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Effects of the probiotic formulation SLAB51 in *in vivo* and *in vitro* Parkinson's disease models

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INTRODUCTION

CHAPTER I

Overview of the thesis

Parkinson's disease (PD) is a progressive neurodegenerative disorder. The prevalence is 1.3%-1.5% for people above the age of 60 years in Europe (Hirsch et al., 2016). The number of individuals with PD will double by the year 2030.

Currently, there is no cure for PD, and the treatment is therefore symptomatic, and primarily involves dopaminergic medication (Bioinformatics Centre, University of Kashmir, Srinagar, J&K, India et al., 2018). Deep brain surgery is an alternative, but this is only available for a selected group of patients whose symptoms are dopamine responsive but experiencing debilitating response fluctuations (Malek, 2019). There is also a wide variety of non-pharmacological treatment options, including physical therapy, occupational therapy, and speech and language therapy (Bioinformatics Centre, University of Kashmir, Srinagar, J&K, India et al., 2018). The evidence to support these interventions is gradually growing, and treatment guidelines (partially based on evidence, partially on practical clinical experience) for some of these health care interventions have been developed. Integrating these different treatment options into a

bundled multidisciplinary approach (along with pharmacological and surgical treatment) is considered to represent an optimal therapeutic strategy for this complex, multifaceted disease.

Parkinson is characterized by motor and non-motor symptoms, including abnormalities in the gut function, which may appear before the motor sign. To date, there are treatments that can help relieve the PD-associated symptoms, but there is no cure to control the onset and progression of this disorder. Altered components of the gut could play a key role in gut-brain axis, which is a bidirectional system between the CNS and the enteric nervous system. Diet can alter the microbiota composition, affecting the gut-brain axis function. Gut microbiome restoration is able to counteract the PD progression and this effect could be exerted by probiotics (Bonfili et al., 2017; Jeong et al., 2015; Klingelhoefer and Reichmann, 2015).

In order to identify potential neuroprotective approaches able to counteract or to be useful as coadjuvant, in this study the potential therapeutic effects of SLAB51 formulation in PD, both *in vitro* and *in vivo* Parkinson's models, was investigated.

Our findings indicate that this probiotic formulation can counteract the detrimental effect of 6-OHDA *in vitro* and *in vivo* models of PD. The results suggest that SLAB51 can be a promising candidate for the prevention or as adjuvant treatment for PD.

CHAPTER II

2.1 Neurodegenerative Disorders

Neurodegenerative disorders trigger progressive loss of brain performances and overlapping clinical conditions. For example, cognitive decline appears not only in Alzheimer's disease (AD), but also in dementia with Lewy bodies (LBD), vascular and mixed dementia and Parkinson's disease (PD). Comparably, the motor impairment occurs in PD, amyotrophic lateral sclerosis (ALS) and Huntington's disease (HD). The common risk factor in all these disorders is aging. Since lifespan is longer, the incidence of these disorders is radically increased, with high social, economic impact (Gan et al., 2018). Neurodegenerative diseases show common mechanisms and features as shown in **figure 1**.

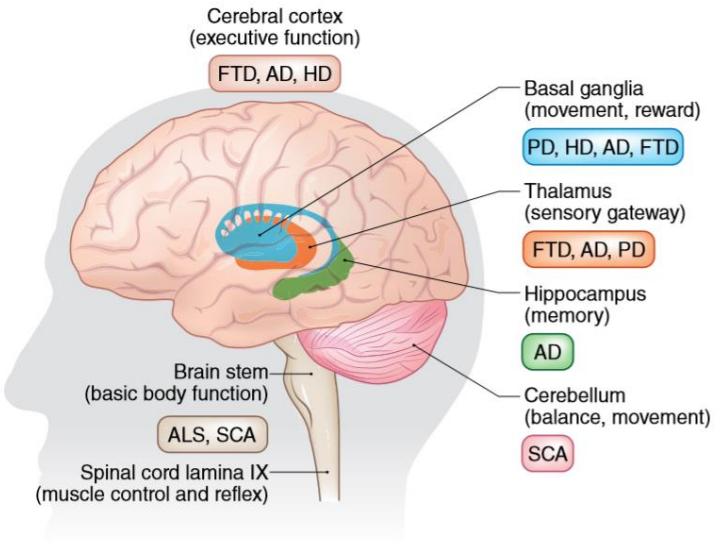

Fig1: *Primary brain regions affected in major neurodegenerative diseases. Clinical manifestations reveal distinct and overlapping neuronal circuits that progressively degenerate in various neurodegenerative disorders* (Gan et al., 2018).

Neurodegeneration is the term used to describe the progressive loss of structure and function of neurons, concomitant with neuronal cell death (Chi et al., 2018). Neurodegenerative disorders are considerate as a group of pathological conditions arising out from slow progressive and irreversible dysfunction of neurons and synapses in specific areas of the central and peripheral nervous systems, which determines the typical symptoms of these diseases. The underlying mechanisms of neurodegeneration are multifactorial; they include genetic, environmental

and endogenous factors related to aging, although the pathogenic role and basic molecular mechanisms are still unclear (Castelli et al., 2019a).

To date, neurodegenerative disorders are subdivided into “familial” and “sporadic”. The first group is related to genetic alterations, while the second group is related to environmental factors (i.e: aging, exposure to risk factors), either way the main feature is abnormal protein aggregation. These diseases are designated as ‘proteinopathies’ or ‘protein misfolding’ diseases (Jellinger, 2010). These multisystemic diseases are often characterized by transcriptional alterations, mitochondrial dysfunctions, alterations in energy metabolism, oxidative stress, neuroinflammation, proteasome dysfunction or impaired autophagy (Castelli et al., 2019a).

Main pathogenic mechanisms involved in Neurodegenerative diseases:

- Atypical protein dynamics with misfolding, defective degradation, proteasome dysfunction and aggregation, often with actions and mutations of molecular chaperones. The lack in proteasome function in neurodegenerative disorder results in diminished autophagy.
- Oxidative stress (OS) and of free radicals/reactive oxygen species (ROS) and -reactive nitrogen species (RNS) generation.
- Impaired bioenergetics, mitochondrial dysfunctions and DNA damage.

- Breaking of cellular/axonal transport.
- Dysfunction of neurotrophins (NTFs).
- Neuroinflammatory/neuroimmune processes.

These mechanisms are involved in a close network of relationships that underlie these diseases beginning and progression (**Fig.2**).

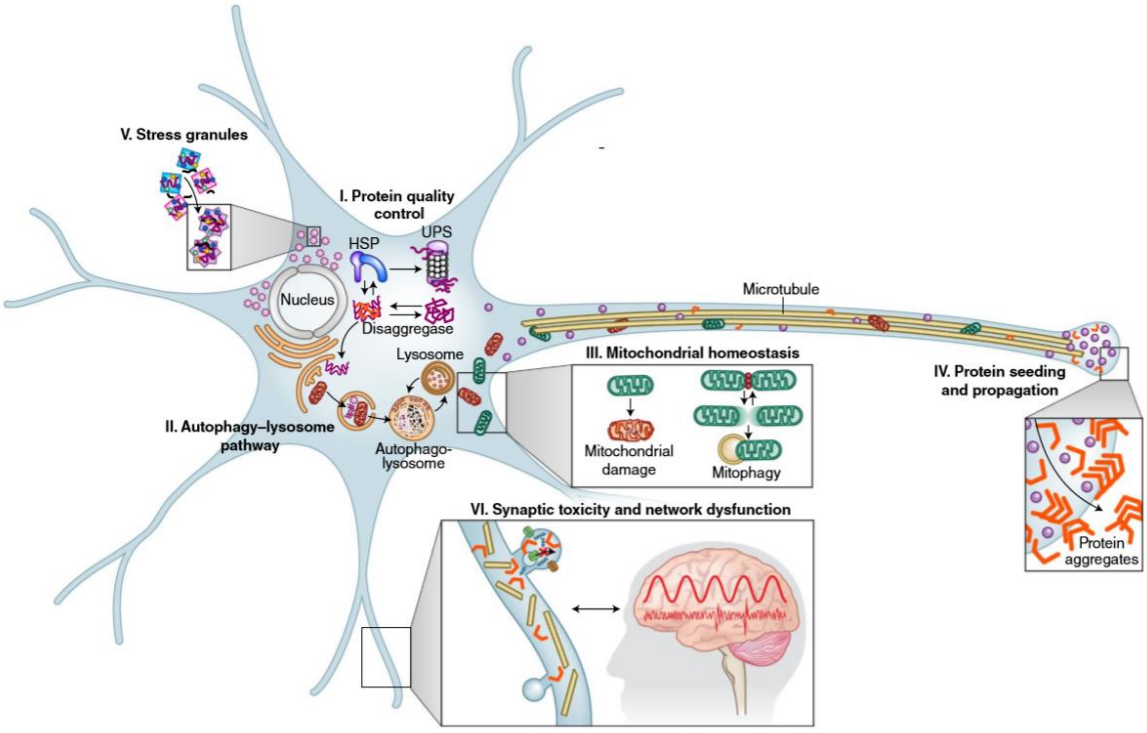

Fig.2: *Common neuronal pathways altered in multiple neurodegenerative diseases* (Gan et al., 2018).

2.2 Parkinson's disease

Neurodegenerative disease etiology is still unclear, but different contributing factors, such as lifestyle and genetic factors are involved (Tan et al., 2015). Parkinson is a common neurodegenerative disease, characterized by loss of dopaminergic neurons and α-synuclein intracellular accumulation, named Lewy bodies (Pozo Devoto and Falzone, 2017; Zeng et al., 2018). The principal lesion that underlies the characteristic motor phenotype of PD patients is unequivocally the loss of dopaminergic neurons in the *substantia nigra pars compacta* (SNpc) of the midbrain, which normally innervates the *striatum* (Surmeier et al., 2007).

SNpc dopaminergic neuronal loss leads to depletion of striatal dopamine (DA), thus an impaired nigro-striatal system, with consequent uncoordinated movements. PD is viewed as a slowly progressive neurodegenerative disorder that begins years before diagnosis can be made, implicates several brain areas, resulting from a combination of genetic and environmental factors. These complexities of Parkinson's disease are accompanied by clinical challenges.

Numerous studies have indicated that the underlying mechanisms of PD involve the inflammatory pathway and the oxidative stress, characterized by an imbalance between protective and detrimental function (Hassanzadeh and Rahimmi, 2019; Miller et al., 2009). Moreover, in neurodegenerative diseases, including PD, reduced neurotrophic support has been reported, such as BDNF

(Brain-derived neurotrophic factor) (Mercado et al., 2017; Sangiovanni et al., 2017).

Neuroinflammation and oxidative stress trigger to α-synuclein aggregation, which, in turn, lead to a stronger release of proinflammatory cytokines and reactive oxygen species (ROS) (Dias et al., 2013; Zeng et al., 2018).

PD pathology is indicated to begin in the *substantia nigra* but could involve also the enteric nervous system, highlighting the interaction between the gut and CNS (Klingelhoefer and Reichmann, 2015; Lionnet et al., 2018; Perez-Pardo et al., 2017, p.). Clinically, PD patients present motor symptoms, including tremor, rigidity, postural instability, and bradykinesia, accompanied by non-motor symptoms, such as depression, abnormalities in the gut function, pain, hyposmia, which may appear before the motor sign (Massano and Bhatia, 2012). Altered components of the gut could represent a key role in gut-brain axis, which is a bidirectional system between the CNS and the enteric nervous system.

The gold standard for PD diagnosis is represented by the post-mortem pathological examination for the presence of SNpc degeneration (Kalia and Lang, 2015).

2.2.1 Dopamine in Parkinson's disease

The death of nigrostriatal midbrain dopaminergic neurons in the SNpc is a defining characteristic of PD. Nigral cell death, and degeneration of nigrostriatal terminals, results in a loss of dopaminergic input into the *striatum* (STR) and causes an imbalance in striatal processing and output that is responsible for action selection and movement (Nordström et al., 2015). Much has been learned from SNpc toxic-lesion models that replicate the end-stage PD cell loss (6-OHDA, MPTP, paraquat, rotenone, etc.) and they have been proved valuable in designing therapies for alleviating PD motor symptoms, e.g., L-dopa, dopamine agonists, and deep-brain stimulation(Duty and Jenner, 2011).

Despite their strengths, animal models mimicking late-stage lesions fail to translate to neuroprotective therapy, given that they cannot inform upon the causes, onset, or progression of PD (Duty and Jenner, 2011)

2.2.2 Striatal circuitry

Axons of nigral cells in the SNpc project to the *striatum* a large subcortical nucleus involved in motor and cognitive action selection. *Striatum* is often referred as the gateway to the basal ganglia, a group of interconnected

subcortical nuclei that regulate a wide range of brain functions(Lanciego et al., 2012). *Striatum* receives input from the cortex, thalamus, and midbrain carrying information for action, selection, salience, and locomotion(Gerfen and Surmeier, 2011). *Striatum* consist of GABAergic spiny-projection neurons (SPNs; 95%), but also aspiny GABAergic and cholinergic interneurons (Melzer et al., 2017). Parkinson's disease affects the dorsal *striatum*, as the target nigral deterioration in late stage of PD and involves DA from the SNpc. SNpc neurons are active at low frequencies, supplying a basal DA tone to the *striatum*, which could be distorted by transient pauses of activity and phasic bursts (Tritsch and Sabatini, 2012). This two-way function of DA neurons is even more dynamic with various DA receptor subtypes and different target cells.

2.2.3 Direct and indirect pathways

The SPNs is able to elaborate incoming glutamatergic information from thalamus and cortex with dopaminergic input from the SNpc, and develop two output pathways, specifically the direct and indirect pathways as indicated in **fig 3**.

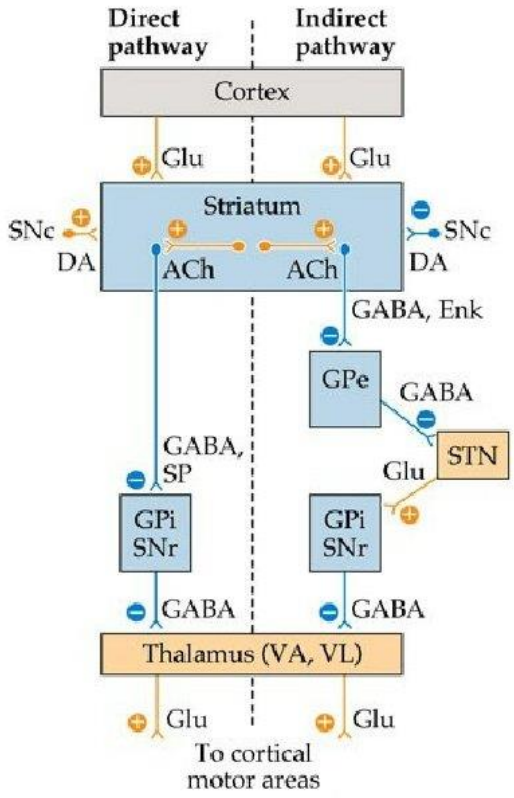

Figure 3: *Circuit diagram for direct & indirect pathways. Neurotransmitters: Ach, acetylcholine; DA, dopamine; Glu, glutamate; Enk, enkaphalin; SP, substance P. Nuclei: SNc, substantia nigra pars compacta; SNr, substantia nigra pars retriculata; GPe, globus pallidus pars externa; GPi, globus pallidus pars interna; STN, subthalamic nucleus; VL, ventral lateral nucleus; VA, ventral anterior nucleus* (Calabresi et al., 2014).

Regarding the direct pathway, the SPNs project until the *substantia nigra* pars reticulate (SNpr) and the internal segment of the Globus pallidus. GABAergic inhibition of the SNpr triggers to disinhibition of excitatory

glutamatergic thalamic neurons that extend to the cortex, resulting in locomotor activation (Calabresi et al., 2014).

Concerning the indirect pathway, SPNs project indirectly to the SNpc via the Globus pallidus *pars externa* (GPe) and subthalamic nucleus. Inhibition of the GABAergic neurons of the GPe, disinhibits the glutamatergic neurons of the subthalamic nucleus. The disinhibition of excitatory subthalamic neurons stimulates the GABAergic SNpr neurons leading to the thalamus, eventually decreasing locomotion. Besides these divergent pathways, intricate striatal microcircuits are composed by interneurons and collaterals, regulating SPN activity (Gerfen and Surmeier, 2011).

Notably, some studies reported a presence of other pathways besides the traditional ones; 60% of labelled neurons project along the direct pathway but presenting collateral terminal in the GPe, possibly supporting a way for the direct pathway to modulate the indirect pathway (Cazorla et al., 2015). In order to initiate an action, these pathways work together in both direct and indirect SPNs (Cui et al., 2013). Initially, the direct and indirect pathways were considered separate, but, recently, it is becoming gradually accepted that these pathways are functionally and structurally interconnected, leading to both the intensity of glutamatergic activation, and the amount and timing of DA release (Lindroos et al., 2018).

2.2.4 D1 versus D2 receptor function

The functions of the D1 and D2 dopamine receptors are not totally clear, with a number of conflicting theories relating to the interactions of the direct and indirect pathways. Furthermore, dopamine receptors show different properties. DA receptors are a family of G protein-coupled receptors (GPCRs), with opposing effects on adenylyl cyclase (AC) signalling pathways, leading to contrasting downstream signals, and, generally, D1DRs behave in an excitatory manner, while D2DRs act in an inhibitory manner (Surmeier et al., 2007).

In addition, these two receptors show different temporal profiles of activity. DA release is divided into two kind: tonic and phasic. Tonic activity indicates the uninterrupted output of the system, while phasic activity indicates shorter term burst activity of the DA-ergic cells. D2DRs have higher affinity and sensitivity to changes in tonic DA levels, while D1DRs are sensitive to phasic changes in the DA levels (Surmeier et al., 2007).

