The Effects of Gut Dysbiosis via Bacteriophages and its Role in Parkinson's Disease

by

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Abstract

Parkinson’s disease (PD) is chronic age-dependent neurodegenerative disorder that has both motor and non-motor symptoms. A “multi-hit” hypothesis for PD suggests that a combination of risk factors (i.e., genetic and/or environmental) may lead to the disease. Increasing evidence is suggesting a link between the gut and PD. Phage-936 is a Lactococcal bacteriophage that targets lactic acid bacteria and may be associated with gut microbiome dysbiosis and inflammation. In this thesis, we sought to determine whether the phage-936 virus exacerbates the impact of inflammatory or neurotoxic stimuli (LPS, paraquat). We assessed changes in bacterial population by constructing a microbiome profile for both control and treated groups. Our results showed that L. lactis alone has an impact on the gut microbiome, but no significant changes were observed in neuronal dopaminergic cell counts with the treatments.
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List of Abbreviations

ASO = Overexpressing alpha-synuclein
BAC = Bacterial artificial chromosome
BBB = Blood-brain barrier
CFU = Colony forming unit
CNS = Central nervous system
ENS = Enteric nervous system
GBA = Gut-brain axis
GF = Germ free
GI = Gastrointestinal tract
*L. lactis = Lactococcus lactis*
LAB = Lactic acid bacteria
LBP = Lipopolysaccharide-binding protein
LPS = Lipopolysaccharide
PD = Parkinson’s disease
PFU = Plaque forming unit
Phage = Bacteriophage
PQ = Paraquat
SCFA = Short chain fatty acids
SNpc = Substantia nigra pars compacta
TH+ = Tyrosine hydrolase positive
WT = Wild-type
Introduction

Overview

Parkinson’s disease (PD) is an adult-onset progressive neurodegenerative disease that is defined by impaired motor coordination characterized by somatic problems such as bradykinesia, resting tremor, rigidity, freezing, and loss of postural reflexes (Fahn, 2003). PD is also linked to non-physical symptoms including cognitive impairment, neuropsychiatric problems, sensory and sleeping abnormalities (Park and Stacy, 2009). In a collective study carried out by De Lau and Breteler in 2006, it was determined that industrialized countries reported a prevalence estimated at 0.3% of the general population and approximately 1% in individuals over 60 years of age (De Lau and Breteler, 2006). In Canada, average onset of symptoms usually occurs at 64 years of age with diagnosis at 66 years (Wong, Gilmour & Ramage-Morin, 2015).

Most cases of PD are sporadic; however, genetic, and environmental factors play a vital role in the pathophysiology of the disease, suggesting that PD is multifactorial in origin (Veldman et al., 1998). Familial cases are rare, with an earlier age of onset and an accelerated disease progression, accounting up to 20-30% of PD patients (Veldman et al., 1998). One twin study for PD depicted that monozygotic twins had approximately 3 times higher concordance rate compared to dizygotic twins (Warner and Schapira, 2003).

Pathophysiology of Parkinson’s Disease

Parkinson’s disease is associated with a loss of dopaminergic neurons and gliosis in the substantia nigra region of the brain as well as the presence of intraneuronal proteinaceous cytoplasmic inclusions referred to as “Lewy bodies” (Dauer and Przedborski, 2003). The
nigrostriatal pathway contains dopaminergic neurons whose cell bodies are in the substantia nigra pars compacta (SNpc) that project to the basal ganglia. In PD, the loss of dopaminergic neurons is associated with the degradation of this pathway (Spillantini et al., 1998). These Lewy bodies are found in various regions of the brain such as the dorsal motor nucleus of the vagus, the nucleus basalis, and the locus coeruleus (Spillantini et al., 1998). One of the components of Lewy bodies is α-synuclein, a protein that binds to lipid membranes and changes the conformation of the previously unfolded N-terminus of the protein to a stable α-helical secondary structure. A missense mutation in α-synuclein results in degradation of dopaminergic terminals and neurons (Dauer and Przedborski, 2003). Studies show that patients have a triplication of the SNCA (gene encoding α-synuclein) on the disease-segregating chromosome, resulting in overabundance of α-synuclein protein. Indeed, there is a relationship between the expression of the protein and disease severity (Houlden and Singleton, 2012). Several studies show that loss-of-function mutation in the PARK2 gene (gene encoding parkin protein) may also lead to the development of Parkinson’s disease, as it inhibits dopamine transmitter release and mediates cell death (Huang et al., 2003; Houlden and Singleton, 2012).

Inflammation is also suggested to be one of the main hallmarks of PD as several studies have shown an increase in microglial activation in the striatum and substantia nigra of PD patients. The brain regions surrounding the substantia nigra have the highest density of microglia that respond with rapid proliferation, hypertrophy, and cytokine release (Whitton, 2007). Research has shown that there is an upregulation of pro-inflammatory cytokines (such as IL-1β, IL-6, TNFα) in the striatum and SNc of PD patients. It has been reported that α-synuclein
activates microglia to generate extracellular superoxide, increase intracellular ROS and further induce morphological changes in microglial cells (Augusto-Oliveira et al., 2019).

Microglia are a specific cell type present in the central nervous system which are responsible for regulating the immune system in response to stressors such as diseases, chemical or physical damage (Whitton, 2007). Microglia act as the first line of defense against a stressor, constantly observing their microenvironment and are sorted into various phenotypes depending on their two main states; resting ramified and activated. Microglia are extremely sensitive to many stressors and can be quickly activated, displaying a low expression of membrane receptors as well as major histocompatibility complex (MHC) antigen and T- and B-lymphocyte markers that are necessary for mediating macrophage functions (Pajares et al., 2020). Once encountering a stress stimulus, activated microglia counteracts via receptors that recognize danger-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs), including adenosine triphosphate (ATP) and lipopolysaccharide (LPS) (Augusto-Oliveira et al., 2019). Microglia also display receptors for various neurotransmitters such as glutamate, GABA, norepinephrine, cannabinoids, and acetylcholine, and respond by releasing immuno-transmitters such as chemokines and cytokines (Augusto-Oliveira et al., 2019). This stimulates an inflammatory state as these immuno-transmitters alert the surrounding cells of a stressor and regulates their functioning. Microglia have several phenotypes, and most of them are sorted distinctively within the M1 or M2 phenotype. M1 microglia is activated by pathogens and pro-inflammatory factors (LPS, tumor necrosis factor-alpha (TNFα), etc.) and M2 microglia is activated by anti-inflammatory factors (interleukin-4, interleukin-10, etc.) (Jurga et al., 2020).

Oxidative stress is considered another major component in the onset of the disease. Free radicals, which are highly reactive molecules with unpaired electrons, are naturally generated by
cells. To counteract their potentially harmful effects, the body produces antioxidants. These reactive oxygen species (ROS) have essential roles in various biological processes, including immune system functions, growth factor regulation, the development of inflammatory responses, and programmed cell death (Zuo and Motherwell, 2013). However, when there is an imbalance between ROS and antioxidants, oxidative stress occurs. Excessive ROS levels can lead to significant cell damage, protein degradation, and the deterioration of neurological function (Onyou Hwang, 2013). In the case of PD, mitochondrial dysfunction contributes to an overproduction of ROS, which in turn triggers the degeneration of dopaminergic neurons due to oxidative stress. ROS also targets the high concentrations of phospholipids and polyunsaturated free fatty acids, altering the structure of neuronal plasma and lipid bilayers. A defect in complex I of the electron transport chain exacerbates mitochondrial dysfunction. This disruption in the electron transport chain leads to the generation of oxidative stress, ultimately causing apoptosis (Zuo and Motherwell, 2013; Blesa et al., 2015).

