Development of qPCR Based Method to Quantify Colonization of an Engineered Bacterium in Gut

A Master's Thesis

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Abstract:

Parkinson's Disease is the leading neurodegenerative disease in the western world, caused by degeneration of neurons in the substantia nigra. This causes a decrease of dopamine in nigrostriatal neural circuitry, leading to motor and nonmotor symptoms which can be incredibly debilitating for the patient. Current dopamine supplementation therapy does not provide a steady state neurotransmitter level and can lead to dyskinesias and dystonia's. However, *Escherichia coli Nissle* (EcN) bacteria have been shown to prove effective therapeutic treatments that can be engineered to specifically treat certain diseases, such as Phenylketonuria. EcN can be engineered to upregulate production of L-DOPA by adding an extra *HpaBC* gene. Administration of L-DOPA through ECN can maintain steady state dopamine level and thereby normalizing dopaminergic neural pathways for patients suffering from Parkinson's Disease. The goal of this study was to monitor EcN-DOPA gut colonization in mouse models of Parkinson's disease using a qPCR method. We generated a unique primer set for ECN-L-DOPA and determined its utility in measuring the colonization. Our results showed that ECN-L-DOPA primer effectively amplifies the gene and detected the engineered bacteria in the fecal sample. Further evaluation in Mitopark transgenic mouse model of Parkinson's disease, qPCR method successfully detected colonization profile of ECN-DOPA and correlated with the plasma level with plasma L-DOPA level. Collectively, our results show qPCR method is very amenable for monitoring gut colonization in microbiome-based therapy.

Introduction:

The goal of this study was to give a steadier administration of L-DOPA to Parkinson's patients through an engineered bacterial treatment and then to monitor the colonization of this bacteria in the gut. The human body has many known commensal bacteria that create the normal flora of the body. These bacteria can reside in the skin, eyes, nostrils, mouth, throat, upper respiratory tract, stomach, and intestines. In the human gut alone, there are 10^{13} to 10^{14} bacteria alone. (Martin et al, 2013) Commensal bacterium exist in a symbiotic mutualistic system with their host, humans. They aid in maintaining homeostasis in the human body by providing a first line of defense against pathogenic bacteria that enter the human body, as well as producing nutrients, breaking down harmful waste products, metabolize indigestible compounds, and is critical for the development of the CNS system (Martin et al, 2013)

The central and enteric nervous systems communicate bidirectionally by neural, endocrine, immune, and humoral means through the Gut-Brain Axis. The Gut-Brain Axis connection between the enteric nervous system and the central nervous system is made up of the autonomic nervous system (parasympathetic, sympathetic and enteric nervous system, as well as the hypothalamic-pituitary-adrenal axis. (Breit et al, 2018 et al, 2018) Enteric microbiota majorly impacts this Gut-Axis by locally interacting with enterochromaffin cells in the intestine as well as directly interacting with the central nervous system through metabolic and neuroendocrine pathways. Animal studies, probiotic and antibiotic studies, as well as infection studies have shown that the absence of microbial colonization is critical for the development and maturation

of the central nervous system and the enteric nervous system. The bacteria of the gut can interact both with the effector cells of the gut as well as directly acting on neuroendocrine and metabolic systems.

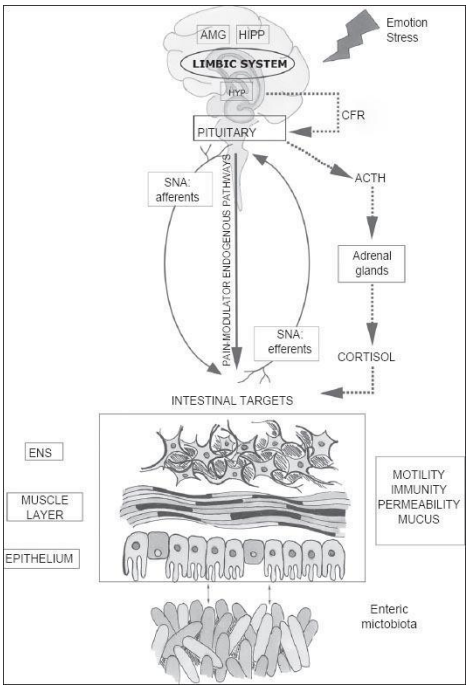

Figure 1. The Microbiome Gut-Brain Axis Structure

As seen in the figure above from the 2015 Carabotti study, the Enteric plexus lies underneath the muscularis externa (muscle) layer. On top of this muscle layer are epithelial cells that are effector cells of the nerve supply to the gut. These effector cells can be smooth muscle cells, enterochromaffin cells, epithelial cells, immune cells, and glandular cells. (Forsythe et al, 2014) The hypothalamic axis is activated in response to many external stimuli and environmental factors, which stimulates interactions between the amygdala, hippocampus, and hypothalamus. The central nervous system will communicate both through afferent and efferent autonomic (parasympathetic and sympathetic) pathways to target these effector cells and the gut microbiota. This creates the bidirectional pathway of interaction between the CNS and microbiota. (Carabotti et al, 2015)