The activation of AC by D1 stimulation induces the protein kinase A (PKA) activation and the cyclic adenosine monophosphate (cAMP) production, on contrary, D2 stimulation inhibits AC activation and ensuing effects. In turn, PKA activation induces improved excitability, thus promoting long-term potentiation, owing to the phosphorylation and regulation of many cellular substrates, comprising ionotropic glutamate

and GABA receptors, transcription factors, and voltage-gated K^+, Na^+ and $Ca2^+$ channels (Keeler et al., 2014).

PKA activation, in turn, targets DA and cAMP-regulated phosphoprotein 32 (DARPP-32), which participates in multiple neurotransmitters, either blocking an inhibitor of PKA after dopamine receptors activation, becoming a potent inhibitor of the PKA pathway (Keeler et al., 2014).

2.2.5 Circuitry gone awry in Parkinson's disease

Human nigral DA cell axons are estimated to be 4m long, forming around 2.5 million striatal synapses, while DA cells in the ventral tegmental area, which are spared in PD, form only around 30,000 synapses (Bolam and Pissadaki, 2012). Due to a massive metabolic request, nigral axons arborized nature may trigger to improved vulnerability; thus, it is clear that the removal of a single nigrostriatal DA neuron projection will target a wide range of neurons (Bolam and Pissadaki, 2012). In PD, DA input to the *striatum* is decreased and the nigrostriatal neurons degenerate, while the GABAergic outputs to the globus pallidus or nigra are affected differently and consequently the originally balanced gateway is disturbed, inducing to hyperexcitability and imbalance of the pathways involved in movement control(**Fig. 4**) (Calabresi et al., 2014; Cazorla et al., 2015).

Indeed, this issue can be improved temporarily by treating with dopamine precursor L-DOPA; however, this approach is not able to counteract PD progression.

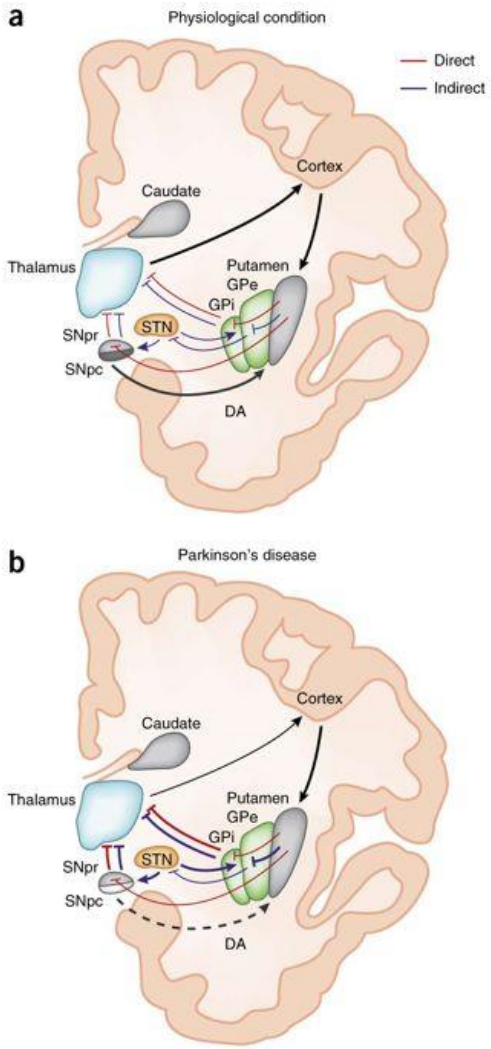

Figure 4: *Schematic representation of the direct/indirect pathway "classical" model in the physiological condition and in PD* (Calabresi et al., 2014).

2.2.6 Genetic Parkinson's disease

PD mainly remains a sporadic disorder, but recently there has been the identification and characterization of several genes involved in inherited Parkinsonism. These mutations are causative of rare familial form of PD, the first gene identified is *α-synuclein (SNCA)*, and mutations are associated with autosomal dominant Parkinsonism. Disease-causing mutations include missense mutations, which result in amino acid substitutions, and multiplications of the gene locus.

Aminoacid substitutions due to these missense mutations, or increased protein expression resulting from gene locus multiplications render, for example, α-synuclein prone to aggregating (Devine et al., 2011). Genes involved in autosomal dominant forms of PD comprise *LRRK2*, *VPS35*, *DNAJC13, CHCHD2, SNCA* and *EIF4G1*.

α-synuclein, 140-aminoacid long protein, belongs to a family of related synucleins, including β and γ-synuclein. The physiological function of α-synuclein is unclear, this synuclein is highly expressed throughout the mammalian brain and is enriched in presynaptic nerve terminals, where it can associate with membranes and vesicular structures. Recent studies have shown that α-synuclein specifically associates with membrane microdomains known as lipid rafts, and this raft association may be required for its synaptic localization. Analysis of mice with a targeted

deletion of the α-*synuclein* gene suggests a role for α-synuclein in synaptic vesicle recycling and DA neurotransmission (Abeliovich et al., 2000). This evidence is further supported by observations *in vitro* that demonstrate that α-synuclein can bind to acidic phospholipid vesicles and can also bind to and inhibit the activity of mammalian phospholipase D (Bendor et al., 2013). Studies in yeast further demonstrate that α-synuclein selectively associates with the plasma membrane, and inhibits PLD activity, inducing lipid droplet accumulation, and modulating vesicle trafficking (Outeiro, 2003). Thus, α-synuclein may play an important role in regulating synaptic vesicle size and recycling with particular relevance to dopamine storage. *LRRK2* encodes the leucine-7rich repeat kinase 2 (LRRK2), a large multidomain protein involved in multiple cellular processes, including neurite outgrowth and synaptic morphogenesis, membrane trafficking, autophagy, and protein synthesis (Hur et al., 2019). LRRK2 may also have a role in the innate immune system and its activity is conferred, in part, by its dual enzymatic functions (GTPase and serine-threonine kinase). *Parkin, PINK1* and *DJY1* are associated with autosomal recessive forms of Parkinson's disease (**Table 1**). Unlike autosomal dominant Parkinson's disease, which tends to have an age of onset similar to sporadic Parkinson's disease, recessively inherited Parkinsonism is more frequently associated with early onset (age less than 40 years). Mutations in *parkin* are the most frequent cause of autosomal recessive Parkinson's disease. Autosomal recessive Parkinson's disease might result from either

homozygous or compound heterozygous mutations in these genes, in some patients, only a single heterozygous mutation is detected (Fang et al., 2019). Parkin is a 465-amino acid long protein with a RING domain and can function as an E3 ubiquitin ligase, therefore Parkin is involved in the cellular machinery that covalently tags target proteins with ubiquitin after conveyed to the proteasome degradation. Several substrates have been reported for parkin on the basis of *in vitro* experiments, including synphilin-1, a rare *O*-glycosylated form of α-synuclein, synaptotagmin XI, cyclin E, the p38 subunit of the aminoacyl-tRNA synthetase complex and α/β-7tubulin. Some of this Parkin's substrates are implicated in enhancing neuronal cell death or toxicity. PINK1 is a 581-amino-acid long protein that contains a mitochondrial targeting sequence at its N-terminus and a highly conserved protein kinase domain similar to serine/threonine kinases of the Ca2+-calmodulin family. Although PINK1 is considered to be a mitochondrial protein kinase, the kinase activity of PINK1 has not yet been demonstrated, but it is involved in mitophagy process. The function of DJ-1 is less well characterized, but it seems to protect mitochondria from oxidative stress. Another significant genetic alteration that occurs in some cases of familial PD is missense mutation in gene *UCHYL1* encoding to ubiquitin carboxyl-terminal hydrolase L1. The inheritance pattern of this gene is unclear. UCH-L1 is a highly abundant, neuron-specific protein that belongs to a family of deubiquitinating enzymes that are responsible for hydrolysing polymeric ubiquitin chains to free ubiquitin monomers. UCH-

L1 can apparently maintain ubiquitin homeostasis by promoting the stability of ubiquitin monomers *in vivo* (Zhang et al., 2018).

	Protein	Pathogenic mutation(s)
Autosomal dominant		
SNCA	α-synuclein	Missense mutations (Ala18Thr, Ala29Ser, Ala30Pro, Glu46Lys, His50Gln, Gly51Asp, Ala53Glu, Ala53Thr); multiplications (duplications, triplications)
LRRK2	Leucine-rich repeat kinase 2	Missense mutations (Ile1371Val, Asn1437His, Arg1441Cys, Arg1441Gly, Arg1441His, Tyr1699Cys, Gly2019Ser [most common], Ile2020Thr)
VPS35	Vacuolar protein sorting 35	Missense mutation (Asp620Asn)
EIF4G1	Eukaryotic translation initiation factor 4-γ 1	Missense mutations (Arg1205His, Ala502Val)
DNAJC13	Receptor-mediated endocytosis 8 (REM-8)	Missense mutation (Asn855Ser)
CHCHD2	Coiled-coil-helix-coiled-coil-helix domain containing 2	Missense mutations (Thr61Ile, Arg145Gln); splice-site alteration
Autosomal recessive		
Parkin	Parkin	Exon rearrangements, including exon deletions or multiplications (most common); missense mutations, nonsense mutations, small deletions or insertions; splice-site alterations
PINK1	PTEN-induced putative kinase 1	Missense or nonsense mutations (most common); exon rearrangements, including exon deletions or duplications
DJ-1	DJ-1	Missense mutations or exon rearrangements (most common); splice-site alterations

Table 1: *monogenic form of PD by gene* (Kalia and Lang, 2015).

2.3 PD pathogenesis mechanisms

The main pathological feature of PD is the loss of dopaminergic neurons within *substantia nigra pars compacta* (SNpc), this damage affects all nigro-striatal connections in the brain. The histopathological hallmark of

PD is the Lewy body inclusions predominantly consisting in misfolded insoluble α-synuclein(Maiti et al., 2017). Lewy bodies are present in cell body and neurites of neurons, but they are also distributed in the spinal cord and peripheral nervous system. Certain assumptions describe the diffusion of Lewy bodies to progress in a stereotyped pattern over the course of Parkinson's disease (Kalia and Lang, 2016, 2015). Braak and collaborators have proposed six stages, starting in the peripheral nervous system and progressively affecting the central nervous system in a caudal-to-rostral direction within the brain(Braak and Del Tredici, 2017). This model has gained attraction since the proposed temporal and spatial progression could explain the clinical course of Parkinson's disease. However, PD pathology is more intricated than neurodegeneration due to the Lewy bodies. Pathogenic mechanisms leading to onset and progression of PD are numerous, including aberrant proteins, mitochondrial dysfunction and impaired calcium homeostasis, increased oxidative stress, neuroinflammation, but also impaired glucose metabolism and insulin desensitisation (**Fig.5**) (Castelli et al., 2019a).

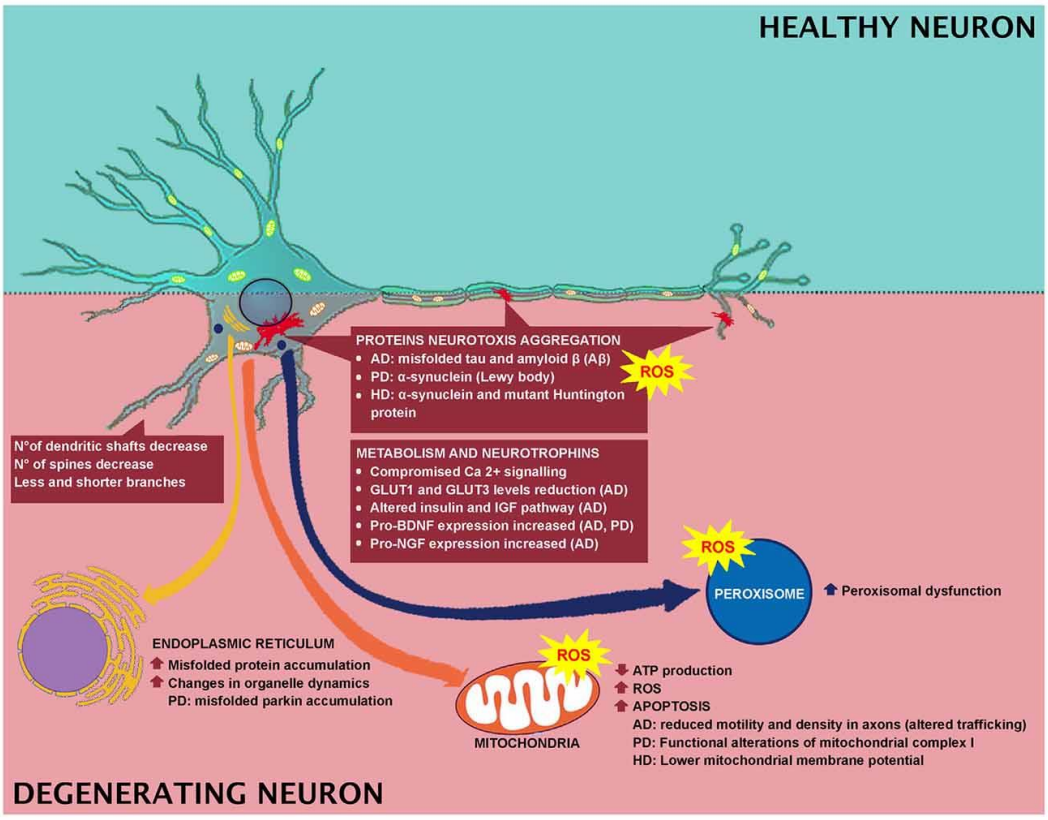

Fig.5*: Effect of degeneration in neuronal cell and involved mechanisms (Castelli et al., 2019a).*

2.3.1 Protein homeostasis, mitochondrial dysfunction and oxidative stress in PD

The presence of Lewy bodies in affected brain areas in PD is the main feature that encourages thinking at the protein homeostasis alterations as a potential cause for Lewy bodies formation. The major component of

Lewy bodies is α-synuclein. When there is the inhibition of the chaperone function, proteasome and autophagy the result is the accumulation of α-synuclein and the progressive formation of α-synuclein positive inclusions. In turn, the accumulation of α-synuclein can exert reciprocal effects on the various proteins of the quality control systems. For example, components of the heat shock proteins chaperone system (Hsp70 and Hsp40) can be depleted through their sequestration within α-synuclein-positive aggregates. Further, aggregated α-synuclein species can selectively interact with the components of the proteasome complex and concomitantly inhibit its function (Roodveldt et al., 2009). Finally, α-synuclein can also inhibit autophagy and chaperone mediated autophagy (CMA), thus further amplifying the amount of misfolded protein (including α-synuclein) and protein aggregates, that can lead to the death of dopaminergic neurons. In line of this concept, CMA inhibition following L-DOPA treatment is more evident in ventral midbrain dopaminergic neurons compared to than non-DA cortical neurons (Post et al., 2018). It is clear that the chaperone, ubiquitin-proteasome and autophagy pathways have all a role in the biogenesis of α-synuclein-positive Lewy bodies and thereby PD.

In PD, oxidative stress triggers to α-synuclein aggregation in dopaminergic neurons and in turn α-synuclein aggregation generates intracellular ROS (Xiang et al., 2013).

In a transgenic model of neurodegenerative diseases, misfolded protein accumulation induces altered organelle dynamics, and in the reticulum stress (Rao et al., 2002; Reddy et al., 1999). Consequently to the parkin function loss, its misfolded substrates accumulate in dopaminergic neurons of the substantia nigra, inducing reticulum stress and cell death (Imai et al., 2001). UPR targeting, through inhibition or inactivation represent a valid therapeutic approach for neurodegenerative diseases(Brown and Naidoo, 2012). Also mitochondria has pivotal functions in neurodegeneration. During mitochondrial impairment, high levels of ROS, apoptosis and low ATP occur (Suárez-Rivero et al., 2016). PD pathogenesis is linked also to Ca^{2+} dysfunction (Surmeier et al., 2012). Maintaining mitochondrial Ca^{2+} buffering capacity through the modulation of L-type channel activity can be a potential therapeutic approach for counteracting PD progression (Calì et al., 2014, 2011). Dysfunctions in mitochondrial complex I are responsible for PD onset and are linked to increased ROS generation (Hu and Wang, 2016); notably, sporadic PD patients showed reduced complex I in different brain regions, neural and extra-neural tissues (Parker et al., 2008).

Dopaminergic neurons in *substantia nigra* are more vulnerable toward oxidative stress that is further enhanced by the abundance of redox-active iron in this region of the brain, as well as by the presence of dopamine, whose oxidation products are potentially cytotoxic (Trist et al., 2019). Moreover, several evidences have reported that markers for lipid

peroxidation, including 4-hydroxynonenal (HNE) and malondialdehyde, protein carbonyl modifications and even DNA and RNA oxidation are markedly elevated in the *substantia nigra* of post-mortem PD brains (Niedzielska et al., 2016). These ROS-induced events induced a strong decrease of reduced glutathione (parallel with reduced antioxidant activity) (Niedzielska et al., 2016). Parkin mutations are related to UPS and autophagy dysfunction, thus also mutations in protein involved in oxidative stress could be related to PD onset and progression. For example, DJ-1 is thought to operate as an atypical peroxiredoxin-like peroxidase that is capable of scavenging mitochondrial H_2O_2, mutations on *DJY1*gene are involved in early onset form PD. DJ-1 is mainly localized in the mitochondria and has a pivotal role in the organelle. Remarkably, a recent study indicates that DJ-1 is able to protect dopaminergic neurons against rotenone-induced apoptosis, through ERK activation (Gao et al., 2012), thus lack of DJ-1 predisposes to oxidative stress and mitochondrial dysfunctions. Finally, it is important to know that besides intrinsic sources of ROS, oxidative radicals can also be produced extracellularly from activated glial cells, which is well documented in affected regions of the PD brain, as well as in genetic and toxin-induced models of PD. Accordingly, glia-mediated inflammatory events can aggravate the pathogenic outcomes and as such may play an instrumental role in promoting neuronal cell death. Overall, oxidative stress, altered mitochondrial function, and impairment of the autophagy and UPS may

underlie the molecular pathogenesis of familial and sporadic PD, and these pathways are linked together at multiple levels, as summarized in **Fig. 6**.

