

Therapeutic potential of microbiome based interventions in a mouse model for Parkinson's disease

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During the writing process of this master's dissertation, ChatGPT3.5 was used to rephrase sentences, and to improve spelling and grammar.

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List of Abbreviations

6-OHDA	6-hydroxydopamine
ANOVA	Analysis of variance
ASO	Alpha-synuclein overexpressing
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
CNS	Central nervous system
CSF	Cerebrospinal fluid
DAB	3,3'-diaminobenzidine
DAPI	4',6-Diamidino-2-phenylindole
DAT	Dopamine transporter
DBS	Deep brain stimulation
DLB	Dementia with Lewy bodies
DMV	Dorsal motor nucleus of the vagus
ELISA	Enzyme linked immunosorbent assay
ENS	Enteric nervous system
FMT	Fecal microbiota transplantation
GABA	Gamma-aminobutyric acid
GFAP	Glial fibrillary acidic protein
GI	Gastrointestinal
GWA	Genome-wide association
IBA1	Ionized calcium-binding adaptor molecule 1
icv	Intracerebroventricular
IHC	Immunohistochemistry
IL-1 β	Interleukin-1beta
IL-6	Interleukin-6
ip	Intraperitoneal
KO	Knockout
LB	Lewy bodies
LBP	Live biotherapeutic product
LC	Locus coeruleus
LN	Lewy neurites

LPS	Lipopolysaccharide
LRRK2	Leucine Rich repeat kinase 2
MAC	Membrane attack complex
MAO-B	Monoamine oxidase B
MDS-PD	Movement Disorder Society PD
Mpi	Months-post injection
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MSA	Multiple system atrophy
NMB	Nucleus basalis of Meynert
OB	Olfactory bulb
PBS	Phosphate buffered saline
PD	Parkinson's disease
PFA	Paraformaldehyde
PFF	Pre-formed fibril
PNS	Peripheral nervous system
pS129	Phosphorylated residue serine 129
RBD	REM sleep behavior disorder
REM	Rapid eye movement
RT	Room temperature
SCFA	Short-chain fatty acid
SEM	Standard error of the mean
SNCA	Synuclein Alpha
SNpc	Substantia nigra pars compacta
SNpr	Substantia nigra pars reticulata
SPF	Specific pathogen free
TGF β	Transforming growth factor beta
TH	Tyrosine hydroxylase
TLR	Toll-like receptor
TNF- α	Tumor necrosis factor-alpha
VTa	Ventral tegmental area
WT	Wildtype
α -syn	Alpha-synuclein

English summary

Parkinson's disease (PD) is the second most common neurodegenerative disorder. At present, there is no cure for the disorder. The current gold standard of symptomatic treatments, levodopa, is associated with the development of involuntary movements or dyskinesias in the majority of patients over the course of years of treatment. Furthermore, over time, many patients experience a reduction in effectiveness of levodopa. Today, the clinical diagnosis is made based on typical motor symptoms, including bradykinesia, muscular rigidity, dyskinesia, rest tremor, and postural and gait impairment. The typical motor symptoms result from the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc), a key pathological hallmark of PD. Other hallmarks include neuroinflammation and the misfolding, aggregation, and accumulation of alpha-synuclein (α -syn). In addition to the motor symptoms, PD is characterized by non-motor symptoms, such as constipation, which can precede motor symptoms by years. Based on primary sites of α -syn accumulation, PD patients are classified into gut-first and brain-first phenotypes. Moreover, recent studies highlight the importance of the gut microbiome in PD, revealing significant differences in gut composition between PD patients and healthy individuals, including a reduction in *Prevotella* bacteria. This gut dysbiosis is thought to trigger or exacerbate PD progression. Understanding the microbiota-gut-brain connection in PD may lead to novel treatments targeting gut microbiome dysbiosis.

In collaboration with MRM Health, this project aimed to assess the therapeutic potential of specific bacterial species, namely *Prevotella* bacterial cocktails, on PD phenotype and pathology. To this end, 2 *Prevotella* live biotherapeutic products (LBPs) with different strain compositions and one consortium LBP (CC113), an 11-strain consortium build with strains selected based on literature and preliminary *in vitro* results, were used. The study used intrastriatal α -syn preformed fibrils (PFF) injection PD mouse models, one representing early-stage and the other late-stage PD. Additionally, to investigate the direct impact on neuroinflammation, the study incorporated an intracerebroventricular (icv) lipopolysaccharide (LPS) injection mouse model. Phenotypic analysis encompassed motoric tests and gastrointestinal (GI) transit time assessment, while pathological analysis involved evaluation of α -syn aggregation and neuroinflammation, specifically microglia characteristics and C3 levels in the brain. Immunohistochemistry (IHC) coupled with arivis Vision4D or Aiforia software was employed for microglial and α -syn aggregation analysis, while C3 levels were determined via enzyme-linked immunosorbent assay (ELISA).

The results indicate that among the tested *Prevotella* LBPs, none exhibited significant effects on motor behavior, α -syn pathology, or neuroinflammation in early-stage PD mice. Conversely, administration of the CC113 cocktail exacerbated α -syn pathology and motor deficits in late-stage PD mice. Notably, *Prevotella* cocktail 1 LBPs demonstrated a small promising trend towards reducing pS129- α -syn levels in the early-stage PD mouse cohort. In addition, the LBPs consisting of the *Prevotella* cocktail 2 strains and consortium CC113 strains did not show conclusive effects on microglial characteristics in an LPS-injected mouse model. However, further research is needed to validate these findings. Analyzing multiple brain sections per mouse is a crucial initial step toward obtaining more robust results, followed by validation in independent cohorts with the same or increased sample size, dependent on practical feasibility.

Part 1: Introduction

1.1. PD

1.1.1. *Epidemiology*

PD, initially characterized as a "shaking palsy" by James Parkinson in 1817, manifests as an age-related progressive movement disorder. It stands as the second most prevalent neurodegenerative disease following dementia.

Over the past quarter-century, there has been a notable twofold increase in the incidence rate of PD. According to research, the prevalence and incidence rates of PD in Europe range between 108–257/100,000 and 11–19/100,000 per year, respectively (Balestrino & Schapira, 2020). Global prevalence rates for PD range from 5 to more than 35 new cases per 100,000 people per year (Poewe *et al*, 2017). PD is infrequent before the age of 50, yet its incidence surges by 5 to 10 times from the 6th to the 9th decade of life. Its prevalence, conservatively estimated at 0.3% worldwide, rises sharply with age and exceeds 3% in people over 80 (Poewe *et al*, 2017). Regarding incidence, studies indicate a higher propensity for men to develop PD compared to women (Wooten, 2004). Potential explanations for the elevated risk in men may include exposure to toxicants, head injuries, neuroprotective effects of estrogen, mitochondrial dysfunction, or genetic risk factors linked to the X chromosome (Hong *et al*, 2020; Li *et al*, 2015). The occurrence of PD also appears to fluctuate across subgroups delineated by factors such as race, ethnicity, genotype, or environmental influences (Poewe *et al*, 2017). For example, there is evidence of a possibly lower prevalence of PD among African-Americans and Asians in the United States. As for mortality, no notable elevation in mortality rates is observed during the initial decade following the onset of the disease (Poewe *et al*, 2017; Pinter *et al*, 2015). However, mortality rates gradually increase thereafter, eventually reaching twice the rate compared to the general population. Improved healthcare services have contributed to extended survival periods, resulting in a corresponding rise in the prevalence of PD over a 20-year study period (Poewe *et al*, 2017; Lix *et al*, 2010).

1.1.2. *Etiology*

For several years, PD was predominantly perceived as sporadic, with genetic factors being deemed insignificant (Bekris *et al*, 2010). However, although the majority cases of PD are idiopathic, 5-10% of all cases are due to an inherited gene abnormality (Pinter *et al*, 2015). Mutations are thought to cause disease directly by inducing abnormal and possibly toxic protein conformations. Approximately 30% of familial cases and 3-5% of sporadic cases of PD can be attributed to a monogenic form, where a mutation in a single gene is responsible for the disease (Chang *et al*, 2013). Nevertheless, no known genetic variant is fully penetrant, indicating the possibility of other genetic or environmental variables influencing the progression of the illness (Andruska & Racette, 2015). Several PD genes have been identified, including PARK2, Leucine Rich repeat kinase 2 (LRRK2) and deglycase DJ-1. The first gene found to be linked to familial PD is Synuclein Alpha (SNCA) (Konnova & Swanberg, 2018). The corresponding α -syn protein is the major component of Lewy bodies, which are a hallmark for familial and sporadic PD. Monogenic variants in SNCA and LRRK2 lead to autosomal dominant PD, while monogenic mutations in Parkin and DJ-1 lead to autosomal recessive PD (Chang *et al*, 2013). Additionally, through the discovery and replication of risk loci, genome-wide association (GWA) studies have provided insight into the genetic basis of this illness (Nalls *et al*, 2014; Poewe *et al*, 2017). The loci found have implicated new proteins in the pathogenic

cascade and confirmed the pivotal function of genes previously associated with PD. The discovery of novel monogenic PD mutations has been used to investigate the effects of mutated proteins and to create new reliable animal models, where most genetic models have succeeded in reproducing only some features of PD (Konnova & Swanberg, 2018).

The environmental hypothesis states that PD-related neurodegeneration results from exposure to an environmental dopaminergic neurotoxin (Salama & Arias-Carrión, 2011), such as herbicides and pesticides. In a meta-analysis study, exposure to pesticides such as paraquat and rotenone was demonstrated to be correlated with a >50% increased risk of developing PD (Tanner *et al*, 2011). Furthermore, after the neurotoxic metabolite 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) was shown to be a trigger for the onset of PD, the importance of pesticide exposure increased (Langston *et al*, 1983). Intoxication with MPTP was shown to lead to a syndrome nearly identical to PD (Sian *et al*, 1999; Meredith & Rademacher, 2011). The neurotoxin elicits its neurotoxic effects by inducing oxidative stress for example, but also mitochondrial apoptosis, inflammation, excitotoxicity, and the formation of inclusion bodies (Mustapha & Mat Taib, 2021). These processes lead to damage of dopaminergic neurons within the SNpc and striatum. Therefore, the MPTP-induced mouse model has been widely used in PD research. Other toxin-based mouse models for PD have also been developed, such as 6-hydroxydopamine (6-OHDA), paraquat and rotenone. Other environmental agents that are thought to be associated with PD and contribute to its progression, are heavy metals, such as iron, copper, aluminum and lead (Pyatha *et al*, 2022; Altschuler, 1999). Dairy product consumption and traumatic head injury have also been shown to be associated with PD (Baixauli *et al*, 2014). Furthermore, consumption of caffeine and tobacco smoking have been shown to decrease the risk of PD (Hong *et al*, 2020; Li *et al*, 2015).

1.1.3. **Diagnosis and symptoms**

Based on the occurring symptoms, PD can be categorized into distinct stages, namely the preclinical stage, the prodromal stage, and the clinical stage which can be divided into the early-stage, mid-stage and late-stage PD (**Figure 1**).

PD is characterized by typical **motor symptoms** such as bradykinesia, muscular rigidity, dyskinesia, rest tremor, and postural and gait impairment (Kalia & Lang, 2015). These symptoms occur because of the loss of dopaminergic neurons in the SNpc, one of the pathological hallmarks of PD (Schaeffer *et al*, 2020). The first clinical symptom, namely bradykinesia, is defined as slowness of movement and speed (Berardelli *et al*, 2001), while dyskinesia refers to uncontrolled movements (Kalia & Lang, 2015). Rigidity is defined by elevated muscular tone upon palpation during rest, diminished compliance to passive movement, and heightened resistance to stretching (Rodriguez-Oroz *et al*, 2009). Postural instability and gait impairment become more prominent after long-term disease progression in advanced stages of PD (Kalia & Lang, 2015; Rodriguez-Oroz *et al*, 2009). The final main clinical symptom is resting tremor, indicative of the presence of rhythmic involuntary movements of a body part while the patient is in a state of relaxation (Baumann, 2012).

PD is also associated with several other **non-motor features**, including olfactory dysfunction, cognitive impairment, psychiatric symptoms, autonomic dysfunction, pain, and fatigue (**Figure 1**). Furthermore, some of the features appear before motor dysfunction. This stage, termed the prodromal phase, is a period during which the loss of dopaminergic neurons occurs prior to the onset of the characteristic motor symptoms (Postuma *et al*, 2012). The prodromal

period is associated with symptoms such as depression, rapid eye movement (REM) sleep behavior disorder (RBD) and constipation (**Figure 1**)(Goldman & Postuma, 2014). The latter already points towards the importance of the gut in PD, which will be discussed more in detail in section 1.4. RBD is defined by the absence of REM sleep paralysis, enabling patients to physically enact their dreams (Postuma *et al*, 2015b; Schenck *et al*, 2013). Idiopathic RBD serves as a strong predictor of neurodegenerative synucleinopathies such as PD, with over 80% of cases progressing to neurodegenerative illness (Iranzo *et al*, 2013; Schenck *et al*, 2013; Postuma *et al*, 2015b). Initially, however, there is a preclinical PD phase in which neurodegeneration occurs without obvious symptomatic signs.

Presently, because of the absence of biomarkers, motor symptoms remain the fundamental characteristic used for clinical diagnosis of PD together with the absence of other neurological impairments, as well as a lack of history indicating exposure to triggering drugs, toxins, or infections (Wirdefeldt *et al*, 2011). According to the Movement Disorder Society PD (MDS-PD) Criteria, parkinsonism is indicated by bradykinesia along with either rest tremor, rigidity, or both (Postuma *et al*, 2015a). Parkinsonism denotes a category of neurological disorders characterized by movement issues resembling those observed in PD, including conditions such as multiple system atrophy (MSA). Therefore, after documentation of parkinsonism, it is determined whether PD is the underlying cause. For this purpose, 3 categories of diagnostic features are used: absolute exclusion criteria (which negate the possibility of PD), red flags (which necessitate additional supportive criteria for a PD diagnosis), and supportive criteria (positive features that enhance confidence in the diagnosis of PD)(Postuma *et al*, 2015a). However, achieving complete diagnostic certainty for PD during life remains challenging. A conclusive diagnosis of idiopathic PD can be established through the post-mortem assessment of dopaminergic neuronal cell loss in the SNpc and the extensive deposition of α -syn, both hallmark features of PD (Poewe *et al*, 2017). Given the absence of specific and sensitive biomarkers, there is a need for such markers to assess disease risk or progression and enhance early diagnosis in both research and clinical settings for individuals with PD (Poewe *et al*, 2017).

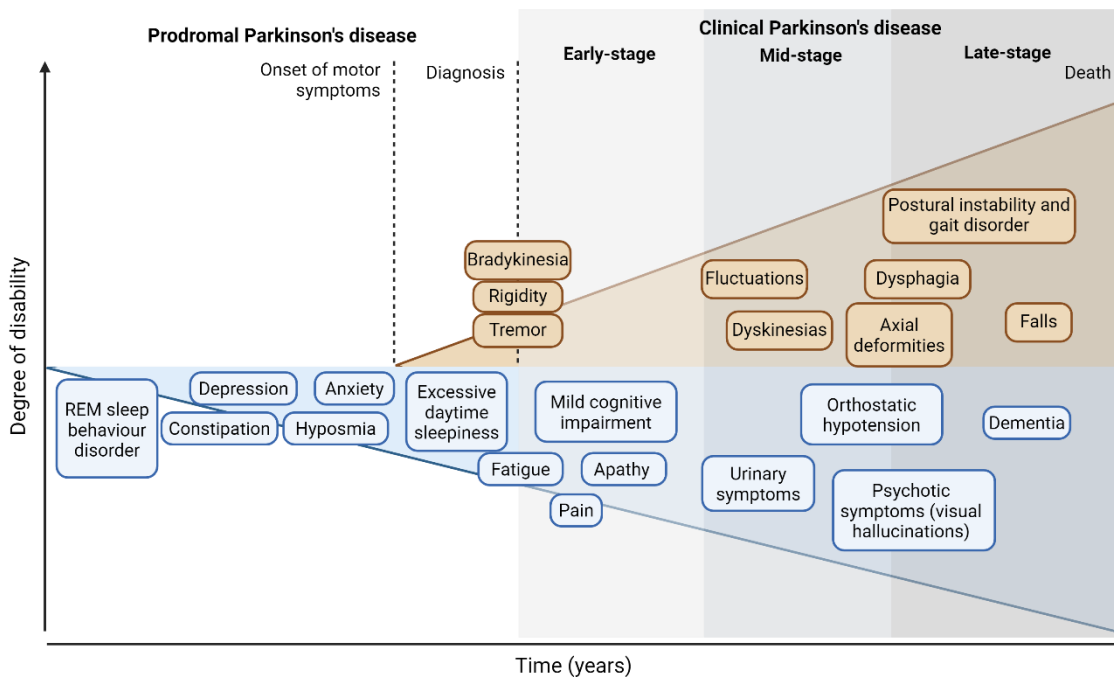


Figure 1: Progression of Parkinson's disease (PD) with time course and clinical symptoms. PD is a neurodegenerative disorder which is characterized by both motor and non-motor features. Parkinsonian motor symptoms include bradykinesia, muscular rigidity, postural instability and tremor. Non-motor features, on the other hand, are pain, fatigue, Mild Cognitive Impairment (MCI) and urinary problems. Some traits appear before the motor dysfunction, including constipation, rapid eye movement (REM) sleep behaviour disorder, excessive daytime sleepiness (EDS) and depression. Figure based on (Poewe et al, 2017) and created with BioRender.

1.1.4. Pathophysiology

PD is distinguished by 3 distinctive pathological hallmarks, namely (1) the loss of dopaminergic neurons in specific regions of the SNpc, (2) the misfolding, aggregation and widespread accumulation of the protein α -syn, and (3) neuroinflammation.

Loss of dopaminergic neurons

The distinctive motor impairments observed in PD seem to stem mainly, if not solely, from dopamine depletion in the striatum, consequent to the degeneration of **dopaminergic neurons** within the SN (**Figure 2**)(Alexander, 2004; Açar *et al*, 2023). The SN is a dopaminergic nucleus located in the midbrain, which plays a crucial role in the modulation of motor movements and can be morphologically and functionally subdivided into the SNpc and the SN pars reticulata (SNpr)(Sonne *et al*, 2024). The latter consists of neurons that transmit signals from the basal ganglia, produce the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) and deliver it to other parts of the brain. The SNpc is a densely clustered core region that, in contrast, consists of dopaminergic neurons responsible for dopamine synthesis. The SNpc neurons are crucial in the nigrostriatal pathway, which

constitutes 1 of the 4 major dopamine pathways. In the nigrostriatal pathway, dopaminergic neurons in the SNpc project to areas within the circuitry of the basal ganglia, such as the striatum, regulating voluntary movement (Luo & Huang, 2016; Gantz *et al*, 2018). The other main dopamine pathways are the mesocortical pathway, the mesolimbic pathway, and the tuberoinfundibular pathway. The latter originates from the hypothalamus and projects dopamine to the pituitary gland (Xu & Yang, 2022). The mesocortical pathway originates from dopaminergic neurons within the ventral tegmental area (VTA) and projects dopamine to the cortex, whereas the mesolimbic pathway originates from the VTA and extends to the nucleus accumbens (Xu & Yang, 2022).

Dopamine is a neurotransmitter that carries out multiple functions in the brain. The chemical messenger has been shown to be involved in modulation of behaviour and cognition, voluntary movement, modulation of sleep patterns and dreaming, mood regulation and facilitation of learning processes (Calabresi *et al*, 2007; Juárez Olguín *et al*, 2016). Dopamine synthesis occurs within the neuronal cytosol through a two-step process. Initially, in dopamine-secreting cells, the enzyme tyrosine hydroxylase (TH) catalyzes the conversion of tyrosine to L-3,4-dihydroxyphenylalanine or levodopa, which is subsequently transformed into dopamine by aromatic L-amino acid decarboxylase (Zahoor *et al*, 2018). Following synthesis, dopamine is packaged into synaptic vesicles and released from axon terminals into the synaptic cleft (**Figure 2**). Within this space, dopamine interacts with dopamine-receptive postsynaptic neurons, initiating a signaling cascade that ultimately triggers motor output. Following activation of the postsynaptic neuron, dopamine dissociates from its receptor and is swiftly taken back up into the presynaptic cell by the dopamine transporter (DAT).

In PD patients, there is notable depletion of dopaminergic neurons and degeneration of nigrostriatal fibers. As a consequence, this loss entails the deprivation of their long-range axonal projections, which play a regulatory role in modulating the activity of striatal neurons (Gordán-Vélez *et al*, 2021; Obeso *et al*, 2010; Struzyna *et al*, 2018). This eventually results in the denervation of the striatum and reduction in dopamine (**Figure 2**). Remarkably, until 70-80% of the dopaminergic neurons have been lost, the symptoms and features of PD do not arise, leading to a considerable delay between initial cell damage and the onset of clinical features (Emamzadeh & Surguchov, 2018). Furthermore, the neurodegeneration extends beyond the nigrostriatal dopaminergic neurons in PD, involving the degeneration of other neuronal populations as well, including the locus coeruleus (LC), dorsal motor nucleus of the vagus (DMV) and nucleus basalis of Meynert (NMB) (Salama & Arias-Carrión, 2011). This broader degenerative process contributes to the emergence of non-motor symptoms, rendering PD a heterogeneous disorder with a variety of symptomatic manifestations (Kouli *et al*, 2018). However, the reason for the selectivity for certain neuron populations such as the dopaminergic neurons in the SNpc remains largely unclear.

