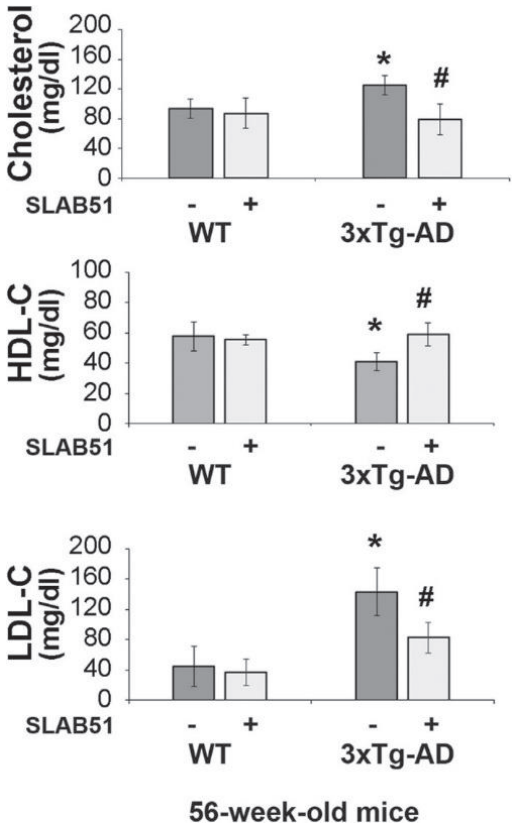


Figure 1. *Blood lipid profile. Plasma concentrations (mg/dL) of total cholesterol, HDL-C and LDL-C in 56-week-old wild type and AD mice treated (light grey) or not (dark grey) with SLAB51 probiotic formulation ($^{*}p > 0.05$ compared with untreated wild type mice; $^{\#}p > 0.05$ compared with untreated age-matched mice of the same group).*

2.3.2 SLAB51 oral administration influences cholesterol biosynthesis and metabolism

Then we explored the ability of probiotics to modulate the functionality of HMGCR, the rate-limiting step of cholesterol biosynthesis. In detail, the activity of HMGCR was measured in liver microsomes of mice administered with SLAB51 or water using a previously described HPLC based method [189].

HMGCR activity was significantly lower in 56-week-old AD mice orally administered with SLAB51 for 12 months, as indicated by the decreased area of the CoA peak (Fig. 2). No significant differences were observed in wt mice (data not shown).

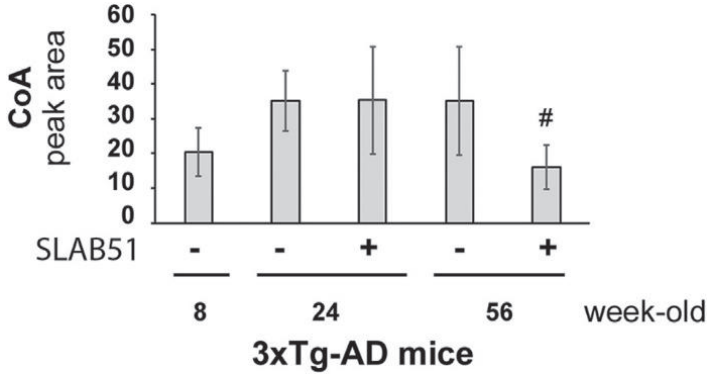


Figure 2. *Inhibition of HMGCR activity in 3xTg-AD mice treated with SLAB51. Mevalonate production calculated from changes in CoA peak area (mevalonate/CoA 1:1 stoichiometric ratio) in 8, 24 and 56-week-old mice orally administered with SLAB51. Data points marked with a hashtag are statistically significant compared to age-matched untreated mice ($^{\#}p < 0.05$).*

Liver homogenates were analyzed through western blotting in order to evaluate the expression levels of HMGCR enzyme. 56-week-old untreated AD mice showed a significant increased expression of HMGCR with respect to young animals (Fig. 3). Interestingly, HMGCR levels significantly decreased in the liver of 56-week-old treated mice, indicating that the cholesterol lowering effect (Fig.1) was due to both decreased HMGCR activity (Fig. 2) and reduced expression of the protein (Fig. 3).

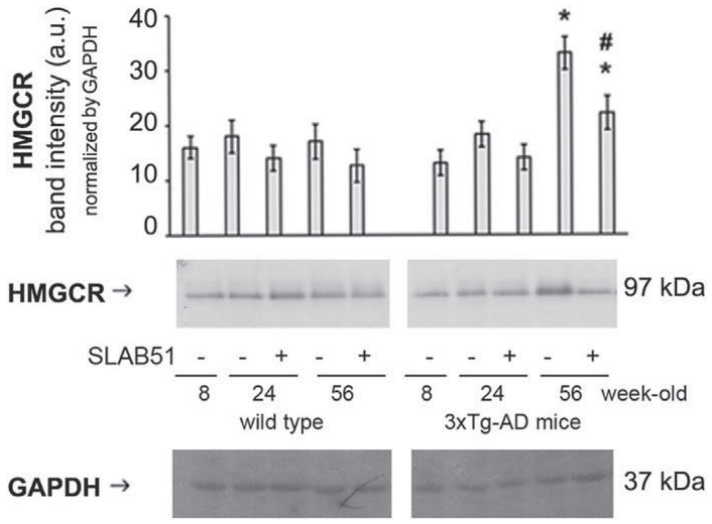


Figure 3. *Effect of SLAB51 on HMGCR expression levels. HMGCR expression levels were detected in 8-week-old mice treated for 4 and 12 months with SLAB51 probiotic formulation. Representative immunoblots of both wt (right) and 3xTg-AD (left) mice is reported and corresponding densitometric analyses derived from six separate blots are shown. Equal protein loading was verified by using an anti-GAPDH antibody and normalized expression of the target protein is reported as arbitrary units (a.u.). Data points marked with an asterisk are statistically significant compared to 8-week-old untreated control mice of the same genotype ($^{*}p < 0.05$). Data points marked with a hashtag are statistically significant compared to age-matched untreated mice ($^{\#}p < 0.05$).*

Amelioration of blood lipid profile can also be explained by the modulated expression of SREBP1c and LXRs in probiotic-treated 3xTg-AD mice. In detail, increased levels of SREBP1c were observed in both liver and brain of untreated AD mice (Fig. 4, upper panels) indicating an increased cholesterol synthesis and in agreement with the enhanced expression and activity of HMGCR (Fig. 3). Interestingly, cerebral and hepatic protein expression of SREBP1c significantly decreased upon 12 months treatment with probiotics (Fig. 4, upper panels), suggesting that HMGCR inhibition can be also modulated through SREBP1c dependent pathways.

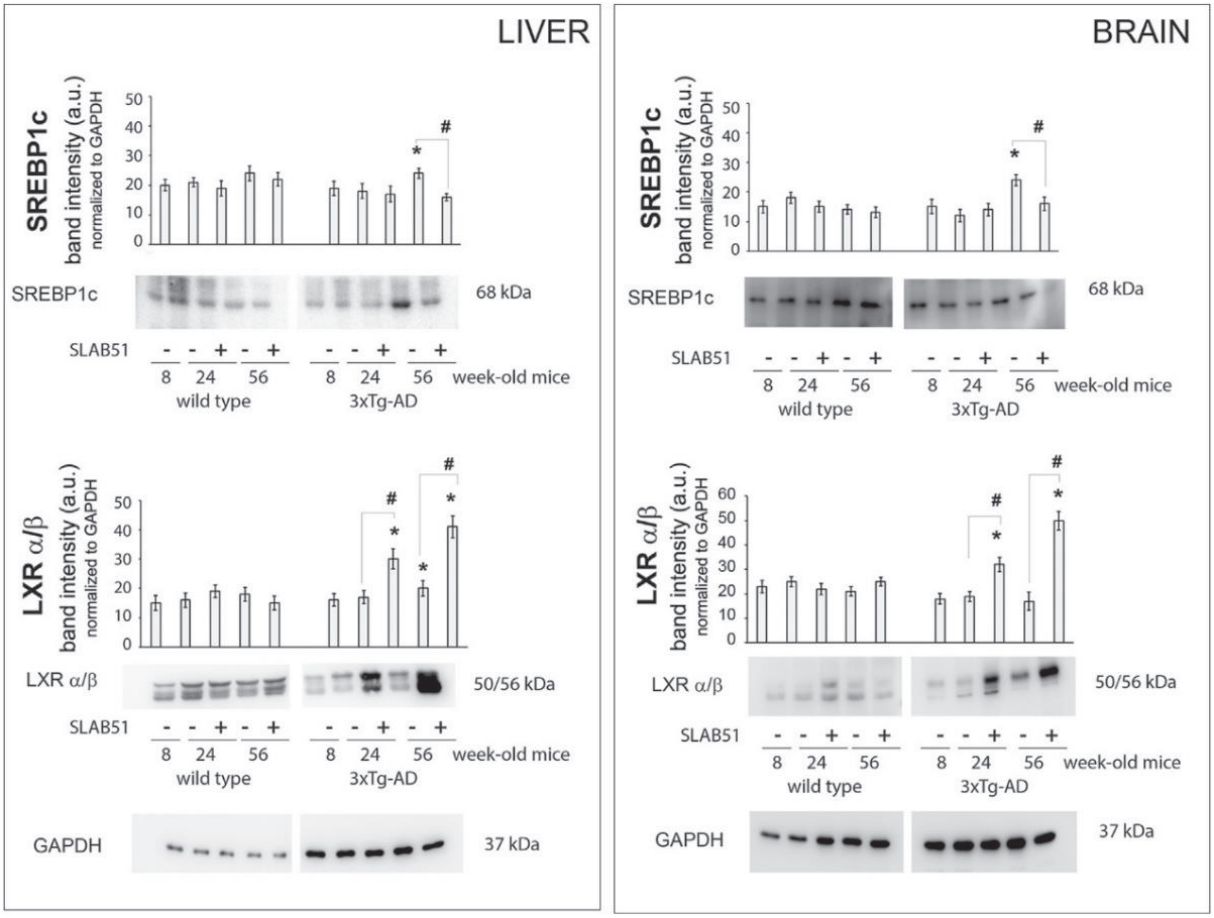


Figure 4*. SREBP1c and LXRs expression levels in wt and AD mice orally administered with SLAB51 for 4 and 12 months. Representative immunoblots and corresponding densitometric analyses derived from six separate blots are shown. Equal protein loading was verified by using an anti-GAPDH*

antibody and normalized expression of the target protein is reported as arbitrary units (a.u.). Data points marked with an asterisk are statistically significant compared to 8-week-old untreated control mice of the same genotype ($^{*}p < 0.05$). Data points marked with a hashtag are statistically significant compared to age-matched untreated mice ($^{\#}p < 0.05$).

Additionally, SLAB51 oral administration significantly upregulated LXRs expression in the brain and the liver of 3xTg-AD mice, at 24- and 56- weeks of age (Fig. 4, lower panels), in line with the previously documented anti-inflammatory effects and cognitive improvement [157], and consistently with the ameliorated blood lipid profile (Fig. 1). This is of significant importance considering that LXRs also regulate the expression of genes involved in cholesterol absorption, transport, efflux, excretion and conversion to bile acids, finally affecting the reverse cholesterol transport [190].

In turn, LXRs can induce the expression of the inducible degrader of LDL receptor (IDOL) which is a E3 ubiquitin ligase that targets lipoprotein receptors for proteasome-mediated degradation and with a key role in metabolism and AD. Figure 5 shows that IDOL increased in the brain of SLAB51-treated mice, in agreement with the ability of probiotics to improve neuronal proteolysis in AD mice [157].

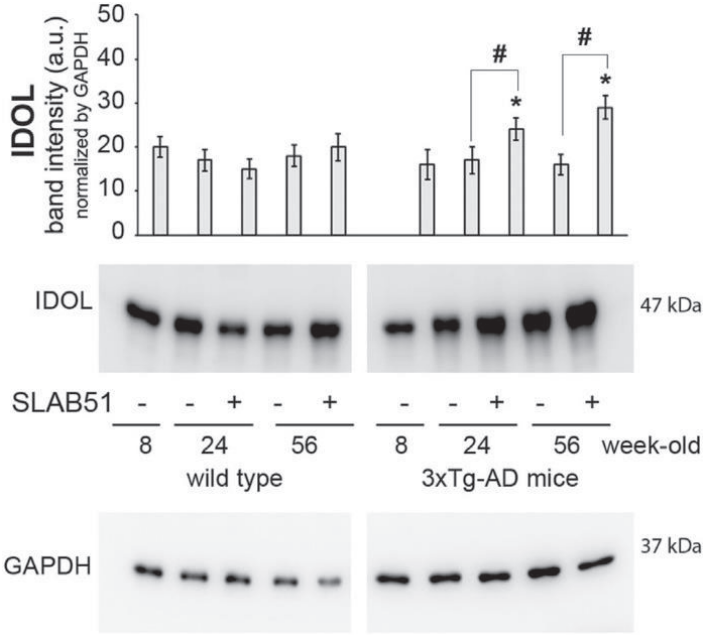


Figure 5. *IDOL expression levels in wt and AD mice orally administered with SLAB51 for 4 and 12 months. Representative immunoblots and corresponding densitometric analyses derived from six separate blots are shown. Equal protein loading was verified by using an anti-GAPDH antibody and normalized expression of the target protein is reported as arbitrary units (a.u.). Data points marked with an asterisk are statistically significant compared to 8-week-old untreated control mice of the same genotype ($^{*}p < 0.05$). Data points marked with a hashtag are statistically significant compared to age-matched untreated mice ($^{\#}p < 0.05$).*

2.3.3 Probiotic supplementation modifies plasma fatty acids composition

The determination of the fatty acid composition of plasma glycerophospholipids was performed to investigate the neuroprotective effects of chronic SLAB51 treatment attributable to the modulation of lipid metabolic pathway.

Plasma lipidomic analyses confirmed the probiotics anti-inflammatory effects as indicated by the significant decrease of cis-linoleic acid (Fig. 7) and the decreased ratio of cis-linoleic acid / (EPA + DHA) in 3xTg-AD mice supplemented with SLAB51 for 12 months (Fig. 6). In fact, the ratio of omega-6 to omega-3 is a recognized indicator of the health status and a lower ratio of n-6 to n-3 fatty acids is associated with reduction in inflammation [191], [192].

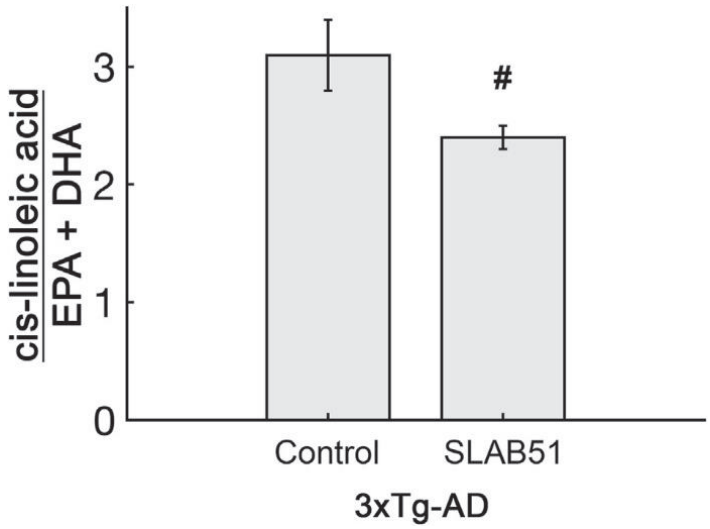


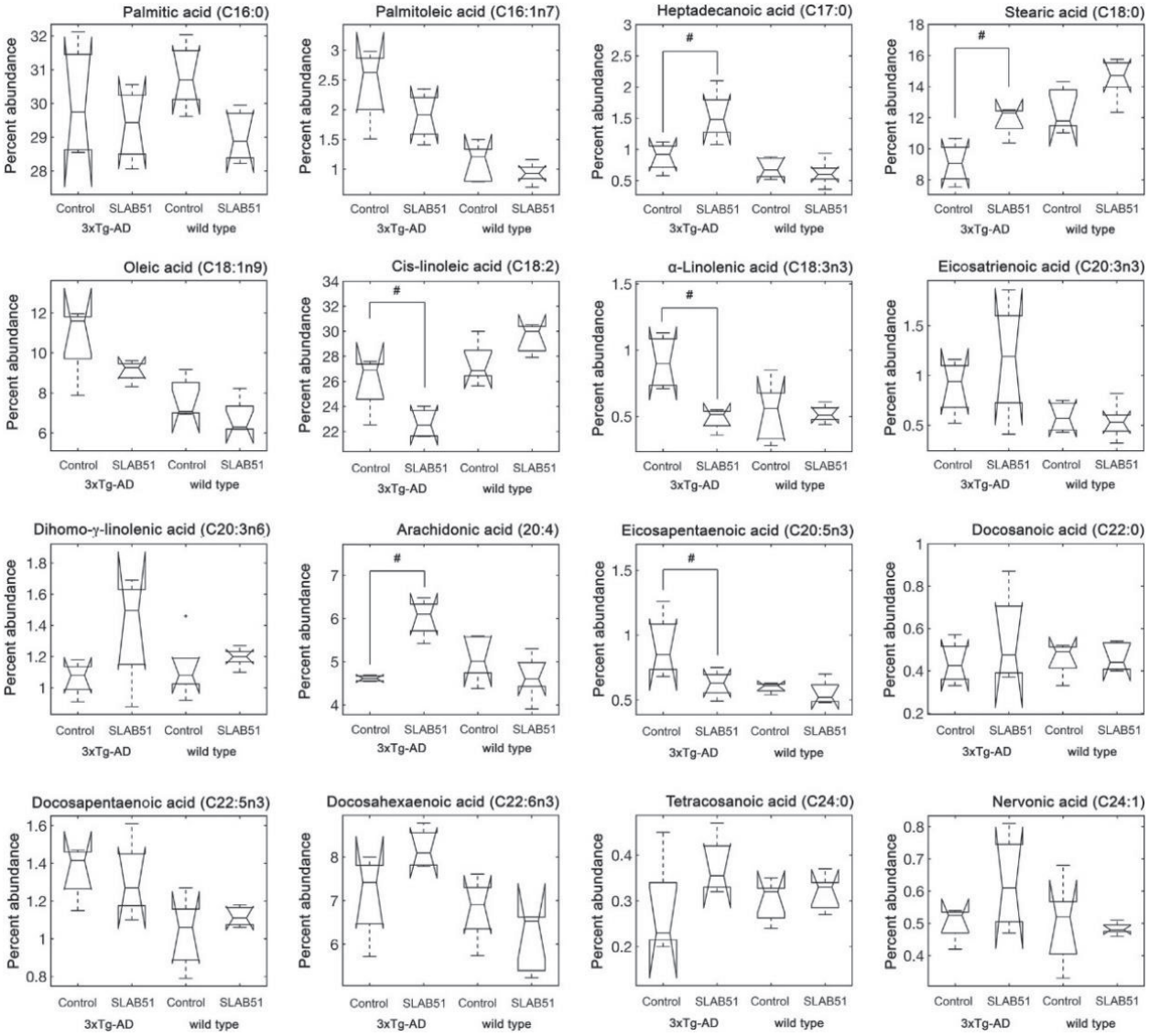
Figure 6. *Decreased omega6/omega 3 polyunsaturated fatty acids ratio in treated AD mice. cis-linoleic acid / (EPA þ DHA) ratio in the plasma of 56-week-old AD mice chronically administered with water (control) or with SLAB51 for 12 months. Data points marked with an asterisk are statistically significant compared to control ($^{\#}p < 0.05$).*

Data reported in Fig. 7 reveal that SLAB51-modified intestinal microbial status can affect plasma fatty acids composition. Although a slight but significant decrease of the omega-3 fatty acids α-linolenic acid and eicosapentaenoic acid was observed in 56-week-old AD mice upon SLAB51 supplementation (Fig. 7), the important reduction of the omega-6 cis-linoleic acid definitively determine the anti-inflammatory effect, by decreasing the omega/omega-3 ratio (Fig. 6).

Interestingly, SLAB51 treated AD mice displayed increased plasma levels of stearic acid, which was previously demonstrated to exert hypocholesterolemic effects [193]. Moreover, probiotic treated AD mice showed higher levels of heptadecanoic acid, which is inversely associated with insulin resistance and type 2 diabetes [194], in line with a correlation with the amelioration of glucose metabolism in the same mice upon SLAB51 chronic administration [195].

Altered composition of plasma fatty acids observed in AD mice was significantly modulated by probiotics

(Fig. 7), indicating dynamic effects of the shifted gut microbiota on the plasma lipid profile of AD mice. In fact, gut microbiota of AD mice has a significantly different structure with respect to wild type mice,



causing disequilibrium in energy homeostasis. No significant differences were observed in SLAB51-treated wt mice, confirming the safety of the probiotic-based approach, most likely due to the higher stability of wt gut microbiota [157].

Figure 7. *SLAB51 affects plasma lipid profile. Fatty acid composition of plasma glycerophospholipids in 56-week-old wt and 3xTg-AD mice treated with water (control) or SLAB51 for 12 months. Results were analyzed using the post hoc Tukey's test and are expressed as percent abundance. Data points marked with an asterisk are statistically significant compared to age-matched control mice of the same genotype ($^{\#}p < 0.05$).*

2.3.4 Decrease of 27-hydroxycholesterol upon probiotic treatment in AD mice

The neurotoxic oxysterol 27-hydroxycholesterol (27-OHCE) can cross BBB thus mediating the effects of hypercholesterolemia on the brain [173]. Interestingly, upon SLAB51 chronic administration, 27-OHCE concentration significantly decreased in both the plasma and the brain of 56-week-old AD mice compared to age-matched controls (Fig. 8).

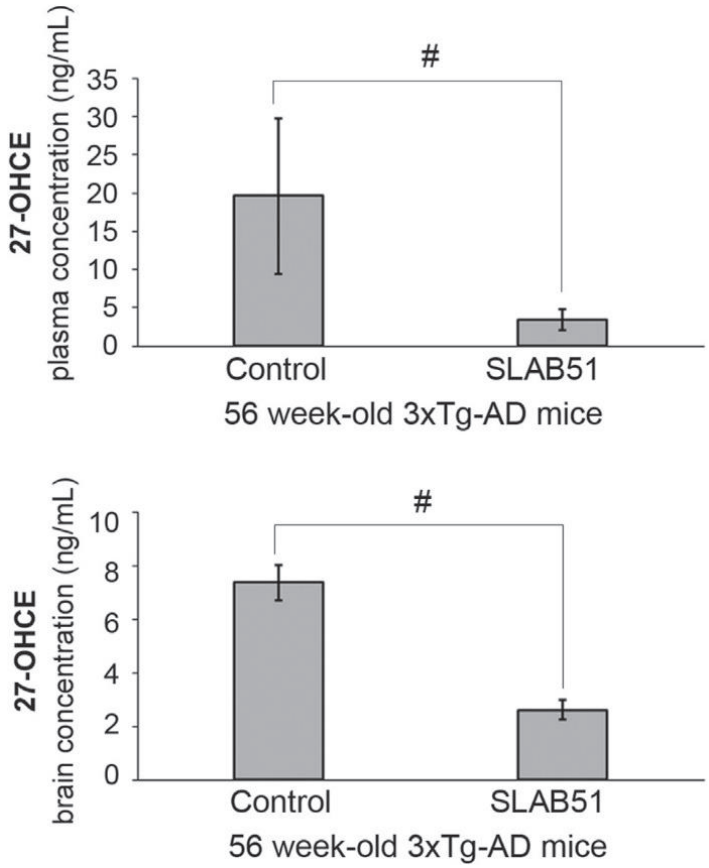


Figure 8. *27-Hydroxycholesterol plasma and brain levels. ELISA determination of 27-Hydroxycholesterol (27-OHCE) in the plasma (upper panel) and the brain (lower panel) of control and treated 56-week-old 3xTg-AD mice. 27-OHCE concentrations are expressed as ng/mL ± SE. Data marked with a hashtag are significantly different with respect to age-matched controls ($^{\#}p < 0.05$).*

2.3.5 SLAB51 increased the brain expression of CYP46A1

CYP46A1 is the rate limiting enzyme in cholesterol degradation and its cerebral restoration implicates neuroprotective effects. The increased expression of CYP46A1 enzyme in the brain of 24- and 56- week-old AD mice chronically administered with SLAB51 suggested the probiotic-dependent induction of cerebral cholesterol turnover [178]. No significant differences were observed in wild type animals (Fig. 9).

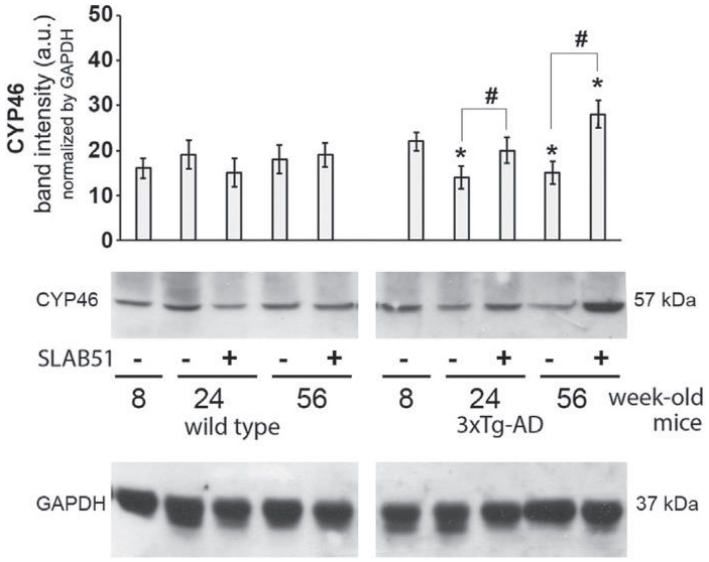


Figure 9. *Brain CYP46 expression levels in wt and AD mice orally administered with SLAB51 for 4 and 12 months. Representative immunoblots and corresponding densitometric analyses derived from six separate blots are shown. Equal protein loading was verified by using an anti-GAPDH antibody. Data points marked with an asterisk are statistically significant compared to 8-week-old untreated control mice of the same genotype ($^{*}p < 0.05$). Data points marked with a hashtag are statistically significant compared to age-matched untreated mice ($^{\#}p < 0.05$).*

2.4 Discussion

NDDs are a common cause of morbidity and cognitive impairment in older adults. One of the main global challenges for health and social care is to identify new strategies aimed at preventing or delaying NDDs onset and development. Potentially modifiable risk factors received increasing attention, with gut microbiota representing an attractive preventative/therapeutic target because of its role in regulating multiple neurochemical pathways through the gut-brain axis [196]. Deregulation of lipid homeostasis substantially contributes to the onset and progression of NDDs, including AD, and cholesterol lowering compounds (like statins), were demonstrated to reduce the risk of dementia. However, mechanistic insights into the link between abnormal lipid metabolism and pathogenesis of NDDs are still unavailable. The ability of statins to improve metabolism can be explained through gut microbiota altered composition [197]. In this context, dietary interventions, including probiotics, which are able to modulate microbiota composition, have been studied for their ability to improve energy homeostasis and immune system in NDDs [155], [198].

Chronic dietary supplementation with SLAB51 probiotic formulation modified gut microbiota in 3xTg-AD mice, ameliorated glucose metabolism, inflammatory and oxidative status and partially recovered the impaired neuronal proteolysis, finally decreasing Aβ and tau aggregates and improving cognitive abilities, with consequent delay of AD progression [157], [195], [199].

Considering the contradictory data on the role of deregulated lipid homeostasis in NDDs and in light of the promising effects of probiotics on energy metabolism, 3xTg-AD mice as a model of NDDs and their wild type counterpart were chronically treated with SLAB51 with the aim of deeply dissecting the role of microbiota modulation in ameliorating NDDs pathology by affecting lipid homeostasis.

High levels of total cholesterol and LDL-C and low levels of HDL-C are associated with vascular dementia and, indirectly, with NDDs [200]. As expected, dyslipidemia was observed in older 3xTg-AD mice but not in wild type animals (Fig. 1). Interestingly, the probiotic mixture exerted hypocholesterolemic effects in AD mice, qualitatively ameliorating plasma lipid composition (Fig. 1), with a consequent reduction of total cholesterol/HDL-C and LDL-C/HDL-C ratios. These results can be partially explained with the significant decrease in HMGCR concentration and activity observed in hepatic microsomes, indicating that this multi-strain probiotic mixture can inhibit cholesterol biosynthesis, consistently with other studies [201], [202]. The present data are in agreement with the increased fecal content of short chain fatty acids induced by SLAB51 in the same animal model [157], particularly for the ability of propionate to inhibit hepatic lipogenesis and cholesterol synthesis, finally alleviating metabolic disorders [203].

Additionally, the increased hepatic expression of the lipogenic SREBP1c in untreated AD mice (Fig. 4, upper panels) indicates an age-dependent increase in cholesterol synthesis, in line with the enhanced expression and activity of HMGCR (Fig. 3). Interestingly, cerebral and hepatic protein expression of SREBP1c significantly decreased upon 12 months treatment with probiotics (Fig. 4, upper panels),

suggesting a probiotic-dependent effect on fatty acid and triglyceride synthesis [204] and indicating that HMGCR levels/activity can be regulated through SREBP1c dependent pathways. Moreover, SREBP1c decreased expression in both the brain and the liver of treated AD mice is in agreement with the ability of SLAB51 to counteract insulin resistance in the same animal model [195], since SREBP1c emerged as a metabolic mediator between insulin/glucose signaling and lipogenesis [205]. In fact, other studies indicated that hyperinsulinemia could mediate the induction of SREBP-1c that, in turn, triggers lipogenesis, suggesting inhibition of SREBP-1c as a therapeutic approach in dyslipidemia-associated disorders [206], [207].