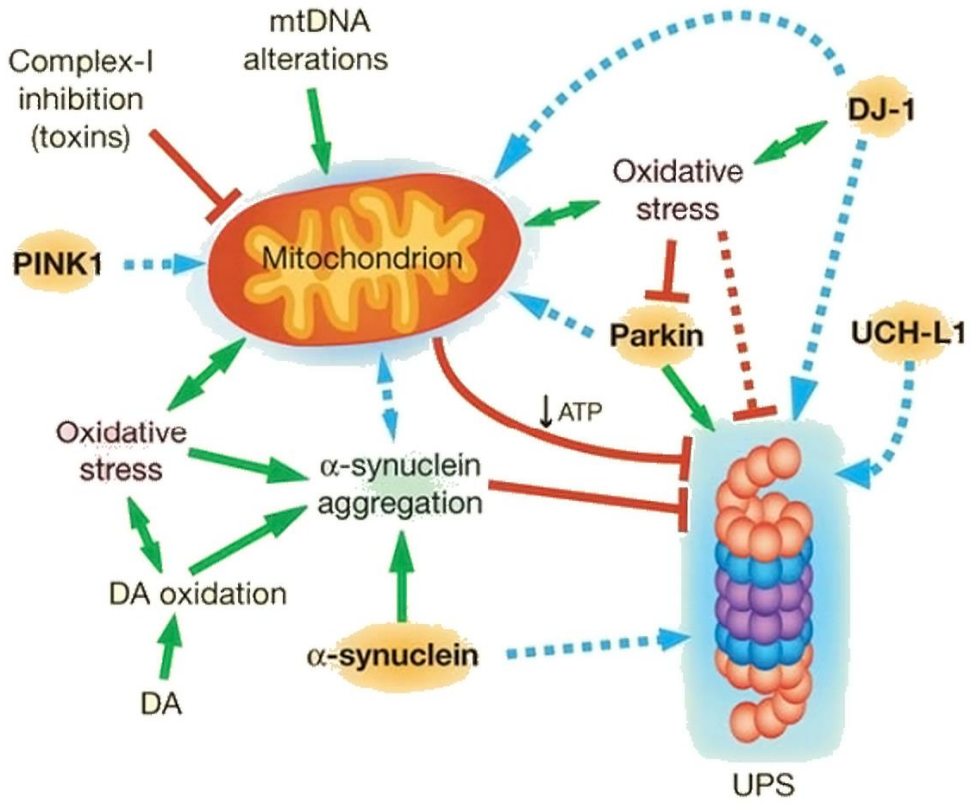

Figure 6: *Common pathways underlying PD pathogenesis. Red lines indicate inhibitory effects, green arrow indicates relationships between components or systems and blue dashed arrow indicates possible relationships* (Moore et al., 2005).

2.3.2 Neuroinflammation and other hypothetical pathogenic mechanisms in PD

Neuroinflammation is another feature of Parkinson's disease pathology, the presence of an active inflammatory response in the brain mediated primarily by resident astrocytes and microglia has been long recognised. Both reactive gliosis resulting from activated astrocytes and microgliosis resulting from microglial activation occur within areas of neurodegeneration in PD (Guzman-Martinez et al., 2019). Astrocytes and microglia are both involved in clearance of extracellular debris, which might aid in the survival of neurons (Jung and Chung, 2018). Indeed, Lewy bodies can be inducing a M1 microglia phenotype, therefore activating a pro-inflammatory response. Activated microglia can release trophic factors, such as brain-derived neurotrophic factor (BDNF) and glial-derived neurotrophic factor (GDNF), but also harmful reactive oxygen and nitrogen species and pro-inflammatory cytokines (Pöyhönen et al., 2019). Moreover, recent study has reported that use of the non-steroidal anti-inflammatory drug (NSAID) ibuprofen, but not other NSAIDs, reduces the subsequent risk of developing PD (d'Angelo et al., 2019).

Oxidative stress is also a key stimulator of microglial activation and the generation of reactive oxygen species (ROS), reactive nitrogen species (RNS) and, consequently, of further dopaminergic neuronal death (Rizor et al., 2019). ROS, RNS can also accumulate in brain tissue in response to

lipopolysaccharide (LPS) and the pro-inflammatory cytokine interleukin-1β (IL-1β), and analysis of post-mortem brains from individuals with PD have identified the presence of enzymes associated with inflammation, including cyclo-oxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) (Bartels and Leenders, 2010). This suggests that neuronal cell death and inflammation are the basis the progression of PD. Interestingly, adaptive immune system also seems to play a role in PD. Some evidences demonstrated the presence of CD4+ and CD8+ T lymphocytes, but not B-lymphocytes, in the *substantia nigra* of MPTP-intoxicated mice and post-mortem human brains (Evans et al., 2019). Furthermore, it is observed an important reduction of MPTP-mediated neuronal loss in absence of mature T cells in Rag1-/- and Tcrb-/- immunodeficient mice (Appel et al., 2010). Thus, PD appears to be a complex interaction between the inherent vulnerability of the nigrostriatal dopaminergic system, genetic predisposition, aging, exposure to environmental toxins, and inflammation.

Aging is a very important risk factor for PD and other neurodegenerative diseases. Aging amplifies the damage induced by the main pathogenic mechanisms known and discussed above (Reeve et al., 2014). During aging an accumulation of lesions in DNA occurs also because the DNA repair system appears downregulated (Milanese et al., 2019). Recently, some studies revealed that DNA repair system could be involved in age-related PD pathogenesis. Fibroblasts from PD patients and from *Ercc1* (Ercc1 is

involved with XPF in endonuclease activity of nucleotide excision repair system, also called NER) mutant mice showed typical PD-like alterations in dopaminergic neurons, such as decreased dopaminergic innervation, increased phosphorylated synuclein levels and mitochondrial dysfunction. *Ercc1* mutant mice are more susceptible to toxic effects induced by MPTP. However, these results are related to dysfunction of NER and therefore there may be a relationship between the accumulation of DNA damage and susceptibility to PD (Sepe et al., 2016).

Parkinson's disease, like Alzheimer's disease, is associated with biochemical changes in insulin signalling in affected brain areas. The levels of insulin receptor (IR) phosphorylation are increased in the basal ganglia and in the *substantia nigra*. Furthermore, there is increased IRS2 (serine) phosphorylation, a marker of IGF1 (insuline-like growth factor) resistance, in the basal ganglia of the 6-hydroxydopamine (6-OHDA) lesioned rats, a model of PD. Animal studies reported similar changes. Indeed, in a mouse model of diabetes type 2, fed with high fat, learning, memory and synaptic plasticity resulted compromised (Hölscher, 2014). In a high-fat-diet rat model of early-stage T2DM, insulin resistance and attenuated dopamine release were observed, thus dopamine clearance is diminished in the basal ganglia, indicating that dopaminergic signalling is compromised in T2DM (Porter et al., 2011).

2.4 Epidemiology

Parkinson is a progressive neurodegenerative disease, that affects 0.3% of the total population and 1% of people over 60 years of age in industrialized countries; this number is set to rapidly growing concomitant with the increasing trend of population aging (Tysnes and Storstein, 2017).

Prevalence represents the proportion of PD affected individual in a population at a given time. Crude prevalence of PD has been reported to vary from 15 (per 100,000 population) in China to 657 in Argentina in door-to-door surveys, and to vary from 100 to 250 in North America and Europe (Rocca, 2018). The prevalence estimates derived by this method are greater than those derived from other methods for comparable populations. Prevalence is easily affected by socioeconomical issues and factors that affect survival rate. The overall prevalence of PD appears to be lower in the Eastern studies compared to Western ones, probably reflecting the combination of genetic and environmental factors, but also the methodological differences.

Incidence is a better estimate frequency, and it calculates the number of new subjects with PD occurring in a given time period for a certain population at risk. The incidence is affected by clinical manifestations and since the symptoms can appear after a long latent stage, an accurate calculation of PD incidence is hard. The variation may be due also to study design differences, such as methods or diagnostic criteria. Both prevalence and incidence of PD

vary greatly across age groups. The average age of PD onset is approximately after 50 years of age, but, recently, is growing mainly due to an increase in aged population. Regarding the gender, PD is slightly more common in men than in women (ratio 1.5:1/ 1.2:1). Furthermore, PD incidence and prevalence are influenced by racial composition of the surveyed population. Commonly, white people and North American showed higher prevalence compared to Asians and Africans, even though it has been reported that PD incidence in African-Americans and Asian-Americans was pretty similar to European-Americans. These findings may explain the effect of environmental factors exposure (Dorsey et al., 2018). Regarding the life span of PD affected is less than normal individuals, even though has been extended and, moreover, PD patient are more prone to other pathologies or infections.

The cause or causes of PD are still unclear, but numerous factors are associated with PD onset. Among demographic history, as mentioned, age, racial origin and gender are linked to increased risk of PD. Several studies indicate that environmental factors exert crucial role in PD, as reported for example in twin studies. Notably, it has been reported an inverse relation between PD incidence and caffeine consumption, since the coffee users had less risk to develop PD compared to the non-drinkers, suggesting that caffeine is able to lower PD incidence (Palacios et al., 2012). Another risk factor is represented by infectious agents. Indeed, some agents or disease, including HIV, influenza B, measles, diphtheria, herpes simplex and rheumatic fever, could predispose to

PD or be associated with postinfectious parkinsonism either acutely or as a long-term complication (Caggiu et al., 2019).

2.5 Clinical diagnosis

PD is characterized by 4 cardinal aspects grouped under the acronym TRAP, stands for Tremor, Rigidity, Akinesia and Postural instability. Since lifestyle and individual characteristic affect Parkinsonism, it is important to include motor but also non motor impairment during clinical diagnosis. To assess motor symptoms Hoehn and Yahr scale is generally used to compare groups of patients and to classify the state of PD progression (from 0, no sign to 5 wheelchair). A well-established scale to evaluate disability and to follow the disease progression is represented by Unified Parkinson's Disease Rating scale (UPDRS), which indicate that PD progression is not linear and is characterized by high variability especially in the early stage (Jankovic, 2008). Overall, since there is no conclusive test for PD diagnosis, the disorder needs to be diagnosed basing on clinical features. Considering only the initial diagnosis could generate misdiagnosis, thus, to improve the diagnostic accuracy follow-up visits are necessary. To facilitate diagnostic-therapeutic approaches, different guidelines and criteria have been established (Marsili et al., 2018). In particular, recently has been published a novel diagnostic criterion, the

International Parkinson and Movement Disorder Society (MDS) (Postuma et al., 2015).

The Movement Disorder Society PD Criteria are intended for use in clinical diagnosis but also for clinical research. These criteria aim to render the diagnostic process more reproducible and applicable by clinicians with less expertise in PD diagnosis. In these criteria also non- motor functions are considered. Like previous criteria, the Movement Disorder Society PD Criteria maintain motor parkinsonism as the cardinal feature, defined as bradykinesia plus rest tremor or rigidity. PD determination depends on three categories of diagnostic characteristics: absolute exclusion criteria (no PD), red flags (supportive exams or visits are necessary), and supportive criteria (positive features that increase confidence of the PD diagnosis). Two levels of certainty are defined: probable and clinically established PD (Postuma et al., 2015). As increasing the knowledge of PD, the Movement Disorder Society criteria will require constant revision to be in line with these advances.

2.6 Symptoms

PD is a progressive neurodegenerative disorder that affects mainly movement. PD sign and symptoms can differ between patients and in the early stage of the pathology can be unnoticed due to the widespread neurochemical and neuroanatomical alterations during PD course. PD is now recognized as a multi-system disorder with motor and non-motor features. PD motor symptoms and sign include tremor often in the hand and during rest; akinesia and

bradykinesia, muscle stiffness accompanied by pain; speech and writing alterations; decreased ability of unconscious movements and uncontrollable movements during sleep (Grayson, 2016). Non-motor symptoms (NMS) are a real burden in PD, and they can appear in early pre-symptomatic stage or during PD course (Stacy, 2011; Tibar et al., 2018). NMS are represented by cognitive impairment (most common) olfactory dysfunction (from earliest stages), depression, sleep disorders, gastrointestinal and urinary dysfunctions, and fatigue pain (Wu et al., 2017). In particular, altered gastrointestinal functions might occur at all PD stages, often preceding the onset and may include reflux, loss of weight, constipation and dysphagia. For this reason, recently is growing the interest in gut-brain axis. The treatment of these symptoms is still inconsistent and inadequate (Wu et al., 2017).

2.7 Treatments

To date, there is no cure for PD. The pharmacological approach used are effective only for symptoms, such as replacement of DA with L-DOPA administration mostly in the initial stage of disease. L-DOPA long-term treatment could induce adverse side effects, intensifying the motor symptoms, including dyskinesia, motor and non-motor fluctuations, and drug-induced psychosis (Kalia and Lang, 2016, 2015).

Another approach is represented by Deep brain stimulation (DBS), which replaced the ablative stereotactic surgery in the treatment of PD and it has become the most used surgical treatment method for advanced PD (Wagle Shukla and Okun, 2014). DBS is effective in reducing tremor, bradykinesia

and rigidity in PD, and it allows a reduction of the antiparkinsonian medication doses (Wagle Shukla and Okun, 2014). In DBS, high frequency pulses are used to stimulate the STN (subthalamic nucleus) or the internal segment of globus pallidus and associated brain regions (Perlmutter and Mink, 2006). The DBS device consists of an implanted pulse generator (electrode placed in a subcutaneous pocket below the clavicle), a connecting wire, and one or two leads with electrode contacts (placed in the STN or globus pallidus) (Peng et al., 2017). Unilateral or bilateral stimulation techniques are possible (Peng et al., 2017). The exact mechanisms of the action of DBS are still unclear. The success of DBS treatment is mainly based on the exact locations of the stimulation electrodes, on the settings of the stimulation parameters (stimulation amplitude, electrode polarity, pulse width, and stimulation frequency) and on biological factors (Steigerwald et al., 2018).

Despite all research efforts, nowadays the PD remains an incurable disorder and the debilitating nature and morbidity of this disease represent significant healthcare, social, emotional, and economic problems.

CHAPTER III

3.1 Gut-brain axis

The concept regarding the capability of gut microbiota to communicate with the CNS and thus regulate behavior is emerging in health and disease. This bidirectional communication between brain and enteric system is mediated by gut-brain axis and it is vital for the maintenance of homeostasis and is regulated at neural, immunological and hormonal levels (**Fig.7**) (Carabotti et al., 2015). Alteration in gut-brain axis could results in altered stress-response (Cryan and O'Mahony, 2011). The importance of the gut-brain axis is highlighted by the fact that psychiatric symptoms are related with co-morbidity at gastrointestinal level, including inflammatory bowel disorder (IBD) and irritable bowel disorder (IBS) (Camara et al., 2009). Further, increasing evidence reported that the gut microbiome significantly impacts on this axis. The underlying mechanism regarding this communication are still uncertain. Indeed, recent studies are investigating on the influence of microbiota on brain health and functions with the impact of antibiotics, infections and probiotics use on brain (Cryan and O'Mahony, 2011).

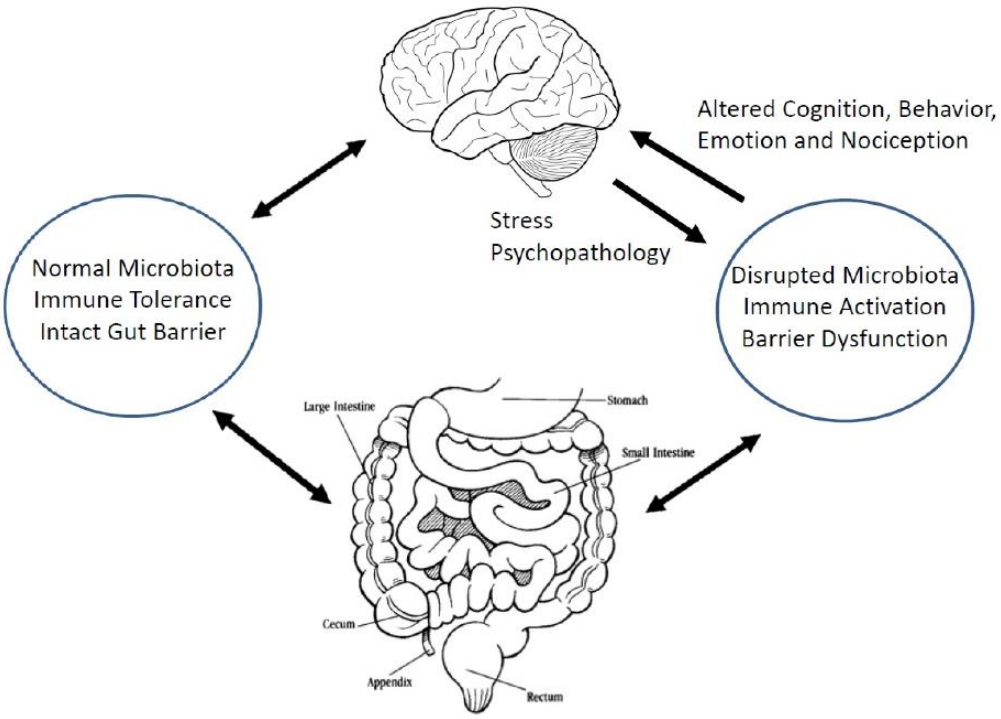

Figure 7: *Gut-brain axis schematic representation* (Quigley, 2018)

3.2 Gut-brain axis in brain disorders

Recent studies have shown a clear link between microbial changes and cognition. Intestinal dysbiosis affects cognitive behavior, learning and memory processes (Gareau et al., 2011). It has been demonstrated that learning performances in germ-free mice affected by intestinal bacterial infections were impaired while restored upon probiotics treatment. This study indicates that the gut-brain axis, which in germ-free is not exposed to stress, involves a neuroendocrine system susceptible to external threats.