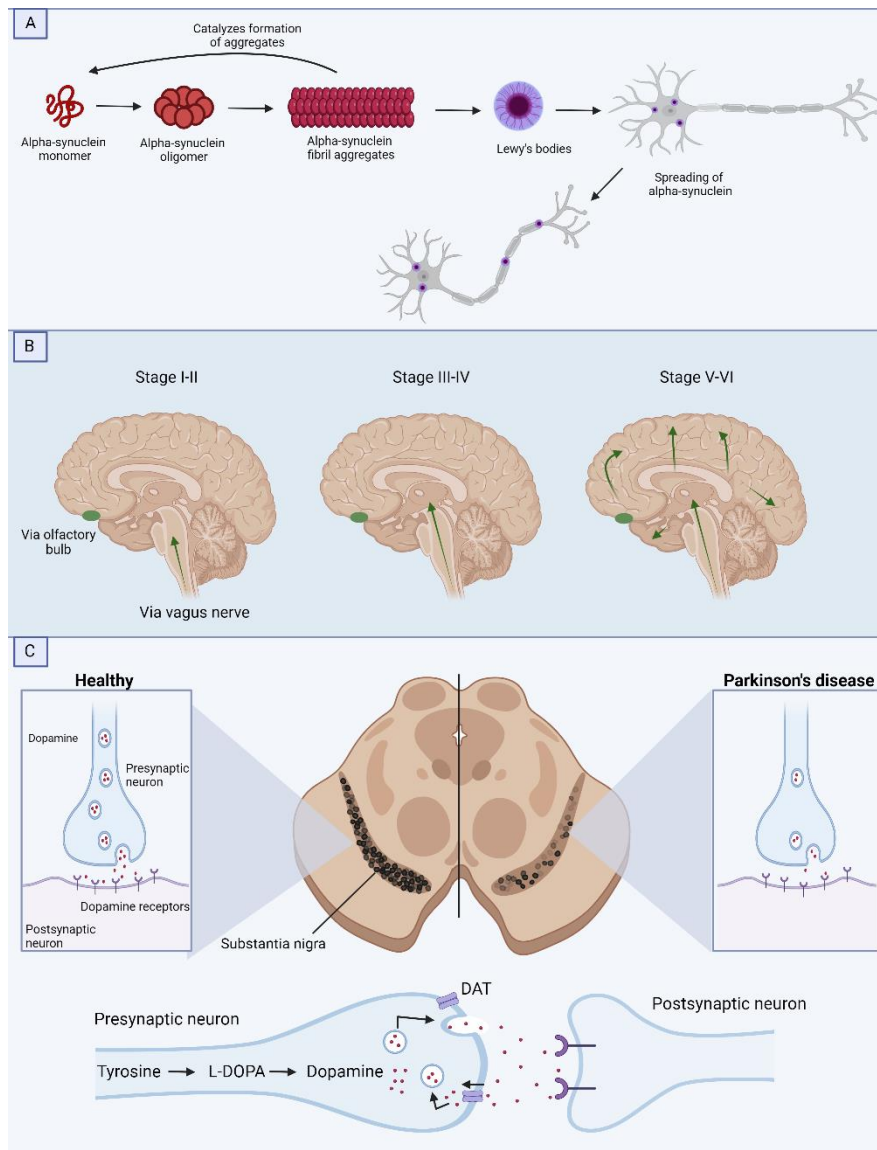


Figure 2. Overview of neuropathological hallmarks in Parkinson's disease (PD), with focus on Lewy body (LB) pathology spreading, alpha-synuclein (α -syn) aggregation, and dopaminergic cell loss in substantia nigra pars compacta (SNpc). (A). A typical hallmark of PD is the misfolding of α -syn monomers, forming oligomers and fibrils. These fibrils further aggregate into protein inclusions of Lewy bodies. The α -syn aggregates spread in a prion-like manner and can induce aggregation of healthy α -syn monomers in neighbouring cells. Figure based on (Kingwell, 2017). **(B).** The Braak stages describe the spreading of Lewy pathology throughout the brain. During stage I, the first inclusions are present in the olfactory bulb (OB) and/or the dorsal motor nucleus (DMV) of the vagal nerve. In the next stage, the inclusions spread to the brainstem, and subsequently, to the amygdala and substantia nigra (stage III) followed by the temporal cortex (stage IV). In last stages, LB pathology appears in the neocortex. Figure based on (Georgiopoulos, 2019). **(C).** Another typical hallmark of PD is the loss of dopaminergic neurons in the SNpc. These neurons project to the putamen and the caudate nucleus within the dorsal striatum via the nigrostriatal pathway. Dopaminergic neurons are responsible for the production of dopamine, which is derived from tyrosine and subsequently converted to levodopa (L-dopa). Dopamine is released from the axon terminals into the striatum, where it interacts with dopaminergic-receptive neurons, initiating a signaling cascade that ends in motor output. Subsequently, dopamine is released from the receptor and taken back up from the synaptic cleft into the cytosol of the presynaptic neuron. In PD patients, there is notable depletion of

dopaminergic neurons and degeneration of nigrostriatal fibers. This eventually results in the denervation of the striatum and reduction in dopamine. Figure based on (Açcar et al, 2023). Full illustration made with BioRender.

Lewy bodies and α -syn

The second characteristic hallmark of PD is the **Lewy body** pathology (Poewe *et al*, 2017). Lewy bodies are neuronal inclusion bodies, initially described by Lewy Fritz Heinrich (Holdorff, 2002). These inclusions can be categorized into cytoplasmic and neuritic forms, referred to as Lewy bodies (LB) and Lewy neurites (LN), which occur in neuronal cell bodies and their axons and dendrites, respectively. The primary constituent of both LB and LN is the aggregated form of the protein **α -syn**.

The **α -syn** protein was initially recognized in Alzheimer's disease as a precursor protein crucial in the formation of amyloid plaques (Culvenor *et al*, 1999). Subsequently, the first genetic aberration was found to be linked to familial PD, namely in the α -syn gene or *SNCA* (Konnova & Swanberg, 2018). The α -syn protein is a 14 kDa small acidic protein composed of 140 amino acids and characterized by 3 distinct domains (Emamzadeh, 2016; Lashuel *et al*, 2013). It possesses an amphipathic lysine-rich amino-terminal alpha-helix, which functions in modulating its membrane interactions through lipid-binding. Additionally, it harbors a central amyloid-binding domain and an acidic carboxy-terminal tail, which governs its nuclear localization and interactions with metals, small molecules, and proteins (Emamzadeh, 2016; Lashuel *et al*, 2013). The typical role of the protein remains poorly comprehended, but it appears to be involved, among other things, in neuronal differentiation, modulating vesicle trafficking and acting as a molecular chaperone (Emamzadeh, 2016; Bendor *et al*, 2013). Though the exact function of α -syn remains unclear, the protein is pivotal in various neurodegenerative processes, including dementia with Lewy bodies (DLB) and MSA (Wong & Krainc, 2017).

Endogenous α -syn is abundantly expressed in the central nervous system (CNS)'s presynaptic terminals and primarily occurs as a monomer in the cellular cytosol (Schaeffer *et al*, 2020). In PD patients, misfolding of α -syn monomers occurs in neurons leading to formation of oligomeric intermediates, which then develop into protofibrils which ultimately further aggregate into large, insoluble fibrils (**Figure 2**)(Kingwell, 2017). Research has shown that, due to its structure, natively unfolded α -syn may have a propensity for self-aggregation, or the ability to induce the aggregation of other proteins or other intracellular structures (Lücking & Brice, 2000; Giasson *et al*, 1999). The α -syn pathology is considered to spread throughout the brain in a prion-like manner, meaning that a pathological aggregate can induce or “seed” the aggregation of healthy α -syn monomers in neighbouring cells by acting like a template to form LB and LN (**Figure 2**)(Murray *et al*, 2023). Interestingly, pathologically aggregated α -syn can be distinguished from unfolded monomeric α -syn, as studies have found unusual accumulation of phosphorylated α -syn on residue S129 (pS129) in the brains of PD patients and transgenic animal models of synucleinopathies (Oueslati, 2016).

The sequential spread of the LB pathology through the brain is described by Braak staging, consisting of VI stages (**Figure 2**)(Braak *et al*, 2004). Braak stages I and II form the presymptomatic stages. During the stage I, Lewy inclusions are confined to the olfactory bulb (OB) and the DMV. This might explain the occurrence of several non-motor symptoms

in prodromal PD, such as constipation and loss of smell. In stage II the pathology in the DMV worsens and the LBs further ascend to the brain stem (Braak *et al*, 2004). Subsequently, during stages III-IV of Braak pathology, the SN and other nuclear grays in the midbrain and forebrain (i.e. amygdala and temporal cortex) undergo progressive pathological alterations. At this stage, most individuals transition into the symptomatic phase of the illness. Finally, end stages lead to high clinical manifestations, with the neocortex being affected (Braak *et al*, 2004).

Research showed that abnormally elevated levels of α -syn protein might be responsible for the death of dopamine neurons in PD, since missense mutations in SNCA gene give rise to production of degradation-resistant mutant proteins (Yasuda *et al*, 2013). It is thought that α -syn promotes dopaminergic neuron dysfunction and death through accumulation in the mitochondria, but the consequence of mitochondrial α -syn localization on the neuronal cells is poorly understood (Ganjam *et al*, 2019). Other than this protein misfolding and aggregation and mitochondrial dysfunction, there are other mechanisms that are suggested to lead to neurodegeneration, including defects in intracellular transport pathways to lysosomes (trafficking), and oxidative stress (**Figure 3**)(Dauer & Przedborski, 2003).

Neuroinflammation

The final characteristic hallmark of PD is chronic **neuroinflammation**. Neuroinflammation refers to a physiological inflammatory response/reaction in the brain or spinal cord to exogenous and endogenous attacks on the CNS, mediated by the production of cytokines, chemokines, reactive oxygen species and secondary messengers (Çınar *et al*, 2022; DiSabato *et al*, 2016). This response can manifest as acute, typically serving a protective function, or chronic, characterized by excessive inflammatory reactions that prove detrimental to the CNS. In general, short-term activation of immune cells is believed to confer neuroprotection. However, prolonged immune activation may lead to adverse effects on neuronal integrity (Araújo *et al*, 2022). **Microglia** serve as the primary immune cells involved in neuroinflammatory reactions. Generally, they play essential roles in brain development and CNS homeostasis, including tasks like programmed cell death and clearance of apoptotic newborn neurons, as well as pruning developing axons and synapses. Moreover, microglia actively survey the CNS for damage. However, when exposed to infectious agents or aggregates, microglia can undergo reactive activation. In various diseases, these mononuclear phagocytes can be activated and may exert neurotoxic effects, such as releasing reactive oxygen species, or beneficial effects, like releasing trophic factors.

In the context of PD, post-mortem investigations have shown close contact between activated microglia and neurons displaying pathological accumulation of α -syn in PD patients (Croisier *et al*, 2005; Ferreira & Romero-Ramos, 2018). Microglia are thought to provide neuroprotection by aiding in the clearance of extracellular α -syn, thereby preventing its excessive buildup. However, infectious agents or aggregates can trigger microglial activation. Research has revealed that misfolded α -syn can directly activate microglia, leading to the production of proinflammatory molecules and changes in the expression of Toll-like receptors (TLRs)(Béraud & Maguire-Zeiss, 2012). Indeed, several studies have shown that extracellular α -syn can provoke a pro-inflammatory response in microglia, resulting in an increased production of cytokines (Ferreira & Romero-Ramos, 2018; Klegeris *et al*, 2008; Couch *et al*, 2011; Roodveldt *et al*, 2010; Su *et al*, 2008; Lee *et al*, 2009). Research indicates that the microglial receptor TLR1/6, along with its associated

signaling pathways, facilitates neuroinflammation mediated by microglia (Chen *et al*, 2023; Hanisch, 2013; Kim *et al*, 2016). Ligands such as α -syn stimulate TLR2-dependent downstream signaling via the adaptor co-receptors TLR1 or TLR6, with α -syn activation ultimately leading to the production of proinflammatory cytokines (Chen *et al*, 2023). Further evidence for contribution of microglia in PD is the observation of reactive microglia, reduced levels of neurotrophins (i.e. proteins inducing the survival, development, and function of neurons) such as brain-derived neurotrophic factor (BDNF), and increased levels of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF- α), interleukin-1beta (IL-1 β), and IL-6 in postmortem brains and/or cerebrospinal fluid (CSF) from PD patients (McGeer *et al*, 1988; Nagatsu & Sawada, 2005). Furthermore, reactive microglia are found in the SN of PD patients, suggesting their involvement in neuroinflammation in PD (McGeer *et al*, 1988). When activated, microglia can exert harmful effects on dopaminergic neurons through the production of pro-inflammatory cytokines, for example (**Figure 3**). The excessive release of pro-inflammatory cytokines in PD contributes to neuropathological and toxic mechanisms (Pajares *et al*, 2020). This occurs through the sustained activation of microglia, which subsequently secrete elevated levels of pro-inflammatory mediators. These mediators damage neurons and further activate microglia, establishing a feedback loop that amplifies inflammation and promotes neurodegeneration (Pajares *et al*, 2020). Next to neuroinflammation, mitochondrial dysfunction, α -syn and LB formation, and blood-brain barrier (BBB) leakage, which has been observed in animal models of PD (Al-Bachari *et al*, 2020), are thought to act together and promote neurodegeneration (**Figure 3**)(Pizarro-Galleguillos *et al*, 2023).

Interestingly, after a pathological stimulus, such as inflammation, microglia undergo morphological alterations with shortening of their processes, reduced branching and compacting their cell body into an amoeboid morphology (Green *et al*, 2022; Vidal-Itriago *et al*, 2022). Furthermore, reactive microglia exhibit increased expression of major histocompatibility complex II (Paolicelli *et al*, 2022), and induce neurotoxic A1 **astrocytes** or neuroinflammatory reactive astrocytes (**Figure 3**). Unlike typical astrocytes that promote neuronal survival, support synapses, and maintain BBB integrity, these reactive astrocytes downregulate supportive functions and commence secretion of neurotoxic factors, complement components such as C3, and chemokines (Lawrence *et al*, 2023). Reactive astrocytes also release proinflammatory cytokines, such as TNF- α and IL-1 β , which further worsen the conditions in PD (**Figure 3**)(Wang *et al*, 2023). Additionally, they exhibit altered morphology characterized by elongated processes and an increased number of segments and branching.

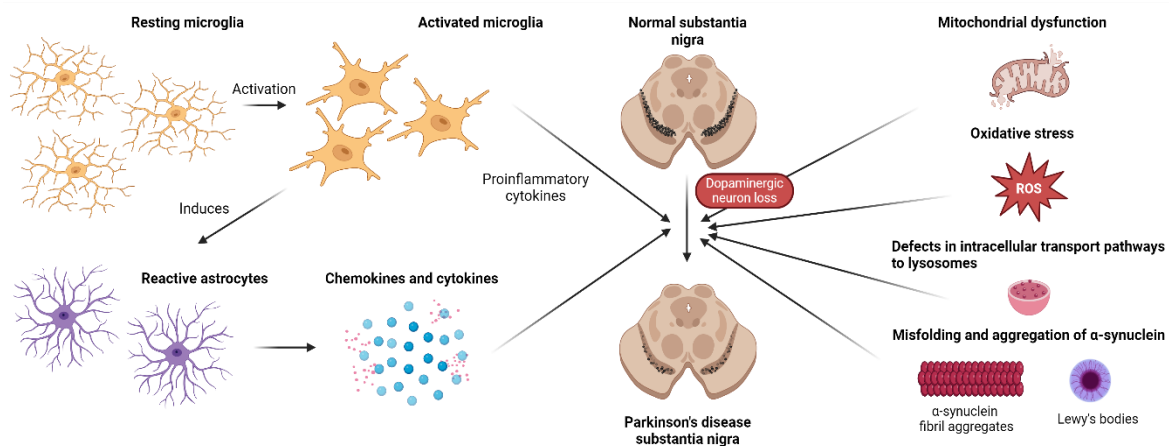


Figure 3. Mechanisms for neurodegeneration as one of the pathological hallmarks of Parkinson's disease (PD). Resting microglia can be directly activated through misfolded α -synuclein (α -syn). Activated microglia contribute to neuroinflammation and loss of dopaminergic neurons, and thus PD pathology, through the production of pro-inflammatory cytokines such as tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β). Activated microglia also induce neurotoxic A1 astrocytes or neuroinflammatory reactive astrocytes. Reactive astrocytes also release proinflammatory cytokines, such as TNF- α and IL-1 β , which further worsen the conditions in PD. Other mechanisms that are suggested to lead to neurodegeneration include α -syn misfolding and aggregation into protein deposits (Lewy bodies), mitochondrial dysfunction, defects in intracellular transport pathways to lysosomes (trafficking), and oxidative stress. Figure based on (Chen et al, 2023) and made with BioRender.

The **complement** system, a fundamental component of the innate immune system, plays an important role in modulating immune reactions and inflammation, encompassing those occurring within the CNS. The complement system serves as the primary defense mechanism against various threats, including microbial invaders. Comprising over 30 proteins, it forms a complex network with multiple initiation and regulatory mechanisms (Sarma & Ward, 2011; Ricklin *et al*, 2010). These proteins are predominantly found in the plasma or are associated with cell membranes. Activation of the complement system can occur through 3 distinct pathways: the classical, lectin, or alternative pathway. Regardless of the pathway involved, all lead to the generation of the C3 convertase. C3 convertase catalyzes the cleavage of multiple C3 proteins, which are pivotal complement proteins, yielding C3a and C3b fragments (Ricklin *et al*, 2010; Cell Biology of the Immune System, 2021). This cleavage event triggers numerous responses. Firstly, it facilitates the opsonization of cells, marking them for recognition and engulfment by phagocytes. This process is facilitated by opsonins such as C3b and C4b (Schartz & Tenner, 2020; Sarma & Ward, 2011; Ricklin *et al*, 2010). Another consequence of complement pathway activation is the recruitment of microglia or leukocytes, including neutrophils, monocytes, and macrophages, to the site of action. This recruitment is mediated by the generation of anaphylatoxins, specifically C3a and C5a, which induce chemotaxis and instigate pro-inflammatory signaling through their respective receptors. The final effect is the formation of membrane attack complex (MAC). The MAC functions by inserting into the lipid bilayer of target cell membranes, disrupting osmotic equilibrium, and ultimately leading to cell lysis. This process of complement-dependent cell lysis serves as a pivotal antimicrobial mechanism mediated by the complement system (Cell Biology of the Immune System, 2021; Ricklin *et al*, 2010; Sarma & Ward, 2011).

As part of the innate immune response, the complement system plays a crucial role in both keeping the brain healthy and contributing to disease processes of various CNS diseases, including PD (Carpanini *et al*, 2019). For example, complement activation is essential for synaptic pruning, a process crucial for refining neural connections during development and in maintaining brain function. The central complement factor C3 has been identified as one of the immune genes that are upregulated in the SN during the early phases of synucleinopathy (Stoll *et al*, 2024). Indeed, elevated C3 levels have been detected in the CSF and various brain regions of PD patients (Zhou *et al*, 2023). This is consistent with another study in which similar elevated CSF levels of complement protein C3 correlated with disease severity in PD patients (Wang *et al*, 2011). Furthermore, in PD, as discussed in section 1.1.4.3, the induction of neurotoxic A1 astrocytes occurs, with C3 being one of the most characteristic and highly upregulated genes. C3-positive astrocytes are found in the SNpc of postmortem human PD tissue (Liddelow *et al*, 2017), as well as in mice injected with α -syn PFFs (Yun *et al*, 2018), following substantial neurodegeneration. However, the upregulation of C3 is not only restricted to A1 astrocytes. In another study, using the α -syn PFF model in rats, it was found that microglia in the SNpc exhibited increased expression of C3 (Stoll *et al*, 2024). This upregulation was specifically observed following the administration of α -syn PFFs, indicating a specificity for α -syn aggregation. Interestingly, C3 expression in the SNpc was initially localized to microglia at 2- and 4-months post-PFF administration. However, by 6-months post-PFF, C3 expression had shifted to astrocytes. This suggests a dynamic cell type switching between microglia and astrocytes throughout the degenerative process. Specifically, microglia seem to be the predominant cellular source of C3 expression prior to the neurodegeneration of nigral dopamine neurons, whereas astrocytes become the primary source following neuronal degeneration (Stoll *et al*, 2024).

1.1.5. Treatment

Currently, there is no cure for PD and treatment typically involves a combination of medications, surgical interventions, and other therapies, which can often alleviate certain symptoms. It is mostly treated with dopaminergic therapies, which are only symptomatic. Today, the gold standard PD treatment is the use of a dopamine precursor, namely L-3,4-dihydroxyphenylalanine or levodopa (Tambasco *et al*, 2018). It has been shown that oral administration of levodopa alleviates most of the motoric symptoms. However, after several years of levodopa treatment most patients develop involuntary movements or dyskinesias (Pezzoli & Zini, 2010). Furthermore, over time, many patients experience a reduction in effectiveness of levodopa. Other medications used to manage PD include dopamine agonists and enzyme inhibitors such as monoamine oxidase B (MAO-B) inhibitors (Tan *et al*, 2022). Dopamine agonists stimulate dopamine production in the brain, while MAO-B inhibitors increase dopamine levels by slowing down the enzymes responsible for dopamine breakdown in the brain. Moreover, to alleviate involuntary movements, tremors, and muscle rigidity, amantadine and anticholinergic drugs may be considered as treatment options (Parkinson's Disease: Causes, Symptoms, and Treatments | National Institute on Aging).

Another example of treatment is deep brain stimulation (DBS). DBS is a neurosurgical intervention involving the implantation of electrodes into precise brain regions. These electrodes are connected to an impulse generator, enabling the delivery of electrical stimuli to the brain tissue. The objective of DBS is to modulate or disrupt patterns of neural signaling within the targeted brain regions (Okun, 2012). However, as with dopamine replacement

strategies, the aim of DBS is to alleviate motor impairment rather than correct the underlying cause of motor symptoms (Struzyna *et al*, 2018).

Recently, the gut microbiome has emerged as a strategy to tackle PD. There are several ways to alter the microbiome, including **fecal microbiota transplantation (FMT)**. In such treatment, feces from a healthy donor is introduced into a patient's gastrointestinal tract. A recent study from the host lab demonstrated that a single FMT resulted in modest yet lasting improvements in motor symptoms among patients with early-stage PD (Bruggeman *et al*, 2024). This underscores the therapeutic promise of altering the gut microbiome and advocates for expanded research on FMT across various stages of PD.

1.2. Modelling PD in vivo

Since PD pathology affects the brain and since it is difficult to examine live brain tissue, experimental models are needed to study the pathology of PD. Cell cultures provide a means to examine the pathological and biochemical facets of PD at the cellular level (Orth & Tabrizi, 2003). However, drawbacks of cell cultures include their reliance on specific culture conditions for neuronal survival and the inability of cells to develop the natural complex neuronal networks (Falkenburger & Schulz, 2006), making animal models more representative. An ideal model of PD would encompass several key characteristics to effectively mimic the progression and pathology of the condition. Firstly, it should begin with a normal amount of dopamine neurons at birth, with the gradual and selective loss of these neurons commencing in adulthood (Beal, 2010). This progressive degeneration should lead to easily detectable motor deficits, including bradykinesia, rigidity, and resting tremor, mirroring the clinical symptoms observed in PD patients. Moreover, the model should faithfully replicate LB neuropathology. If the model is genetically based, it should ideally be centered around a single mutation to ensure the robust propagation of the mutation throughout the model population. Lastly, to allow rapid and cost-effective screening of potential therapeutic agents, the model should have a relatively short disease course, spanning a few months (Beal, 2010).

Rats and mice are frequently used as model organisms for studying PD due to their genetic similarity to humans and the ability to manipulate their genomes. Over the years, numerous PD models have been developed in these species. The discovery of novel monogenic PD mutations has been used to investigate the effects of mutated proteins and to create new reliable animal models, where most genetic models have succeeded in reproducing only some features of PD (Konnova & Swanberg, 2018). Environmental dopaminergic neurotoxins are proposed to play a role in sporadic PD (Salama & Arias-Carrión, 2011). Consequently, toxin-based mouse models have been developed as well to mimic PD, such as MPTP, which induces a syndrome closely resembling PD upon systemic administration (Meredith & Rademacher, 2011). Nevertheless, this project will make use of a PFF-injection mouse model.