In parallel, SLAB51 improved blood lipid profile and cholesterol homeostasis also by triggering LXRs-mediated pathways. In fact, these receptors can induce genes that positively regulate bile acid synthesis and apolipoprotein metabolism, and that decrease Aβ production, exerting neuroprotection [208], as previously reported [157]. Considering the recognized anti-inflammatory effects of LXRs [199], their increased levels upon treatment (Fig. 4, lower panels) better explain the ameliorated inflammatory status in SLAB51 treated AD mice [157]. Additionally, SLAB51 anti-inflammatory properties were confirmed by the decreased ratio of cis-linoleic/(EPA + DHA) in the plasma of 3xTg-AD mice (Fig. 6). This is a crucial information, because the ratio of omega-6 to omega-3 has been previously described as a fundamental indicator of human health and a lower ratio of omega-6 to omega-3 fatty acids is associated with reduction in inflammation [191]. Inflammation-associated diseases, such as diabetes, obesity, mental disorders, cardiovascular and autoimmune diseases are associated to an increased omega-6/omega-3 ratio leading to the production of pro-inflammatory molecules [209]. Consequently, the ameliorated omega-6/omega-3 balance in AD mice chronically supplemented with SLAB51 can represent one possible mechanism through which oral bacteriotherapy can modulate inflammatory processes.

Interestingly, the augmented expression of LXRs in the brain of treated mice contribute to elucidate the partial restored functionality of the ubiquitin-proteasome system (UPS) observed in the same mice upon SLAB51 treatment [157]. In fact, LXRs can trigger the expression of the E3 ubiquitin ligase IDOL, promoting the lipoprotein receptors proteolysis, affecting the structural and functional plasticity of synapses and neural circuits with effects on memory and behavior [170]. Moreover, they can also limit cholesterol uptake, complementary to SREBP mediated effects [171]. Improved cognitive abilities are also confirmed by the significant decrease of EPA plasma concentrations in treated AD mice, in line with a recent study suggesting that this n-3 polyunsaturated fatty acid contributed to impair memory and learning in animal models [210].

Other significant variations in plasma fatty acid composition were detected upon SLAB51 chronic supplementation. For example, heptadecanoic acid increased in probiotic-treated AD mice, suggesting a correlation with the improved glucose homeostasis and decreased insulin resistance in the same animal model chronically supplemented with SLAB51 for 12 months [195], since heptadecanoic acid is inversely associated with insulin resistance and type 2 diabetes mellitus [194].

On the other hand, the slight but significant reduction of α-linolenic acid and the increase of arachidonic acid detected in the plasma of 56-week-old treated AD mice are apparently in contrast with the previously documented decrease of pro-inflammatory cytokines in the plasma of SLAB51 treated mice at 24 weeks of age [157]. Actually, these variations highlight the dynamic interactions among numerous actors of a complex inflammatory response, in which polyunsaturated fatty acids can compete each other [211] and simultaneously co-operate in resolution of inflammation. In particular, the role of arachidonic acid in neurodegenerative conditions is controversial [212]; importantly, it is essential for the development of the brain, as it regulates cell membrane fluidity and ion channels activity and guarantees optimal cognitive functions, in agreement with the ameliorated behavioral performances observed in 3xTgAD mice chronically administered with SLAB51 [157]. Additionally, it stimulates type 2 immune response [213] and possesses anti-diabetic properties [214] in agreement with the previously documented effects of SLAB51 on glucose uptake and metabolism in 3xTgAD mice [195]. Consequently, arachidonic acid represents one of the key mediators in the interactions among probiotic-induced lipid profile changes, insulin sensitivity and inflammation.

An important linker among hypercholesterolemia, oxidative stress and neuropathology of AD in the brain is the cholesterol oxidation product 27-hydroxycholesterol [173], which is an intermediate of the alternative pathway of bile acids biosynthesis [215] and a recognized biomarker for impaired glucose homeostasis [149]. As expected, 27-OHCE was significantly increased in both the plasma and the brain of AD mice (Fig. 8, upper panel), confirming a correlation between circulating 27-OHCE and cholesterol [216] and in agreement with the altered BBB permeability, enhanced oxidative status and reduced glucose uptake in the brain of 3xTg-AD mice with respect to wild type animals [195], [199]. Upon SLAB51 supplementation, decreased concentrations of the oxysterol were measured both in the plasma and the brain of transgenic mice confirming the probiotics antioxidant efficacy [199] and the ability to restore glucose homeostasis [195]. Decreased 27-OHCE, together with the increased levels of CYP46A1 enzyme in the brain of SLAB51 treated AD mice (Fig. 8) indicated an effect on alternative pathways of bile acid production and confirm the activation of LXRs dependent pathways that maintain cholesterol turnover and homeostasis and support neuronal function [215], [217]. Considering the role of CYP46A1 in regulating memory functions [179] and Aβ deposition [180], its increase in SLAB51 treated AD mice contribute to elucidate the restored cognitive abilities and the reduced number of Aβ plaques previously observed in younger animals [157].

Collectively, these data contribute to address the unclear microbiota-influenced changes in the plasma lipids and demonstrate that probiotics can significantly affect the gut microbial composition with positive impact on the host lipid profile.

2.5 Conclusion

Our studies are part of the enormous effort that scientists are making to identify a definitive preventative and/or therapeutic approach for the uncurable, progressive and persistent dementia associated with NDDs. The etiology of these multifactorial diseases, including AD, still lacks a global consensus, as various factors such as proteinopathies, oxidative stress, inflammation, and metabolic disorders coexist in their pathogenesis. The emerging understanding of the gut-brain axis has shed light on the link between imbalanced gut microbiota and NDDs. Both preclinical [157] and human [218] studies have demonstrated the association, although the precise involvement of metabolic pathways is yet to be fully understood. Our evidence contributes to define the currently ambiguous mechanisms through which gut microbiota manipulation can ameliorate specific NDDs characteristics, and suggest that by identifying a successful combination of probiotic strains it is possible to strategically shift gut microbiota composition with positive consequences on the brain oxidative and inflammatory status, by regulating plasma lipid profile, cholesterol biosynthesis, and bile acid synthesis.

Concluding, our data unequivocally demonstrate that by targeting the gut-brain axis through a probiotic-based approach, it is possible to ameliorate lipid metabolism in this preclinical model of AD, mimicking effects exerted by statins, demonstrating which pathways are involved.

Achieving effective prevention of NDDs by finely regulating their associated risk factors, particularly through modulation of the gut-brain axis, will continue to be a persistent goal in research. Therefore, our findings should inevitably be considered in future preventative and therapeutic protocols targeting these devastating conditions.

CHAPTER 3

FLAVAN-3-OL MICROBIAL METABOLITES MODULATE PROTEOLYSIS IN NEURONAL CELLS REDUCING AMYLOID - BETA (1 - 42) LEVELS

Abstract

Background & aims: Aberrant proteostasis, characterized by impaired protein clearance and the accumulation of misfolded or aggregated proteins, is a pathogenesis and characteristic of neurodegenerative disorders (NDDs), including Alzheimer's disease (AD). The dysregulation of proteolytic pathways, such as the proteasomal and autophagic-lysosomal systems, contributes to the accumulation of protein aggregates in the brain, leading to neuronal dysfunction and cognitive decline. Since current drugs can only reduce specific symptoms, the identification of novel treatments is a major concern in NDDs research. Among natural compounds, (poly)phenols and their derivatives/metabolites are emerging as candidates in NDDs prevention due to their multiple beneficial effects. This study aims to investigate the ability of a selection of phenyl-γ-valerolactones, gut microbiota-derived metabolites of flavan-3-ols, to modulate the functionality of cellular proteolytic pathways. Understanding how these phenyl-γ-valerolactones can influence cellular proteolytic pathways may offer valuable insights into the development of innovative treatments targeting aberrant proteostasis in NDDs.

Methods and Results: Neuronal SH-SY5Y cells transfected with either the wild-type or the 717 valine-to-glycine amyloid precursor protein mutated gene are used as an NDDs model. And treated with 5-(4′-hydroxyphenyl)-γ-valerolactone, 5-(3′,4′-dihydroxyphenyl) -γ-valerolactone and 5-(3′-hydroxyphenyl)-γ-valerolactone-4′-sulfate, which is microbial metabolites of flavan-3-ol. Combining *in vitro* and in silico studies, it is observed that the phenyl-γ-valerolactones of interest modulated cellular proteolysis via proteasome inhibition and consequent autophagy upregulation and inhibited cathepsin B activity, eventually reducing the amount of intra- and extracellular amyloid-beta (1-42) peptides.

Conclusion: The findings of this study establish, for the first time, that these metabolites exert a neuroprotective activity by regulating intracellular proteolysis and confirm the role of autophagy and cathepsin B as possible targets for NDDs including AD preventive/therapeutic strategies.

Keywords: Neurodegenerative disorders; Alzheimer's disease; amyloid; autophagy; polyphenol metabolites; proteasome

3.1 Introduction

The proper synaptic connections are crucial for optimal brain function, and it heavily relies on the regulation of proteostasis, which governs the functional integrity of neuronal proteomes. Proteostasis encompasses a network of pathways that control the fate of proteins from their synthesis to degradation. Disruptions in proteostatic signaling have been implicated in age-related functional decline and the development of Neurodegenerative disorders (NDDs).

Alzheimer's disease (AD), in particular, is a neurodegenerative condition characterized by the accumulation of protein aggregates, such as amyloid-beta (Aβ) plaques and tau neurofibrillary tangles. The sequential cleavage of the amyloid precursor protein (APP) through the so-called amyloidogenic pathway is responsible for the release of amyloid beta (Aβ) peptides. A large part of these proteins are fragments of 40 amino acid residues in length, Aβ (1-40), whereas a remaining portion is a 42-residues protein, Aβ (1-42), known as the most toxic and particularly prone to aggregation [219]. Pathological protein aggregation processes, such as the formation of protein aggregates, represent a fundamental hallmark of NDDs. These aggregates result from defects in proteolytic pathways, leading to the accumulation of misfolded proteins. Additionally, these aggregated proteins are directly associated with oxidative stress and inflammatory responses, thereby exacerbating the underlying pathology of NDDs [220], [221]. The ubiquitin proteasome system (UPS) and the autophagy-lysosomal pathway (ALP) are the two major catabolic pathways in eukaryotic cells. The UPS is in charge of the degradation of cytosolic and nuclear proteins, including short-lived proteins, whereas autophagy is responsible for the clearance of protein aggregates and damaged organelles. Due to an age-dependent decline in their activity, aberrant proteins accumulate, contributing to the onset and development of NDDs [222].

A growing number of studies has associated the intake of foods rich in (poly)phenols with a reduced risk of developing NDDs, including AD. Dietary products such as green tea, apples, berries, cocoa, and chocolate are extremely rich in flavan-3-ols, a common subclass of flavonoids, and their consumption was associated with healthy brain aging, neuronal protection against disorders, and ameliorated cognitive function [223]–[225]. Flavan-3-ols exist either as simple monomers called catechins or as oligomeric and complex polymeric structures known as proanthocyanidins [226]. Only a small fraction of these molecules is absorbed in the upper part of the intestine, whereas most of the ingested flavan-3-ols are transformed by the colonic microbiota into low molecular phenolic compounds, mainly phenyl-γ-valerolactones (PVLs) and their related phenylvaleric acids, and subsequently conjugated to glucuronide and sulfate groups in the liver [227]. Recently, increasing attention has been directed to the possible biological effects exerted by this class of molecules, being them one of the most abundant phenolic metabolites in the circulation upon consumption of a (poly)phenol-rich diet, but still few data are available in the literature [226], [228]. In this regard, it was demonstrated that PVLs, particularly (R)-5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, and their sulfated forms, protect brown adipocytes from increased production of reactive oxygen species (ROS) [227]. 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone,

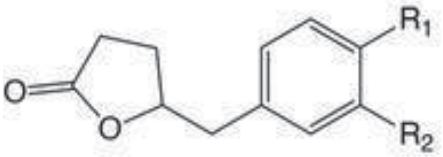
an epigallocatechin gallate-derived microbial metabolite, significantly induced neurites outgrowth and elongation in human neuroblastoma SH-SY5Y cells [229]. In addition, given the neuroprotective role of flavonoids, several studies investigated also the ability of these molecules and their derivatives to cross the blood brain barrier (BBB). The metabolite 5-(3′,5′-dihydroxyphenyl)-γ-valerolactone showed a slightly higher BBB permeability than its parental compound epigallocatechin-3-gallate (EGCG) [229]. Similarly, Angelino et al. recently evidenced that the 5-(hydroxyphenyl)-γ-valerolactone-sulfate (3′,4′ isomer), another key microbial metabolite of flavan-3-ols, is able to reach the brain [230].

In this study, human neuroblastoma SH-SY5Y cells were stably transfected with either the wild-type amyloid precursor protein gene (APPwt) or the 717 valine-to-glycine AβPP-mutated gene (APPmut), serving as a model for AD, the most prevalent NDDs. These cells were treated with 5-(4′-hydroxyphenyl)-γ-valerolactone, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, and the conjugated form 5-(3′-dihydroxyphenyl)-γ-valerolactone-4′-sulfate. Interestingly, the mutation in the APP gene sequence is associated with familial forms of AD and promotes the increased brain accumulation of Aβ (1-42/43) resulting in the enhancement of amyloid fibril formation from soluble Aβ [231], making these cells a suitable model to investigate the molecular mechanisms involved in AD. Here, we explored if PVLs could modulate the functionality of cellular proteolytic pathways, dissecting both proteasomal and autophagy functionality, and how the presence of the wild-type or mutated APP form can influence the final effect. In silico analyses were performed to better elucidate the interaction between the considered metabolites and proteasome catalytic subunits and cathepsin B. In addition, the effect of proteolysis regulation on Aβ (1-42) production and release by neuronal cells was investigated.

3.2 Material and methods

3.2.1 Reagents and Chemicals

5-(4′-hydroxyphenyl)-γ-valerolactone (C1), 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (C2) and 5-(3′-hydroxyphenyl)-γ-valerolactone-4′-sulfate (C3) (Figure 1) were synthesized and kindly provided by Prof. C. Curti (University of Parma, Italy) [232], [233]. The substrates Suc-Leu-Leu-Val-Tyr-AMC, Z-Leu-Ser-Thr-Arg-AMC, Z-Leu-Leu-Glu-AMC for assaying the chymotrypsin-like (ChT-L), trypsin-like (T-L), and peptidyl glutamyl-peptide hydrolyzing (PGPH) activities of the proteasomal complex were purchased from Sigma-Aldrich S.r.L. (Milano, Italy). The substrate Z-Gly-Pro-Ala-Leu-Ala-MCA to test the branched chain amino acids preferring (BrAAP) activity was obtained from Biomatik (Cambridge, Ontario). Aminopeptidase N (EC 3.4.11.2) for the coupled assay utilized to detect BrAAP activity was purified from pig kidney as reported elsewhere [234]. Membranes for western blot analyses were purchased from Millipore (Milan, Italy). Proteins immobilized on films were detected with the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Milan, Italy). All chemicals and solvents were of the highest analytical grade available.



	R_1	R_2
C1	OH	H
C2	OH	OH
C3	SO_4	OH

Figure 1. *Structure of the PVLs used in this study. C1, 5-(4'-hydroxyphenyl)-γ-valerolactone; C2, 5-(3',4'-dihydroxyphenyl)-γ-valerolactone; C3, 5-(3'-hydroxyphenyl)-γ-valerolactone-4'-sulfate.*

3.2.2 Molecular Docking

The molecular models of complexes between the PVLs and the catalytic subunits of human proteasomes were obtained according to flexible ligand-receptor docking using Autodock 4 [235]. 3D structures of the molecules of interest were built and optimized with Avogadro [236] and docked onto crystallographic structures of human constitutive (PDB ID: 6rgq [237]) and immuno-proteasomes (PDB ID: 6e5b [238]) retrieved from the RCBS Protein Data Bank [239]. Specifically, a grid box (20Í20Í20 Å) was individually placed around the accessible internal portions of catalytic subunits β1, β2 and β5 and β1i, β2i and β5i immune counterparts, respectively, each box being centered on the catalytic Thr-1 residues and covering the entire surface extending 10 Å in each direction. Likewise, the valerolactones-cathepsin B models were obtained. The grid box was placed around the catalytic site of 3D structure of human cathepsin B (PDB ID: 1csb [240]), spanning 10 Å in each direction around the catalytic Cys-29. Unless stated differently, default settings were used throughout. Resulting models were rendered with PyMOL (The PyMOL Molecular Graphics System, Version 2.4 Schrödinger, LLC).

3.2.3 Measurements of Isolated 20S Proteasome Activity

PVLs effects on the 20S constitutive and immunoproteasome peptidase activities were measured through *in vitro* assays performed with fluorogenic peptides as substrates. Isolation and purification of the 20S proteasome from bovine brain and thymus were performed as previously reported [241]. The incubation mixture contained the compound at concentrations ranging from 0.0 to 10 μM, 1 μg of the isolated 20S proteasome, the appropriate substrate (5 μM final concentration), and 50 mm Tris/HCl (pH 8.0), up to a final volume of 100 μL. Purified proteasomes were always preincubated with each compound at 37°C for 30 min before the fluorogenic peptide substrates were added to the reaction mixture. Final incubation was performed at 37°C, and after 60 min the fluorescence of the hydrolyzed 7-amino-4-methyl-coumarin (AMC) and 4-aminobenzoic acid (pAB) was detected (AMC, λexc = 365 nm, λem = 449 nm; pAB, λexc = 304 nm, λem = 664 nm) on a SpectraMax Gemini XPS microplate reader.

3.2.4 Cell Culture and Transfections

SH-SY5Y cells were cultured in 1:1 Dulbecco's modified Eagle's medium and Nutrient Mixture F12 containing 10% fetal bovine serum (FBS), 2 mM glutamine, 100 units mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin at 37°C in a 5% CO_2-containing atmosphere. The SH-SY5Y cells stable transfection with wild type AβPP 751 (APPwt) and AβPP (Val717Gly) mutations (APPmut) was prepared as described elsewhere [242]. These cells were a kind gift of Prof. Daniela Uberti from the University of Brescia. Stably transfected cells expressing either the APPwt or the APPmut construct were maintained in SH-SY5Y medium added with G418 at a final concentration of 600 μg mL^{-1}.

3.2.5 Cell Treatment and Cytotoxicity Assay

Cell viability was evaluated with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) [243]. Upon 6 h and 24 h treatment with increasing concentrations (0-10 μM) of C1, C2, and C3 dissolved in DMSO, cells were washed in PBS, pH 7.5, and then MTT (final concentration 0.5 mg mL^{-1}) was added to the culture medium without FBS and incubated for 2 h at 37 °C. The medium was then removed and replaced with 100 μL of DMSO. The optical density was measured at 550 nm in a microtiter plate reader. At least six cultures were utilized for each time point. Neuroblastoma cells, control and transfected cells, were then treated with C1, C2, and C3 for 6 and 24 h at the concentrations 0–1–5 μM. Compounds were dissolved in DMSO and then added to cell culture media. Control cells were included in each time point. After removing the medium and washing with cold phosphate buffered saline (PBS), cells were harvested in 4 mL of PBS and centrifuged at 1600 × g for 5 min. The pellet was resuspended in lysis buffer (20 mM Tris, pH 7.4, 250 mM sucrose, 1 mM EDTA, and 5 mM β-mercaptoethanol) and passed through a 29-gauge needle at least ten times. Lysates were centrifuged at 12 000×g for 15 min and the supernatants were stored at -80°C until use. Protein concentration was determined by the method of Bradford using bovine serum albumin (BSA) as standard [244].

3.2.6 Proteasome Activity

The effects on the proteasome system were evaluated through fluorimetric assays, as previously reported [245], using the following synthetic substrates: Leu-Leu-Val-Tyr-AMC for ChT-L, Leu-Ser-Thr-Arg-AMC for T-L, Leu-Leu-Glu-AMC for PGPH, and Gly-Pro-Ala-Leu-Ala-AMC for BrAAP, whose test is performed with the addition of the aminopeptidase-N (AP-N). The incubation mixture contained 1 μg of cell lysate, the appropriate substrate, and 50 mM Tris/HCl pH 8.0, up to a final volume of 100 μL. Incubation was performed at 37 °C, and after 60 min, the fluorescence of the hydrolyzed 7-amino-4-methyl-coumarin (AMC) was recorded (AMC, λexc = 365 nm, λem = 449 nm) on a SpectraMax Gemini XPS microplate reader. The 26S proteasome ChT-L activity was tested using Suc-Leu-Leu-Val-Tyr-AMC as substrate and 50 mM Tris/HCl pH 8.0 buffer containing 10 mM $MgCl_2$, 1 mM dithiothreitol, and 2 mM ATP. The effective 20S proteasome contribution to short peptide cleavage was evaluated with control experiments performed using specific proteasome inhibitors, Z-Gly-Pro-Phe-Leu-CHO and lactacystin (5 μM in the reaction mixture). The fluorescence values of lysates were subtracted of the values of control assays in the presence of the two inhibitors.

3.2.7 Cathepsin B Activity

Cathepsin B proteolytic activity was measured using the fluorogenic peptide Z-Arg-Arg-AMC at a final concentration of 50 μM, as previously described [242]. The mixture for cathepsin B, containing 1 μg of cell lysate, was pre-incubated in 100 mM phosphate buffer pH 6.0, 1 mM EDTA, and 2 mM dithiothreitol for 5 min at 30°C. Upon the addition of the substrate, the mixture was incubated for 15 min at 30°C. The fluorescent signal released by the hydrolyzed 7-amino-4-methyl-coumarin (AMC, λexc = 365 nm, λem = 449 nm) was detected on a SpectraMax Gemini XPS microplate reader.

3.2.8 Western Blotting Analysis

Proteins were resolved on SDS–PAGE and electroblotted onto PVDF membranes. Membranes with transferred proteins were incubated with the primary monoclonal antibody and successively with the specific peroxidase conjugated secondary antibody. Monoclonal antibodies against Ub and p27 were obtained from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). SQSTM1/p62 (sequestosome 1, herein p62) mouse monoclonal antibody was from Sigma-Aldrich S.r.L. (Milano, Italy) and the anti-LC3B antibody was purchased from Cell Signaling Technology, Inc. The immunoblot detection was performed with ECL Western blotting detection reagents using a ChemiDoc MP system. Each gel was loaded with molecular weight markers in the range of 12 to 225 kDa (GE Healthcare). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was utilized as a control for equal protein loading: membranes were stripped and re-probed with anti-GAPDH monoclonal antibody (Santa Cruz Biotechnology, Heidelberg, Germany). Stripping buffer contained 200 mM glycine, 0.1% SDS, and 1% Tween 20. Immunoblot images were quantified using ImageJ 1.52p software (NIH, USA).

3.2.9 Monodansylcadaverine Assay

Upon treatment with PVLs, the formation of autophagic vacuoles was monitored with monodansylcadaverine assay (MDC, Sigma-Aldrich S.r.L. Milano, Italy). In detail, 1 μM MDC was added to cell medium. After 10 min incubation at 37 °C, cells were washed three times with phosphate buffered solution (PBS) and immediately analyzed with a fluorescence microscope (Olympus IX71).

3.2.10 Quantification of Aβ (1-42)

Levels of Aβ (1-42) secreted into the medium and present in cellular extracts were determined after PVLs treatment using the Human Aβ42 solid-phase sandwich ELISA Kit from Invitrogen, following the manufacturer's instructions. For Aβ quantification in cell medium, culture medium was collected after neuronal cells treatment, centrifuged at 300 × g for 10 min to remove non adherent cells and debris and then treated with protease inhibitors.

3.2.11 Statistical Analysis

Data are expressed as mean values ± S.D. Statistical analysis was performed with one way ANOVA, followed by the Bonferroni post hoc test using Sigma-stat 3.1 software (SPSS, Chicago, IL, USA) and $p < 0.05$ was considered statistically significant.

3.3 Results

3.3.1 Effects of PVLs on Isolated Proteasomes Activity

PVLs were first tested on isolated constitutive and immunoproteasomes purified from bovine brain and thymus, respectively. The constitutive 20S proteasome is a barrel-shaped complex of four stacked rings, made of seven α subunits in the two outer rings and seven β subunits in the two inner rings, the latter bearing the catalytically active sites in the β1, β2, and β5 subunits. Treatment with inflammatory cytokines induces the transcription of three additional active subunits, known as β1i, β2i, and β5i, that replace constitutive homologues during proteasome assembly [246]. Proteasome proteolytic activities depend on the hydroxyl group of the N-terminal threonine (Thr-1) residue, responsible for cleaving peptides through a nucleophilic attack [247].

Increasing concentrations of the PVLs (0-10 μM in DMSO) were used in the fluorescent assays, as described in materials and methods. An evident inhibitory effect on the catalytic components of the two enzymes, namely the ChT-L (associated with the β5 subunit), T-L (β2 subunit), PGPH (β1 subunit), and BrAAP (β5 subunit), was observed, with the highest tested concentration inducing an almost complete inhibition. As reported in Table 1, the three PVLs showed a subunit-dependent specificity of inhibition. Considering the constitutive proteasome, C1 and C3 were particularly effective in inhibiting the T-L component whereas C2 showed the highest inhibitory effect toward the ChT-L. Globally, the catalytic components of the constitutive proteasome were more susceptible to the action of the three PVLs, showing lower IC_{50} values (down the nanomolar range) compared to the values obtained for the components of the immunoproteasome. The only exception was the BrAAP activity, whose inhibition was more evident in the immunoproteasome (see Table 1).

Table 1. *IC_{50} values obtained from in vitro activity assays on isolated proteasomes*

	IC_{50} [μM]		IC_{50} [μM]	
	ChT-L		T-L	
	Constitutive proteasome	Immunoproteasome	Constitutive proteasome	Immunoproteasome
C1	0.1196 ± 0.0121	0.1258 ± 0.0113	0.0130 ± 0.0021	$0.2272 \pm 0.0132^{\#\#}$
C2	$0.0162 \pm 0.0012^{**}$	$0.2379 \pm 0.0167^{*,\#\#}$	$0.0874 \pm 0.0079^{**}$	$0.3344 \pm 0.0289^{*,\#}$
C3	0.1090 ± 0.0110	$1.4520 \pm 0.1530^{**,\#\#}$	$0.0568 \pm 0.0023^{*}$	$0.5876 \pm 0.0476^{*,\#\#}$

	PGPH		BrAAP	
	Constitutive proteasome	Immunoproteasome	Constitutive proteasome	Immunoproteasome
C1	0.0628 ± 0.0587	0.0418 ± 0.0038	0.2427 ± 0.0201	0.2179 ± 0.0196
C2	0.0858 ± 0.0078	$0.1136 \pm 0.0197^{*,\#}$	0.2558 ± 0.0144	$0.0613 \pm 0.0057^{*,\#}$
C3	0.1044 ± 0.0110	$0.0727 \pm 0.0057^{*,\#}$	$0.3024 \pm 0.0278^{*}$	$0.0884 \pm 0.0070^{*,\#}$

Asterisks refer to statistically significant differences obtained comparing C1 effects with C2/C3 effects

on constitutive proteasomes or immunoproteasomes, respectively. Hashtags refer to statistically significant differences obtained comparing the effects of a single metabolite on the constitutive and immunoproteasome. $^{*,\#}$ $p < 0.05$, $^{**,\#\#}$ $p < 0.01$.

3.3.2 Effects of PVLs on Neuronal Cells Proteasomes

Next, SH-SY5Y control and transfected cells were exposed to increasing concentration of PVLs (0-10 μM) and viability was checked with the MTT assay. No cytotoxic effect was detected, with only a minor reduction in the number of APPmut viable cells upon 24 h exposure to 10 μM C2 (data not shown). Neuronal cells were then treated with 1 and 5 μM of each PVLs for 6 and 24 h.

The effects of the three compounds on the functionality of the proteasomal system, both the 20S core and the 26S proteasome, the latter consisting of a 20S core and one or two 19S regulatory particles, were then evaluated with fluorometric tests and immunoassays (Fig. 2). The inhibitory activity previously observed on isolated complexes was confirmed in the assays on cellular lysates. In detail, the metabolites induced the most remarkable effect upon 24-h exposure displaying a concentration- and subunit-dependent pattern of proteasome inhibition, with the BrAAP activity being the least affected (Fig. 3, 4, and 5). Among the three tested compounds, the dihydroxylated metabolite C2 was the least efficient whereas C1 and C3 displayed a similar behavior strongly altering the functionality of the enzymatic complex, mainly the ChT-L component of both the 20S and 26S proteasome (C1: 30% ChT-L residual activity for both enzymes, C3: 50 and 60% ChT-L residual activity for the 20S and 26S proteasome, respectively). Interestingly, the presence of the wt or mut APP sequence influenced the results and APPmut cells showed the highest extent of proteasomal inhibition. The inhibitory effect of the PVLs was confirmed by immunodetection of two proteasome cellular substrates, ubiquitin-protein conjugates and p27. As shown in Figures 6 and 7, no particular effect was observed upon 6 h exposure to C1, C2 and C3 whereas an evident increase in the levels of both Ub-conjugates and p27 was obtained upon 24 h treatment, mainly in APPmut cells exposed to C1, confirming the data observed in the fluorescent assays. Together, these data show that these metabolites strongly affect proteasome functionality, selectively inhibiting its catalytic activity and favoring the accumulation of its related substrates.