Age is considered to be one of the greatest risk factors for several neurodegenerative diseases that are associated with neuronal loss. Under normal physiological conditions, microglia are neuroprotective and the activation of it is regulated through neuronal electrical activity and neurotransmitters (Conde and Streit, 2006). Research has shown that there is a reciprocal relationship between increased microglial activation and aging. In a normal healthy aging brain, there is an upregulation in microglial expression of MHC class antigens, pro-inflammatory cytokines, and the phagocytic activity of microglia (Jyothi et al., 2015). This suggests that the microglia are essentially “primed” to be over-responsive to stressors which eventually lose control, creating a hyper-inflammatory state in the aging brain compared to the young brain. However, the mechanisms behind this are still unknown (Luo et al., 2010).
**Parkinson’s Disease and the Gut**

The Gut-Brain Axis (GBA) is a bidirectional communication network between the gut and brain, consisting of physical and chemical connections that influence the central nervous system (CNS), the autonomic nervous system (ANS) and the enteric nervous system (ENS) (Carabotti et al., 2015). There is a direct neural link between the gut and the brain via the vagus nerve, which can impact the function of several organs and transmits immune signals from the periphery to the CNS. The human gut houses numerous microorganisms including bacteria, viruses, fungi, parasites, and bacteriophages and all of these have a beneficial symbiotic relationship with the host (Kho and Lal, 2018). This gut microbiome influences several physiological functions such as gut motility, energy production, protection against pathogens, regulating host immunity, and maintaining general homeostasis (Carabotti et al., 2015).

Recent studies have shown that PD patients may exhibit non-motor symptoms such as gastrointestinal issues prior to the development of the motor symptoms, suggesting that the gut microbiome may differ between healthy individuals and PD patients (Zhu et al., 2017). Approximately 30% of PD patients do indeed develop gastrointestinal symptoms including nausea, colonic dysmotility, impaired gastric emptying and an onset of constipation that precedes the development of motor symptoms (Preiffer, 2003). Oropharyngeal dysphagia is one of the most common symptoms of PD and research has suggested that the onset can occur during any stage of the disease, including prodromal stages. Furthermore, it’s been reported that esophageal motility disorders also occur in the premotor stages of PD and constipation is prevalent in up to 80% of PD patients (Warnecke et al., 2022). Studies have reported that individuals who experience constipation are at an increased risk of developing Parkinson’s disease in the long term, with the risk being two to three times higher over a period of 10 years (Chen et al., 2022).
Changes in the gut microbiota have been marked in patients with PD, proposing that there may be prodromal microbiome disturbances prior to the clinical diagnosis of the disease. One experiment by Gerhardt and Mohajeri (2018) hypothesized that different types of pro-inflammatory bacteria significantly increased, whereas various probiotic bacteria decreased in the intestines of PD patients (Lei et al., 2021). A meta-analysis by Romano et al. (2021) analyzed ten 16S microbiome datasets and reported differences within PD patients compared to the healthy microbiota, with significant changes in specific bacterial taxa and potential links between intestinal inflammation and changes between the production of short-chain fatty acids (Romano et al., 2021). Butyrate, a SCFA that is produced by the gut during dietary fermentation with anti-inflammatory properties, has shown to be significantly decreased in PD patients. At the normal physiological intestinal levels, butyrate regulates oxidative status and intestinal barrier function (Canani et., 2011; Nuzum et al., 2020).

The gastrointestinal tract (GI) contains a barrier composed of mucus and epithelial layers, as well as biochemical and immunological factors that limit exposure between the host internal microbiome and the outside environment (Zhu et al., 2017). The intestinal barrier functions to prevent the entry of harmful microorganisms and toxic substances from the gut while controlling the absorption of nutrients, electrolytes, and water from the gut into the bloodstream (Fukui et al., 2016). The microbiota composition can be disrupted by internal and external factors, leading to dysbiosis and can have a negative impact on the gut barrier function. This can result in the development of various diseases associated with metabolic disturbances, and possibly even neurodegenerative disorders and autoimmune illness (Kho and Lal, 2018). A case-controlled study by Schepersjans et al, (2014) observed the differences between PD microbiome and control subjects and reported that there was significant reduction of Prevotellaceae, a microbe that
contributes to polysaccharide breakdown, and may be associated with increased gut permeability by reducing mucin synthesis. Furthermore, an increase in the presence of various pathogenic gram-negative bacteria was also reported (Lei et al., 2021).

The “leaky gut” hypothesis states that dysfunction of the gut barrier induces chronic low-grade inflammation in various organs (Fukiu et al., 2016). Individuals with Parkinson's disease display low levels of tight junction proteins, such as zonula occludens-1 and occludin, in the epithelial cells of the colon (Chen and Lin, 2022). Studies have linked increased intestinal permeability and reduced tight junction proteins to the accumulation of alpha-synuclein in the colon, heightened fecal inflammatory markers, and gut dysbiosis in individuals with Parkinson's disease as well as in animal models of the disease (Perez-Pardo et al, 2018). In both transgenic models of Parkinson's disease and peripheral endotoxin-induced models, disrupted intestinal permeability has been observed in the early stages of the disease, prior to the onset of brain pathology (Kelly et al., 2013). This suggests that the development of a leaky gut could result in the release of pathogens from the intestinal lumen into the systemic circulation, which could then spread across the blood-brain barrier (BBB) and contribute to the development of Parkinson's Disease neuropathology in the CNS (Fukiu et al., 2016; Santos et al., 2019).

It has also been suggested that disturbances in the gastrointestinal microbiome can cause misfolding and abnormal aggregation of localized α-synuclein in the gut. Several studies have also reported that in both prodromal and symptomatic stages of PD, there is a widespread of Lewy body pathology in enteric neurons located throughout the intestinal mucosa, submucosa, and myenteric plexus. These neurons are known to interconnect with the vagus nerve and dorsal motor nucleus of the vagus nerve in the brainstem (Chen and Lin, 2022). One experimental study carried out by Sampson et al. (2016) examined the changes within the microbiome in PD in
which mice overexpressing α-synuclein (ASO) were colonized with specific-pathogen-free (SPF) microbiome. Results showed that the microbiota in the mouse model exhibited significant deficits in gut motility as well as motor dysfunction compared to wild-type (WT) mice. Interestingly, a cohort of germ-free (GF) ASO-SPF mice displayed similar phenotypes as the WT-SPF mice, and there was an increase in aggregated α-synuclein within the substantia nigra (Sampson et al., 2016) (Gallop et al., 2021). This further indicates that there may be a link between the PD pathogenesis and the gut.

