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DYSBIOSIS PREVENTS AGE-DEPENDENT PHENOTYPES IN DROSOPHILA *PINK1*^{B9} A FLY MODEL FOR PARKINSON´S DISEASE

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LIST OF ABBREVATIONS IN THE TEXT

Bz: Benzaldehyde
CI: Centrophobism Index
CNS: Central Nervous system
DA: Dopamine
DBS: Deep brain stimulation
ENS: Enteric nervous system
IMM: Inner mitochondrial membrane
MTS: Mitochondrial translocation signal
NMS: Non motor symptoms
OTU: Operative taxonomic unit
PD: Parkinson disease
PI: Preference Index
PINK1: PTEN-induced putative kinase 1
Pink1^{B9}: *Drosophila* PD model
ROS: Reactive oxygen species
SNpc: Substancia nigra pars compacta
5-HT: Serotonin
αSyn: αSynuclein protein

RESUMEN

La interacción entre el sistema nervioso entérico (SNE) y el sistema nervioso central (SNC), comúnmente conocido como el eje Intestino-Cerebro, consiste en una comunicación bidireccional regulada a nivel endocrino, inmunológico y neuronal, el desequilibrio en su normal funcionamiento se ha asociado recientemente con varios trastornos neuropsiquiátricos y neurodegenerativos. La investigación sobre este eje podría ofrecernos nuevos blancos terapéuticos para una amplia gama de enfermedades, incluida la enfermedad de Parkinson (EP). La EP es un desorden neurodegenerativo caracterizado por alteraciones motoras, las cuales son precedidas por lo que se denomina una etapa prodrómica o premotora donde es posible observar síntomas no motores. En esta tesis se propone manipular la microbiota intestinal en etapas premotoras tempranas de un modelo de EP en mosca generado por una deleción en el gen Pink1 (*Pink1*^{B9}), para evaluar si esta manipulación podría afectar la progresión temporal de los síntomas de la EP.

En este trabajo, se analizó si existían diferencias en la composición de la microbiota del intestino medio a los 8-9 días posteriores a la eclosión y se generó un modelo de disbiosis (desequilibrio microbiano) intestinal en Drosophila mediante un tratamiento crónico con antibiótico (kanamicina, 0,5 mM), durante un máximo de 16 días. Finalmente, mediante el uso de un sistema de registro, evaluamos individualmente en moscas macho diversos parámetros de comportamiento motores y no motores en las moscas tratadas y no tratadas con kanamicina.

Los resultados obtenidos en esta tesis evidencian una diferencia sustancial en la composición de la microbiota intestinal del mutante *Pink1*^{B9} en comparación con las moscas control. Los datos también muestran que el tratamiento con kanamicina induce la recuperación de algunos de los parámetros no motores en etapa premotora estudiados en la EP, mientras que no hay cambios en los parámetros locomotores en la etapa. Por otro lado, el tratamiento específicamente durante la etapa premotora tiene un efecto prolongado induciendo una mejora en la capacidad locomotora de las moscas de control y mutantes.

ABTRACT

The interaction between the enteric nervous system (ENS) and the Central nervous system (CNS), often described as the Gut-Brain Axis, consists in a bidirectional communication regulated at the endocrine, immunological and neuronal levels. The imbalance in the normal functioning of the Axis has been recently associated with several neuropsychiatric and neurodegenerative disorders. Additionally, the research about this axis could offer us new therapeutic targets for a wide range of diseases including Parkinson's disease (PD). This is a neurodegenerative disease characterized by motor alterations, which is preceded by what is called a prodromal or premotor stage where it is possible to observe non-motor symptoms. In this thesis it is proposed to manipulate the gut microbiota at early premotor stages of a fly PD model generated by deletion in the Pink1 gene (*Pink1*^{B9}), to assess whether this manipulation could affect the temporal progression of PD symptoms.

In this work, we analyzed whether there are differences in the composition of the mid-gut microbiota al 7-8 days post eclosion and we generated a *Drosophila* model for gut dysbiosis (microbial imbalance) by feeding flies one antibiotic (kanamycin, 0.5 mM concentration, for 14 days). By using a tracking setup we previously described we evaluated in single male flies several motor and non-motor behavioural parameters in thekanamycin-treated flies.

Results obtained in this thesis show a substantial difference in the composition of mid-gut microbiota in the *Pink1*^{B9} mutant compared with control flies. Data also show that the kanamycin treatment induces the recovery of some of the non- motor parameters studiedin premotor stage PD, although there is no substantial change in the locomotor parameters at the premotor stage. On the other hand, a specific treatment only during thepremotor stage has a longer effect inducing improvement in the locomotor capacity of control and mutant flies.

BACKGROUND

The Gut-Brain axis is known as a bidirectional communication between the brain and the gastrointestinal system. Their main actors are the brain, the gut, and the gut microbiota (the microbial community that lives in the gut). The integration of these components is known to occur via different mechanisms that include the activity of the autonomic nervous system, neurotransmitters, fatty acid, immune and endocrine signals. These different pathways act continuously over time to maintain body homeostasis. Importantly, imbalance in the microbiota-gut-brain axis is proposed as the gateway to many diseases including neurodegenerative diseases such as Parkinson's Disease (PD). Thus, it has been proposed that changes in the bacteria community in the intestine, results in alterations in the organism's physiology, which then is associated with brain disorders.

Parkinson´s disease (PD) is a monoaminergic disorder in which dopamine (DA) and serotonin (5-HT), play important roles in regulating classic motor behaviors and also non-motor behaviors. Historically, it has been studied with a focus on the brain, although this has changed with the new evidence on the contribution of the Gut-Brain axis to this disease. A better understanding of how the brain and the gut communicate could provide information for the search for

new novel therapeutic targets.

This thesis is focused on the microbiota and the effect of dysbiosis (an alteration of normal microbiota) over behavioral features associated with a *Drosophila* model for PD. Below we will review some key information to support this idea.

1. Parkinson´s disease

PD is the second most common neurodegenerative disease after Alzheimer's disease, and it highly affects the elder population. Actually, aging is associated with increased PD incidence. It is considered that people with a PD diagnosis belong to a groupwith double vulnerability: old age and PD. It has been proposed that “for PD the median age-standardized annual incidence rates in high-income countries is 14 per 100 000 people in the total population, and 160 per 100 000 people aged 65 years or older” (Ascherio & Schwarzschild, 2016). There is no accurate data about PD incidence in Chile. However, it was estimated that about 40.000 people were suffering from PD in Chile in 2013 (Pedro et al., 2013) and since the group of people over 60 years is increasing, it has been projected that these numbers will increase in the following years.

As mentioned above, age is the principal risk factor for the disease. Other factors associated with higher PD incidence are male gender, exposure to environmental elements (e.g., Pesticides), and family history. Most PD cases are

idiopathic, while familial/genetic PD reaches 5%-15% of the total cases (Balestrino & Schapira, 2020). There are several genes associated with PD. Those genes encode for proteins associated with different cellular functions and activities, including synaptic communication, ubiquitin ligase, mitochondrial kinase, cellular sensors of oxidative stress, lysosomal proteins, and in some cases, there are genes linked to PD incidence with unknown functions. One of the most frequent genes associated with PD is PINK1 (about 2-5% of the frequency of all PD cases). Mutations in PINK1, which is linked to autosomal recessive PD, is considered the second most common cause of early-onset PD (Balestrino & Schapira, 2020)

At the brain level, the main feature of PD is the loss of dopaminergic neurons present in the Substantia nigra pars compacta (SNpc) that project to the Caudate- Putamen (Striatum) in the basal ganglia in the brain. The loss of these neurons results inthe typical movement disorder.

2. Symptoms of PD

2.1 Motor symptoms of PD

The deficit of dopamine causes a decrease in the ability of movement, and from that point and onwards, patients will show gait impairment, postural instability, rigidity, slowness, and tremor, among other behavioural problems

(Peterson & Horak, 2016). These symptoms are classified as classical motor-stage PD symptoms (Kalia & Lang,2015).

Dopaminergic neurons seem to be one of the most susceptible cell populations to be damaged as compared to other neuronal types. Despite this, the dopaminergic system generates compensatory mechanisms to maintain the release of dopamine at physiological levels. Thus, it is possible for the dopaminergic nigrostriatal pathway to maintain the modulation on the circuitry formed between the different nuclei in the basal ganglia, avoiding locomotor impairment for a long time (Kuter et al., 2019). When about 60% of dopamine neurons have died, the compensatory mechanisms are not enough to maintain dopamine release in the striatum and the system collapses. The lack of dopamine in the striatum has a direct effect on the other structures of basal ganglia, including the globus pallidum, provoking a severe imbalance in the circuitry, evidenced in the onset of classical motor alterations associated with PD. With time, patients fail to keep the ability to do precise voluntary movements, losing control over body movements (Jagadeesan et al., 2017)

Currently, there is no cure for PD. One of the most common therapeutic approaches is based on the increase of dopamine signaling in the brain. This is accomplished by oral administration of Levodopa, a dopamine precursor, or alternatively, by administration of dopamine agonists (pramipexole, ropinirole, and rotigotine) that stimulate dopaminergic receptors in the central nervous system.

Usually, levodopa or dopaminergic agonists are used in combination with other drugs that improve the efficacy or the potency of these molecules in the brain. Unfortunately, these treatments can only mitigate PD symptoms for a few years and some treatments can even cause side effects in the long term; thus, there are several cases of patients developing dyskinesias induced by L- dopa (Hayes, 2019). The alternative in advanced PD cases is an invasive surgical intervention, deep brain stimulation (DBS). This is a neurosurgical alternative that consistsof the stimulation of a critical nucleus in the basal ganglia (either the subthalamic nucleus or internal globus pallidum), by using an electrode (Okun, 2012). DBS helps to decrease the doses of medication to control the motor impairment but it does not contribute to modify the course of the disease (Hayes, 2019).

2.2 Non-motor symptoms (NMS) of PD

Due to the poor prognosis of PD patients, the discovery of physiological hallmarks before the onset of movement impairment is crucial to modify the diagnosis and management of the disease. It has been reported that NMS could be present even 20 years before locomotor impairment (Kalia & Lang, 2015), and include anxiety, autonomic dysfunction, gastrointestinal dysfunction, fatigue, sensory alterations and sleep disturbance. All these manifestations occur in what is called the premotor, presymptomatic, or prodromal stage of the disorder (Figure 1). While many people experience NMS as they age, in people with PD these manifestations tend to be more frequent and more severe, and at younger ages (Okun, 2012). However, the individuals may be unaware of these

symptoms.

One of the most frequent sensory dysfunctions is the olfactory impairment which includes anosmia or hyposmia. This symptom is present in more than 70% of PD cases in the early premotor stage. Although some investigators report that impaired olfaction is independent of the disease stage, others report that it is associated with higher disease severity, progression, and dementia (Okun, 2012). The olfactory impairment could be due to the loss of dopaminergic neurons that innervate the olfactory bulb and are crucial for odorant perception. Also, it has been found protein aggregates in both the olfactory bulb and tract. Some studies have suggested that olfactory testing could be useful to diagnose PD in the pre-motor stage, but confirmation with additional studies would be still necessary (Reichmann, 2017).

Additionally, anxiety is catalogued as a pre-motor stage symptom of PD and it is present in 20%-50% of patients. However, it can co-exist in the motor stage with depression. The presence of anxiety in PD patients worsens their quality of life, affecting their mental stability and cognitive performance (Khatri et al., 2020). Therefore, it is highly important to investigate and treat anxiety in PD. Anxiety is the result of the monoaminergic disorder observed in PD progression and could be treated with classical anxiolytics in parallel with treatment for PD. It is also recommended the use of complementary non- pharmacological treatments like good nutrition and physical activity (Khatri et al., 2020; Ray &

Agarwal, 2020). PET and SPECT studies confirm the link between anxiety and reduced levels of dopamine. They also show the relationship between anxiety and the dysfunction in other aminergic pathways such as serotonergic and noradrenergic. However, sometimes the results are difficult to understand due to different patientinclusion criteria in the studies (Ray & Agarwal, 2020; Wen et al., 2016).

PD patients also are affected by gastrointestinal disturbances that affect PDpatients several years before the onset of motor impairments. These include a wide number of symptoms like swallowing problems, delays in gastric emptying, and constipation. There is evidence of accumulation of αSynuclein (αSyn) in the gastrointestinal tract in PD patients. αSyn is a very abundant synaptic protein and it is highly expressed in the brain. When αSyn is defective it forms aggregates that damage cells; this synucleinopathy affects mostly the myenteric and submucosal plexuses and mucosal nerve fibers. These fibers are highly connected with the autonomic system and the brain through the vagus nerve (Fasano et al., 2015). In this context, αSyn aggregatescould be a good biomarker for PD. Although it has been reported a wide range of gastrointestinal alterations in PD, constipation is one of the most frequent disturbances.

Evidence shows that the pre-motor PD stage is accompanied by changes in serotoninergic pathways and compensatory phenomena in the dopaminergic system inside and **outside of the brain** (Rao et al., 2006). The demonstration of

the early contribution of the gut to PD progression could open an opportunity to explore new ways to modify the course of the disease before the point of no return.

3. Brain hallmarks in PD

PD is a neurodegenerative disorder highlighted by a severe and progressive loss of dopaminergic neurons located in the SNpc, as explained above. At the histological level, it has been observed the accumulation of protein aggregates named Lewy bodies in dopaminergic neurons, which is an important factor proposed to be involved in the lossof these neurons. The main component of Lewy bodies is the presynaptic protein αSyn. The formation and maturation of Lewy Bodies are implicated in several cellular impairments like temporal sequestration of synaptic and mitochondrial proteins and mitochondrial alterations (Daniel E Shumer, 2017).

Though protein aggregates and oxidative stress are manifested in the whole brain, dopaminergic neurons seem to be particularly affected by these events. This is explained because of the extensive axonal arbor of dopaminergic neurons, the reactive properties of the neurotransmitter, and the high production of reactive oxygen species (ROS) during cellular respiration (Mahul-Mellier et al., 2020). Thus, oxidative stress seems to play a central role in the neurodegenerative process.