Altered microbiota is correlated with cognitive impairment and hepatic encephalopathy (Bajaj et al., 2012). The dysbiosis induced by antibiotics is associated with BDNF decrease, serotonin transported dysfunction, thus leading to cognitive decline, including novel object recognition (Fröhlich et al., 2016). It has been demonstrated *in vivo* that probiotic formulation is able to improve cognitive tests by chronic restraint stress (Liang et al., 2015). Moreover, in rat hepatic encephalopathy model, characterized by hyperammonia, the *Lactobacillus helveticus* administration induced improved spatial memory performances and anxiety-like signs (Luo et al., 2014). Further, *Bifidobacterium* administration was effective in enhanced non-spatial and spatial memory (Savignac et al., 2015).

Enhanced gut permeability caused by microbial dysbiosis could indirectly or directly affect neurodegenerative disorders. Indeed, gut bacteria can release large quantities of lipopolysaccharide and amyloid, involved in the regulation of signaling pathways and the production of neuroinflammation in Alzheimer's disease. Further, altered gut microbiota is directly associated with other factors implicated in AD pathogenesis, including type 2 diabetes and obesity (Kim and Shin, 2018).

Gut microbiome it might be involved in the vulnerability associated with the aging process. Firmicutes and Bacteroidetes, dominant phyla, are progressively prominent with age. During aging, pathogenic bacteria (i.e. Proteobacteria) increase at the expense of beneficial bacteria (*Bifidobacterium* species), probably inducing chronic low-grade inflammation. Mice treated with

supplement of *Lactobacillus curvatus* and *plantarum*, age-dependent memory impairments ameliorated via inhibition of NF-κB pathway (Jeong et al., 2015).

3.2.1 Gut-brain axis in Parkinson's disease

Numerous clinical investigations reported that PD patients expressed α-synuclein accumulation in the enteric nervous system (ENS) (Clairembault et al., 2015; Perez-Pardo et al., 2017). α-synuclein accumulations are linked with injured enteric neurons and cause GI abnormalities (Gold et al., 2013; Sánchez-Ferro et al., 2015). They affect the gut submucosal and myenteric plexuses in PD patients and are distributed in the GI tract (Arizona Parkinson's Disease Consortium et al., 2010).

Braak and collaborators suggested that α -synuclein pathologies could initiate in either the olfactory bulbs (OB) and/or in the ENS probably through unknown environmental and pathogen toxins and then progresses towards the SN and other areas in the CNS (**Fig.8**). Probably the vagal nerve represent the connection for α -synuclein (Braak et al., 2003; Hawkes et al., 2007), and the initiation of the pathological process in the OB can more directly affect the brain via the olfactory tract (Hawkes et al., 2010, 2009; Klingelhoefer and Reichmann, 2015). Recent findings (Forsyth et al., 2011) suggest that gut-initiated pathological processes in PD do not necessarily require a pathogen and/or an environmental toxin since they can be triggered by the intestinal microbiota.

Environmental factors such as nasal/gut microbiota, and toxins can trigger oxidative stress and inflammation (Hawkes et al., 2009), and thus initiating alpha-synuclein accumulation (Hawkes et al., 2010).

Enteric nervous system of PD patients, α-synuclein aggregates are more prevalent respect aging subjects (Barrenschee et al., 2017). Recent *in vivo* studies showed that accumulation of α-synuclein aggregates in the enteric nervous system can be induced by alterations in the gut microbiome (Chen et al., 2016). Notably, mice overexpressing α-synuclein showed altered gut microbiota (Sampson et al., 2016). It has been also demonstrated that transplanting feci of PD patients on these mice, they showed impaired motor performances, suggesting that gut microbiome exerts a crucial role in the onset of synucleinopathies, including PD (Sampson et al., 2016). Some components of the gut microbiome release microbial amyloids in extracellular area and the neighboring cells, comprising neurons, and induce the formation of pathological aggregates of endogenous α-syn via permissive templating (Friedland and Chapman, 2017; Soto and Pritzkow, 2018). Altered clearance mechanisms of the ubiquitin-proteasome system, as occur in familial and idiopathic PD (McNaught et al., 2001), to degrade the misfolded protein, may facilitate the seeding process.

The bacteriophage components of the microbiome should be included in microbiome dysbiosis (Manrique et al., 2017). Bacteriophages are viral parasites of bacteria and are important regulators of host-microbiome interactions but can also impact human health by involving on intestinal

inflammatory processes (Gogokhia et al., 2019) and possibly causing α-syn misfolding (Tetz and Tetz, 2018). Early signs of PD in the gut are altered gut permeability and dopamine production, concomitant with a reduction of *Lactococcus* bacteria (Darby et al., 2019; Houser and Tansey, 2017; Tetz et al., 2018). An alternative antimicrobial strategy is represented by phage therapy (Chen et al., 2016), which through manipulating the microbiome could contribute to counteract PD (Tetz et al., 2018).

Probiotic formulations improved the CNS activity through the modulation of inflammation and positive interactions with the commensal gut microbiota(Wang et al., 2016). PD patients microbiota is rich of pro-inflammatory cytokines (Bedarf et al., 2017; Petrov et al., 2017) due to enhanced intestinal permeability to endotoxins (lipopolysaccharide) (Forsyth et al., 2011). Bacterial amyloids may also support a pro-inflammatory environment in the gut (Miraglia and Colla, 2019). In multiple sclerosis patients, probiotics were able to stimulate an anti-inflammatory peripheral immune response (Tankou et al., 2018), thus they could be advantageous for PD individuals. Lactobacilli are able to prevent biofilms formation pathogenic bacteria (Vuotto et al., 2014), however, probiotics effect is strongly changing, being person-specific (Zmora et al., 2018). Recently, it has been developed different probiotics formulations but only few studies reported about its efficacy (Cohen, 2018). More investigations are necessary concerning the potential therapeutic effect of probiotics in maintaining protein and oxidative homeostasis in enteric nervous system.

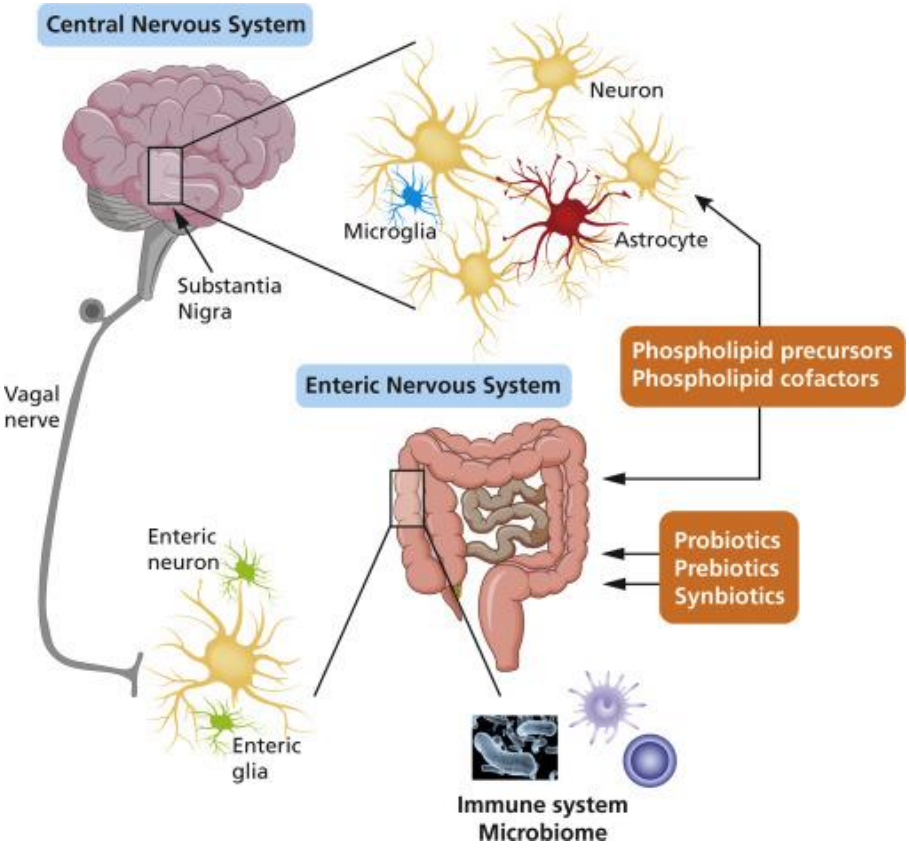

***Fig 8**: A schematic representation of alpha-synuclein accumulation and spreading from the brain to ENS* (Kim and Shin, 2018).

3.2.2. Gut-brain axis in Schizophrenia and Bipolar Disorder

Schizophrenia is a serious mental disease and the affected are characterized by abnormal motor behavior, confused speech and hallucinations. It has been demonstrated that schizophrenia is related to gastrointestinal comorbidities, including IBS, IBD and celiac disease (Filipovic and Filipovic, 2014; Kirkpatrick and Miller, 2013), suggesting that gut and microbiota may participate in schizophrenia disorder.

The onset and progression of schizophrenia are characterized by chronic inflammation (Kirkpatrick and Miller, 2013; Miller et al., 2011). As mentioned above, altered gut microbiome could lead to chronic inflammation. Indeed, in schizophrenia patients high instability of intestinal structure concomitant with inflammatory bowel disorders has been reported (Kim and Shin, 2018). *Toxoplasma gondii* infection in the GI tract alters the commensal bacteria inducing gut microbiota dysbiosis. Indeed, this infection represents a risk factor for early-onset schizophrenia (Mortensen et al., 2007). Another risk factor for schizophrenia onset is represented by bacteriophages since they can alter bacteria metabolism and microbiome composition (Mills et al., 2013).

Even though inflammation can be a factor to bipolar disorder onset, the role of gut microbiota is uncertain (Goldstein et al., 2009). Numerous observational studies reported that gut microbiota is linked with bipolar disorders. As in schizophrenia, *T. gondii* infection or altered gut-brain axis function have been detected in patients with manic behavior (Dickerson et al., 2014; Hamdani et al., 2015). Notably, non-psychotropic charcoal treatment dampens the inflammation and contains manic symptoms.

3.2.3 Gut-brain axis in Autism Spectrum Disorder

The term autism spectrum disorder (ASD) indicates different neurodevelopmental conditions in which the patients show difficulties in communication, social interaction, and repetitive stereotyped activities. The link between gut microbiome and ASD is due to the fact that the neurotoxic effects of *Clostridium* were implicated in ASD onset (Bolte, 1998). Indeed, it has been reported higher number of species belonging to the *Clostridium* in the gut microbiome of autistic children (Finegold et al., 2002; Parracho et al., 2005). This hypothesis was strengthened by studies regarding the benefit of using vancomycin in ASD patients (Sandler et al., 2000). Since ASD patient show gastrointestinal abnormalities, other hypothesis enhance that the gut microbiome is implicated in the late onset of ASD. Indeed, it has been reported that in late-onset autism patients the gut microbiome is characterized by an increase in *Sutterella* and a reduction in Bacteroidetes compared to control group (Williams et al., 2011). This discrepancy is caused by a malabsorption of carbohydrate, due to a decline in gray matter transcription of disaccharidases and hexose transporters (Williams et al., 2011). This malabsorption could be due to changes in nutritional sources available to the intestinal bacteria, which, consequently, triggers to microbiota dysbiosis.

Subsequent molecular-based studies have found larger numbers of microorganisms that are altered in autistic children. In autistic children modified quantities of *Sutterella*, *Bifidobacterium*, *Prevotella*, *Lactobacillus*, Alcaligenaceae and *Ruminococcus* have been found (Adams et al., 2011; Kang et al., 2013; Williams et al., 2012).

CHAPTER IV

4.1 Gut microbiota

In the intestinal tract reside a “microbiota” composed by abundant and various microorganism, comprising archaea, bacteria, viruses, and fungi (Kang et al., 2013). The term indicates the microbial communities. In the microbiota bacteria are abundant, as reported in a metagenomics analysis of the gut microbiome. Moreover, human gut microbiome is characterized by three different enterotypes, *Bacteroides*, *Prevotella*, and *Ruminococcus* (Eckburg et al., 2005) and the different population depends on age, nutrition, genetical predisposition, environmental stressors, antibiotic use, physical activity and presence of disease (Arumugam et al., 2011).

4.1.1 Development

Gut microbiome is generally formed in three phases and until the birth is sterile since the uterus is aseptic. With the birth, through the vagina, the infant is subjected to different bacteria that colonize the skin, conjunctiva, mucosa and gastrointestinal tract. The microbiome acquired from vaginal passage include mainly *Lactobacillus, Prevotella and Bifidobacterium* (Karlsson et al., 2011), while *Staphylococcus* and *Corynebacterium* from cesarean section (Dominguez-Bello et al., 2010). Thus, the kind of birth, the external environment and the maternal skin influence the gut microbiome composition

of a newborn (Biasucci et al., 2010; Corey et al., 2019). During breastfeeding, gut microbiome show a lower diversity compared to the initiation with solid foods (Favier et al., 2002; Roger and McCartney, 2010), indeed, the proportion of anaerobic bacteria (Firmicutes) is increased(Favier et al., 2002), and at three years of age the microflora is close to adult gut (Biasucci et al., 2010).

Between mother-baby microbiome there is a deep interaction, indeed, different studies reported a relation between lactation and Body Mass Index (BMI) and different microbiota. The different milk formula derived from breastfeeding analyzed in 47 different mothers suggested that BMI influenced the different gut infant flora. A normal microbiome is characterized by firmicutes, Bacteroidetes and proteobacteria, but parallel with weight gain and insulin desensitization (third trimester), levels of firmicutes and Bacteroidetes levels considerably diminished, while proteobacteria and gut inflammation increased. The various BMI categories, obese, overweight, and normal weight contributed to the different population of the mother's gut microbiome. Obese mothers have reduced levels of microbiome compared to normal-weight mothers. Moreover, non-exclusive breastfeeding was associated with higher abundance of gut microbiota over exclusively breastfeeding type. Alterations due to the feeding method may influence in later years and adulthood. Specifically, it has been demonstrated increased levels of fecal calprotectin and ingested chemokine IP-10 (which promotes intestinal T cell migration and activation) in exclusively breast-fed infant (Groer et al., 2016; Takahata et al., 2003; Yee et al., 2019).

4.1.2 Gut microbiome in children

Several factors could influence gut microbiome, such us prenatal and postnatal illnesses, use of antibiotic, amount and quality (IgA rich) of milk received and weekly levels of fecal calprotectin. The individual “signature” microbiome is different between small babies who showed slow growth and bigger babies with faster growth and as we mentioned above, human milk amount and pre and post-natal factors as well fecal calprotectin level. This difference between microbiome and the relationship between growth could help in managing pediatric health and diseases. In fact, microbiome dysbiosis induces cytokines release and trigger to detrimental inflammation (Yee et al., 2019).

Targeting the microbiota by managing pre- and post-natal elements, human milk amount and quality, and fecal calprotectin amounts may improve the health and quality of life of these children.

4.1.3 Factors Affecting the Structure of the Gut Microbiome

Gut microbiome composition is influenced by long-term dietary habits. Diet rich in protein and fat induce elevated levels of *Bacteroides,* while diet rich in high fiber stimulates *Prevotella* enterotypes (Wu et al., 2011). Prebiotics are indigestible food ingredients that selectively promote the growth and activities

of helpful microorganisms, such as *Bifidobacterium* and *Lactobacillus* (Roberfroid et al., 2010).

Other factors that influence gut microbiome are represented by antibiotics use, infection, and can be detrimental for the host (Forsythe et al., 2010). Indeed, even the short-term antibiotic treatment can induce long-term dysbiosis, with diseases exacerbation (Lange et al., 2016).

Increasing data reported that gut microbiota, participating in the physiology and pathology of cellular organisms, is implicated in health and disease (Maynard et al., 2012). Inherited gut microbiome change with diet habits and environmental signals (Gomez de Aguero et al., 2016; Koh et al., 2016). Gut microflora is also influenced by the immune system (Belkaid and Hand, 2014) and dysbiosis is due to immune signaling that occur during cancer, inflammatory bowel disease and autoimmune disease (Blander et al., 2017; Roy and Trinchieri, 2017).

The development and maturation of human CNS is controlled by extrinsic and intrinsic components. Different investigations reported a correlation between CNS neurochemistry and physiology and gut microbiota, as demonstrated in animals treated with broad-spectrum antibiotics and in germ-free animals (GF) (Ma et al., 2019). In fact, GF mice showed a strong alteration in NMDA, 5-HT and BDNF, which trigger to neurological impairment in memory, recognition, learning, and emotional behaviors (Bercik et al., 2011; Foster et al., 2017; Gareau et al., 2011; Heijtz et al., 2011). As mentioned above, neuropathology

are related with gut microbiome dysbiosis (Foster et al., 2017; Fung et al., 2017), but it is also implicated in homeostasis and development of CNS(Tremlett et al., 2017).