One way that the microbiota interacts with the central nervous system is through the Vagus nerve, cranial nerve 10. This bidirectional communication via the Vagus nerve is responsible for regulating and communicating with a very broad range of visceral organ activities and functions. These activities include many vital reflex functions such as sneezing, coughing, vomiting, and swallowing. Regulatory functions include heart rate and respiratory rate, vasomotor activity, and gastrointestinal activity. The Vagus nerve contains both sensory and motor fibers, and its pathway helps to explain why it has such an extensive reach in the body. Being the largest nerve in the human body, cranial nerve X originates in the medulla where the preganglionic neurons of the efferent fibers emerge from the dorsal motor nucleus and then exits the cranium through the jugular foramen with two other nerves. In the cranium, a branch arises called the auricular branch which provides sensation to the external ear and auditory canal. In the cervical region, the three branches arise from the Vagus nerve, the pharyngeal branch, the

superior laryngeal nerve, and the recurrent laryngeal nerve. These branches provide motor stimulation to the pharynx and soft palate, innervates the cricothyroid muscle in the larynx and provides sensory innervation, and innervates the larynx (causing speech). As the nerve moves posteriorly through the neck, the Vagus will split into left and right branches which will both enter the thorax. In the thorax, cardiac plexus forms and innervates the heart to regulate heart rate and provide parasympathetic supply, as well as sensation. This vagal trunk will enter continue to enter the abdomen through the diaphragm, where it will terminate into branches that innervate the esophagus, stomach, small intestine, and large intestine. In the small and large intestines, up to the splenic flexure, the Vagus nerve will innervate the muscular and mucosal layers in the lamina propria and the muscularis externa layers, giving rise to the enteric plexus. Here, this controls smooth muscle contractions and glandular secretions. The celiac branch specifically innervates the intestines. (Leanage et al, 2019) (Breit et al, 2018 et al, 2018) The branches of the Vagus nerve located in the lumen of the gut transmit information up to the central nervous system through the pathway described above.

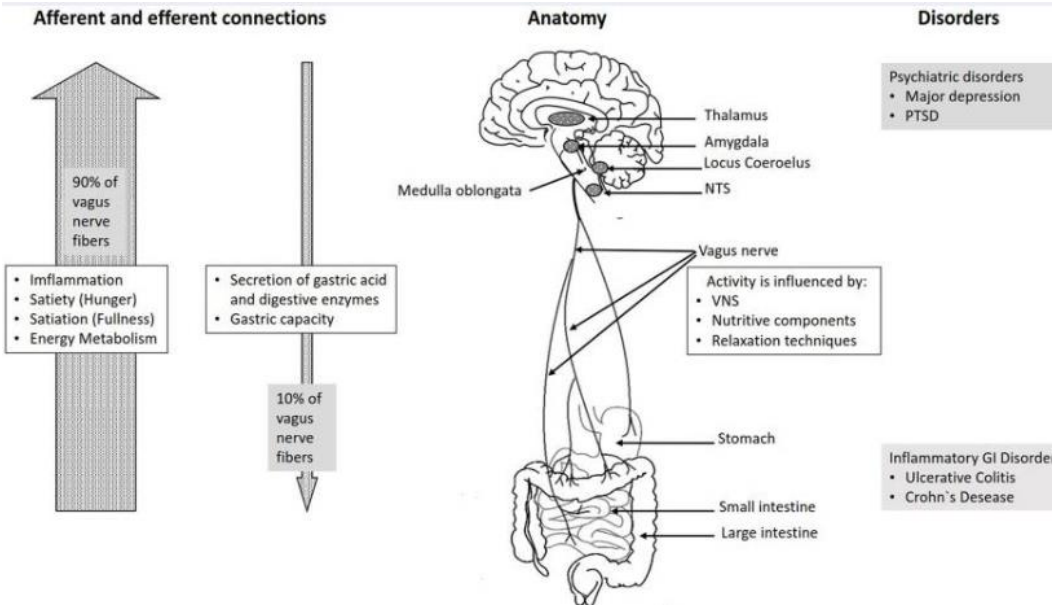

Figure 2. Overview of the Basic Anatomy and Functions of the Vagus Nerve

Looking at the chart from the 2018 Breit study in Figure 2, the Vagus nerve provides the major communication pathway between the microbiota and the brain. The many effector cell types of the Vagus nerve in the intestine are affected by the microbiota, these cells are immune cells, enteric neurons, interstitial cells of Cajal, enterochromaffin cells, smooth muscle cells, and epithelial cells. These cells are sensitive to pathogens and bacteria that surround them. (Breit et al, 2018) With the wide range of effects of the Vagus nerve listed, it is not difficult to see how gut Microbiota could affect a large range of functions in the body

Other mechanisms of interactions between the brain-gut microbiota axis can be from either the gut microbiota to the brain or from the brain to the gut microbiota. From the gut, mechanisms include production, expression, and turnover of neurotransmitters and neurotrophic factors, modulation of enteric sensory afferents, bacterial metabolites, and mucosal immune

regulation. (Carabotti et al, 2015) In the reverse direction, the microbiota can undergo alterations in mucus and biofilm production, alteration in motility, alterations in intestinal permeability, and alterations in immune function with modulation from the central nervous system.