1.2.1. *Intrastriatal PFF-injection mouse model*

The PFF-injection model serves as a prevalent model for studying the biology of PD and evaluating therapeutic interventions (Polinski, 2021). In this model, wildtype (WT) recombinant **α -syn PFF** which resemble aggregates similar to those found in PD patients, are injected into WT rodents. Prior to their injection, the PFFs are sonicated to produce short fragments (<50 nm) capable of neuronal uptake (Polinski *et al*, 2018; Luk *et al*, 2012). As already mentioned in 1.1.4, α -syn aggregates spread in a prion-like manner and can induce aggregation of healthy α -syn monomers in neighbouring cells. PFFs also have self-propagating

properties, allowing even a small amount of PFFs to catalyze the transformation of endogenous α -syn proteins into insoluble aggregates (Polinski, 2021; Chung *et al*, 2019). This transformation can propagate sequentially through the brain via interconnected neural pathways. Important to consider is the species of the PFF. In general, the degree of homology between the injected host and the PFF species correlates with their seeding capacity and pathogenicity, meaning that injection of murine PFF in mice results in a larger seeding capacity compared to injection of human PFF in mice (Polinski *et al*, 2018). Therefore, the host lab works with murine α -syn PFFs. Other factors to consider include the route of administration, the quantity of injected PFF, and their size. PFFs should ideally be 50 nm or smaller, as larger fibrils exhibit reduced pathogenicity or may even fail to induce pathology (Pieri *et al*, 2016; Abdelmotilib *et al*, 2017). Moreover, to increase the reproducibility of this model, several quality controls have been established to which PFF must comply such as analyzing fibril size and seeding capacity (Polinski *et al*, 2018).

Unilateral intrastriatal PFF-injection in mice results in the formation of α -syn-containing LBs within mouse neurons (Volpicelli-Daley *et al*, 2011). Furthermore, PFF-injection leads to bilateral dopaminergic neuronal cell death and pS129- α -syn (Polinski, 2021), which previous studies have also observed in the brains of PD patients (Oueslati, 2016). Studies showed absence of visible pS129- α -syn following PFF-injection in α -syn knockout (KO) mice, indicating the requirement of endogenous α -syn for transmissibility (Volpicelli-Daley *et al*, 2011; Luk *et al*, 2012). In addition to pS129- α -syn and neuronal dysfunction, the injected mice also exhibited behavioral deficits (Polinski, 2021). The host lab successfully replicated the pathogenic effects of PFF-injection in mice using commercially available murine α -syn PFFs (StressMarq), leading to the aggregation of α -syn from 1 month after intrastriatal injection onwards and degeneration of dopaminergic neurons at 8 month post-injection (mpi) in the mouse model.

In contrast to other models which are based on the overexpression of human WT or mutant α -syn, this PFF model offers the advantage of more physiological α -syn levels (Chung *et al*, 2019). Furthermore, this technique permits the induction of α -syn pathology in PD-related brain areas comparable to what is observed in human conditions and the progression of aggregates can be followed from the moment of formation to the point of neuron death, giving this model great temporal and spatial resolution (Chung *et al*, 2019). The flexibility of the model permits the injection of various forms of PFFs, such as mouse or human α -syn PFFs (Polinski, 2021). It also allows for unilateral or bilateral injections, targeting of different brain regions, and administration through different peripheral routes. Thus, these injections are administered not only in the brain, but also in several other areas of the body, including the muscle, intestines, OB, eye and peritoneum (Chung *et al*, 2019; Polinski, 2021). However, this flexibility also serves as a weakness. The use of different protocols can result in varying pathologies, complicating cross-study comparisons and potentially hindering the ability to draw consistent conclusions across research studies (Polinski, 2021). Maintaining the reproducibility of studies utilizing the PFF model poses a significant challenge. Variations in experiment implementation, such as monomer preparation, PFF seed generation, and the quantity of injected PFFs, can also impact the consistency of results.

1.2.2. Icv LPS-injection mouse model

Neuroinflammation is one of the pathological hallmarks of PD and can be studied with the PFF-injection mouse model mentioned above. However, results from the host lab indicate that

neuroinflammation is present from 2 mpi onwards and in a rather mild state. As an alternative method to study neuroinflammation, this project will use a different model where neuroinflammation can be induced more quickly and drastically, namely by the icv injection of LPS. Peripheral administration of LPS, a component of the gram-negative bacterial cell wall, is a widely used model for systemic inflammation (Skrzypczak-Wiercioch & Sałat, 2022). The intraperitoneal (ip) route is the most used method for systemic administration of LPS. Upon ip administration of LPS, the innate immune system within the CNS becomes activated, leading to the activation of reactive glial cells and the production of pro-inflammatory cytokines including IL-6, TNF- α , and IL-1 β (Skrzypczak-Wiercioch & Sałat, 2022). Microglia have been identified as the primary LPS-responsive cells in the CNS, as studies have shown that these glial cells predominantly express the specific receptor for LPS, known as TLR4 (Lehnardt *et al*, 2003; Hanafy, 2013). Studies revealed that LPS administration initiates an inflammatory response primarily mediated by TLR4, leading to microglial reactivation and subsequent neuroinflammation (Skrzypczak-Wiercioch & Sałat, 2022). This cascade of events ultimately culminates in neuronal degeneration, synaptic loss, and ultimately, neuronal cell death. In addition to ip injections, LPS can be administered by icv injection. The icv injection of LPS is a frequently used model to study meningitis, but can also be used to investigate neuroinflammation. Indeed, a study demonstrated that icv injection of LPS in mice resulted in microglial activation within the hippocampus (Zhao *et al*, 2019), which was also shown by data of the host lab one month after LPS-injection.

1.3. The link between the gut and PD

1.3.1 Gut-microbiota-brain axis

The human GI tract contains a heterogeneous microbial community, playing a pivotal role in both the physiological functions and pathological conditions of the host (Rutsch *et al*, 2020). Alterations in gut microbiota, termed dysbiosis, are linked not only to gastrointestinal disorders but also to diseases impacting distant organs. Recent findings suggest that the microbiota residing in the intestines can exert an influence on the physiology and inflammatory responses of the CNS. Indeed, there is communication between the CNS and the gut microbiome, called the **microbiota-gut-brain axis**, connecting the emotional and cognitive regions of the brain with the peripheral functions of the intestines (Carabotti *et al*, 2015; Loh *et al*, 2024). Despite the anatomical separation between the gut and brain, numerous pathways have been proposed through which the gut microbiota communicates with the CNS. These pathways encompass modulation of the immune system, the vagus nerve, influence on the enteric nervous system (ENS), interaction with the neuroendocrine system, and impact on the circulatory system (Loh *et al*, 2024; Carabotti *et al*, 2015). This communication appears to be bidirectional and is mediated through the production of neuroactive substances, metabolites, and hormones. For instance, studies have shown that the gut microbiota can produce or stimulate the production of neurotransmitters such as serotonin, dopamine and GABA (Strandwitz *et al*, 2019; Loh *et al*, 2024). During dysbiosis, these pathways become dysregulated, leading to alterations in the permeability of the BBB and neuroinflammation. However, the precise mechanisms underlying the influence of the gut microbiota on neurodevelopment and neuropathogenesis remain poorly understood (Rutsch *et al*, 2020).

1.3.2. Brain-first versus body-first PD

In the early phase of PD, α -syn aggregates are found at 2 sites in the brain, namely, the olfactory nerve on the one hand and the lower brainstem on the other, specifically the nucleus of another nerve, the vagal nerve (Zhu *et al*, 2022). Remarkably, the vagal nerve communicates information from the gut to the brain by connecting them, via the gut-brain axis. Furthermore, α -syn and its aggregates were also discovered in the GI tract of PD patients (Schaeffer *et al*, 2020). Moreover, a study demonstrated that the injection of human α -syn fibrils into the GI tissue of healthy rodents is adequate to initiate the aggregation of α -syn pathology within the vagus nerve and brainstem (Holmqvist *et al*, 2014; Zhu *et al*, 2022). This, along with the fact that there were symptoms outside the CNS described including constipation, gave rise to the idea that PD pathology can spread from the gut to the brain.

Recently, 2 PD subtypes were described depending on the origin of the pathology, namely: gut-first and brain-first PD (**Figure 4**)(Horsager *et al*, 2020; Borghammer & Van Den Berge, 2019). The latter subtype is also known as the CNS-first phenotype, in which the autonomic peripheral nervous system (PNS) is initially spared. Indeed, this phenotype is typified by early, noticeable α -syn pathology in CNS tissues, including the SN, that subsequently descends anterogradely to the autonomic PNS. In contrast, in the gut-first or PNS-first subtype it is hypothesized that α -syn inclusions originate in the enteric nervous systems' synapses before ascending to the CNS via the vagal nerve (Horsager *et al*, 2020, 2022; Borghammer & Van Den Berge, 2019). Direct evidence for this theory comes from animal research demonstrating gut-to-brain transmission of α -syn aggregates via the vagus nerve into the brain (Kim *et al*, 2019). Not only α -syn transmission, but also loss of dopaminergic neurons, motor and non-motor symptoms were observed. Moreover, truncal vagotomy and α -syn deficiency inhibited the propagation of α -syn pathology from the gut to the brain, along with the related neurodegeneration and behavioral impairments (Kim *et al*, 2019). Further support for the concept of the gut-first PD phenotype is the observation of LB pathology in PD patients' GI tracts up to years prior to onset of clinical PD (Stokholm *et al*, 2016; Shannon *et al*, 2012). Based on these data, it was hypothesized that microbiome perturbations may trigger α -syn aggregation in the gut whereafter the pathology spreads through the brain (Hawkes *et al*, 2007; Johnson *et al*, 2019).

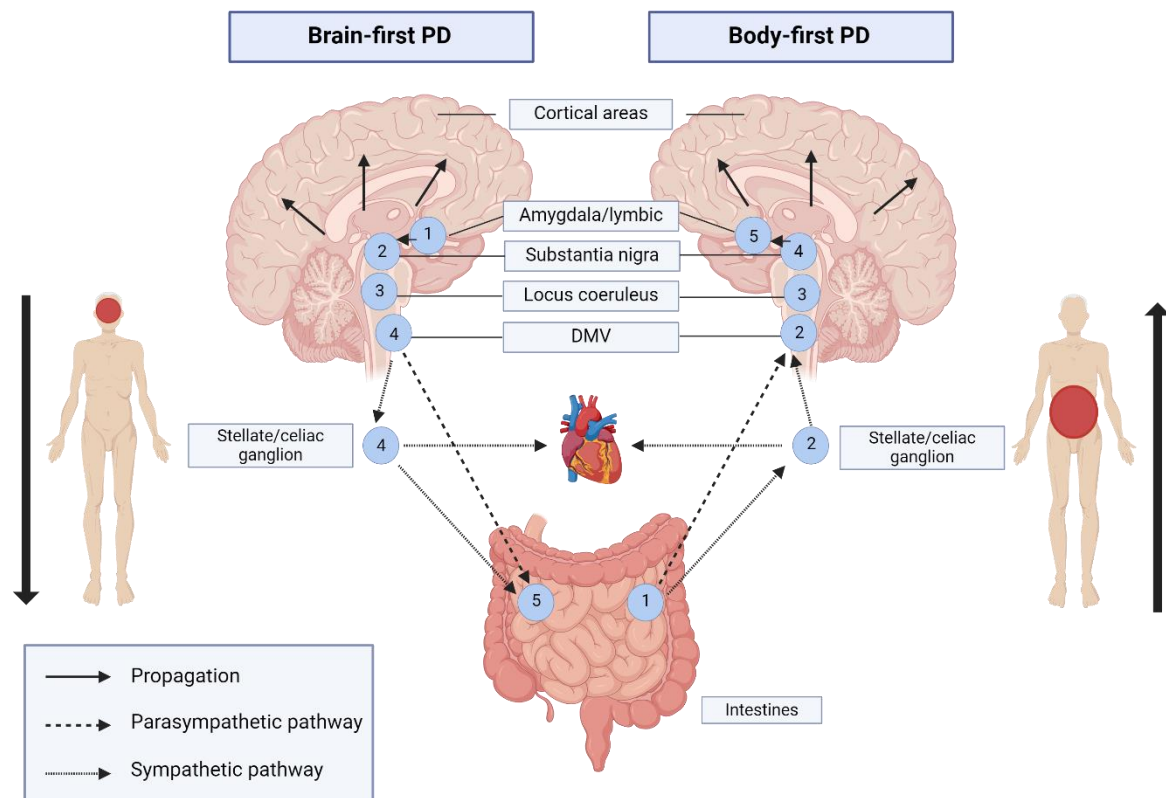


Figure 4. Brain-first versus body-first spreading of α -synuclein (α -syn) in Parkinson's disease (PD). The figure illustrates 2 contrasting pathways for the spread of α -syn aggregates, which is a pathological hallmark of PD. In the "body-first" type, the initial α -syn pathology emerges in the enteric or peripheral autonomic nervous system. Subsequently, it spreads through the sympathetic pathways to the heart and via the vagus nerve to the dorsal motor nucleus of the vagus (DMV). Ascending pathology affects the locus coeruleus, substantia nigra, amygdala and eventually cortical areas. In the "brain-first" type, the initial α -syn pathology originates in the central nervous system (CNS). The most probable site of origin appears to be the amygdala or its connected structures, or in some cases, the pathology may enter via the olfactory bulb. Subsequently, the pathology is thought to spread to the substantia nigra, locus coeruleus and then to DMV. Next, through the stellate/cealic ganglion and sympathetic pathways the pathology reaches the heart. Eventually, α -syn will emerge in the intestines. From the initial site, the pathology spreads to the brainstem and cortex. Figure based on (Horsager et al, 2022) and created with BioRender.

1.3.3. Gut dysbiosis in PD

As mentioned, constipation is a common complication in PD, and in addition, α -syn and its aggregates were also found in the digestive tract of PD patients. Additionally, it was observed that GI dysfunctions, such as constipation, bloating, nausea, dysphagia, sialorrhea, vomiting, and gastroparesis, are prevalent in PD, occurring in over 80% of PD cases (Zhu *et al*, 2022). This led to more research into the link between the gut and PD. Interestingly, the gut bacterial composition is significantly different in PD patients in comparison to healthy individuals. The alterations in gut microbiota in PD patients have been consistently demonstrated in multiple studies. In contrast to healthy people, lower and higher abundance of short-chain fatty acid producing bacteria and bacteria associated with intestinal barrier dysfunction were found in

PD patients, respectively (Kleine Bardenhorst *et al*, 2023; Heintz-Buschart *et al*, 2018). For example, a reduction in Lachnospiraceae bacteria was observed in PD patients (Romano *et al*, 2021). Furthermore, individuals with RBD, one of the non-motor symptoms of PD that can occur years before clinical PD onset, already have an altered gut microbiome (Heintz-Buschart *et al*, 2018). This indicates that microbiome alterations precede the motor symptoms of PD.

Other consistent PD gut microbiome alterations include depletion of *Prevotella* bacteria and bacteria belonging to the *Faecalibacterium* genus and enrichment of *Lactobacillus*, *Akkermansia*, and *Bifidobacterium* bacteria (Scheperjans *et al*, 2015; Romano *et al*, 2021). Notably, the intestinal microbiota are related to the clinical phenotype. For example, there exists a positive correlation between increasing levels of *Enterobacteriaceae* in fecal samples and the heightened severity of motor symptoms experienced by PD patients (Scheperjans *et al*, 2015). Furthermore, *Prevotella* bacteria have been identified as a highly specific prodromal marker for PD (Heintz-Buschart *et al*, 2018). Moreover, *Prevotella* has been linked to RBD and progressive PD motor symptoms over a 2-year period (Aho *et al*, 2019). A previous study, in addition, indicated that individuals with a *Prevotella*-enriched enterotype experience the lowest rates of constipation and the least frequent occurrence of subthreshold parkinsonism (Heinzel *et al*, 2021). *Prevotellaceae* degrades mucin, contributing to the formation of intestinal mucus (Wang *et al*, 2021). It also influences the normal production of short-chain fatty acids (SCFAs), which is an example of gut-microbiota-brain axis metabolites, through fiber fermentation. Reduced levels of *Prevotellaceae* in PD patients could lead to a decrease in mucus and SCFA production, which may increase barrier permeability, making the intestine more susceptible to inflammation. This decrease in SCFA levels has also been reported in the gut microbiome of PD patients (Zhu *et al*, 2022; de Theije *et al*, 2014; Sampson & Mazmanian, 2015). In PD, patients display diminished levels of SCFAs, which result in elevated occurrences of endotoxin and neurotoxin. These factors are both potentially associated with the initiation and advancement of PD (Zhu *et al*, 2022).

The concept of an unbalanced gut microbiota is also supported by animal research. Significant alterations of the gut bacteria were noted in PD mouse models compared to healthy mice (Perez-Pardo *et al*, 2018; Sampson *et al*, 2016). Moreover, transplantation of fecal material from PD patients into germ-free mice leads to increased α -syn-mediated motor impairment (Sampson *et al*, 2016). This is consistent with another study in which human alpha-synuclein overexpressing (ASO) mice that received FMT from PD patients exhibited more severe symptoms compared to those that received fecal microbiota from healthy individuals (Zhu *et al*, 2022). This direct evidence suggests that gut microbiota dysbiosis may play a causal role in the development of PD. Important to take into account is the fact that the composition of the gut microbiota is influenced by various exogenous factors, including age, sex, medications, and lifestyles, for example (Zhu *et al*, 2022). These factors need to be considered when interpreting the diverse findings across studies. However, case-control comparative studies can still offer insights into the potential roles of gut microbiota in the development of PD.

The gut dysbiosis is thought to trigger and/or exacerbate the disease progression (Zhu *et al*, 2022). Dysbiotic gut bacteria are believed to play pivotal roles in the onset and progression of PD. This is primarily through increased intestinal permeability, heightened neuroinflammation, abnormal aggregation of α -syn fibrils, oxidative stress, and reduced neurotransmitter production (Zhu *et al*, 2022). Changes in bacterial by-products associated with dysbiosis are thought to play a role in the inflammatory processes observed in people

with PD (**Figure 5**)(Hill *et al*, 2021). Indeed, the functional elevation of intestinal epithelial barrier permeability (Davies *et al*, 1996; Kelly *et al*, 2014), and the diminished expression of intestinal barrier tight junctions in PD have been associated with inflammatory processes (Perez-Pardo *et al*, 2017; Clairembault *et al*, 2015; Hill *et al*, 2021). The heightened permeability, also known as “leaky gut”, facilitates the passage of bacteria and their products into the lamina propria, initiating additional inflammatory responses. Inflammation, driven by oxidative stress, operates through both neural and humoral pathways, serving as a mechanism for α -syn misfolding (**Figure 5**) (Lema Tomé *et al*, 2013; Hashimoto *et al*, 1999; Hill *et al*, 2021). Peripheral inflammation promotes the misfolding of α -syn, which then spreads to the brain via neural pathways. Concurrently, systemic inflammation induces neuroinflammation in the brain, exacerbating α -syn misfolding (Hill *et al*, 2021; Mogi *et al*, 1994). α -syn, in turn, further amplifies oxidative stress, establishing a self-sustaining cycle that ultimately leads to neurodegeneration within the brain (**Figure 5**)(Hill *et al*, 2021).

Taken together, these findings suggest alterations in the gut microbiota of PD patients and their potential role in the disease process. While the precise causal relationship between gut microbiota and PD remains elusive, recent findings from animal model studies provide growing support for the notion that dysregulated gut microbiota can exacerbate PD pathology, whereas restoration of gut microbiota can potentially delay or ameliorate the onset of PD (Zhu *et al*, 2022). These observations suggest that gut microbiota could serve as both a diagnostic indicator and a therapeutic target for PD.

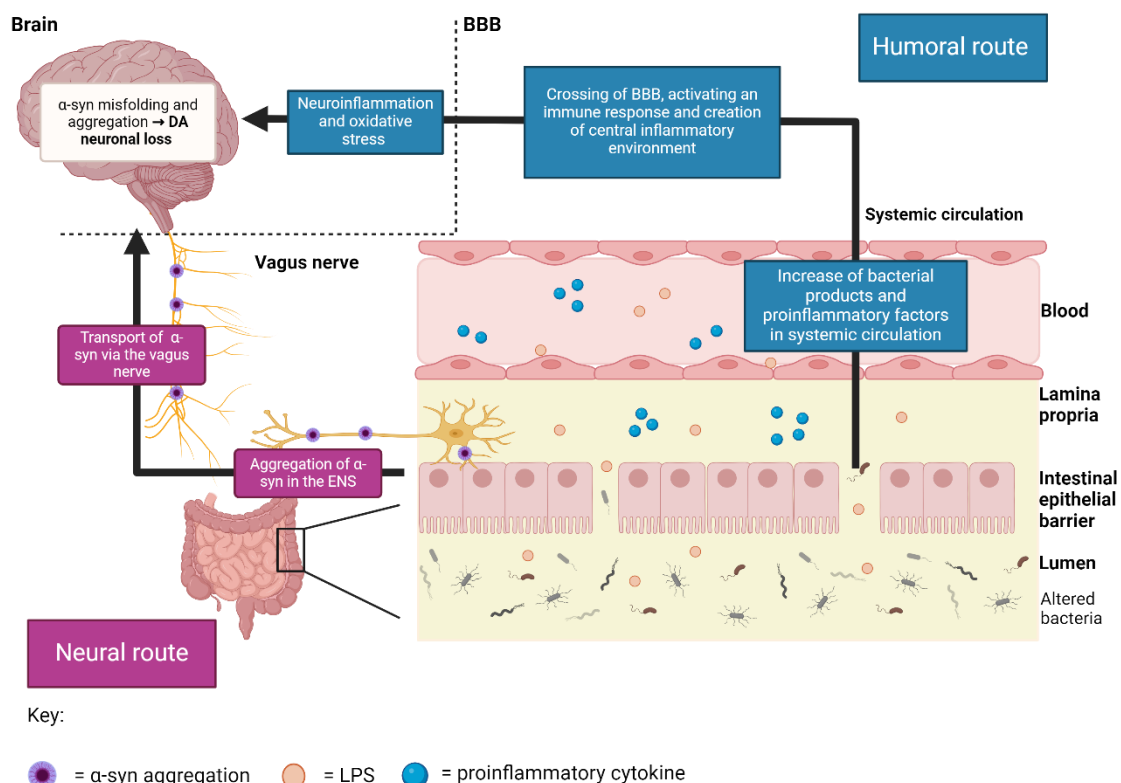


Figure 5. Schematic overview: Mechanisms underlying the potential role of bacterial gut microbiota dysbiosis in Parkinson's disease (PD) pathogenesis. Changes in bacterial by-products associated with

dysbiosis (i.e. altered gut bacteria) are thought to play a role in the onset and progression of PD. The heightened permeability, also known as “leaky gut”, facilitates the passage of bacteria and their products, such as lipopolysaccharide (LPS), into the lamina propria, initiating inflammatory responses. Inflammation, driven by oxidative stress, operates through both neural and humoral pathways, serving as a mechanism for α -synuclein (α -syn) misfolding and aggregation. Peripheral inflammation promotes the misfolding of α -syn, which then spreads to the brain via the vagus nerve via neural pathways. Concurrently, in the humoral route, there is an increase of bacterial products and pro-inflammatory factors in the systemic circulation. Some bacterial products are able to bypass the blood-brain barrier (BBB) and activate an immune response. This induces neuroinflammation in the brain, exacerbating α -syn misfolding. α -syn, in turn, further amplifies oxidative stress, establishing a self-sustaining cycle that ultimately leads to neurodegeneration within the brain causing dopaminergic (DA) neuron loss, a typical pathological characteristic in PD patients. Figure based on (Hill et al, 2021) and created with BioRender.