Other neurons that are highly affected are serotoninergic neurons. The somas of these cells are located in the Raphe nucleus and receive neural afferents from the substantia nigra. Additionally, dorsal raphe nuclei project axons to different nuclei in the basal ganglia, including the SNpc. The Raphe nucleus is also susceptible to neurodegeneration and a post-mortem study has shown the presence of Lewy bodies in this structure (Stocchi & Torti, 2017). PET scan analysis has demonstrated that SERT binding is reduced in patients with PD in different areas in the brain, including the rostral and caudal raphe, putamen, caudate, ventral striatum, thalamus, and hypothalamus. Moreover, reduced SERT binding could be crucial for an early diagnosis (de Natale et al.,2021).

4. PTEN-induced putative kinase 1 (PINK1)

Mitochondria are the most important energy-generator organelles in cells. Imbalances in energy-handling processes make this organelle vulnerable to ROS-induced damage. This organelle has several mechanisms responsible for counteracting this kind of damage, maintaining mitochondrial quality control and homeostasis. Importantly, in many of these mechanisms, the mitochondrial protein PINK1 is involved. A defect in this protein produces serious oxidative damage in neurons. Mutations in the PINK1 gene (PARK6) are the second most prevalent cause for early-onset familiar PD. It corresponds to an autosomal-recessive inheritance; heterozygous mutations in PINK1 are possible genetic risk

factors for PD (Borsche et al., 2021).

In a physiological context, after PINK1 is translated it is directed from the cytoplasm to the mitochondria. Then, it can move across the outer mitochondrial membrane, and when PINK1 is in the intermembrane spaces and reaches the inner mitochondrial membrane (IMM) the mitochondrial translocation signal (MTS) directs PINK1 to the mitochondria is cleaved and PINK1 translocation stops. A protease in the inner membrane cleaves PINK1 to generate a c-PINK1 which is released into the cytoplasm (c-PINK1). Then, PINK1 is degraded while a small amount of c-PINK1 remains in the cytoplasm to regulate mitochondrial functions such as mitogenesis, mitochondria fission, fusion, and mitophagy (Voigt et al., 2016; Wang et al., 2020).

When there is mitochondrial damage and/or a change in the membrane potential of the mitochondria, PINK1 is accumulated on the mitochondrial surface attached to the outer mitochondria membrane. This would result in an imbalance between PINK1 and c-PINK1. If the damage is moderated the amount of c-PINK1 is higher than PINK1 and therefore mitophagy will not occur. On the contrary, under strong mitochondrial damage PINK1 overcomes c-PINK1 and mitophagy occurs (Wang et al., 2020). When there are mutations in PINK1, the loss of effective control over oxidative stress plays a crucial role in the progression of neurodegeneration. On the other hand, PINK1 attached to the outer membrane could recruit and activate parkin (other gene associated with PD), which would

induce a signaling cascade responsible for mitophagy.

As discussed above, it is thought that dopaminergic neurons in SNpc are more susceptible to little changes in redox state or to stress situations, which would affect mitochondria metabolism. Thus, in PD there could be affected mitochondria in dopaminergic cells which would be one of the molecular mechanisms involved in PD progression (Subramaniam SR & Chesselet MF., 2013; Surmeier DJ et all., 2018).

5. The Gut-Brain axis and its relationship with monoamine disorders

The study of monoaminergic disorders has recently focused on the gut microbiota. Thus, it is known that the gut microbiota can use the precursor in serotonin biosynthesis (tryptophan) reducing its availability to the host; some of the bacterial species that can metabolize tryptophan include Escherichia coli, Achromobacter liquefaciens, and Paracolobacturm coliforme (O'Mahony et al., 2015). Also, several reports involve tryptophan metabolism and the serotonergic system as an important regulator not only of the brain and centrally-mediated behaviors but also for the microbiota. Then, it is possible to define a bidirectional crosstalk between the brain and the gut, where serotonin levels can change according to the types of bacteria living in the gastrointestinal tract (Cryan & Dinan, 2012).

Certain bacterial strains express a tryptophanase enzyme that produces indole from tryptophan. Other bacteria synthesize tryptophan via tryptophan synthase, and some specific bacterial strains can also produce serotonin from tryptophan, at least *in vitro* (Ozogul F. et al 2012 and Ozogul F., 2004). Moreover, there are some microorganisms, mainly gram-positive bacteria, susceptible to serotonergic drugs administered to the host,including selective serotonin reuptake inhibitors (SSRIs) (O'Mahony et al., 2015). Then, serotonin could be thought as one of the links between the emotional and cognitive centers of the brain and the peripheral functioning of the digestive tract. Actually, it has been proposed that serotonin can act as a mediator between the gut, the brain, and the microbiota, which could be relevant in the progression and prognosis of conditions like autism spectrum disorders (Israelyan N, Margolis KG 2019).

On the other hand, dopamine is linked with microbiota mainly through the immune system and the changes that occur in the gastrointestinal epithelium. Supporting this idea, dopamine and tyrosine hydroxylase are found in non-neuronal cell bodies of the intestine, immunocytes, and other cells of the gastric mucosa (Eisenhofer G. et al, 1997). Also, there are bacteria like Bacillus that can produce dopamine and this microbially-synthesized neurotransmitter can cross the mucosal layer present in the gut (Dinan T., and Cryan J., 2017).

Considering all this evidence, it is easy to think about microbiota as a system that modifies biogenic amines availability in the blood. Besides, other

researchers have shown a positive correlation between the complexity of the gut microbiota and brain maturation. Conversely, deterioration of cognitive functions is often accompanied by an important decrease in microbial richness. Interestingly, the evidence shows that biogenic amines, their related proteins including transporters and receptors, and the microbiome of the organism, changes during aging as well (O'Mahony S. et al, 2015 and Jucaite A. et al, 2010). Furthermore, modification in gut microbiota during early childhood or aged population increases the likelihood of brain dysfunction (Dinan T., and Cryan J., 2017). Finally, impairment in the normal fluctuation of microbiota composition affects the correctphysiological functioning. These changes can affect each stage of the host life cycle changes in metabolism, immunity, and neurologic functions, which are important to try to modify the progression of different pathologies like PD. However, how the regulation of the gut-brain axis by biogenic amines is involved in neurodegeneration is far from being understood.

6. Parkinson´s disease and Gut-Brain axis.

Neurodegenerative diseases have provided important new information about the Gut-Brain axis. It is now known that before motor symptoms are evident, it is possible to observe cognitive and sensory impairments related to changes in the levels of different neurotransmitters including dopamine and serotonin. Around 50% of patients present gastrointestinal disorders like

constipation, appetite loss, weight loss, dysphagia (difficulty swallowing), sialorrhea (excessive salivation), and gastroesophageal reflux which often precedes PD diagnosis (Felice et al., 2016). Moreover, most of the patients exhibit reduced intestinal barrier function that increases their exposure to microbial metabolites. It has been detected changes in the microbiota of patients as well as alterations in microbial function including the alteration in the rate of production of certain bacterial metabolites that could be associated with specific behavioral features (Scheperjans et al., 2015). Also, α-Synuclein aggregates, a classical marker of PD, are present in the submucosal and myenteric plexuses of the enteric nervous system before their detection in the brain (Cox & Weiner, 2018; Dinan & Cryan, 2017) According to some researchers, all these evidence pose the gut as the starting point in PD although this is still under debate.

The literature suggests that PD non-motor and motor symptoms depend on the oscillations of biogenic amines. However, it remains undetermined whether intestinal microbiota contributes to regulating the metabolism of these molecules and their biosynthetic precursors. Also, some of the phenotypes related to monoaminergic diseases like PD are very similar to those obtained in murine models when the animals suffer dysbiosis.

7. *Drosophila* melanogaster as a model for the study of the contribution of the gut-brain axis to neurodegenerative diseases

The fruit fly is a small invertebrate widely used in genetic studies. It has been recently proposed as an animal model for the study of complex behaviors including anxiety and addiction (Devineni & Heberlein, 2013; Mohammad et al., 2016a). The use of *Drosophila melanogaster* has many advantages. Their short life cycle allows to evaluate in a brief period of time different biological events like development and aging. On the other hand, flies are physiologically very similar to mammals despite their anatomical differences, and some of the key neurotransmitters, neuromodulators, and hormones are well conserved between these species.

As in mammals, biogenic amines act as neuromodulators and neurotransmitters in the insect CNS. Although serotoninergic neurons represent a small fraction of the entire *Drosophila* brain neuronal population, serotonin is widely distributed in the brain (Vallés & White, 1988). It plays an important role in locomotion and complex behaviors such as learning, memory, and anxiety. Moreover, there are serotonergic clusters in the thoracic CNS, and serotonin can be also produced in enterochromaffin cells of the mid-gut like in mammals (Apidianakis & Rahme, 2011). Serotonin receptors and transporter share sequence similarities with the orthologous proteins in mammalian species (Haddad et al., 2016; Saudou & Hen, 1994). On the other hand, dopamine has

been involved in the locomotor system and complex behaviors in insects (e.g., olfactory discrimination, social space, memory system punishment/learning), and dopaminergic neuronal clusters are described in the brain and thorax. Finally, several drugs widely used to manipulate the dopaminergic system in vertebrates are also effective in *Drosophila* (Monastirioti, 1999).

Currently, it is known that the levels of dopamine and serotonin in male adults fly brains change over aging as well as their behavior, although dopamine remains more stable during early adulthood than serotonin which suggests different roles of both amines during aging (Molina-Mateo et al., 2017). In the same way, *Pink1*^{B9} mutant fly a fruit fly with a deletion in the PINK1 gene, has been extensively studied from its behavioral characteristics up to brain neurochemistry.

7.1. *Pink1*^{B9} mutant fly

In flies the CG4523 gene codes for the PINK1 protein, which is a 721 amino acid polypeptide with two main motives, the mitochondrial targeting motif and a serine/threonine kinase domain very similar to that in the human gene (Park et al., 2006). The mutant *Pink1*^{B9} *Drosophila* strain was created in 2006 by Park and collaborators. It is a mutant strain generated by a deletion of 570pb. It has been described that this fly exhibits loss of dopaminergic neurons and locomotor deficit at 30 days post eclosion (Parket al., 2006).

Posterior studies showed that *Pink*^{B9} mutant flies present several behavioral impairments at different points in their life including movement slowness, olfactory disfunction, and changes in the circadian rhythm (Julienne et al., 2017; Molina-Mateo et al., 2017). Also, in this model it was evidenced two phases in the progression of behavioral impairment: a premotor stage (0-24 days post eclosion) and a motor stage (over 24 days post eclosion). It was finally reported a molecular compensatory mechanismto delay the locomotor impairment in the premotor stage (Molina-Mateo et al., 2017). These features make the *Pink1*^{B9} *Drosophila* strain a very good model for the study of PD progression (Figure 1).

7.2 Features of *Drosophila* gut

Drosophila melanogaster is a very useful model for the study of the gut-brain axis because its intestinal tract shares several characteristics with vertebrate animals, at the molecular and cellular levels. For instance, the fly intestine exhibits the same types of transporters, receptors, and aminergic innervation as compared to mammals (Apidianakis& Rahme, 2011). As in humans, the *Drosophila* gut is an epithelium surrounded by a layerof muscles and nerves. At the cellular and tissue levels, there are other similarities: both vertebrates and invertebrates have stem cells and enterochromaffin cells that play the same role. The main difference between *Drosophila* and humans is that *Drosophila* carryout the digestion process at a more basic pH while humans make it in an actual acid

environment (Capo et al., 2019).

Drosophila intestine is divided into three main parts: foregut, mid-gut, and hindgut. Digestion and absorption occur mainly in the mid-gut, where several digestive enzymes (proteases, lipases, and carbohydrases) are contained in the gastric fluid and it is in this section where water and nutrients are absorbed (Kitani-Morii et al., 2021). The matrix of the luminal side of the gut filters the content from de lumen to be carried towards internal organs in the fly. Therefore, this structure only allows the passage to digested food leaving out bacteria (Kitani-Morii et al., 2021; Miguel-Aliaga et al., 2018).

In the lumen, bacterial families that are present in humans such as Lactobacillaceae, Acetobacteraceae, and Enterobacteriaceae are also observed in *Drosophila* microbiota. There are different strains along the *Drosophila* gastrointestinal track which are crucial to keeping the bacterial diversity (Miguel-Aliaga et al., 2018). The specific pH, anti-microbial peptides (AMPs), and dual oxidase present in mid-gut are important for maturation and operation of specific mid-gut microbiota (Xuefeng Chen, et al, 2011). Importantly, there are a few studies that relate neurodegenerative diseases with the gut-brain axis in flies. It has been already demonstrated that intestinal dysbiosis aggravates the progression of Alzheimer's disease in *Drosophila* (Wu S., Cao Z., Chang K. & Juang J., 2017) and there is evidence that models for Alzheimer and PD have different microbiota at the motor stage (Kong et al., 2018; Xu et al., 2020). But the microbiota in the pre-motor stage of PD and the specific mechanisms that

underlie the link between microbiota and progression of neurodegenerative diseases remain undiscovered. These antecedents make the fruit fly an excellent model to study the topic of the present thesis.

Finally, it is expected that impairment in the normal fluctuation of gut microbiota composition affects the correct physiological functioning of the animal causing changes in metabolism, immunity, and neurologic functions, which are important in different pathologies, including PD. However, whether the regulation of the gut-brain axis by biogenic amines is involved in the progression of neurodegenerative diseases is far from being understood. The present thesis has focused on dysbiosis as an approach to assess changes in the behavioral phenotype in a *Drosophila* PD model.