CHAPTER V

5.1 Nutraceutical intervention

Different nutraceutical interventions were devoted to normalizing gut microbiome dysbiosis and to improve biological outcome in different pathological conditions. These nutraceutical approaches include probiotics, ginko biloba, green tea, alpha lipoic acid, vitamin A, biotin, and curcumin. Nutraceuticals are defined as "*food or food product that provides medical or health benefits including the prevention and treatment of diseases*" (Abd El-Salam and El-Shibiny, 2017). This definition is partially overlapping with "bioactive compounds".

Indeed, neuro-nutraceuticals represent active compounds, derived from plants or food products that influence CNS functions. Neuro-nutraceuticals could include amino acids, minerals, vitamins and so on with positive effects on health and disease conditions. Brain aging and neurodegenerative diseases are characterized by redox metals homeostasis, oxidative stress, and inflammation, thus antioxidant and anti-inflammatory molecules can represent a valid strategy for numerous brain diseases, including PD, Alzheimer's disease, depression and dementia (Mandel et al., 2012; Sarubbo et al., 2017).

5.1.1 Probiotics and Prebiotics

Prebiotics are defined as “nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth or the activity of one or a limited number of bacteria (*Bifidobacteria* and *Lactobacilli*) in the colon” (Delzenne and Williams, 2002). Chemically, prebiotics are fructoligosaccharides, enzymatically metabolized in the colon generate short-chain fatty acids with benefit for commensal flora (Shen et al., 2012).

Probiotics are “viable microorganisms, sufficient amounts of which reach the intestine in an active state and thus exert positive health effects” (de Vrese and Schrezenmeir, 2008). The most utilized probiotics are bacterial species (*Bifidobacterium and Lactobacillus*, *E. coli* and *Bacillus* species), but also the yeast *Saccharomyces cerevisiae* (de Vrese and Schrezenmeir, 2008).

These kinds of nutraceuticals positively affect intestinal environment. Indeed, prebiotics affected lipid metabolism, including lower cholesterol (Enciu et al., 2018), and maintained blood-brain barrier integrity, modifying positively gut microbiome through gut-brain axis.

Probiotics are live microorganisms that exert beneficial to humans or animals when consumed (Morgan et al., 2014). *Bifidobacterium* and *Lactobacillus* species are the most used probiotics. Increasing evidences reported the beneficial role of probiotics in cognition and brain health in different neurological disorders. These evidences indicated that probiotics are able to

modulate neurotrophic factors, such as BDNF, dampen neuroinflammation and restore gut permeability and gut microbiome composition. With the term prebiotics it has been indicated non-digestible fibers that are selectively processed in the small intestine and stimulate the colonization of positive gut microbiome, including *Bifidobacterium* and *Lactobacillus*.

Examples of these prebiotics are inulins, oligofructose, fructo-oligosaccharide and galacto-oligosaccharide, which enhance *Bifidobacterium* levels, normalize the composition of *Lactobacillus*, *Bacteroides*, promoting the gut-brain axis and improving gut microbiome composition (Akbari et al., 2016; Depeint et al., 2008; Vulevic et al., 2015). As demonstrated in experimental studies but also in clinical trials, prebiotics dampen inflammation and alleviate psychological distress (Nyangale et al., 2014).

A recent clinical study reported the effects of prebiotics on stress level, measuring the cortisol levels and performing neuropsychological tests. The prebiotics were able to lower cortisol levels and to increase attentional vigilance (Schmidt et al., 2015).

5.1.2 Gut-brain axis and nutraceuticals

Improper diet and gut dysbiosis, concomitantly with age-related variations in gut microbiome and a weakened immune system (Rondanelli, 2015; Salazar et al., 2014) cause an intestinal low-grade inflammation. Finally, this process

induce neuroinflammation, glial cells activation, and to cognitive impairment in the aged individuals (Caracciolo et al., 2014).

The most used model to study gut-brain axis is the GF mouse, which showed socially impaired behavior (Enciu et al., 2018) and present an amplified stress response (Desbonnet et al., 2015) that can be directly correlated with changes occurring in different regions of the brain (Heijtz et al., 2011), low neurogenesis (Collins et al., 2013) and prefrontal cortical hypermyelination (Stilling et al., 2015). Preclinical studies showed that nutraceutical administrations (including probiotics) is able to diminish anxiety and depression and to reestablish brain chemistry (Bercik et al., 2011; Messaoudi et al., 2011). Indeed, the impairment in GF animals was ameliorated upon short-chain fatty acids (Erny et al., 2015). Interestingly, in aged rats, probiotic formulation VSL#3 (Distrutti et al., 2013) was able to ameliorate gut microbiome composition and brain performances, through dampening neuroinflammation and stimulating BDNF and synapsin protein, involved in neural plasticity.

Gut microbiome is fundamental also for nutraceuticals bioavailability, which concomitantly help counteracting neuronal and cell aging under normal circumstances (Rogers et al., 2016). Notably, blueberries (rich in polyphenols) ameliorated spatial memory and motor performances in aged animals (Spencer, 2010), increased neural stem cells proliferation, and insulin-like growth factor-1 (IGF-1) level, the key modulator of hippocampal neurogenesis (Casadesus et al., 2004; Shukitt-Hale et al., 2015).

Animal treated with Omega-3 polyunsaturated acids (n-3 PUFA) showed improved synaptic plasticity, diminished oxidative stress and neuroinflammation and inhibited microglial activation (Cutuli et al., 2014; Hussain et al., 2013). The mechanism by which PUFA counteract cognitive decline, brain atrophy and emotional dysfunctions is anti-inflammatory and antioxidant (Cutuli et al., 2016, 2014). Some evidence in animal models suggested that long-term consumption of fish oil (rich in n-3 PUFA) may predispose the brain to lipid oxidation. Plant nutraceuticals, i.e. phytosterols esters are able to lower cholesterol level, to inhibit oxidative stress and to ameliorate cognitive performances in aged rats (Morris Water Maze). Notably, phytosterols improved cholinergic activity, decreasing acetylcholinesterase activity, increasing choline acetyl transferase and restoring the acetylcholine level (van Kessel et al., 2019). Overall, preclinical investigations suggest that nutraceutical are able to improve brain functions during aging or neurodegeneration, taking benefits from antioxidant, anti-inflammatory and neuroprotective properties of enriched diet.

A recent *in vitro* study reported that probiotics reduced oxidative stress, pro-inflammatory cytokines, and counteracted pathogenic bacterial overgrowth in PD patients. This study was performed in peripheral blood mononuclear cells isolated from PD patients testing different probiotic microorganisms belonging to the *lactobacillus* and *bifidobacterium* genus (Magistrelli et al., 2019).

Specific probiotic strains could counteract pathogens and produce tyrosine decarboxylase (TD) (van Kessel et al., 2019). This enzyme converts levodopa

to dopamine in the gut, even in the presence of a competitive substrate. Indeed, levodopa levels are reduced by the high presence of TD in PD individuals (Nicola et al., 2016).

Probiotic formulations may dampen the inflammation through cytokines production (Nowak et al., 2019), and decrease the oxidative stress through a reduction in ROS (Gazerani, 2019). This aspect is of high interest since PD progression is accelerated in presence of infections (Su et al., 2018). It has been demonstrated that a probiotic formulation VSL#3 is able to control the expression of different genes in the brain cortex of aging animals, dampening the inflammation and improving neuronal performances (Distrutti et al., 2014).

5.1.3 SLAB51 probiotic formulation

Slab51 (sold in Europe as SivoMixx) is a commercial multi-strain probiotic containing 200 billion lactic acid bacteria per 1.5 grams of product, contained of the following strains: Streptococcus thermophilus DSM 32245, Lactobacillus brevis DSM 27961, Lactobacillus acidophilus DSM 32241, Lactobacillus paracasei DSM 32243, Lactobacillus helveticus DSM 32242, Lactobacillus plantarum DSM 32244, Bifidobacterium lactis DSM 32246 and Bifidobacterium lactis DSM 32247.

It has been reported the effect of SLAB51 in counteracting the activity of the parasite Giardia Dudenalis in *in vitro* and *ex vivo* mouse model, by inducing apoptosis (Perrucci et al., 2019). Moreover, SLAB51 treatment in cats with

chronic constipation and idiopathic megacolon induced clinical ameliorations after treatment, by exerting an anti-inflammatory effect, as demonstrated from a decrease of mucosal infiltration, and restoration of the number of interstitial cells of Cajal (Rossi et al., 2018).

This innovative formulation beside exerting these intestinal protective activities, showed beneficial effect on cognitive performances.

Indeed, transgenic 3xTg-AD mice upon SLAB51 presented partial restoration of autophagy and the ubiquitin-proteasome system, concomitant with an improvement in cognitive impairment due to reduced accumulation of amyloid plaques and brain injury. This novel formulation reduced plasma inflammatory cytokines and gut metabolic hormones, therapeutic targets in neurodegeneration. In this study Bonfili and collaborators demonstrated that SLAB51, modulating the microbiota, influenced neuroprotective pathways, counteracting the progression of Alzheimer's disease (Bonfili et al., 2017).

Further, the same research group demonstrated that SLAB51 formulation was able to significantly decrease oxidative stress in AD mice brain, by stimulating SIRT1-dependent mechanisms, thus this formulation could represent a potential adjuvant in AD treatment (Bonfili et al., 2018).

CHAPTER VI

6. AIM OF THE THESIS AND STUDY OBJECTIVES

To date, there are treatments that can help relieve the PD-associated symptoms, but there is no cure to control the onset and progression of this disorder. Altered components of the gut could represent a key role in gut-brain axis, which is a bidirectional system between the CNS and the enteric nervous system. Diet can alter the microbiota composition, affecting the gut-brain axis function. Gut microbiome restoration is able to counteract the PD progression and this effect could be exerted by probiotics. Indeed, during the last 10 years, in clinical trials the protective role of probiotic formulations in neurological disorders, such as depression and autism, was investigate. More recently, a new probiotic formulation (SLAB51) was tested in AD transgenic mice model. The formulation was able to counteract cognitive decline and neuronal injury, to reduce β-amyloid plaques through a SIRT1-mediated mechanism.

Thus, the aim of this thesis work was to investigate the effects of the probiotic formulation SLAB51 in PD. In order to identify potential neuroprotective approaches able to counteract cognitive decline or to be useful as coadjuvant, in this study both *in vitro* and *in vivo* Parkinson's models were used, and the potential therapeutic effects of SLAB51 formulation in PD was investigated. To this purpose, we first tested the

formulation on a PD *in vitro* model and after obtained interesting data, the potential therapeutic of SLAB51 was examined *in vivo* through dopaminergic neurons analyses and behavioral tests.

CHAPTER VII

7. MATERIALS AND METHODS

7.1 In vitro experiments

7.1.1 Preparation of SLAB51 bacterial lysates

Amount of 1 g of Slab51 formulation has been suspended in 10 ml of Phosphate Buffer Saline (PBS, Euroclone, West York, UK) for bacterial lysates preparation, subjected to centrifuge and sonication processes and at the final step the lysates were also filtrated to remove whole bacteria remaining as previously described by (Castelli et al., 2018).

7.1.2 MTS assay

To test cell viability, Cell Titer Cell Proliferation kit according to manufacturer's instructions were used (Promega Corporation Madison, USA). The index of viability, which is dependent on formazan generated, was evaluated using an ELISA reader, Infinite F200 (Tecan, Swiss). Test was performed in quadruplicate. The results were reported as absorbance at 492 nm.

7.1.3 *In vitro* model

The human SH-SY5Y cell line has been purchased from ECACC and cultivated in Dulbecco's minimum essential medium, completed with 10% heat-inactivated FBS and 1% penicillin/streptomycin (Corning, USA) at 37°C in 95% O_2 and 5% CO_2 incubator (Thermo, USA). For the PD in vitro model, the SH-SY5Y cell line was differentiated with all trans-retinoic acid (10mM) and 3 days with phorbol (80nM). At 6 DIV, cells were treated with SLAB51 0.1 mg/ml of extract for 2 hours and then added 6-OHDA (Sigma Aldrich, USA) (35μM) for 24 hours. All experiments were performed at 19th passage and the cell culture were tested to Mycoplasma presence (Mycoplasma PCR, abm, USA).

7.1.4 Immunofluorescence

After culturing the cells as described above, were fixed in 4% PFA in PBS for 15 min and permeabilized in CH_3OH for 7 minutes at −20° C. Cells were incubated in 4% BSA for 30 minutes then with the subsequent primary overnight at 4° C: rabbit polyclonal anti-β-Tubulin III (1:1000 Abcam, Cambridge, UK), rabbit polyclonal anti-TH (1:200, Novus Biologicals, Centennial, USA), mouse monoclonal anti-GAP43 (1:200, Abcam, Cambridge, UK). After several washings, coverslips were incubated with secondary antibodies, goat anti-mouse or anti-goat IgG Alexafluor 488 or 633 or 546 (1:2000 Life Technologies, California, USA), for 1h at RT. After different washes, Vectashield mounting with DAPI (Vector Laboratories

Burlingame, USA) were used. All the samples were observed using confocal laser microscope (Leica, Wetzlar, Germany).

7.1.5 Western blotting

Control and treated cells were collected and lysated as previously described and the protein amount were evaluated (Castelli et al., 2019b). 30 μg of proteins were loaded and separated on precast 4–20% gradient Bis-Tris gel in running buffer at 100 mV for 70 min followed by transfer to PVDF membranes using a semi-dry device (Thermo scientific), then blocked in 5% no-fat milk for 30 minutes. Membranes were incubated with the subsequent primary antibodies overnight: anti-p-AKT (1:1000), anti-PI3K (1:1000 Cell Signaling), anti p-CREB (1:500 Cell Signaling), anti-pTRKb (1:2000 Cell Signaling), anti-mBDNF (1:500 Abcam), anti-pJNK (1:1000 Santa Cruz), anti-p75(1:1000 Abcam), anti-pro-BDNF(1:1000 Millipore), anti-pERK5 (1:1000 Cell Signaling), anti p-ERK1,2 (1:1000 Santa Cruz). After different washes, membranes were incubated with 1:10000 horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG. The protein bands were detected, normalized and analyzed to actin (housekeeping). Anti-β-actin (HRP-conjugate) (1:10000) has been used. To reprobe, membranes have been stripped with Restore stripping buffer (Thermo Scientific, UK) following manufacturer's instructions.

7.2 In vivo experiments

7.2.1 Animals

Animal handling and surgical procedures were performed in order to minimize discomfort and pain, according to the ethical regulations of the European Communities Council (Directive 2010/63/EU, prot #542/2019-PR).

For 6-OHDA lesion experiments, male C57BL/6 mice purchased from Charles River (Massachusetts, USA) 9-week-old were used. Animals (n=30) were kept in ventilated cages (Tecniplast, Germany) under a 12-hour light/12-hour dark cycle with water and food ad libitum. Stereotaxic (Stoelthing, USA) injections of 6-OHDA were performed as previously reported [43]. Briefly, mice were subjected to anesthesia (xylazine (10 mg/kg) and ketamine (200 mg/kg) and then 4 µg of 6-OHDA containing 0.2% L-ascorbic acid (Sigma Aldrich, USA) in saline solution or saline solution with 0.2% L-ascorbic acid (SHAM group) were injected into the right region of the striatum (coordinates relative to bregma: medial-lateral +0.18 cm; anteroposterior + 0.04 cm; dorsal-ventral +0.35 cm) with a rate of 0.5 µl/minute using single syringe nano Infusion KDS 310 (KD Scientific, USA). After injection, we waited for 5 minutes before removal.

7.2.2 Treatment

SLAB51 formulation was provided by Mendes Sa (Lugano, Switzerland). SLAB51 was freshly prepared dissolving one sachet (1,5g/200 billion of bacteria) in 10 ml of water and the treated mice received 270 µl using oral

gavage (corresponding at around 5,4 billion, based on a weight human/weight mice ratio). SLAB51 was administered daily for 2 weeks previous 6-OHDA inoculation and followed for further 3 weeks. Control group received SLAB51 only.

7.1.3 Behavioral Tests

7.1.3.1 *Elevated body swing test*

All investigators performing the behavioral tests were blinded to the treatment condition. To perform EBST, mice were gently picked up at the base of the tail and the direction of the swing, either left or right, was considered until 20 swings as described by (Borlongan and Sanberg, 1995).

5.1.3.2 *Cylinder test*

Cylinder rearing test (Schallert et al., 2000)(was adjusted for use in mice to evaluate forelimb use during normal exploratory behavior and was conducted before 6-OHDA lesion and 1, 2, 3 weeks after the first lesion. Each mouse was positioned in a Plexiglass cylinder 25 cm in height and 11.48 cm in diameter. Spontaneous forelimb contacts while rearing were recorded, until 20 contacts for each animal. The number of paired and impaired forelimb contacts are evaluated as percentage of total contacts observed in the entire observation time.

7.1.4 Morphological analysis

Animals were deeply anesthetized with ketamine/xylazine, before being sacrificed by transcardial perfusion. Mice were perfused at RT with phosphate buffer saline (PBS), followed by 4% PFA in 0.12M phosphate buffer, pH 7.6. Brains were placed overnight in 4% PFA, then cryoprotected in 30% sucrose solution in 0.1M phosphate buffer (PB). Brains from each mouse were embedded in the OCT (Sigma Aldrich, Saint Louis, USA). The blocks were cut by a cryostat to obtain coronal 40 μm thick sections following "Paxinos and Franklin's the Mouse Brain in Stereotaxic Coordinates" (Elsevier).