Parkinson’s has been proposed to progress through 6 stages, each characterized by the spread of Lewy bodies and neurites between cells in different body and brain regions. During the first stage, intraneuronal lesions containing α-synuclein begin in the dorsal motor nucleus of the vagal nerve and proceed upwards in an active retrograde manner, progressing to lower brain regions and eventually spreading to the cerebral cortex (Braak et al., 2004; Neilsen et al., 2021). One study conducted by Holmqvist et al. (2014), explored this by injecting α-synuclein collected from PD patient brain lysate into the gastrointestinal tract of adult wild-type mice. It was observed that when α-synuclein was injected in form of fibrils, it actively spread from the intestines to the dorsal motor nucleus of the vagal nerve in a time dependent manner, suggesting that aggregated α-synuclein is transported through fast and slow axonal transports (Holmqvist et al., 2014). Another experiment carried out by Van Der Berge and colleagues (2019) also explored this theory in which bacterial artificial chromosome (BAC) transgenic rats were injected with pre-formed α-synuclein fibrils which led to trans-synaptic propagation of α-synuclein, supporting the “gut-first” hypothesis of PD (Van Der Berge et al., 2019). Hilton and colleagues (2013) assessed the aggregation of α-synuclein in the bowels of pre-clinical PD
patients by comparing gastrointestinal tissues obtained from 62 patients to healthy 161 controls. Immunocytochemistry showed that all patients with positive biopsies had an onset of autonomic symptoms compared to negative controls, suggesting a possible biomarker for the progression of PD (Hilton et al, 2013). Several epidemiological studies have shown that truncal vagotomy can also reduce the risk of PD pathogenesis (Svensson et al., 2015; Liu et al, 2017).

Misfolded α-synuclein may trigger an innate immune response, increasing the production of pro-inflammatory cytokines such as TNFα and IL-1β, inducing inflammation and compromising the integrity of the GI barrier (Chen and Lin, 2022). This was supported by an in vivo study rodent model study carried out by Pelligrini and colleagues (2022) in which it was demonstrated that male transgenic mice expressing human A53T mutant of α-synuclein had a significant increase in enteric glia activation, along with altered TLR-2 and zonulin-1 expression, through the direct activation of canonical caspase-1-dependent inflammasome signaling. Furthermore, a decrease in butyrate and propionate, involved in the regulation of gut epithelium, was also observed (Pellegrini et al., 2022)

Lipopolysaccharide (LPS) are bacterial surface glycolipids that are produced by Gram-negative bacteria and is known to activate surface pattern recognition receptors to initiate innate immune responses in the gut. LPS plays an important role in the modulation of gut microbiota and acts as an “alarm molecule” that alerts the body of any potential pathogens and triggers the release of pro-inflammatory cytokines (Candelli et al., 2021). LPS binds to TLR-4 receptors on the microglia and generates a robust immune response, promoting the activation of NFkB, COX-8 and IL-2 (Salguero et al., 2019). At baseline levels, LPS is thought to contribute to the modulation of tight junction proteins and maintaining the barrier function, blocking the passive
diffusion of hydrophobic solutes (Zhang et al, 2013). The intestinal epithelial cells regulate the transport of ions and nutrients whereas bacterial products are restricted by tight junction proteins such as occludin, claudin, and ZO-1 (Ghosh et al., 2020). Furthermore, homeostatic expression of intestinal alkaline phosphate (IAP) ensures that LPS in the luminal space is continuously detoxified through dephosphorylation. Several factors including disease-associated gut dysbiosis can lead to inadequate breakdown of LPS, causing the tight junctions to be disordered, leading to an accumulation of LPS and allowing paracellular transport of LPS and other endotoxins into systemic circulation (Ghosh et al., 2020). As a response to these events, inflammatory cytokines and macrophages are activated, causing an increase in systemic inflammation.

A study by Hasegawa and colleagues (2015) measured lipopolysaccharide-binding protein (LBP) serum markers and reported that serum levels were lower in PD patients compared to control (Hasegana et al., 2015). LBP is an acute-phase polypeptide that is synthesized in the liver and released into the bloodstream after glycosylation. LBP plays an essential role in the innate immune response by binding to LPS in the bloodstream and facilitating its recognition by macrophages (Zweigner et al., 2006). In PD, LBP levels are significantly lower, affecting the internalization of LPS within intestinal cells, leading to subsequent intestinal inflammation, impaired gut barrier function, and bacterial pathogen invasion, resulting in systemic inflammatory responses (Chen et al, 2021).

Several studies have reported that gut-associated LPS facilitates α-synuclein aggregation within the microbiome and impairs its biological function. LPS modulates α-synuclein kinetics by increasing growth rate and decreasing half-life, which leads to the formation of beta-sheet fibrillar forms, stabilized by LPS (Bhattacharyya et al, 2019). A study by Kelly and colleagues (2013) demonstrated that systemic exposure to low levels of LPS causes progressive increase in
α-synuclein and gut leakiness in mice intestines at 5 months post administration, predominately in the large intestine (Kelly et al., 2013).

**Phage as a human pathogen**

World Health Organization (WHO) classifies probiotics as a mixture of live bacteria and yeasts that are known to have beneficial health effects when consumed in an adequate amount. Probiotic mechanisms involve various processes, such as manipulating the microbial communities within the intestine, suppressing the growth of pathogens, immunomodulation, stimulating the differentiation and proliferation of epithelial cells, and strengthening the intestinal barrier. They are identified as beneficial microorganism that colonize the microbiome and can change the intestinal flora to improve host immunity (Hemarajata and Versalovic, 2013; Wang et al., 2021). Lactic acid bacteria (LAB) are a type of gram-positive bacteria that have several beneficial metabolism features such as producing acid, hydrolyzing proteins, production of short-chain fatty acids, amines, and offering protection against infectious agents (Mathur et al., 2020). Lately, researchers have been targeting the effects of probiotics, including LAB, and their role with immune modulation within neurodegenerative diseases such as Parkinson’s disease. Perez-Visnuk and colleagues (2020) demonstrated via a MPTP model of PD that LAB significantly improved motor behaviour by increasing tyrosine hydrolase (TH)-positive cells and IL-10 in the brain while decreasing levels of pro-inflammatory cytokines (Perez Visnuk et al., 2020). Another study done by Yue et al., (2022) investigated the effects of probiotic strain *Lactococcus lactis* (*L. lactis lactis*) MG1363-pMG36e-GLP-1 in a murine mice model and reported that the LAB improved motor deficits and rescued dopaminergic neurodegeneration in
the substantia nigra by suppressing ferroptosis via the activation of the Keap1/Nrf2/GPX4 signaling pathway, thus decreasing oxidative stress. Furthermore, the strain reduced gut dysbiosis by promoting the production of certain genera of bacteria (Yue et al., 2022). Tetz and colleagues (2018) analyzed faecal samples from drug-naive PD patients and healthy participants to identify alterations between the gut microbiota and reported that dopamine-producing Lactococcus spp. was significantly decreased in PD patients, with an increase in their lytic bacteriophages 936 (Tetz et al., 2018).

Bacteriophages (phage) are viruses that also reside in the gut microbiome and infect specific bacteria, sometimes constituting as an alternate to the use of antibiotics and antibiotic-resistant bacterial infections. Before the widespread use of modern antibiotics, bacteriophages were used clinically for a variety of bacterial infections and they are still in use in some countries for certain conditions. The minimum bacteriophage titers necessary for clinical applications remain uncertain. Bacteriophage administration typically represents an 'active' treatment, involving multiplication within the bacterial host and factors such as treatment timing, dosage, and the composition of the bacteriophage formulation (single versus multiple strains) need careful consideration (Merabishvili et al., 2009; Parracho et al., 2012).