1.3.4. Modulators of gut dysbiosis

Investigating gut dysbiosis in PD holds significant promise, as it may serve as a prospective biomarker for early disease detection and the development of therapeutic interventions targeting the gut microbiota. Thus, novel therapeutic options at modifying the gut microbiota composition and modulating the microbiota-gut-brain axis using for example probiotics, prebiotics, specific bacterial species, FMT or dietary changes may influence the neurodegeneration cascade in PD (Zhu *et al*, 2022).

A meta-analysis study has suggested that probiotics may improve constipation and motor symptoms in PD patients, potentially by reducing inflammation and enhancing gut-brain axis neuron function (Xie *et al*, 2022). The effect of prebiotics is also investigated, as consumption of prebiotic fibers affects the composition of the microbiota and SCFA levels (Cantu-Jungles *et al*, 2019; Hall *et al*, 2023). Another intervention that has been studied is the impact of dieting. Diet strongly influences the intestinal microbiome, with the Western diet (i.e. high caloric intake diet, high in high in saturated and omega-6 fatty acids, refined sugars, excessive salt intake, and low consumption of omega-3 fatty acids and fiber) being associated with an increased risk of PD (Jackson *et al*, 2019). In contrast, the Mediterranean diet (i.e. daily consumption of vegetables, fruits, nuts, whole grains, and healthy fats; weekly consumption of fish, poultry, beans, and eggs; moderate consumption of dairy products; and limited intake of red meat, characterized by a high intake of dietary fiber) is associated with a decreased risk of PD. Lastly, as already mentioned in 1.1.5., a recent study demonstrated that a single FMT resulted in modest yet lasting improvements in motor symptoms among patients with early-stage PD (Bruggeman *et al*, 2024). This underscores the therapeutic promise of altering the gut microbiome and advocates for expanded research on FMT across various stages of PD. However, the FMT technique is very labor intensive and there is always a risk that a fecal transplant could transfer a harmful pathogen to the recipient. Hence, specific bacterial strains are now being looked at, which is the focus of this master dissertation.

Part 2: Aim of Research Project

2.1. Setting of the research problem

PD is the most common neurodegenerative movement disorder. Globally, the prevalence of PD has doubled in the past 25 years with global estimates in 2019 showing over 8.5 million individuals living with PD, according to World Health Organization. PD is generally characterized as a neurological motor disorder, with primary symptoms including progressive tremor, bradykinesia, postural instability, and muscular rigidity. The disorder is distinguished by 3 distinctive pathological hallmarks, namely the loss of dopaminergic neurons in the SNpc, the misfolding, aggregation and widespread accumulation of the protein α -syn, and neuroinflammation. Despite extensive research, many questions still linger about various aspects of the disease, such as its etiology and how to diagnose patients in the early stage. Furthermore, there is currently no cure for the disorder and all present treatments are only symptomatic. Replenishment of striatal dopamine through oral administration of the dopamine precursor levodopa, the gold standard of symptomatic treatments, alleviates most of the motoric symptoms. However, after several years of levodopa treatment most patients develop dyskinesias (Pezzoli & Zini, 2010). Furthermore, over time, many patients experience a reduction in effectiveness of levodopa.

In addition to motor symptoms, PD is characterized by specific non-motor symptoms, such as constipation, which can occur years or even decades prior to the onset of motor symptoms. Furthermore, it was found that PD pathology can spread from the gut to the brain via the vagal nerve. This led to the description of 2 PD subtypes, depending on the origin of the pathology, namely: gut-first and brain-first PD (Horsager et al, 2020; Borghammer & Van Den Berge, 2019). Additionally, the gut composition is significantly different in PD patients and it is thought that the gut dysbiosis might trigger and/or exacerbate the disease progression (Zhu et al, 2022). Consequently, targeting the dysbiosis of the gut microbiome could potentially offer a new treatment avenue to combat PD.

2.2. Aims and objectives of the research project

In this project, I want to investigate whether supplementing the gut microbiome has a beneficial effect in a mouse model for PD using LBPs. More specifically, I will work with LBPs provided by the company MRM Health, of which the detailed microbial composition is confidential. Since research showed a reduction in *Prevotella* bacteria in PD patients (Romano et al, 2021), 2 different *Prevotella* LBPs with different strain compositions will be used. In addition, I will also use another LBP (CC113), an 11-strain consortium build with strains selected based on literature and preliminary *in vitro* results obtained by MRM health.

Firstly, my research project aims to investigate the effect of *Prevotella* and CC113 LBPs on the pathology in a brain-originating mouse model of PD, based on unilateral intrastratial injection of murine PFF in WT mice. To this end, 2 experiments with different time courses will be performed. In the first experiment, mice will be treated with *Prevotella* cocktails at an early disease stage, from 1 week after PFF-injection to 2 mpi. In the second experiment, mice will be treated with *Prevotella* cocktail 2 or CC113 LBPs for 1 month starting at 6 mpi, i.e. later disease stage.

To investigate the effect of LBPs on PD pathology, phenotypic and pathological analyses will be performed. For the phenotypic analysis, motor function will be assessed by analyzing the

beam traversal assay. Also, GI transit time will be examined by performing the carmine red assay since constipation is a typical complication of PD (Poewe *et al*, 2017), and a transgenic mouse model of PD showed an increase of colon transit time and a decrease in fecal water content, suggesting constipation (Ghaisas *et al*, 2019). In addition, brain pathology will be examined. Thereto, pS129- α -syn in mouse brain, specifically in striatum, is studied, since abnormal accumulation of pS129- α -syn is observed in the brains of PD patients (Oueslati, 2016). For this purpose, brain samples will be collected followed by agarose embedding of the samples, vibratome sectioning to obtain brain sections containing the striata and IHC. Using the Aiforia Ai-tool, the amount of pS129 aggregates will be quantified. Furthermore, possible neuroinflammation will be analyzed by performing microglia staining, followed by quantification and morphological analysis using the Aiforia and the Vision4D software. In addition, the C3 levels will be examined in the early disease stage mice by performing a C3 ELISA, as C3 is known to be involved in CNS inflammatory processes.

To more directly study the impact of microbiome supplementation on neuroinflammation, I will use the icv LPS-injection mouse model, which induces strong neuroinflammation. Thereto, mice will receive oral gavage with *Prevotella* cocktail 2 and CC113 LBPs from 1 week before LPS-injection up to 1 mpi. Next, brain samples will be collected followed by agarose embedding of the samples, vibratome sectioning to obtain brain sections containing the hippocampi and IHC. Neuroinflammation is analyzed by performing microglia staining, followed by quantification using the Aiforia software.

Part 3: Results

3.1 Investigating the effect of LBPs consisting of *Prevotella* strains in a mouse model of early-stage PD

In the host laboratory's α -syn PFF intrastratial injection mouse model of PD, commercially available recombinant murine α -syn PFFs (2.5 mg/ml, Stressmarq) are sonicated and injected into the left dorsal striatum of the mouse brain. This injection site was chosen because the striatum is densely innervated by dopaminergic neurons, which are lost in PD. The α -syn PFF intrastratial injection mouse model of PD was used to investigate the impact of supplementation of the gut microbiome with specific bacterial strains. Since research has shown a decrease in *Prevotella* bacteria in PD patients (Romano *et al*, 2021), the aim of this experiment is to investigate the impact of *Prevotella* supplementation on motor behaviour and pathological features of PD at an early stage in the disease spectrum. To this end, 30 α -syn PFF mice received oral gavage with *Prevotella* cocktail 1 (n=10), *Prevotella* cocktail 2 (n=10) or vehicle (n=10), starting 1 week after injection. During the first week of treatment, the mice were orally gavaged daily for 5 consecutive days followed by oral gavage 3 times a week for a further 6 weeks (**Figure 6**). The remaining 8 phosphate buffered saline (PBS)-injected mice were given vehicle by oral gavage and will be referred to as control group. Two months after brain injection, the treatment was terminated whereafter mice were phenotypically characterized and sampled to investigate PD pathology (**Figure 6**).

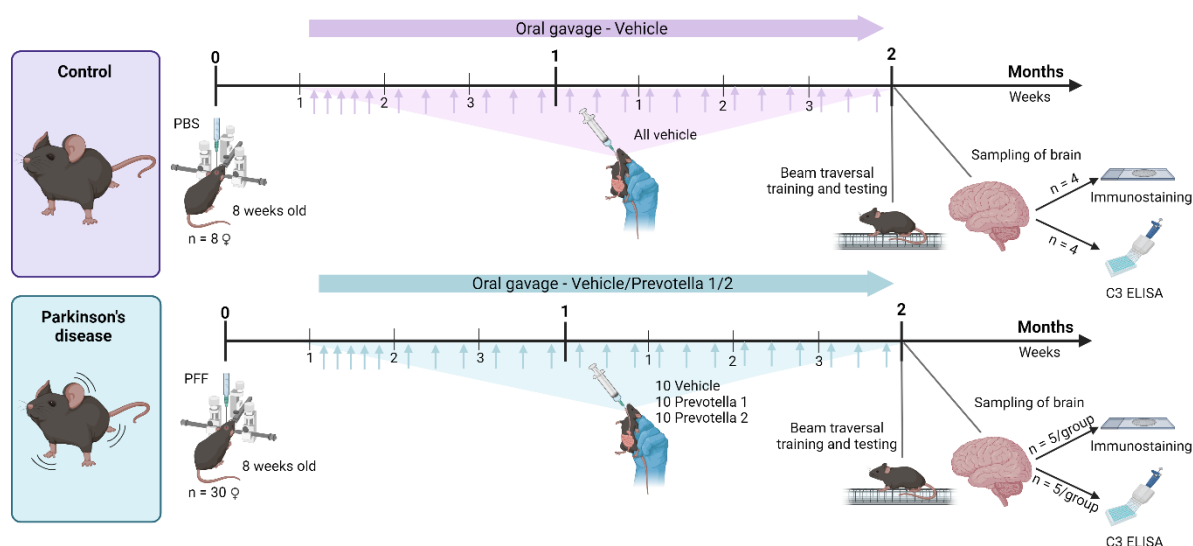


Figure 6. Experimental set-up of the study on the effect of live biotherapeutic products (LBPs) consisting of *Prevotella* strains in an early-stage mouse model of Parkinson's disease (PD). In this set-up, 30 female mice were injected into the left dorsal striatum at 8 weeks of age with commercially available recombinant murine α -synuclein (α -syn) preformed fibrils (PFF). To include controls, 8 mice were injected with phosphate buffered saline (PBS) into the left dorsal striatum. One week after injection, mice were given either vehicle, *Prevotella* cocktail 1 or *Prevotella* cocktail 2 by oral gavage for 5 consecutive days. This was followed by oral gavage 3 times a week for 6 weeks. At the end of the treatment period, the mice were subjected to beam traversal to test motor disturbances. Two months after PFF/PBS injection and 3 days after the last oral gavage, the mice were euthanized and their brains, cecal content and plasma were collected for further analysis. Stool samples

were collected at various time points and sent to MRM Health, who supplied the LBPs, for validation of engraftment. Illustration made with BioRender.

3.1.1. Investigating the effect of LBPs consisting of *Prevotella* strains on motor function

To investigate the effect of *Prevotella* LBPs on the pathology of PD, phenotypic studies were performed. The motor behaviour of PBS- and PFF-injected mice was assessed after oral gavage to determine the effect of *Prevotella* supplementation on motor disturbances. The C57BL/6J mice were analyzed using the beam walking assay. This beam traversal test is a method of assessing mice as they navigate an increasingly narrow bar, enabling the detection of impairments in fine motor coordination and balance.

No significant differences were observed between vehicle-treated PFF-injected mice and control mice in the full beam test and the final section only (**Figure 7**). Furthermore, mice treated with *Prevotella* cocktail 1 took significantly longer to complete the full test than control mice (**Figure 7**). Upon examination of the final section only, this significant increase in completion time was also observed in comparison to the results of the PFF mice administered a vehicle treatment and those administered a *Prevotella* cocktail 2 treatment.

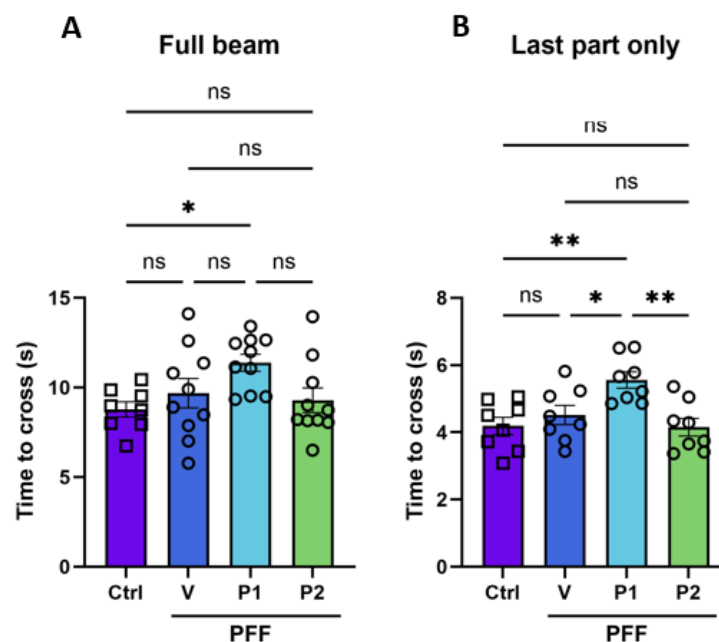


Figure 7. Administration of *Prevotella* cocktail 1 impairs motor function in an early-stage α -synuclein (α -syn) preformed fibril (PFF) intrastriatal injection mouse model for Parkinson's disease. Eight PBS-injected mice that had received vehicle (V) treatment (Ctrl) and 30 PFF-injected mice, of which 10 mice received V treatment, 10 mice received *Prevotella* cocktail 1 (P1) and 10 mice received *Prevotella* cocktail2 (P2), were subjected to the beam traversing assay after the treatment regimen. Mice were timed to assess how long they took to cross the full beam (**A**) and only the last and most narrow part of the beam (**B**). For each mouse, 5 trials were performed and the average of the 3 best trials was calculated. Graphs were made using the GraphPad Prism 10 software and outliers were identified using the ROUT test ($Q = 1\%$). A one-way ANOVA test was utilized, followed by Tukey's multiple comparison testing to determine statistical significance. The height of each bar represents the group mean, with error bars indicating $\pm$ standard error of the mean (SEM).

Significance levels: ns: $p > 0.05$; * $0.01 \leq p < 0.05$; ** $0.001 \leq p < 0.01$; *** $0.0001 \leq p < 0.001$; and **** $p < 0.0001$

3.1.2. Investigating the effect of LBPs consisting of *Prevotella* strains on brain pathology

As α -syn aggregation is one of the pathological features of PD, the effect of *Prevotella* LBPs on α -syn aggregation was investigated. To this end, mice were euthanized at 2 mpi (i.e early in disease spectrum) and their brains were sampled. The brains of 14 PFF-injected mice (5: vehicle-treated; 5: *Prevotella* cocktail 1-treated; 5 *Prevotella* cocktail 2-treated) and 4 PBS-injected mice were then sectioned to obtain brain slices containing the striatum, which were immunostained for the presence of α -syn aggregates. pS129 has emerged as the predominant marker for the formation and propagation of α -syn pathology in PD, as research has shown that the majority (>90%) of α -syn in PD-affected human brains is phosphorylated at S129 (Lashuel *et al*, 2022). After staining and imaging, brain sections were analyzed using the Aiforia software. This software was trained to recognize the different brain regions, as well as the LBs and LNs. To investigate the effect of *Prevotella* LBPs on α -syn aggregation, we focused on the striatum, as this is the injection site.

The number of cellular inclusions or LBs, and the area occupied by LBs and LNs were significantly higher in the striata of vehicle-treated and *Prevotella* cocktail 2-treated PFF-injected mice compared to the striata of PBS-injected control mice (**Figure 8D-F**). Furthermore, we observed a decreasing trend in the number of LBs and size of the area occupied by LBs in the striata of *Prevotella* 1-treated PFF mice compared to the striata of vehicle- and *Prevotella* cocktail 2-treated PFF-injected mice (**Figure 8D-E**).

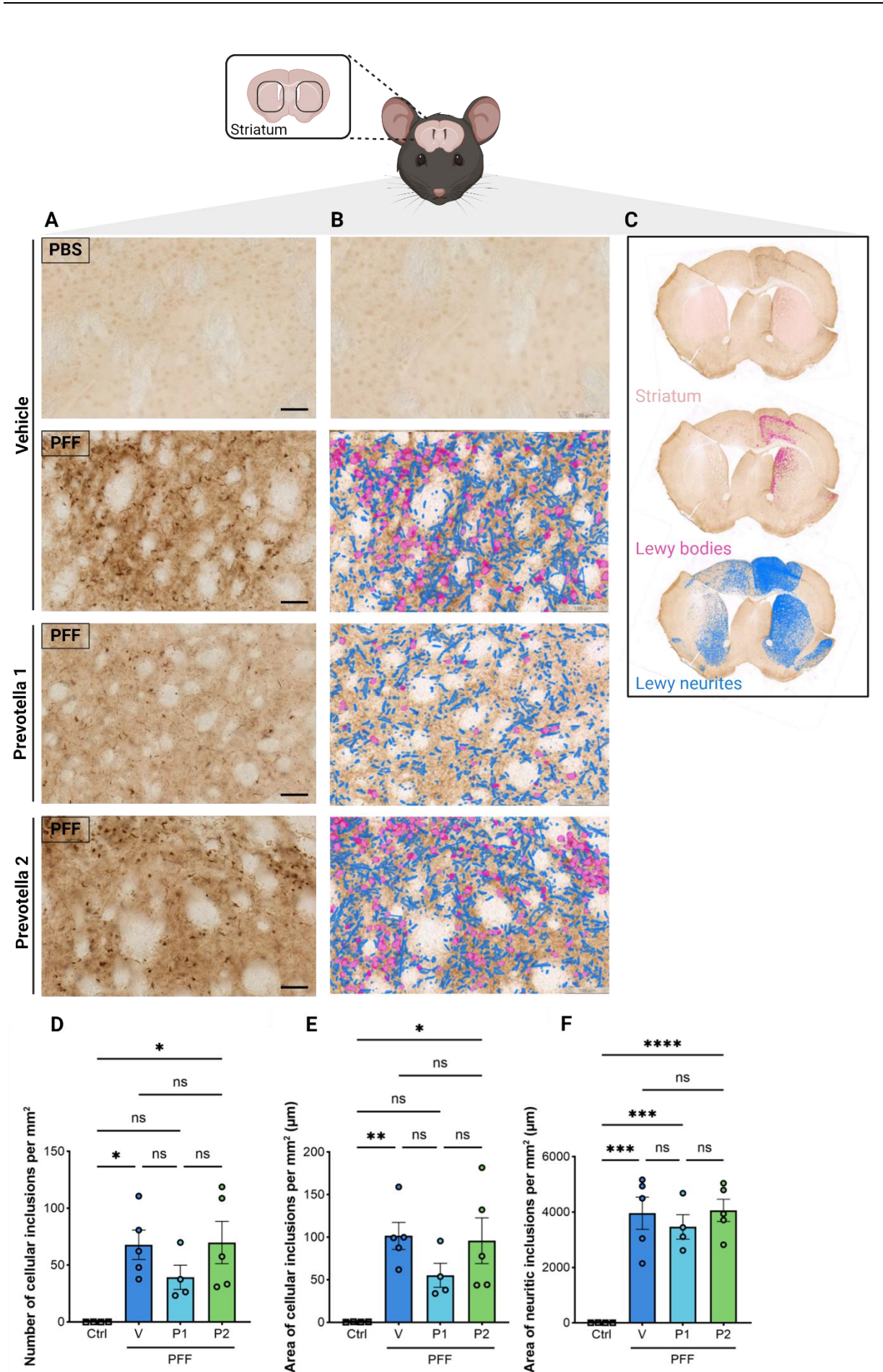


Figure 8. Live biotherapeutic products (LBPs) consisting of *Prevotella* cocktail 1 strains show a trend towards a beneficial effect on α -synuclein (α -syn) pathology in an α -syn preformed fibril (PFF) intrastriatal injection model in an early disease stage. (A) Representative images of phosphoS129 (pS129)- α -syn aggregates (brown) in the left striatum (injected site) of PBS-injected, vehicle-treated mice (ctrl; n=4) and PFF-injected mice treated with vehicle (V; n=5) or LBPs consisting of *Prevotella* 1 strains (P1; n=5) or *Prevotella* 2 strains (P2; n=5). **(B).** Examples of how the Aiforia software analyses the representative images from panel A. The color coding employed in the images allows for the identification of cellular inclusions (shown in pink) and neuritic inclusions (shown in blue). Scale bar: 100 μ m. **(C).** Example of how the Aiforia software recognizes the striatum regions (light pink), the Lewy bodies (dark pink) and Lewy neurites (blue) in a brain section. This brain section represents the vehicle-treated PFF-injected mice. **(D-F).** Quantification of the number of cellular inclusions (i.e. Lewy bodies)**(D)**, and area covered by Lewy bodies **(E)** and neuritic inclusions (i.e. Lewy neurites) **(F)**. Each data point represents the average of 2 brain sections, whereby for each brain section data from both striata are analyzed and pooled. Graphs were made using the GraphPad Prism 10 software and outliers were identified using the ROUT test (Q = 1%). A one-way ANOVA test was utilized, followed by Tukey's multiple comparison testing to determine statistical significance. The height of each bar represents the group mean, with error bars indicating $\pm$ standard error of the mean (SEM). Significance levels: ns: $p > 0.05$; * $0.01 \leq p < 0.05$; ** $0.001 \leq p < 0.01$; *** $0.0001 \leq p < 0.001$; and **** $p < 0.0001$. Illustration made with BioRender.