Altered levels of expression and synthesis of neurotransmitters in both nervous systems, especially Dopamine, Serotonin, Acetylcholine, and Noradrenaline alters sensory and motor functions and decreases the amount of muscular contractile proteins present in the organism. The microbiota modulates the intestinal barrier and if disturbed, can affect the function and integrity of tight junctions which participate in the protection of said barrier. The microbiota also affects the modulation of afferent sensory nerves by inhibiting calcium-dependent potassium channels. This increases excitability and controls gut motility as well as pain perception. Bacterial metabolite short-chain fatty acid will stimulate the sympathetic nervous system, serotonin release in the mucosa, and can even influence learning and memory. (Carabotti et al, 2015)

Dysbiosis of the gut microbiota has been shown to cause a plethora of diseases and pathologies ranging anywhere from locomotor dysfunction to mood disorders to gastrointestinal disorders. The equilibrium of gut microbiota is important for producing vitamins, metabolizing nutrients, and degrading toxic products like bile salts, carcinogens, food additives, and cholesterol. (DeMaargd) The Vagus nerve itself can differentiate between harmful and non-pathogenic bacteria present in the gut, even in the absence of inflammation, and induce both anxiogenic and anxiolytic effects. This causes anti-inflammatory and immodulatory actions which also alters brain function and mood as well as immune function. (Forythe)

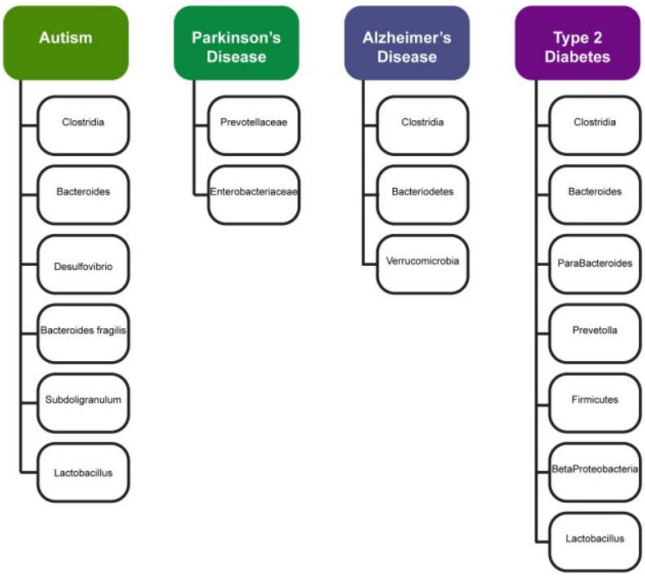

Figure 3. Dysbiosis of Commensal Bacteria in Specific Diseases

In the above chart from the 2016 Ghaisas review, it is shown that certain strains of the commensal bacterium, when in altered states, can contribute to major pathologies. Besides Parkinson's Disease, Alzheimer's Disease, and Autism, many Gastrointestinal diseases and cardiovascular diseases are directly impacted by the microbiome, the bacteria that colonize the linings of the vasculature and gastrointestinal system create a barrier, that when altered, can either cause irritation, like in the gastrointestinal system, or plaque formation, in the cardiovascular system. (Thursby et al, 2017)

Escherichia coli Nissle (EcN), is a strain that is being more sought after to treat dysbiosis of human microbiome. EcN, serogroup O6:K5:H1, was first isolated by Professor Alfred Nissle of Germany in 1917. After a young German soldier was spared from developing diarrhea, unlike the rest of his troop who developed Shigella, Nissle isolated the nonpathogenic ECN strain. This strain was used to create a probiotic drug called Mutaflor. What makes ECN so particularly useful as a probiotic, is that it lacks virulence factors such as alpha-hemolysin and P and S-fimbral adhesins. ECN does express Type 1 and F1C Fimbriae, which are important surface organelles that help mediate receptor-specific binding to the host surface, especially for the gut and urinary environments. (Klemm et al, 1994) EcN expresses Microcin H47 and Microcin M, which causes its direct antagonistic effect. (Sonnenborn et al, 2016) The strain also expresses fitness factors, adhesins, and proteases that help to potentiate its survival and make it a particularly good colonizer in human gut environment. ECN exhibits a semi-rough lipopolysaccharide phenotype, meaning it lacks O-antigen chains which would make it a higher target for host cell antibodies. Without this chain, ECN is more penetrable for antibiotics however, giving it a somewhat high serum sensitivity. (Scaldaferri et al, 2016) The Nissle strain does not produce protein toxins but expresses microcins and produces curli and cellulose independent of temperature. Microcins are involved in microbial competition, which causes antimicrobial effects of the strain by inhibiting Enterohepatic *E. Coli* and Shiga-Toxin producing *E. coli.* (Scaldaferri et al, 2016) Because of its adept ability to colonize in the human gut, it has been seen that it establishes commensalism within a few days of administration and remains for up to a few months. (Scaldaferri et al, 2016, Gladsinov). This combined with the direct and indirect antagonist properties make it a very desirable biotherapeutic agent that may be modified to produce -LDOPA. Importantly, this strain of *E. coli* only colonizes for a short period of time after administration, not long term which could unintentionally cause pathogenesis. (Isabella et al, 2018)