Phages bind to specific targets on the bacterial cell surface, limiting the range of bacterial species that each individual phage can attach to (Dalmasso et al., 2014). Phages replicate via two alternate cycles: lytic and lysogenic cycles. During the lytic cycle, phages bind and infect their host bacteria, resulting in the bacterial cell to be reprogrammed as a “phage factory”, leading to the production of new phage particles which are later released from the cell to repeat the cycle. In the lysogenic cycle, phages infect the bacterial cell but do not trigger a complete degradation (lysis) of the bacterial cell and instead causes it to harbour the prophages of the viral genome.
However, certain stressors such as oxidative stress, UV light, DNA damage, and temperature can induce the lysogenic cells to enter the lytic cycle (Campbell, 2003; Lusiak-Szelachowska et al., 2017). Even in lysogenic state, phages can increase pathogenesis by altering gene expression regulated by their host bacteria as well as lysing competitor bacterial strains, resulting in dysbiosis (Howard-Varona et al., 2017). Lysogenic phages constitute approximately 20%-50% of free phages in the gut and it has been suggested that the disease-associated inflamed gut may increase prophage induction, resulting in an increase in bacterial lysis and thereby promoting dysbiosis, re-enforcing the inflammatory loop (Sausset et al., 2020).

While bacterial translocation is dependent on increased permeability of the gut and immune deficiencies, little to none is known about the mechanics of viral translocation across the epithelial cells (Gorski et al., 2006). Experimental animal studies have reported the phages may be able to translocate using M cells, dendritic cells, and epithelial cells to obliterate to systemic sites (Gorski et al., 2006). Additionally, phages may be able to interact with eukaryotic cells and induce transcytosis by binding to mucin glycoproteins. It has also been suggested that dendritic cells may also be involved in phage translocation by phagocytizing and extending dendrites through the intestinal epithelium, however, this may be a rare event (Sausset et al., 2020). Nguyen and colleagues (2017) reported that estimated 31 billion bacteriophages are passed through the epithelial cell layers each day and identified that phages can translocate through the Golgi apparatus (Nguyen et al., 2017; Marongiu et al., 2021). In a study by Tetz and Tetz (2018), a phage cocktail was administered in mammalian animal models, which resulted in leaky gut and an altered inflammatory response. Furthermore, phages were detected in the cerebrospinal fluid (CSF), suggesting (in accordance with the phage-eukaryotic interactions) that phages may be able to pass the BBB as well (Tetz and Tetz et al., 2018).
Tetz and Tetz (2018) also reported that bacteriophages can induce protein misfolding via their prion-like domains, with a strong capability to turn into prions (Tetz and Tetz, 2018). Recently, new evidence has suggested that α-synuclein pathology within PD propagates through the CNS in a prion-like manner. Prions are distinguished by their ability to self-replicate, change their conformation, generate motifs resembling β-sheets, and produce misfolded proteins (Daley and Brundin, 2019; Liddle, 2019). The prion domains are presented within the attachment protein, G3P, in phages and could potentially interact with eukaryotic proteins, leading to misfolding of α-synuclein (Tetz and Tetz, 2018; Daley and Brundin, 2019).

**Phage exposure through diet**

Dietary products also play a vital role in influencing the gut microbiome, maintaining gut homeostasis, and reducing risk of developing diseases. However, diet also affects the physiology of bacteriophages and can modulate the activity of phages, thus affecting the microbiome as well (Marongiu et al., 2021). Dairy products are a source for both probiotic LAB *Lactococcus lactis* and their host-specific bacteriophage 936, most common belonging to the *Siphoviridae* family. Within the dairy industry, most of the starter cultures contain *L. lactis* to promote fermentation but this process is hindered by bacteriophage infections (Chmielewska-Jeznach et al., 2018). Within dairy factories, phages have a constant source including originating from raw milk, and spontaneous prophage induction. In some cheese factories, bacteriophage 936 has also been reported to be airborne as well, thus making it difficult to control phage infections (Verreault et al., 2011). Genome sequencing projects have reported that many LAB strains contain prophages,
especially among lactococci and lactobacilli, and putting these lysogenic LAB under certain environmental conditions can induce lysis (Marco et al., 2012).

More and more studies have been exploring the link between diary exposure and prevalence of PD, reporting that large amounts of dairy consumed increases the risk of developing PD. Chen and colleagues (2007) assessed the American Cancer Society’s Cancer Prevention Study II Nutrition Cohort, identifying 250 men out of 57,689 and 139 women out of 73,175 that developed PD five to nine years later. The prevalence of PD was found to be 1.6-fold increase for women and 1.8-fold increase for men who consumed large amount of dairy, suggesting that dairy consumption poses a high risk for PD pathogenesis, particularly in men (Chen et al., 2007). Similarly, Chen, Zhang, Harnan and colleagues (2002) reported a positive association between dairy and PD in 2 cohorts in which PD cases were identified in 210 men and 184 women, especially within men (Chen et al., 2002). Another meta-analysis study done by Hughes et al., (2017) assessed cohorts from the Nurses’ Health Study (n=80,736) and Health Professionals Follow-up Study (n=48,610) with a total of 26 and 24 years of follow-up. The findings revealed that individuals who consumed low-fat dairy products at least 3 times a day had a hazard ratio increase of 1.34 compared to those who consumed none (Hughes et al., 2017). According to Jiang, Ju, Jiang, and Zhang, who assessed 7 different cohort studies comprising 1,083 PD cases among 304,193 subjects, reported that there was a 17% rise in the PD incidence with every 200g/day increment in milk consumption and a 13% increase with every 10g/day increment in cheese consumption (Jiang et al., 2014). Furthermore, a study by Abbott et al., (2015) assessed relationship between midlife dairy consumption and PD and reported that neuronal density was lowest in men who consumed more than 16oz of milk per day, with a 2.3-fold increase in the incidence of Parkinson’s disease (Abbott et al., 2015; Parks et al., 2005).
Bacteriophages found in dairy products may contribute to the increased risk of Parkinson’s disease (Tetz et al., 2018). The dairy industry has been facing challenges in controlling Phage 936, which persists as the most troublesome phage species in the industry, with many species being airborne, resistant to heat and containing lytic prophages (Verreault et al., 2011).

**Objective**

The aim of this study is to assess whether bacteriophages, particularly bacteriophage 936, can influence the PD-like pathophysiology via gut dysbiosis. To this end, an initial study was conducted to first determine if the bacteria *Lactococcus lactis* was able to survive and colonize the mice gut following ingestion and if it alone could produce some degree of measurable changes (e.g., changes in the bacterial colonization). Next, the main study of this thesis involved six groups of old (22-24 months) mice receiving phage (or vehicle), followed by an inflammatory/neurotoxic stimulus (LPS or paraquat) as illustrated by the figure below:
The effects of gut dysbiosis via gut bacteriophages and its role in PD

Figure 1: Study design of the experiment, with mice receiving phage (or vehicle) and an inflammatory stimulus (LPS or paraquat or vehicle). All cohorts received *L. lactis* prior and during phage and treatment administration. Group 9 was excluded from this study as the mice were only sacrificed 90 minutes after injection, as compared to the rest of the cohorts that were sacrificed 24h after final injection.