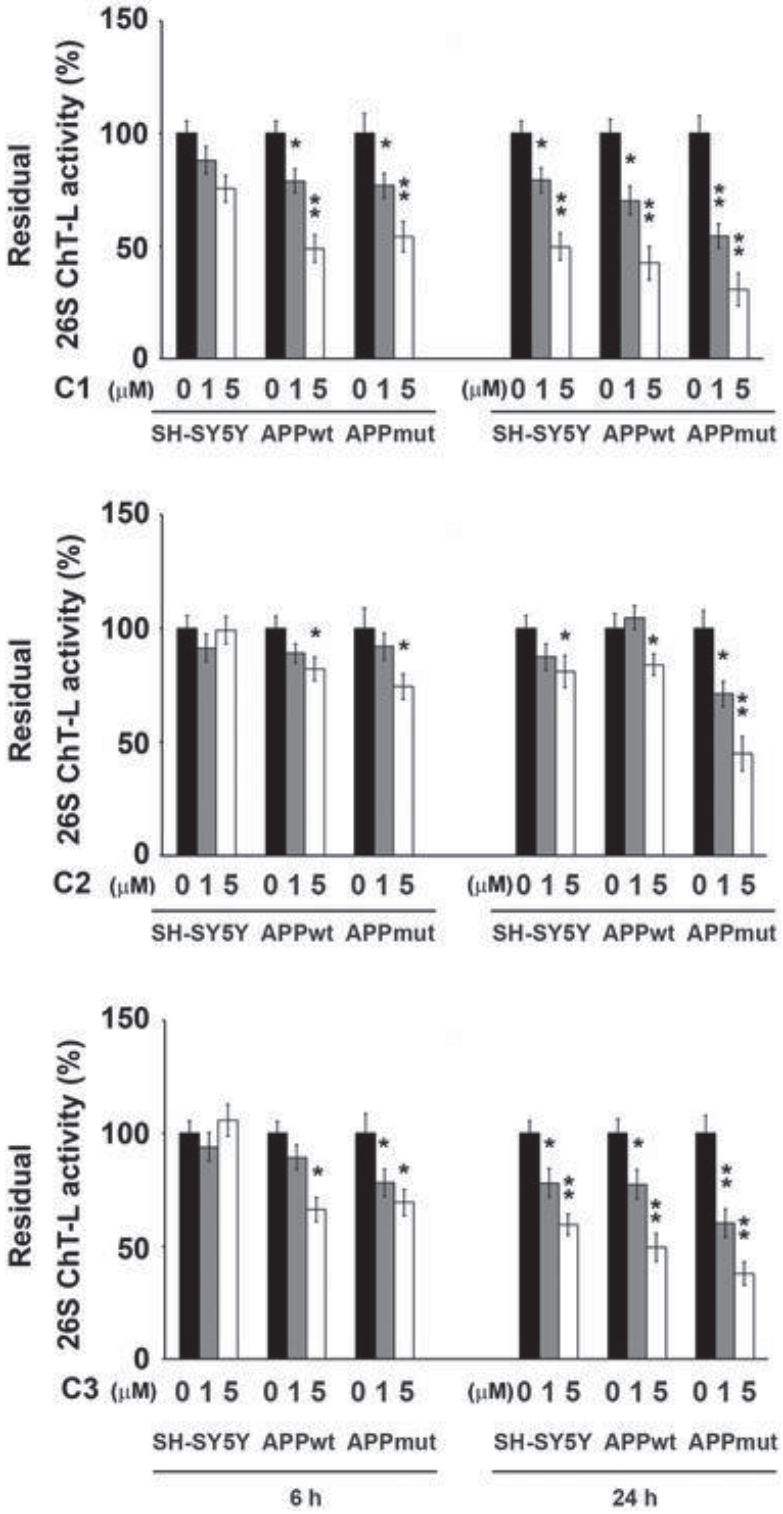


Figure 2. *ChT-L activity of the 26S proteasome measured in control and transfected SH-SY5Y cells upon 6 and 24 h exposure to the three tested PVLs (C1, C2, and C3). Activities were measured using a fluorogenic peptide as a substrate as described in the Materials and methods section. Data are indicated as percentage versus untreated control/transfected cells ($^{*}p < 0.05$, $^{**}p < 0.01$).*

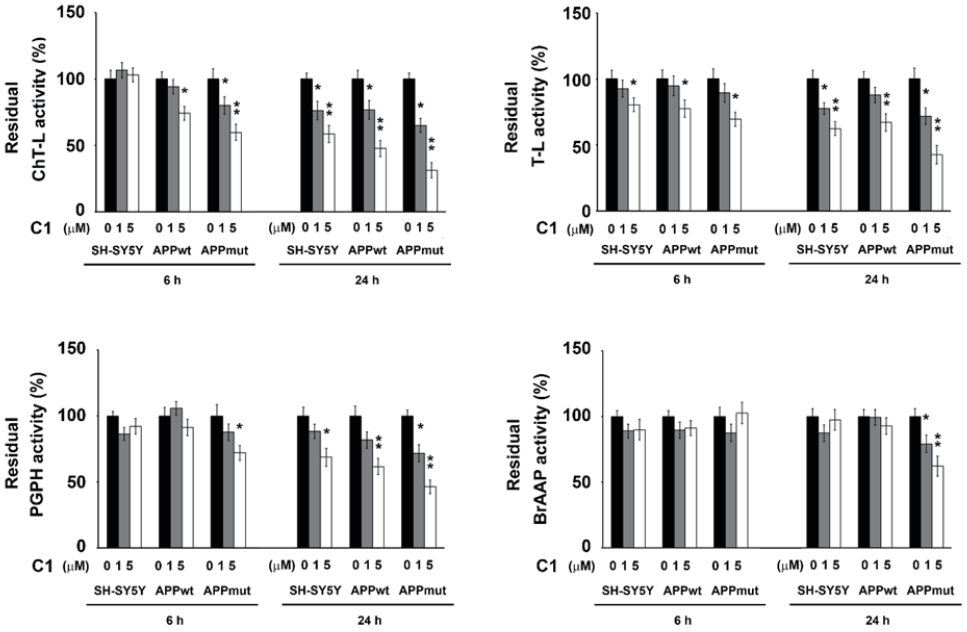


*Figure 3. Proteasome ChT-L, T-L, PGPH and BrAAP activities measured in control and transfected SH-SY5Y cells upon 6 and 24 h exposure to C1. Activities were measured using a fluorogenic peptide as a substrate as described in the Materials and methods section. Data are indicated as percentage vs. untreated control/transfected cells (*p<0.05, **p<0.01).*

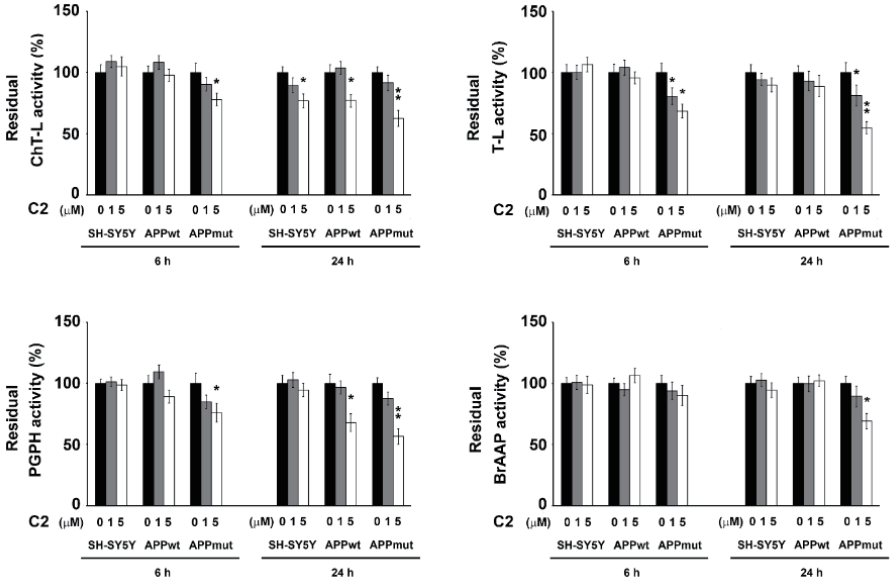


*Figure 4. Proteasome ChT-L, T-L, PGPH and BrAAP activities measured in control and transfected SH-SY5Y cells upon 6 and 24 h exposure to C2. Activities were measured using a fluorogenic peptide as a substrate as described in the Materials and methods section. Data are indicated as percentage vs. untreated control/transfected cells (*p<0.05, **p<0.01).*

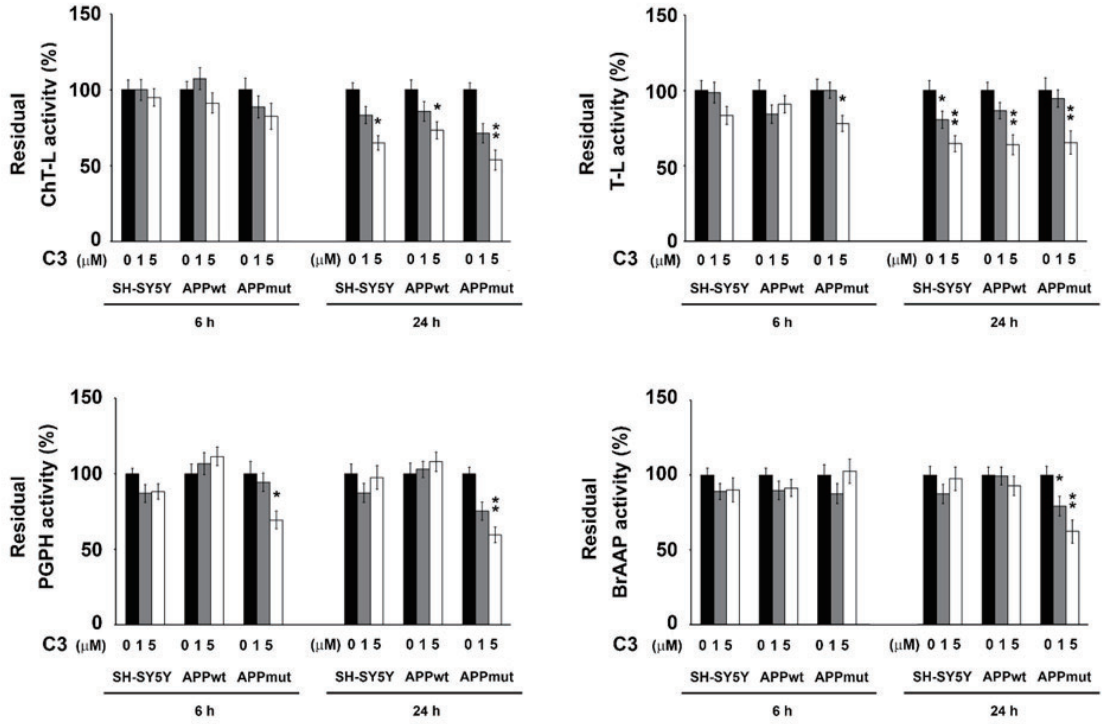


*Figure 5. Proteasome ChT-L, T-L, PGPH and BrAAP activities measured in control and transfected SH-SY5Y cells upon 6 and 24 h exposure to C3. Activities were measured using a fluorogenic peptide as a substrate as described in the Materials and methods section. Data are indicated as percentage vs. untreated control/transfected cells (*p<0.05, **p<0.01).*

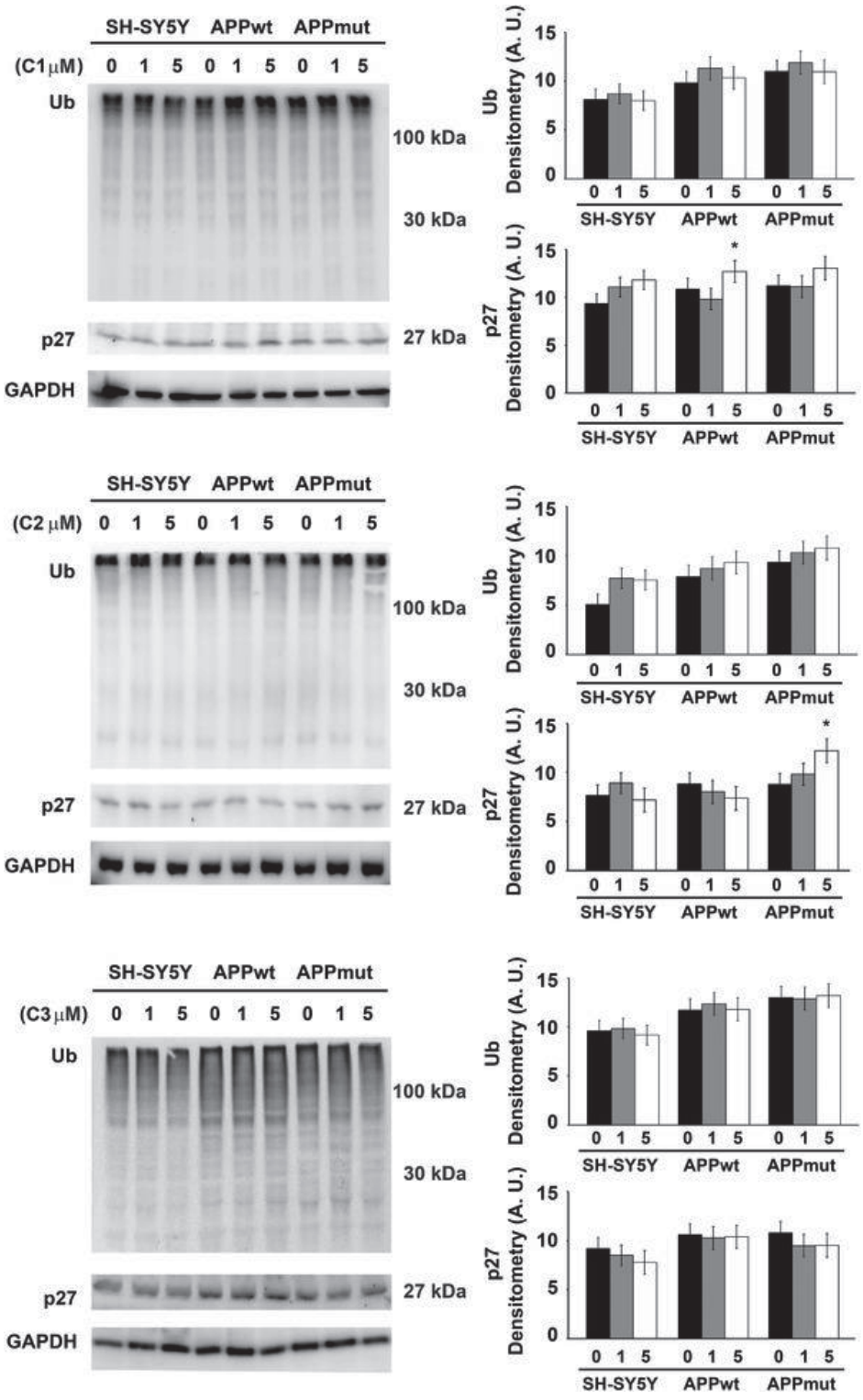


Figure 6. *p27 and ubiquitin-conjugates detected in control and transfected SH-SY5Y cells upon 6 h exposure to PVLs. Representative immunoblots and densitometric analyses obtained from five separate experiments are shown (A.U. arbitrary units). Equal protein loading was verified by using an anti-GAPDH antibody. Data points marked with an asterisk are statistically significant compared to the respective untreated cell line ($^{*}p < 0.05$).*

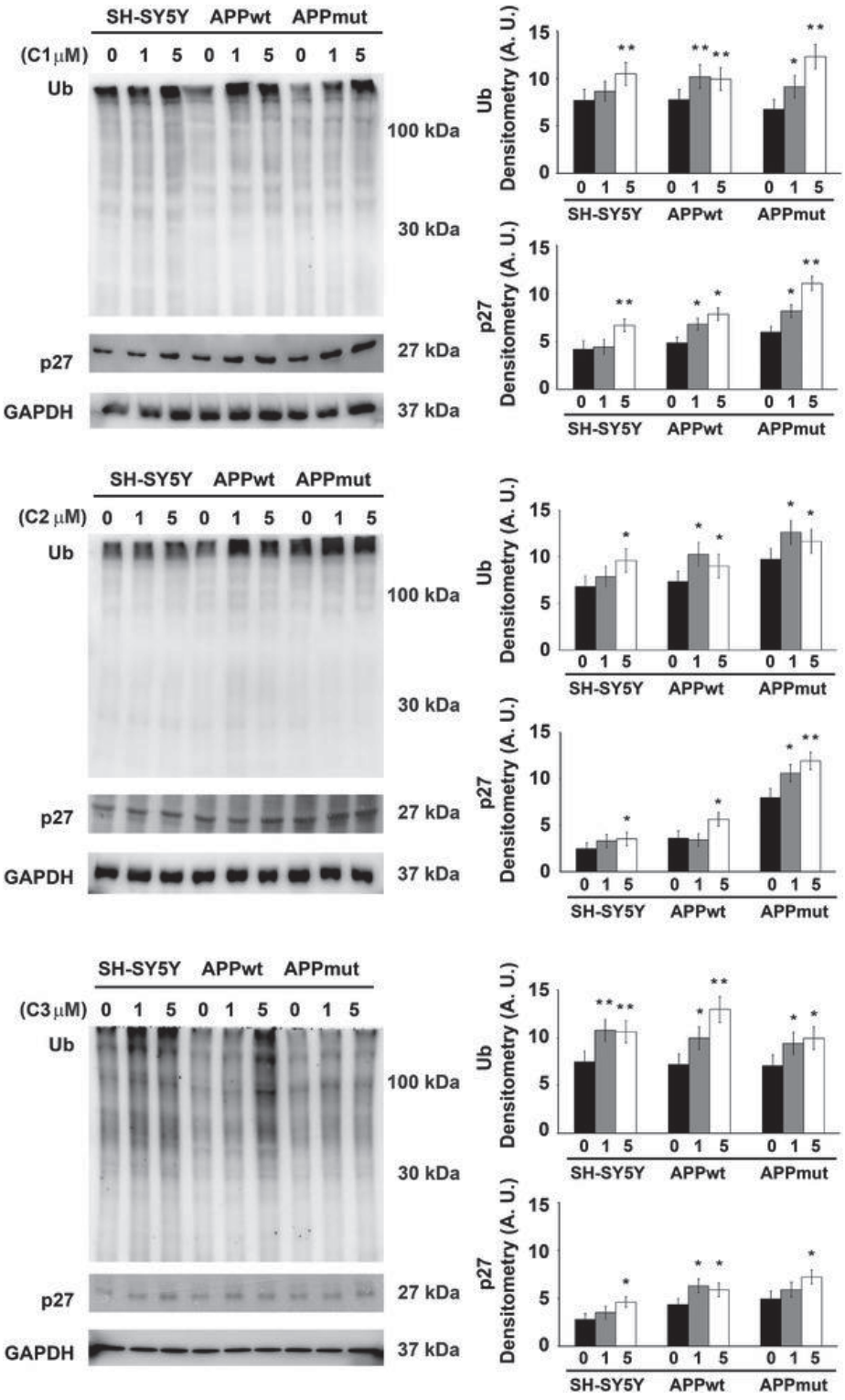


Figure 7. *p27 and ubiquitin-conjugates detected in SH-SY5Y control and transfected cells upon 24 h exposure to PVLs. Representative immunoblots and densitometric analyses obtained from five separate experiments are shown (A.U. arbitrary units). Equal protein loading was verified by using an anti-GAPDH antibody. Data points marked with an asterisk are statistically significant compared to the respective untreated cell line ($^{*}p < 0.05$, $^{**}p < 0.01$).*

In silico analyses were also conducted to dissect the mechanisms that mediate the interaction between the

active site of the enzyme and the metabolites and to explain the observed inhibitory trend. The compounds of interest showed moderate binding affinities for proteasome catalytic subunits, with predicted equilibrium constants in the range 3–23 μM and a general conserved trend (C1>C2>C3). K_D values and energy contributions are summarized in Table 2A and 2B.

Table 2. *Computationally predicted affinities and energy contribution values for the complexes formed between human constitutive 20S proteasome catalytic subunits and C1, C2, and C3*

A	Compound	$K_{D,pred}$ [μM]	ΔG [kcal mol^{-1}]	T. Energy [kcal mol^{-1}]	I. Energy [kcal mol^{-1}]	vdW Energy [kcal mol^{-1}]	Electrostatic Energy [kcal mol^{-1}]	Thr-1-Lactone distance [Å]
β1	C1	3.3	−7.475	−7.22	−23.971	−15.386	−8.585	6.8
	C2	19.09	−6.435	−6.754	−23.422	−10.742	−14.044	7.3
	C3	22.87	−6.328	−6.213	−26.784	−3.132	−24.939	6.9
β2	C1	7.12	−7.019	−19.119	−26.17	−13.22	−12.95	3.9
	C2	9.02	−6.879	−6.001	−24.086	−10.042	−14.044	4.5
	C3	12.86	−6.669	−6.931	−28.253	−3.314	−24.939	6.9
β5	C1	8.9	−6.887	−23.549	−32.468	−1.96	−30.508	6.9
	C2	12.43	−6.689	−7.364	−23.271	−2.321	−20.95	7.5
	C3	21.3	−6.37	−9.559	−30.029	4.666	−34.695	6.2

B	Compound	$K_{D,pred}$ [μM]	ΔG [kcal mol^{-1}]	T. Energy [kcal mol^{-1}]	I. Energy [kcal mol^{-1}]	vdW Energy [kcal mol^{-1}]	Electrostatic Energy [kcal mol^{-1}	Thr-1-Lactone distance [Å]
β1i	C1	7.959	−6.953	−19.898	−27.529	−13.506	−14.023	8.8
	C2	8.067	−6.945	−4.830	−21.318	−9.904	−11.414	6.1
	C3	22.87	−6.328	−5.438	−21.534	−0.924	−20.610	5.2
β2i	C1	9.487	−6.849	−22.519	−29.889	−8.034	−21.855	8.6
	C2	16.84	−6.509	−8.364	−24.732	−3.414	−21.318	8.6
	C3	16.87	−6.508	−12.007	−33.114	−1.473	−31.641	5.3
β5i	C1	5.973	−7.123	−25.270	−32.410	−12.876	−19.534	5.6
	C2	10.91	−6.766	−11.075	−28.657	−11.490	−17.167	5.9
	C3	10.99	−6.762	−13.963	−37.543	−8.997	−28.546	7.0

A, constitutive proteasome subunits; B, immunoproteasome subunits.

Structurally, these molecules were predicted to establish a variable number of H-bonds with amino acid residues in close proximity to the proteasome catalytic sites, with the carbonyl group of the lactone ring being always favorably positioned for a nucleophilic attack by the proteasome catalytic Thr-1 residue, resulting in the opening of the γ-lactone ring and acylation of the hydroxyl group, as expected for candidate proteasome inhibitors carrying lactone moieties [248] (Fig. 8 and Fig. 9), and as supported by covalent docking analysis (Fig. 10). Interestingly, the predicted distances between the hydroxyl group of Thr-1 and the carbonyl group of the lactone and the consequent different tendency to form a covalent bond (more than the calculated binding affinity values) were in agreement with the general higher inhibitory effect toward constitutive proteasome observed in the *in vitro* studies, in particular with T-L and BrAAP activities of constitutive and immuno-proteasomes, respectively.

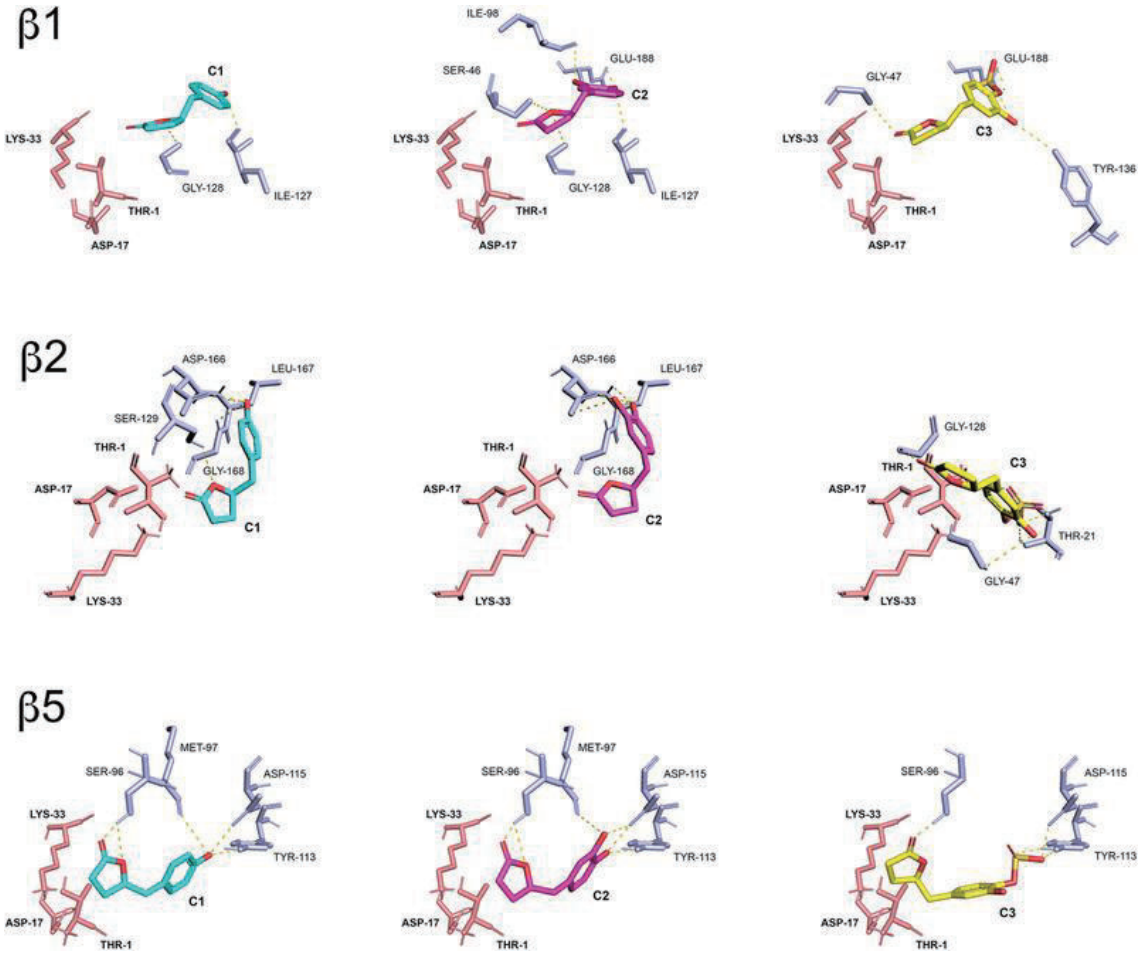


Figure 8. *Comparative visualization of computed binding modes of PVLs C1, C2, and C3 to β1, β2, and β5 subunits of human constitutive 20S proteasome (pdb ID: 6rgq). Only the catalytic residues (Thr-1, Asp-17 and Lys-33) and other residues in close proximity to the active site that are directly involved in the formation of H-bonds are displayed as pink and light blue sticks, respectively. H-bonds are indicated as yellow dashed solid lines.*

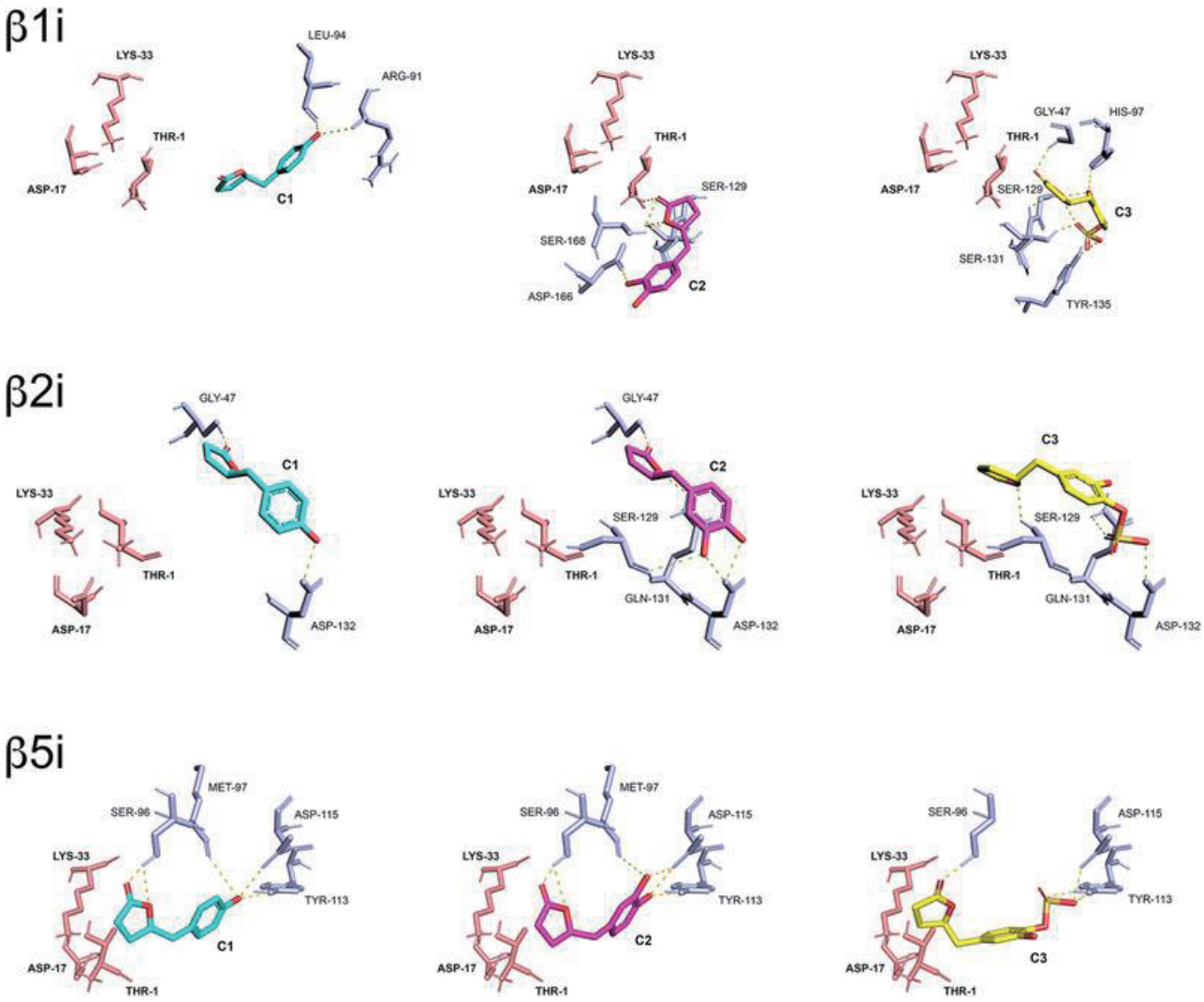


Figure 9. *Comparative visualization of computed binding modes of PVLs C1, C2, and C3 to β1i, β2i, and β5i subunits of human immunoproteasome (pdb ID: 6e5b). Only the catalytic residues (Thr-1, Asp-17, and Lys-33) and other residues in close proximity to the active site that are directly involved in the formation of H-bonds are displayed as pink and light blue sticks, respectively. H-bonds are indicated as yellow dashed solid lines.*

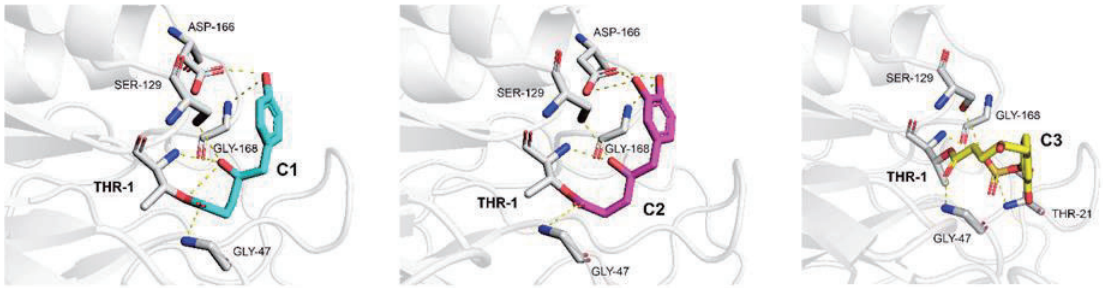


Figure 10. Binding models of C1, C2 and C3 bound to Thr-1 catalytic residue of constitutive 20S proteasome. Covalent docking was performed using Dockovalent (Covalent docking of large libraries for the discovery of chemical probes. Nir London, Rand M Miller, Shyam Krishnan, Kenji Uchida, John J Irwin, Oliv Eidam, Lucie Gibold, Peter Cimermančič, Richard Bonnet, Brian K Shoichet & Jack

Taunton Nature Chemical Biology volume 10, pages 1066–1072(2014)). Amino acids involved in the formation of covalent and H-bonds are highlighted as sticks.