Probiotic effects on animals and humans with dysbiosis of the commensal microbiome have been showing increasingly positive outcomes in research studies. Tight junctions in the gut have been shown to be restored, increasing barrier integrity and mending the hypothalamic-pituitary-adrenal axis and autonomic nervous system activities. These combined causes increased protection of the intestinal barrier. (Carabotti et al, 2015) Therapy by introducing live bacteria as therapeutics has been shown to be very beneficial. After first discovering the ECN strain 1917, Dr. Nissle created one of the first widely used probiotic treatments, called Mutaflor, in the same year. This is still commonly used in Germany and Italy as a digestive support aid. Mutaflor is just one of many probiotics used. With the increasing need for treatments of bacterial infections other than antibiotics due to acquired antibiotic resistances, probiotics are being south to both prevent and treat many pathologies and symptoms of. Probiotics are routinely used to support digestive health and bacterial infections due to their antagonistic properties which inhibit the growth of pathogenic bacteria, and their production of glutamine which helps maintain mucosal barrier integrity. There are many indications for the use of probiotics such as Rotavirus diarrhea, antibiotic-associated side effects, ulcerative colitis, urinary tract infections, irritable bowel syndrome, cystic fibrosis, and food allergies. Probiotics have a strong presence in periodontal disease treatment since many of these infections are caused by bacteria in the mouth cavity, mucosal lining, and on the teeth themselves. Probiotics may be used as vaccine adjuncts to amplify the response of the immune system to the vaccine itself. Since probiotics cause an assimilation of lipids, this can be very useful in managing cholesterol level in patients predisposed or already suffering from cardiovascular issues. Colorectal cancer caused by

mutations and carcinogens can be help be prevented by using probiotics or can help the effectiveness and safety of chemotherapy treatment for it. (Hendler et al, 2018)

Parkinson's Disease, originally known as shaking palsy, was first noted by Dr. James Parkinson in 1817. Parkinson's Disease (PD) is one of the most common neurodegenerative disorders in humans, with more than ten million people living with PD worldwide, one million of which live in the US. PD is characterized by both motor and nonmotor symptoms, arising from degeneration of the striatal dopaminergic neurons in the substantia nigra pars compacta (SNpc) of the midbrain. Motor symptoms include resting tremors, postural instability bradykinesia, and muscle rigidity. Some secondary motor symptoms of PD are speech difficulties, mask-like expressions, and shuffling gait. (Downward et al, 2017) Nonmotor symptoms include depression, difficulty urinating, constipation, sleep problems, chronic pain, fatigue, and cognitive problems. (Downward et al, 2017). The motor symptoms are attributed to loss of dopaminergic function in the motor structures of the basal ganglia. The nonmotor symptoms are due to the involvement of other neurotransmitters of the cholinergic, glutaminergic, adrenergic, and serotonergic systems. (DeMaagd et al, 2015) There is evidence that may suggest that PD originates in the brain stem and ascend to higher levels of the cortical regions, starting in the dorsal motor nucleus of the vagal (Cranial Nerve X) and glossopharyngeal (Cranial nerve IX) nerves and in the anterior olfactory nucleus. Symptoms of PD typically occur first as gastrointestinal symptoms such as constipation and reduced gastric motility, the characteristic motor symptoms typically associated with the disease do not present in patients until approximately 50-80% of the neurons have been lost. (DeMaagd et al, 2015) The lack of motor symptoms seen up to this point suggest that there are compensatory mechanisms before severe degeneration takes place. Seven genes mutations are currently known the be associated with Parkinson's Disease, Alpha-synuclein, Eukaryotic Translation Initiation Factor 4 Gamma 1, Glucocerebrosidase, Leucine-rich Repeat Kinase 2, PTEN-induced Putative Kinase 1, Superoxide Dismutase 2, and Vacuolar Protein Sorting 35 Homolog. Mutations in the Alpha-Synuclein protein cause the protein to aggregate and form insoluble fibrils. These aggregates form Lewy Bodies, "intracellular cytoplasmic aggregates composed of proteins, lipids, and other materials." (DeMaagd et al, 2015). It has been seen that the misfolded alpha-synuclein aggregates are first found in the gut wall before they are found in the brain. The hypothesis is that these aggregates in the gut will interact with the gut brain access through the Vagus nerve to then cause alpha-synuclein in the brain to form the insoluble fibrils that cause the lesions of Parkinson's Disease. (Chandra et al, 2017) Parkinson's Disease itself has been linked to changes in the colonic flora composition. There are known increases in *Lactobacillus, Bifidobacterium, Verrunomicrobiaeceae, and Akkermansia,* while there are decreases in *Faecalibacterium, Coprococcus, Blautia, Prevotella, and Prevotellaceae* in patients suffering from Parkinson's Disease. (Gerhardt et al, 2018) A probiotic treatment for the disease may prove beneficial if it can help to restore the gut homeostasis as well as upregulating L-DOPA production.