7.2.5 Immunohistochemistry

Free floating sections were incubated in a 0.3% hydrogen peroxide solution, for 10 min, protected from the light, to block internal peroxidases, and then in PBS 0.5% Triton X-100, 4% BSA for 1 h, RT. Sections were then incubated overnight at 4°C with rabbit polyclonal anti-TH (1:500), in PBS containing 0.4% Triton X-100. In control sections, the primary antibody was omitted. After incubation for 2h at RT with goat anti-rabbit IgG-HRP (Sigma, B7401), 1:100 in PBS containing 0.4% Triton X100, immuno-complexes were revealed using 3,3'-diamino-benzidine (DAB Substrate Kit for Peroxidase, Vector) as the chromogen. After extensive washing, sections were dehydrated and mounted with Permount (Fisher Scientific, US). Slides were observed with a Leica S8 Apo microscope equipped with EC3 camera.

7.2.6 Immunofluorescence

For immunofluorescence experiments, sections were processed as reported in "immunohistochemistry" section and incubated for 24 hours at 4°C with the subsequent primary antibodies: rabbit polyclonal anti-TH (1:500), anti-NeuN (1:1000), anti DAT (1:1000), anti-Iba1 (1:500), anti-GFAP (1:500). Sections were rinsed with PBS and then incubated for 2h at RT in BSA containing 0.4 % Triton X-100, Alexa488 conjugated donkey anti-rabbit IgG 1:500 or Alexa633 conjugated donkey anti-mouse IgG 1:500 (Invitrogen, US). Controls were performed by omitting the primary antibody. Image acquisition in a Leica TCS SP5 confocal microscope was performed and then analyzed by ImageJ software.

7.2.7 Western blotting

Under stereomicroscope, *substantia nigra* and *striatum* were isolated and the different regions were freshly lysate using pestles, and protein extracted and dosed as previously described (Castelli et al., 2019b). Tissues lysates containing 10µg of protein have been separated on 4–13% gradient Bis-Tris gel in running buffer at 100 mV for 80 min. Proteins were transferred into PVDF membranes using a semi-dry device (Thermo scientific, UK). Membranes were washed in tris-buffered saline with 0.05% Tween20, and blocked in 5% no-fat milk for 1 h at RT. Membranes were then incubated overnight at 4°C with the following primary antibodies, diluted in the same blocking solution: anti p-NRF2 (1:5000 Abcam, UK), anti-NFKB (1:2000

Abcam,UK) anti-p-TRKB (1:2000 Cell Signaling), anti-BDNF (1:500 Abcam, UK), anti- PPARγ (1:500, Thermo, USA) anti-HO1(1:1000 Santa Cruz, USA) at 4°C overnight and then incubated with 1:10000 HRP-conjugated anti-rabbit IgG or anti-mouse IgG. The protein bands have been detected with West Pico luminol (Thermo scientific) following kit's datasheet. Through Alliance Q9 (Uvitec, Cambridge, UK) image chemiluminescent bands were detected and using ImageJ program we analyzed each band intensity normalized as indicated in the "Wester Blotting" *in vitro* section.

TUNEL assay

The *substantia nigra* (SN*)* region of the mouse brain was cut at 40 μm on a cryostat and stored at −80 °C. To perform terminal transferase-mediated dUTP nick end-labeling (TUNEL) analyses, sections were fixed in 4% PFA for 30minutes and then washed several times with room temperature PBS. Then, the sections were incubated in cold ethanol/acetic acid 2:1 for 5mins and washed in PBS again. The labeling of neuronal apoptosis in SN sections was performed using the apoptosis detection kit purchased from ThermoScientific (USA), which is based on the *in situ* TUNEL technique using terminal deoxynucleotidyl transferase (TdT) and the images though confocal microscope were acquired (Leica TCS SP5).

7.2.8 Statistics

Statistical analysis has been performed by t-test using PRISM 6 software. For statistical studies, *P<0.05 has been valued as statistically significant.

CHAPTER VIII

8. RESULTS

8.1. In vitro

To investigate if the probiotic formulation SLAB51 contained neuroprotective components, an *in vitro* model of PD was developed, and neuroprotective and neuronal death pathways were analyzed. The induction of the dopaminergic phenotype of SH-SY5Y neuroblastoma cells, described in the Methods section, was analyzed by contrast phase microscopy, Tyrosine Hydroxylase (TH) expression, dopamine production and immunofluorescence (**Fig. 9**). It is possible to observe that, upon retinoic acid (RA)/phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) treatment, cells displayed neuronal morphology, expressed higher levels of TH, produced dopamine and expressed neuronal markers, such as β-tubulin III and growth associated protein-43(GAP-43).

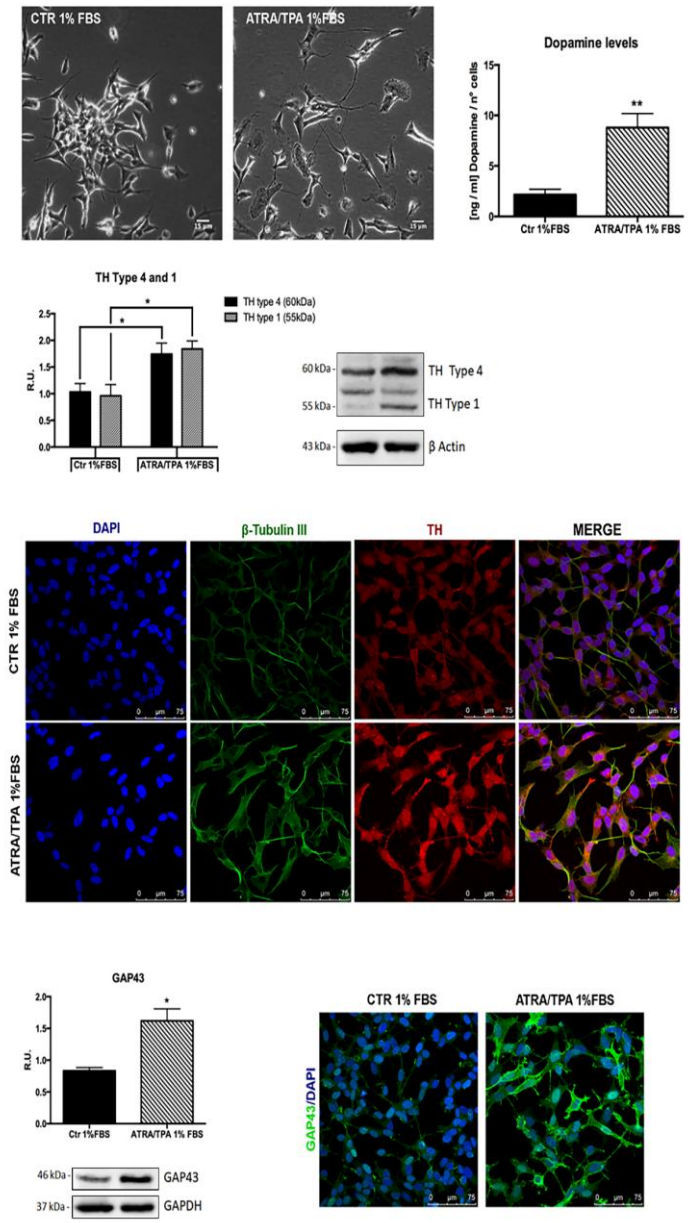

***Fig9**: Dopaminergic phenotype of SH-SY5Y neuroblastoma cells. Contrast phase microscopy of differentiated with ATRA/TPA and not differentiated SH-SY5Y cells and histogram showing dopamine production. Western blotting for TH. Immunofluorescence of β-tubulin III and TH. Western blotting and*

*immunofluorescence for GAP43. Results are mean ± SE of 3 different experiments (n=3). *p< 0,05, **p< 0,005 vs. ATRA/TPA.*

6-hydroxydopamine (6-OHDA) concentration able to induce a significant decrease of cell viability by MTS assay was evaluated (not shown). On this basis, 35 μM 6-OHDA was chosen for the subsequent experiments, since a 50% mortality with this dosage was obtained.

SLAB51 lysates do not shown toxic effect, as demonstrated by cell viability test on differentiated SH-SY5Y; thus, basing on these preliminary results, 0.1 mg/ml of extract was set as testing concentration for the subsequent experiments. In **Fig. 10,** the MTS assay of cells treated with 35 μM 6-OHDA and SLAB51 is shown. As evident, 6-OHDA led to 50% mortality, while SLAB51 was able to counteract 6-OHDA injury and to restore control conditions.

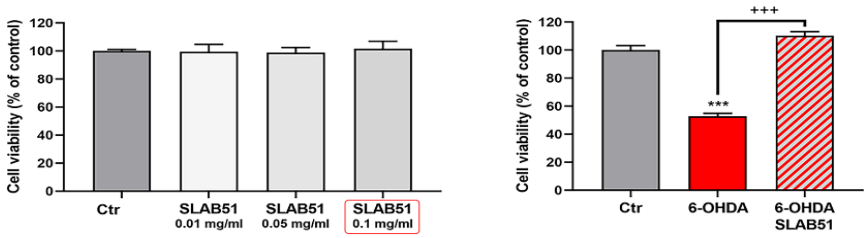

Fig.10*: MTS assay of cells treated with different concentration of SLAB51 (left). MTS assay of cells treated with 35 μM 6-OHDA and 35 μM 6-OHDA and*

*SLAB51 0.1mg/ml (right). Data are mean ± SE of three different experiments run in quadruplicate (n=3). *** p< 0.0005 vs Ctr; +++ p< 0.0005 vs 6-OHDA.*

Since it is worth noting that BDNF pathway is involved in neuroprotection and survival, we first analyzed, by Western blotting, the protein levels of mature BDNF (mBDNF), phosphorylated tyrosine receptor kinase B (p-TrkB), phosphorylated cAMP response element-binding protein (p-CREB) and phosphorylated extracellular-signal-regulated kinase 5 (p-ERK5). In the presence of 6-OHDA, mBDNF and its specific receptor TrkB as well the survival kinase ERK5 were significantly decreased with respect to control neurons, while the presence of SLAB51 restored the control levels (**Fig. 11**). Further, p-CREB, which is known to control mBDNF levels and the survival pathway phosphoinositide 3-kinase (PI3K)/ phosphorylated protein kinase B (p-Akt), was dramatically decreased upon 6-OHDA and restored at control levels by SLAB51 (**Fig. 11**).

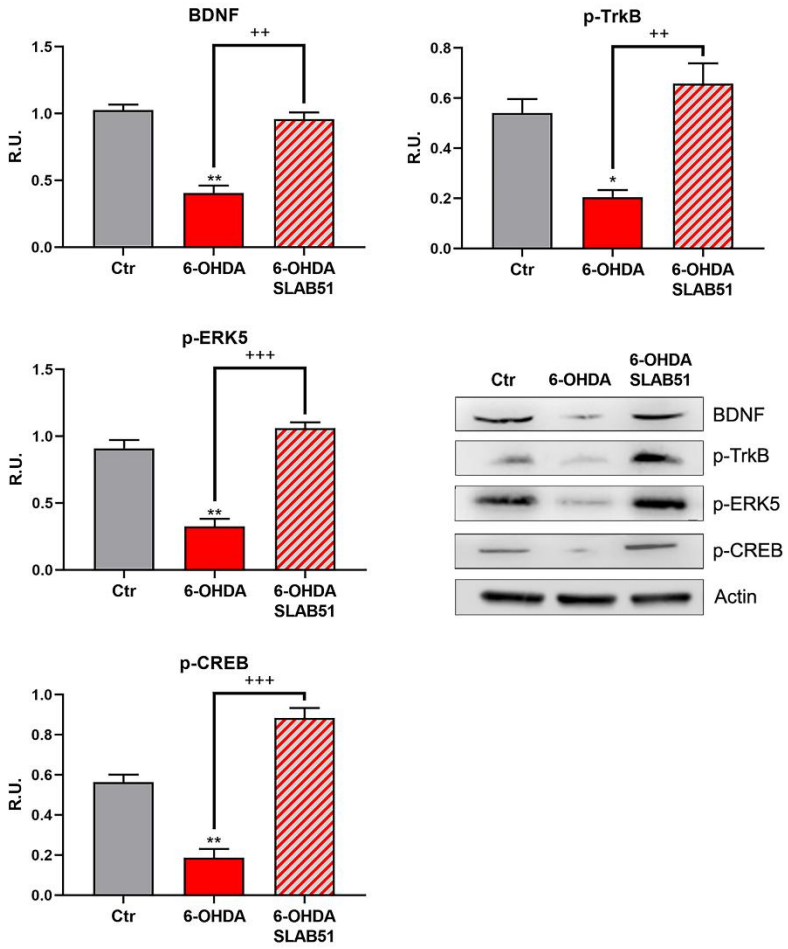

Fig.11*: WB and relative densitometric analysis for Ctr, 6-OHDA and 6-OHDA+SLAB51 for mBDNF, p-TrkB, p-ERK5, p-CREB. Results are mean ± SE of 3 different experiments (n=3). *p< 0.05; ** p< 0.005 vs Ctr; ++ p< 0.005, +++ p< 0.0005 vs 6-OHDA. Representative WB figures are shown.*

Finally, PI3K/Akt pathway, which is involved in neuronal survival and CREB phosphorylation, as well as the postsynaptic density protein 95 (PSD95) appeared strongly downregulated by 6-OHDA, while the presence of SLAB51

counteracted this effect (**Fig. 12**), thus suggesting that the lysate is able to ameliorate the neuronal synaptic plasticity as well the neuronal survival.

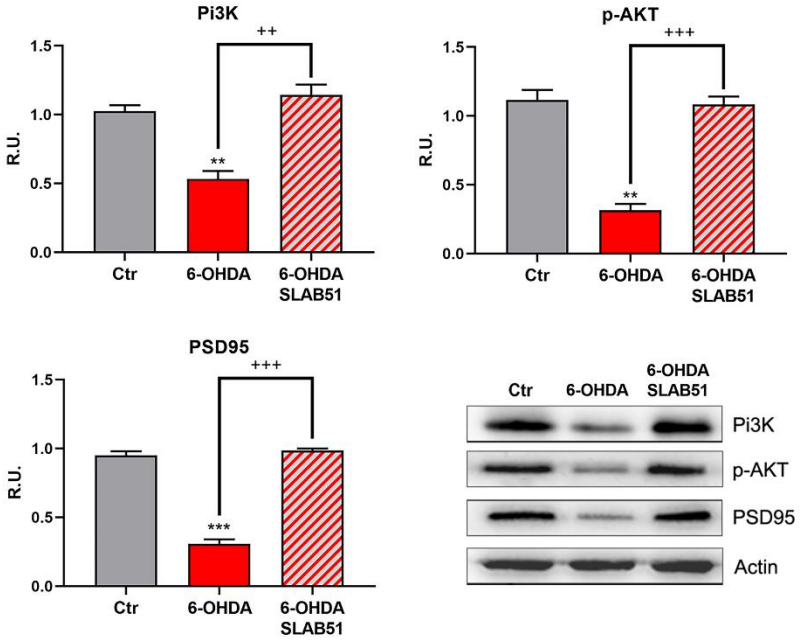

***Fig.12**: WB and relative densitometric analysis for Ctr, 6-OHDA and 6-OHDA+SLAB51 for PI3K, p-Akt, and PSD95. Results are mean ± SE of 3 different experiments (n=3). ** p< 0.005, ***p< 0.0005 vs Ctr; ++ p< 0.005, +++ p< 0.0005 vs 6-OHDA. Representative WB figures are shown.*

The neuronal death pathway, comprising pro-BDNF, phosphorylated c-Jun N-terminal kinase (p-JNK), phosphorylated extracellular signal–regulated kinase (p-ERK1,2) and P75 was then analyzed. All these proteins were significantly increased by 6-OHDA, while the presence of SLAB51 restored the control

conditions, thus confirming a neuroprotective role exerted by the probiotic (**Fig. 13**).

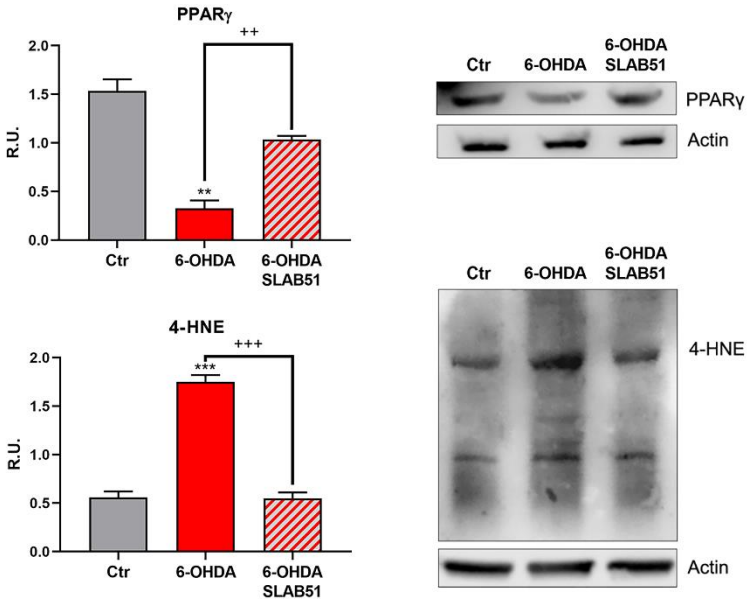

***Fig 13**: WB and relative densitometric analysis for Ctr, 6-OHDA and 6-OHDA+SLAB51 for pro-BDNF, p-JNK, p-ERK1,2 and P75. Results are mean ± SE of 3 different experiments (n=3). ** p< 0.005 vs Ctr; ++ p< 0.005, +++ p< 0.0005 vs 6-OHDA. Representative WB figures are shown.*

Recent evidences reported the involvement of activated peroxisome proliferator activated receptor γ (PPARγ) in modulating BDNF levels in different pathologies, including PD (d'Angelo et al., 2019). Thus, on light of the results collected so far, we assayed PPARγ level through Western blotting analysis. Indeed, in 6-OHDA-treated cells a significant reduction of the

transcription factor was apparent; while SLAB51 lysate increased PPARγ protein levels (**Fig. 14**). Further, since it is known that a product of lipid peroxidation, 4-hydroxynonenal (4-HNE) is generally increased during oxidative stress, as occur in neurodegeneration processes, and to highlight a potential role of probiotics in counteracting 6-OHDA oxidative injury, 4-HNE protein adducts by Western blotting in *in vitro* samples were analyzed. It is possible to observe that the probiotic formulation significantly reduced the level of 4-HNE proteins adducts, thus suggesting a protective role of SLAB51 against oxidative damage (**Fig. 14**). Once obtained the above promising results *in vitro*, in order to evaluate if this formulation was able to modulate neuroprotective pathways in a more complex model, we tested the probiotic in unilateral 6-OHDA-lesioned animal model.