We assessed the weight, sickness behaviour gut microbiome changes, along with dopamine neuronal counts. It was hypothesized that mice receiving the phage and inflammatory treatment would display the greatest degree of pathology. However, it was also plausible that the bacteriophage could reduce the effects of the treatments by neutralizing endogenous bacteria, potentially reducing the pathogenesis of PD.

Aim and Hypothesis

The Aim was to assess whether LPS and paraquat treatments induce neuronal damage and changes in the gut and whether exposure to phage will modify pathologies (reduction in dopaminergic neurons) produced by toxin stimuli (i.e., LPS or PQ). We hypothesize that phage will exacerbate the neuronal cell changes produced by the toxin-immune treatments.
Methods

Experiment 1

During a period of 7 days, mice were trained to consume pudding as a method of administering the bacteriophage. This involved placing the pudding on a plastic dish within the mouse's enclosure and repeating this process every 24 hours. The mice were randomly assigned to two conditions: control and treatment. The control cohort only received pudding with vehicle and the treatment group received pudding with L. lactis. The mice were maintained on their regular diet of Harlan mouse chow and water ad libitum.

Experiment 2

During a period of 7 days, mice were trained to consume pudding as a method of administering the bacteriophage. This involved placing the pudding on a plastic dish within the mouse's enclosure and repeating this process every 24 hours. The mice were randomly assigned to one of 6 experimental groups, which included those that were given plain pudding without the phage, those that received pudding with the phage once a day, and those that received pudding with the phage a day with toxin treatment. The mice were allowed to consume their regular diet of Harlan mouse chow and water ad libitum throughout the study. All experimental procedures were approved by the Carleton University Committee for Animal Care, which followed the guidelines set out by the Canadian Council for the Use and Care of Animals in Research.

Following the procedure previously carried out by Tetz and Tetz (2016), 1 microliter of phage 936 was mixed with 0.1 ml of pudding and administered to the mice for a period of 10-12 days. The mice were sacrificed by rapid decapitation after assigned toxin treatments and their
brain tissue, upper intestinal section, and fecal pellets were collected and frozen for later analysis. The SNC was analyzed for neuronal dopamine counts to determine the effects of phage.

**Lactococcus lactis culture**

One litre of M17 broth was cultured and sterile filtered through a vacuum flask with pore size 0.22 μm. Ten millilitres of the broth was added into a glass culture tube with 10mL of cryopreserved *L. lactis* stock and cultured overnight in an incubated shaker at 30 degrees Celsius. The next day, 5mL of overnight *L. lactis* culture was added to two 500mL of M17 each for overnight culture. Following the incubation, one flask of 500mL of overnight was spun down to obtain a bacterial pellet, which was added to the second 500mL of overnight *L. lactis* culture to achieve a dose of 1x10⁷ CFU. The bacterial concentration was determined by serial dilution of *Lactococcus lactis*.

**Phage 936 replication and purification**

*Lactococcus lactis* (University of Laval, Montreal, QC) was inoculated into Medium M17 containing 0.5% lactulose and incubated overnight at 30°C. The next day, Phage 936 (University of Laval, Montreal, QC) was added to the bacteria at a concentration of 1x10⁶ pfu/ml. The resulting phage lysate was used to infect a fresh culture of host bacteria, and after five hours of incubation, the phage was purified by centrifugation at 10,000rpm for 15 minutes at 4°C. Supernatant was collected, and NaCl and PEG800 were added before the solution was incubated overnight at 4°C. The solution was again centrifuged, and the resulting pellet was resuspended in phage buffer before being placed in a 15 ml conical tube. A gradient of CsCl in phage buffer was created using three solutions of different densities (4.2g/8ml, 6.2g/8ml, 8.2g/8ml), and the
resuspended phage was added to the top of the gradient to fill the tube. The tube was then centrifuged at 35,000rpm for three hours at 20°C in a swinging bucket rotor. The extracted phage was centrifuged again at 40,000rpm for 18 hours at 20°C before being extracted from the side of the tube as before. The extracted phage was then dialyzed three times for 20 minutes each time against phage buffer using a dialysis cassette. The resulting purified phage was stored in a screw cap tube at 4°C.

**Immunohistochemistry DAB Staining**

The SNc and striatal sections were washed with 10mM PBS (pH 7.2) and then treated with 0.3% H₂O₂. After additional washes with PBS, the sections were blocked using 5% NGS solution. They were then incubated with specific anti-TH (mouse) antibody, in a modified NGS solution. The next day, the sections were washed and incubated with secondary antibody, anti-mouse HR (conjugate). After more PBS washes, the sections were incubated with Strp HRP in a modified NGS solution. A DAB solution was prepared and applied to visualize the specific antigens. The sections were then mounted and allowed to dry overnight. After 24 hours, the sections were dehydrated using a series of ethanol and clearene solutions. Finally, coverslips were added using DPX, and the samples were left to dry for 24 hours in a fume hood.

**Polymerase Chain Reaction for bacteria detection**

To extract DNA, the DNeasy Blood & Tissue Kit (Qiagen, MD) and its provided protocol were utilized. The DNA primer recommended by Labrie and Moineau (2000) for detecting *L. lactis* was used to confirm the presence of the bacteria in the fecal pellets. Amplification of the
DNA was carried out using a PTC-200 thermocycler from MJ Research.

**Animals and general experimental design**

The study design of the experiment is shown in **Figure 1**. A total of 90 animals, 22-24 months, C57BL6 mice from Charles River Laboratories (Montreal, QC) were used and arrived 18 months prior to the start of the experiment to be aged in the vivarium. The mice were provided with a Harlan mouse chow diet and water *ad libitum* while maintaining the room temperature at approximately 21°C. They were kept in standard polypropylene cages, with 4 mice per cage, measuring 27×21×14 cm. Additionally, the mice were given an enriched environment consisting of nestlet and a house. A week prior to the start of the experiment, the mice were separated and housed individually in cages to get acclimated.

The timeline of the experiment is shown in **Figure 2**. The mice were divided into 6 groups and randomly assigned to receive phage (or vehicle) and treatment (LPS or paraquat or vehicle). The mice were randomly selected to receive a dose of 1 mg/kg LPS (**Figure 2A**) or 3 doses of 10 mg/kg paraquat, each a week apart (**Figure 2B**). Three days prior to the experiment, all mice were trained on pudding administration to get acclimated. The mice received 0.1 ml of dairy-free chocolate with L. Lactis (at a concentration of 3.00 x 10⁹ CFU) for the first 10 days. For the next 10 days (Day 11-21), the mice received 0.1 ml of dairy-free chocolate pudding (with or without phage at the concentration of 1x10⁷ PFU). The LPS cohort received 1 dose of LPS on Day 22 and were sacrificed 24 hours after the treatment injection (**Figure 2A**). The paraquat cohort received the first injection of PQ on Day 22, the second one on Day 29, and the third injection on Day 36, and sacrificed 24 hours after the last treatment injection (**Figure 2B**).
**Timeline**

**Figure 2**

**Figure 2A:** LPS timeline. Pudding training occurred for three days followed by *L. lactis* administration for 20 days. On the 21st day, LPS was given concurrently. After 10 days of phage and 1 day of LPS, the mice were sacrificed 24hrs after the LPS injection.

**Figure 2B:** Paraquat timeline. Pudding training occurred for three days followed by *L. lactis* administration for 20 days. On the 21st day, the first dose of PQ was given concurrently. The second dose was given Day 27, and the third dose on Day 34, all three injections were administered a week apart. Following the last PQ injection, the mice were sacrificed after 24h.