Current treatments available for Parkinson's Disease only treat the symptoms of Parkinson's Disease unfortunately. The gold standard of treatment is currently Levodopa, which is a synthetic L-DOPA compound that is then converted into dopamine in the brain. In order to prevent premature conversion of Levodopa, the drugs are given with Carbidopa or Benserazide, which inhibits the conversion but is not able to cross the blood brain barrier, which allows for the conversion to take place at its desired target. There are drawbacks to the supplemental L-DOPA drugs. The drug exists in three states, "ON with dyskinesia", "ON", and "OFF". The "ON" state

is the desired state, but unfortunately oral medications can cause fluctuations of dosage. The "ON with Dyskinesia" is too much administration of L-DOPA, which produces dyskinesias and dystonia's. Dyskinesia and dystonia occur when there is overactivation of D1 and D2 receptors in dopaminergic pathways other than the target systems. The "OFF" stage produces no relief from side effects at all. (Schrock et al, 2016) For patients who have progressed further into their disease but who still are able to respond to therapy, a gel injection of Duopa (a synthetic L-DOPA drug) is given via a pump similar to an insulin pump. Dopamine agonists can also be given due to longer lasting effects as opposed Levodopa so they may be used in conjunction with Levodopa to offset the off and on effects. Dopamine agonists are not as effective on their own for treating Parkinson's Disease. Other drugs such as Monoamine Oxidase B inhibitors and Catechol O-methyltransferase inhibitors prevent the breakdown of dopamine in the nerve terminal, allowing more to be present to exert its effects on the body. Anticholinergic drugs. can be used to control the tremor associated symptoms but these drugs come with many CNS effects such as impaired memory, hallucinations, and confusion. Amantadine, an antiviral drug, is used sometimes in early treatment of Parkinson's Disease in order to moderate the extrapyramidal motor effects. (Mayo Clinic Staff et al, 2018)

Looking at the current therapies, the best options don't provide steady states of Dopamine dispersion and may be invasive, in reference to the pump. Parkinson's Disease has been recently linked to dysbiosis of gut bacteria, so supplemental EcN engineered probiotic therapy will be used to increase dopamine production by increasing L-DOPA synthesis. To monitor this delivery, there needed to be a way to measure dispersion Knowing that the bacteria themselves are successful in upregulating the synthesis of L-DOPA, the colony forming units found in the fecal samples should indicate the level of L-DOPA synthesis. However, a better quantitative measure of EcN-L-DOPA is needed. Thus, the goal of this study was to monitor EcN-Dopa colonization using a qPCR method.

Methods:

Fecal DNA from mice was isolated using the Qiagen DNeasy Powersoil kit. Before starting the isolation protocol, fecal matter was weighed into 1.5mL collection tubes. The weight recorded here was used after qPCR results were generated to normalize the colony forming units. The weight was generally between 40mg and 100mg per sample. While weighing fecal matter, contamination was eliminated by sterilizing the metal spatula with 70% alcohol between uses. After the fecal matter had been weighed out, the samples were prepared by loading each into a PowerBead tube that contained 60uL of Solution C1 and vortexed for approximately 10-20 minutes. Mouse fecal matter was not as easily broken down due to being more of a dry material, so this was always vortexed for 20 minutes to ensure complete breakdown. The canine fecal matter was easily broken down, so it was vortexed for 10 minutes, unless there were over twelve samples, then the samples were vortexed for 15 minutes. The cells from the supernatant were then lysed by adding 250uL of Solution C2, vortexing, and then incubating at four degrees Celsius for five minutes. The inhibitor was removed from the cells in the supernatant by adding 200 uL of Solution C3, vortexing, and incubating at four degrees Celsius for five minutes again. In order to bind the DNA to the MB Spin Column, 1200uL of Solution C4 was added to the supernatant, and then this sample was loaded onto the MB Spin Column. The column and samples were washed using Solution C5, which is an ethanol solution. A drying step was performed by centrifuging the samples a second time after washing. Finally, the samples were eluted from the Column using Solution C6 and then the DNA in this final sample was quantified

for concentration (ng/uL) using a NanoDrop Microvolume Spectrophotometer. This quantification was done both to ensure that the DNA isolation was successful and contained some level of DNA, and that the sample had adequate purity.

In order to quantify the nucleic acids in the fecal DNA samples, quantitative real time PCR, was performed on each sample using dye-based fluorescent labeling. This allows for the dsDNA binding of the dye to the samples in order to signify the amount of replicated DNA in real time. SYBER green (Applied Biosystems SYBR Safe) was the fluorescent dye used in the master mix for each sample and negative control. Three primer sets were used, N2 (B-3F) and N2 (B-3R) primers were used first on the mice DNA for all AA-LDOPA-17-19 studies in order to target and detect the plasmid containing the extra *HpaBC* gene. Then another set of primers HpaBC-gBlock FWD and REV Set 2 and HpaBC-gBlock FWD and REV Set 4 were repeated on the data for the mice studies. These two sets of primers, Set 2 and Set 4, specifically targeted the *HpaBC* gene itself rather than the plasmid. Firstly, efficiency of the qPCR method had to be determined by running the desired Master Mix on a set of serial dilutions. qPCR was started by determining the Master Mix preparation and standards. Three Master Mix sets that all targeted the same standard, JK-STD 1 and 2, were tested that had varying primer concentrations. The first Master Mix, Master Mix 1, contained 10uL of SYBER Green (Applied Biosystems), 2uL of N2 Primer (1uL of Forward and 1uL of Reverse), and 6uL of Molecular Grade RNAase free Water per sample (11.1% primer). Master Mix 2 contained 10uL of SYBER Green dye, 0.2uL of N2 primer (0.1uL of Forward and 0.1uL of Reverse), and 7.8uL of Molecular Grade RNAase free Water per sample (1.11% Primer). Master Mix 3 contained 10uL of SYBER Green dye, 0.5uL of N2 Primer (0.25uL of Forward and 0.25uL of Reverse), and 7uL of Molecular Grade RNAase free Water per sample (2.78% of primer). 8 standard preparation serial dilutions were prepared using the GBlock Gene fragments in a one to nine ratio with Molecular Grade water to test the three Master mix Samples. Each standard was run in triplicate.