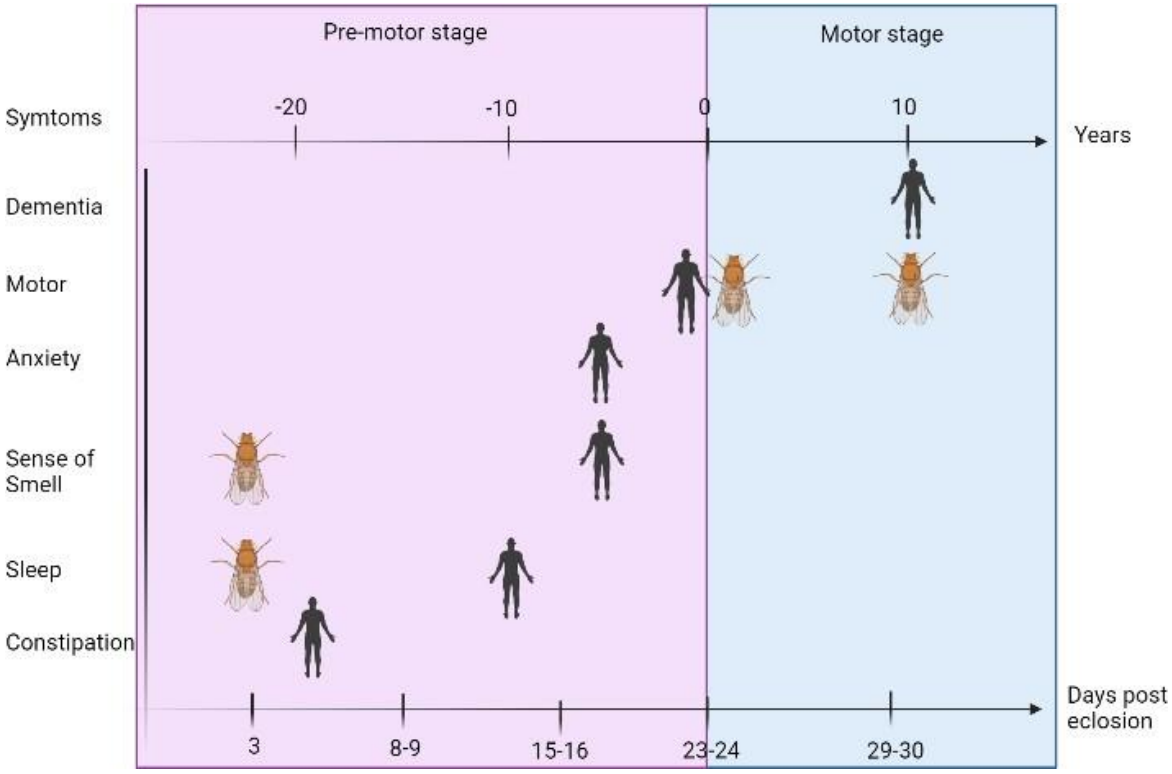

Figure 1: Clinical symptoms and disease progression: humans and the Pink1^{B9} Drosophila model.

The onset of motor symptoms is time 0 corresponding to the diagnosis of PD (upper time scale). This time is preceded by a premotor stage, which is characterized by a variety of non-motor symptoms such as constipation, sleep disorder, anxiety, and disturbances in the sense of smell. After diagnosis, there is cognitive impairment, like dementia. In *Pink1^{B9}* flies, motor disturbances start at 23-24 days post eclosion (lower time scale) and they get worse at 29-30 days post eclosion, while non-motor impairments are present since 3 days post eclosion.

HYPOTHESIS

1. General hypothesis

Dysbiosis induces a worsening of the phenotypes associated with a *Drosophila* model forPD.

2. Specific hypothesis

Dysbiosis treatment aggravates the non-motor and motor characteristics of the parkinsonian phenotype in the *Pink1*^{B9} mutant *Drosophila* strain.

OBJECTIVES

1. General objective

To advance our understanding of how dysbiosis in the gut affects the behavioral manifestations in a PD model.

2. Specific objectives

i) To determine the composition of intestinal microbiota, and changes induced by Kanamycin treatment.

ii) To evaluate behavioral changes in the Drosophila *Pink1*^{B9} PD model compared to control animals, as flies age.

- To assess non-motor behaviors in *Pink1*^{B9} mutant flies after kanamycin-induced dysbiosis, as compared to control flies.

- To study locomotor behavior in *Pink1*^{B9} mutant flies after kanamycin-induced dysbiosis, as compared to control flies.

METHODS

1. Bioethical and biosafety issues

All experimental procedures (ID 180629003) were approved by the Bioethical and Biosafety Committeeof the Pontificia Universidad Católica de Chile, specifically the "comité institucional de seguridad en investigación" and were conducted in accordance with the guidelines of the National Committee for Scientific and Technological Research (ANID) and the Servicio Agrícola y Ganadero de Chile (SAG).

2. Flies

All experiments were carried out with male flies. Animals were raised at 19 °C on a 12/12 h light/dark cycle and maintained on a standard yeast meal diet. The *Pink1*^{B9} mutant strain (w[*], PINK1[B9]/ FM7i, P{w[+mC] = ActGFP}JMR3) was obtained from the Bloomington *Drosophila* Stock Center (line # 34749). As genetic controls, we use *w*^{1118} flies, which were obtained from the Bloomington *Drosophila* Stock center (line # 5905). All animals used in experiments were obtained from at least three independent cohorts.

3. Antibiotic treatment:

Kanamycin, a broad-spectrum antibiotic able to kill Gram-positive, Gram-negative,mycoplasma, and bacterial pathogens in *Drosophila* (Jin, Y. et al, 2017), was used to generate dysbiosis in flies, by using a protocol modified from Wu et al (2017). Briefly, flies are fed with fresh regular food or regular food mixed with Kanamycin (at a final concentration of 0.5 mM), from day 1 post-hatching until day 15-16 as adult animals (Supplementary figure1). The food is prepared each week and stored in the lab cold room at 4°C. Flies were moved to new vials containing new food every 2 days.

4. Lifespan and Chronic exposure to Antibiotic

Newly eclosed flies were raised in standard food (control) or kanamycin (0.5 mM)supplemented food (experimental), in groups of 20 flies. The total number of flies per condition was 200 animals. Flies were changed every 48 hours to a new vial. The numberof dead flies was registered daily until there were no flies alive. Assays were performed at25°C. For data analysis, results were expressed as % survival rates.

5. Video recording, centrophobism index, and chemotaxis assay

For behavioural assays, we used a setup previously described by the groups of Drs. Colomb and Brembs to assess naïve motor behaviour in flies (Colomb et al 2012), whichwe modified with their support (Molina-Mateo et al., 2017; Hidalgo et al., 2017) and have been used in several works in our lab in recent years (Fuenzalida-Uribe and Campusano, 2018; Hidalgo et al., 2020; Hidalgo et al., 2021). In brief, single flies were placed in a circular arena (39 mm diameter, 2 mm high), with two pieces of cotton placed on opposite sides (Figure 2A). Fly behaviour was recorded in this arrangement for 3 min at room temperature (Figure2B). Afterward, one of the pieces of cotton was soaked with 100 µl of Benzaldehyde (Bz, 1% v/v in water), while the other was soaked with 100 µl of distilled water. Fly behaviour was recorded for another 3 min, indicated from here on as the “Bz” experimental group (Figure2D). All video recordings were analyzed offline, using the tracking analysis software Buridan tracker (Colomb et al 2012).

In addition, heat maps that reflect the position of flies during the control and Bz conditions were generated. These maps were processed with ImageJ to calculate Performance Index (P.I.), a value that reflects the innate aversion of flies to Bz. To do this,the heat map was divided in two halves and P.I. was calculated according to the followingformula:

$$PI = \frac{AUC_{H2O} - AUC_{Bz}}{AUC_{H2O} + AUC_{Bz}}$$

Where AUC_{H2O} and AUC_{Bz} correspond to the area under the curve of time spent in the half of the arena closer to water, and the area under the curve of time spent in the halfof the arena closer to Bz, respectively.

Also, it is possible to calculate centrophobism indices (CI). For doing this, the circular arena is divided into two concentric circular areas of equal surface: a smaller circle and an outer ring (Figure 2C). The software records the amount of time spent in eachsubdivision. Both are calculated using the following formula:

$$CI = \frac{TE - TI}{TI + TE}$$

Where CI is the centrophobism index, TI is the time that the fly spends in the internal area and TE is the time that the fly spends in the external area. Therefore, an index of 1 means that the fly spent the entire experiment in the outer area, -1 is obtainedif the fly spends the entire experiment in the centre and 0 denotes an equal distribution between outside and inside areas.

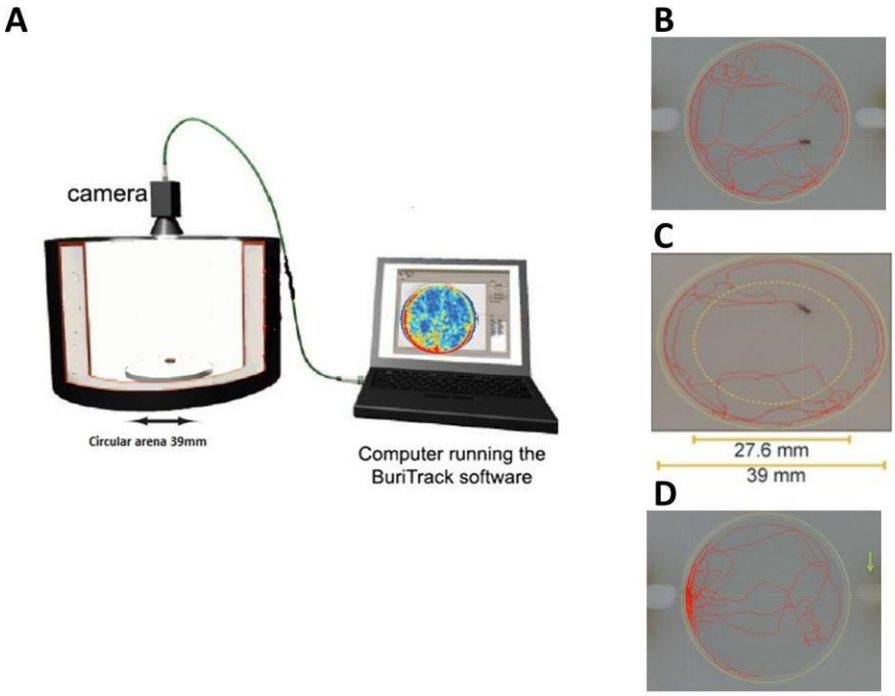

Figure 2: Figure Set up for innate behavioral performance.

A single fly was put in a circular arena with a camera on top to record the innate behavior (A-B). From the recording, it is possible to calculate a centrophobism index a measure about how much time spend flies avoiding the center (C). Also, it is possible to calculate a preference index a measure about fly aversive response to benzaldehyde(D). The green arrow marks the cotton wet with the aversive odorant.

5.1 Data analysis of video recordings

All behavioural data were compared using One way ANOVA or Two-way ANOVA and Tukey's or Sidak's multiple comparisons test respectively, in Prism software (GraphPad). Also, we perform the comparison and obtain the median survival data by Mantel-Cox test in Prism software (GraphPad).

6. Analysis of *Drosophila* microbiota

6.1 Fly rinsing:

To avoid contamination of gut samples with bacteria from the cuticle, flies were rinsed in three different solutions in the following order: sodium hypochlorite 2,5% (twice, one minute each time), Ethanol 70% (twice, one minute each time), and sterile water (twice, one minute each time). 40 male flies were included in each sample and 17-22 biological repeats of each group).

6.2 DNA extraction:

Intestines were dissected out in sterile conditions and placed in a 0.5 mL tube containing sterile water (180 □L). Phenol/Chloroform extraction was used to prepare DNA. Before DNA extraction, we add lysis buffer and solid glass beads (1 mm diameter) to the tube to homogenize the microbial cells by bead beating, by

three pulses of 20 s. Quality and quantity of the extracted nucleic acids were checked by Qubit Fluorometric (Table 1) Quantification and by inspection after separation in a 1% agarose gel. Samples were briefly kept at −20^{o}C. Library construction and sequencing were performed by NOVOGENE. PCR of the hypervariable regions V3 and V4 of bacteria 16 S, were performed by specific primers connecting with barcodes. The construction of the DNA libraries included end repairing, adding poli-A to tails, purification, etc. Libraries were generated in a paired-end Illumina platform to generate 250bp paired-end raw reads.

6.3 Analysis of 16S regions

The raw tag-sequences were processed using QIIME 2. Multiplexed reads were first trimmed, quality-filtered, and assigned to the corresponding sample. The filtering criteria and a minimal average quality score. Wolbachia sequences were removed. To identify chimeras, the dataset was processed using usearch61. The number of reads per sample was normalized by rarefaction and reads clustered in OTUs. A representative sequence from each OTU was selected. Then, taxonomy assignment was done with QIIME 2 by searching the representative sequences of each OTU against the SILVA 16SrDNA. We used PCoA (MDS) analysis Weighted UniFrac distances metric to determine the distance between samples, and separate the subjects in distinct clusters considering their abundances.

7. Mid-gut microbiota culture

Four intestines of each condition were disaggregated in plates of enriched culture medium. The plates were kept in an oven for 24 hours at a temperature of 30°C. The qualitative results were obtained by taking pictures of bacteria growing on mMRS or GYC plates, with a Leica Camera and microscope. Composition of each agar-based medium here below.

7.1. Man, Rogosa y Sharpe agar médium (mMRS)

Distilled water , universal peptone (0.125g/ml), yeast extract (0.075g/ml), glucose (0,20g/ml), dipotassium phosphate (0.02g/ml), ammonium citrate (0.02g/ml), sodium acetate (0.05g/ml), magnesium sulphate (0.001g/ml), manganous sulphate (5ug/ml) and agar (0,12g/ml).

7.2. GYC agar medium (mGYC)

Distilled water , Glucose (0.5g/ml), yeast extract (0.03g/ml), calcium carbonate (0.015g/ml) and agar (0.06g/ml).

RESULTS

1. Characterization of microbiota from young flies

In order to investigate whether there is a difference in microbiota in the early stages of the fly PD model as compared to control animals, we performed 16S rRNA gene (16S iTag V3-4 amplicons) sequencing of both *Pink*^{B9} and *w*^{1118} *Drosophila* genotypes.

The general analysis of the samples shows the sequence depth in the rarefaction curves indicated as Species richness v/s Sample size. It is important to highlight that every curve reaches a plateau, which means that all the species of each sample have been processed (Supplementary figure 2). After the taxa assignation for whole sequences, it was evidenced that the most prevalent groups are acidobacteriota, actinobacteriota, firmicutes, proteobacteria, and cyanobacteria (Supplementary figure 3). Interestingly, there is a big amount of "no assigned taxa" in these samples (Supplementary figure 3).

The analysis by taxa represented in an alluvial diagram (Figure 3) describes the relative abundance of bacteria in each genotype from phylum to family. The results obtained from the microbiota analysis indicate that the *w*^{1118} strain is characterized by proteobacteria and cyanobacteria representing almost

50% and 25% of the phylum, respectively (Figure 3A). On the other hand, *Pink1*^{B9} is characterized by proteobacteria, actinobacteria, and cyanobacteria, although unlike the control strain the proteobacteria phylum reaches almost 70% of the total bacterial composition while actinobacteria and cyanobacteria represent about 10% each (Figure 3B). Thus, in both control and PD flies the proteobacteria phylum is the most abundant (Figure 3).