3.3.3 Effects of PVLs on the Autophagic Pathway

Together with the proteasome, autophagy represents a major quality control system responsible for the maintenance of cellular homeostasis. The effect on autophagy of the three PVLs was evaluated measuring the expression of proteins involved in this pathway, such as LC3II, the lipidated form of LC3 that localizes in autophagosomal membranes, and p62, a substrate of autophagy that accumulates in cells when autophagy is inhibited [221]. Upon 6 h treatment with the three metabolites (1 and 5 μM), SH-SY5Y control cells showed increased levels of LC3II but almost no change in the expression of the p62 protein, suggesting the activation of the early steps of the autophagic pathway in this cell line (Fig. 11). A minor effect was observed at this time point in the two transfected clones, as LC3II increased in APPwt and APPmut cells upon exposure to C2 and C1, respectively and no variations in p62 amounts were detectable. Interestingly, the 24 h treatment markedly modified the levels of both proteins not only in untransfected cells but also in APPmut cells, demonstrating the complete activation of autophagy with a significant downregulation of the p62 protein (Fig. 12). APPwt cells were the least sensible to the action of the compounds. In details, upon long-term exposure to the three PVLs, this clone showed increased levels of LC3II but only 5-(3′-hydroxyphenyl)-γ-valerolactone-4′-sulfate (C3) favored p62 degradation, suggesting the complete activation of autophagy. To further confirm data on the activation of the autophagic pathway, we measured the amounts of autophagic vacuoles staining PVLs-treated cells with the autofluorescent dye MDC [249]. Figure 13. shows the increased number of autophagosomes in the three cell lines treated for a period of 24 h with the PVLs of interest. Increased numbers of autophagic vacuoles were detected in all the treated cell lines, but they were particularly evident in normal SH-SY5Y and APPmut cells.

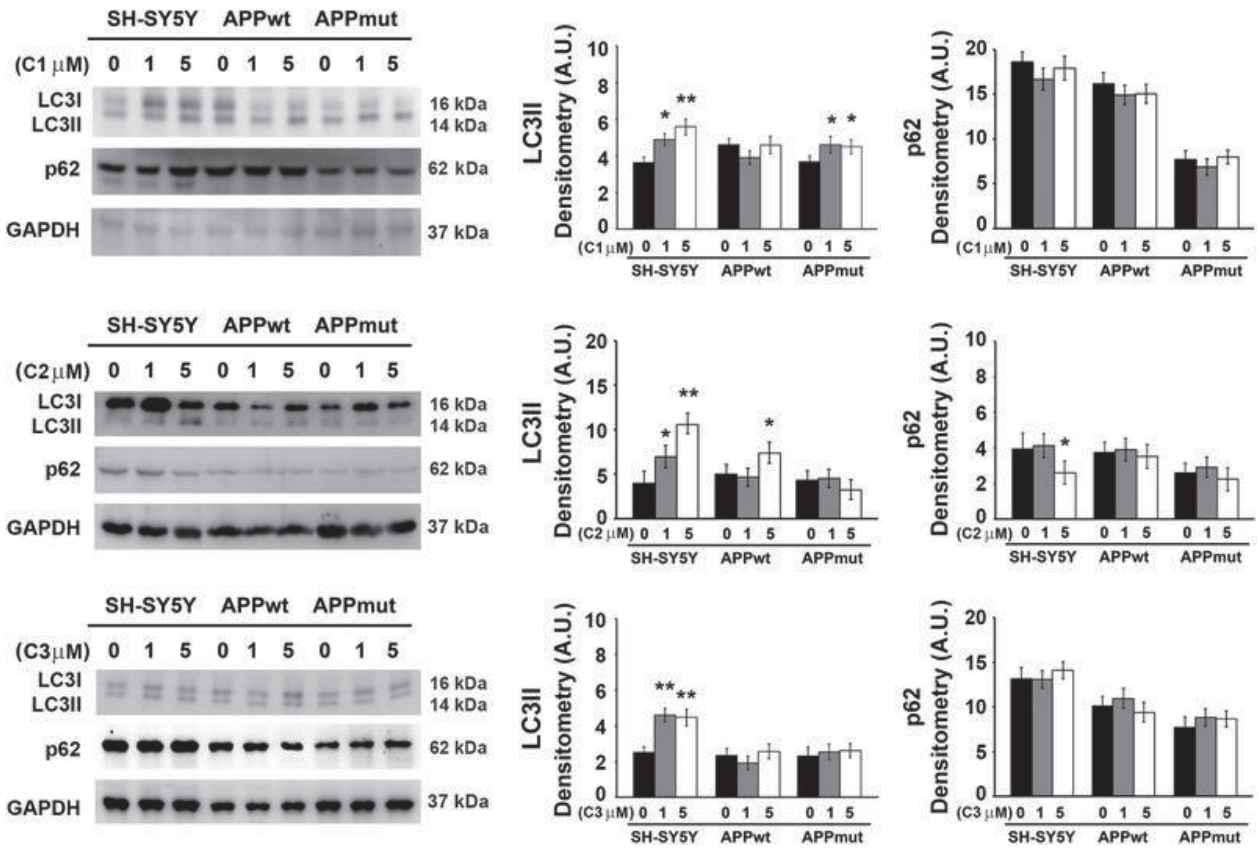


Figure 11. *LC3II and p62 detected in SH-SY5Y control and transfected cells upon 6 h exposure to PVLs. Representative immunoblots and densitometric analyses obtained from five separate experiments are shown (A.U. arbitrary units). Equal protein loading was verified by using an anti-GAPDH antibody. Data points marked with an asterisk are statistically significant compared to the respective untreated cell line ($^{*}p < 0.05$, $^{**}p < 0.01$).*

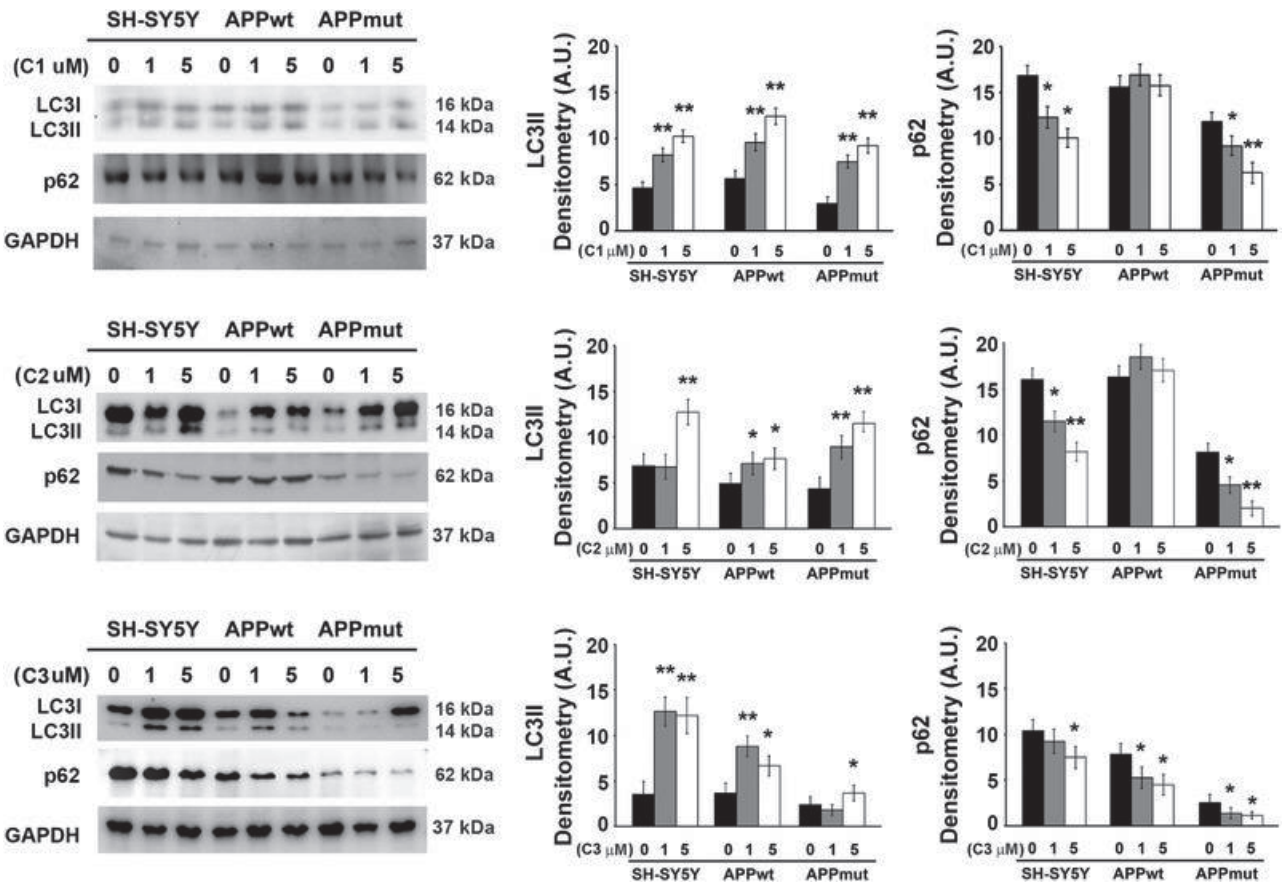


Figure 12. *LC3II and p62 detected in SH-SY5Y control and transfected cells upon 24 h exposure to valerolactones. Representative immunoblots and densitometric analyses obtained from five separate experiments are shown (A.U. arbitrary units). Equal protein loading was verified by using an anti-GAPDH antibody. Data points marked with an asterisk are statistically significant compared to the respective untreated cell line ($^{*}p < 0.05$, $^{**}p < 0.01$).*

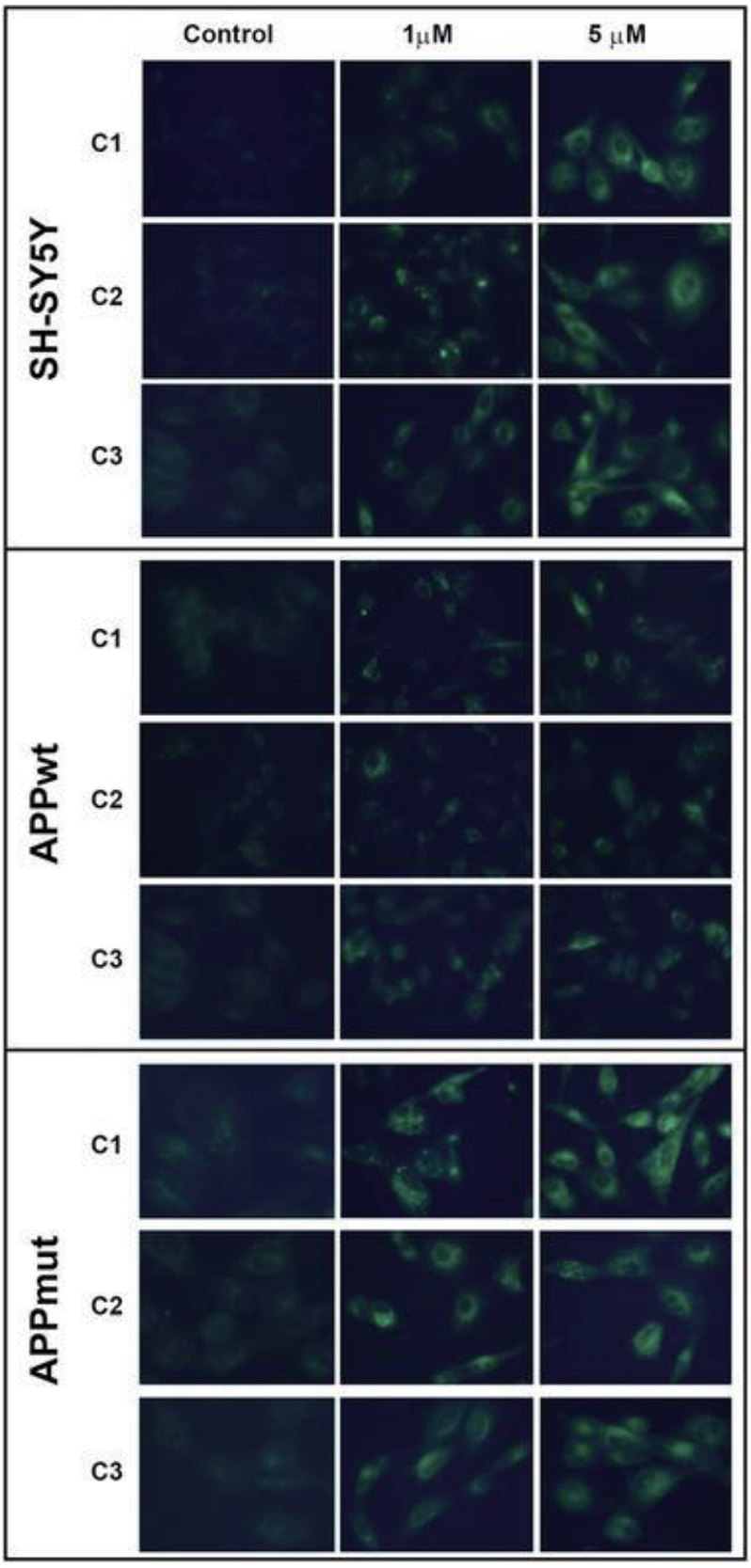


Figure 13. *MDC staining of induced autophagic vacuoles in neuronal cells treated with C1, C2, and C3 (Control, 1 and 5 μM). Cells were treated with PVLs for 24 h and then incubated with MDC dye as indicated in the Materials and methods section.*

3.3.4 PVLs Effect on Cathepsin B Activity

Cathepsin B is a lysosomal cysteine protease that was shown to be upregulated in AD subjects and to play a role in plaques formation and consequent behavioral deficits and neuropathology of AD [250]. We previously characterized the three cell lines in terms of cathepsin B activity, highlighting that it is strongly upregulated in APPmut cells, with no differences between APPwt cells and control neuronal cells [242]. Growing evidence suggests that cathepsin B inhibition is able to reduce Aβ levels [251]–[253]. Cathepsin B functionality was measured in cell lysates after the exposure to the three PVLs. Upon 6 h treatment, only APPmut cells showed an inhibited cathepsin B activity (20% inhibition upon 5 μM C1 and C2 treatment and 30% inhibition upon 5 μM C3 treatment), whereas after 24 h, a decreased functionality of the enzyme was observed also in control and APPwt cells (Figure 14, panel A). Similarly, a computational predictive study was conducted to characterize the interaction between C1, C2 and C3 and cathepsin B. This in silico analysis indicated interactions with moderate affinities (K_D in the range 1–5 μM, with the same trend displayed for proteasome (Table 3)). Structurally, the valerolactones were predicted to establish H-bonds with amino acid residues of the active site cleft of the enzyme, the resulting binding pose being likely to prevent substrate access to the catalytic Cys-29 [254], consistently with our experimental evidence. Again, the lactone carbonyl was favorably positioned for a nucleophilic attack by the thiol group of Cys-29 (Figure 14, panel B), in line with the possible acylation of cysteine (Fig. 15) [255].

Table 3. *Predictive affinities and energy contribution values for the complexes formed between human cathepsin B and C1, C2, and C3*

Compound	$K_{D,pred}$ [μM]	ΔG [kcal mol^{-1}]	T. Energy [kcal mol^{-1}]	I. Energy [kcal mol^{-1}]	vdW Energy [kcal mol^{-1}]	Electrostatic Energy [kcal mol^{-1}]	Cys-29-Lactone distance [Å]
C1	1.61	−7.899	−10.161	−26.132	−20.005	−6.127	8.8
C2	4.26	−7.323	−11.223	−27.287	−12.506	−14.781	6.1
C3	5.53	−7.169	−25.500	−35.483	−15.572	−19.911	5.2

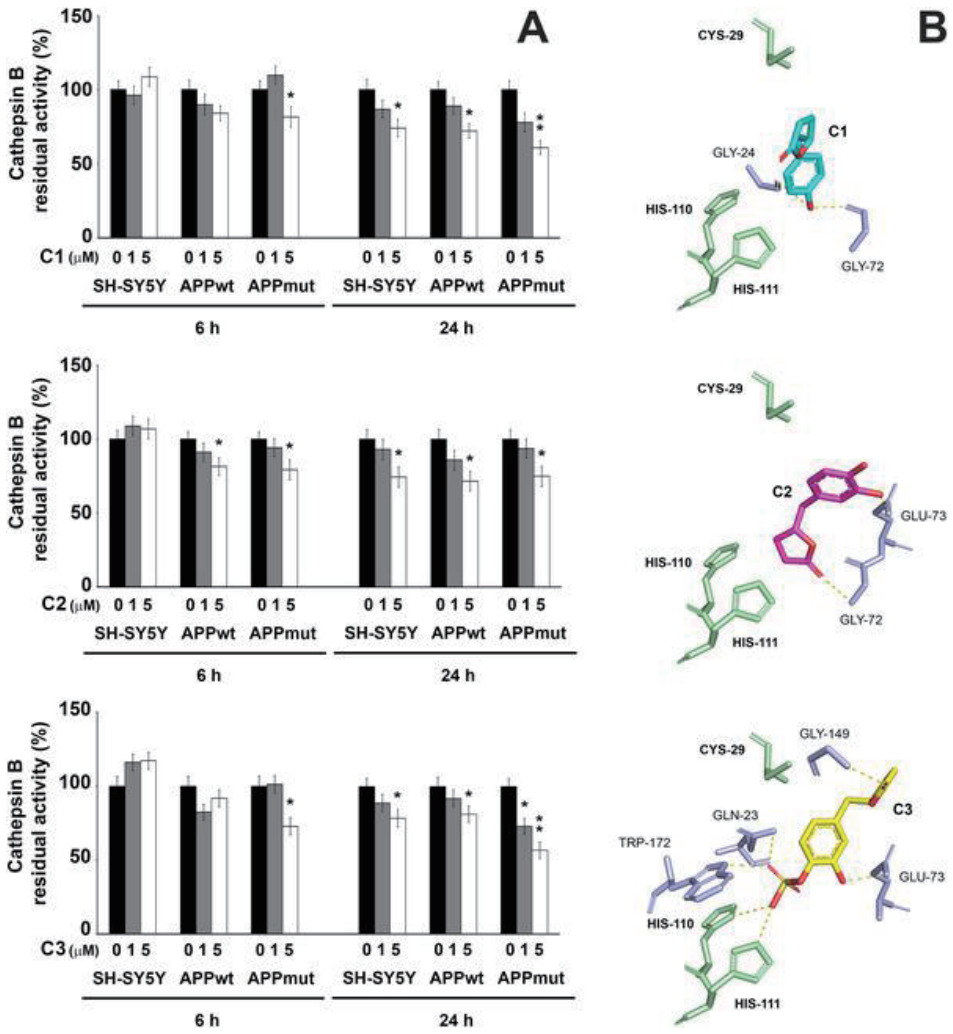


Figure 14. *A) Cathepsin B activity measured in SH-SY5Y control and transfected cells upon 6 and 24-h exposure to PVLs. Activity was measured using a fluorogenic peptide as substrate as described in the Materials and Methods section. Data are indicated as percentage versus untreated control/transfected cells ($^{*}p < 0.05$, $^{**}p < 0.01$). B) Comparative visualization of computed binding modes of valerolactones C1, C2, and C3 to human cathepsin B (pdb ID: 1csb). Only the residues involved in the catalysis (Cys-29, His-110, and His-111) and other residues in close proximity to the active site that are directly involved in the formation of H-bonds are displayed as light green and light blue sticks, respectively. H-bonds are indicated as yellow dashed solid lines.*

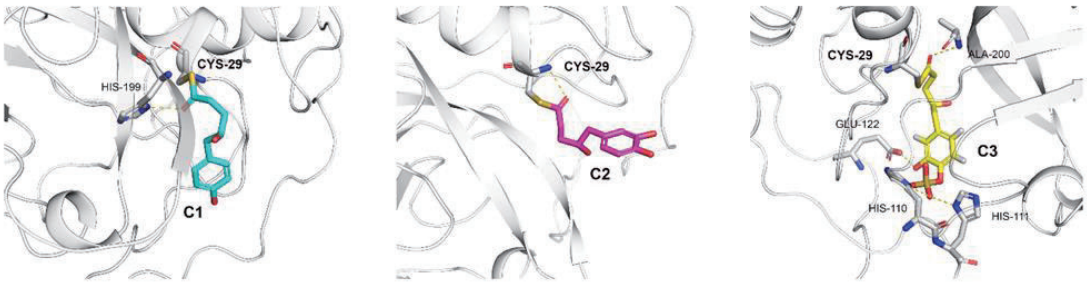


Figure 15. Binding models of C1, C2 and C3 bound to Cys-29 catalytic residue of cathepsin B. Covalent docking was performed using Dockovalent (Covalent docking of large libraries for the discovery of chemical probes. Nir London, Rand M Miller, Shyam Krishnan, Kenji Uchida, John J Irwin, Oliv Eidam, Lucie Gibold, Peter Cimermančič, Richard Bonnet, Brian K Shoichet & Jack Taunton Nature Chemical Biology volume 10, pages 1066–1072(2014)). Amino acids involved in the formation of covalent and H-bonds are highlighted as sticks.

3.3.5 Amyloid-β (1-42) Levels Upon Exposure to PVLs

As previously reported, APPwt and APPmut clones show comparable APP expression levels, higher than control SH-SY5Y cells, and generate and release elevated amounts of Aβ (1-42) peptide in their culture media [242]. Furthermore, APPmut cells produced and released significant higher levels of Aβ (1-42) peptide than APPwt cells [256]. The levels of this peptide were detected both in the medium and cellular extracts of treated control and transfected SH-SY5Y cells using an ELISA kit.

PVLs treatment significantly reduced the amounts of intracellular and extracellular Aβ (1-42) with comparable final effects in the two tested samples. In detail, C3 was the most effective in reducing the amount of the toxic peptide in APPmut cells (35% reduction both in cell extracts and cell medium) (Fig. 16). The monohydroxylated metabolite (C1) exerted similar effects on the three cell lines whereas C2, the less active among the three metabolites, did not induce any change in Aβ (1-42) levels in APPwt cells. Interestingly, these data on the amount of intracellular and released amyloid peptide reflect the activation of the autophagic pathway induced by the compounds with the exception of C1 and its effect on APPwt cells. In fact, although it was not able to completely activate autophagy in this cell line, it significantly reduced the amounts of Aβ (1-42) peptide.

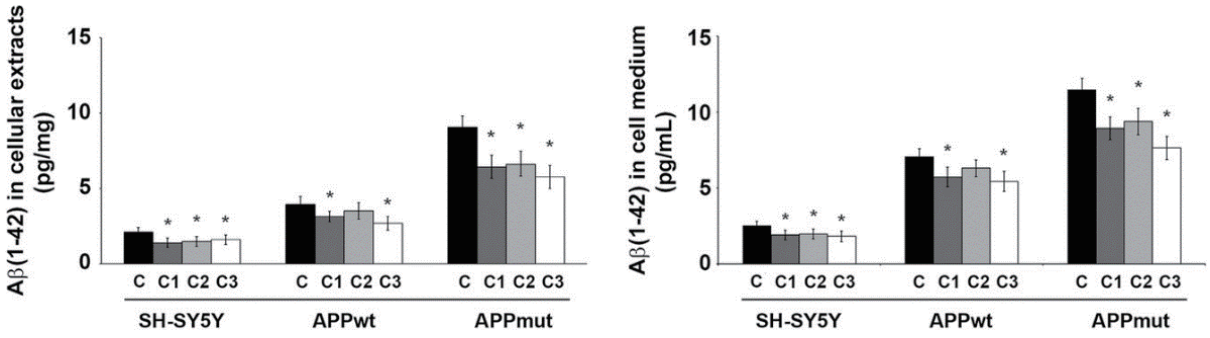


Figure 16. *Levels of Aβ (1-42) measured in cellular extracts and cell medium upon 24 h treatment with C1, C2, and C3 (5 μM). Protein levels were measured using an ELISA kit as described in the Materials and Methods section. Data are indicated as percentage versus untreated control/transfected cells ($^{*}p < 0.05$, $^{**}p < 0.01$).*

3.4 Discussion

Neurodegenerative disorders (NDDs), such as Alzheimer's disease (AD), are characterized by faulty proteolytic pathways and accumulation of pathological peptides in brain. These pathological features contribute to altered cognition and memory. A proper modulation of intracellular proteolysis could therefore help in ameliorating NDDs condition. On this regard, dietary polyphenols and their derivatives, besides the antioxidant and anti-inflammatory activity, were reported to regulate proteolysis and to act as anti-amyloid agents, making them interesting candidates in preventative strategies against AD [257]. An increasing number of studies is now dissecting the biological properties of PVLs, products of intestinal microbial metabolism of flavan-3-ols, that constitute a rich portion of phenolic metabolites in the circulation of subjects exposed to the widely spread dietary sources of this subclass of compounds [226].

In this study, we investigated the ability of three PVLs to modulate proteolytic disturbances in SH-SY5Y neuronal cells, a key hallmark of NDDs. Additionally, we investigated its potential to mitigate the production and release of the toxic Aβ (1-42) peptide, a hallmark of AD. Notably, we evaluated these effects at levels readily achievable in body fluids upon moderate consumption of flavonoid-rich foods or beverages [258], [259]. In details, two microbial metabolites with different hydroxylation patterns, the 5-(4′-hydroxyphenyl)-γ-valerolactone and the 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, and the conjugated derivative 5-(3′-hydroxyphenyl)-γ-valerolactone-4′-sulfate, were tested.

The three compounds similarly affected the functionality of isolated 20S proteasomes, both the constitutive and the immunoproteasome, inducing an almost complete inhibition at the highest doses. They were particularly effective in inhibiting the catalytic subunits of the constitutive complex, with the exception of the BrAAP subunit that showed the highest inhibition in the immunoproteasome. In silico computational studies better clarified the mechanisms behind the observed inhibitory trend, underlining the central role of the lactone moiety, produced by the action of intestinal bacteria that open the C-ring of the flavan-3-ol precursor and convert it into the PVL [260], [261]. This group is likely to undergo a nucleophilic attack by the N-terminal threonine of the active site of proteasomes and covalently inhibits the enzyme, like previously reported for proteasome inhibitors carrying the same functional group [248]. This newly generated lactone group strongly increases the inhibitory potency of PVLs with respect to parental catechins such as (−)-epicatechin and EGCG [262].

We then treated human SH-SY5Y neuroblastoma cells, control and APPwt/APPmut transfected cells, with the three metabolites and evaluated their effects on cellular proteolysis. Previous studies from our laboratory indicated the presence of a less functional proteasome in APP transfected cells, mainly the APPmut clone [242]. PVLs inhibited the 20S and 26S complexes in a time- and subunit-dependent manner, with the monohydroxylated form (C1, 5-(4′-hydroxyphenyl)-γ-valerolactone) and the sulfated derivative (C3, 5-(3′-hydroxyphenyl)-γ-valerolactone-4′-sulfate) resulting the most effective. APPmut cells showed the highest degree of proteasome inhibition likely due to an already compromised enzymatic complex, as a consequence of the higher amounts of amyloid peptides released. These results indicate

that PVLs can act like proteasome modulators as widely reported for their flavan-3-ol precursors and other polyphenols [263]–[266]. While comparisons with other compounds may be biased by the different experimental settings, the prospects of these PVLs have been demonstrated. Further works should be designed to understand the relationship among these microbial-derived phenolic metabolites and phase II-conjugated flavan-3-ols able to cross the BBB, identifying the most active metabolites upon flavan-3-ol consumption, and investigating their synergistic, additive, and/or antagonistic effects occurring in presence of multiple metabolites.

The coordinated action of proteolytic systems is fundamental for protein quality control and for the maintenance of cellular homeostasis, especially in conditions where protein aggregates easily tend to accumulate. Numerous studies described a marked alteration of both the proteasome and autophagy in NDDs, including Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, frontotemporal dementia, and AD, with these defects favoring aggregates-mediated toxicity [267]–[269]. Considering the complex and the dynamic nature of the autophagic pathway [242], we monitored several markers associated with different steps of this process. Our data show that PVLs treatment upregulated autophagy in the three cell lines, mainly in untransfected and APPmut cells, as shown by the increase in autophagosomes formation and the simultaneous reduction of p62 levels. The complete activation of the autophagic process in these two cell lines was observed upon 24 h exposure whereas only SH-SY5Y control cells showed an increased amount of LC3II upon the short time treatment, suggesting an earlier response of untransfected cells to the treatment. These findings indicate that the presence of the wild-type or mutated sequence of the APP influences the final effect of the metabolites on this proteolytic pathway. Among the tested compounds, only 5-(3′-hydroxyphenyl)-γ-valerolactone-4′-sulfate (C3) was able to trigger complete autophagy in APPwt cells.

We then dissected the effects of the three metabolites on cathepsin B activity. This enzyme was associated with the amyloidogenic APP processing and the consequent release of amyloid peptides [252] and we previously found that the expression of the V717G mutated sequence of APP in SH-SY5Y cells strongly elevated the activity of the hydrolase [242]. Our in silico and experimental data indicate that these PVLs can effectively inhibit the activity of this enzyme preventing the substrate access to the catalytic Cys-29 residue. The slight differences observed in cathepsin B inhibition between control and transfected cells suggest that the presence of the wt or mut APP sequence does not significantly alter the final effect of these metabolites on the protease.