Next, we investigated the potential effects of *Prevotella* microbiome supplementation on another important pathological feature of PD, namely neuroinflammation. This was investigated through morphological analysis of microglial cells, which play a pivotal role in regulating central inflammation (Xie *et al*, 2021). Neuroinflammation is characterized by the activation of microglia, in which microglia undergo morphological changes (Green *et al*, 2022; Vidal-Itriago *et al*, 2022). The general classification of microglia morphology characterizes resting microglia as small, round cell bodies with extensive long branching (**Figure 9**)(Davis *et al*, 2017). In contrast, activated microglia are described as larger, more amoeboid cell bodies with retracted, shorter processes, leading to a smaller total volume compared to resting microglia (**Figure 9**).

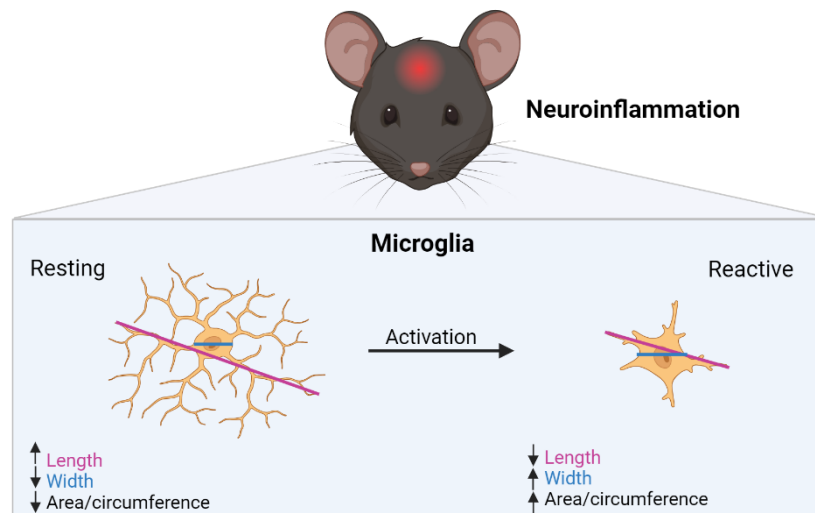


Figure 9. Microglial cells show morphological changes upon activation. Neuroinflammation is characterized by the activation of microglia, in which microglia undergo morphological changes. The general classification of microglia morphology characterizes resting microglia as small, round cell bodies with extensive and long branching, resulting in a low area/circumference ratio, which serves as an indicator of their activation status. Reactive microglia exhibit swollen cell bodies, leading to an increased width. Furthermore, the activated microglia show reduced branching, leading to a decrease in total length. Collectively, these morphological changes result in a higher area-to-circumference ratio. Illustration made with BioRender.

Consequently, to identify activated microglial cells, mouse brains were sectioned to obtain brain slices containing the striatum, and the ionized calcium-binding adaptor molecule 1 (IBA1) staining was performed. Indeed, IBA1 is mainly confined to microglia (Hopperton *et al*, 2018), and it is a suitable marker for morphological differentiation of ramified (i.e. resting) vs. amoeboid (i.e. activated) microglia (Davis *et al*, 2017). Subsequently, the immunostaining is followed by morphological analysis and quantification using the arivis Vision4D software, with the objective of studying the characteristics of microglia. In particular, the total length of cells, the number of branches, the number of terminals, the number of neurites, the volume, and the branch length were analyzed.

No significant differences were observed in the number and length of microglial dendrites between the vehicle-treated PBS- and PFF-injected groups, nor between the PFF mice in the different treatment conditions (**Figure 10D-E**). The same was true for the number of terminals, number of branch points and length of branches (**Figure 10F-H**). Furthermore, no significant differences in total microglial volume or dendritic volume were observed between the mice in different treatment groups (**Figure 10I-J**).

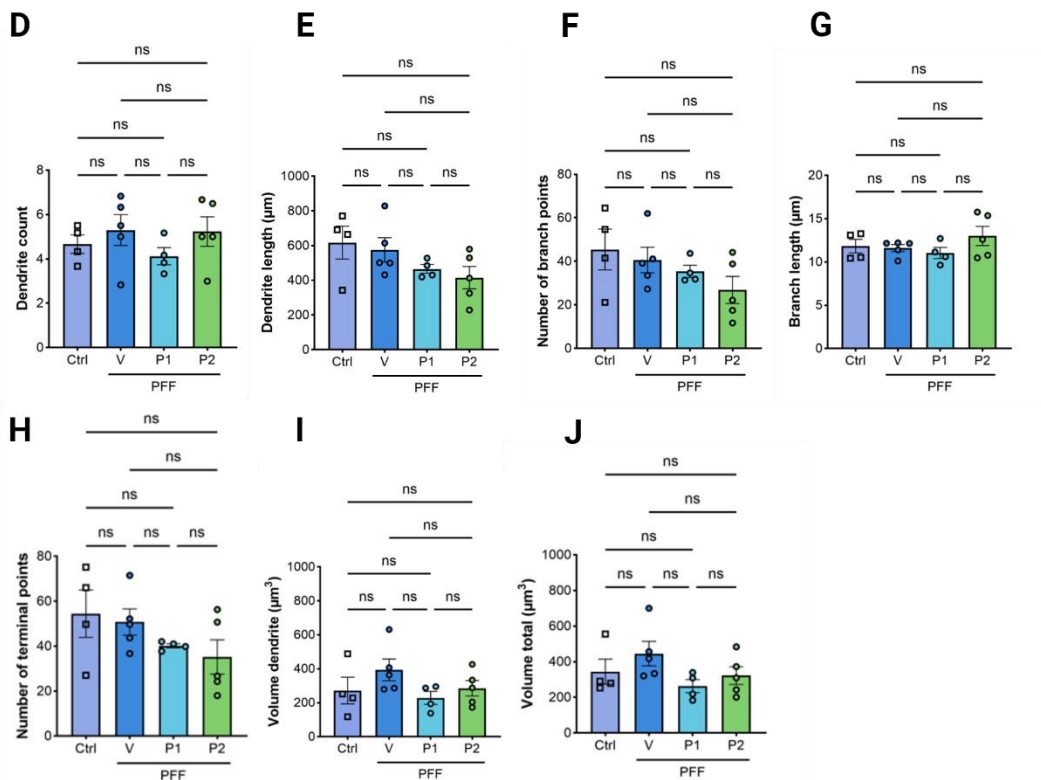
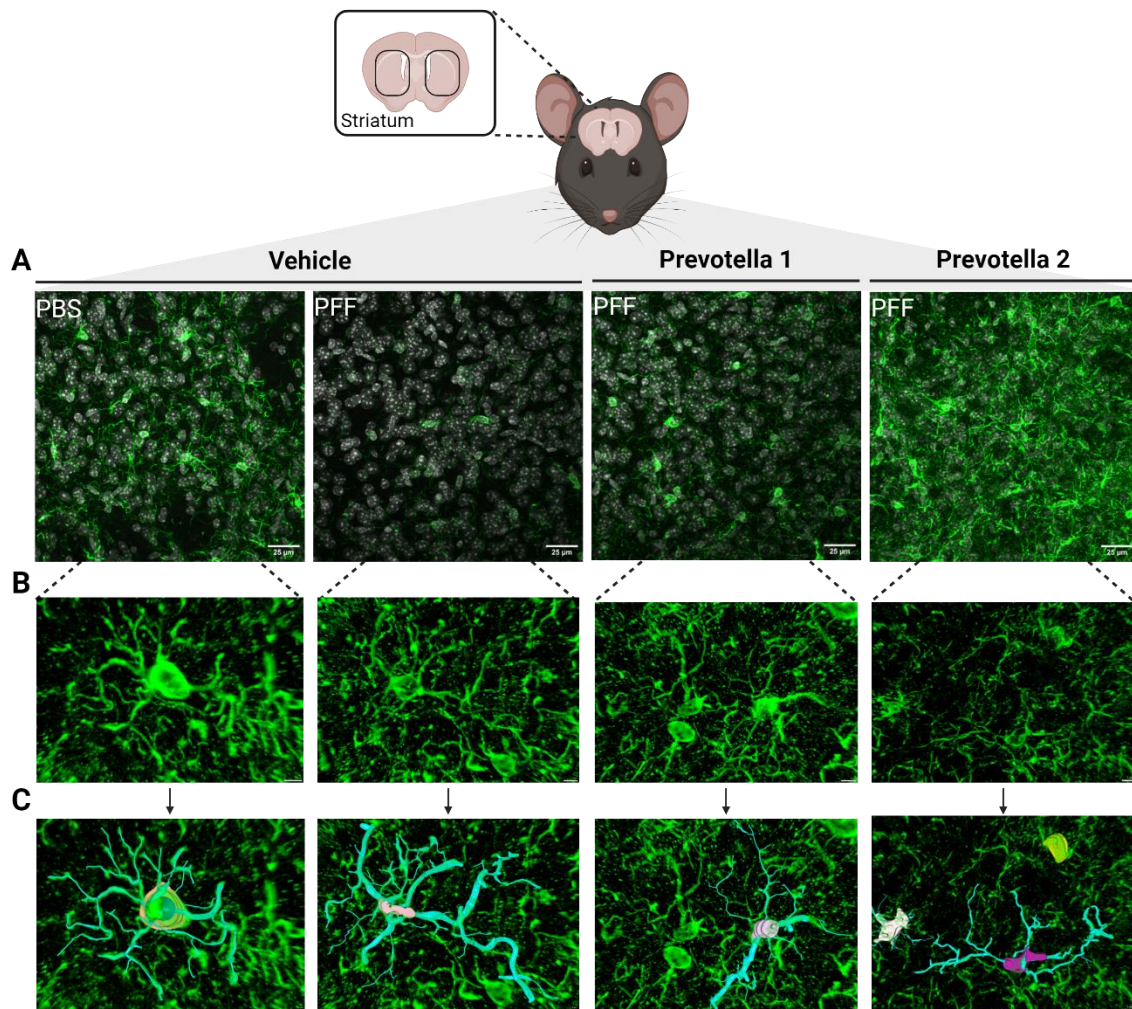


Figure 10. Live biotherapeutic products (LBPs) consisting of *Prevotella* strains do not lead to significant differences in microglial characteristics in an α -synuclein (α -syn) preformed fibril (PFF) intrastriatal injection model in an early disease stage. (A) Representative images of ionized calcium-binding adaptor molecule 1 (IBA1) stained microglia (green) and 4',6-diamidino-2-phenylindole (DAPI)-stained nuclei (white) in the striatum of PBS-injected, vehicle-treated mice (ctrl; n=4) and PFF-injected mice treated with vehicle (V; n=5) or LBPs consisting of *Prevotella* 1 strains (P1; n=5) or *Prevotella* 2 strains (P2; n=5). Scale bar: 25 μ m. (B). A zoom-in on the microglia from images of panel A. Scale bar: 5 μ m. (C). Examples of how the arivis Vision4D software analyses the images from panel B. Scale bar: 5 μ m. (D-J). The number of dendrites (D), branch points (F), and terminal points (H), as well as the length of dendrites (E), length of branch points (G), and dendrite volume (I) and total volume (J), were quantified. The data points represent the mean data from both striata for each mouse. Per striatum, 3 microglia were analyzed. Graphs were made using the GraphPad Prism 10 software and outliers were identified using the ROUT test (Q = 1%). A one-way ANOVA test was utilized, followed by Tukey's multiple comparison testing to determine statistical significance. The height of each bar represents the group mean, with error bars indicating $\pm$ standard error of the mean (SEM). Significance levels: ns: $p > 0.05$; * $0.01 \leq p < 0.05$; ** $0.001 \leq p < 0.01$; *** $0.0001 \leq p < 0.001$; and **** $p < 0.0001$. Illustration made with BioRender.

The effect of *Prevotella* microbiome supplementation on C3 expression was also investigated, as C3 and its activated fragments (C3a, C3b) are involved in inflammatory processes within the CNS. For this purpose, the left striatum was sampled followed by C3 ELISA. No significant differences in C3 levels were observed between the striata of the different groups of mice, except for the *Prevotella* cocktail 2-treated mice (**Figure 11**). These mice had significantly higher C3 levels in the left striatum than the vehicle-treated PFF-injected mice (**Figure 11**).

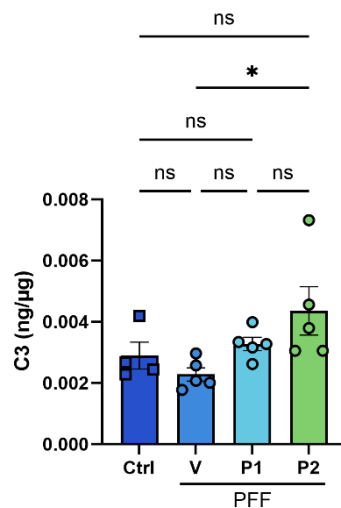


Figure 11. Oral administration of live biotherapeutic products (LBPs) consisting of *Prevotella* cocktail 2 strains increases C3 levels in the left striata in an α -synuclein (α -syn) preformed fibril (PFF) intrastriatal injection model in an early disease stage. C3 levels in the striatum of PBS-injected, vehicle-treated mice (ctrl; n=4) and PFF-injected mice treated with vehicle (V; n=5) or LBPs consisting of *Prevotella* cocktail 1 strains (P1; n=5) or *Prevotella* cocktail 2 strains (P2; n=5) was analyzed using a C3 enzyme-linked immunosorbent assay (ELISA). The C3 ELISA was performed with 200 μ g loaded protein using the Complement C3 Mouse ELISA Kit (Abcam) in accordance with the manufacturer's instructions. Graphs were made using the GraphPad Prism 10 software and outliers were identified using the ROUT test (Q = 1%). A one-way ANOVA test was utilized, followed by Tukey's multiple comparison testing to determine statistical significance. The height of each bar

represents the group mean, with error bars indicating $\pm$ standard error of the mean (SEM). Significance levels: ns: $p > 0.05$; * $0.01 \leq p < 0.05$; ** $0.001 \leq p < 0.01$; *** $0.0001 \leq p < 0.001$; and **** $p < 0.0001$.

3.2 Investigating the effect of LBPs consisting of *Prevotella* cocktail 2 and consortium CC113 strains in a mouse model of late-stage PD

The α -syn PFF intrastratial injection mouse model of PD was also used to investigate the impact of supplementation of the gut microbiome with specific bacterial strains. Again, the aim of this experiment was to investigate the impact of *Prevotella* supplementation on motor behaviour and pathological features of PD. Based on a preliminary QuPath analysis of the IBA1 staining from the experiment in the early-stage cohort, conducted by another researcher in the lab preceding my master thesis, the decision was made to use the *Prevotella* cocktail 2 in this experiment. The impact of another LBP, namely CC113 LBP was also examined. To this end, 24 α -syn PFF mice received oral *Prevotella* cocktail 2 ($n=8$), CC113 ($n=8$) or vehicle ($n=8$) cocktails at 6 mpi according to the treatment schedule in (Figure 12). During the first week of treatment, the mice were orally gavaged daily for 5 consecutive days whereafter they were orally gavaged 3 times a week for a further 4 weeks (Figure 12). The remaining 8 PBS-injected mice were administered vehicle cocktails via oral administration and will be referred to as control group. Phenotypic tests were conducted at baseline (i.e prior to initiation of the oral gavage treatment). Seven mpi, mice were subjected to a second round of phenotypic characterization followed by brain sampling, with the objective of investigating PD pathology (Figure 12).

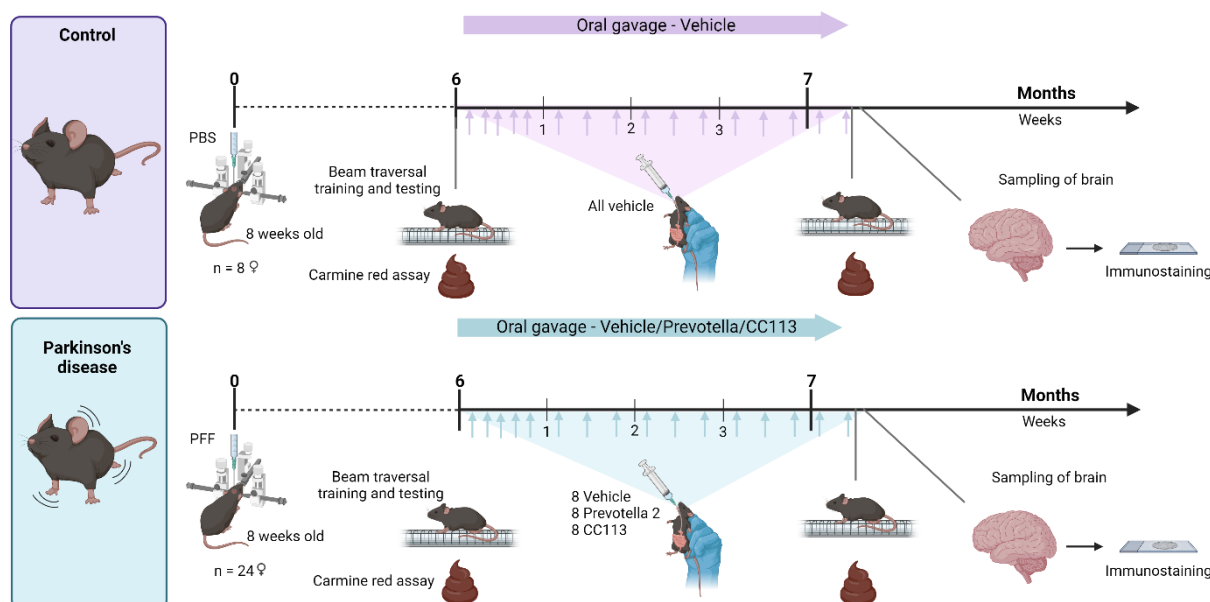


Figure 12. Experimental set-up of the study on the effect of live biotherapeutic products (LBPs) consisting of *Prevotella* cocktail 2 and consortium CC113 strains in a late-stage mouse model of Parkinson's disease (PD). In this set-up, 24 female mice were injected into the left dorsal striatum at 8 weeks of age with commercially available recombinant murine α -synuclein (α -syn) preformed fibrils (PFF). To include controls, 8 mice were injected with phosphate buffered saline (PBS) into the left dorsal striatum. Six months after injection, mice were given either vehicle, *Prevotella* cocktail 2 or consortium CC113 cocktails by gavage for 5 days. This was followed by oral gavage 3 times a week for 4 weeks. Before and after the oral treatments, the mice were subjected to beam traversal and carmine red assay to test motor disturbances and gastrointestinal

dysfunction, respectively. Seven months after injection, the mice were euthanized and their brains, cecal content and plasma were collected for further analysis. Stool samples were collected at various time points and sent to MRM Health, who supplied the LBPs, for validation of engraftment. Illustration made with BioRender.

3.2.1. Investigating the effect of LBPs consisting of *Prevotella* cocktail 2 and consortium strains on motor and non-motor function

To investigate the effect of *Prevotella* 2 and CC113 LBPs on the pathology of PD, phenotypic studies were performed. The motor behaviour of PBS- and PFF-injected mice was assessed after oral gavage to determine the effect of microbiome supplementation on motor disturbances. To this end, the C57BL/6J mice were subjected to the beam walking assay. This was also assessed before the oral treatment, at 6 mpi. Indeed, analysis at this timepoint is already relevant since the host lab has demonstrated significant differences between PFF- and PBS-injected mice on the beam traversal test from 2 mpi onwards in other cohorts.

However, no significant differences were observed between vehicle-treated PBS- and PFF-injected mice in the full beam traverse test and last section only (**Figure 13A,C**). Furthermore, it was shown that mice injected with PBS showed a trend towards a faster crossing time on the full beam after vehicle-treatment compared to baseline, potentially indicating a training effect over time (**Figure 13B**). In contrast, the performance of the PFF-injected mice on the complete beam test remained consistent before and after the oral intervention (**Figure 13B**). Upon examining the last part only, the CC113-treated PFF mice showed a slower crossing time before and after oral gavage (**Figure 13D**). Mice given CC113 cocktails took significantly longer to complete the full test and last section only compared to the control mice (**Figure 13A,C**).

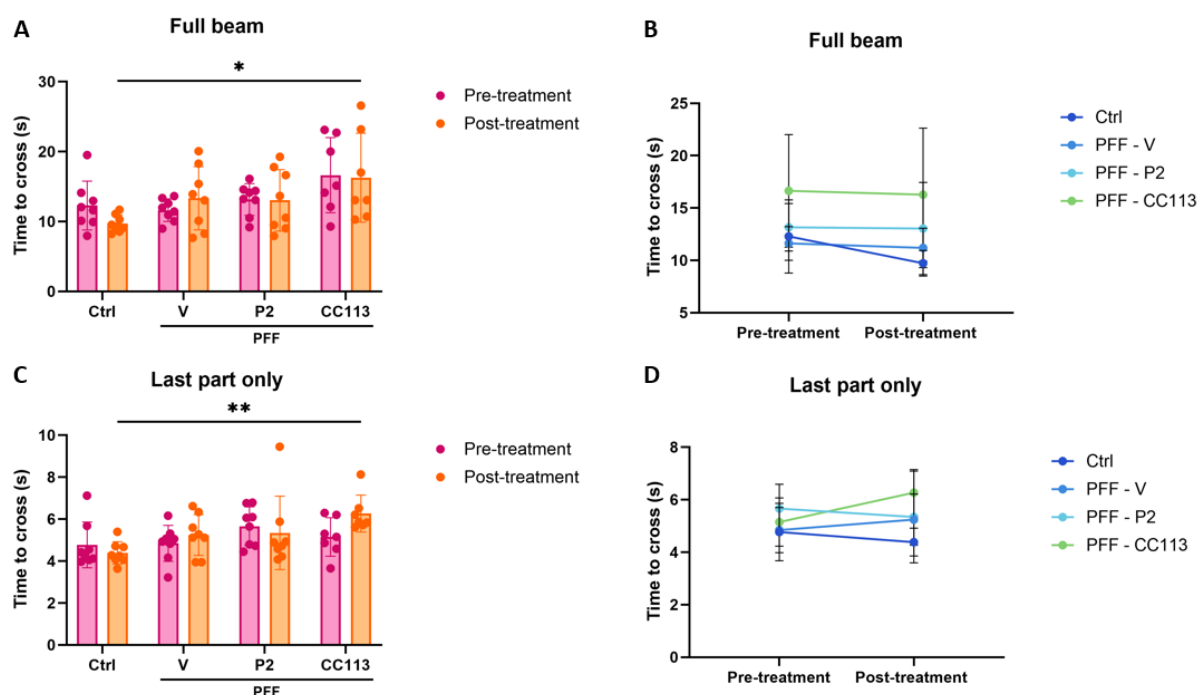


Figure 13. Live biotherapeutic products (LBPs) consisting of consortium CC113 strains have a detrimental effect on motor function in an α -synuclein (α -syn) preformed fibril (PFF) intrastriatal injection model in a late disease stage. Eight PBS-injected mice that had received vehicle (V) treatment (Ctrl) and 24 PFF-injected

mice, of which 8 mice received V treatment, 8 mice received *Prevotella* cocktail 2 (P2) and 8 mice received CC113 cocktail, were subjected to the beam traversing assay before after treatment. Mice were timed when they crossed the full beam (A, B) and the last narrow part of the beam (C, D). For each mouse, 5 trials were performed and the average of the 3 best trials was calculated. Graphs were made using the GraphPad Prism 10 software and outliers were identified using the ROUT test (Q = 1%). A two-way ANOVA test was utilized, followed by Šídák's multiple comparisons test to determine statistical significance. The height of each bar represents the group mean, with error bars indicating $\pm$ standard error of the mean (SEM). Significance levels: ns: $p > 0.05$; $*0.01 \leq p < 0.05$; $**0.001 \leq p < 0.01$; $***0.0001 \leq p < 0.001$; and $****p < 0.0001$

In addition to typical motor symptoms, PD patients also experience non-motor symptoms, with GI dysfunction being a common complaint. Therefore, constipation was assessed using the carmine red assay (Figure 14). This simple approach entails the oral administration of carmine red dye, followed by a visual examination of the fecal pellet output from singly housed mice until the emergence of a red pellet. No significant differences were observed in GI transit time between vehicle treated PBS- and PFF-injected mice in the carmine red test, nor between the PFF-injected mice with different treatments (Figure 14).