After determining that the 1.11% primer ratio (Master mix 2) was the best for reducing background primer dimerization in the PCR tests, Fecal samples from AA-LDOPA-17, AA-LDOPA-19, and AA-LDOPA-22 were run in triplicate using this same master mix formula. DNA samples isolated from fecal matter were then run on a 96 well plate (Applied Biosystems), 2uL of the DNA sample was added to 18uL of Master Mix (per sample). This N2 Primer targeted the plasmid containing the extra *HpaBC* gene. The qPCR was run in three stages, a Hold Stage, a PCR Stage, and a Melt Curve Stage. The Hold stage ran in two steps, in step one the samples were heated at 1.6 °C/s and then held at 50.0 °C for 2 minutes. In step two, the samples were heated again at 1.6 °C/s and then held at 95.0 °C for five minutes. The PCR stage was run in two stages. In stage one, the samples were held at 95.0 °C for ten seconds. Then in stage two, the samples were cooled at a rate of 1.6 °C/s and then held at 60 °C for one minute. The Melt Curve Stage had three steps. In the first step, the samples were heated at a rate of 1.6 °C/s and then held at 95.0 °C for 15 seconds. In step two, the samples were cooled to 60.0 °C at a rate of 1.6 °C/s and then held for one minute. In the third dissociation step, the samples were heated at a rate of 0.1 °C/s until the temperature reached 95.0 °C, and then held for fifteen seconds. This process was repeated for 40 cycles. A standard curve was generated for genes coding for the desired target by creating 10-fold serial dilutions. After running PCR, each standard curve was analyzed for the R^2 value and then the melt plot curve was assessed for purity. The R^2 had a desired value of over 0.95 (preferably around 0.99). The amplification plot was assessed for purity by looking for the number of peaks. One peak for all samples showed that they all had the same melting

point. After running samples with the N2 primer, the HpaBC-gBlock Set 2 and Set 4 primers were used to select for the *HpaBC* gene itself. It was determined that the same Master Mix formula of 1.11% primers was the most productive formula. The quantity values of the samples themselves were normalized to CFU per gram of fecal matter using the weights of each fecal sample. These new normalized quantity per gram of fecal matter values were compared in order to assess colonization and then used Prism software to create visual representations both in CFU/g and in logarithmic forms.

Results:

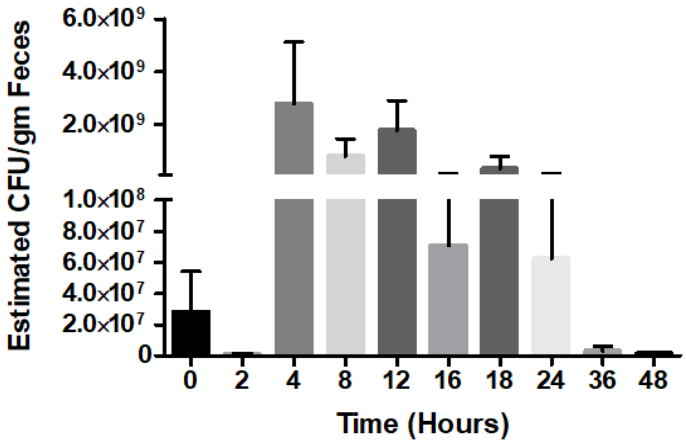

Figure 4. AA-LDOPA-19 Fecal Colonization Profile of EcN2-LDOPA in Mice

In figure 4, mice were administered EcN by gavage and their fecal samples were collected on hours 0, 2, 4, 8, 12, 16, 18, 24, 36, and 48. DNA was isolated from the fecal samples and used for real time-qPCR to produce the colonization profile seen above. The colonization shows to be increased after administration, staying mostly study until hour 36 after administration where it drops off.