Considering the ability of PVLs to modulate cellular proteolytic systems involved in Aβ generation and processing, we investigated possible effects on the production and release of the Aβ (1-42) peptide. Autophagy activation in response to inhibition of the proteasome is frequently observed in cells and it is considered as a compensatory protective mechanism to guarantee the elimination of protein aggregates and alleviate associated proteotoxic stress [270]–[272]. This crosstalk between protein degradative pathways assumes a central role in neurodegenerations, including AD, and it was demonstrated to help

neurons getting rid of detrimental Aβ (1-42) peptides [251], [273], [274]. In addition, it was demonstrated that cathepsin B exhibits β-secretase activity in secretory vesicles of neuronal chromaffin cells and that its inhibitors can lower amyloid levels produced from APP in guinea pigs, suggesting that a reduction in cathepsin B functionality can ameliorate AD condition [250], [252], [253]. In line with these observations, the three tested PVLs diminished the amounts of Aβ (1-42) in the medium and lysates of neuronal cells with the only exception of C2 that did not modify the levels of the toxic peptide in APPwt cells. On this regard, metabolites C1 and C2 exhibited a comparable effect in this cell line showing a limited activity in upregulating autophagy and inducing a significant inhibition of cathepsin B functionality. It is therefore reasonable to think that, beyond cathepsin B inhibition, 5-(4′-hydroxyphenyl)-γ-valerolactone (C1) exerts its effect against amyloid peptides through additional mechanisms, likely involving other enzymes responsible for APP processing and amyloid production.

These results on the anti-amyloid and neuroprotective effects of the considered metabolites are in line with previous findings on PVLs ability to detoxify amyloid-β oligomers and prevent memory impairment [275]. Also relevant in this context are data showing that these metabolites can cross the blood-brain barrier, reach the brain parenchyma and promote neurogenesis in the brain [229], [230]. Together, these data further confirm the beneficial role of a diet rich in catechins and proanthocyanidins, whose metabolites can successfully reduce the presence of molecular markers associated with the onset and progression of NDDs.

3.5 Conclusion

Our findings provide, for the first time, evidence for the neuroprotective activity of PVLs, microbiota-derived metabolites of flavan-3-ols, associated with the modulation of intracellular proteolytic systems and suggest their use in preventative strategies against NDDs, particularly AD. Future experiments should be performed to enrich the evidence gathered so far. In this regard, a better understanding of the journey of flavan-3-ol metabolites into the brain, addressing transportation mechanisms through the BBB, and taking into account their effects in the framework of animal models of NDDs may serve to provide effective, tailored dietary recommendations.

CHAPTER 4

PROBIOTICS SUPPLEMENTATION ATTENUATES INFLAMMATION AND OXIDATIVE STRESS INDUCED BY CHRONIC SLEEP RESTRICTION

Abstract

Background: Insufficient sleep is a serious public health problem in modern society. It leads to increased risk of chronic diseases, such as neurodegenerative disorders (NDDs) and it has been frequently associated with cellular oxidative damage and widespread low-grade inflammation. Probiotics have been attracting increasing interest recently for their antioxidant and anti-inflammatory properties. Here, we tested the ability of probiotics to contrast oxidative stress and inflammation induced by sleep loss to avoid or delay the risk of NDDs.

Methods: We administered a multi-strain probiotic formulation (SLAB51) or water to normal sleeping mice and to mice exposed to 7 days of chronic sleep restriction (CSR). We quantified protein, lipid, and DNA oxidation as well as levels of gut-brain axis hormones and pro and anti-inflammatory cytokines in the brain and plasma. Furthermore, we carried out an evaluation of microglia morphology and density in the mouse cerebral cortex.

Results: We found that CSR induced oxidative stress and inflammation and altered gut-brain axis hormones. SLAB51 oral administration boosted the antioxidant capacity of the brain, thus limiting the oxidative damage provoked by loss of sleep. Moreover, it positively regulated gut-brain axis hormones and reduced peripheral and brain inflammation induced by CSR.

Conclusions: Probiotic supplementation can be a possible strategy to counteract oxidative stress and inflammation promoted by sleep loss, thereby effectively preventing the initiation and progression of NDDs.

Keywords: neurodegenerative disorders; probiotics; sleep deprivation; inflammation; oxidative stress; microglia

4.1 Introduction

Sleep is a fundamental behavior that fills approximately one-third of a human's lifetime and is critical for both physical and mental well-being [276]. Chronic sleep restriction (CSR), defined as insufficient/inadequate sleep over a prolonged period of time, is prevalent in contemporary society owing to professional obligations and lifestyle habits [277], [278]. Epidemiological investigations have estimated that about 30% of adults and adolescents regularly experience insufficient sleep [279]. CSR can lead to a range of brain deficits, including impaired attention and learning, and is associated with increased risk of neurodegenerative disorders (NDDs), but also cardiovascular diseases and metabolic alterations [279]–[282]. Growing evidence has demonstrated that CSR is linked to a low-grade inflammation, as reflected by increased inflammatory plasma cytokines and by the presence of other markers of inflammation in the brain, such as activation of microglia cells [283], [284]. In addition, insufficient sleep can lead to the accumulation of intracellular reactive oxygen species (ROS) and/or reactive nitrogen species (RNS), resulting in an unbalance between the oxidant and antioxidant systems of the body [285]. Excessive ROS and RNS can react with carbohydrates, proteins, lipids, and DNA, and therefore, causes oxidative stress-related cellular damage and increased risk of disease, and in extreme cases, even death [285], [286]. Sleep deprivation also affects energy homeostasis and has been associated with perturbed blood levels of peptide hormones, including ghrelin, leptin, and glucagon like peptide 1 (GLP-1) [287], [288].

Furthermore, Studies have revealed that sleep loss can affect several physiological processes that are critical to brain health, such as the clearance of toxic proteins, the regulation of oxidative stress and inflammation, and the restoration of neuronal function. Lack of sleep triggers brain and systemic inflammation and is a potential risk factor for NDDs such as AD, PD and multiple sclerosis.

Probiotics have been attracting increasing interest in recent years for their ability to ameliorate inflammation-related illness. Numerous studies suggested that probiotics can effectively reduce both peripheral and central inflammation through multiple pathways. The underlying mechanism is associated with rebalancing of gut flora alteration, improvement of gut permeability, and modulation of immune function with lower production of proinflammatory cytokines [289]–[291]. Furthermore, probiotics can regulate microglia maturation and activity, and may also prevent neuroinflammatory processes, with positive impact in a series of diseases, such as inflammatory bowel disease, obesity, and neurodegenerative conditions [292]–[294]. Furthermore, it has been observed that probiotics and/or bacterial metabolites can interact with the host by modulating the level of both endogenous and exogenous ROS, ultimately improving oxidative status [295]–[297]. Long-term supplementation with multi-strain probiotic formulation exerted antioxidant and neuroprotective effects in a transgenic AD mouse model by activating the silencing information regulator 2 related enzyme 1 (SIRT1) pathway.

Several studies have provided evidence that sleep deprivation can perturb the composition of gut microbiota [298], [299]. By inducing a breakdown of the intestinal epithelial barrier, sleep disruption

may favor the passage of bacteria and their end-products, thus affecting the host and promoting immune reaction and inflammation [300]. Thus, sleep loss-associated inflammation may depend, at least in part, on an alteration of the gut microbiota physiology. There is also evidence that administration of probiotics can improve sleep. Manipulation of the gut microbiota through the administration of single or multi-strain probiotics can ameliorate sleep quality by reducing the Pittsburgh Sleep Quality Index (PSQI), a common indicator reflecting the impairment of sleep quality [301], [302].

Here, we tested the hypothesis that chronic oral supplementation with a multi-strain probiotic formulation can reduce oxidative stress and inflammation induced by CSR. To this end, we administered a mixture of several probiotic strains (SLAB51) or vehicle in normal sleeping mice and in mice exposed to CSR, and we assessed the extent of oxidative damage and inflammation in the brain and at systemic level using biochemical and morphological methods.

4.2 Material and methods

4.2.1 Materials

SLAB51 was provided by Ormendes SA (Jouxtens-Mezery, Switzerland, https://agimixx.net). SLAB51 is a multistrain probiotic formulation that contains eight different live bacterial strains: *Streptococcus thermophilus* DSM 32245, *Bifidobacterium lactis* DSM 32246, *Bifidobacterium lactis* DSM 32247, *Lactobacillus acidophilus* DSM 32241, *Lactobacillus helveticus* DSM 32242, *Lactobacillus paracasei* DSM 32243, *Lactobacillus plantarum* DSM 32244, *Lactobacillus brevis* DSM 27961. Polyvinylidene difluoride (PVDF) membranes and reagents for western blotting analyses, and the oxyblot protein oxidation protein detection kit for carbonyl groups introduced into proteins were obtained from Merck KGaA, (Darmstadt, Germany). All antibodies used for western blotting, including nitrotyrosine, dityrosine, 4-hydroxynonenal (4-HNE), 8-oxoguanine DNA Glycosylase (OGG1), 8-oxo-2'-deoxyguanosine (8oxodG), ionized calcium-binding adapter molecule 1 (IBA-1), Interleukin 6 (IL-6), Interleukin 10 (IL-10), tumor necrosis factor alpha (TNFα), Interleukin 1 beta (IL-1β) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody were purchased from AbCam (Milano, Italy). Anti-IBA-1 for immunohistochemistry was purchased from Wako (019-19741), while fluorescent secondary antibodies were purchased from Thermo Fisher (Monza, Italy). ELISA Kits for IL-1β, TNF-α, IL-10 and IL-6 cytokines determination in plasma were obtained from Thermo Fisher Scientific Inc. (Italy). ELISA Kits for ghrelin, leptin and GLP-1 measurement in plasma were from Merk-Millipore (Milan, Italy). Proteases inhibitors tosyl phenylalanyl chloromethyl ketone (TPCK) were from Merck KGaA, (Darmstadt, Germany). Proteins immobilized on films were detected with the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Milan, Italy).

4.2.2 Animals

Eight-week-old wild-type male B6128SF2 (n=28, weight 25-35 g) mice were acquired from the Jackson Laboratory (Bar Harbor, ME, USA). All mice were housed in groups of four in environmentally controlled cages for the duration of the experiment (12 h light/dark cycle, light on at 8:00 P.M., the temperature of 24 ± 1 °C; food and water available ad libitum and replaced daily at 9:00 A.M.). The mouse body weight was measured before and after experimental conditions. All the experiments were performed according to the local Institutional Animal Care and Use Committee and the European Communities and the European Communities Council Directives (2010/63/EU). All appropriate measures were taken to minimize pain and discomfort in experimental animals.

4.2.3 Experimental Design and SLAB51 Administration

Mice were divided into two weight-balanced groups, the water group (w), and the probiotic (p) group. Both the water and the probiotic groups were further separated into chronic sleep restriction (CSR-w, n=7, CSR-p, n=7) and normal sleep groups (S-w, n=7, S-p, n=7). CSR-p and S-p were administered with

SLAB-51 dissolved in the drinking water, while CSR-w and S-w received only water. After 8 weeks of treatment, mice started the CSR experiment, during which the probiotic group was still fed with probiotics until the end of the experiment (Figure 1).

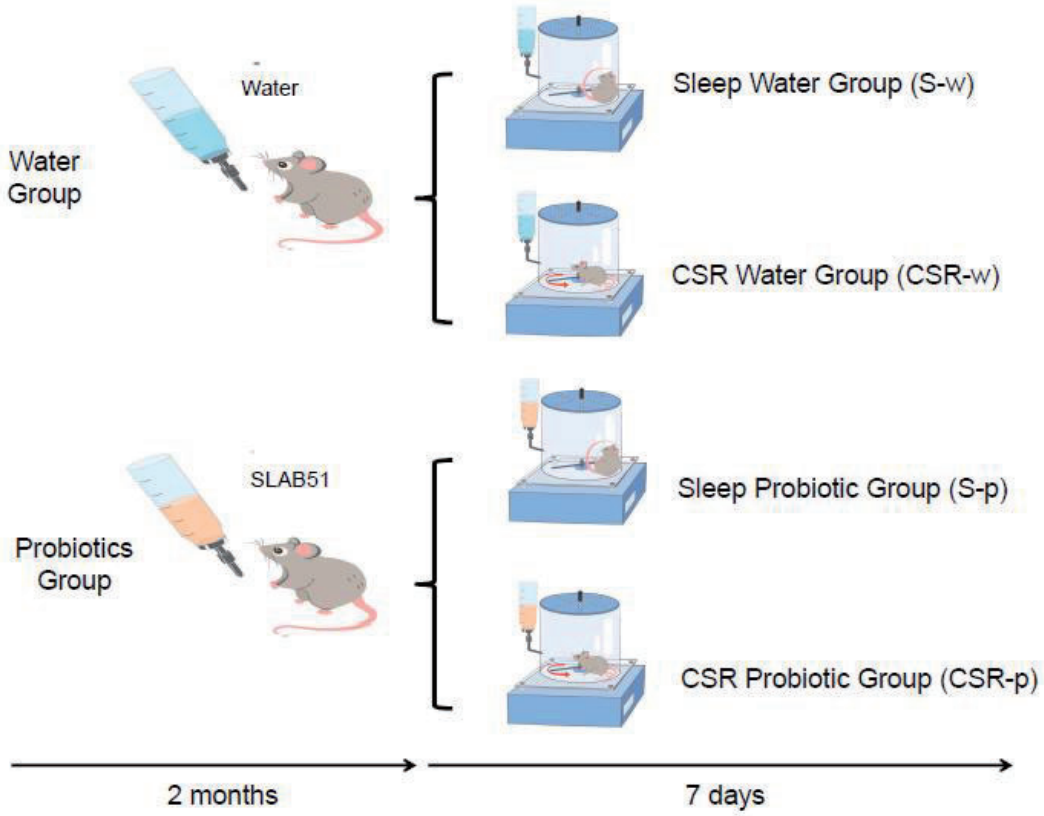


Figure 1. *Experimental design.*

The dosage of SLAB51 (200 billion bacteria/kg/day) was calculated using the body surface area principle based on our previous experiments. Before starting the experiment, we estimated the daily water intake and dissolved the proper amounts of probiotics into the drinking water to reach the desired concentration. We have previously checked the viability and stability of the probiotic formulation after dissolution in water at 21 ± 5 °C. Fluorescence microscopy was used to measure the proportion of vital bacteria, which indicated that 88 percent of the strains survived after 30 h under the aforementioned conditions. The fresh drinking solution was changed every day. The body weights of mice were monitored every 2 weeks before treatment and subsequently weekly during the experiment to ensure normal experimental food consumption.

4.2.4 CSR Procedure

CSR was achieved by an automated sleep deprivation chamber (Pinnacle Technology inc.). The effectiveness of this automated sleep deprivation method has been proved in previous experiments using EEG recording in rodents [303], [304]. The procedure consists of a slow rotating bar placed at a short distance above the cage floor, lightly nudging the animal from sleep and encouraging low levels of

activity until the animal maintains wakefulness on its own. Mice were sleep restricted for 7 consecutive days. To ensure a modest but persistent sleep restriction, mice were exposed to the rotating bar for 24 h/day at a velocity of 2 rpm (one turn each 30 s). Control mice were placed in the same sleep deprivation chambers and allowed to sleep undisturbed, except for 3 h/day (during the dark period, when mice are usually awake) during which the bar rotation was activated to expose the mice of this group to the experience of the bar movement and to the stress associated with it. Mice were housed in groups of four with ad libitum access to food and water or drinking bottle containing SLAB51. Animal behavior was daily assessed by direct visual observation. After 7 days, all mice were sacrificed between 9:00 and 11:00 A.M. to maintain the time of tissue collection within the same 2-h time of day window for all experimental groups.

4.2.5 Tissue Collection

Mice were anesthetized with isoflurane (1-1.5% volume) and sacrificed by cervical dislocation. Brains were extracted, one hemisphere was processed for biochemical assessments, while the other one was immersed in cold 4% paraformaldehyde dissolved in 0.1M phosphate buffer for fixation. Blood samples were collected from the abdominal aorta with a heparinized syringe connected to a 26 G needle and collected in EDTA tubes. They were centrifuged at 3500 rpm for 10 minutes at 4 °C, and obtained plasma was promptly supplemented with Pefabloc 1 mM for subsequent cytokine ELISA detection.

4.2.6 Western Blotting Analyses

Mouse brain tissue was homogenized in a solution of 50 mM Tris buffer, 150 mM KCl, 2 mM EDTA, and pH 7.5 (1:5 weight/volume of buffer). Brain homogenates were immediately centrifuged, at 13,000 g for 20 min at 4°C, and the supernatants were collected upon adding proteinase inhibitors (1 mM tosyl phenylalanyl chlorome-thyl ketone (TPCK) and Pefabloc). Protein concentration was determined using the Bradford protein assay [305]. Brain homogenates were analyzed through western blotting to investigate the following protein expression levels: 3-Nitrotyrsosine (3-NT), Dityrosine, 4-HNE, OGG1, 8-oxodG, and IBA-1. In detail, brain samples (30 µg total protein) were loaded on 10-12% sodium dodecyl-sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes. Following incubation with the specific antibodies, the immunoblot detection was performed with an enhanced chemiluminescence (ECL) Western Blotting ChemiDocTM System (Biorad, Milano, Italy). Molecular weight markers (6.5 to 205 kDa) were included in each gel. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to ensure equal protein loading and to normalize western blot data. ChemiDoc acquired images or scanned autoradiographs (16 bit) were processed through ImageJ software (NIH) to calculate the background mean value and its standard deviation. The background intensity mean was then subtracted from the raw digital data to obtain a background-free image. For each band, the integrated densitometric value was determined as the sum of the density values for all pixels belonging to the analyzed band with a density value larger than the

background standard deviation. The ratios of band intensities were calculated within the same western blot. All the calculations were carried out using Matlab (The MathWorks Inc., Natick, MA, USA).

4.2.7 Oxyblot Analysis

The Oxyblot protein oxidation protein detection kit was used to determine protein carbonyl groups. According to the manufacturer's instructions, brain homogenates (15 μg total proteins) were incubated at room temperature with 2,4-dinitrophenylhydrazine (DNPH) to generate 2,4-dinitrophenylhydrazone (DNP-hydrazone). The DNPH-derivatized samples were subsequently separated by SDS-PAGE and electroblotted onto the PVDF membrane. Then, the membrane was incubated with an anti-DNP antibody followed by a specific secondary antibody. The ECL system was utilized for the detection. To examine the same protein load, prior to incubation with an anti-DNP primary antibody, a reversible Ponceau stain was applied. The statistical significance was determined by comparing the densitometric values of oxyblot bands (oxidation level) to those stained with Ponceau red (protein content).

4.2.8 Plasma Cytokines Levels

The levels of inflammatory cytokines IL-1β, TNF-α, IL-6, and IL-10 in plasma were measured using an enzyme-linked immunosorbent assay NOVEX® ELISA kit (Invitrogen™, Waltham, MA, USA), according to the manufacturer's instructions and detected using a SpectraMax ABS Plus microplate reader (Molecular Devices, Germany).

4.2.9 Ghrelin, Leptin, and GLP-1 Determination

Concentrations of gut-brain axis hormones were measured through ELISA in mouse plasma treated with protease inhibitors (Pefabloc and TPCK). We used a sandwich ELISA based on the capture of ghrelin, leptin, or GLP-1 (active form) in the plasma by specific monoclonal IgG. After the binding of a second biotinylated antibody to ghrelin, leptin, or GLP-1, the unbound material was washed. The remain complex was conjugated to horseradish peroxidase and the quantification of immobilized antibody-enzyme conjugates was performed by monitoring horseradish peroxidase activities in the presence of the substrate 3,3,5,5-tetra-methylbenzidine. The enzyme activity was measured spectrophotometrically by the increased absorbance at 450nm, corrected from the absorbance at 590nm, after acidification of formed products using a SpectraMax ABS Plus microplate reader (Molecular Devices, Germany).

4.2.10 Immunohistochemistry

Brain tissue was allowed to fix for 10 days at 4 °C and then was cut on a vibratome in 50 μm coronal sections. Sections were rinsed in a blocking solution [3% bovine serum albumin (BSA) and 0.3% Triton X-100] for 1 h and incubated overnight (4°C) in the same blocking solution containing anti-IBA-1 (1:500). Sections were then probed with secondary antibodies: anti-rabbit Alexa Fluor 594 (1:600)-conjugated secondary antibodies. Sections were examined with a confocal microscope (Nikon Eclipse Ti, Tokyo,

Japan). For IBA-1, microscopic fields (n = 5 per section, 1 section per mouse) were randomly acquired as 1024 x 1024-pixel images (pixel size, 561 nm; Z-step, 750 nm) in mouse frontal cortex using a UPlan FL N 40x objective (numerical aperture, 1.3). To improve the signal/noise ratio, two frames of each image were averaged.

Image analysis. For IBA-1 staining, all analyses were performed on maximum-intensity projections (Z-project, Maximum Intensity function in ImageJ) of the 21 images constituting the Z-stack. Individual microglial cells were counted and manually segmented using the Single Neurite Tracing plug-in of FIJI [306]. Microglia process arborization was quantified using Sholl analysis by measuring the number of intersections between microglial branches and each Sholl ring.

4.2.11 Statistical Analysis

Statistical analysis was performed using Graphpad prism software (La Jolla, CA, USA) and Matlab (The MathWorks Inc., MA, USA). One-way ANOVA was used for western blotting, ELISA experiments, and microglia density, while two-way ANOVA was used for microglial morphological branching analysis where the between-subject factor were the groups, and the within-subject factor were the Sholl rings. One or two-way ANOVA was followed by the Tukey post-hoc test. Alpha was set to 0.05 and appropriately corrected for multiple comparisons.

4.3 Results

4.3.1 Probiotics Administration Ameliorates CSR-Induced Protein and Lipid Oxidation

To verify the antioxidant effect of SLAB51, we measured the levels of protein and lipid oxidation by quantifying carbonyls, nitrotyrosine, dityrosine, and 4-HNE in the brain homogenates of all groups using western blotting. We found that levels of carbonyl groups, nitrotyrosine, dityrosine, and 4-HNE considerably increased in the CSR-w relative to the S-w group (p=0.003, p=0.0092, p=0.0054, and p=0.0118, respectively), confirming the reported role of CSR in promoting oxidative stress. SLAB51 administration reduced the CSR-induced effects on oxidative stress as the levels of nitrotyrosine, dityrosine, and 4-HNE were significantly lower in CSR-p than CSR-w (p=0.0052, p=0.0217, and p=0.032, respectively) and no longer significantly different between CSR-p and S-p (p>0.05), with the only exception of carbonyl levels that showed only a trend (CSR-p vs CSR-w, p=0.0666, Figure 2).

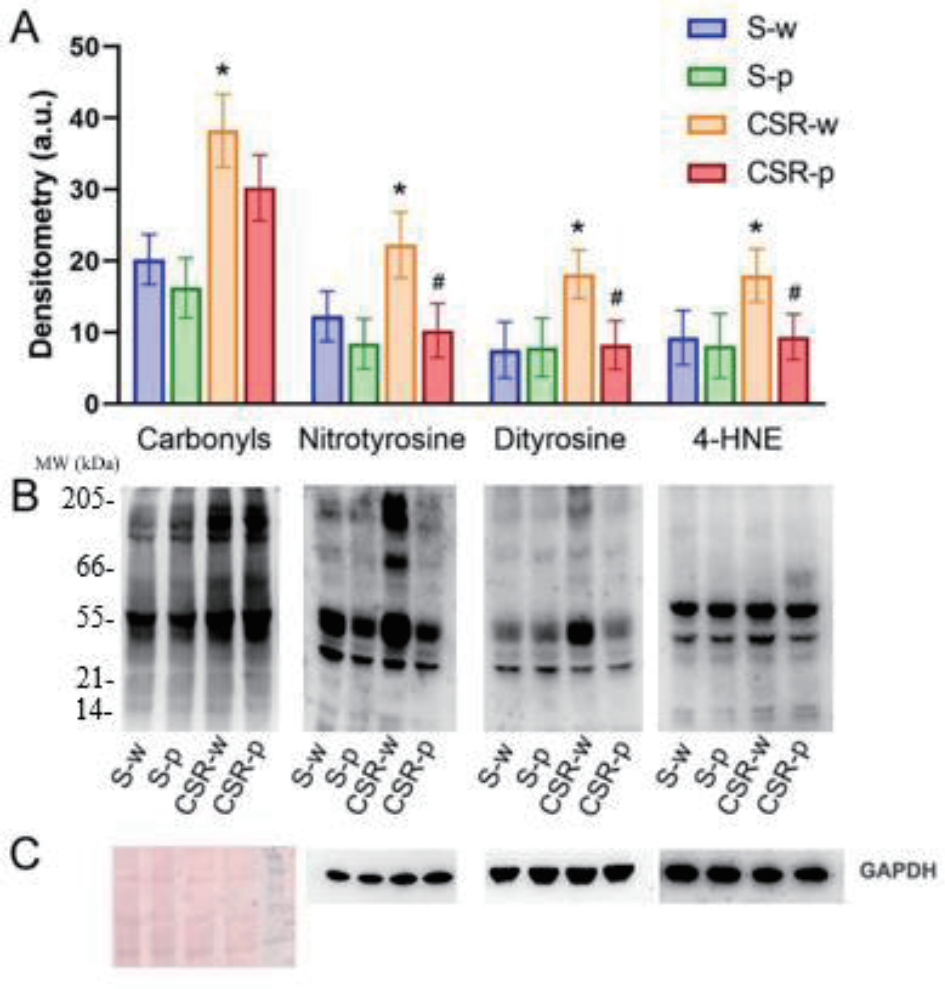


Figure 2. *Effects of CSR and SLAB51 on protein and lipid oxidation. (A). Quantification of protein carbonyls, nitrotyrosine, dityrosine, and lipid 4-HNE adduct in brain homogenates in all groups of mice. The densitometric analyses obtained from three separate blots. Values are expressed as mean ± standard error. One-way ANOVA results were (F (3,24) = 9.277; p = 0.0003) for carbonyls, (F (3, 24)*

*= 7.550; p = 0.001) for nitrotyrosine, (F (3, 24) = 5.431; p = 0.00539) for dityrosine, and (F (3, 24) = 3.967; p = 0.01183) for lipid 4-HNE. * indicates statistical significance between S-w and CSR-w, while # indicates statistical significance between CSR-w and CSR-p. (B). Representative immunoblots for protein carbonyls, nitrotyrosine, dityrosine, and lipid 4-HNE adduct. (C). Equal protein loading for nitrotyrosine, dityrosine and 4-HNE adducts were verified by using an anti- GAPDH antibody. Ponceau staining has been used to check loading in oxyblot. Molecular weight standards (6–205 kDa) were used for molar mass calibration.*

4.3.2 Probiotics Treatment Improves DNA Antioxidant Capacity

In order to determine the effect of SLAB51 on DNA oxidation, the expression of the DNA base excision repair enzyme OGG1 and the DNA oxidation product 8-oxodG were measured in brain homogenates of all groups of mice. In the water group mice, CSR had no significant effect on OGG1 levels compared to control, whereas the probiotic treatment significantly increased OGG1 levels in both S-p (p = 0.029) and CSR-p mice (p = 0.0043), confirming the antioxidant effect of SLAB51 treatment. By contrast, we found higher levels of 8-oxodG in CSR-w mice compared to S-w (increased by 27 ± 9.4%; p = 0.0491). This difference was no longer present in CSR mice treated with SLAB51 (CSR-p vs. S-w, p=0.85; Figure 3).

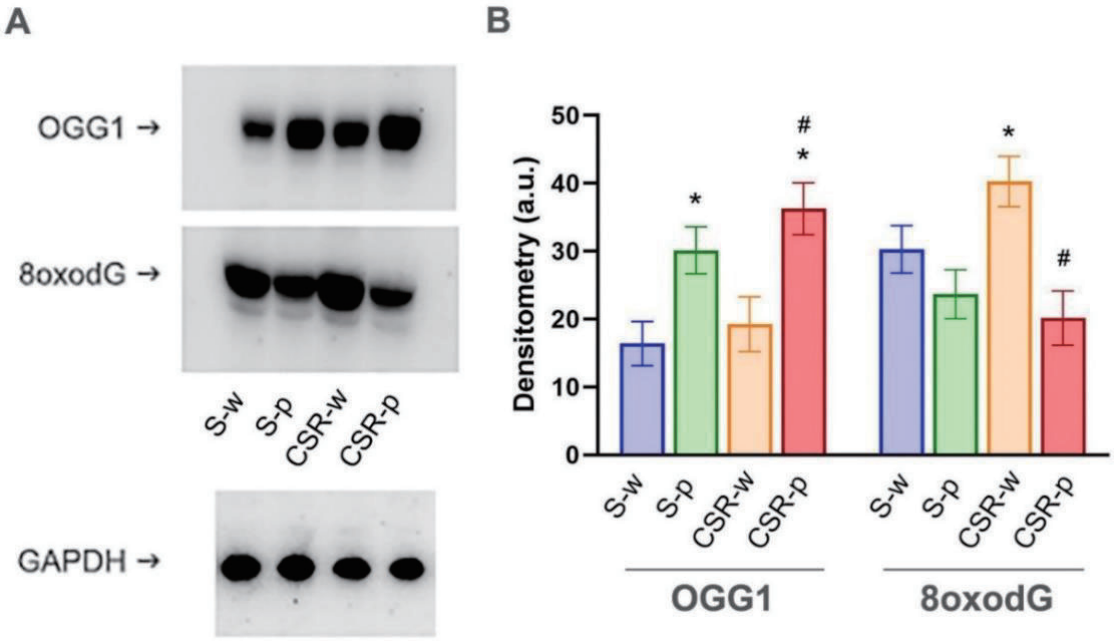


Figure 3. *Effects of CSR and SLAB51 on DNA oxidation. (A). Representative bands for OGG1 and 8-oxodG levels in brain homogenates of all groups. Equal protein loading was verified by using an anti-GAPDH antibody. (B). Quantification of OGG1 and 8-oxodG levels. The densitometric analyses obtained from three separate blots. Values are mean ± SEM. One-way ANOVA results were (F (3, 24) = 6.304; p = 0.00262) for OGG1, (F (3, 24) = 6.698; p = 0.00192) for 8oxodG. * indicates statistical significance relative to S-w, while # indicates statistical significance between CSR-w and CSR-p.*

4.3.3 Probiotics Reduces CSR-induced Neuroinflammation and Systemic Inflammation

We probed the effects of SLAB51 on neuroinflammation by measuring the expression level of several markers of inflammation, including IL-1β, TNF-α, IL-6 and IL-10 cytokines. In brain homogenates, CSR-w mice showed significantly enhanced expression of TNF-α ($p = 0.011$) and IL-1β ($p = 0.048$), and attenuated expression of IL-6 (by 56 ± 9.8%, $p=0.023$) relative to S-w. By contrast, SLAB51 treatment reduced the expression of IL-1β and TNF-α (CSR-p vs. CSR-w, IL-1β: $p=0.024$; TNF-α: $p= 0.0013$), while it increased the expression of IL-6 (CSR-p vs. CSR-w, $p = 0.014$) and IL-10 (CSR-p vs. CSR-w, $p = 0.017$). We also measured the levels of the microglia-specific expression marker IBA-1 in brain homogenates. We found that CSR increased the expression of IBA-1 by 28 ± 8.9% (S-w vs. CSR-w, $p = 0.040$) in the water group; this effect was blunted by probiotic administration (CSR-p vs. CSR-w, $p = 0.013$, Figure 4). At the systemic level, SLAB51 administration had no effect on plasma cytokines concentrations in S-w mice. Similar to the brain compartment, CSR significantly increased the plasma concentration TNF-α ($p = 0.005$) and IL-1β ($p = 0.015$), while it decreased the concentrations of IL-6 ($p = 0.002$), and IL-10 ($p = 0.001$). Long-term SLAB51 supplementation effectively restored changes in the plasma cytokines levels in CSR mice (S-w vs. CSR-w, TNF-α: $p = 0.0031$; IL-1β: $p = 0.0023$; IL-6: $p = 0.016$; IL-10: $p = 0.0089$, Figure 5).