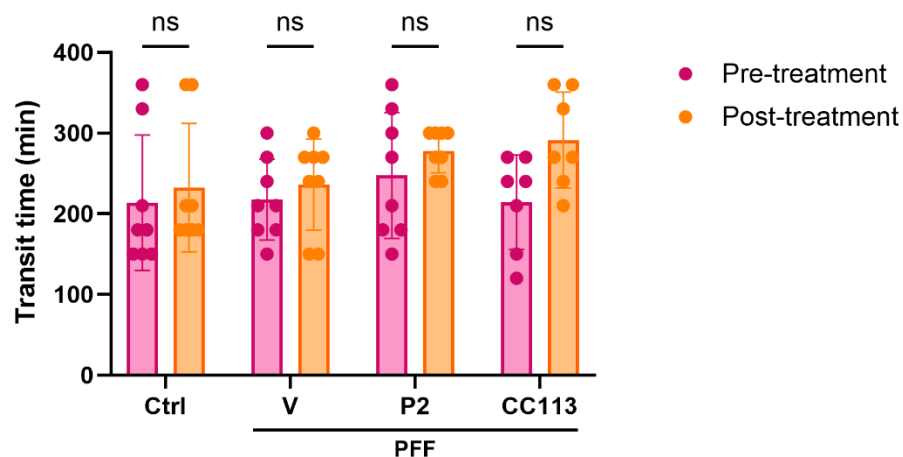


Figure 14. Live biotherapeutic products (LBPs) consisting of *Prevotella* cocktail 2 and consortium CC113 strains have no effect on the gastrointestinal transit time in an α -synuclein (α -syn) preformed fibril (PFF) intrastriatal injection model in an late disease stage. PBS-injected mice that had received vehicle treatment (Ctrl; n=8) and PFF-injected mice treated with vehicle (V; n=8), *Prevotella* cocktail 2 (P2; n=8), and CC113 (C; n=8) were subjected to the carmine red assay before and after oral gavage treatment. The mice were placed in individual cages and administered carmine red dye orally, followed by visual examination of pellet release for up to 6 hours after administration or until the red pellet emerged. Graphs were made using the GraphPad Prism 10 software and outliers were identified using the ROUT test (Q = 1%). A two-way ANOVA test was utilized, followed by Šídák's multiple comparisons test to determine statistical significance. The height of each bar represents the group mean, with error bars indicating $\pm$ standard error of the mean (SEM). Significance levels: ns: $p > 0.05$; $*0.01 \leq p < 0.05$; $**0.001 \leq p < 0.01$; $***0.0001 \leq p < 0.001$; and $****p < 0.0001$

3.2.2. Investigating the effect of LBPs consisting of *Prevotella cocktail 2* and consortium strains on brain pathology

Again, the effect of LBP administration in a PD mouse model was studied on α -syn aggregation. To this end, mice were euthanized at 7 mpi (i.e. late in disease spectrum) and their brains were sampled. The brains were then sectioned to obtain brain slices containing the striatum, which were immunostained for the presence of α -syn aggregates. After staining and imaging, brain sections were analyzed using the Aiforia software. Control mice were excluded from this experiment since intrastriatal PBS-injection does not lead to formation of pS129- α -syn aggregates. The striata were manually marked and then used for detection of LBs and LNs with the AI tool. This time, additional parameters were studied, including mean length, mean width and circumference of the LBs. One of the key advantages of the Aiforia software is its capability to automatically identify various brain regions in immunohistochemically stained 40 μ m agarose brain sections. However, this specific version of the tool did not perform optimal yet for this staining and will require further training of the AI model. Consequently, the annotation of the striata was performed manually. As the striata were manually marked, left (i.e. injected) and right (i.e. non-injected) striata could be distinguished from each other.

The results showed that the right striatum regions contained a significantly lower amount of α -syn aggregates compared to the left brain regions (**Figure 15G-I**). This was to be expected as we injected α -syn PFF into the left dorsal striatum. Furthermore, no statistically significant differences were observed in the mean length, width, and circumference of the LBs among the different mouse groups (**Figure 15D-F**). The injected striata of the consortium-treated PFF mice exhibited a significantly higher level of LBs in comparison to those of vehicle treated PFF mice (**Figure 15G**). Furthermore, the left striatum of mice administered CC113 cocktails exhibited a significantly elevated area percentage covered by LBs and LNs in comparison to those of PFF mice treated with vehicle (**Figure 15H,I**). Despite manual annotation, the entire tissue and some aggregates were not always accurately recognized (**Figure 15C**). Thus, further optimization of the Aiforia tool is necessary. However, we anticipate a consistent error rate across all sections.

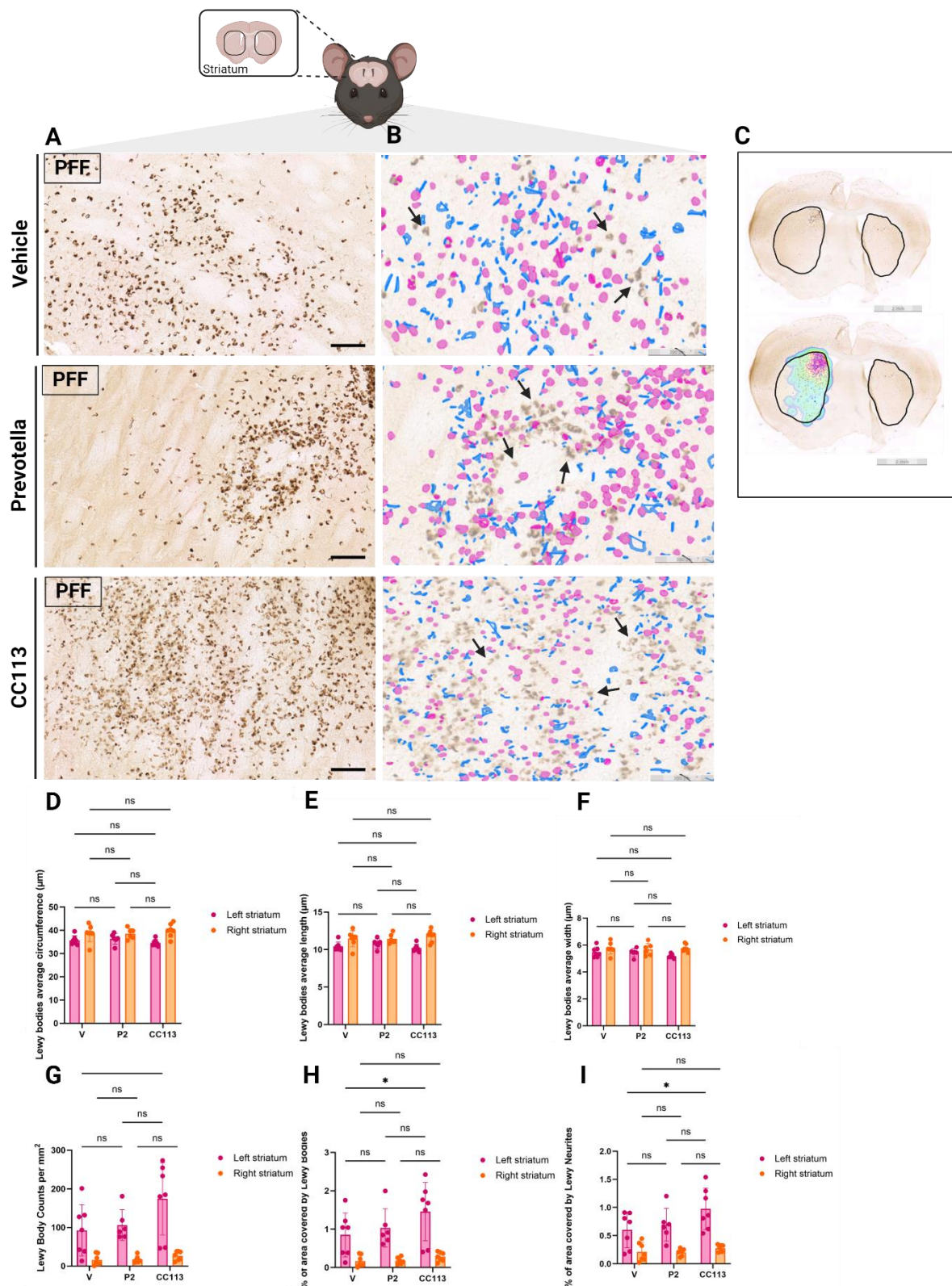


Figure 15. Live biotherapeutic products (LBPs) consisting of consortium CC113 strains worsen the α -synuclein (α -syn) pathology in an α -syn preformed fibril (PFF) intrastriatal injection model in a late disease stage. (A) Representative images of phosphoS129 (pS129)- α -syn aggregates (brown) in the left striatum (injected site) of PFF-injected mice treated with vehicle (V; n=8) or LBPs consisting of *Prevotella* cocktail 2

strains (P2; n=8) or CC113 strains (n=8). **(B)**. Examples of how the Aiforia software analyses the representative images from panel A. The color coding employed in the images allows for the identification of cellular inclusions (shown in pink) and neuritic inclusions (shown in blue). Some pS129- α -syn aggregates were not identified by the AI tool (as indicated by the arrows). Scale bar: 100 μ m. **(C)**. Example of how the Aiforia software recognizes the Lewy bodies (dark pink) and lewy neurites (blue) in the black manually marked striatum regions in a brain section. This brain section represents the V treated PFF injected mice. Scale bar: 2 mm. **(D-I)**. Quantification of the number of cellular inclusions (i.e. Lewy bodies) **(G)**, the area covered by Lewy bodies **(H)** and neuritic inclusions (i.e. Lewy neurites) **(I)**, and the average Lewy body circumference **(D)**, length **(E)** and width **(F)**. Graphs were made using the GraphPad Prism 10 software and outliers were identified using the ROUT test (Q = 1%). A one-way ANOVA test was utilized, followed by Tukey's multiple comparison testing to determine statistical significance. The height of each bar represents the group mean, with error bars indicating $\pm$ standard error of the mean (SEM). Significance levels: ns: $p > 0.05$; * $0.01 \leq p < 0.05$; ** $0.001 \leq p < 0.01$; *** $0.0001 \leq p < 0.001$; and **** $p < 0.0001$. Illustration made with BioRender.

The impact of the *Prevotella* cocktail 2 and CC113 LBPs on neuroinflammation was also examined through their influence on microglial characteristics using the α -syn PFF intrastriatal injection mouse model. As explained earlier, microglia show morphological changes upon activation. Upon activation, microglia show shorter processes and less branching, resulting in reduced process length (Green *et al*, 2022; Vidal-Itriago *et al*, 2022). In addition, the soma swells, resulting in increased soma width. Together, these changes result in an increased area to circumference ratio, which can be seen as an indication of activation status (**Figure 9**). In addition to these morphological changes, microglia begin to proliferate in response to injury, often leading to increased numbers and activity in the affected areas (Vidal-Itriago *et al*, 2022). Therefore, the number of microglia was assessed as well. For this experiment, mouse brains were sectioned to obtain brain sections containing the striatum, and IBA1 staining was performed. Subsequently, the slides were imaged and the immunostaining was followed by morphological analysis and quantification using the Aiforia software, with the objective of studying the characteristics of microglia. In particular, the total amount, average length, average width, and activation status of the microglia were analyzed. Also, this particular version of the tool did not yet perform optimally for this staining and will require further training of the AI model. Therefore, the annotation of the striata was performed manually.

No significant differences were observed between the vehicle-treated PBS- and PFF-injected groups, nor between the PFF mice in the different treatment conditions regarding the number, average length and average width, or activation status of microglia (**Figure 16E-H**).



Figure 16. Live biotherapeutic products (LBPs) consisting of *Prevotella* cocktail 2 and consortium CC113 strains do not lead to significant differences in microglia properties in an α -synuclein (α -syn) preformed fibril (PFF) intrastriatal injection model in a late disease stage. (A). Representative images of ionized calcium-binding adaptor molecule 1 (IBA1) stained microglia (brown) in the striatum of PBS-injected, vehicle-treated mice (ctrl; n=8) and PFF-injected mice treated with vehicle (V; n=8) or LBPs consisting of *Prevotella* cocktail 2 strains (P2; n=8) or consortium CC113 strains (n=8). Scale bar: 100 μ m. **(B).** Examples of how the Aiforia software analyses the representative images from panel A. Scale bar: 200 μ m. **(C).** Example of how the black manually marked striatum regions in a brain section of a P treated PFF mouse. **(D).** Example of an activated and non-activated microglia of a P2-treated PFF mouse with its respective measured parameters by the Aiforia software. **(E-H).** Quantification of the number **(E)**, average length **(F)**, average width **(G)**, and activation status **(H)** of microglia. The data points represent the mean data from both striata for each mouse. Microglial characteristics were obtained with the Aiforia software. Graphs were made using the GraphPad Prism 10 software and outliers were identified using the ROUT test ($Q = 1\%$). A one-way ANOVA test was utilized, followed by Tukey's multiple comparison testing to determine statistical significance. The height of each bar represents the group mean, with error bars indicating $\pm$ standard error of the mean (SEM). Significance levels: ns: $p > 0.05$; * $0.01 \leq p < 0.05$; ** $0.001 \leq p < 0.01$; *** $0.0001 \leq p < 0.001$; and **** $p < 0.0001$. Illustration made with BioRender. Illustration made with BioRender.

3.3 Investigating the effect of LBPs consisting of *Prevotella* cocktail 2 and consortium strains in a mouse model of LPS-induced neuroinflammation

Neuroinflammation is one of the pathological hallmarks of PD and can be studied with the PFF-injection mouse model mentioned above. However, results from the host lab indicate that neuroinflammation is present from 2 mpi onwards and in a rather mild state. As an alternative method to study neuroinflammation, neuroinflammation can be induced more quickly and drastically by the icv injection of LPS. Using this model, the effect of supplementing the gut microbiome with *Prevotella* cocktail 2 and CC113 LBPs on brain inflammation was investigated. To this end, 24 LPS-injected WT mice received oral gavage of *Prevotella* cocktail 2 (n=8), CC113 (n=8) or vehicle (n=8) cocktails according to the treatment schedule in **(Figure 17)**. In brief, 1 week prior to icv injection, mice received oral gavage for 5 consecutive days. After icv injection, this was followed by oral gavage 3 times a week for 4 weeks. The remaining eight PBS-injected mice were administered vehicle cocktails and will be referred to as control group. One mpi, mice were euthanized and sampled, with the objective of investigating neuroinflammation through microglial analysis **(Figure 17)**.

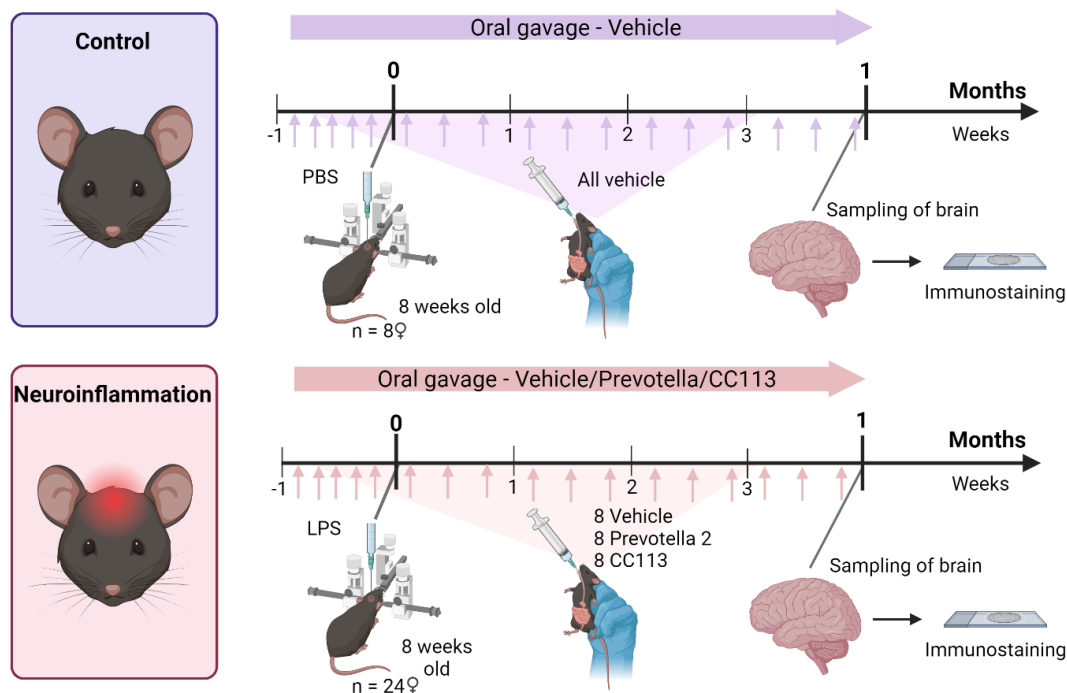


Figure 17. Experimental set-up of the study on the effect of live biotherapeutic products (LBPs) consisting of *Prevotella* cocktail 2 and consortium CC113 strains in a mouse model of neuroinflammation. In this set-up, 24 female mice were received an intracerebroventricular (icv) injection of lipopolysaccharide (LPS) at 8 weeks of age. To include controls, 8 mice were injected with phosphate buffered saline (PBS). One week prior to injection, mice were given either vehicle, *Prevotella* cocktail 2 or consortium CC113 cocktails by oral gavage for 5 consecutive days. This was followed by oral gavage 3 times a week for 4 weeks. One month after injection, the mice were euthanized and their brains and plasma were collected for further analysis. Stool samples were collected at various time points and sent to MRM Health, who supplied the LBPs, for validation of engraftment. Illustration made with BioRender.

The impact of supplementing the gut microbiome with *Prevotella* cocktail 2 and CC113 LBPs on brain inflammation was investigated through analysis of microglia. For this purpose, mice were euthanized and samples were taken again from the brain, followed by vibratome sectioning to obtain brain slices containing the hippocampus, as the hippocampus is located near the ventricle, where LPS was administered. The hippocampus was then subjected to IBA1 IHC. The brain sections were imaged and analyzed with the Aiforia software. In particular, the total amount, average length, average width, and activation status of the microglia were analyzed. Again, this particular version of the tool did not yet perform optimally for this staining and will require further training of the AI model. Therefore, the annotation of the hippocampi was performed manually.

No significant differences were observed between the different mouse groups with regarding the number, average length and average width, or the activation status of microglia (**Figure 18E-H**).

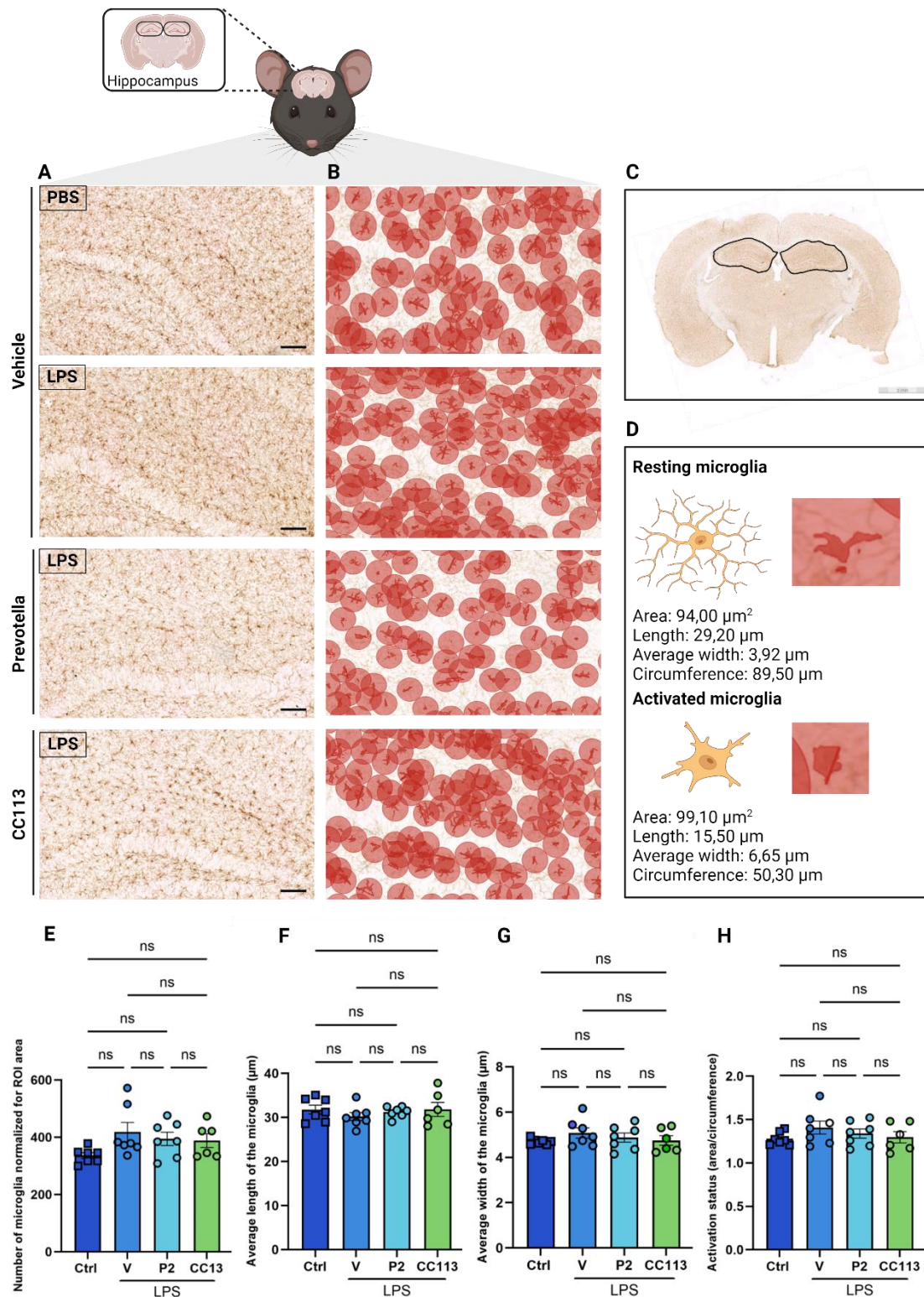


Figure 18. Live biotherapeutic products (LBPs) consisting of *Prevotella* cocktail 2 and consortium CC113 strains do not lead to significant differences on microglial properties in intracerebroventricular lipopolysaccharide (LPS)-injected mice. **(A).** Representative images of ionized calcium-binding adaptor molecule 1 (IBA1) stained microglia (brown) in the hippocampus of PBS-injected, vehicle-treated mice (ctrl; n=8) and LPS-injected mice treated with vehicle (V; n=8) or LBPs consisting of *Prevotella* cocktail 2 strains (P2; n=8) or consortium CC113 (CC113; n=8).

n=8) or consortium CC113 strains (n=8). Scale bar: 100 μ m. **(B)**. Examples of how the Aiforia software analyses the representative images from panel A. Scale bar: 200 μ m. **(C)**. Example of how the black manually marked striatum regions in a brain section of a P2-treated PFF mouse. **(D)**. Example of an activated and non-activated microglia of a P2-treated PFF mouse with its respective measured parameters by the Aiforia software. **(E-H)**. Quantification of the number **(E)**, average length **(F)**, average width **(G)**, and activation status **(H)** of microglia. The data points represent the mean data from both hippocampi for each mouse. Microglial characteristics were obtained with the Aiforia software. Graphs were made using the GraphPad Prism 10 software and outliers were identified using the ROUT test (Q = 1%). A one-way ANOVA test was utilized, followed by Tukey's multiple comparison testing to determine statistical significance. The height of each bar represents the group mean, with error bars indicating $\pm$ standard error of the mean (SEM). Significance levels: ns: $p > 0.05$; * $0.01 \leq p < 0.05$; ** $0.001 \leq p < 0.01$; *** $0.0001 \leq p < 0.001$; and **** $p < 0.0001$. Illustration made with BioRender.