At the class level, the same trend observed at the phylum level is maintained. In *w*^{1118} alphaproteobacteria of the phylum proteobacteria and cyanobacteria class of the phylum cyanobacteria, represent about 50% and 25% of the bacteria classes, respectively (Figure 2A). In *Pink1*^{B9} flies, alphaproteobacteria is also the most prevalent bacterial class in the phylum proteobacteria, reaching almost 70% of the bacterial composition, while actinobacteria and cyanobacteria classes represent about 10% each (Figure 3B).

The most detailed analysis is at the family level. In *w*^{1118} the most abundant families are acetobacterareae (20%) and sphingomonadaceae (25%), which belong to the proteobacteria phylum, and nostocaceae family (25%) which belongs to the cyanobacteria phylum. However, in *Pink1*^{B9} Acetobacteraceae represents about 40% of the family bacterial composition, followed by sphinomonadaceae (20%) and nostocaceae (10%).

Beta diversity analysis between samples by PCoA (MDS) analysis Weighted

UniFrac distances (Figure 4A) shows that PD model flies (yellow dots) are distinguishable from control flies (blue dots) demonstrating a difference in microbiota of both strains. The analysis shows that two components are able to explain about 77% of the variability between groups (Figure 4A). The final dendrogram which was computed as WUnifarc distances reveals two distinct clusters: the vast majority of blue dots are included in the left group. This group exhibits a long distance from the right group where the vast majority of yellow dots are observed (Figure 4B).

All features of intestinal microbiota indicate important differences between strains at the time window studied, which is critical in the progression of PD features in this animal model. In order to evaluate whether we can modify the progression of PD-associated features in the *Pink1*^{B9} strain by a perturbation in intestinal microbiota, we decided to generate dysbiosis by feeding young flies with kanamycin.

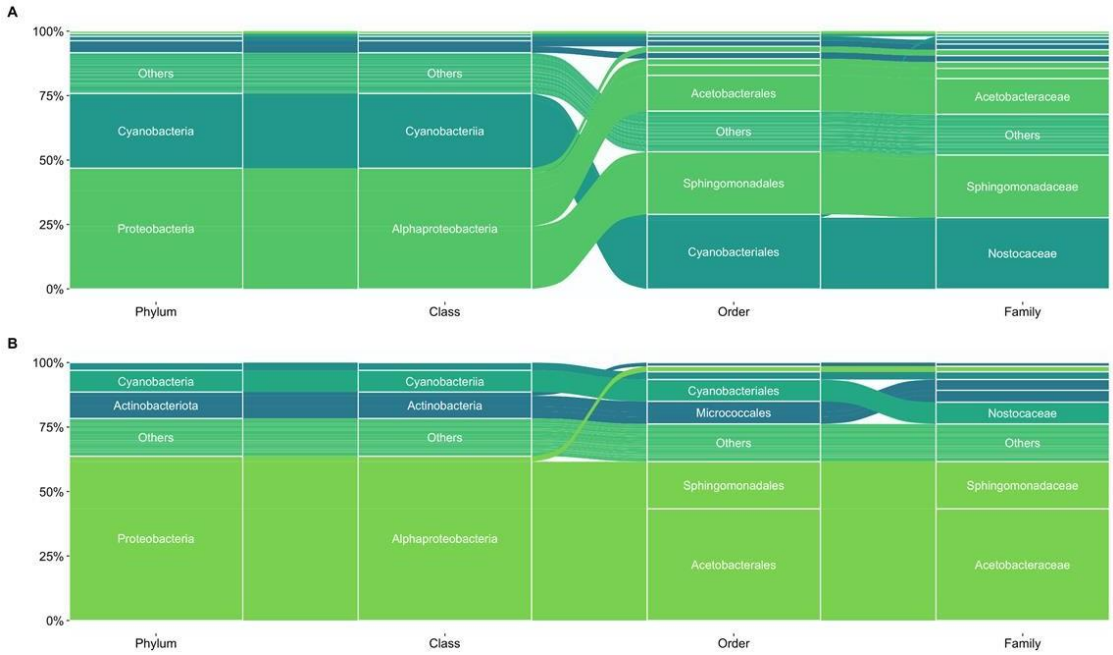

Figure 3: Characterization of microbiota from critical lifetime window.

16S v3-v4 sequencing from *Pink1*^{B9} and w^{1118} young flies were analyzed to evaluate whether there aredifferences between intestinal microbiota from both strains. Taxa analysis of (A) *w*^{1118} and (B) *Pink1*^{B9}.

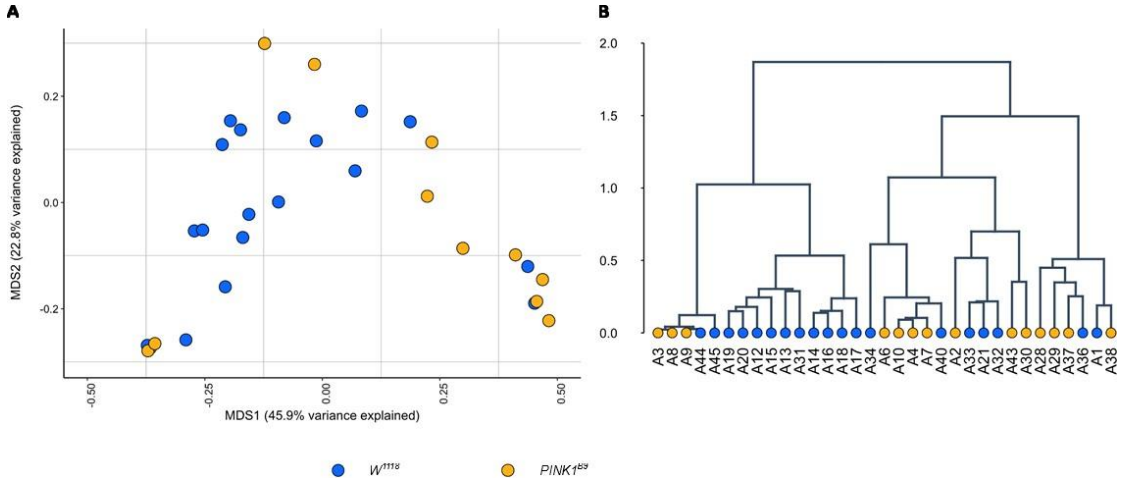

Figure 4: Pink1^{B9} exhibits different microbiota respect control flies.

16S v3-v4 sequencing from *Pink^{B9}* and w^{1118} young flies were analyzed to evaluate whether there are differences between intestinal microbiota from both strains. (A) The β diversity of the microbiota composition was shown as PCoA based on unweighted UniFrac distances. (B) WUnifarc distances clustering of the microbiota from Control and PD *Drosophila*. PCoA: Principal co-ordinates analysis.

2. Kanamycin effect over young flies

2.1 Survival assay

Before beginning to assess the effect of kanamycin on fly behaviors, it was evaluated the effect of this antibiotic on fly survival in the two genotypes: w^{1118} control and *Pink1*^{B9} mutant fly. For this, flies were maintained since the eclosion until death in fly food supplemented with kanamycin (0.5 mM). Results show no effect of the antibiotic on lifespan in control or mutant flies (Figure 5B and 5C). It is already known that there is a difference in lifespan between the mutant and control genotypes (Liu et al., 2020). Therefore, as an internal control, we confirmed that *Pink1*^{B9} flies exhibit a shorter lifespan than *w*^{1118} control flies (Figure 5A). Results show a median lifespan of 7 and 9 weeks, respectively.

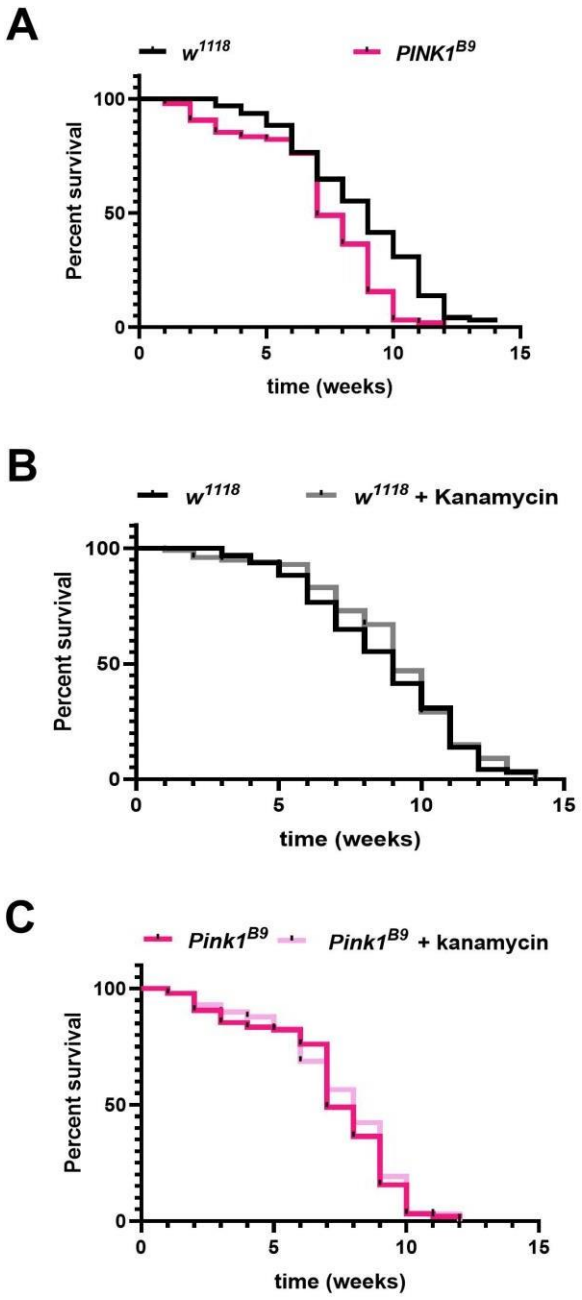

Figure 5: Effects of Kanamycin treatment on lifespan in different genotypes.

Lifespan in (A) w^{1118} v/s $Pink1^{B9}$ mutant flies; (B) w^{1118} exposed or not to the antibiotic, and (C) $Pink1^{B9}$ mutant strain when exposed or not to the antibiotic. Data are presented as % survival rates. Mantel-Cox test show differences between strains in (A) ($p < 0,0001$; n=100 flies), while no significant differences were observed between groups in B or C ($p>0.05$; n=100 in each experimental group).

2.2 Kanamycin treatment cleans the mid-gut from bacteria

According to literature flies contain in their gut both Gram-negative bacteria like Acetobacteraceae and Gram-positive like Lactobacillus (Bost A. et al, 2017). Data obtained in this thesis show that control and *Pink1*^{B9} flies principally contain Gram- negative bacteria of the Acetobacteraceae, sphinomonadaceae, and nostocaceae (Figure 3). It was carried out a general evaluation of the effect of a chronic kanamycin treatment on intestinal microbiota. For doing this, it was isolated a piece of *Drosophila* mid-gut and evaluated whether bacteria are growing from this tissue in classical media mMRS and mGYC.

Particularly, flies were separated in four groups according to genotype (*w*^{1118} and *Pink1*^{B9}) and according to diet (standard food, or standard food + antibiotic). Mid-guts weredissected from 4 animals in each experimental condition, mechanically disaggregated, and material placed on mMRS or mGYC culture plates during 24 hours at 30°C (Figure supplementary 1).

Results show that a chronic 8-9 days old kanamycin treatment results in no bacteriagrowing in either phenotype (Figure 6). There is no evidence of colonies when we extendthe chronic treatment up to 15-16 days post eclosion (Figure 7). This data suggests that kanamycin treatment is effective at eliminating all

bacteria in the fly gut.

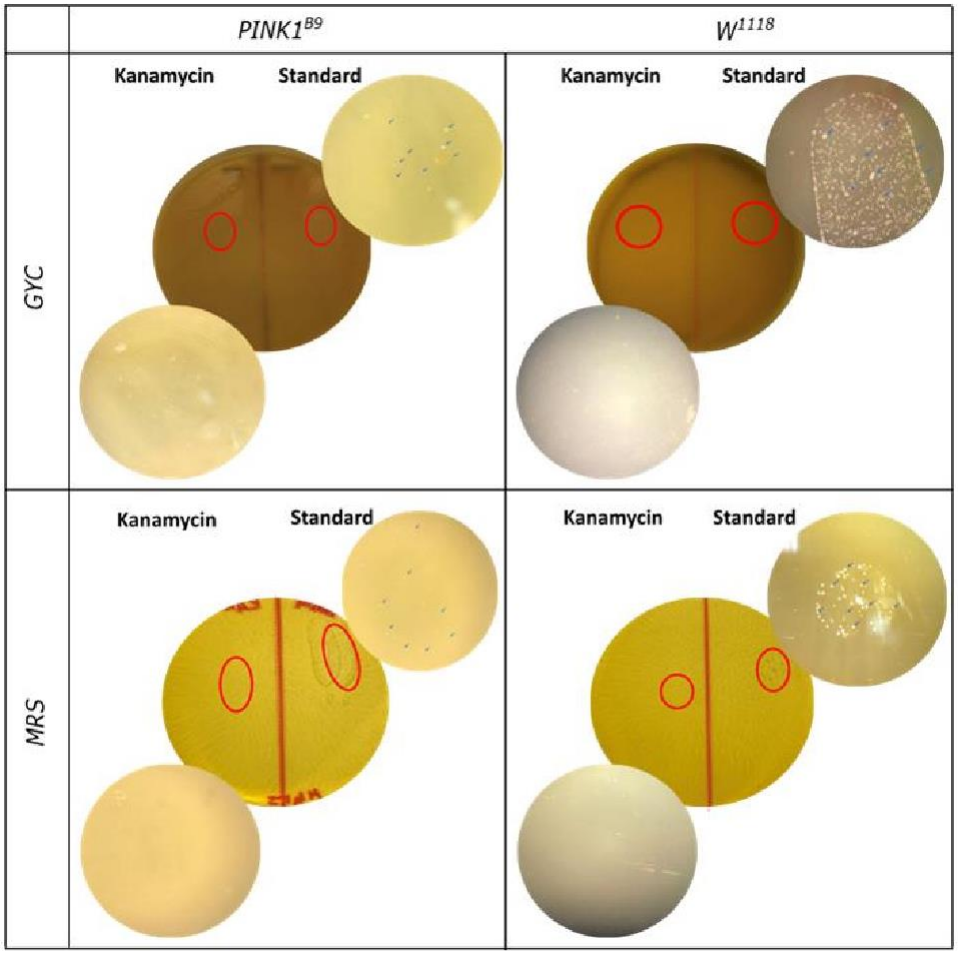

Figure 6: Kanamycin prevents the growth of intestinal bacteria in flies 8-9 days old.