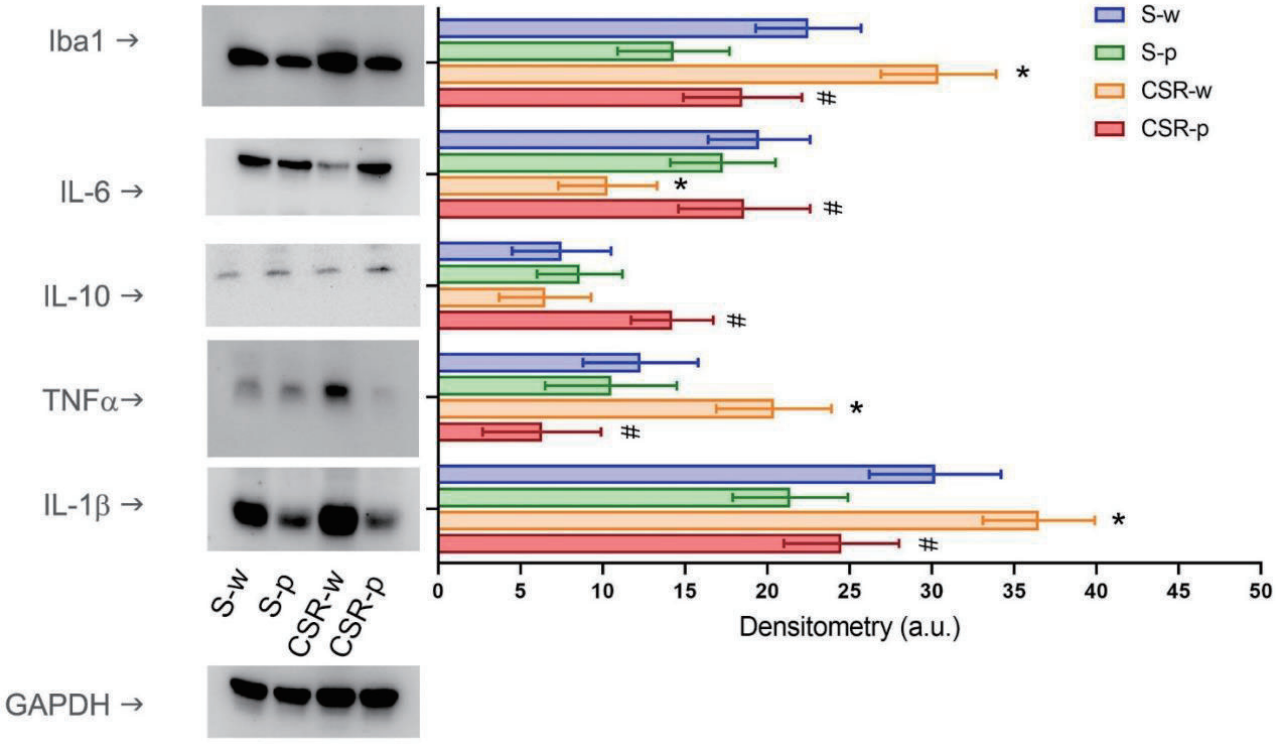


Figure 4. *Effects of CSR and SLAB51 on neuroinflammation. Neuroinflammation cytokines levels measured in brain homogenates of all groups. The densitometric analyses obtained from three separate blots and representative immunoblots are shown. Equal protein loading was verified by using an anti-GAPDH antibody. Values are mean ± SEM. One-way ANOVA results were ($F (3, 24) = 6.239$; $p = 0.00276$) for Iba1, ($F (3, 24) = 3.379$; $p = 0.03473$) for IL-6, ($F (3, 24) = 3.847$; $p = 0.02220$) for IL-*

*10, (F (3, 24) = 7.774; p = 0.00085) for TNF, and (F (3, 24) = 4.534; p = 0.01179) for IL-1β. * indicates statistical significance between S-w and CSR-w, while # indicates statistical significance between CSR-w and CSR-p.*

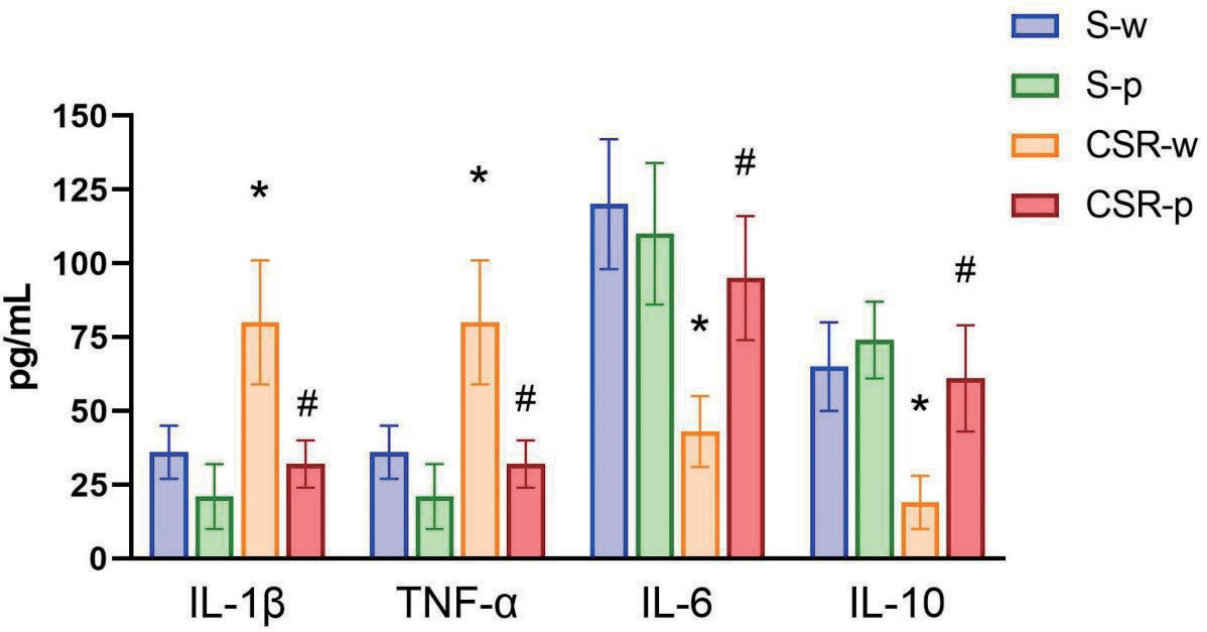


***Figure 5**. Effects of CSR and SLAB51 on systemic inflammation. Proinflammatory and anti-inflammatory cytokines levels in blood plasma of all groups of mice. Analytes concentrations are expressed as mean ± SEM. One-way ANOVA results were (F (3, 24) = 3.379; p = 0.03473) for IL-6, (F (3, 24) = 7.816; p = 0.00082) for IL-10, (F (3, 24) = 6.376; p = 0.00248) for TNF_, and (F (3, 24) = 9.891; p = 0.0002) for IL-1β. * indicates statistical significance between S-w and CSR-w, while # indicates statistical significance between CSR-w and CSR-p.*

4.3.4 Probiotics Attenuate Morphological Microglial Changes Promoted by Sleep Loss

Low-grade neuroinflammation can be associated with morphological changes of microglia, such as process retraction. Hence, we quantified microglia process arborization using Sholl analysis (Figure 6). We manually segmented 2108 microglial cells in the frontal cortex (S-w, n=606; S-p, n=520; CSR-w, n=383; CSR-p, n=599) and found an effect of condition (F (3, 1289) = 4.355; p = 0.0046). Specifically, the administration of probiotics did not promote morphological changes in microglial cells in the normal sleeping mice (S-w vs. S-p, p = 0.4025; Figure 7A). When comparing CSR-w with S-w, we found a significant difference in the level of process arborization between these groups, with CSR-w mice showing a net decrease in the number of processes relative to S-w (p=0.0032, Figure 7B). By contrast, the mice exposed to CSR that received probiotics showed comparable levels of process arborization to those of normal sleeping mice (CSR-p vs. S-p, p=0.1359, Figure 7C) and significantly more than CSR-w (p=0.0489; Figure 7D). Furthermore, we quantified microglia density, finding no changes in the density of cells in all groups of mice (F (3, 24) = 0.7390; p = 0.55; Figure 8). These results indicate that probiotic administration can attenuate the microglia morphological changes induced by sleep loss.

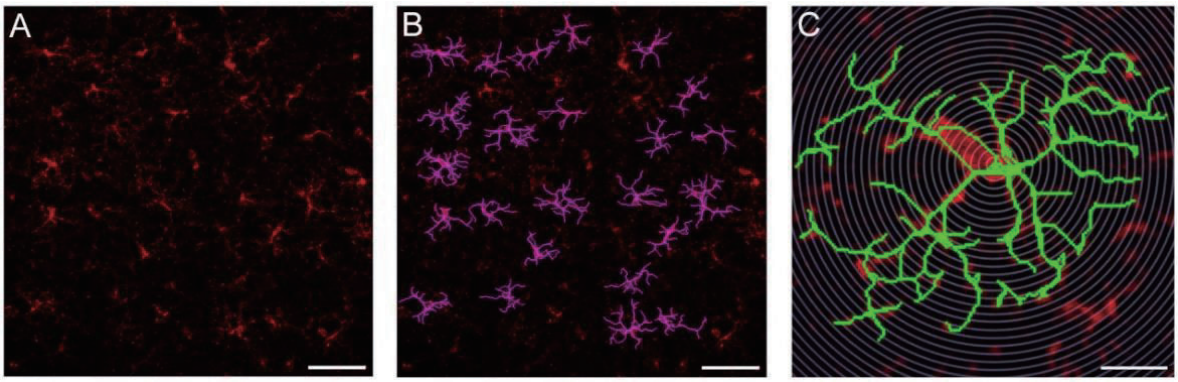


Figure 6. *Microglia cells segmentation and Sholl analysis. (A) Confocal image of microglia IBA-1 positive cells. Scale bar = 50µm. (B) Examples of manually segmented microglial cells. Scale bar = 50µm. (C) Sholl analysis of a representative microglial cell. Scale bar = 5µm.*

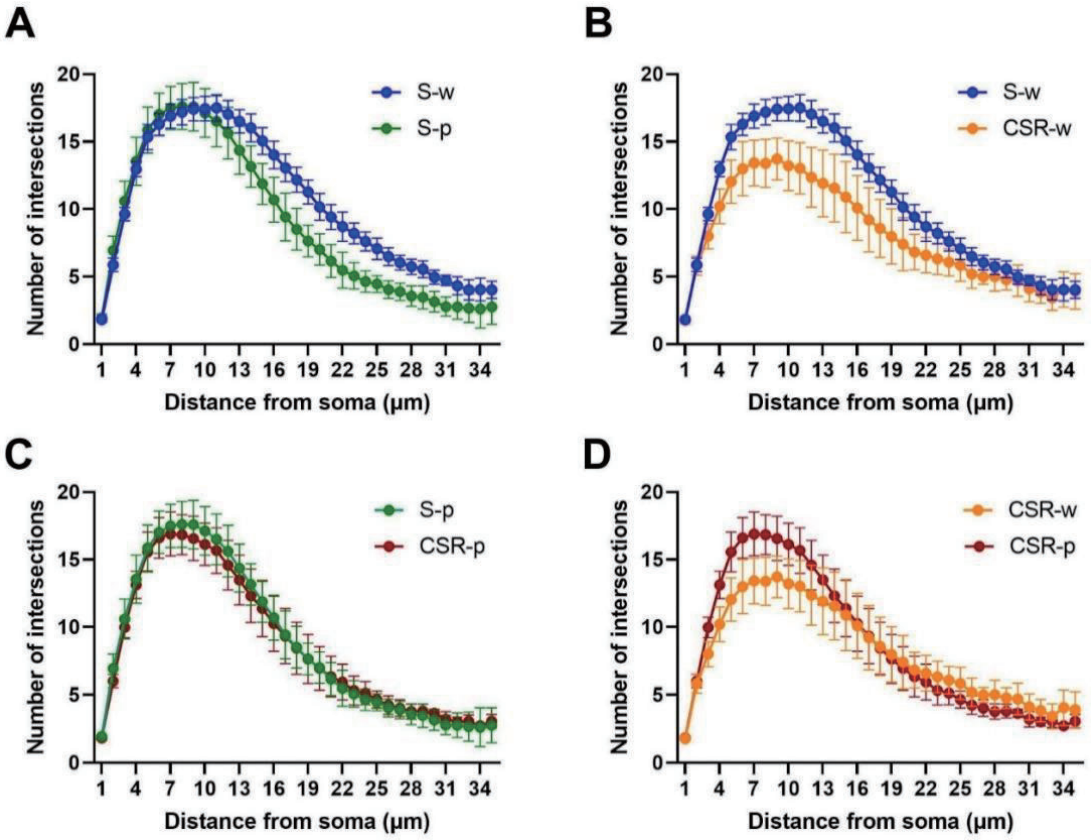


Figure 7. *Quantification of the Sholl analysis of microglia process arborization. Number of intersections in S-w and S-p (A), in S-w and CSR-w (B), in S-p and CSR-p (C), and in CSR-w and CSR-p (D). N = 7 for each group, values are mean ± SD.*

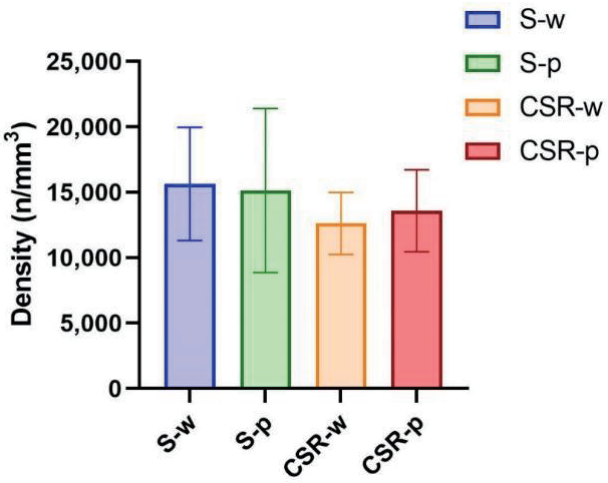


Figure 8. *Density of microglia. Quantification of the density of microglial cells in all groups. Values are mean ± SD.*

4.3.5 Probiotics Restored Blood Concentrations of Gut-Brain Axis Hormones

To confirm a role of gut-brain axis modulation by probiotics, we measured the blood concentrations of key hormones involved in the gut-brain axis. Concentration of ghrelin, leptin, and GLP-1 were importantly modified by CSR. Specifically, we found an increase of ghrelin (p=0.0025) and GLP-1 (p=0.0068) and a decrease of leptin (p=0.0004) in CRS-w relative to S-w. These changes were dampened by SLAB1 supplementation, with ghrelin, leptin, and GLP-1 concentration levels being comparable to those of the sleeping mice and statistically different from those of CSR-w mice (Ghrelin: p=0.047; Leptin: p=0.0045; GLP-1: p=0.04, Table 1).

Table 1. *Plasmatic levels of ghrelin, leptin, and GLP-1. Values are mean ± SD. One-way ANOVA results were (F (3, 24) = 8.231; p = 0.00061) for Ghrelin, (F (3, 24) = 10.952; p = 0.00010) for Leptin, and (F (3, 24) = 10.269; p = 0.00015) for GLP-1. * indicates statistical significance between S-w and CSR-w, while # indicates statistical significance between CSR-p and CSR-w.*

	S-w	**S-p**	**CSR-w**	**CSR-p**
Ghrelin (pg/mL)	458.26 ± 56.45	476.01 ± 46.58	689.37 ± 44.00*	562.25 ± 42.21#
Leptin (ng/mL)	11.12 ± 3.40	13.02 ± 2.90	1.99 ± 0.70*	7.41 ± 3.22#
GLP-1 (pg/mL)	102.36 ± 32.56	112.23 ± 26.65	602.36 ± 100.23*	303.26 ± 53.65#

4.4 Discussion

In this study, we found that a week of CSR induced oxidative stress in the mouse brain, promoted brain and systemic inflammation, and altered the gut brain-axis. The oral administration of SLAB51 boosted the antioxidant capacity of the brain, thus limiting the oxidative damage provoked by loss of sleep. Moreover, it restored the levels of gut-brain axis hormones and attenuated the development of peripheral and brain inflammation induced by CSR.

Sleep is critical for maintaining body and brain functions, and insufficient sleep has been related to increased risk for a variety of diseases (e.g., neurodegeneration, type 2 diabetes, obesity, depression, anxiety, etc.), for which oxidative damage and inflammation have been proposed as potential underlying mechanisms [307], [308].

Oxidative stress is the result of an unbalance between the production of reactive oxygen species (ROS) or reactive nitrogen species (RNS) and the antioxidant capacity of the cells [309]. Proteins, lipids, and DNA are major targets of ROS or RNS in biological systems [310], [311]. There is no general consensus on the role of sleep loss in promoting oxidative stress [312]. Some studies found that sleep loss can either increase the production of oxidative radicals or lower antioxidant responses, while others did not find any change in oxidative stress markers or antioxidant capacity in peripheral blood or brain regions following sleep loss [313]–[317]. This discrepancy has been ascribed to the different sleep deprivation procedures (e.g., gentle handling vs. disk over the water) and the different duration of the sleep deprivation. While acute sleep loss appears to up-regulate the antioxidant cellular machinery, chronic loss of sleep weakens the antioxidant response, thus suggesting that extended wakefulness may be more likely associated with oxidative stress. In our study, we found that 7 days of chronic sleep restriction were capable of increasing the brain levels of carbonyls, nitrotyrosines, and dityrosines, all well-established markers of protein oxidation. In parallel, we found augmented levels of 4-Hydroxynonenal (HNE), a major end product that is derived from the oxidation of lipids. Furthermore, studies on people over age 60 have shown that even one night of sleep loss can induce the expression of genes involved in DNA damage and aging [318]. Animal studies have also shown that sleep deprivation can cause genetic damage in a variety of organs [319]. The accumulation of DNA damage has been linked to DNA mutations, altered gene expression in the brain, and cognitive decline [320]. In our study, we observed increased DNA oxidation in CSR mice compared to control animals, as detected by the decreased OGG1 expression and the increased 8-oxodG levels in the brain homogenates. We also found increased plasma levels of ghrelin in the CSR-w group. Besides its role in regulating appetite, ghrelin has been recently proposed as a systemic oxidative stress sensor [321]. Collectively, these data suggest that chronic sleep loss can lead to oxidative damage of proteins, lipids, and DNA.

Sleep loss has also been repeatedly associated with heightened inflammation. Increased plasma levels of numerous cytokines including IL-1, TNF-α, IL-6, IL-17, nuclear factor-kappa B (NFkB), and altered numbers and activity of macrophages and natural killer cells have been found after both acute and chronic

sleep deprivation in healthy individuals [322]–[324]. These findings were also supported by preclinical studies, in which the inflammatory markers IL-1, IL-6, and TNF-a were found elevated in the peripheral blood and several brain regions [325], [326]. While the mechanisms through which sleep loss leads to an inflamed state are unclear, oxidative stress may contribute to inflammation by stimulating the release of proinflammatory cytokines including TNF-α and IL-1β. Furthermore, ROS and RNS can activate other inflammatory mediators such as NF-kB and vascular cell adhesion molecule-1(VCAM-1) [327]. In this study, CSR was associated with an increase of TNF-α and IL-1β and a decrease of IL-6 and IL-10 in the brain and peripheral blood. While IL-10 is a well-recognized anti-inflammatory cytokine, the role of IL-6 in sleep deprivation-related inflammation remains unclear. Some studies showed that total sleep deprivation or sleep fragmentation led to increases in plasma IL-6 levels, which were interpreted as a marker of inflammation [328], [329]. However, other works demonstrated that IL-6 has a crucial anti-inflammatory role in local and systemic inflammatory responses by modulating levels of proinflammatory cytokines [330], [331]. Our previous study showed that CSR could activate microglia without affecting the levels of cytokines in the cerebral spinal fluid [283]. Sustained microglia activation could potentially increase the brain's vulnerability to various types of damage, ultimately causing NDDs [283]. In this study, we confirmed the activation of microglia induced by CSR, but we also found an increase of IBA-1 expression in the brain of CSR mice. IBA-1 has been demonstrated to have a role in actin-crosslinking of microglial membrane ruffling, and its expression is related to the microglial activation since membrane ruffling is required for the shift from quiescent ramified to activated amoeboid microglia [331]. All together, these findings confirm that sleep loss is associated with increased systemic and brain inflammation.

Probiotics are live microorganisms intended to change the composition of the flora of the gastrointestinal tract of the host and provide health benefits when consumed [332]. The mechanisms through which probiotics improve health are numerous and include the modulation of the host immune system, modification of the intestinal microbiota, protection against physiological stress, pathogen antagonisms, and improvement of the barrier function of the gut epithelium [333]. In this study, we used SLAB51, a multi-strain probiotic supplementation. Previous research has shown that SLAB51 could restore normal eubiosis in animal models of NDDs [334], while it had little/no effect on the microbiota of healthy wild-type mice. The effects of probiotics on inflammation and oxidative stress biomarkers have been extensively investigated in animal models and clinical trials. Most recent studies confirmed the role of probiotics in decreasing the levels of CRP, high-sensitivity(hs)-CRP, and TNF-a levels [333]–[336]. Previous work using SLAB51 found that this formulation increased the relative abundance of gut anti-inflammatory bacteria such as *Bifidobacterium* spp. and decreased the concentrations of pro-inflammatory *Campylobacterales*, consequently regulating inflammatory pathways. Moreover, it promoted the proliferation of bacteria that produced short-chain fatty acids (SCFAs). It is, thus, possible that the enriched gut concentration of anti-inflammatory and neuroprotective SCFAs contributed to reduce the plasma levels of pro-inflammatory cytokines and to enhance the concentrations of anti-

inflammatory cytokines. Consistent with these results, a recent meta-analysis evaluating 42 controlled trials demonstrated that levels of several pro-inflammatory cytokines (i.e., IL-12, IL-4, etc.) were significantly lowered by probiotic supplementation [337]. By contrast, levels of IL-10, glutathione, nitric oxide, total antioxidant status, and total antioxidant capacity were significantly increased with probiotics administration [333], [334], [338], [339]. It is worth noting, however, that several studies have reported no effect of the probiotic treatment in regulating inflammation or the oxidative status relative to the placebo group [335], [337], [340], [341].

The anti-inflammatory effects of probiotics are not limited to the gut. Indeed, chronic low-grade inflammatory processes are now thought to play an etiological role in the pathogenesis of several NDDs and probiotics have been proposed as potential compounds capable of mitigating these pathologies by modulating the immune-to-brain signaling and alleviating the chronic immune activation in the brain [342], [343]. In that perspective, administration of multi-strain probiotics including SLAB51have resulted in reduced neuroinflammation in animal models of AD [344], [345]. Moreover, a recent meta-analysis of 5 studies involving 297 subjects has found improved cognitive performance in AD or MCI patients following probiotic supplementation, likely through decreasing inflammatory and oxidative stress levels [346]. In our study, we found that administration of SLAB51 per se affected neither the levels of inflammatory cytokines in the brain and plasma nor the morphology of microglia cells of sleeping animals (S-p similar to S-w), whereas it induced the overexpression of OGG1 in both sleeping and sleep restricted mice (S-p and CSR-p). Thus, SLAB51 supplementation not only did not trigger an inflammatory response per se, but it enhanced cellular antioxidant capacity. More importantly, when administered in mice later exposed to CSR, it contrasted the rise of central and peripheral inflammation and oxidative stress levels, thus indicating that probiotics can abolish the immune and inflammatory response associated with the loss of sleep. These findings are consistent with a recent study carried in acutely sleep-deprived monkeys, where supplementation of GABA-producing probiotics reduced the proinflammatory cytokines IL-8 and TNF-alpha, with no effect on the circulating levels of IL-6 and IL-10 [347]. Similarly, supplementation of *Lacticaseibacillus paracasei* was capable of restoring memory deficits in mice subjected to partial sleep deprivation [348]. Although it was not directly tested in this study, it is possible that the effect on cognition was mediated by the immunomodulation properties of probiotics. The mechanism through which probiotics exert their protective role on neuroinflammation is unclear, but it could be attributed, at least in part, to their direct effects on the gut-brain axis [349]–[351]. For example, numerous reports over the past decades have described ghrelin to be a potent anti-inflammatory mediator [84], while high levels of leptin have been related to inflammation [352]. In this regard, we found that probiotic supplementation restored nearly normal plasma concentration of ghrelin, leptin, and GLP-1, whose levels were remarkably altered by CSR. Although indirect, this evidence supports the hypothesis that probiotics can modulate neuroinflammation via the production and release of specific gut hormones [349]–[353].

In humans, probiotics have been mostly administered to improve sleep quality rather than counteract the

effects of sleep loss. A recent systematic review analyzed a total of 14 studies finding that probiotics supplementation significantly reduced Pittsburgh Sleep Quality Index (PSQI) score (i.e., improved sleep quality) relative to baseline, while no significant changes were reported for other subjective sleep quality metrics or objective sleep parameters, such as efficiency and latency. Although not significant, subsequent analysis found that healthy participants had a greater benefit on sleep quality than those with a medical condition and the use of single-strain probiotics was better than multi-strain probiotics in improving sleep quality [354]. The possibility of counteracting the deleterious effects of sleep deprivation with probiotics is intriguing and could be relevant for populations who by necessity have a disrupted sleep schedule, such as shift workers. The few available studies along this direction showed that probiotics have the potential to reduce the magnitude of the stress response that anticipates the beginning of the night shift [355] and alleviate anxiety and fatigue in shift-workers [356], but there are still no available data on the role of probiotics in reducing the proven long-term risk in developing shift work-associated diseases.

A number of limitations to the study should be acknowledged. First, we did not use oral gavage to administer probiotics to mice, but we dissolved SLAB1 in the drinking water. This methodological aspect could have generated variability in the intake of probiotics within the probiotic group. However, we treated the animals for overall 9 weeks (8 weeks before starting the sleep experiment and during the week of the experiment). This long treatment duration ensured adequate intake of probiotics and may have limited heterogeneous effects among animals. Additionally, since we administered probiotics before and during the CSR, we currently cannot distinguish whether probiotics had preventive or therapeutic effects on CSR-induced inflammation and oxidative stress, and future research with a different experimental design is needed to clarify this ambiguity. Furthermore, we did not assess cognitive functions in our mice, and therefore, we do not know whether probiotics were able to restore potential sleep loss-associated cognitive deficits and whether these effects were related to systemic and brain inflammation. Finally, we only studied male mice, and it is possible that probiotics exerted a different influence on inflammation in female mice, although other studies have found that sex had only a minor effect on the immune modulation of probiotics [357].

4.5 Conclusion

Our study provides direct support to the growing evidence that probiotics can attenuate oxidative stress and inflammation in the brain and at systemic level via the gut-brain axis. In addition, it indicates that probiotic supplementation can represent a viable strategy to counteract oxidative stress and inflammation related to sleep loss, thus possibly limiting its negative consequences on health and well-being, including neurodegenerative disorders.

CHAPTER 5

PREBIOTIC-BASED COOKIE ENRICHED WITH PROBIOTICS AMELIORATED SHORT-TERM MEMORY, BLOOD GLUCOSE LEVELS AND LIPID PROFILE IN ALZHEIMER'S DISEASE

Abstract

Background: Age-related cognitive decline and neurodegeneration represent a heavy burden on society due to the increasing aging population. Alzheimer's disease (AD) is the most common form of dementia among old people. It is an incurable and progressive pathology with a multifactorial pathogenesis including disturbances of the gut-brain axis, a bidirectional communication between central nervous system and gastrointestinal tract. Accumulating evidence suggests that metabolic dysfunctions such as dyslipidemia, and hyperglycemia often coexist with AD and may exacerbate the pathogenesis.

Preservation or strategic manipulation of the composition and functional characteristics of the gut microbiota is a key condition for healthy longevity and a promising perspective to prevent or delay neurodegeneration, with prebiotics and probiotics counteracting gut dysbiosis by producing neuroprotective hormones and anti-inflammatory metabolites. Strategic changes of gut microbiota can affect cognition, but the exact mechanisms are not fully clarified.

Interestingly, oral administration of a multi-strain formulation of *lactic acid bacteria* and *bifidobacteria* (Slab51®) counteracted cognitive decline, reduced amyloid aggregates and brain damages, and partially restored the impaired neuronal proteolytic pathways in a mouse model of Alzheimer disease. Driven by global dramatic demographic and lifestyle changes and based on the growing interest on on-the-go healthy snacks, we have designed and developed a prebiotic-based cookie prototype enriched with probiotics with proved neuroprotective properties.