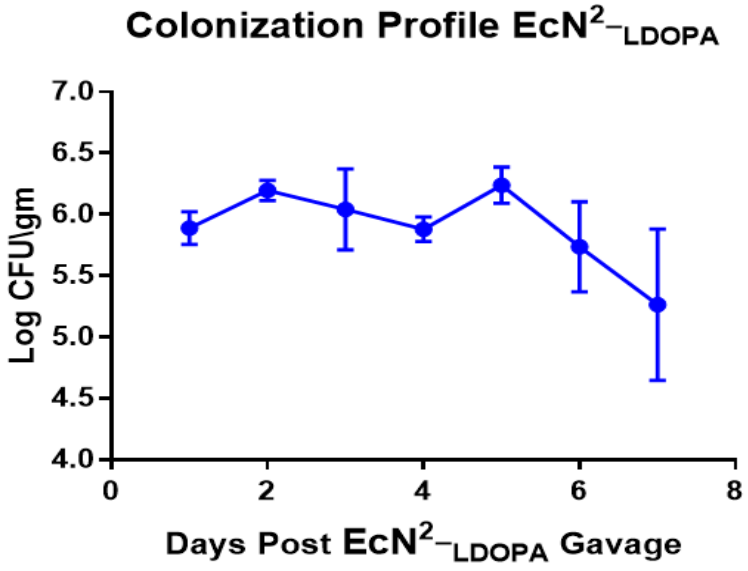

Figure 5. AA-LDOPA-22 Fecal Colonization Profile of EcN2-L-DOPA in Mice

Figure 5 shows the gut colonization profile of a mouse given an administration of EcN-LDOPA via gavage. The mice were given one administration on day zero, then fecal samples were collected every day for seven days after. The colonization profile shows that there was a steady colonization level that lasted for approximately six days, where after this the bacteria were no longer colonized and were excreted.

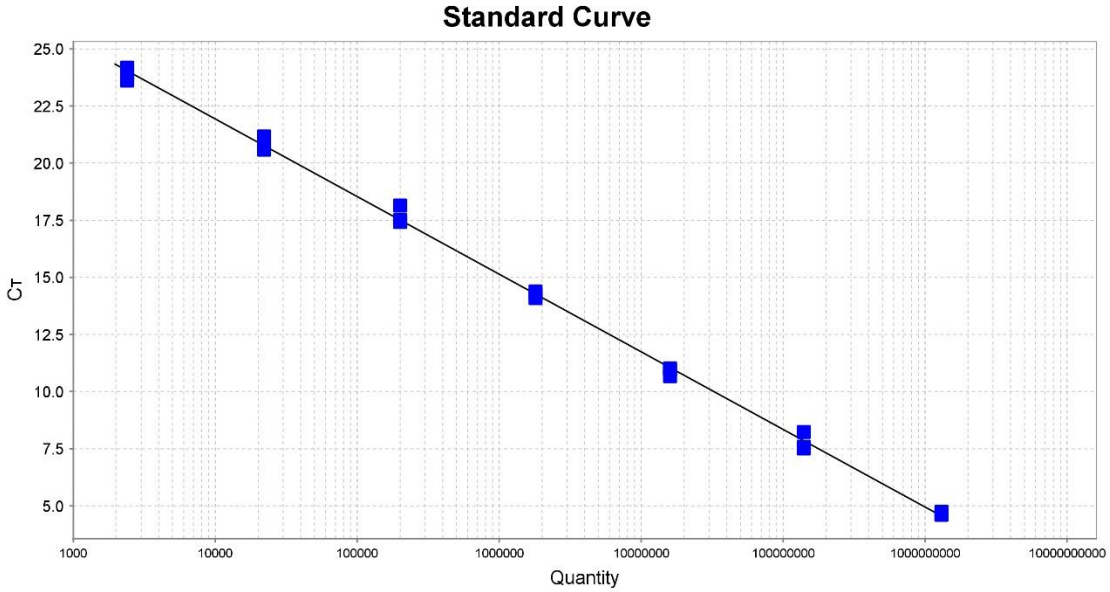

Figure 6. Standard Curve For AA-LDOPA-19 Mice Study using Set 4 Primers

Figure 6 above shows the standard curve with an R^2 of 0.99. Standard curves were run with each qPCR test. This shows that the standard curve worked; and that the results of the PCR

will be consistent and accurate. This serves as a control for qPCR and checks for the efficiency of your primers. By running a standard curve as a control, this tests the primers for efficiency as well as background to rule out false results.