**Sacrifice**

Half of each group received an injection of sodium pentobarbital and underwent transcardial perfusion to collect tissue for immunostaining. The remaining mice were sacrificed by rapid decapitation and their tissue was collected for Western blot analysis. Specifically,
regions of the ST, SNC, prefrontal cortex, and hippocampus were excised from coronal brain sections using a micro-dissecting block with 0.5mm slots and stored in vials of PB on ice. For immunohistochemistry, the blood was flushed out of the tissue using 5ml of saline followed by 40ml of 4% paraformaldehyde through a needle in the right ventricle of the heart. The brain and intestine were then extracted and placed in a vial of 4% paraformaldehyde, which was stored on ice. The brains were subsequently refrigerated at -20 degrees Celsius and transferred to a vial containing a 10% sucrose 0.1M PB solution (pH 7.4) after 24 hours. After an additional 24 hours, the brains were transferred to a 30% sucrose 0.1M PB solution.

**Statistical analyses**

The data on TH+ counts, obtained through immunohistochemistry, was analysed using 2 x 3 ANOVAs; phage/no phage vs. saline/LPS/PQ. Significant interaction effects between factors were analyzed using Tukey’s HSD post-hoc pairwise comparisons. In addition, Fisher post hoc tests (α = 0.05) using statistical software GraphPad Prism 5 were carried out where appropriate, with differences being considered significant at p <0.05.

**Results**

**Microbiota**

In Experiment 1, the control group and the group treated with *L. lactis* did not show any significant differences in the abundance of most bacteria, except for two species of *Clostridium* bacteria: *Clostridium* species ASF502 and *Clostridium fusiformis*. The abundance of these two species was significantly lower in the treatment group compared to the control group (p > 0.05),
indicating that the administration of *Lactococcus lactis* at a dose of $3.00 \times 10^9$ CFU influenced the bacterial population, particularly in reducing the abundance of these two Clostridium species. It should be mentioned that we did detect evidence of the actual *L. lactis* bacteria in the gut (after 7 days) using PCR. However, this was only an $n = 1$ and it still needs to be analyses further.

**Figure 3:** Microbiota variations of bacteria at family taxonomic classification. Mice subjected to *L. lactis* bacterial treatment for 10 days exhibited a decrease in the relative frequency of Clostridium species ASF502 (2.1%) and *Clostridium fusiformis* (1.6%), in contrast to the control group where these frequencies were 6.6% and 11.2% respectively. Relative frequency in bacterial populations refers to the proportion or percentage of a specific bacterial strain or type within a larger population of bacteria. It indicates how common or abundant a particular bacterial group is compared to other groups in the same population (Tsang and Yung, 1991). See Appendix for a complete bacterial taxonomy frequency microbiome legend.

**Weight Loss**

The mice were weighed every day from the beginning of this experiment to assess the effects of both *L. lactis* and phage-936 until sacrifice. The cohort receiving saline + phage did show a slight but statistically significant decrease in body weight during the 22-day experimental
period (F (2, 54) = 12.2, p < 0.0001). There were no interactions observed between phage, LPS and PQ treatments, however, multiple comparison tests revealed that phage alone had a main effect on the weight of the cohort compared to cohorts that received PQ and LPS along with phage (or no phage).

Figure 4: Mice were given a combination of phage/vehicle plus LPS, PQ and saline treatments and weighed over a period of 10 days, prior to sacrifice. Only the cohort that received phage as a treatment along with vehicle (saline) exhibited a decrease in body weight compared to the rest of the groups *p < 0.05, relative to vehicle treated controls.

Sickness score

The mice exposed to both paraquat and LPS displayed sickness symptoms when compared to the groups that only received saline, regardless of whether they received phage treatment or not (F (2, 54) = 6.3, p = 0.0032). This indicates that there was a treatment main effect and there were no interactions observed within the groups. Additionally, the mice treated with LPS alone exhibited slightly greater sickness symptoms compared to the group that received PQ alone.
Figure 5: Mice were given a combination of phage/vehicle together with LPS, PQ and saline treatments for a period of 10 days. Sickness score was assessed 24 hours after administration. Mice that received LPS and PQ as a treatment, irrespective of phage administration, displayed sickness symptoms (i.e., lethargy, piloerection, ptosis) when compared to the saline control group, with mice treated with LPS showing a more pronounced level of sickness.

Immunohistochemistry

Tyrosine Hydroxylase (TH) positive cell counts in the substantia nigra (SNc) were analyzed using DAB staining. The overall number of counted TH+ cells were multiplied by a stereological factor of 14.348 calculated by section interval, section thickness and counting frame. There was no significant Phage x Treatment interaction observed (F (2, 24) = 0.6, p = 0.5250) (Figure 6A & 6B). However, there was a significant treatment main effect F (2, 24) = 4.631, p = 0.0199). The follow up comparisons indicted that the LPS treatment reduced the overall number of TH+ neuron in the SNc (p < 0.05). However, this was a very small effect (approximately 4-5 %) and is likely not biologically significant.
**Figure 6A:** Mice were administered saline or Phage 936, together with either vehicle, lipopolysaccharide (LPS) or paraquat (PQ) for a period of 10 days, and after TH+ neuron counts within the SNc were assessed. A very slight but significant (4-5%) reduction of TH+ neurons was apparent for the LPS-treated mice, regardless of phage administration. *p <0.05, relative to vehicle treated controls.
Figure 6B: The TH+ dopamine cell counts imaged at 4.25x at bregma level -3.28 within cohorts that received a combination of phage (or no phage) and LPS or PQ or saline treatments using immunohistochemical DAB staining. An approximately 4-5% decrease in TH+ neurons was observed in mice treated with LPS, independent of phage administration (p < 0.05), when compared to mice treated with PQ or saline.

Since the overall TH+ neuronal reduction was so modest, we further assessed the number of TH+ over a series of bregma levels. Indeed, neuronal loss is not necessarily consistent across anatomical site. The number of TH+ neurons were analyzed at four different bregma levels of the substantia nigra (SNe) - 3.08mm, -3.16mm, -3.28mm, and -3.40mm (Figure 7). However, there
was no significant interaction or treatment main effect in TH+ dopamine neuron numbers across the different bregma levels (\( F(2, 60) = 0.3296, p = 0.994 \)).

**Figure 7**: TH+ dopamine cell counts were assessed between bregma levels -3.08, -3.16, -3.28 and -3.40 of the brain at the SNc region, using immunohistochemical DAB staining. No statistical differences were observed between TH+ neuronal counts within groups that received phage (or no phage) in combination with LPS, PQ or saline.