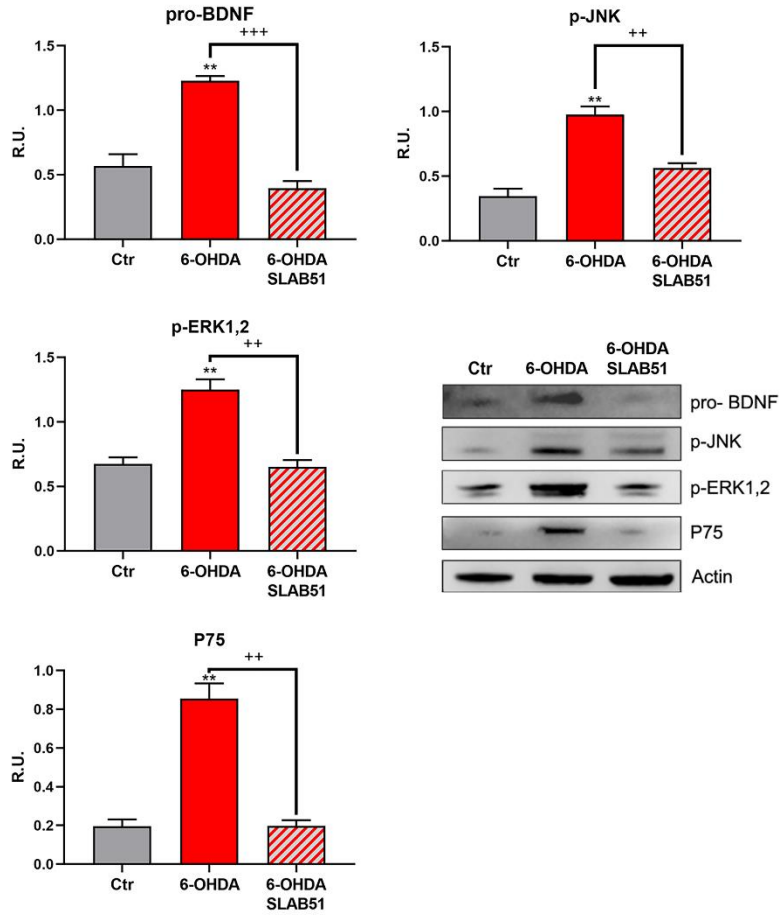

Fig.14: *WB and relative densitometric analysis for Ctr, 6-OHDA and 6-OHDA+SLAB51 for PPARγ and 4-HNE proteins adducts. Results are mean ± SE of 3 different experiments (n=3). ** p< 0.005, ***p< 0.0005 vs Ctr; ++ p< 0.005, +++ p< 0.0005, vs 6-OHDA. Representative WB figures are shown.*

8.2. In vivo

To test the formulation *in vivo*, SLAB51, diluted in drinking water, was daily administered *via* oral gavage for 2 weeks previous 6-OHDA injection and followed for further 3 weeks (as indicated in "Material and Methods" section and in the timeline **Fig. 15A**, total 5 weeks). Oral administration of the probiotic formulation did not generate neither mortality nor significant variances in the average body weights, in control and treated mice (**Fig. 15B**). In **Fig. 15B** also behavioral tests are shown. Notably, the cylinder test, considered to evaluate locomotor asymmetry in rodent models of CNS diseases, showed that striatum lesion induced a robust and significative decrease in mice motor performance, in particular, it was possible to appreciate a decrease of the use of contralateral paw. Interestingly, SLAB51 was able to counteract behavioral impairment induced by 6-OHDA inoculation, restoring the control conditions.

The Elevated Body Swing Test (EBST), index of asymmetrical motor performance of hemi-parkinsonian models in a drug-free state, in the same figure is shown. 6-OHDA-lesioned mice showed right-biased swings of 70% or greater respect to control animals, while probiotic formulation treatment was able to counteract this effect. These findings were confirmed by apomorphine test, a tool to monitor motor impairment and functional improvement (Björklund and Dunnett, 2019). In particular, the dopamine (DA) receptor agonist apomorphine (APO), acting post-synaptically and hyperstimulating supersensitive DA receptors in the denervated *striatum*, leads the animal to

rotate in the opposite contralateral direction, i.e., away from the lesioned side. Indeed, in our experimental conditions, upon 6-OHDA mice showed increased contralateral rotations, while the 6-OHDA mice treated with the probiotic formulation had a similar behavior to Sham groups (**Fig. 15B**).

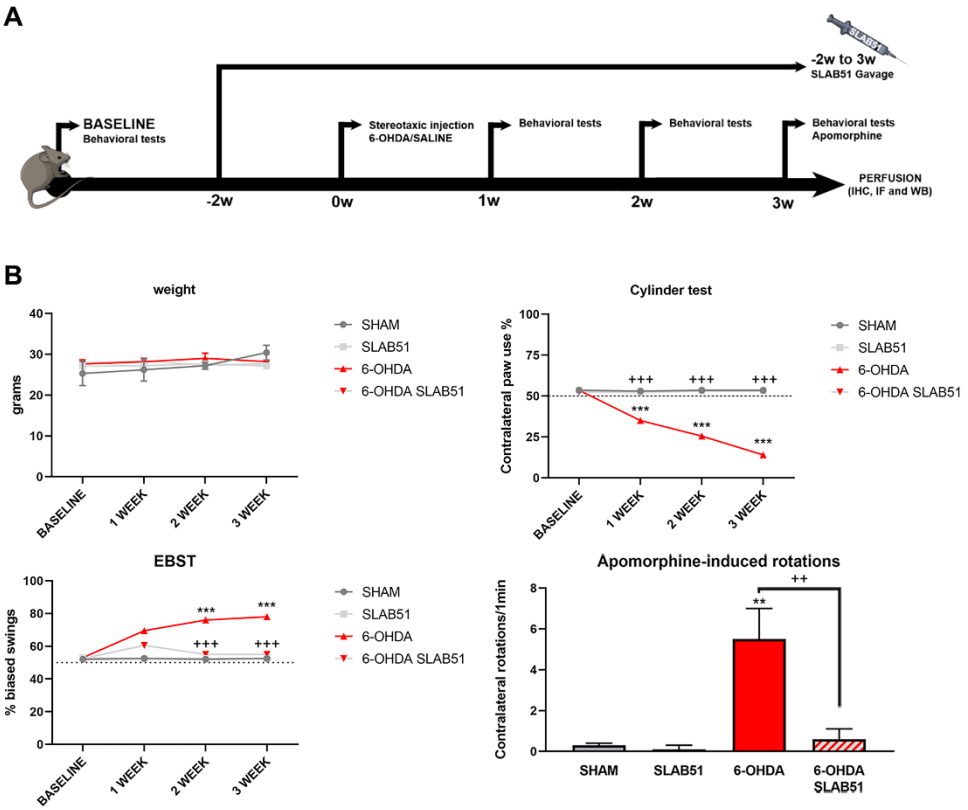

Fig. 15*: A) Procedural timeline with specific timepoints. B) Body weight and behavioral tests in sham, SLAB51, 6-OHDA and 6-OHDA+SLAB51 animals. ** p< 0.005, *** p< 0.0005 vs Ctr; ++ p< 0.005, +++ p< 0.0005 vs 6-OHDA.*

Further, the immunostaining of TH in dopaminergic neurons was performed; it was possible to observe in 6-OHDA-treated animal a marked decrease of TH immunoreactivity, while SLAB51 rescued dopaminergic neurons in both *substantia nigra* and *striatum* (CPu) (**Fig. 16**).

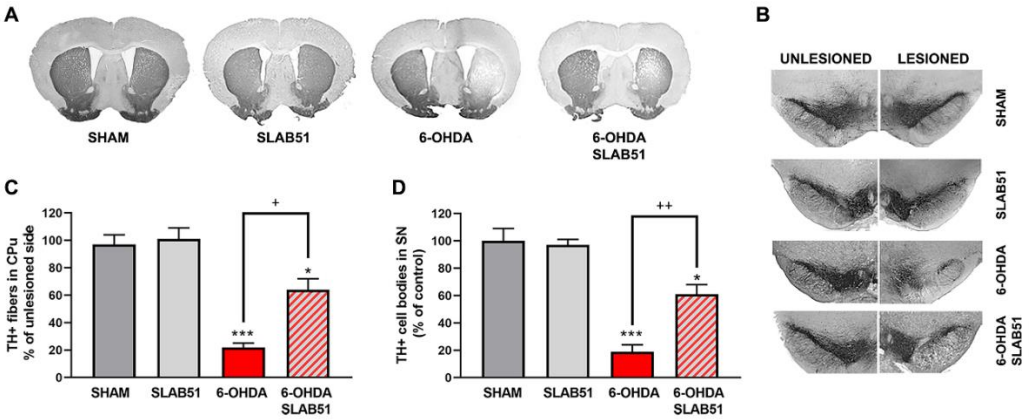

***Fig. 16**: Immunostaining of TH in dopaminergic neurons. Transverse section taken through the substantia nigra pars compacta (SNc) and the ventral tegmental area (VTA), immunostained for TH to evaluate the dopaminergic-induced injury by stereotaxic injection of 6-OHDA in the right side. Histograms shows the percentage of TH+ fibers loss in striatum (CPu) and TH+ cell bodies in substantia nigra (SN) (expressed in percentage of unlesioned side). * $p < 0.05$, *** $p < 0.0005$ vs Ctr; + $p < 0.05$, ++ $p < 0.005$ vs 6-OHDA.*

To further validate these data, immunofluorescence analyses for Dopaminergic Transporter (DAT) in mice *substantia nigra* were performed. As reported in **Fig. 17**, a massive reduction of DAT fluorescence intensity was observed upon

6-OHDA inoculation (in the right side), while the probiotic formulation counteracted this effect, thus suggesting that SLAB51 exerted neuroprotective activities.

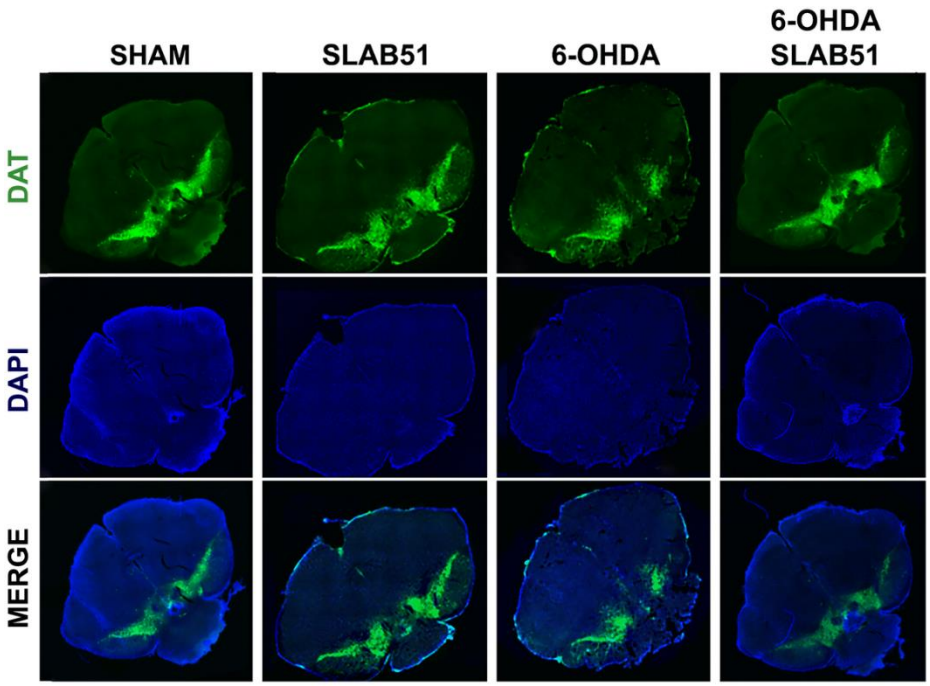

***Fig. 17**: Immunofluorescence for DAT in mice Substantia nigra. Images were taken at confocal microscope at 20x magnification.*

Recent studies have demonstrated neuroinflammation and microglia activation in PD (d'Angelo et al., 2019). For this reason, immunofluorescence analyses and quantification for the specific marker of microglial activation, microglia ionized calcium-binding adapter molecule 1 (Iba1) and for astrogliosis, glial

fibrillary acid protein (GFAP) in brain slices were performed (**Fig. 18**). It is possible to observe a significant increase of Iba1 and GFAP fluorescence intensity in 6-OHDA slices, while SLAB51 was able to counteract 6-OHDA-induced effects, thus indicating a reduction in neuroinflammation.

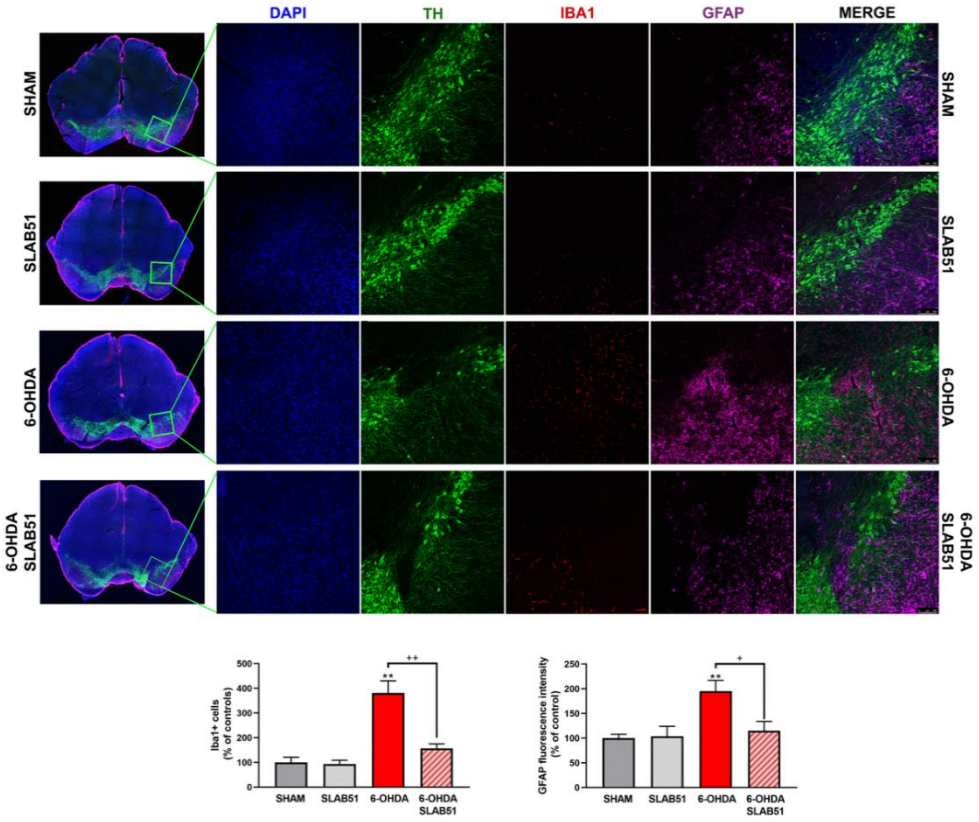

Fig.18*: Triple immunostaining at 20x magnification for Iba1, TH and GFAP, nuclei were counterstained with DAPI. On the left it is possible to appreciate mosaic figures, while on the right inset at higher magnification for TH, Iba1 and GFAP staining and merge figures were reported. Histograms for Iba1 show the number of Iba1 + cells, while for GFAP the fluorescence intensity, as % of controls, is plotted. ** p< 0.005 vs Ctr; + p< 0.05, ++ p< 0.005 vs 6-OHDA*

On this basis, PPARγ, a ligand-dependent transcription factor involved in neuroinflammation, oxidative stress and energetic metabolism, that is also able to stimulate neurotrophins release (including BDNF) (d'Angelo et al., 2019), was analyzed by immunofluorescence in brain slices (**Fig. 19**).