Control or *Pink1*^{B9} flies 8-9 days old were treated or not with antibiotic (Kanamycin or standard food condition). Mid-gut of 4 flies was dissected out, disaggregated and the preparation was seeded on agar plates. Two different culture media were used for these experiments, GYC (Top panels) and MRS (Bottom panels), in both genotypes *w*^{1118} (right) and *Pink1*^{B9} (left). Each panel presents a representative experiment from an agar plate which was divided into two parts to culture the bacteria of mid-gut from flies fed with standard food (right side) or standard food with antibiotic (left side). For both sides, there is a magnification of results in each condition. After 24 hrs at 30°C, it is not possible to observe bacteria growing in samples from the intestines of kanamycin-treated flies.

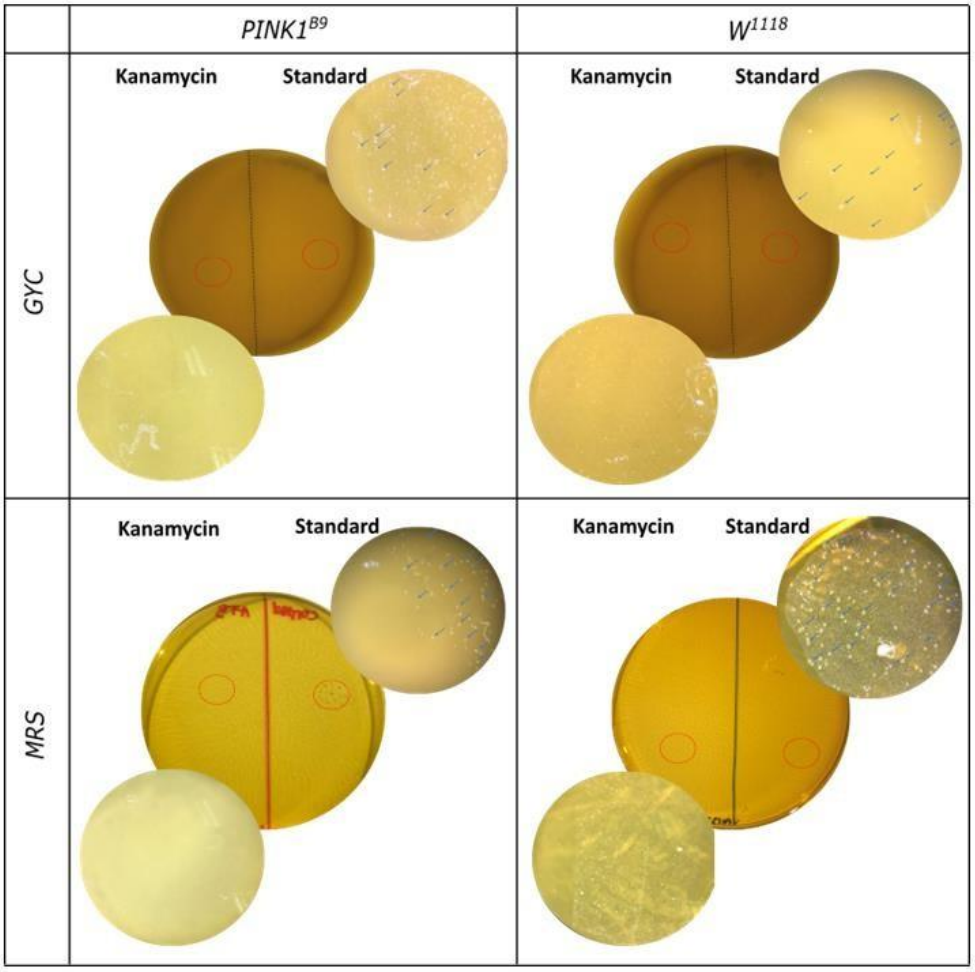

Figure 7: Kanamycin prevents the growth of intestinal bacteria in flies 15-16 days old.

Control or *Pink1*^{B9} flies 15-16 days old were treated or not with antibiotic (Kanamycin or standard food condition). Mid-gut of 4 flies was dissected out, disaggregated and the preparation was seeded on agar plates. Two different culture media were used for these experiments, GYC (Top panels) and MRS (Bottom panels), in both genotypes *w*^{1118} (right) and *Pink1*^{B9} (left). Each panel presents a representative experiment from an agar plate which was divided into two parts to culture the bacteria of mid-gut from flies fed with standard food (right side) or standard food with antibiotic (left side). For both sides, there is a magnification of results in each condition. After 24 hrs at 30°C, it is not possible to observe bacteria growing in samples from the intestines of kanamycin-treated flies.

3. Behavioral characterization of *Drosophila Pink1^{B9}* mutants treated with kanamycin toinduce dysbiosis in the presymptomatic stage of PD model

One of the hallmarks of PD is the detection of non-motor behavioral phenotypes 10or more years before any deficit in locomotion (Kalia & Lang, 2015). Among the symptomsassociated with the prodromal phase of PD, it is described anxiety, depression, constipation, sleep behavior disorder, and hyposmia (Elbaz, 2016).

Once it was clear that the kanamycin treatment does not affect fly survival, it was decided to characterize different behavioral parameters in *Drosophila Pink1^{B9}* mutant flies fed kanamycin. This study was carried out at strategic time points, in the presymptomatic and symptomatic stages of this PD animal model according to what we have previously reported (Molina-Mateo et al., 2017). For this, it was used a tracking system we previously reported (Molina-Mateo et al., 2017; Hidalgo et al., 2017; Fuenzalida-Uribe et al., 2018; Hidalgo et al., 2020). One of the advantages of the use of this tracking system is that we are able to study motor and non-motor behavioral parameters in flies. In this work, the behavioral features were studied in control and *Pink1^{B9}* animals after treatment with kanamycin.

3.1 Dysbiosis reverts the deficit in naïve aversive olfactory responses observed in *Pink1*^{B9}.

Hyposmia is a promising biomarker of the premotor stage of PD; however, it is a poorly understood phenomenon (Sui et al., 2019). Hyposmia has been previously reported in early, non-motor stages of PD models in flies (De Rose et al., 2016; Molina-Mateo et al., 2017). Therefore, we decided to carefully study this parameter to assess whether there could be a modification of this behavioural feature after kanamycin feeding. We recorded the PI, as a parameter to assess olfactory responses, in animals in different experimental conditions.

First, we observed the expected impairment in olfactory response in young mutant *Pink1*^{B9} flies in control conditions (not fed the antibiotic), as compared to *w*^{1118} animals that did not receive the chemical (F (1, 78) = 12.22; P=0.0008; Figure; 8A).

Kanamycin treatment 3 and 8-9 post eclosion respectively, improved the olfactory response measured in mutant flies, while did not modify the aversive response recorded in control flies (F(1, 76)=5.875; P=0.0177 Figure 6 and F(1, 77) = 14.52; P=0.0003 Figure 6B). Interestingly, the mutant flies treated with kanamycin for 8-9 days, exhibit an olfactory response that reaches a magnitude similar to that

recorded in younger control animals ($F(1, 77) = 14.52$; $P=0.0003$ Figure 8B). A longer treatment of 15-16 days does not exhibit the same response; it is possible to observe the difference in PI between control and *Pink1*^{B9} flies that was observed in 0-3 days old flies. However, the kanamycin treatment losses the ability to increase PI in *Pink*^{B9}. Kanamycin does not have any effect in control flies (Figure 8C).

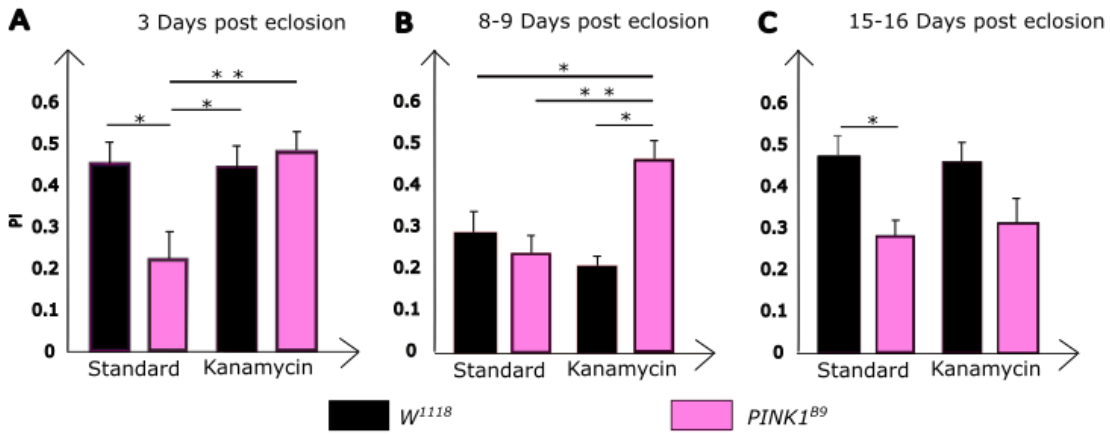

Figure 8: Kanamycin treatment reverts olfactory deficits in Pink1^{B9} mutant animals.

Mutant *Pink1^{B9}* and control (*w^{1118}*) flies were fed for (A) 3 (B) 8-9 and (C) 15-16 days kanamycin 0.5 mM. Then, they were exposed to an aversive odorant, Bz (1%, 3 min), and behavior was videorecorded. Response to this odorant stimulus was measured as preference index (P.I.). Video recordings were analyzed offline using the Buridan tracker software. Data obtained from n=19-22 independent animals per experimental group; two-way ANOVA followed by Bonferroni post-test, * and ** indicates $p < 0.05$ and <0.01.

3.2 Anxiety-like behavior as a hallmark of premotor stage in *Pink1*^{B9}

Fluctuations in anxiety levels are understood as a symptom of several neurological and neurodegenerative human diseases. This phenomenon has been poorly studied in the context of neurodegenerative diseases (Thobois et al., 2017). Anxiety is one of the most common non-locomotor symptoms of PD that highly affect the quality of life of the patients. It has been shown that the average prevalence of an anxiety disorder is 31% or more depending on the criteria utilized to measure this parameter (Broen et al., 2016; Marinus et al., 2002). Anxiety is highly associated with the serotonergic system. In invertebrates, it has been used the centrophobism behavior as a proxy of anxiety-like behavior, high levels of centrophobism index indicate a high levels of anxiety like behavior (Hidalgo et al., 2017; Mohammad et al., 2016b).

From the analysis by genotype, it is possible to detect an effect of age on centrophobism: it is detected a decrease in centrophobism index as animals age in control flies ($F(2, 57) = 5{,}058$ $P=0.0095$) while it is observed an increase of centrophobism index in *Pink1*^{B9} mutant flies of 15-16 days post eclosion ($F(2, 58) = 16{,}11$; $P<0.0001$). Thus, both *Pink1*^{B9} and control flies exhibit changes in this behavior over aging in opposite directions.

Also, the comparison between genotypes shows that there are

differences in the Centrophobism index in the first and second time windows between genotypes, showing the *Pink1*^{B9} mutant animals lower index as compared with control flies ($F(2, 115) = 16.24$; $P<0.0001$, Figure 9). Given the dynamic changes in this parameter, no statistical differences are observed between genotypes at the final time window (P= 0,3332; Figure 9).

While in Molina-Mateo *et al* (2017) it was reported olfactory dysfunction, the present thesis demonstrates for the first time anxiety-like behavior as a non-locomotor early-stage feature in *Pink1*^{B9} mutant fly.

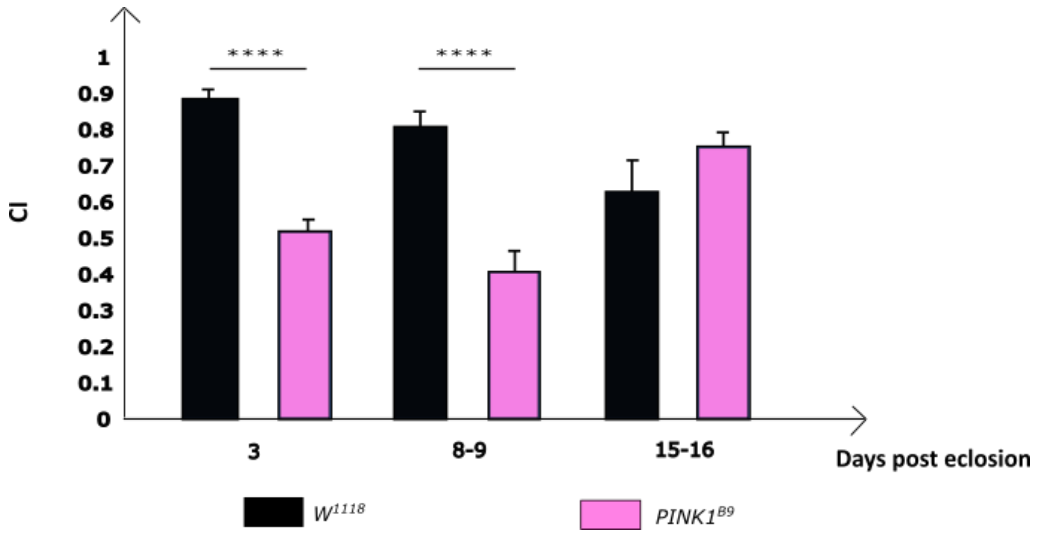

Figure 9. Change in centrophobism, an anxiety-like behavior in flies, over aging.

Centrophobism is measured as it was explained in Materials and methods. This behavior is measured asCentrophobism Index, and it was calculated at 3, 8-9, and 15-16 days after eclosion. Data represent mean + SEM of n=19-22 flies per experimental condition. **** represent p<0.0001, two-way ANOVA with Tukeypost-test when comparing indicated experimental groups.