In addition to assessment of SNc soma counts, we also assessed TH+ striatal terminals. In this regard, there was no visual difference whatsoever apparent in the groups. Accordingly, we did not perform further statistical analyses. **(Figure 8).**
Figure 8: TH+ dopamine striatal terminals were imaged at x4.25 magnification for cohorts that received phage (or no phage) in combination with LPS, paraquat or saline. No visible visual differences were noted and thus, no further analysis was carried out for TH+ striatal terminals.
Discussion

Numerous studies have indicated a potential connection between the underlying mechanisms of Parkinson's disease and gastrointestinal disturbances (Kho and Lal, 2018; Zhu et al., 2017; Scheperjans et al, 2014; Gerhardt and Mohajeri, 2018). According to the "dual-hit" theory, it is hypothesized that the development of PD may begin in the gut well before the appearance of clinical symptoms, and dysbiosis in gut bacteria could be fundamental for the disease (Hawkes et al., 2009). The role of diet as a contributing factor in PD has been widely studied, and some studies suggest a positive link between the consumption of dairy products and PD. Dairy products contain a beneficial probiotic bacterium called Lactococcus lactis, which is thought to aid in immune regulation and the suppression of pathogens in the gut. However, dairy products also harbor specific virulent phages that target L. lactis, leading to imbalances in gut bacteria and weakening of the gut barrier, which can contribute to dysbiosis and the development of disease (Verreault et al., 2011). In fact, Tetz and colleagues (2018) reported that patients with PD have a significant increase in lactococcal phages, particularly group 936. Accordingly, we hypothesize that phage 936 will exacerbate the neuronal changes produced in a mouse model of Parkinson’s disease.

Lactic acid bacteria are widely used for fermented food productions and are considered probiotics that regulate host metabolism and promote immune modulation (Chmielewska-Jeznach et al., 2018; Hemarajata and Versalovic, 2013; Wang et al., 2021). Lactococcus lactis is the most widely used lactic acid bacteria in starter cultures for manufacturing dairy fermented products. Despite the common association of L. lactis with dairy fermentation, the bacterium originally was isolated from plants where it remains dormant only and becoming active in the
gastrointestinal tract after being consumed by a ruminant (Bolotin et al., 2001). *L. lactis* is may not be present in the murine gut and the ingestion of *L. lactis* through food has demonstrated a survival rate of 10-30% within the gastrointestinal (GI) tract. However, its viability significantly diminishes as it progresses towards the colon due to bacterial lysis (Drouault et al; 1999).

The first aim of this thesis was to assess whether *Lactococcus lactis* is able to colonize and/or alter the composition gut bacteria since this bacterial species is not a natural habitant of the mice microbiome (Song et al., 2017). We found that *L. lactis* did appear to survive in the gut (as indicted by PCR; data not shown) and the *L. lactis* treatment induced a significantly lower relative frequency of *Clostridium* ASF502 and *Clostridium fusiformis* bacterial species compared to the control group. Clostridia in the phylum *Firmicutes* are a gram-positive predominant bacterial species present within the intestinal microbiome and have been reported to exert widely beneficial effects on gut health (Guo et al., 2020). *Clostridium* species play a vital role in gut homeostasis by breaking down indigestible nutrients and producing large amounts of SCFAs (Guo et al., 2020). They also exhibit a variety of functions including metabolizing starch and proteins into organic acids (Grenda et al., 2022). The “altered Schaedler flora” (ASF) is a community of eight bacterial species, including two lactobacilli, one Bacteroides, one spiral bacterium from the *Flexistipes* genus, and four fusiform species with a fusiform shape that are highly sensitive to oxygen (EOS) (Dewhirst et al., 1999). *Clostridium* ASF502 is one of the oxygen-sensitive members that is selected to be part of this standardized microbiota and are chosen for its dominance in the normal gut flora (Brand et al., 2015). *Clostridium fusiformis* are from family *Erysipelotrichaceae* that have only recently been isolated from healthy human feces and the physiological features are yet to be explored (Higashimura et al., 2017; Ghimir et al., 2019).
One possible reason for the decrease of these two Clostridial species in our experiment could be due to bacterial lysis in the gut and exposure to lysed bacteria could contribute to reduced population density of competing bacteria (Smakman and Hall, 2022). Although they are considered as beneficial probiotics, Clostridium bacteria are also potential contributors to dysbiosis and potential risks to other serious health benefits such as chronic intestinal inflammation, colorectal cancer, and irritable bowel syndrome (Grenda et al., 2022; Lopetuso et al., 2013). It is also possible that administration of L. lactis exerted protective effects against Clostridium species in the mice microbiota in our experiment. In 2012, Bolla and colleagues demonstrated that L. lactis isolated from cheese had an inhibitory effect on Clostridium difficile in vitro (Bolla et al., 2012). Clostridium difficile is an opportunistic pathogenic clostridial species that release two toxins which promote intestinal inflammation, thus contributing to dysbiosis and development of inflammatory diseases (Anjuwon-Foster and Tamayo, 2017). Another study by Le Lay et al., (2016) determined that nisin, which is a bacteriocin produced by several strains of L. Lactis, also had a protective effect against C. difficile, reducing spore viability by 50% (Le Lay et al., 2016). Furthermore, Garde and colleagues (2014) showed that antimicrobials produced by lactic acid bacteria, such as nisin and Reuterin, also reduced Clostridium perfringens, which releases toxins that contribute to neurotoxicity, hemolytic and enterotoxigenic activity (Garde et al., 2014; Guo et al., 2020). Bacteriocin-producing LABs are thought to modulate the suppression of pathogens, immunomodulation, and regulating the gut-brain axis (Anjana and Tiwari, 2022).

In the second part of this experiment, we assessed weight loss and sickness behaviour among mice that received a combination of phage and treatment. Our observations revealed that the mice treated with the phage alone exhibited a significant decrease in body weight. Similarly,
Tetz and colleagues (2017) reported a trend towards weight reduction after bacteriophage treatment compared to control; however, it is important to note that the experimental mice were administered a phage cocktail with various viruses that may have played a role in weight reduction (Tetz et al., 2017). In contrast, we found no impact of the phage on sickness, but PQ and LPS did induce significant signs, compared to saline control. This is consistent with previous studies showing that LPS readily induces lethargy, reduced appetite, and drowsiness (Bassi et al., 2011; Biesmans et al., 2013) and that PQ promotes mild sickness behaviour when injected intraperitoneally (Jadavji et al., 2019; Rudyk et al; 2015).

To our knowledge, no study to date has been conducted to analyze the effects of bacteriophages on neuronal cell survival and whether they can contribute to neurodegeneration. In this regard, our results found that the phage did not have any impact on the number of TH+ neurons within the SNc. But surprisingly, none of the experimental treatments induced much in the way of neurodegeneration of the nigrostriatal dopaminergic system. The LPS treatment, irrespective of phage administration, produced an approximately 4-5% decline in SNc TH+ cells. Although this surprisingly was statistically significant, it does not reflect much of a genuine biological impact, wherein at least 30-50% SNc dopaminergic neuron loss is required for clinically meaningful behavioral deficits.

Although LPS administered intraperitoneally can induce nigrostriatal neurodegeneration (Qin et al., 2010; Batista et al., 2019; Liu et al., 2008), various studies have also consistently reported that the impact of LPS is dependent on both time and dose. Qin et al., (2007) reported that a single LPS ip injection (5 mg/kg) caused a reduction in TH+ neurons in the SNc by 23% post-treatment, but only after 7 months and by 47% post-treatment at 10 months (Qin et al., 2007). Bodea and colleagues (2018) demonstrated a notable decrease in TH+ cells in the SNc
when they administered systemic LPS (4 x 1 mg/kg) for four consecutive days. On the contrary, when a single injection was given (4 mg/kg) and the mice were sacrificed after 19 days, no decline in TH+ cells was observed, similar to what we presently observed (Bodea et al., 2018). Moreover, delivering LPS intraperitoneally once weekly for five weeks or once monthly for five months could lead to the loss of TH+ neurons in the SNc of mice brain after a further 9 and 20 months (Batista et al., 2019). In our study, we observed a slight decline of approximately 4-5% in TH+ cells after a single systemic injection (1 mg/kg). This indicates that the dose and time may not have been adequate to induce neurodegeneration, or the time between treatment and sacrifice was insufficient to trigger time-dependent neurodegenerative changes. A major limitation of our study was the advanced age of the mice, which made administering LPS at a higher dosage, or systemically, fatal to them.