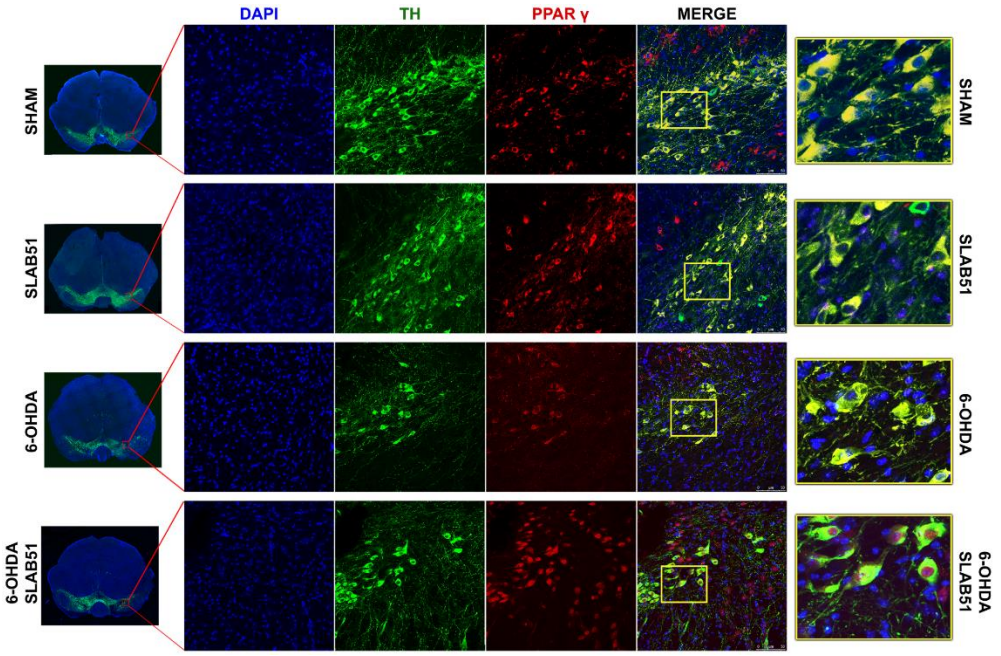

Fig.19*: Immunofluorescence analysis for PPARγ in substantia nigra. On the left, the mosaic images obtained using confocal microscopy at 20x magnification were shown. In the center, double immunostaining at 40x magnification with TH and PPARγ as well as the merge figures were reported. On the right it is possible to observe the inset of the indicated boxes.*

Interestingly, SLAB51-treated samples showed the transcription factor at nuclear level, while in 6-OHDA-treated animals, the fluorescence intensity for PPARγ was strongly decreased and, further, localized at cytoplasmic level. In control and SLAB51-treated samples the transcription factor was present at the same level with a cytoplasmic localization. In 6-OHDA-treated animals, the fluorescence intensity for PPARγ was strongly decreased and localized at cytoplasmic level. Interestingly, the probiotic formulation was able to restore PPARγ levels in 6-OHDA-animals to those of control animals, but in this case, PPARγ was localized to the nucleus, thus suggesting its activation probably responsible for the reduced neuroinflammation and for neuroprotection by the modulation of BDNF pathway, as confirmed by Western Blot analysis performed on *in vivo* samples. Indeed, BDNF and its receptor TrkB showed the same behavior of PPARγ, suggesting that SLAB51 was able to counteract the toxin-induced lesion both in *substantia nigra* and *striatum* (**Fig. 20**).

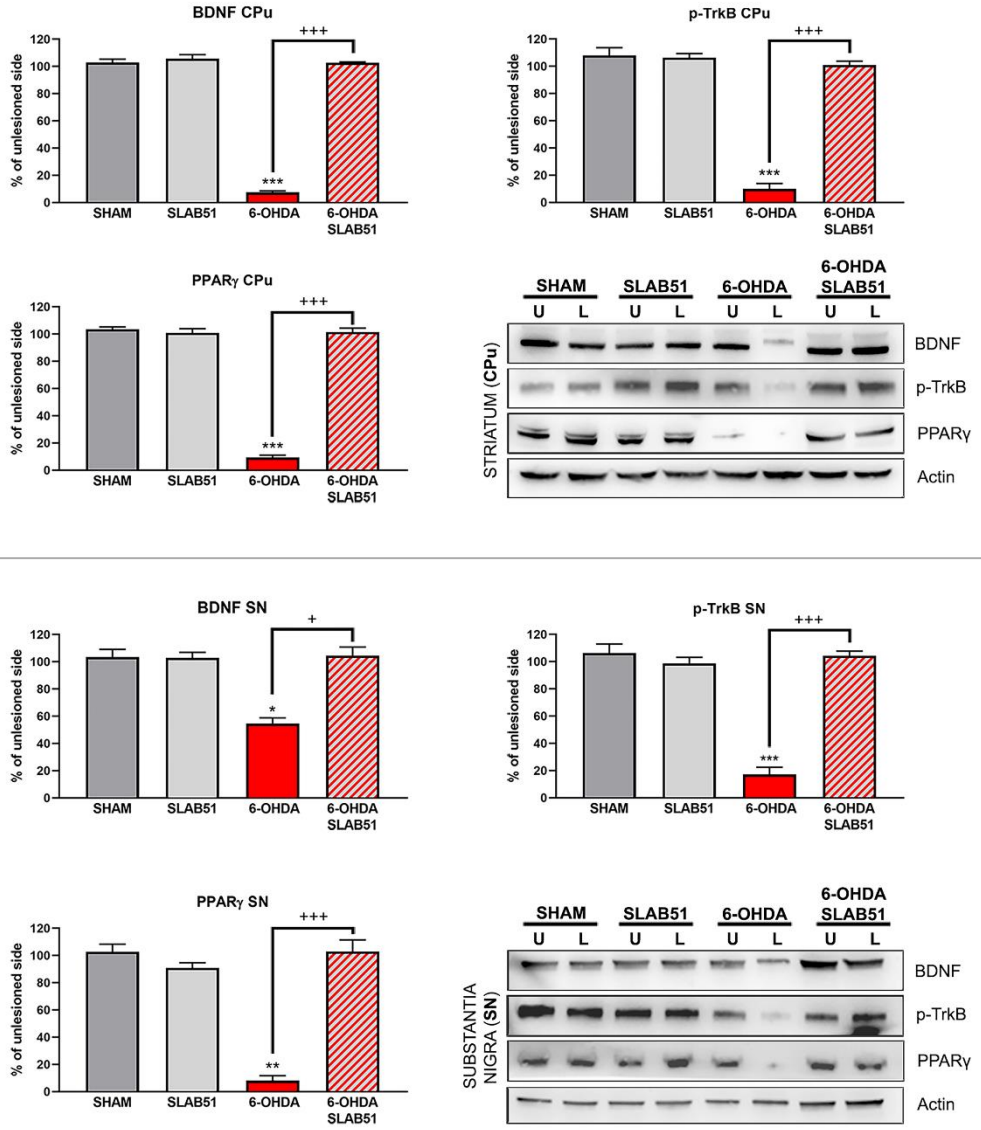

Fig.20: *Western blotting and relative densitometric analysis for mBDNF, p-TrkB and PPARγ in substantia nigra (SN) and striatum (CPu). Results are mean ± SE of 3 experiments (n=3). * p< 0.005, ** p< 0.005, *** p< 0.0005 vs Ctr; + p< 0.005, +++ p< 0.0005 vs 6-OHDA. Representative WB images are shown.*

Recent findings indicated that hemeoxygenase-1 (HO-1) is regulated by upstream regulators of PPARγ (Cho et al., 2018). The antioxidant enzyme HO-1 with established cytoprotective effects has been demonstrated to modulate several pathological processes, including PD. Notably, HO-1 is involved in the release of neurotrophic factors, in the sustainment of dopaminergic neuronal survival in *substantia nigra*, and in preventing α-synuclein aggregation (Zakaria et al., 2019). Indeed, in our experimental conditions, 6-OHDA significantly decreased HO-1, while the probiotic formulation was able to counteract 6-OHDA effects, reverting the levels of HO-1 to those of control condition as shown in **Fig. 21.**

In addition, nuclear transcription factor-erythroid 2 related factor (Nrf2) is able to bind the antioxidant response element (ARE) present in the HO-1 promoter region (Park et al., 2013). Nrf2 activity decreases with aging and represent one of the main PD risk factors, indeed, the increase of Nrf2 provides protection to dopaminergic neurons by counteracting oxidative stress injury (Jing et al., 2016). As shown in **Fig. 21**, p-Nrf2 (transcriptionally active) protein levels were significantly downregulated in 6-OHDA-injured animals, while SLAB51 was able to counteract 6-OHDA effects, both in *substantia nigra* and *striatum*.

Further, NRF2-ARE system interacts with nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a protein complex involved in cell survival and cytokine release, related to neurodegenerative and

neuroinflammatory conditions. Indeed, in our experimental conditions, NF-κB protein levels were upregulated upon 6-OHDA challenge, while the probiotic treatment reverted this protein to control conditions both in *substantia nigra* and *striatum* (**Fig.22**).

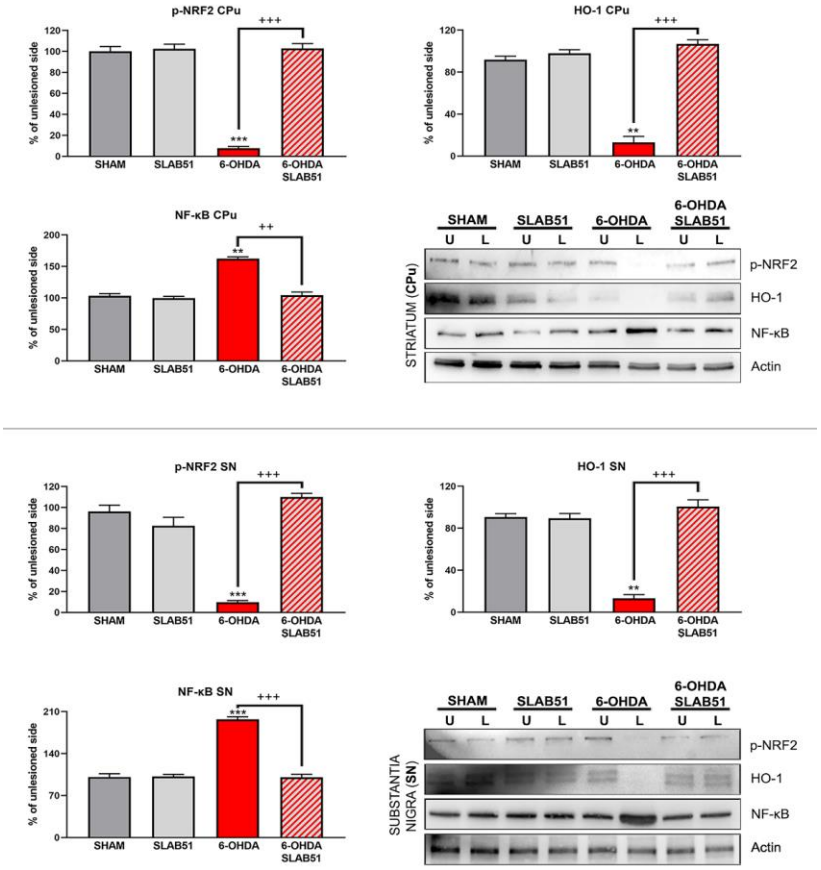

Fig.21*: Western blotting and relative densitometric analysis for p-Nfr2, HO-1 and NF-KB in SN and CPu. Results are mean ± SE of 3 experiments (n=3). ** p< 0.005, *** p< 0.0005 vs Ctr; ++ p< 0.0005, +++ p< 0.0005 vs 6-OHDA. Representative WB images are shown.*

Finally, to confirm the potential pro-survival effect of SLAB51, the apoptosis promotion in dopaminergic neurons of *substantia nigra* was analyzed by TUNEL assay. As reported in **Fig 22,** apoptosis dramatically increased in 6-OHDA treatment, while the presence of the probiotic mixture reduced apoptotic nuclei index (to about 10%), thus suggesting that the probiotic formulation protects against 6-OHDA-mediated apoptosis.

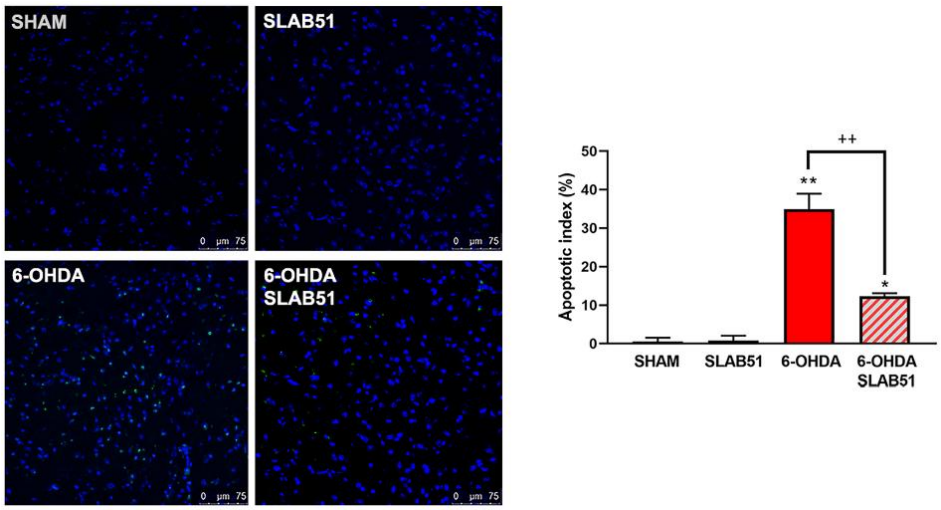

Fig.22*: TUNEL assays in mice substantia nigra. Figures were taken at confocal microscope at 40x magnification. The graph shows apoptotic index obtained by counting positive nuclei. * p< 0.005, ** p< 0.005 vs ctr; ++< 0.005 vs 6-OHDA. Representative figures are reported.*

CHAPTER IX

9. DISCUSSION AND CONCLUSION

PD is a common neurodegenerative disorder, characterized by motor and non-motor symptoms, including abnormalities in the gut function, which may appear before the motor sign (Cersosimo et al., 2013). From a molecular point of view, PD underlying mechanisms include increased oxidative stress and inflammation (Castelli et al., 2019a). To date, there available treatments can help relieve PD-associated symptoms, but there is no cure to control the onset and progression of this disorder.

A growing body of evidence reported that the use of probiotics can have positive influences on CNS disease, altering the gut microbiota, through the gut-brain axis (Martin et al., 2018), mediated by numerous pathways such as immune, neural, inflammatory, and hormonal signaling (Kim and Shin, 2018).

Probiotics are live microorganisms residing in the intestine and are beneficial for their hosts and avoid certain diseases (Khalighi et al., 2016).

Recently, it has been reported that modulating the gut microbiota, by using SLAB51, a mixture of bifidobacteria and lactic acid bacteria, affected different neuronal pathways, delaying the Alzheimer's disease progression by affecting different neuronal pathways (Bonfili et al., 2018).

On this basis, the aim of our study was to investigate the effects of SLAB51 formulation in PD. The effect of this formulation in 6-OHDA were first studied in the *in vitro* model

In neurodegenerative diseases, including PD, reduced neurotrophic support has been reported (Mercado et al., 2017; Sangiovanni et al., 2017). BDNF, a member of the neurotrophin family, maintains the survival and the differentiation of dopaminergic neurons. *In vitro,* BDNF counteracts the dopaminergic neurons death, indicating its potential use in the advance of neuroprotective therapeutic approaches for PD (Sarkar et al., 2016). Accordingly, our *in vitro* studies indicated that the probiotic formulation SLAB51 modulates the BNDF pathway, increasing neuroprotective protein levels and decreasing the neuronal death proteins, confirming a neuroprotective effect exerted by the probiotics.

Thus, we investigated the probiotic *in vivo* assessing behavioral tests and, interestingly, SLAB51 was able to counteract the detrimental effect of 6-OHDA. The behavioral benefits using a therapeutic approach can be achieved through direct stimulation of the dopaminergic receptor or by protecting the dopaminergic neurons from 6-OHDA toxicity. Basing on the immunohistochemistry results, we can propose an involvement of a direct protection against dopaminergic neurons loss in the *substantia nigra*, thus protecting the nigro-striatal pathway.

Besides the anti-inflammatory activity of SLAB51 that we detected by immunofluorescence, the antioxidant activity of the probiotic may also contribute to its neuroprotective effects *in vivo,* as suggested by the decrease of lipid peroxidation. Nrf-2 is a transcription factor involved in PD pathogenesis that controls cellular redox status via endogenous antioxidant systems concomitant with anti-inflammatory effect. HO-1 is a Nrf2 target gene, which is at the core of Nrf2-mediated NFκB inhibition (Sivandzade et al., 2019). Indeed, in our *in vivo* experiments the probiotic formulation tested was able to counteract 6-OHDA-induced dysfunction, reestablishing the activity of Nrf2/HO-1 pathway and inhibiting NFκB. In agreement, in a recent work on a progressive neurodegeneration mouse model induced by lipopolysaccharide, the treatment with a known PPARγ agonist, pioglitazone, was able to increase Nfr2 and HO-1, while reducing NFκB (Zakaria et al., 2019), thus supporting the pivotal role of activated-PPARγ in counteracting oxidative stress as well as neuroinflammation.

It is worth noting that the two models utilized in this research are quite different. In particular, the *in vivo* is a model that most resembles the clinical conditions, while the *in vitro* model is an isolated model, useful to dissect what happens in a single dopaminergic neuron. It is intriguing that, however, we obtained almost the same results in both models. It is possible to speculate that the common denominator may be the PPARγ that both in vivo and in vitro might be activated by some products or derivatives of bacteria metabolism. In

this way, activated PPARγ may trigger anti-inflammatory and antioxidant activities as well as the increase in BDNF and its pro-survival pathways.

Our *in vivo* study showed that the probiotic administration was able to protect dopaminergic neurons and to improve behavioral impairments. This novel probiotic formulation was able also to counteract neuroinflammation and oxidative stress, characteristics of PD, reverting some underlying molecular pathways to control conditions, both *in vivo* and *in vitro.*

Overall, our findings propose SLAB51 as a promising candidate for PD prevention or treatment or as coadjuvant therapy, confirming that the modulation of the gut microbiota affects different pro-survival pathways, possibly leading to a delay of PD progression. Further studies will be necessary to analyze the microbiota composition of PD group *versus* probiotic-treated group, through amplification sequencing methods or hybridization on microarrays.

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