Part 4: Discussion

PD is the most common neurodegenerative movement disorder, with age being the main risk factor as the prevalence of PD increases with the ageing population. PD is characterized by 3 typical pathological hallmarks, namely the loss of dopaminergic neurons in the SNpc, the misfolding, aggregation and widespread accumulation of the protein α -syn, and neuroinflammation. Today, the clinical diagnosis is made based on typical motor symptoms, including bradykinesia, muscular rigidity, dyskinesia, rest tremor, and postural and gait impairment. However, these motor symptoms only manifest when approximately 50% of the dopaminergic neurons in the SN have already been lost, at a relatively advanced stage of the pathological process. At present, the definitive diagnosis of PD patients can only be made post-mortem by examining the brain for the presence of LB pathology with the main principal component aggregated α -syn, associated with loss of dopaminergic neurons. Current therapeutic interventions are limited to the alleviation of symptoms, with no disease-modifying therapies available at the moment. The gold standard PD treatment is the use of a dopamine precursor, namely levodopa. Levodopa is associated with the development of involuntary movements or dyskinesias in the majority of patients over the course of years of treatment (Pezzoli & Zini, 2010). Furthermore, over time, many patients experience a reduction in the effectiveness of levodopa.

In addition to the typical motor symptoms, PD is characterized by non-motor symptoms, including olfactory dysfunction and GI issues. Several of these non-motor symptoms, such as constipation, can manifest years before diagnosis, emphasizing the gut's significance in PD. GI dysfunctions, including constipation, bloating, nausea, dysphagia, sialorrhea, vomiting, and gastroparesis, affect over 80% of PD patients (Zhu *et al*, 2022). Additionally, α -syn aggregates have been found in the digestive tract of PD patients, supporting Braak's hypothesis that α -syn pathology may propagate from the gut to the brain via the vagus nerve (Braak *et al*, 2003). Nevertheless, not all PD patients exhibit this mode of propagation, leading to the proposal of an alternative hypothesis that differentiates PD into 2 subtypes: brain-first and body-first (Borghammer & Van Den Berge, 2019). Other evidence highlighting the importance of the gut in PD is that the gut microbiome of PD patients differs significantly from that of healthy individuals. Furthermore, gut dysbiosis has been linked to the triggering or exacerbation of disease progression (Zhu *et al*, 2022). A greater understanding of the microbiota-gut-brain connection in PD may provide an opportunity to develop novel treatments for PD by using agents targeting gut microbiome dysbiosis.

Recently, the gut microbiome has emerged as a strategy to tackle PD. There are several ways to alter the microbiome, including FMT. A recent study from the host lab demonstrated that a single FMT resulted in modest yet lasting improvements in motor symptoms among patients with early-stage PD (Bruggeman *et al*, 2024). This underscores the therapeutic promise of altering the gut microbiome and advocates for expanded research on FMT across various stages of PD. However, the FMT technique is very labor intensive and there is always a risk that a fecal transplant could transfer a harmful pathogen to the recipient. Consequently, this dissertation focuses on the examination of specific bacterial strains as a potential solution.

Consistent alterations in the gut microbiome of PD patients compared to healthy individuals include the depletion of *Prevotella* bacteria (Scheperjans *et al*, 2015; Romano *et al*, 2021). Research indicated that individuals with a *Prevotella*-enriched enterotype experience the lowest rates of constipation and the least frequent occurrence of subthreshold parkinsonism

(Heinzel *et al*, 2021). Based on this observation, MRM Health developed *Prevotella* LBPs with the objective of investigating the effect of microbiome *Prevotella* supplementation in a mouse model for PD. Additionally, another LBP, namely an 11-strain consortium constructed with strains selected based on literature and some in vitro results obtained by MRM Health, was included. The effect of the gut microbiome supplementation was examined at both the early and late stages of the disease. Analyzing PD mouse models at different stages is informative because it provides insight into disease progression and underlying mechanisms. To illustrate, treatment in the early stages of the disease, when motor deficits are often mild and loss of dopaminergic neurons is minimal, may have an effect on the α -syn pathology. Conversely, treatment in the later stages of the disease may have an effect on motor deficits and dopaminergic neuron loss, as these features are more pronounced at this stage.

While various mouse models of PD have struggled to capture the 3 key characteristics simultaneously, the α -syn PFF intrastriatal injection leads to α -syn aggregation, reduction of dopaminergic neurons in the SNpc and striatum, leading to altered motor function (Luk *et al*, 2012), and neuroinflammation reflected by glial activation (Earls *et al*, 2019). Therefore, the intrastriatal α -syn PFF injection mouse model, which had already been successfully introduced in the host lab, was used for this purpose.

LBPs consisting of Prevotella strains did not show conclusive effects on motor behavior, α -syn pathology, or neuroinflammation in an early-stage PD mouse model

The first objective of this dissertation was to assess the effect of *Prevotella* supplementation on motor behaviour and pathological features of PD at an early stage. To this end, mice were treated with *Prevotella* cocktails, starting 1 week after PFF-injection until 2 mpi.

First, the influence of LBPs on motor function was assessed. Motor function can be assessed using various tests such as the rotarod, inverted grid, pole and beam traversal test. In this thesis, only the beam traversal assay was performed for this purpose, because previous research from the host laboratory showed that only beam traversal yielded significant differences between α -syn PFF- and PBS-injected mice. The results showed that mice treated with *Prevotella* cocktail 1 had a significantly longer time to complete the full-beam traversal test than control mice. When examining only the final section, this significant increase in completion time was also observed in *Prevotella* cocktail 1-treated mice compared to vehicle-treated PD mice and mice orally gavaged with a *Prevotella* cocktail 2. These results suggest that administration of *Prevotella* 1 LBPs leads to impairment of motor behaviour in α -syn PFF-injected mice. No significant differences were observed between vehicle-treated PFF-injected mice and control mice in this motor test. This was not expected, as research from the host laboratory has shown that PFF-injection leads to an increasing time to cross the beam in comparison with PBS-injected mice from 2 mpi onwards. However, the extent of this difference is not always consistent across different cohorts and requires high n-values due to variability inherent to cognitive testing. The attempts by the mice to cross the beam show considerable variability. This observed variability in motor function can be attributed to several factors, including stress. In this experiment, the mice received oral gavage, which induces stress and can consequently influence the outcome of motor function tests. The sensitivity of the beam test can be increased by, for example, using an inclined beam instead of a horizontal one, as mice naturally tend to move upwards to escape (Brooks & Dunnett, 2009). In addition, the non-significant differences observed between vehicle-treated PFF mice and control mice may suggest that more time is needed to develop motor symptoms. Motor

symptoms in PD typically result from dopamine deficiency, specifically due to the degeneration of dopaminergic neurons in the SNpc. Therefore, TH immunostaining or Western blot analysis might provide informative insights into the extent of dopaminergic neuron loss in this experiment. Research from the host laboratory has demonstrated TH loss 8 mpi in this mouse model using TH Western blot, but this has not yet been demonstrated 2 mpi.

Next, the impact of the *Prevotella* LBPs on α -syn aggregation was investigated in the striata through pS129- α -syn immunostaining and Aiforia analysis. The Aiforia AI system is based on a machine learning paradigm and was used to automatically analyze colorimetrically colored brain sections. The number of LBs and the area occupied by LBs and LNs were significantly larger in the striata of vehicle-treated and *Prevotella* cocktail 2-treated PFF-injected mice compared to the striata of control mice. This is in line with data from the host laboratory where they showed pS129- α -syn positive aggregates in the striata of PFF-injected mice from 1 mpi onwards. Moreover, we observed a decreasing trend in the number of LBs and size of the area occupied by LBs in the striata of *Prevotella* cocktail 1-treated PFF mice compared to the striata of vehicle- and *Prevotella* cocktail 2-treated PFF-injected mice. These results showed that the *Prevotella* cocktail 2 had no beneficial effect on α -syn pathology in the early-stage PD mouse model. Supplementation with *Prevotella* cocktail 1 demonstrated a potential therapeutic effect on PD pathology. This observation was further substantiated by the lack of significant differences between control mice and *Prevotella* cocktail 1-treated PFF-injected mice. However, these results are contradictory to the motor function results, where *Prevotella* cocktail 1 leads to impairment of motor function. This makes it very important to validate the obtained results in the future. To further validate the obtained data, it is essential to repeat the experiment on additional sections of the striatum to obtain a more representative result for the entire striatum. Additionally, the experiment should be extended to include additional brain regions such as the SN and cortex, to obtain a more complete image of the mouse brain. In-house studies have identified the presence of α -syn aggregates as early as 1 mpi in the cortex and SN following intrastriatal PFF-injection. The propagation of α -syn to diverse brain regions upon intrastriatal PFF-injection has also been shown in research using synthetic murine α -syn PFFs (Luk *et al*, 2012). Analysis of α -syn pathology in mice at 1, 3, and 6 mpi revealed a progressive, time-dependent spread of LBs and LNs. Initially limited at 1 mpi, the pathology extended to previously unaffected regions such as the ventral striatum, thalamus, occipital cortex, and various commissural and brainstem fibers by 1 mpi (Luk *et al*, 2012). It is important to note, however, that a different source of PFF had been utilized compared to the one employed in this thesis. This distinction is crucial, as variations in fibril structure, purity, reproducibility, or biological effects could influence the outcomes (Polinski *et al*, 2018).

The impact of *Prevotella* LBPs on potential neuroinflammation during the early stages of disease in PFF-injected mice was investigated as well. Microglia represent the primary immune cells engaged in neuroinflammatory responses. Following the onset of a pathological stimulus, such as inflammation, these mononuclear phagocytes undergo morphological changes (Green *et al*, 2022; Vidal-Itriago *et al*, 2022). The general classification of microglia morphology characterizes resting microglia as small, round cell bodies with extensive branching (Davis *et al*, 2017). In contrast, activated microglia are described as larger, more amoeboid cells with retracted processes. Consequently, the influence of LBPs on the morphological characteristics of microglia in the striata was investigated by IBA1 immunostaining and Vision4D analysis. No

significant differences were observed in the number and length of microglial dendrites between the vehicle-treated PBS- and PFF-injected groups, nor between the PFF mice in the different treatment conditions. The same was true for the number of terminals, number of branch points and length of branches. Furthermore, no significant differences in total microglial volume or dendritic volume were observed between the mice in different treatment groups. These results indicate that microglia in striata from neither the vehicle-treated PFF mice nor the *Prevotella*-treated PD mice were activated compared with the control group. However, a parallel study performed in the host laboratory using the same mouse model did reveal a trend towards microglial activation in the striatum of PFF-injected compared to PBS-injected mice at 2 mpi. Of note, in that study, analysis was done using colorimetric IBA1 staining and Aiforia analysis. The Aiforia analysis offers the advantage of evaluating all stained microglia in the striata, whereas Vision4D analysis only examined 3 microglia per striatum, potentially explaining the discrepancy between the results of my study and the parallel study in the lab. Hence, it would be informative to perform the Aiforia analysis on the striatum samples from this experiment. In addition, research showed that microglia contribute to PD pathology through the production of pro-inflammatory cytokines such as TNF- α and IL-1 β (Nagatsu & Sawada, 2005; McGeer *et al*, 1988). Moreover, activated microglia contribute to the elevated levels of TNF, IL-1 β , transforming growth factor beta (TGF β), IL-6, ROS, nitric oxide species, and pro-apoptotic proteins observed in the SNpc, striatum, and CSF of patients with PD (Tansey *et al*, 2022). In this experiment, the striata had already been isolated and the left striata were lysed for the C3 ELISA. In a parallel study from the host laboratory, a trend toward microglial activation was demonstrated at 2 mpi, specifically in the left injected striatum. Consequently, the remaining lysates from the left striata in this experiment can be utilized to perform an ELISA to quantify the levels of TNF- α , IL-1 β , and/or IL-6. An additional experiment that can be performed to check the effect on possible neuroinflammation, is to analyze astrocytes. Reactive microglia induce neurotoxic A1 astrocytes by producing TNF- α , IL-1 α and complement component C1q (Liddel *et al*, 2017). A1 astrocytes exhibit altered morphology characterized by elongated processes and an increased number of segments and branching and may actively contribute to neurotoxicity. Consequently, it could be of interest to study the effect of *Prevotella* LBPs on astrocyte activation, e.g. via glial fibrillary acidic protein (GFAP) immunostaining, a class-III intermediate filament considered the best marker for studying complex astroglial morphology (Jurga *et al*, 2021). After performing immunostaining, the striatum can be analyzed using the Aiforia software to obtain information on the number, morphological parameters and activation status of astrocytes. For instance, analysis conducted by the host laboratory using Aiforia has revealed an increased number of astrocytes in the striata 2 mpi, although no differences in activation status were observed.

The impact of *Prevotella* microbiome supplementation on potential neuroinflammation during the early stages of disease in PFF-injected mice was also evaluated by examining C3 levels. The complement system, of which C3 is a central protein, critically modulates immune responses and inflammation, including within the CNS, and is essential for synapse formation and elimination in the brain (Carpanini *et al*, 2019). Consequently, the influence of the LBPs on C3 levels was investigated by performing C3 ELISA on the left PFF- or PBS-injected striata. No significant differences in C3 levels were observed between the striata of the different groups of mice, except for the *Prevotella* cocktail 2-treated mice. The C3 levels in the striatum of these mice were found to be significantly higher than those of the vehicle-treated PFF mice,

suggesting a greater degree of complement activation. No increased C3 levels were observed in the left striatum of the vehicle-treated PFF group compared to the control group. This was not expected, as a parallel study performed in the host laboratory using the same mouse model did reveal increased C3 levels in the left striatum of PFF-injected compared to PBS-injected mice at 2 mpi. However, it is possible that C3 in the vehicle-treated PFF group was activated and cleaved into its active fragments (i.e. C3a and C3b), which could not be detected by the ELISA designed to measure intact C3. Furthermore, once generated, the fragments of C3 may be rapidly removed from the circulation or local environment, resulting in lower detectable levels of C3 in the sample being tested. Therefore, as an alternative, C3a and C3b ELISAs could be performed on the same samples to validate previous results. Alternatively, droplet digital PCR (ddPCR) can be used to detect C3 mRNA levels in brain tissue, allowing for precise quantification of gene expression. For this purpose, the obtained CSF samples could be used.

Overall, none of the tested *Prevotella* LBPs showed pronounced effects on motor behavior, α -syn pathology, and neuroinflammation in this early-stage PD model, although we did observe a trend towards reduced pS129- α -syn levels after treatment with *Prevotella* cocktail 1. These data should be confirmed by repeating the α -syn aggregates analysis on brain sections containing the SN and cortex. Furthermore, to validate the obtained data regarding neuroinflammation, microglia and astrocyte immunostaining experiments should be performed followed by analysis with the Aiforia software.

LBPs consisting of the consortium strain CC113 appear to exacerbate PD phenotype and pathology in late-stage PD mouse model

The second objective of this dissertation was to assess the effect of *Prevotella* cocktail 2 and CC113 supplementation on motor behaviour and pathological features of PD at a more advanced stage. To this end, mice were treated with *Prevotella* cocktail 2 or CC113 cocktails, starting at 6 mpi until 7 mpi.

First, the motor behaviour of PBS- and PFF-injected mice before and after oral gavage treatment was assessed to determine the effect of microbiome supplementation on motor disturbances. Again, only the beam traversal assay was performed for this purpose, because previous research from the host laboratory showed that only beam traversal yielded significant differences between α -syn PFF- and PBS-injected mice. In this motor test, no significant differences were observed between the vehicle-treated mice injected with PBS and those injected with PFF. This finding was unexpected, as previous research in our laboratory demonstrated that mice injected with PFF intrastrially required more time to complete the beam compared to mice injected with PBS, starting from 2 mpi. However, as mentioned earlier, the extent of this difference is not always consistent across different cohorts and requires high n-values due to variability inherent to cognitive testing. The variability observed in motor function may be attributed to several factors, including stress. This is also the reason why female mice were employed in this thesis. Male mice are known to engage in fighting behaviour within their cages, which can result in elevated stress levels. This, in turn, can influence the outcome of motor function tests. Additionally, conducting motor tests in a quiet environment is crucial to minimize stress induced by noise. The results also showed that mice injected with PBS showed faster crossing time on the full beam after vehicle treatment compared to baseline, which may indicate a training effect. In contrast, the performance of the PFF-injected mice on the complete beam test remained consistent before and after the

oral gavage. This stability might suggest the presence of motor deficits in the PFF mice. Upon examining the last part only, the CC113-treated PFF mice showed a slower crossing time before and after oral gavage. In addition, mice that received CC113 cocktails took significantly longer to complete the full test compared to the control mice, suggesting that the consortium LBP has a detrimental effect on motor function. This hypothesis was again confirmed when analyzing the last section only.

A second phenotypic test was conducted based on one of the common pre-motor symptoms of PD patients, namely GI dysfunction. GI symptoms occur in 60-80% of PD patients and constipation, one of the common GI symptoms, can occur several years before the onset of motor symptoms (Poirier *et al*, 2016; Bhattarai *et al*, 2021). For this purpose, the carmine red test was performed before and after LBP treatment. This method involves the oral administration of carmine red dye, followed by visual inspection of the pellet output from individually housed mice until the appearance of a red pellet. No significant differences were observed in GI transit time between vehicle-treated PBS- and PFF-injected mice, nor between the differently treated PFF mice. These results suggest that PFF-injected mice do not have prolonged gut transit times and therefore do not exhibit increased constipation. It is possible that a longer period may be required to elicit these GI symptoms. Previous studies, where mice were injected with synthetic mouse α -syn PFF into the duodenum, have demonstrated GI dysfunction at 7 mpi (Kim *et al*, 2019). The discrepancy between these and our results might be due to the different PFF injection site. Consequently, further characterization of the in-house intrastriatal PFF mouse model regarding GI symptoms is essential to draw robust conclusions about the efficacy of LBP supplementation on GI dysfunction.

Next, the impact of the *Prevotella* cocktail 2 and CC113 LBPs on α -syn aggregation was investigated in the striata through pS129- α -syn immunostaining and Aiforia analysis. Control mice were excluded from this analysis since intrastriatal PBS-injection does not lead to formation of pS129- α -syn aggregates. One of the key advantages of the Aiforia software is its capability to automatically identify various brain regions in immunohistochemically stained agarose brain sections. However, this specific version of the tool did not perform optimal yet for this staining and will require further training of the AI model. Consequently, the annotation of the striata was performed manually. No statistically significant differences were observed in the mean length, width, and circumference of the LBs among the different treatment groups. The injected striata of the consortium-treated PFF mice exhibited significantly elevated levels of LBs in comparison to those of vehicle-treated PFF mice, indicating that the CC113 cocktail exacerbates α -syn pathology. This hypothesis is further confirmed by the observation of a significantly higher percentage of the surface covered with LBs and LNs in the left striata of mice treated with the consortium cocktail compared with that of mice injected with PBS. Despite manual annotation, the entire tissue and some aggregates were not always accurately recognized, but we anticipate a consistent error rate across all sections. However, further optimization of the Aiforia tool is required. This will allow the analysis to be repeated and the results to be validated. To further validate the obtained data, it is essential to repeat the experiment on additional sections of the striatum to obtain a more representative result for the entire striatum. Additionally, the experiment should be extended to include additional brain regions such as the SN and cortex to obtain a more comprehensive understanding of the mouse brain. In-house studies have identified the presence of α -syn aggregates as early as 1 mpi in the cortex and SN following intrastriatal PFF injection. Additionally, other research has

demonstrated the accumulation of pathological α -syn in different brain regions using the intrastriatal PFF injection model (Luk *et al*, 2012; Earls *et al*, 2019). For instance, intrastriatal synthetic murine α -syn PFF injection has been shown to lead to the accumulation of α -syn pathology in the ventral striatum, thalamus, occipital cortex, and various commissural and brainstem fibers from 6 mpi onwards (Luk *et al*, 2012). However, it is again important to note the differences in the choice of PFFs, as variations in fibril structure, purity, reproducibility, or biological effects could influence the outcomes (Polinski *et al*, 2018). Other than α -syn pathology, the impact of LBPs on the loss of dopaminergic neurons can be evaluated. For this purpose, TH immunostaining can be on striatum sections. This can be further validated by performing a TH western blot in parallel. Indeed, studies from the host laboratory have already shown a loss of TH 8 mpi using a TH Western blot. However, the mice in this experiment were euthanized at 6 mpi, but the TH loss effect could potentially also be visible as early as 6 mpi.