The functional cookie contains the optimal combination of selected prebiotics and probiotics resulting in a natural, safe, appealing and sustainable people-centred food counteracting cognitive decline.

Chronic consumption of the functional cookie improved glucose and lipid profiles, restored neuroprotective gut hormones plasma levels in 3xTg-AD mice, ultimately reducing Aβ load and improving cognitive performance, thereby delaying AD progression.

Keywords: Alzheimer's disease; prebiotics; probiotics; functional cookie; lipid metabolism; glucose metabolism; gut hormones

5.1 Introduction

Alzheimer's disease (AD) is an age-related neurodegenerative disease, although biologically defined by the intracellular amyloid-β (Aβ) plaques and extracellular neurofibrillary tau tangles (NFT) in the brain, the exact etiological mechanism is unclear, probably involving genetic predisposition and environmental factors [358]. Accumulating evidence suggests that metabolic dysfunctions such as dyslipidemia, impaired glucose uptake and utilization, and metabolic hormone dysfunction often coexist with AD and may exacerbate the pathogenesis [359], [360]. Diet impacts human metabolism [361]. Experimental and clinical data have shown that a healthy diet can control modifiable risk factors of AD, including dyslipidemia and hyperglycemia [362].

Being the brain the richest organ in terms of lipid content and diversity, dyslipidemia can increase the risk of AD onset and can exacerbate the pathology [363]. In particular, hypercholesterolemia can disrupt the blood-brain barrier (BBB), leading to infiltration of peripheral macrophages, activating microglia and astrocytes, causing neuroinflammation. Additionally, *in vitro* functional cell biology studies indicate that cellular and membrane-bound cholesterol can modulate β-secretase and γ-secretase activity influencing Aβ formation. 27-hydroxycholesterol (27-OHCE), an oxidized cholesterol metabolite that can penetrate the BBB, increases beta-site amyloid precursor protein cleaving enzyme 1 (BACE1 or β-secretase), a key player in Aβ production [364]. High-density lipoprotein (HDL) is responsible for reversing cholesterol transport by combining with cholesterol to form HDL-C and has an anti-inflammation and anti-oxidation ability, playing a beneficial role in AD pathogenesis, but it was reported to be reduced in AD and obesity. In contrast, LDL mediates cholesterol transport to the tissues, causing brain Aβ accumulation and cholesterol metabolism-related damage. Moreover, dyslipidemia-induced elevated triglycerides can influence leptin transport across the BBB, with a positive impact on hippocampus neuron synaptic plasticity, memory, and cognition.

Aβ and NFT drive the early decline of AD while glucose hypometabolism drives the late decline [365]. A tight regulation of glucose metabolism is critical for brain. Evidence suggests chronic hyperglycemia reduces glucose absorption and utilization in the brain by downregulating glucose transporters (GLUTs) expression [366]. These phenomena are frequently observed in AD, resulting in impairments in energy supply and impaired neuronal activity, contributing to cognitive decrease. Specifically, glucose transporter 1 (GLUT1) and 3 (GLUT3) are reduced in the hippocampus and cortex of AD brains. One recent research demonstrated that chronic hyperglycemia impairs hippocampal neurogenesis and memory in the 3xTg-AD mouse model [367]. Moreover, excess of 27-OHCE can impair insulin-mediated glucose uptake in the brain by modulating the expression and activity of GLUT4, impacting brain function [368]. Clinical studies have shown that AD patients have low levels of resistin, a hormone regulating insulin metabolism, glucose uptake and inflammation [369]. Aging and impairment of energy metabolism have been associated to changes in levels of circulating hormones. In the body, leptin and ghrelin are engaged in lipid metabolism, while GLP-1 and GIP are responsible for glucose metabolism. These neuroprotective

hormones are often reduced in AD patients, and modulating the release of such gut hormones can positively interfere with blood glucose, lipid profiles, and cognition [370]–[372]. Aging, infections, unhealthy diet, and lifestyle can cause dysbiosis, an altered gut microbiota composition, diversity, and functionality, favouring the onset and progression of neurodegenerative disorders including AD. Since dysbiosis is strictly correlated with alterations of intestinal permeability, dysfunctions of BBB and neuroinflammatory processes [373], strongly participating in AD development, gut microbiota represents a key target to tackle AD. Preservation of a healthy microbiota is a necessary condition for longevity and rational manipulation of its composition is a promising perspective to prevent or delay neurodegeneration. Among the approaches aimed to ameliorate microbiota in elderlies there are prebiotics, which are non-viable substrates, selectively utilized by host microorganisms or administered probiotic strains, eliciting a metabolism biased towards health-promoting microorganisms within the indigenous ecosystem. An increasing number of probiotic- and prebiotic-based nutritional interventions in humans were shown to exert positive effects on gut microbiota [374], [375]; these studies primarily involved old adults (including AD patients) with memory deficits, insulin resistance, diabetes, obesity, and cardiovascular disease that are strongly interconnected [376]. The results encourage us to further explore this dietary strategy to contrast age-associated cognitive decline and to explore the multi-level mechanism of action of prebiotics and probiotics.

In this context, increasing scientific interest focused on lentils as functional food ingredients due to their excellent nutritional and bioactive composition and potential beneficial effects for metabolism diseases. It possesses various phenolic compounds and vitamins, which show high antioxidant capacity. Lentils can reduce intestinal digestion and absorption of cholesterol, glucose, and fat by inhibiting α-glucosidase and pancreatic lipase, probably due to being rich in flavonoids and fibers, thereby improving blood lipid profile and glucose metabolism. Lentils were demonstrated to reduce hyperlipidemia and glucose levels, and to raise HDL levels in a rat model of diabetes. Lentils reduced total cholesterol, triglycerides and LDL plasma levels in a hypertensive animal model. Furthermore, lentils contain important prebiotic carbohydrates that support beneficial bacteria growth and promote the production of short-chain fatty acids through microbiota fermentation, fostering a favorable gut environment, enhancing the intestinal barrier, and reducing inflammation [377]–[379]. Regarding probiotics, SLAB51 multi-strain probiotic formulation counteracted cognitive decline, reduced amyloid aggregation, and attenuated brain damage in 3xTg-AD mice. In these animals, SLAB51 significantly reduced cerebral inflammation and oxidative stress, partially restored impaired neuronal protein degradation pathways, increased the plasma levels of neuroprotective gut peptides, and ameliorated energy homeostasis.

Development of snacks to promote healthy aging, with positive action on gut-brain axis, must focus on the delivery of probiotics and expertly selected prebiotics through proper food design [380]. Driven by global dramatic demographic and lifestyle changes and based on the growing interest on on-the-go healthy snacks, a snack prototype was recently developed at Unicam for a pre-clinical pilot study. The snack prototype consisted in a red lentils-based cookie (prebiotic carrier [381]) coated with probiotic

(Slab51®) enriched chocolate. The cookie was administered to a triple-transgenic mouse model of AD, B6; 129-Psen1tm1Mpm Tg (APPSwe, tauP301L)1Lfa/J (named 3xTg-AD), to investigate the ability of the functional cookie to improve short-term memory in AD mice and qualitatively ameliorate plasma lipid profile and glucose metabolism. Plasma concentration of gut hormones (ghrelin, leptin, GIP, GLP-1 and resistin) were monitored for their potential neuroprotective effects associated with the gut-brain axis.

5.2 Material and methods

5.2.1 Reagents and chemicals

SLAB51 probiotic formulation contains eight different live bacterial strains: *Streptococcus thermophilus* DSM 32245, *Bifidobacterium lactis* DSM 32246, *Bifidobacterium lactis* DSM 32247, *Lactobacillus acidophilus* DSM 32241, *Lactobacillus helveticus* DSM 32242, *Lactobacillus paracasei* DSM 32243, *Lactobacillus plantarum* DSM 32244, *Lactobacillus brevis* DSM 27961, was kindly provided by Ormendes SA (Jouxtens-M_ezery, Switzerland, https://agimixx.net).

Polyvinylidene difluoride (PVDF) membranes and reagents for western blotting analyses were obtained from Merck KGaA (Darmstadt, Germany). Antibodies for detecting amyloid oligomers, glucose transporters (GLUT 1, 3, 4) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), were purchased from AbCam (Milan, Italy). Proteases inhibitors tosyl phenylalanyl chloromethyl ketone (TPCK) and 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF or Pefabloc) were obtained from Sigma-Aldrich S.r.L. (Milano, Italy). All solvents and reagents used in this experiment were the highest purity available.

5.2.2 Animal model

B6;129-Psen^{1tm1Mpm} Tg (APPSwe, tauP301L)1Lfa/J (named 3xTg-AD) were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA). 3xTg-AD are a reliable triple transgenic model of AD containing three mutations (amyloid precursor protein [APP]Swe, tau MAPT P301L, and presenilin-1 M146V) associated with frontotemporal dementia or familial AD. As early as 3 to 4 months of age Aβ intracellular immunoreactivity can be detected in certain brain regions and at 10-12 months of age tau hyperphosphorylation occurs.

Before the experiment, all mice were housed in plastic (Makrolon) cages (4 animals per cage) in a temperature-controlled room (21 ± 5 °C), 60% humidity, 12 h light/dark inverted cycle (light was switched on at 8:00 P.M.) and were fed laboratory diet (Mucedola, Italy) with water ad libitum. 8-week-old male mice (weight 15–25 g) were used for experiments. All experimental procedures were performed according to the local Institutional Animal Care and Use Committee (Approval n° 687/2023-PR (Risp. a prot. 1D580.43) of Italian Ministry of Health) and following the European Communities Council Directives (2010/63/EU). All appropriate measures were taken to minimize pain and discomfort in experimental animals.

5.2.3 Functional cookies preparation

The functional snack consists of three components: a main phase (cookie with prebiotic-rich ingredients, specifically red lentils), a probiotic carrier (dark chocolate coating), and Slab51® probiotic mix (*bifidobacteria* and *lactic acid bacteria* with documented positive effect on gut-brain axis health)

obtaining a chocolate-coated cookie. The main phase (cookie with prebiotic-rich ingredients) design is based on a "pasta frolla" standard recipe [382] (control) modified by substituting wheat flour with legume flour and by using specialty fibers with documented applications in cookies to easy sugar (e.g. Meltec® [383]; Microsin®) and fat/saturated fat (e.g. HI-FIBREWF® [382], [384], [385]) reduction in the final product. Probiotic vitality retention in the chocolate carrier have been ensured by the use of mild processing conditions during snack production, and it has been tested in the final product.

Preliminary experiments evaluating the prebiotic activity of the different legumes-based cookies on Slab51® have been tested upon processing with the internationally standardized INFOGEST *in vitro* digestion protocol [386] (data not shown).

As controls a prebiotic cookie (chocolate-coated) without probiotic and a reference cookie (made with wheat refined flour, sugar, and butter) have been produced.

5.2.4 Experimental design

8-week-old 3xTg-AD male mice (n = 60) were divided into five groups, namely water group (Water, n=12), SLAB51 group (SLAB51, n=12), reference group (Ref, n= 12), prebiotic group (Prebiotic, n=12) and prebiotic+SLAB51 group (Prebiotic+SLAB51, n=12), and fed with different food for 4 months, Water group was provided with normal drinking water and normal laboratory diet; SLAB51 group was provided with SLAB51 dissolved in drinking water and normal laboratory diet, according to the principle of body surface area, the amount of SLAB51 2×10^{11} bacteria/kg/day was calculated; Ref group was fed with reference cookie and normal drinking water; prebiotic group was fed with prebiotic cookie and normal drinking water; and Pre+SLAB51 group was treated with the functional cookie and normal drinking water. The fresh drinking solution was replaced every day. The body weights of mice were monitored every two weeks prior to treatment and then weekly during the treatment to ensure adequate consumption of experimental food.

Table 1. *Experimental groups*

GROUPS	3xTg-AD mice
Water	n=12
Slab51® in water	n=12
Control cookie	n=12
Prebiotic cookie	n=12
Prebiotic + probiotic (Slab51®) cookie (best prototype)	n=12

A preliminary study was performed to assess the probiotics viability in dark chocolate and functional cookies stability. Viable cell counts of the probiotic bacteria in chocolate were determined by the standard plate method and values were expressed as colony forming units per gram (CFU/g) of chocolate. Ten grams of chocolate was homogenized in a 90 mL saline solution (0.9% NaCl). Serial dilutions were prepared and appropriate dilutions were plated on a generic, rich agar (Merck, Darmstadt, Germany) based growth medium. Total bacterial counts were determined after 48h of incubation at 37 °C. The viability of both bacterial strains was analyzed in triplicate immediately after production and after 1-68 weeks of storage at room temperature (RT) or at 4°C.

5.2.5 Behavioral assessments

The NOR test which relies on the innate curiosity of mice towards novel stimuli was employed to assess the recognition memory capability of mice. The test was conducted between 8:00 a.m. to 3:00 p.m. (dark phase of the light/dark cycle), and investigators were blinded to group allocation. The test spanned two days, the first day was to familiarize the mice with the experimental environment with a five-minute acclimation period in an empty arena. On the second day, two 10-minute trials were performed, a training phase and a test phase, separated by 3-hour intervals. During the training phase, mice were allowed to explore two identical objects, followed by the test phase where they encountered a familiar and a novel object.

To evaluate memory integrity, the total exploration time during the test trial was recorded, and the preference index was calculated as (seconds spent with the novel object - seconds spent with the familiar object) / (seconds spent with both objects). Lower scores in this task indicate potential memory impairment. To ensure replicable data, the novel objects differed from the familiar objects in shape, color, and texture and the objects were strictly cleaned after each set of experiments to eliminate the smell interference.

5.2.6 Tissue and plasma collection and preparation

Mice were euthanized at 24 weeks of age following behavioral assessment by CO_2 overdose. Brain tissues were properly collected for subsequent biochemical analysis. In detail, mouse brain was extracted on ice-cold glass plates. Then tissues were homogenized in 50 mM Tris buffer, 150 mM KCl, 2 mM EDTA, and pH 7.5 (1:5 weight/volume of buffer), homogenates were promptly centrifuged at 13,000× g for 20 min at 4 ℃, and the supernatants were collected upon adding proteinase inhibitors (1 mM TPCK and Pefabloc).

Blood samples were extracted from the abdominal aorta using a heparinized syringe fitted with a 26 G needle and transferred to EDTA tubes, after centrifugation at 3500 rpm for 10 min at 4 ℃. Obtained plasma samples were immediately supplemented with protease inhibitors (1 mM TPCK and Pefabloc). Protein concentration was determined using the Bradford protein assay. Then all samples were stored at

-80 °C.

5.2.7 Plasma lipid analysis

Plasma lipid profile, including total cholesterol, HDL-C, LDL-C, and triglycerides were enzymatically measured using colorimetric kits obtained from Chema Diagnostica (Italy). The assays were performed following the manufacturer's instructions. Data are expressed as mg/dL ± SD.

5.2.8 Plasma glucose level determination

Glycosylated hemoglobin (HbA1c) level is a reliable retrospective glycemic index. Mice HbA1c plasma level were determined using a mouse HbA1c solid-phase ELISA kit (My BioSource, San Diego, California, USA) and following the manufacturer's instructions. Data are expressed as ng/mL ±SD.

5.2.9 Hormones determination

The plasma levels of ghrelin, leptin, GIP, GLP-1 were determined by ELISA kits from Merk-Millipore (Milan, Italy) respectively, following the manufacturer's instructions. Resistin was measured using the RayBio® Mouse Resistin ELISA Kit (Ray biotech Inc.). Data are expressed as pg/mL ±SD.

5.2.10 Western blotting

Amyloid oligomers and glucose transporters were analyzed through western blot. In detail, 30 μg total protein of brain homogenates were loaded on 10–12% sodium dodecyl-sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred onto PVDF membranes. After incubation with the specific antibodies, the immunoblot detection was carried out with an ECL western blotting ChemiDocTM System (Biorad, Milan, Italy). Each gel contained molecular weight markers ranging from 6.5-205 kDa. GAPDH was utilized to guarantee equal protein loading and to normalize western blot data. ChemiDoc acquired images or scanned autoradiographs (16 bit) were processed through ImageJ software (NIH) to calculate the background mean value and its standard deviation. The background intensity mean was then subtracted from the raw digital data to obtain a background-free image. The integrated densitometric value for each band was determined as the sum of the density values for all pixels belonging to the analyzed band with a density value larger than the background standard deviation. The ratios of band intensities were calculated within the same western blot.

5.2.11 ELISA assay for Aβ levels determination.

Brain homogenates (supernatant fraction) promptly supplemented with protease inhibitors (Pefabloc and TPCK) were used to measure $A\beta_{1-40}$ and $A\beta_{1-42}$ levels using enzyme-linked immunosorbent assay NOVEX® ELISA kits (Invitrogen). Based on preliminary tests, samples were diluted at 1:5 with diluent buffer provided by the kit. Assays were performed according to the manufacturer's directions.

5.2.12 Statistical analysis

Results of behavioral tests and biochemical data were presented as mean values ± SE. Statistical analysis was performed with one-way ANOVA, followed by the Bonferroni test using Sigma-stat 3.1 software (SPSS, Chicago, IL, USA). P-Values $p < 0.05$ were considered to be significant.

5.3 Results

5.3.1 Administration of probiotics and functional cookie improved short-term memory of AD mice

A characteristic feature of AD is the deterioration of short-term memory function. To assess this aspect, we employed the NOR test to evaluate the ability of treatments to improve short-term memory in AD mice. Remarkably, the administration of both probiotics and probiotic-enriched functional cookies showed a significant enhancement in the preference index of mice compared to the water group (Fig. 1), indicating a SLAB51® dependent effect.

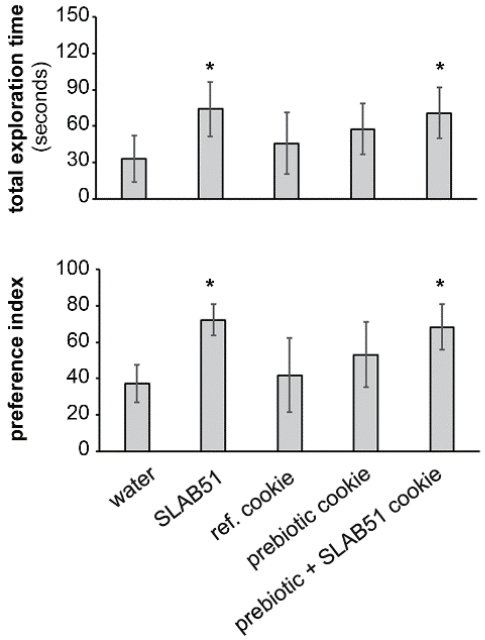


Figure 1. *NOR test. Total exploration time: The total time the mice spent exploring two different objects. Preference index: (seconds spent with the novel object - seconds spent with the familiar object) / (seconds spent with both objects). Data points marked with an asterisk are statistically significant compared to water group mice (*p < 0.05).*

5.3.2 Probiotics and functional cookies ameliorated blood lipid profile of AD mice

The effect of functional cookies on lipid profile was assessed by measuring plasma levels of cholesterol, HDL-C, LDL-C, and triglycerides. Supplementation with SLAB51 alone and with the SLAB51 enriched prebiotic cookie significantly reduced total cholesterol concentration and increased HDL-C concentration compared to both water group and the group receiving the reference cookie. SLAB51 treatment also decreased LDL-C compared to both water and reference cookie treatment, functional cookie treatment decreased LDL-C compared to reference cookie treatments. Prebiotic cookie also reduced total cholesterol, LDL-C and increased HDL-C compared to the reference cookie group, and the increase in

HDL-C was significant compared to the water group. Triglycerides were not affected by treatments.

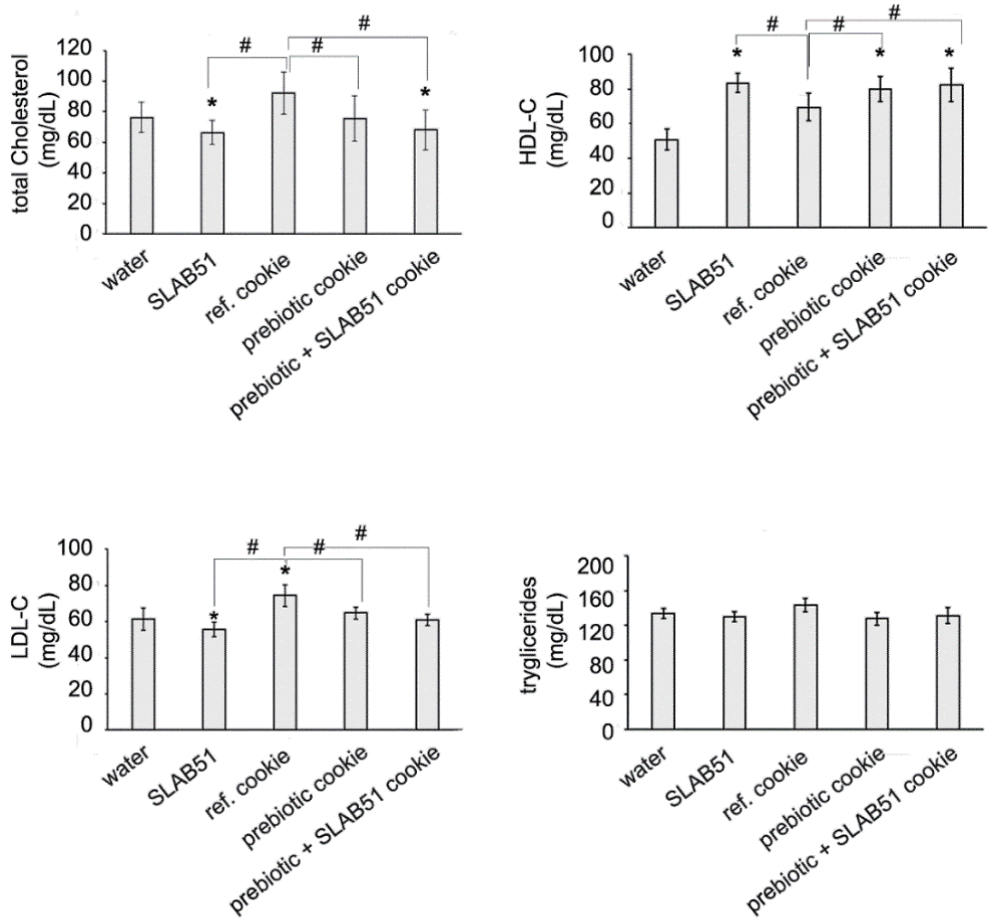


Figure 2. *Blood lipid profile. Plasma concentrations (mg/dL) of total cholesterol, HDL-C, LDL-C, and triglycerides in 3xTg-AD mice after 4-month supplementation with water, SLAB51, reference cookie, prebiotic cookie, SLAB51 enriched prebiotic cookie. Data points marked with an asterisk are statistically significant compared to water group mice (*p < 0.05). Data points marked with a hashtag are significantly different compared to mice treated with the reference cookie (#p < 0.05).*

5.3.3 Administration of probiotics and functional cookies improved glucose metabolism

Hyperglycemic states have been associated with poor cognitive function (such as learning and memory), increased risk of dementia and alterations in key brain structures, such as the hippocampus [387]. Being HbA1c an indicator of long-term glycemic control, the plasma concentration of this parameter was measured upon 4-month treatments. As expected, this indicator was significantly reduced in the SLAB51 group compared with the water group, while plasma HbA1c concentrations were significantly increased in AD mice supplemented with all cookies (Ref, Prebiotic, and Prebiotic+SLAB51 groups) compared to

water group (Fig. 3). Compared to reference cookie group, both prebiotic and Prebiotic+SLAB51 groups showed significantly lower concentrations of HbA1c (Fig. 3). Interestingly, the functional cookie treatment significantly reduced HbA1c levels compared to prebiotic cookie treatment (Fig. 3), suggesting an enhanced positive effect on glucose metabolism upon the association of prebiotics and probiotics (synergistic effect).

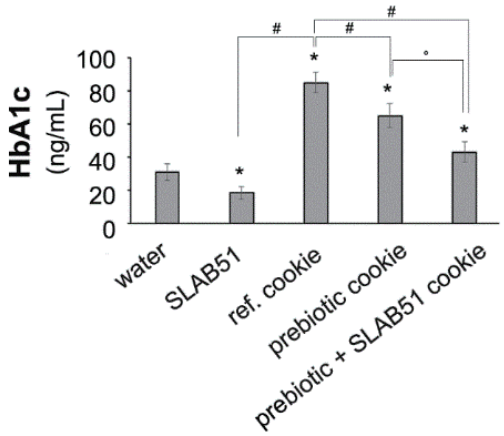


Figure 3. *Glycated hemoglobin (HbA1c) plasma concentrations. HbA1c plasma concentration of 3xTg-AD mice are expressed as ng/mL. Data points marked with an asterisk are statistically significant compared to water group mice (*p < 0.05). Data points marked with a hashtag are significantly different compared to mice treated with the reference cookie (#p < 0.05). ° indicate a significative difference (p<0.05) between prebiotic cookie and prebiotic-SLAB51 group.*

SLAB51 based treatment positively affected GLUTs expression (Fig. 4). As shown in Fig. 4. supplementation with the prebiotic and probiotic functional cookie increased the expression of cerebral glucose transporters GLUT1 and GLUT3, the major proteins responsible for brain glucose delivery and utilization. Interestingly, a synergistic effect was observed combining SLAB51 and the prebiotic component. This is an interesting result, considering that GLUT3 has a higher affinity for glucose than GLUT1, -2, or -4 and it has at least a fivefold greater transport capacity than GLUT1 and -4 [388]. No significant variation in GLUT4 expression was observed upon treatments.

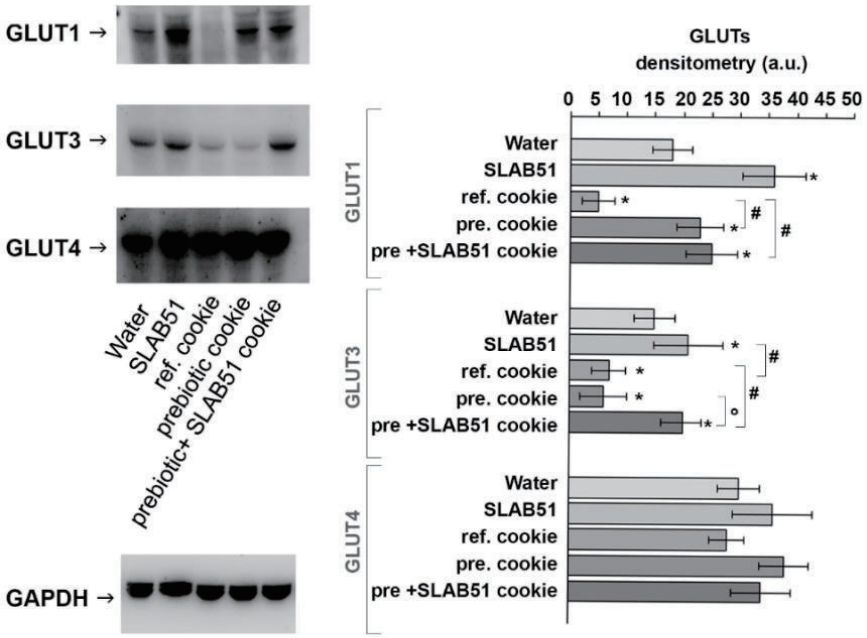


*Figure 4. Glucose transporters brain expression in 3xTg-AD mice supplemented with the functional ccokies.. Data points marked with an asterisk are statistically significant compared to water group mice (*p < 0.05). Data points marked with a hashtag are significantly different compared to mice treated with the reference cookie (#p < 0.05). ° indicate a significative difference (p<0.05) between prebiotic cookie and prebiotic-SLAB51 group.*

5.3.4 Probiotics and functional cookie restored plasma levels of neuroprotective hormones

The plasma concentration of the gut peptide hormones ghrelin, leptin, GLP-1 and GIP were measured due to their neuroprotective effects and potential as therapeutic targets. No changes in hormone plasma levels were observed in mice supplemented with the reference cookie compared to the water group, except for GIP, which significantly decreased (Fig. 5). As expected, compared to water group mice, treatment with SLAB51 significantly increased the plasma concentration of ghrelin, leptin and GIP hormones, and functional cookie treatment significantly increased concentration of ghrelin, GIP and GLP-1, confirming the positive effect of probiotics and functional cookie on neuroprotective hormones (Fig. 5). The functional cookie significantly increased all four gut neuroprotective hormones compared to the reference cookie treatment (Fig. 5). The group receiving the functional cookie had considerably higher ghrelin concentration compared to the group supplemented with prebiotic cookie, and higher levels of GIP compared to both prebiotic and SLAB51 treatment (Fig. 5), suggesting a synergistic effect of prebiotics and probiotics. Notably, GLP-1 concentrations were significantly increased only after supplementation with functional cookie compared to reference cookie treatment (Fig. 5). These findings indicated that SLAB51 and functional cookie are able to restore dysregulation of gut hormones, with the

functional cookie being more effective.