3.3 Antibiotic treatment reverts the normal centrophobism phenotype in *Pink1^{B9}*.

Currently, it has not been possible to thoroughly study anxiety in prodromal phases of the disease, as it progresses. The *Pink1^{B9}* mutant model provides an opportunity to do it.

Mutant flies exhibit reduced centrophobism as compared to control flies at 3 and 8-9 days time points ($F(1, 76) = 7.728$; $P=0.0069$ Figure 10A and $F(1, 77) = 19.40$ $P<0.0001$ Figure 10B). The antibiotic treatment eliminates the differences between control and mutant strains, although this seems to be explained by a dual effect on control and the mutant strains: while it reduces centrophobism in control flies, it is possible to detect an increase in this behavioral feature in mutant animals ($F(1, 76) = 7.728$; $P=0,0069$ Figure 10A and $F(1, 77) = 19.06$; $P<0.0001$ Figure 10B). The 15-16 days long chronic treatmentwith kanamycin does not have any effect over the anxiety-like behavior in both control ($P=0.1063$) and PD model strains ($P= 0.9086$) (Figure 10C).

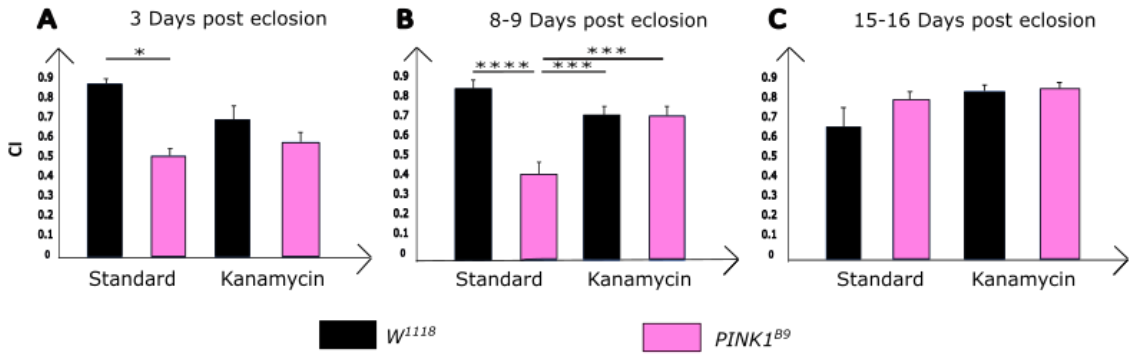

Figure 10: Kanamycin treatment affects anxiety-like behavior in both mutant and control strains.

The innate centrophobism behavior of flies was recorded for 3 min. Videos were analyzed offline using the Buridan tracker software. The centrophobism index represents the time that the fly spends avoiding the center (Hidalgo et al., 2017). Data from n=19-22 flies; two-way ANOVA followed by Bonferroni post- test. *, ***; **** indicate $p<0.05$; $p<0.005$ and $p<0.001$ respectively when comparing data from experimental groups identified.

3.4 Locomotor activity in pre-motor stages of the PD model.

Both centrophobism and PI depend on the ability of flies to execute motor programs. Thus, we decided to assess whether there is any motor alteration in flies studied at the experimental conditions analyzed that could explain the results presented above. To assess motor behavior, three motor parameters were used: distance traveled, speed, and activity time, as previously reported (Molina-Mateo et al., 2017; Hidalgo et al., 2017).

First, data obtained show no differences in motor parameters in young *Pink1*^{B9} flies as compared with age-matched control (Figure 11). This is similar to what has been previously reported (Molina-Mateo et al., 2017). It has been described that dysbiosis is able to change locomotor activity in flies (Schretter et al., 2018a). For this reason, it was evaluated motor behavior in the different conditions of interest, at the time points that have been previously studied in this thesis work. In general, results show no differences in motor behavior in the different genotypes, regardless kanamycin treatment (Figure 11). The only consistent effect that it is possible to observe is a change in speed in kanamycin-treated control 15-16 days old flies as compared to control flies that did not receive the antibiotic (Figure 11F $F(1, 76) = 8.663$; $P=0.0043$).

Overall, these results show that no changes in locomotion are observed in

Pink1^{B9} flies as compared to control animals, in these time windows that correspond to the pre- symptomatic motor stage. Importantly, the kanamycin treatment does not greatly affect locomotion in these flies.

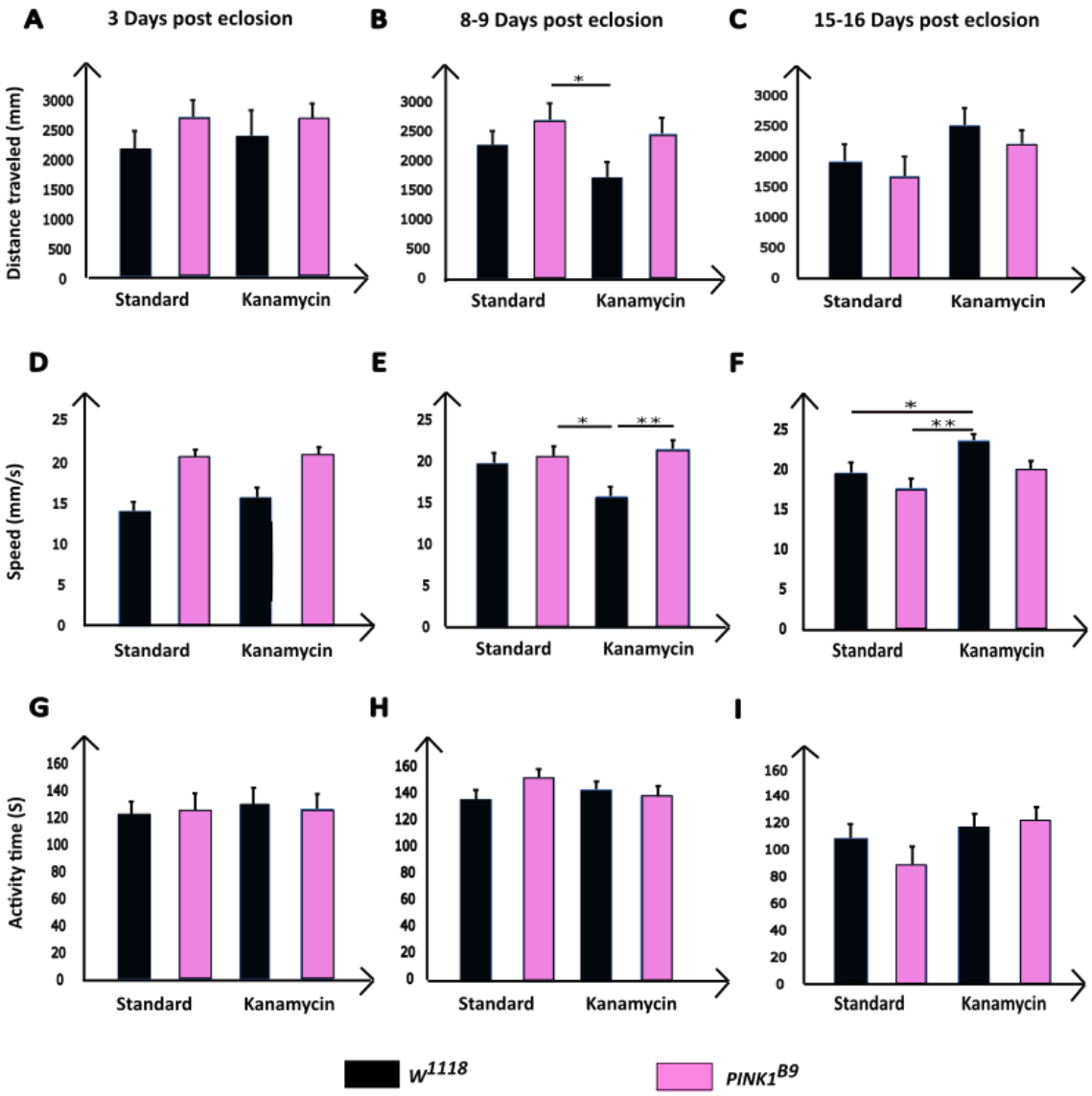

Figure 11: No differences are detected in control or mutant flies at the premotor phases of the PD model, regardless antibiotic treatment.

3; 8-9- and 15-16 days post eclosion flies, both w^{1118} and $Pink^{B9}$ mutant flies were fed with kanamycin or standard food (A-C) distance traveled, (D-F) Speed and (G-I) Activity time was recorded in (A, D, G) 3 days post eclosion, (B, E, H) 8-9 days post eclosion and (C, F, I) 15-16 days post eclosion. Innate motor behaviour of single male mutant or control flies was recorded at different time points. Video recordings were analyzed offline using the Buridan tracker software. Data are shown from n=19-22 flies per experimental group. Two-way ANOVA followed by Bonferroni post-test indicates * and ** $p<0.01$ and $p<0.05$ respectively, between experimental groups identified.

4. Characterization of motor behavioral features in kanamycin-treated flies at premotor stages of the PD *Drosophila* model.

One of the hallmarks of PD is the deficit in motor behaviour which is age-related. Then, it was decided to study parameters that reflect the execution of motor programs in old flies (29-30 days post eclosion) (Figure 12A), which is a time window at which alterations in locomotion are evident in mutant flies (Molina-Mateo et al., 2017). Results were obtained from animals in control conditions or in animals where kanamycin treatment was carried out from days 1 to 16 days post eclosion (supplementary figure1).

As it has been reported, it is observed decreased locomotor parameters in *Pink1*^{B9} mutant flies as compared to control flies in regular food (Figure 12B and 12C), evidence supports the occurrence of a motor alteration linked to this PD model.

Concerning the kanamycin treatment, the results show an increase in locomotor parameters *Distance traveled,* and *Speed* in control animals fed with the antibiotic as compared to control flies maintained in normal food (Figure 12 B and Figure 12D). Activity time does not present significant changes (Figure 12C). Also, *Pink1*^{B9} mutant flies exhibitan increase in distance traveled as compared to

what is measured in mutants not receiving the antibiotic (Figure 12B) whereas activity time and speed remain stable despiteantibiotic treatment.

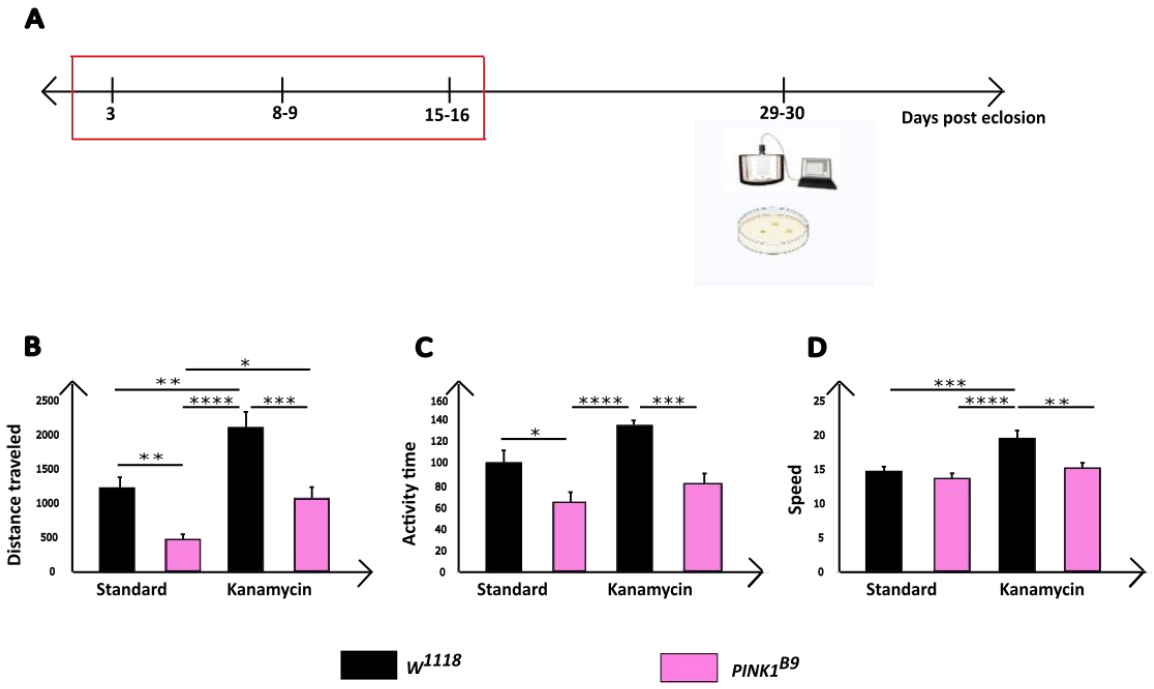

Figure 12: Kanamycin treatment in early adulthood increase locomotor activity in both control and PD model flies.

Flies were fed with standard food or standard food supplemented with Kanamycin for 16days since eclosion. After 16 days all flies were fed with standard food. Innate behavior of single flies was recorded at 29-30 days post eclosion and compared with control flies. Videos were analyzed offline using the Buridan tracker software. Data shown are: A Distance traveled, B Speed, and C Activity time. *; **; *** and **** indicates $p < 0.05$; <0.01; <0.005 and <0.0001 respectively, when comparing data from control flies and *Pink1*^{B9} mutant animals; two-way ANOVA followed by Bonferroni post-test.

5. Two weeks after stopping kanamycin treatment it is possible to find microbiota in the *Drosophila* gut

To get more information about the condition of *Drosophila* microbiota two weeks after the suspension of kanamycin treatment, we performed bacteria culture from 29-30 days old flies. Intestines were dissected from the four conditions previously mentioned (two genotypes and two treatments), in which flies were fed kanamycin-supplemented food up to day 16 post eclosion and then kept in regular food. The control condition is maintaining flies in regular food up to the moment when the experiment is carried out. Then, 29-30 days old flies were killed to dissect out the intestines.

As expected, after stopping kanamycin treatment there is a process of repopulation of the gut microbiota as results show bacterial colonies in all conditions (Figure 13).