The influence of *Prevotella* cocktail 2 and CC13 LBPs on neuroinflammation was also investigated in this late-stage PD mouse group by analyzing microglial characteristics using the Aiforia software. Again, this specific version of the tool did not perform optimal yet for this staining and will require further training of the AI model. Consequently, the annotation of the striata was performed manually. No significant differences were observed between the PFF mice in the different treatment conditions regarding the number, average length and average width, or activation status of microglia. In addition, the results showed no significant differences between the vehicle-treated PBS- and PFF-injected groups regarding the different microglial characteristics. However, a parallel study performed in the host laboratory using the same mouse model did reveal a trend towards increased activation status and a significant decrease in length of microglia in the left striatum of PFF-injected compared to PBS-injected mice at 2 mpi. It is conceivable that microglia are activated shortly after injection, but that this activation effect is lost over time. In this experiment, only 1 brain section was analyzed per mouse, to further validate the data obtained, the experiment can be repeated on more brain sections per mouse. An additional experiment that can be performed to check the effect on possible neuroinflammation, is to analyze astrocytes using the GFAP immunostaining and Aiforia software analysis.

Overall, the CC113 cocktail LBPs exacerbated α -syn pathology and motor deficits in the late-stage PD mouse model. These data should be confirmed by repeating the α -syn aggregates analysis on brain sections containing the SN and cortex, after optimization of the AI tool. Furthermore, to validate the obtained data regarding neuroinflammation, the microglia analysis should be repeated including more brain sections per mouse and an astrocyte immunostaining experiment should be performed followed by analysis with the Aiforia software.

LBPs consisting of the *Prevotella* cocktail 2 strains and consortium CC113 strains did not show conclusive effects on microglial characteristics in an LPS-injected mouse model

To more directly study the impact of microbiome supplementation on neuroinflammation, the icv LPS-injection mouse model was used, which induces strong neuroinflammation. To this end, mice received oral gavage with *Prevotella* cocktail 2 and CC113 LBPs, starting from 1 week before LPS-injection up to 1 mpi. Again, the impact of microbiome supplementation was studied by analyzing microglial characteristics using the Aiforia software. In this instance, the striata were not subjected to investigation, but the hippocampi were investigated, since the hippocampus is situated in the vicinity of the left lateral ventricle, where LPS was

administered. The hippocampi were previously studied in the host lab 1 month after icv LPS injection, showing that there was an increase in microglia in the hippocampus of LPS-injected mice compared with PBS-injected mice.

No significant differences were observed among the mouse groups in the number, average length, average width, or activation status of microglia. In this experiment, only 1 brain section was analyzed per mouse, to further validate the data obtained, the experiment can be repeated on more brain sections per mouse. Again, in addition, a GFAP immunostaining can be performed, to check astrocytic characteristics. Additionally, the application of a C3 ELISA assay on isolated hippocampi presents an opportunity to assess potential alterations in C3 levels following LPS-administration. It could be interesting to perform a follow-up experiment, with more mice included per treatment, so that additional analyses such as C3 ELISA could be performed.

In summary, LBPs consisting of the *Prevotella* cocktail 2 strains and consortium CC113 strains did not show conclusive effects on microglial characteristics in an LPS-injected mouse model. These data should be confirmed by repeating the microglia analysis including more brain sections per mouse and an astrocyte immunostaining followed by the Aiforia software analysis.

Overall conclusion and future perspectives

In this thesis, the overarching aim was to unravel the effects of gut microbiome supplementation on PD phenotype and pathology using *Prevotella* and CC113 LBPs provided by MRM Health. This was examined in an early- and late-stage PD mouse model, as well as in the LPS-induced neuroinflammation mouse model. The validation of engraftment will be conducted through 16S sequencing of fecal samples, carried out by MRM Health. The mice were subjected to a treatment regimen which was established in the early-stage PD experimental mouse group, which commenced 1 week after PFF-injection, with analyses planned at 2 mpi (i.e. known motor deficits in the beam traversal test, presence of pS129- α -syn accumulation, and microglial activation). The frequency of treatments per week was chosen based on the available amount of treatment cocktail and the workload associated with oral gavage. In the other experimental groups of mice, the treatment schedule was kept similar to the first experiment in the early-stage PD mouse group. For future studies, treatment could be extended to month-long treatment whereby intermediate evaluations of motor function could be added, for example, to assess the efficacy of the treatment cocktails.

The results indicate that among the tested *Prevotella* LBPs, none exhibited significant effects on motor behavior, α -syn pathology, or neuroinflammation in early-stage PD mice. Conversely, administration of the CC113 cocktail exacerbated α -syn pathology and motor deficits in late-stage PD mice. Notably, *Prevotella* cocktail 1 LBPs demonstrated a small promising trend towards reducing pS129- α -syn levels in the early-stage PD mouse cohort. In addition, the LBPs consisting of the *Prevotella* cocktail 2 strains and consortium CC113 strains did not show conclusive effects on microglial characteristics in an LPS-injected mouse model. However, as mentioned earlier, further research is needed to validate the obtained results. Subsequently, validation can be pursued in independent cohorts maintaining the same n-value, or ideally in larger independent cohorts, dependent on practical feasibility. If the LBPs would have beneficial effects on PD progression compared to healthy control mice, several steps need to be taken before the LBPs can be commercialized by the company. Firstly, the

results will need to be validated in an independent cohort. Hereby, also the administration method and/or treatment regimen can be further optimized. Subsequently, clinical evaluation also consists of multiple steps, beginning with safety testing in healthy individuals in a phase I study. Next, a phase II study can be conducted in which the efficacy and side effects of the LBPs are tested on PD patients. In the next step, which is phase III, the therapeutic effect and safety should be assessed in a group of diseased patients. Finally, long-term effects can be monitored where surveillance prevails after marketing the LBPs.

In addition to evaluating the therapeutic potential of LBPs, MRM Health aims to develop a novel biocontrol LBP to enhance the efficacy and mitigate the adverse effects associated with levodopa treatment. Levodopa, the golden standard therapy for PD, is susceptible to degradation by specific intestinal bacteria, thereby diminishing its bioavailability in the brain and compromising therapeutic outcomes (van Kessel *et al*, 2019; Maini Rekdal *et al*, 2019). Studies have elucidated that gut microbiota, including *Enterococcus faecalis*, *Eggerthella lenta*, and *Clostridium sporogenes*, possess diverse enzymatic pathways capable of metabolizing levodopa (Xu *et al*, 2023). The peripheral metabolism of levodopa not only diminishes its bioavailability but also undermines its therapeutic efficacy. Notably, certain gut bacteria can decarboxylate levodopa to dopamine through the action of tyrosine decarboxylases, resulting in a reduction of levodopa's ability to traverse BBB (van Kessel *et al*, 2019). To address this challenge, MRM Health is looking for bacterial strains that can outcompete levodopa-metabolizing bacteria to assess the potential impact of LBPs on levodopa pharmacokinetics. This entails administering levodopa to mice via oral gavage and subsequently analyzing blood samples at distinct time intervals to quantify levodopa concentrations in the bloodstream. Such methodology thus enables the evaluation of LBPs' influence on the systemic availability of levodopa, offering insights into their potential therapeutic utility.

It is estimated that by the year 2040, 14.2 million individuals will be affected by PD worldwide (Dorsey & Bloem, 2018). This highlights the crucial societal importance of PD research. Finding new treatments for PD will not only benefit individuals suffering from the disease, but will also have far-reaching positive implications for society as a whole. Dysbiosis in the gut microbiome represents a promising therapeutic target for PD, whereby a bacterial pill may be developed in the future with the potential of benefitting many PD patients.

Part 5: Materials and Methods

5.1 Mice

For all experiments, female WT C57BL/6J mice were purchased from Janvier. The mice intended for PFF-injections were randomly distributed in cages within the dedicated pathogen-free (SPF) facility of the Flemish Institute of Biotechnology in Ghent. After brain injections at 8 weeks of age, these mice were moved to a conventional animal facility where a dedicated motor function procedure room is present. The mice intended for LPS-injections were continuously housed in a conventional animal facility. Each cage accommodated groups of 3 to 5 mice, with unrestricted access to water and food in a controlled light-dark cycle of 14 hours of light and 10 hours of darkness. All animal studies were conducted in compliance with governmental and EU guidelines for the care and use of laboratory animals and were approved by the ethical committee of the Faculty of Sciences, Ghent University, Belgium. An overview of the animals can be found in addendum (**Supplementary Table 1**).

5.1.1 Intrastriatal injections

The technical procedure for intrastriatal injection was performed as previously described in publications (Luk *et al*, 2012). In brief, at 8 weeks of age, mice were anesthetized with 4% isoflurane and secured onto a stereotactic frame (Stoelting). A constant body temperature of 37°C was maintained via a heating pad. Isoflurane concentration was subsequently reduced to 2%, while sedation depth was assessed via the pinch reflex. Prior to surgery, the surgical site was prepared by shaving and disinfecting the head with 70% ethanol. A 1.5 cm incision was made along the medial axis of the skull. Injection coordinates, referenced to the bregma intersection (anteroposterior +0.7 mm, mediolateral +2.0 mm, dorsoventral –3.0 mm), were determined using the Franklin and Paxinos mouse brain atlas. Using a Hamilton™ Neuros™ syringe, mice unilaterally injected into the left striatum with 5 µg (2 µl; 2.5 mg/ml) of PFF at a constant rate of 0.4 µL/min. Commercially available murine α -syn PFFs (Stressmarq) were used for this purpose, whereas control mice were injected into the left striatum with 2 µl of PBS. After injection, the needle was left in place for a further 4 minutes before being slowly withdrawn (1 mm/1 min) to prevent reflux. The burr hole and skin were closed using bone wax and nylon sutures. Each mouse was individually housed for post-operative recovery under a heat lamp before being reintroduced to its cage. Following surgical procedures and subsequent recovery, mice were transferred to the conventional facility at the INW animal house of Ghent University. At this location, the oral gavage, phenotypic assessments, and sampling were conducted.

5.1.2 Icv injections

The technical procedure for icv injection was performed as previously described in host lab publications (Vandendriessche *et al*, 2024). In brief, at 8 weeks of age, mice were anesthetized with 4% isoflurane and secured onto a stereotactic frame (Stoelting) to maintain a constant body temperature of 37°C via a heating pad. Isoflurane concentration was subsequently reduced to 2%, while sedation depth was assessed via the pinch reflex. Prior to surgery, the surgical site was prepared by shaving and disinfecting the head with 70% ethanol. A 1.5 cm incision was made along the medial axis of the skull. Injection coordinates, referenced to the bregma intersection (anteroposterior –0.7 mm, mediolateral +1.0 mm, dorsoventral –2.0 mm), were determined using the Franklin and Paxinos mouse brain atlas. Utilizing a Hamilton™ Neuros™ syringe, mice were injected with 5 µl (1 µg/µl; 5 µl) LPS into the left lateral ventricle.

LPS from *S. abortus equi* (L-5886, Sigma-Aldrich) was used for this purpose. To include controls, mice were injected with 5 μ l PBS. After injection, the needle was left in place for a further 4 minutes before being slowly withdrawn (1 mm/1 min) to prevent reflux. The burr hole and skin were closed using bone wax and nylon sutures. Each mouse was individually housed for post-operative recovery under a heat lamp before being reintroduced to its cage.

5.1.3 Oral gavage of LBPs

The LPS mice had already received a 5-day pre-treatment with LBP 1 week prior to the LPS-injections. Following the administration of LPS-injections, the mice were subjected to a course of LBPs 3 days a week for a period of 4 weeks. The PFF-injected mice received pretreatment with LBPs for a period of 5 consecutive days, with this occurring 1 week after PFF-injection (early-stage PD) or 6 mpi (late-stage PD). Subsequently, the mice underwent administration of LBP on 3 days per week for either 4 (late-stage PD) or 6 weeks (early-stage PD). The LBPs were always thawed just before administration and dissolved in buffer A, delivered by MRM Health. The composition of buffer A is confidential. In the oral gavage procedure, a curved metal needle with a spherical tip was used to administer 200 μ l per mouse. Animals were observed during and for 5 minutes after the procedure to evaluate stress indicators like irregular breathing, shivering, and immobility.

5.1.4. Tissue sample isolation

Mice were anesthetized by PBS/ketamine/xylazine (6ml/2ml/2ml) injection. Full sedation was confirmed using the pinch reflex test. Blood and CSF were obtained through cardiac puncture and cisterna magna puncture techniques, respectively. Mice were then perfused transcardially using ice-cold 1x PBS-heparin. Following decapitation, either the complete brain or brain regions (i.e. striata) were isolated. Brain regions were snap-frozen in liquid nitrogen and then stored at -80°C. The complete brains were further processed for staining. The brains were collected and post-fixed in 4% paraformaldehyde (PFA) at 4°C overnight. The brains were then put in 1x PBS. The brains were cut on the coronal plane at the hypothalamus and embedded in 5% agarose. After the agarose solidified, 0.01% sodium azide was added. Brain samples were kept at 4°C until sectioning. The brain sections were cut using a Leica VT1200 S vibratome (Leica Biosystems)(speed: 0.8 mm/sec, amplitude: 1, auto feed: 40 μ m). Subsequently, the sections were stored in 0.01% sodium azide at 4°C until staining.

5.2 Phenotypical analysis

5.2.1 Beam traversal assay

For the beam traversal test, a challenging beam spanning a total length of 1 m was utilized. This beam consisted of 4 sections, each with sequentially thinner widths (3.5, 2.5, 1.5, and 0.5 cm) and featured 1 cm overhangs positioned 1 cm below its surface at both ends. Placed atop 3 inverted clean mouse cages, the beam was oriented to direct its narrowest end into the home cage of the test mice of which most of the bedding was put in a separate, empty cage. The testing procedure comprised 2 training days and 1 testing day, with 24-hour intervals between them. During the initial training day, each mouse traversed the beam 5 times. In the first trial, the home cage, containing cage mates, guided the mouse along the beam. Subsequent trials involved minimal guidance, with mice gently oriented in the correct direction as needed. On the second training day, mice received 5 additional trials, requiring minimal assistance to traverse the beam. The testing phase commenced on the following day.

A mesh grid with dimensions corresponding to each beam segment was placed atop the beam, and mice traversed it 5 times while on the grid surface. Trials were conducted per cage, with an approximate 1 minute inter-trial interval. Fecal pellets and urine were removed with dry paper between the mice of the same cage. Between cages, the beam was cleaned with 20% ethanol. All trials during the testing phase were recorded and manually timed. Successful traversal was defined as both front paws entering the home cage. The mean of the 3 best trials per mouse was used for performance comparison.

5.2.2. Carmine red assay

Each mouse was orally gavaged with 6% carmine red (Sigma) in methylcellulose solution. Hereafter, each mouse was placed in a separate clean cage without bedding, but with a tissue, and with 3 pellets of food and water. The first 1,5 hours no pellets were checked. Afterwards, the fecal pellets were checked and counted every 20-30 minutes. When the pellets clearly contained red dye, the assessment was stopped and the mouse was returned to its original cage. This assessment continued for 6 hours or until red dye was assessed.

5.3 Post-mortem pathological analysis

5.3.1. IHC

For immunostainings on the mouse brain intended for Aiforia analysis, 40 μ m brain sections were made of agarose embedded brains (Leica Vibratome). First, peroxidase blocking was conducted under the hood using 5% H₂O₂ in methanol. Then, the sections were washed 3 times with PBS. Next, for the pS129 α -syn staining, proteinase K was added to the sections. Following blocking with blocking buffer (i.e. 5% goat serum in PBT (0.5% BSA, 0.02% Triton-X-100 in PBS)) for 30 min at room temperature (RT), sections were incubated with rabbit monoclonal recombinant anti-alpha-synuclein (phosphor S129)(anti-p(S129)- α syn) primary antibody (1/20 000; Stressmarq; SMC-600) or recombinant anti-IBA1 primary antibody (1/10 000; Wako Chemicals; 019-19741) diluted in blocking buffer. After an overnight incubation at 4°C, the brain sections were washed 3 times with PBS. The goat anti-rabbit biotinylated secondary antibody (1/1000; Invitrogen) was added to all sections to incubate for 2 hours at RT. After 3 more washes, the Avidin-Biotin Complex (ABC) mix (Vector Laboratories, Vectastain Elite ABC kit, Peroxidase) was made, incubated for 30 min in dark at RT, and then added to the brain sections in the dark for another 30 min. After washing 3 times with PBS, 3,3'-diaminobenzidine (DAB)(Vector Laboratories, ImmPACT DAB Substrate Kit, Peroxidase) was added, which was incubated for either 5 min (pS129- α -syn) or 2 min (IBA1), after which the sections were submerged in PBS to stop the reaction. Subsequently, after 2 additional washing steps, the sections were put on SuperFrost Plus slides which were then dehydrated using the Leica ST5010 Autostainer XL (Leica Biosystems) program 4 (bidi, 90% ethanol, 100% ethanol, and xylene). The coverslips were mounted on the glass slides with Entellan and dried overnight. Hereafter, imaging was conducted using the Zeiss Axioscan.Z1 microscope. Hereafter, the α -syn aggregates and IBA1 microglia were automatically identified with the Aiforia software.

For the immunostaining on the mouse brain sections intended for arivis Vision4D analysis, 50 μ m brain sections were made of agarose embedded brains (Leica Vibratome). The sections were first washed twice with PBS. Following blocking with blocking buffer (i.e. PBS, 0.5% TritonX and 5% normal goat serum) for 1 hour at RT, sections were incubated with recombinant anti-IBA1 primary antibody (1/500; Wako Chemicals) diluted in blocking buffer. After an overnight incubation at 4°C, the brain sections were washed 4 times with PBS. After

washing, staining was continued in the dark. The goat anti-rabbit secondary antibody DyLight™ 633 (1/1000; Invitrogen) was added to all sections to incubate for 2 hours at RT. After washing 3 times with PBS, a 4',6-diamidino-2-phenylindole (DAPI; 1/1000; D1306; Invitrogen) staining was performed to counterstain the nuclei. Subsequently, after an additional washing steps, the sections were put on SuperFrost Plus slides. The coverslips were mounted on the glass slides with DABCO (Merck) and dried overnight. Imaging was conducted using the ZLSM880 FastAiry microscope. The analysis was performed using the arivis Vision4D software.

5.3.2. C3 ELISA

The striatum samples were lysed in RIPA lysis buffer (50 mM Tris HCl pH 8; 150 mM NaCl; 1% NP-40 Tergitol; 0,5 mM EDTA pH8; PBS; protease inhibitor cocktail tablet) through mechanical homogenization using a Qiagen Tissue Lyser II (Qiagen)(5 min, 20 Hz), followed by centrifugation (highest speed, 5 min, 4°C). The supernatant was then collected to determine protein concentration using the Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific). The C3 ELISA was then performed with 200 µg of loaded protein using the Complement C3 Mouse ELISA Kit (Abcam) was in accordance with the manufacturer's instructions.

5.4. Statistical analysis

Statistical analyses were conducted using GraphPad Prism 10 software. Outliers were identified using the ROUT test ($Q = 1\%$). For comparisons among more than 2 groups, a one-way analysis of variance (ANOVA) test was utilized, followed by Tukey multiple comparison testing. For the grouped data, a two-way ANOVA test was utilized, followed by Šídák's multiple comparisons. Data are presented as means $\pm$ standard error of the mean (SEM), and statistical significance was defined as a p -value < 0.05 . Significance levels are denoted on the graphs as follows: ns: $p > 0.05$; * $0.01 \leq p < 0.05$; ** $0.001 \leq p < 0.01$; *** $0.0001 \leq p < 0.001$; and **** $p < 0.0001$.

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Attachments

Supplementary Table 1: Overview of all mice. PBS: Phosphate Buffered Saline. PFF: Preformed fibrils. V: Vehicle.
P1: *Prevotella* 1. P2: *Prevotella* 2. C: Consortium CC113.

	Mouse ID	Injection	Oral gavage	Notes
PD early-stage experiment 1	587L	PBS	V	
	587R	PBS	V	
	587LR	PBS	V	
	587NT	PBS	V	
	637L	PBS	V	
	637R	PBS	V	
	637LR	PBS	V	
	637NT	PBS	V	
	646L	PFF	V	
	646R	PFF	V	
	646LR	PFF	V	
	646RR	PFF	V	
	646NT	PFF	V	
	650L	PFF	V	
	650R	PFF	V	
	650LR	PFF	V	
	650RR	PFF	V	
	650NT	PFF	V	
	647L	PFF	P1	
	647R	PFF	P1	
	647LR	PFF	P1	
	647RR	PFF	P1	
	647NT	PFF	P1	
	653L	PFF	P1	
	653R	PFF	P1	
	653LR	PFF	P1	
	653RR	PFF	P1	
	653NT	PFF	P1	
	649L	PFF	P2	

	649R	PFF	P2	
	649LR	PFF	P2	
	649RR	PFF	P2	
	649NT	PFF	P2	
	645L	PFF	P2	
	645R	PFF	P2	
	645LR	PFF	P2	
	645RR	PFF	P2	
	645NT	PFF	P2	
PD late-stage experiment 2	388L	PBS	V	
	388R	PBS	V	
	388LR	PBS	V	
	388NT	PBS	V	
	389L	PBS	V	
	389R	PBS	V	
	389LR	PBS	V	
	389NT	PBS	V	
	392L	PFF	V	
	392R	PFF	V	
	392LR	PFF	V	
	392NT	PFF	V	
	395L	PFF	V	
	395R	PFF	V	
	395LR	PFF	V	
	395NT	PFF	V	
	396L	PFF	P2	
	396R	PFF	P2	
	396LR	PFF	P2	
	396NT	PFF	P2	
	398L	PFF	P2	
	398R	PFF	P2	
	398LR	PFF	P2	

	398NT	PFF	P2	
	399L	PFF	C	
	399R	PFF	C	
	399LR	PFF	C	Died prior to PFF injection
	399NT	PFF	C	
	401L	PFF	C	
	401R	PFF	C	
	401LR	PFF	C	
	401NT	PFF	C	
LPS neuroinflammation	416L	PBS	V	
	416R	PBS	V	Died due to oral gavage
	416LR	PBS	V	
	416NT	PBS	V	
	417L	PBS	V	
	417R	PBS	V	
	417LR	PBS	V	
	417NT	PBS	V	
	424L	LPS	V	
	424R	LPS	V	
	424LR	LPS	V	
	424NT	LPS	V	
	425L	LPS	V	
	425R	LPS	V	
	425LR	LPS	V	
	425NT	LPS	V	
	428L	LPS	P2	
	428R	LPS	P2	
	428LR	LPS	P2	
	428NT	LPS	P2	
	431L	LPS	P2	Died due to unknown reason
	431R	LPS	P2	
	431LR	LPS	P2	

	431NT	LPS	P2	
	432L	LPS	C	Sacrificied due to fluid accumulation from oral gavage
	432R	LPS	C	
	432LR	LPS	C	
	432NT	LPS	C	
	433L	LPS	C	
	433R	LPS	C	
	433LR	LPS	C	
	433NT	LPS	C	