The lack of effect of paraquat (PQ) on SNc neuronal survival was highly unexpected and completely contradicted previous research findings. PQ acts through several mechanisms to induce PD-like pathology, including formation of α-synuclein fibrils, increased oxidative stress, impaired dopamine catabolism, mitochondrial dysfunction, and cell apoptosis (See et al., 2022). It has been previously reported that PQ induces dopaminergic neuronal loss in the substantia nigra following repeated exposure in male mice (Xia et al., 2005; Dwyer et al., 2021; Somayajulu-Nitu et al., 2009; Yang et al., 2007; Wang et al; 2017; Jiao et al., 2012). Brooks et al., (1999) and McCormack et al., (2003) administered three ip injections of PQ at 10 mg/kg/ every 7 days for 3 weeks and reported a significant reduction in TH+ neuron cells. In contrast, but similar to our results, Breckenridge and colleagues (2013) found no significant neurotoxic effects of weekly intraperitoneal injections of PQ to the mice for a period of 3 weeks, using doses of 10, 15, or 25 mg/kg/week. Likewise, Smeyne and colleagues (2016) also assessed PQ-
induced neurodegeneration by administering the toxin either once (20 mg/kg) or twice (10 mg/kg) weekly for 3 weeks and failed to find a significant reduction in TH+ neurons within the SNc or striatum of the brain.

It is unclear as to why our experiment did not find any differences in TH+ neurons in PQ-treated mice. One plausible explanation could be that the stereological techniques used in our study might not have been adequate to detect a PQ-induced decrease in the number of dopaminergic neurons. It is also possible that PQ-induced neurodegeneration is observed only after a previous stressor, or with co-exposure, that primes the CNS to the effects of the toxin. For example, Kumar et al., (2010) demonstrated that zinc and paraquat co-exposure triggers a significant reduction in striatal dopamine (DA) levels and TH+ immunoreactivity after 8 weeks of administration. Similarly, Wang and colleagues (2009) reported a significant increase in neurodegeneration in the SNc of male mice after a combined exposure of PQ and maneb compared to mice that only received PQ or maneb or saline alone (Wang et al., 2009). Brooks et al., (1999) administered fluorogold as a tracer before PQ injections, which could potentially be toxic itself and may have primed the CNS for PQ-induced damage (Smeyne et al., 2016). Further investigations are necessary to resolve the differences between our findings and those of other researchers.

**Limitations**

This study was a follow-up experiment to a previous study conducted in Hayley lab by Beauchamp et al., (2019). This study reported significant behavioral and biological differences between young mice who received phage + LPS compared to old animals that received phage + LPS. Our objective was to investigate the impact of phage coupled with toxin treatment in both
old and young mice, considering that PD is a disease that manifests with age. We aimed to explore the mechanism of phage on the aging brain. However, due to limitations set by COVID-19, we were unable to acquire young animals for the experiment.

It is a possibility that the concentration of *L. lactis* bacteria at $3.00 \times 10^9$ CFU/ml was insufficient for the lytic actions of phage-936, thus impacting the results. To date, only a handful of studies have been conducted on the survival and viability of *L. Lactis* in the digestive system. Klijn et al., (1995) and Drouault et al., (1999) demonstrated that *L. lactis* at $10^9$ CFU/ml can survive the gastrointestinal environment for up to 3 days mixed with food and increasing the bacteria to a concentration of $10^{12}$ CFU/ml positively impacts its survivability in gastric conditions. However, only 2% of ingested bacteria was observed after a period of 3 days at both concentration levels (Klijn et al., 1995; Drouault et al., 1999).

Another possible problem could be spontaneous mutations occurring during the preparation of *L. Lactis* batch cultures. A standard growth curve of lactic acid bacteria (LAB) includes five well-defined growth phases: the lag phase, exponential phase, stationary phase, and death phase, which occurs when conditions become unfavorable for growth and cell viability diminishes (Arjumand et al., 2022). Depending on the bacterial strain, LABs enter log phase at 6-12h incubation in which there is an exponential increase in the number of living bacteria. Afterwards, the bacteria enter a stationary phase in which the number of living cells reach a plateau and rapidly enter the death phase, where there is an exponential decrease in live viable bacteria (Arjumand et al., 2022). To achieve the desired concentration for our study, we centrifuged a 500ml batch culture and added the pellet to a second 500ml batch culture to achieve a concentration of $3.00 \times 10^8$ CFU/ml. This may have resulted in limited nutrients available in the medium for the bacteria to be sustained in the stationary phase. The composition
of the media encompasses various factors that can influence the growth and functionality of lactic acid bacteria. These limitations include the availability of essential nutrients required for cell metabolism, the production of organic acids leading to a decrease in media pH and subsequent antimicrobial effects, insufficient nutrients during the exponential growth phase, inadequate supply of essential minerals like Fe2+ and Ca2+ required by certain LAB strains, as well as lack of diverse carbon sources preferred or necessary for the growth of specific LAB strains (Hayek and Ibrahim, 2013).

Several study design limitations should also be taken into consideration. Firstly, we lacked a pure control group, meaning a cohort of mice that did not receive L. lactis, phage, or any toxin. Although our results indicated that L. lactis administration did not significantly affect the microbiome, having a pure control group would have helped eliminate potential confounding variables, such as interactions between L. lactis and PQ or LPS. Another limitation is that we did not allow sufficient time after treatment for the toxins (LPS and PQ) to exert their full effect, as previous studies have reported. We sacrificed the mice only 24 hours after the last injection, which might not have provided adequate time for the toxins' effects to manifest properly. A third limitation could be the lack of considering the injections themselves as a stressor. We did not include a group of mice that received three saline injections for comparison with the PQ group, which was treated with three injections. This comparison would have helped to assess the specific impact of the toxins apart from any potential stress effects induced by the injection procedure itself.
**Future Direction**

Our results demonstrate that *L. lactis* may be able to survive in the gut but clearly don’t likely appreciably colonize it, thus limiting phage ability to promote dysbiosis. That said, the phage might be able to impact other biological processes independent of its host bacteria. To gain a deeper understanding of phages and their mechanisms in the gut, an alternative approach could be considered. For example, lysogenizing lactococcal bacteria with its specific virulent and subsequently administering the prophages may offer valuable insights. This method may have the potential to bypass the requirement for host cells to be present, thus allowing for further exploration of phage functions in the gut (Davidson et al., 1990; Bao et al., 2020; Ruiz-Cruz et al., 2020). Additionally, studies have shown some success when phage is administered intraperitoneally rather than orally (Rouse et al., 2020; Biswas et al., 2002; Shivshetty et al., 2014). Finally, it will be important to conduct studies using different sacrifice times, particularly after longer intervals following toxin + phage treatments in order to allow sufficient time for and neurotoxic effects to be manifested.
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