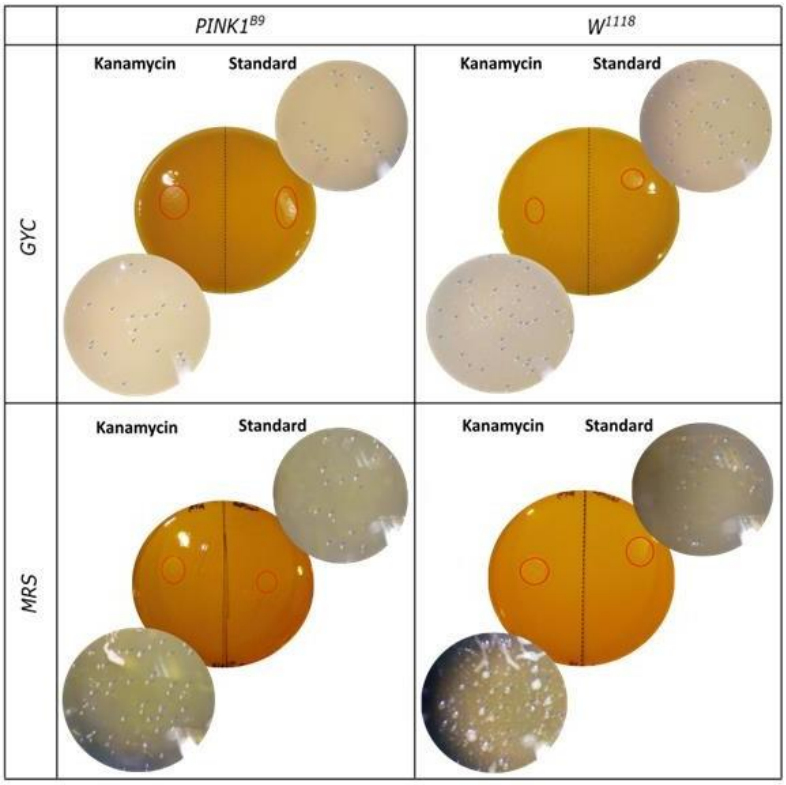

Figure 13: After stopping kanamycin treatment gut is repopulated with bacteria.

Control or *Pink1*^{B9} flies were treated or not with antibiotic (Kanamycin or standard food condition inside the box) during 15-16 days from the moment of eclosion. Mid-gut of 4 flies 29-30 days post eclosion were dissected out, disaggregated and the preparation was seeded on agar plates. Two different culture media were used for these experiments, GYC (Top panels) and MRS (Bottom panels), in both genotypes *w*^{1118} (right) and *Pink1*^{B9} (left). Each panel presents a representative experiment from an agar plate which was divided into two parts to culture the bacteria of mid-gut from flies fed with standard food (right side) or standard food with antibiotic (left side). For both sides, there is a magnification of results in each condition. After 24 hrs at 30^{o}C, it is possible to observe bacteria growing in samples from intestines of kanamycin- treated flies, suggesting repopulation of microbiota.

DISCUSSION

PD is a neurodegenerative disorder highly prevalent for which we do not understand its causes. It has been recently suggested that even though this is a brain disease, it could begin in the gut. Here we proposed to assess this idea by affecting gut microbiota and see how this manipulation affects PD phenotypes in a well-studied PD model, the *Pink1*^{B9} mutant fly.

1. Characterization of gut microbiota in young flies

1.1 General analysis of samples of gut microbiota

Considering that it is the first time that we perform a technique for DNA extraction from intestinal samples in the laboratory, the general exploratory analysis showed interesting things. First, all the rarefaction curves (Supplementary figure 2) reach a plateau suggesting that the sequencing was deep enough, and the samples are suitable for analysis. Moreover, as we expected it was possible to observe a substantial difference in the species richness between samples. This is completely normal for samples from biological systems. Second, we performed an exploratory analysis from the taxa assignation (Supplementary figure 3). This examination included all the sequences and evidenced the classical composition of *Drosophila* gut microbiota. From the taxonomic assignation, the most abundant

phyla were acidobacteriota, actinobacteriota, firmicutes, proteobacteria, and cyanobacteria (Supplementary figure 3). There are several *Drosophila* gut microbiota studies that have reported the presence of these phyla regardless of the genotype analyzed, although the main differences are the proportion of them (studies in laboratory lines and flies collected from the wild) (Erkosar & Leulier, 2014; Walters et al., 2020; Wong et al., 2013). However, it has not been previously reported cyanobacteria like an abundant phylum in the *Drosophila* gut.

Interestingly, there was a vast number of sequences without assigned taxa (Supplementary figure 3). To do the taxa assignation it was used the last version of the Silva database and until now it is the most complete database for the alignment of rRNA16S of bacteria and archaea (Quast et al., 2013). Therefore, a good number of bacteria in *Drosophila* microbiota remain uncharacterized yet. Unfortunately, it is not a common practice to report what is the percentage of the total sample that does not have a match in databases, for this reason it is difficult to know if others group of researchers have found non-identify bacteria in their samples of *Drosophila* gut.

It is important to mention that the sequences corresponding to Wolbachia were excluded from all the analysis. Wolbachia is a type of endosymbiotic bacteria that can be found in a wide range of Arthropoda including *Drosophila melanogaster* in lab strains like ours. Wolbachia is not part of the gut microbiota. However, its rRNA16S is amplificated along that detected in the other bacteria (Ilinsky, 2013; Simhadri et al., 2017). All the analysis of this thesis were

performed with the portion of sequences with assigned taxa and without the sequences of the Anaplasmataceae family because all of them corresponded to the *Wolbachia* genus.

1.2 Main differences between w^{1118} and $Pink^{B9}$ in the gut microbiota of young flies

Several scientific articles are demonstrating the effect of gut alterations over brain. Specifically, it has been shown that enteric microbiota can affect central nervous system functioning in models for neurodegenerative diseases in flies, representing an open window for the understanding and treatment of the pathophysiology of this kind of disorder. However, there are no reports regarding early-stage interventions in microbiota to evaluate changes in the progression of neurodegenerative disease symptoms. Considering this, identifying bacteria families is a key first step for the characterization of mid-gut microbiota from young flies 8-9 days old. At this age, it is possible to observe clear evidence of non-motor features before the onset of motor impairments, in what we called the pre-motor stage.

The operative taxonomic unit (OTU) for this research is the family level because the database for taxa assignation is not able to discriminate well between species when only one site of the 16S rDNA gene is used (Hyland, N. 2016).

B diversity analysis establishes two different clusters, which means that *Pink1*^{B9} and *w*^{1118} have different mid-gut microbiota composition (Figure 4A-B). Due to the fact that microbiota interacts constantly with the environment, blood, and neural system, its community is highly dynamic. It is modified by several factors, such as genes, stress, and inflammation, all present in several pathologies including PD (Adak & Khan, 2019; Vandenplas et al., 2020). Therefore, we expected both *Pink1*^{B9} and control flies presenting a different microbiota. Supporting this idea, a report showed that microbiota species diversity differed between control and *Pink1*^{B9} flies at 20 days post eclosion just in the onset of movement impairment, similar to what we observe at this early life stages (Xu et al., 2020). Our results strongly suggest that this difference starts early in the life of flies.

Concerning bacterial abundance at the phylum level, Proteobacteria leads for both genotypes, although is higher in proportion in *w*^{1118} compared to mutant flies. Proteobacteria has been reported before as the most common phyla for wild type flies (Broderick & Lemaitre, 2012). The most radical differences were regarding the abundance at the family level into the proteobacteria phyla. Acetobacteraceae leads in *Pink1*^{B9} flies, with almost 50% from the total, while its abundance does not exceed 25% in control flies (Figure 3). This family includes *Acetobacter pomorum* and *Acetobacter pasteurianus*, which are described as the

most dominant strains in fruit fly strains at laboratories worldwide. Stenotrophomonas, also proteobacteria, has been related to neurodegenerative pathways, and an increase in Stenotrophomonas is involved in the prevention of Alzheimer's disease in a *Drosophila* model for this disease (Tan et al., 2020). Interestingly, inhibition of the growth of *Acetobacter pomorum*, among others, result in the rescue of locomotive and neuronal defects of PINK1-null mutant (Xu et al., 2020). Lastly, it is frequent to observe in different genotypes or lines of *Drosophila* at the most highly taxonomic level a dominance of the most common groups like proteobacteria. Therefore the most important differences should be present at the genus or species level; this is why it is crucial to perform more experiments that could better help on characterizing microbiota at most deep levels (Wong et al., 2013). Actually, the use of probiotics or the recolonization of the gut with one single bacteria strain has been found enough to induce changes in behaviors (Gómez et al., 2019; Jia et al., 2021). Likewise, it is clear that hyperlocomotion is induced by *lactobacillus brevis* (Douglas, 2018; Schretter et al.,2018a).

Taking the differences observed in our results and previous evidence, it would be possible to suggest that modifying intestinal microbiota could be enough to change the course of the progression of PD in *Drosophila melanogaster*.

2. Chronic Kanamycin treatment is effective to generate dysbiosis

For the generation of a dysbiosis protocol, flies were exposed to a chronic Kanamycin treatment. There is no literature about eventual noxious effects of this antibiotic in invertebrate models. Due to this, it was evaluated how Kanamycin could affectthe survival of both control and *Pink1*^{B9}. Kanamycin did not have any effect on the survival of flies suggesting that it is a non-toxic antibiotic at the concentration used in this work (Figure 5B and 5C). Kanamycin can attack different types of microorganisms including gram-negative, gram-positive, and mycoplasma. Even more, it could be used to kill intestinal fly pathogens like *P. entomophila* (Jin et al., 2017). We showed that the use of kanamycin effectively eliminates bacteria from the mid-gut in both *Pink1*^{B9} and control flies(Figure 6 and Figure 7). Stopping treatment for two weeks is enough for recovering bacteria in mid-gut (Figure 13). All of this suggests that Kanamycin is a safe, non-toxic, antibiotic with the potential to induce dysbiosis in our fly PD model.

3. Behavioural characterization of *Drosophila Pink1*^{B9} mutant in premotor stages indysbiosis conditions

Impairment in the execution of motor programs is the most well-known symptom of PD. Nonetheless, the molecular mechanism associated with non-motor symptoms involved in the progression of PD remains largely unknown. In

Pink1^{B9} flies, as in humans suffering from PD, it is possible to describe two stages associated with disease progression: a pre-symptomatic, pre-motor stage, followed by the motor symptomatic stage (Molina-Mateo et al., 2017) (Figure 1). Considering this, we evaluated at different time windows the P.I., a parameter that measures olfactory responses, and centrophobism as a proxy to anxiety, two behavioral features associated with pre-motorsymptoms in PD. We also recorded activity time, speed and distance traveled, to assessthe motor performance in PD flies (Supplementary figure 1).

3.1 Anxiety-like behaviour as a hallmark of the pre-motor stage in *Pink1*$^{B9.}$

Anxiety is a pre-existent disorder in several cases of PD, including patient with autosomal-recessive early-onset Parkinsonism due to *Pink1* mutations (Ephraty et al., 2007; Ricciardi et al., 2014). Here, we described for the first time that *Pink1*^{B9} mutant flies exhibit a reduced anxiety-like behavior compared with control animals. These results arestrikingly similar to those observed in rat models for early-onset PD (Pink1−/−). Additionally, germ-free animals spend more time in the open arms in the elevated plus maze, indicating lower anxiety levels (Heijtz et al., 2011; Marquis et al., 2020). However, as flies age, there is an increase in this behavioral feature in *Pink1*^{B9} mutant flies (Figure 9), similar to impairments in centrophobism index in a α-synuclein mutant model for PD (Chen et al., 2014). It was reported in Zárate *et al* (submitted article) that serotonin levelswere reduced

in *Pink1*^{B9} mutant flies as compared to control flies, which could explain this behaviour.

Interestingly, even though flies are born with reduced centrophobism, as flies age and the symptomatic stage gets closer in time, this behavioural feature is increased. Thisis similar to what is observed in humans.

3.2 Kanamycin recovers anxiety levels in the pre-motor stage.

Antibiotic-induced dysbiosis increased the centrophobism index (Figure 10B). Some bacteria (lactobacillus and Bifidobacterium) influence the level of neurotransmitters in thebrain (Skolnick & Greig, 2019; Strandwitz, 2018). Thus, it is possible that changes in microbiome composition/levels in flies gut trigger changes in neurotransmitters that could explain this phenomenon in centrophobism. Additionally, it is known that anxiety and depressive disorders are characterized by lower abundance of short chain fatty acid (SCFA) produced by some bacteria, including proteobacteria, the most abundant phylumin *Pink1*^{B9} mutant flies (Simpson et al., 2021). Changes in the content of proteobacteria could also participate in the phenotype here described. Interestingly, differences in the abundance of this particular bacteria were found in *Pink1*^{B9} compared to control flies, supporting this idea (Figure 3). However, it is difficult to propose a better relationship between dysbiosis and the recovery of anxiety levels, because

the bacteria lost by the antibiotic remains unidentified.

These results confirm the importance of intestinal microbiota to regulate complex behaviors related to mental health, including anxiety. Future non-invasive treatments to change specific OTU could be useful, as a mean to increase or decrease some specific metabolites, thus modulating anxiety levels.

3.3 Kanamycin treatment recovers innate olfactory discrimination behaviour in thepremotor stage of the *Drosophila* PD model.

In PD patients and also in asymptomatic heterozygotes carriers of mutations in the *Pink1* gene it has been reported olfactory dysfunction, possibly indicating an underlying preclinical process (Eggers et al., 2010; Ferraris et al., 2009).

Several reports support the idea of gut, oral, and/or nasal dysbiosis in PD patients, which could have effects in both taste and smell senses (Pereira et al., 2017; Shen, 2020). On the other hand impairment in the sense of smell is determined principally by genetic factors and could be observed in different animals among them in humans, rats, and flies (Fullard et al., 2017; Molina-Mateo et al., 2017; Zhang et al., 2020). However, the studies that evaluated the nasal microbiome in PD are not conclusive (Melis et al., 2021).

The results show that two days and one week of Kanamycin treatment re-establishes the normal olfactory behavior in *Pink1^{B9} Drosophila melanogaster* PD model (Figure 8). In flies, intestinal microbiota modulates aversive olfactory responses by regulation of the octopamine pathway in the intestine. This is an aminergic neurotransmitter that is considered the physiological orthologue of noradrenaline in flies, related to attention, stress, and fight or flight responses (Fox et al., 2021). On the other hand, gut dysbiosis changes food preference in *Drosophila melanogaster*, a behavior that relies on smell (Qiao et al., 2019). This suggests that microbiota and smell are closely related. However, the exact physiological mechanism underlying this interaction remains unknown. Thus, it is possible that the imbalance in the gut microbiota produced by Kanamycin treatment caused the recovery of the normal levels of PI (Figure 8A and 8B). Also, it is possible that dysbiosis changed the internal state of flies while producing a remodeling in circuits related to the perception and learning about odorants, as previously suggested (Sayin et al., 2018). An eventual remodeling of circuits could explain why treated *Pink1^{B9}* flies respond more to the same aversive stimulus than *Pink1^{B9}* non-treated. More experiments are needed to test this hypothesis.