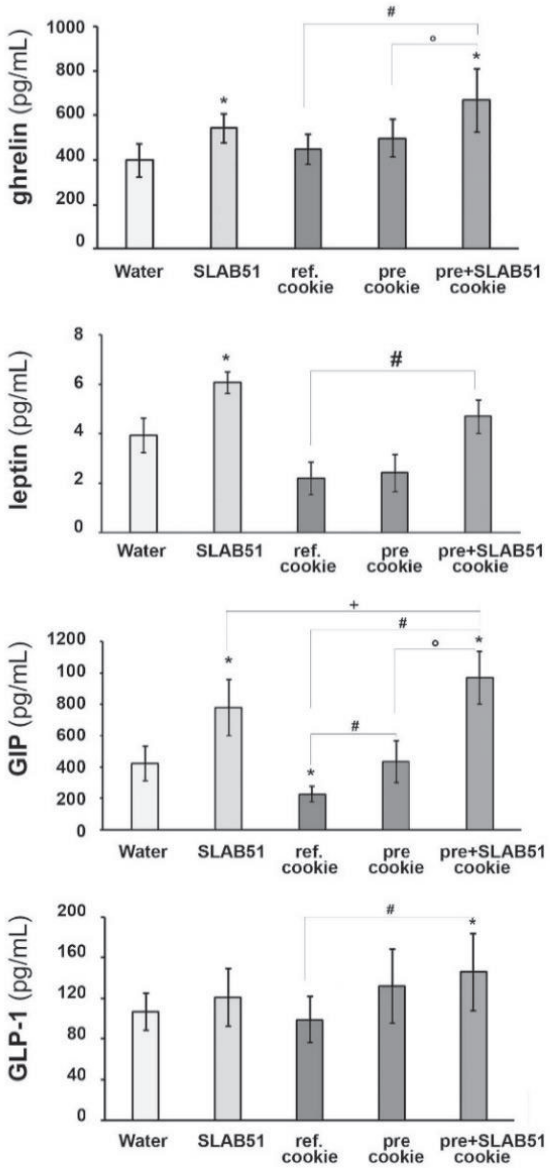


Figure 5. *Plasma concentrations of gut hormones. Ghrelin, Leptin, GIP and GLP-1 were determined in the plasma of 3xTgAD mice supplemented with different treatment. After one way ANOVA, followed by the Bonferroni test, data points marked with an asterisk are statistically significant compared to water group (*p<0.05). Data points marked with hashtag are statistically significant compared to the group supplemented with the reference cookie (# p<0.05). pre+SLAB51 cookie vs prebiotic cookie is indicated with ° (p<0.05); prebiotic+SLAB51 vs to SLAB51 is indicated with +(p<0.05).*

5.3.5 Increased resistin concentrations upon treatments

Resistin is a small secreted protein playing a pivotal role in various metabolic, inflammatory, and autoimmune diseases, and it is mainly secreted by adipose tissue macrophages. Reduced resistin concentrations have been observed in AD patients, with further impairment of glucose metabolism. Interestingly, resistin concentrations were significantly increased upon SLAB51 supplementation compared to the water group. Also, consumption of prebiotic cookie or functional cookie enhanced resistin levels in the plasma of AD mice compared to both water group and reference cookie group.

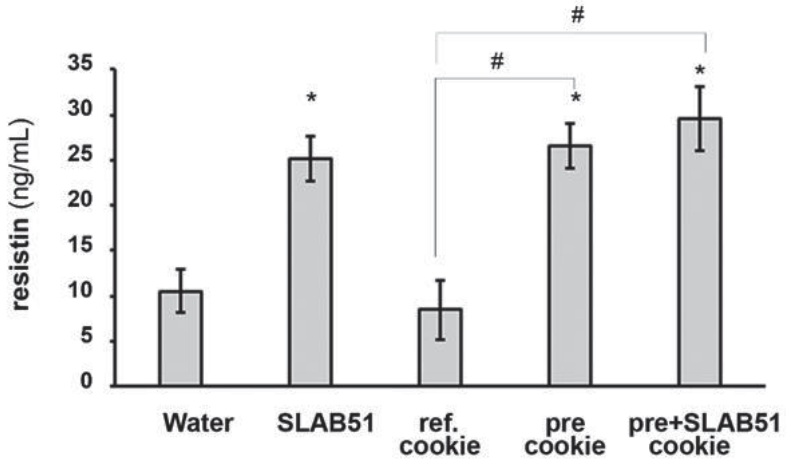


Figure 6. *Resistin plasma levels. Resistin concentration was determined in the plasma of 3xTgAD mice supplemented with water or with Slab51 in water or with control/normal cookies (ref. cookie), or with prebiotic-enriched cookies (pre cookie) or with prebiotic and Slab51®-enriched functional cookies (pre + SLAB51 cookie). After one way ANOVA, followed by the Bonferroni test, data points marked with an asterisk are statistically significant compared to untreated (water) group (*p<0.05). Data points marked with hash are statistically significant compared to the group supplemented with the reference cookie (# p<0.05). pre+SLAB51 cookie vs pre cookie is indicated with ° (p<0.05); pre+SLAB51 vs to SLAB51 is indicated with + (p<0.05).*

5.3.6 Probiotics and functional cookie decreased amyloid load in AD mice brain

To assess whether the functional cookie treatment affected brain Aβ load, the levels of $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ peptides were measured. No significant changes on $A\beta_{1\text{–}40}$ level was observed (Fig. 7, panel A). Interestingly, $A\beta_{1\text{-}42}$ load was significantly reduced in both treated with SLAB51 and functional cookie compared to water group and reference group, while prebiotic cookie had no significant effect (Fig. 7, panel A). Furthermore, the therapeutic effect of functional cookie was also significant compared to the prebiotic treatment (Fig. 7, panel A), suggesting that probiotics play a key role or synergistic enhancement in functional cookie.

The accumulation of amyloid oligomers was estimated by western blotting and found that these toxic structures were significantly reduced only in SLAB51-treated AD mice compared to the water group (Fig.

7, panel B); a decreasing trend is observed in functional cookie treatment, but not significant. However, both prebiotic and functional cookie treatment significantly reduced amyloid oligomers compared to the reference cookie treatment.

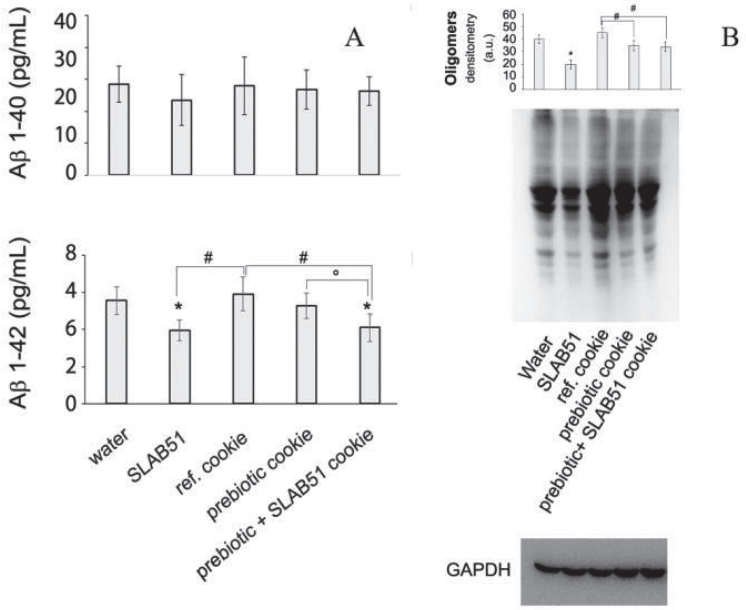


Figure 7. *Aβ load. Panel A: $Aβ_{1-40}$ and $Aβ_{1-42}$ levels expressed as pg/ml determined by ELISA in the brains of AD mice with different treatment. Panel B: Expression levels of amyloid oligomers detected by western blot. The densitometry from three separate blots and a representative immunoblot are reported. Equal protein loading was verified by using an anti-GAPDH antibody. The detection was executed by ECL. Data points marked with an asterisk are statistically significant compared to water group mice (*p < 0.05). Data points marked with a hashtag are significantly different compared to mice treated with the reference cookie (#p < 0.05). ° indicate a significative difference (p<0.05) between prebiotic cookie and prebiotic-SLAB51 cookie treatment.*

5.4 Discussion

For the first time in history, the elderly (with age > 65 years) represents the fastest growing segment of the global population, with an increasing number of individuals over age 65 [389]. Consequently, managing the substantial increase in the prevalence of patients with dementia and reducing disease burden are among the main global priorities. Gut microbiota dysbiosis and dysmetabolism contribute to AD pathogenesis with not completely defined mechanisms. In this context, dietary interventions, including prebiotics and/or probiotics, have become a mainstay of microbiota–gut–brain axis research as potentially interesting preventative and therapeutic approaches in neurodegenerations. The idea behind the developed functional snack is to obtain food for supporting the cognitive function which would help to prevent the NDDs in the general population, thus contributing to decrease the burden on the national/international health care system. Based on previous studies on the neuroprotective properties of SLAB51® multi-strain formulation and with the aim to improve the survival of the probiotics in the gastrointestinal tract and to obtain a synergistic beneficial effect on the host energy metabolism and clinical outcome in the preclinical model of AD, a prebiotic-based cookie enriched with SLAB51® has been designed and developed and a chronic (4-month) supplementation of 3xTg-AD has been performed.

As expected SLAB51® alone or in the functional cookie improved both glucose and lipid profiles, with restored plasma concentration of neuroprotective gut hormones in 3xTg-AD mice, ultimately reducing Aβ load and improving animals short term memory. These data confirm previously published data and support the hypothesis of a healthy innovative snack as a simple and affordable dietary approach to prevent the onset of age-related neurodegenerations.

Behavioral NOR testing highlighted the positive effects of SLAB51 and probiotic-enriched functional cookie on short-term memory in AD mice, but no improvement was observed with prebiotic cookie treatment, suggesting the importance of the probiotic ingredient in improving short-term memory in AD mice, consistent with published evidence supporting the notion that cognitive function is affected by bacteria acting through the gut-brain axis [390].

High levels of total cholesterol and LDL-C and low levels of HDL-C are associated with vascular dementia and, indirectly, with AD. The prebiotic cookie (in the absence of SLAB51) significantly increased HDL-C and decreased LDL-C in the plasma of 3xTg-AD mice compare to water group and to mice supplemented with the reference cookie, respectively, indicating that lentil-based ingredients have a role in ameliorating lipid profile. Large prospective epidemiological studies reported that the consumption of phenolic-rich lentils was inversely associated with the incidence of obesity and diabetes [391]. Rats following a lentil-based diet showed increased HDL-C and decreased triglycerides. In addition, SLAB51 and the functional cookie reduced the total cholesterol level showing the same effects as the prebiotic cookie on the HDL-C and LDL-C, indicating that the supplementation of prebiotics enhances the potential of probiotics in ameliorating lipid profile, suggesting a synergistic effect most likely due to the positive effect of the prebiotic component on the bacteria vitality. Consistent with these

results, a study showed a synergistic effect of jackfruit seed sourced resistant starch (JSRS) and *Bifidobacterium pseudolongum* subsp. *globosum* on ameliorated hyperlipidemia in mice, JSRS as a prebiotic showed limited preventive effects on body weight and blood lipid profiles, but the synergistic effect of JSRS and probiotic *B.pseudolongum* can improve hyperlipidemia [392]. Interestingly, *in vitro* functional cell biology studies support cellular and membrane-bound cholesterol modulating β-secretase and γ-secretase activity and promoting Aβ formation [393]. Hypercholesterolemia increases 27-OHCE, which can penetrate the BBB, and boost beta-site amyloid precursor protein cleaving enzyme 1, a key Aβ synthesis step [364]. In line with our observed SLAB51 and functional cookie reduced total cholesterol levels, these interventions correspondingly correlate with a reduction in $A\beta_{1-42}$ formation. Similarly, many epidemiological studies have shown that circulating HDL-C levels are associated with decreased AD risk [394], and an inverse correlation between plasma HDL-C and brain amyloid burden measured by positron emission tomography (PET) [395]. Consistent with our findings, probiotics and functional cookie also simultaneously showed enhanced HDL-C and reduced Aβ load. In addition, a meta-analysis study showed that LDL-C levels are closely related to AD and mild cognitive impairment [396]. Combining Aβ load and cognitive performance results, the improvement of AD pathological features by probiotics and functional cookie treatment can be partially explained by improving lipid profile, consist with previous conclusion that probiotics can ameliorate lipid profiles and improve AD.

Improvement of cognitive function is also supported by increased plasma concentration of gut hormones such as ghrelin, leptin, GLP-1 and GIP. Previous studies have shown that AD patients have a time-dependent reduction in plasma peptide hormones that play a role in regulating neural functions such as learning and memory. Ghrelin has been shown to counteract memory deficits and synaptic degeneration in animal models of AD [397], while leptin has been shown to act as a neurotrophic factor and exert neuroprotective effects against Aβ oligomer-induced toxicity *in vitro* [398]. GIP and GLP-1 are peptide hormones of the incretin family with growth factor properties that reactivate energy utilization. In progressive NDDs, energy utilization is greatly reduced, and GIP and GLP-1 have the potential to reverse this, showing promising neuroprotective effects [399]. Interestingly, oral administration of SLAB51 and functional cookie showed higher plasma levels of such hormones, and the combination of these prebiotics with SLAB51 potentiated the ability of SLAB51 probiotics to restore GIP and GLP-1 levels. In general, prebiotics promote the production of SCFA by probiotics [400], and SCFA (mainly butyrate, acetate and propionate) can increase GLP-1 and GIP in plasma [401]. Consistent with the results of blood lipid profile, since such gut hormones have ability in affecting energy metabolism and neuroprotection, it is suggested that probiotics and functional cookies may improve gut microbiota and restore the secretion of such hormones to improve lipid metabolism and cognitive behavior in AD mice.

Furthermore, these data correlate with the decreased Aβ load in the brains of SLAB51 and functional cookie treated AD mice. Both in vivo and *in vitro* studies have demonstrated that GLP-1 can reduce Aβ load [402], while leptin or ghrelin can ameliorate amyloid and tau pathologies [403]. In our study, $A\beta_{1-42}$ level was significantly reduced in both treated with SLAB51 and functional cookie compared to water

group and reference group. Consequently, a decreased expression of oligomers was observed upon SLAB51 treatment, while the functional cookie showed only a reduction compared to prebiotics and reference cookie. This phenomenon may be attributed to the disruptions in glucose metabolism, as elevated blood glucose levels have been shown to promote amyloid aggregation and accelerate disease progression in transgenic animal models of AD [404]. Our observations revealed that probiotic treatment mitigated the glycemic index HbA1c, whereas cookie treatment, including reference cookie, prebiotic cookie, and functional cookie, all exhibited a propensity to elevate blood HbA1c levels, this may be due to differences between cookie ingredients (including chocolate and sugar) and normal laboratory diet. Flavonoids in lentils have the potential to inhibit the action of α-glucosidase and lipase, suggesting that consuming lentils may control post-meal blood glucose and body weight [405]. Consistent with our observations, the prebiotic lentils cookie reduced blood glucose levels. Studies have shown that the combination of prebiotic (oligofructose) and probiotic (*Bifidobacterium animalis*) shows a synergistic effect in lowering blood glycemia [406]. As expected, our results also demonstrated that cookie containing prebiotics and probiotics showed a synergistic enhancing effect, significantly reducing blood glucose levels compared to reference cookie and prebiotic cookie. This superiority of functional cookie may be associated with enhanced expression of GLUTs. Evidence suggests that chronic hyperglycemia reduces glucose absorption and utilization in the brain by downregulating glucose transporters (GLUTs) expression, ultimately leading to impaired energy supply and impaired neuronal activity, contributing to cognitive decrease [367]. Our results showed that probiotics and the functional cookie treatment increased the expression of GLUT1 and GLUT3. It is noteworthy that functional cookies significantly increased GLUT3 compared to reference and prebiotic cookie, which has a higher affinity for glucose than GLUT1, 2, 4 and has at least fivefold greater transport capacity than GLUT1 and -4 [388], help improving glucose utilization and cognitive ability. In parallel, SLAB51 and functional cookie supplementation enhanced resistin level. In fact, this small secreted protein plays a pivotal role in glucose metabolism [407], and reduced level have been observed in AD patients [408]. It further proved that the improvement of pathological features of AD mice by probiotics is related to the improvement of glucose metabolism.

Further experiments are needed to establish the ability of the innovative cookie to delay dysbiosis derived cognitive disorders associated to neuroinflammatory markers. This tailored functional food possesses improved properties with respect to commercially available healthy cookies, contributing to maintain an innovative, sustainable and globally competitive health-related industry.

6. FINAL CONCLUSION

NDDs are a class of progressive neuronal degenerative diseases that exhibit similar pathological characteristics, including metabolic disorders, sleep dysfunction, and gut dysbiosis, strictly interconnected. Increasing evidence suggests that gut microbiota is associated with these pathological characteristics through the neural, immune, and metabolic pathways of the gut-brain axis. Therapeutic approaches targeting the gut-brain axis offer promising preventive and therapeutic opportunities for NDDs.

Consistently, abnormal lipid metabolism was observed in a 3xTg-AD mouse model, with SLAB51 probiotic mixture reducing cholesterol biosynthesis and improving plasma fatty acid composition, qualitative ameliorating plasma lipid profile.

Furthermore, sleep disturbance is a common feature in NDDs and is associated with metabolic disorders [409]. Chronic probiotics treatment improved inflammation and oxidative stress caused by chronic sleep restriction (CSR) at the brain and systemic levels, and restored the CSR-induced imbalance of key hormones such as ghrelin, leptin and GLP-1 within the gut-brain axis. These gut hormones are known to play important roles in lipid and glucose metabolism, as well as possess neuroprotective effects. In addition, studies have shown that dietary polyphenols and their gut microbial metabolites exert significant antioxidant and anti-inflammatory effects, and contribute to the metabolic and neuronal health. The investigation conducted on AD cellular model treated with microbial polyphenol metabolites phenyl-γ-valerolactones (PVLs) showed that three tested PVLs inhibited UPS and activated autophagy, consequently diminishing the amount of $A\beta_{1-42}$ in cell lysates and growth medium. This pioneering research elucidates the neuroprotective potential of these microbial metabolites, associated with the modulation of intracellular proteolytic systems and suggests that dietary supplementation with polyphenols and their microbial metabolites have beneficial effects on NDDs. Accordingly, we designed a functional cookie based on polyphenol-rich prebiotic lentils and probiotics with proved neuroprotective properties. This functional cookie is more in line with the people centered nutrition, improving short-term memory, lipid and blood glucose profiles, decreasing Aβ load and enhancing the production of neuroprotective gut hormones in a mouse model of AD. A synergistic effect due to the combination of prebiotics and probiotics was obtained thanks to the positive effect of prebiotic components on bacteria vitality.

Collectively, our studies provide important contribution in the development of new therapeutic options for NDDs targeting the gut-brain axis and unraveled deeper molecular mechanisms underlying therapeutic approaches targeted to gut microbiota intervention, centered on the gut-brain axis to prevent and treat NDDs.

Future research should focus on developing targeted personalized approaches based on gut microbiota characteristics associated with different NDDs, exploring optimal combinations of prebiotics and

probiotics, and considering the impact of treatment duration and disease stage on intervention strategies.

7. LIST OF PUBLICATIONS

1. Bonfili, L., Cuccioloni, M., Gong, C., Cecarini, V., Spina, M., **Zheng, Y**., ... & Eleuteri, A. M. (2022). Gut microbiota modulation in Alzheimer's disease: Focus on lipid metabolism. Clinical Nutrition, 41(3), 698-708.

2. Cecarini, V., Cuccioloni, M., **Zheng, Y**., Bonfili, L., Gong, C., Angeletti, M., ... & Eleuteri, A. M. (2021). Flavan-3-ol Microbial Metabolites Modulate Proteolysis in Neuronal Cells Reducing Amyloid-beta (1-42) Levels. Molecular Nutrition & Food Research, 65(18), 2100380.

3. **Zheng, Y**., Zhang, L., Bonfili, L., de Vivo, L., Eleuteri, A. M., & Bellesi, M. (2023). Probiotics Supplementation Attenuates Inflammation and Oxidative Stress Induced by Chronic Sleep Restriction. Nutrients, 15(6), 1518.

4. Gong, C., Bonfili, L., **Zheng, Y**., Cecarini, V., Cuccioloni, M., Angeletti, M., ... & Eleuteri, A. M. (2023). Immortalized Alzheimer's Disease Astrocytes: Characterization of Their Proteolytic Systems. Molecular Neurobiology, 60(5), 2787-2800.

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Original article

Gut microbiota modulation in Alzheimer's disease: Focus on lipid metabolism

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SUMMARY

Background & aims: Alzheimer's disease (AD) and age-related dementias represent a major and increasing global health challenge. Unhealthy diet and lifestyle can unbalance the intestinal microbiota composition and, consequently energy metabolism, contributing to AD pathogenesis. Impairment of cerebral cholesterol metabolism occurs in both aging and AD, and lipid-lowering agents have been associated to a lower risk of neurodegenerative diseases, but the link between blood lipid profile and AD remains a matter of debate. Recently, probiotics have emerged as a promising and safe strategy to manipulate gut microbiota composition and increase the host health status through a multi-level mechanism that is currently under investigation. Specifically, oral supplementation with a multi-strain probiotic formulation (SLAB51) reduced amyloid beta aggregates and brain damages in a triple transgenic mouse model of AD (3xTg-AD). Treated mice showed improved cognitive functions in response to an enrichment of gut anti-inflammatory metabolites, increased plasma concentrations of neuroprotective gut hormones, and ameliorated glucose uptake and metabolism.
Methods: This work focuses on the evaluation of the effects of SLAB51 chronic administration on lipid metabolism in 3xTg-AD mice and the respective wild-type counterpart. On this purpose, 8 weeks old mice were orally administered with SLAB51 for 4 and 12 months to analyze the plasma lipid profile (using lipidomic analyses and enzymatic colorimetric assays), along with the cerebral and hepatic expression levels of key regulators of cholesterol metabolism (through Western blotting and ELISA).
Results: Upon probiotics administration, cholesterol biosynthesis was inhibited in AD mice with a process involving sterol regulatory element binding protein 1c and liver X receptors mediated pathways. Decreased plasma and brain concentration of 27-hydroxycholesterol and increased brain expression of cholesterol 24S-hydroxylase indicated that alternative pathways of bile acid synthesis are influenced. The plasmatic increase of arachidonic acid in treated AD mice reflects dynamic interactions among several actors of a complex inflammatory response, in which polyunsaturated fatty acids can compete each other and simultaneously co-operate in the resolution of inflammation.
Conclusions: These evidence, together with the hypocholesterolemic effects, the ameliorated fatty acids profile and the decreased omega 6/omega 3 ratio successfully demonstrated that microbiota modulation through probiotics can positively change lipid composition in AD mice, with arachidonic acid representing one important hub metabolite in the interactions among probiotic-induced lipid profile changes, insulin sensitivity, and inflammation.

1. Background

Alzheimer's disease (AD) is a debilitating neurodegenerative disorder that afflicts millions of adults worldwide and represents the main cause of late-life dementia. AD is characterized by neuronal cell loss associated with memory and cognitive decline,

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RESEARCH ARTICLE

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Flavan-3-ol Microbial Metabolites Modulate Proteolysis in Neuronal Cells Reducing Amyloid-beta (1-42) Levels

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Introduction: Alzheimer's disease (AD) is a progressive neurodegeneration characterized by extensive protein aggregation and deposition in the brain, associated with defective proteasomal and autophagic-lysosomal proteolytic pathways. Since current drugs can only reduce specific symptoms, the identification of novel treatments is a major concern in AD research. Among natural compounds, (poly)phenols and their derivatives/metabolites are emerging as candidates in AD prevention due to their multiple beneficial effects. This study aims to investigate the ability of a selection of phenyl-γ-valerolactones, gut microbiota-derived metabolites of flavan-3-ols, to modulate the functionality of cellular proteolytic pathways.
Methods and Results: Neuronal SH-SY5Y cells transfected with either the wild-type or the 717 valine-to-glycine amyloid precursor protein mutated gene are used as an AD model and treated with 5-(4′-hydroxyphenyl)-γ-valerolactone, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone and 5-(3′-hydroxyphenyl)-γ-valerolactone-4′-sulfate. Combining in vitro and in silico studies, it is observed that the phenyl-γ-valerolactones of interest modulated cellular proteolysis via proteasome inhibition and consequent autophagy upregulation and inhibited cathepsin B activity, eventually reducing the amount of intra- and extracellular amyloid-beta (1-42) peptides.
Conclusion: The findings of this study establish, for the first time, that these metabolites exert a neuroprotective activity by regulating intracellular proteolysis and confirm the role of autophagy and cathepsin B as possible targets of AD preventive/therapeutic strategies.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative condition mostly characterized by extensive protein aggregation with amyloid-beta deposition in senile plaques and tau aggregation in neurofibrillary tangles. The sequential cleavage of the amyloid precursor protein (APP) through the so-called amyloidogenic pathway is responsible for the release of amyloid-beta (Aβ) peptides. A large part of these proteins are fragments of 40 amino acid residues in length, Aβ(1-40), whereas a remaining portion is a 42-residues protein, Aβ(1-42), known as the most toxic and particularly prone to aggregation.[1] Defective proteolytic pathways, oxidative and inflammatory processes further exacerbate AD pathology.[2,3] The ubiquitin proteasome system (UPS) and the autophagy-lysosomal pathway are the two major catabolic pathways in eukaryotic cells. The UPS is in charge of the degradation of cytosolic and nuclear proteins, including short-lived proteins, whereas autophagy is responsible for the clearance of protein aggregates and damaged organelles. Due to an age-dependent decline in their activity, aberrant proteins accumulate, contributing to the onset and development of age-related disorders, such as AD.[4]

A growing number of studies has associated the intake of foods rich in (poly)phenols with a reduced risk of developing neurodegenerative diseases, including AD. Dietary products such as green tea, apples, berries, cocoa, and chocolate are extremely rich in

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Article

Probiotics Supplementation Attenuates Inflammation and Oxidative Stress Induced by Chronic Sleep Restriction

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Abstract: Background: Insufficient sleep is a serious public health problem in modern society. It leads to increased risk of chronic diseases, and it has been frequently associated with cellular oxidative damage and widespread low-grade inflammation. Probiotics have been attracting increasing interest recently for their antioxidant and anti-inflammatory properties. Here, we tested the ability of probiotics to contrast oxidative stress and inflammation induced by sleep loss. **Methods:** We administered a multi-strain probiotic formulation (SLAB51) or water to normal sleeping mice and to mice exposed to 7 days of chronic sleep restriction (CSR). We quantified protein, lipid, and DNA oxidation as well as levels of gut–brain axis hormones and pro and anti-inflammatory cytokines in the brain and plasma. Furthermore, we carried out an evaluation of microglia morphology and density in the mouse cerebral cortex. **Results:** We found that CSR induced oxidative stress and inflammation and altered gut–brain axis hormones. SLAB51 oral administration boosted the antioxidant capacity of the brain, thus limiting the oxidative damage provoked by loss of sleep. Moreover, it positively regulated gut–brain axis hormones and reduced peripheral and brain inflammation induced by CSR. **Conclusions:** Probiotic supplementation can be a possible strategy to counteract oxidative stress and inflammation promoted by sleep loss.

Keywords: probiotics; sleep deprivation; inflammation; oxidative stress; microglia

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1. Introduction

Sleep is a fundamental behavior that fills approximately one-third of a human's lifetime and is critical for both physical and mental well-being [1]. Chronic sleep restriction (CSR), defined as insufficient/inadequate sleep over a prolonged period of time, is prevalent in contemporary society owing to professional obligations and lifestyle habits [2,3]. Epidemiological investigations have estimated that about 30% of adults and adolescents regularly experience insufficient sleep [4]. CSR can lead to a range of brain deficits, including impaired attention and learning, and is associated with increased risk of neuropsychiatric disorders, but also cardiovascular diseases and metabolic alterations [4–7]. Growing evidence has demonstrated that CSR is linked to a low-grade inflammation, as reflected by increased inflammatory plasma cytokines and by the presence of other markers of inflammation in the brain, such as activation of microglia cells [8–10]. In addition, insufficient sleep can lead to the accumulation of intracellular reactive oxygen species (ROS) and/or reactive nitrogen species (RNS), resulting in an unbalance between the oxidant and antioxidant systems of the body [10,11]. Excessive ROS and RNS can react with carbohydrates, proteins, lipids, and DNA, and therefore, causes oxidative stress-related cellular damage

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Immortalized Alzheimer's Disease Astrocytes: Characterization of Their Proteolytic Systems

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Abstract
Alzheimer's disease (AD) is a progressive neurodegeneration with dysfunctions in both the ubiquitin–proteasome system (UPS) and autophagy. Astroglia participation in AD is an attractive topic of research, but molecular patterns are partially defined and available in vitro models have technical limitations. Immortalized astrocytes from the hippocampus of 3xTg-AD and wild-type mice (3Tg-iAstro and WT-iAstro, respectively) have been obtained as an attempt to overcome primary cell line limitations and this study aims at characterizing their proteolytic systems, focusing on UPS and autophagy. Both 26S and 20S proteasomal activities were downregulated in 3Tg-iAstro, in which a shift in catalytic subunits from constitutive 20S proteasome to immunoproteasome occurred, with consequences on immune functions. In fact, immunoproteasome is the specific complex in charge of clearing damaged proteins under inflammatory conditions. Parallelly, augmented expression and activity of the lysosomal cathepsin B, enhanced levels of lysosomal-associated membrane protein 1, beclin1, and LC3-II, together with an increased uptake of monodansylcadaverine in autophagic vacuoles, suggested autophagy activation in 3Tg-iAstro. The two proteolytic pathways were linked by p62 that accumulated in 3Tg-iAstro due to both increased synthesis and decreased degradation in the UPS defective astrocytes. Treatment with 4-phenylbutyric acid, a neuroprotective small chemical chaperone, partially restored proteasome and autophagy-mediated proteolysis in 3Tg-iAstro. Our data shed light on the impaired proteostasis in 3Tg-iAstro with proteasome inhibition and autophagic compensatory activation, providing additional validation of this AD in vitro model, and propose a new mechanism of action of 4-phenylbutyric acid in neurodegenerative disorders.

Keywords Alzheimer's disease · Astrocytes · Ubiquitin–proteasome system · Autophagy · 4-Phenylbutyric acid

Introduction

Alzheimer disease (AD) is the most common type of dementia that has become a rapidly increasing public health concern. The biological construct that helps defining AD comprises the deposition of amyloid-β (Aβ) plaques, pathological tau phosphorylation, and neurodegeneration [1, 2]. A growing body of evidence identified that oxidative stress, chronic inflammation, mitochondrial dysfunction, and endoplasmic reticulum (ER) stress have a role in AD development [3, 4].

Astrocytes, the most abundant glial cells in the central nervous system (CNS), are involved in numerous aspects of CNS physiology. Specifically, astrocytes act as scavengers for reactive oxygen species and supply cysteine precursor for neuronal glutathione [5]. Astrocytes are involved in the removal of toxins, production and release of trophic factors, regulation of neurotransmitters, and ion concentrations, thereby maintaining the overall cell homeostasis, including optimal synaptic glutamate levels and neuroimmune status [6, 7]. During AD progression, astrocytes undergo complex alterations becoming first asthenic and hypotrophic, while later they turn to be reactive and hypertrophic, mostly around developing senile plaques [7, 8]. During

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