As fly locomotion can modify its ability to perform in the assays here described, it was possible to suggest that some of the differences observed could arise from poor locomotor performance triggered by the antibiotic-induced dysbiosis. Nonetheless, no changes in locomotion were found that could affect the interpretation of the results for non-motor parameters (Figure 11). Longer

treatment could cause antibiotic resistance or microbial adaptation that would explain no effects of antibiotic over olfactory discrimination at later ages (Figure 8C).

4. Behavioral characterization of old *Drosophila Pink1^{B9}* mutant with dysbiosis

The hallmark of PD is movement impairment as aging advances. Here, dysbiosis in the premotor stage improves the ability of old flies to move (Figure 12), increasing the distance travelled and activity time in both control and mutant animals. There are evidence about the increase in locomotor activity in germ-free vertebrate and invertebrate animals (Heijtz et al., 2011; Schretter et al., 2018b) supporting the idea that elimination of gut microbiota in a specific time window plays a crucial role in the brain, which strongly impacts on the locomotor behavior. These changes have been associated with high levels of dopamine, serotonin, and norepinephrine in the striatum, as well as changes in octopamine in invertebrate brains (Heijtz et al., 2011; Schretter et al., 2018a). Also, it has been identified the bacteria involved in the hyperlocomotion behavior in Drosophila, Lactobacillus brevis. Nonetheless, we did not perform the analysis of microbiota after repopulation phenomena. It could be crucial to establish some causality relationship between the recovery of motor capabilities and microbiota

repopulation.

CONCLUSIONS

Finally, in this thesis the hypothesis was refused and surprisingly dysbiosis prevents age-dependent PD phenotypes. This work adds new information about the premotor stage in *Drosophila melanogaster*, while it was characterized the microbiota of *Pink1*^{B9} at 8-9 days post eclosion. This new insight offers us a new temporal window to try to change the progression of different behavioral features of PD models. According to this, intervention with Kanamycin at the premotor stage was enough for the recovery of the anxiety-like and olfactory behaviors during antibiotic treatment. Surprisingly, only two weeks of treatment at the premotor stage have long-term effects: 29-30 days old flies that were treated with kanamycin during their early life increase their motor performance. All this data supports modifying gut microbiota as a non-invasive treatment for PD. Despite our efforts, the current sanitary situation abruptly interrupted this research and it was impossible to investigate more deeply the microbiota over aging and the repopulation phenomena. We hope to keep working on all these new questions in the near future to contribute to PD research.

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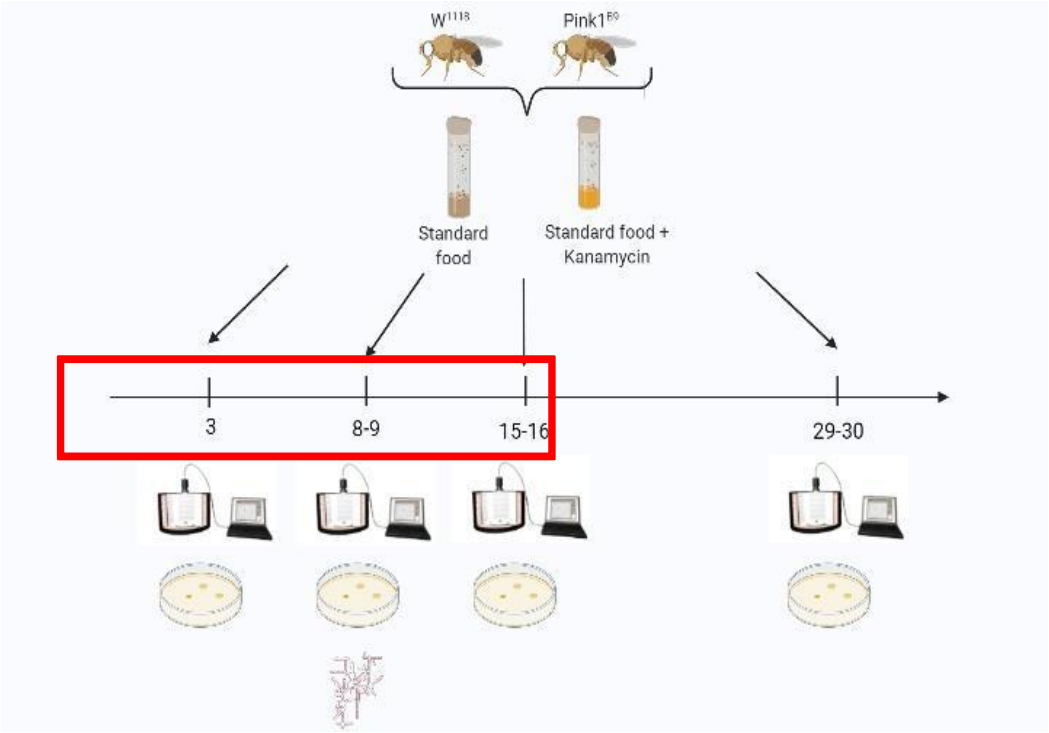
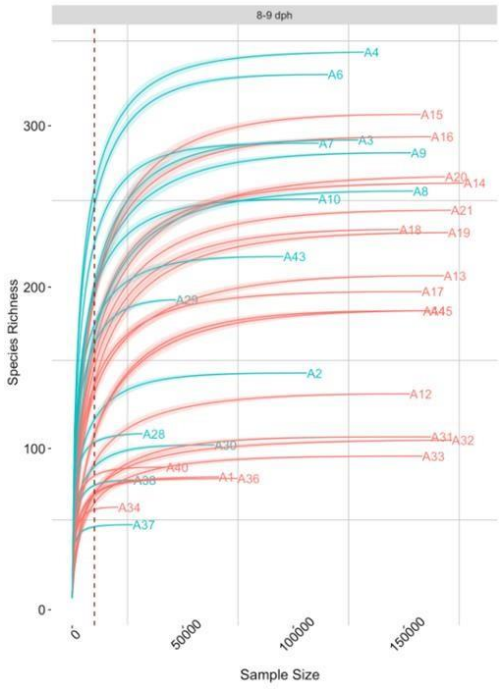
SUPPLEMENTARY FIGURES



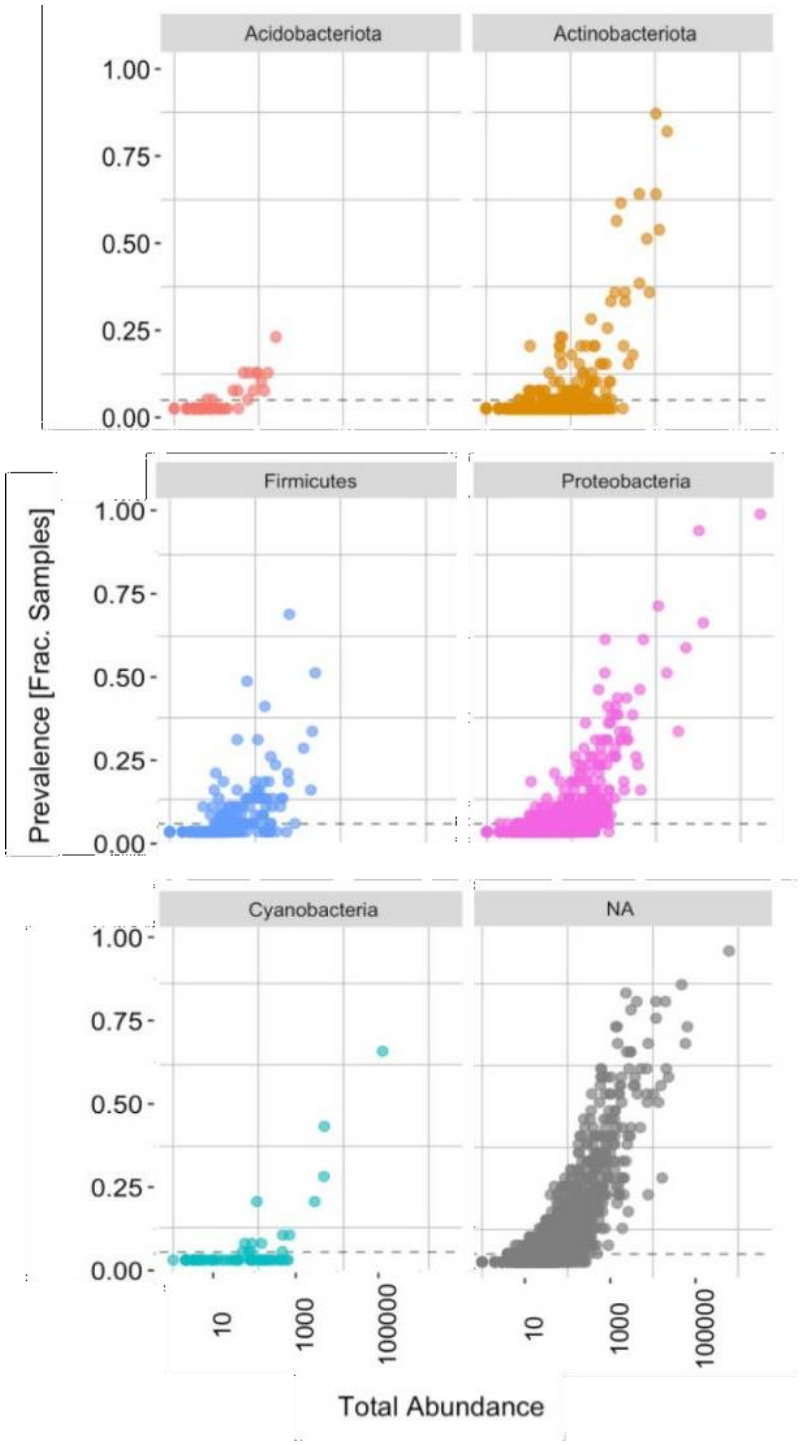
Supplementary figure 1: Experimental design to evaluate the progression of PD features in flies.

Flies were exposed to two types of diet standard food or standard food supplemented with antibiotic. The maximum extension for the antibiotic treatment was 15-16 days after that all flies were fed with standard food. Flies were recoding at different ages in premotor stage and motor stage, in all these point times also were cultivated the bacteria from mid-gut for all conditions. Flies were scarified after each experiment. Only in one point of the premotor stage were performed the microbiota analysis from 16S rRNA sequences to identify differences between *Pink1*^{B9} and control flies.



Supplementary figure 2: species richness for w1118 and Pink1^{B9} mutant fly.

The rarefaction plot shows the number of species of each sample against the size of them. Blue and red curves represent *Pink1^{B9}* and w^{1118} genotypes respectively.



Supplementary figure 3: General analysis of fly microbiota.

Pots represent the relative abundance of the most common phyla for drosophila melanogaster (*Pink1*^{B9}and w^{1118} included). NA corresponds to non assigned taxa.

NUMBER	GENOTYPE	NIPS	AGE	ng/uL
A1	W^{1118}	40	8-9 days post eclosion	2.04
A2	$Pink1^{B9}$	40	8-9 days post eclosion	–
A3	$Pink1^{B9}$	40	8-9 days post eclosion	4.62
A4	$Pink1^{B9}$	40	8-9 days post eclosion	–
A5	$Pink1^{B99}$	40	8-9 days post eclosion	2.70
A6	$Pink1^{B99}$	40	8-9 days post eclosion	3.98
A7	$Pink1^{B9}$	40	8-9 days post eclosion	3.74
A8	$PINK1^{B9}$	40	8-9 days post eclosion	7.50
A9	$Pink1^{B9}$	40	8-9 days post eclosion	3.98
A10	$Pink1^{B9}$	40	8-9 days post eclosion	4.34
A11	$Pink1^{B9}$	40	8-9 days post eclosion	2.12
A12	W^{1118}	40	8-9 days post eclosion	4.04
A13	W^{1118}	40	8-9 days post eclosion	5.28
A14	W^{1118}	40	8-9 days post eclosion	3.86
A15	W^{1118}	40	8-9 days post eclosion	2.92
A16	W^{1118}	40	8-9 days post eclosion	3.98
A17	W^{1118}	40	8-9 days post eclosion	6.28
A18	W^{1118}	40	8-9 days post eclosion	–
A19	W^{1118}	40	8-9 days post eclosion	4.24
A20	W^{1118}	40	8-9 days post eclosion	2.06
A21	W^{1118}	40	8-9 days post eclosion	7.56

A28	*Pink1*^{B9}	40	8-9 days post eclosion	7.15
A29	*Pink1*^{B9}	40	8-9 days post eclosion	0.50
A30	*Pink1*^{B9}	40	8-9 days post eclosion	–
A31	*W*^{1118}	40	8-9 days post eclosion	7.00
A32	*W*^{1118}	40	8-9 days post eclosion	8.25
A33	*W*^{1118}	40	8-9 days post eclosion	5.30
A34	*W*^{1118}	40	8-9 days post eclosion	5.80
A35	*W*^{1118}	40	8-9 days post eclosion	0,50
A36	*W*^{1118}	40	8-9 days post eclosion	1,20
A37	*Pink1*^{B9}	40	8-9 days post eclosion	3.70
A38	*PINK1*^{B9}	40	8-9 days post eclosion	4.23
A39	*Pink1*^{B9}	40	8-9 days post eclosion	2.78
A40	*W*^{1118}	40	8-9 days post eclosion	2,82
A41	*W*^{1118}	40	8-9 days post eclosion	0.40
A42	*W*^{1118}	40	8-9 days post eclosion	5.30
A43	*Pink1*^{B9}	40	8-9 days post eclosion	7.30
A44	*W*^{1118}	40	8-9 days post eclosion	8.25
A45	*W*^{1118}	40	8-9 days post eclosion	7.00

Table 1: Sample information.
The table indicates the identification of each sample, the genotype of flies control (*w*^{1118}) and Parkinson model (*Pink1*^{B9}), the number of intestines for sample, and the age of flies asdays post eclosion. "–" Correspond to non-detected DNA by Qubit.