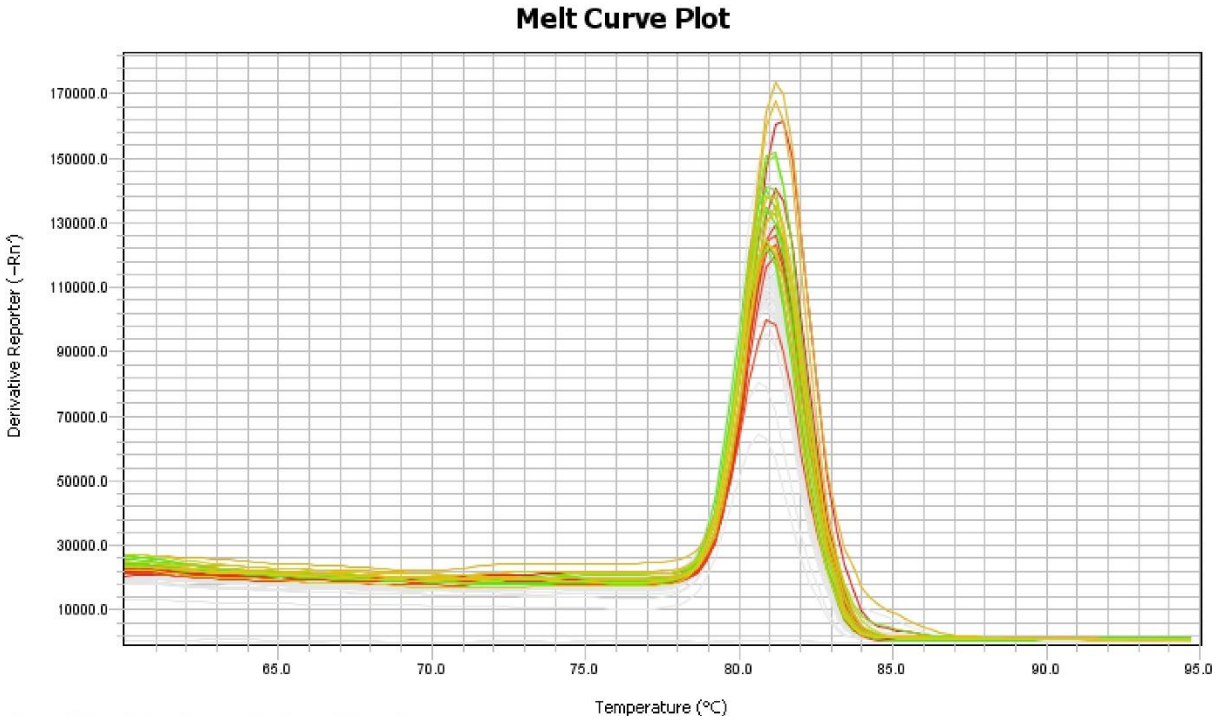

Figure 7. Melt Plot Curve for AA-LDOPA-19 Using Set 4 Primers

Figure 7 shows the Melt Curve Plot for Days one through three of the AA-LDOPA-19 mice study. A melt curve plot was created for every qPCR set as a control in order to determine purity and whether a single product was produced. Since intercalating dyes, SYBR green, used for PCR tests can bind with any dsDNA product, they may not be bound directly to the target sequence. Melting temperatures are specific for a given product and when only one peak is observed like seen above, this represents a single, pure amplicon.

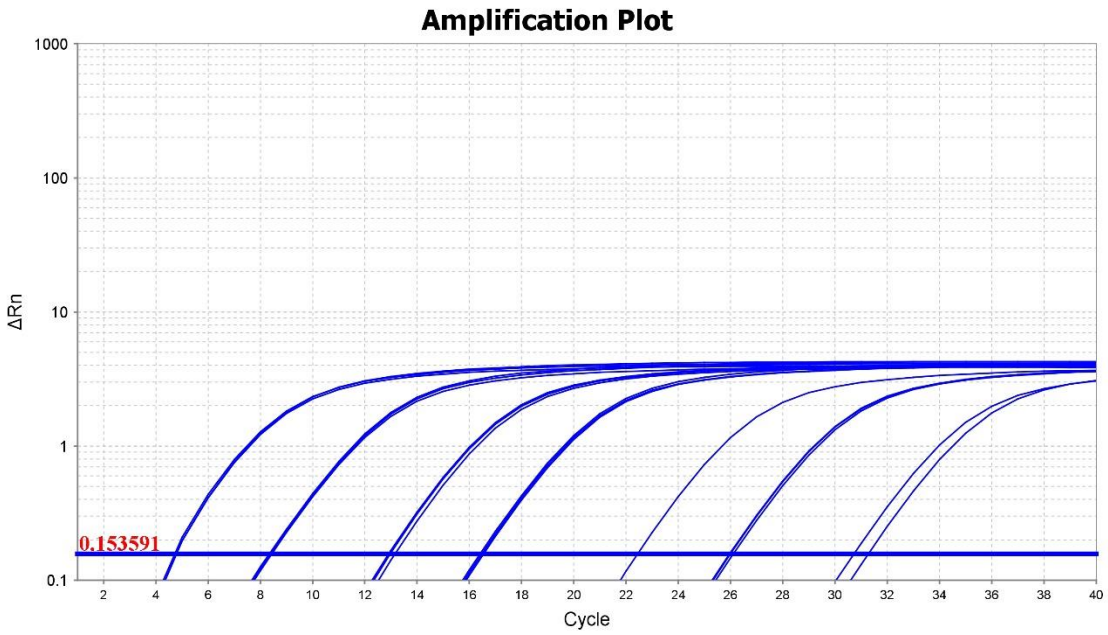

Figure 8. Amplification Plot for AA-LDOPA-19 using Set 4 Primers

The Threshold line appears at 0.153591, which is the Rn. This Rn value is the reporter signal normalized to the fluorescence signal (SYBR green), minus the baseline. This ΔRn is plotted above against the cycle number. The positive amplification signals indicate that there is indeed presence of the desired target, in this case the *HpaBC* gene.

Discussion:

Research studies such as the 2018 Isabella study concerning EcN and Phenylketonuria, the genome editing study performed by Wei to produce L-DOPA, and the Munoz study producing L-DOPA from glucose, have already proven that EcN is a productive therapeutic vector. The Wei study and Munoz also showed that inserting an additional *HpaBC* gene into the EcN organism causes increased L-DOPA production both in ex vivo and in vivo studies after administration. (Munoz et al, 2011) (Wei et al, 2016) For the purpose of this study, it can be assumed that if the bacteria are present in the model organism, then there is an upregulation of the L-DOPA being produced in said organism as well. Using this theory, a method was needed to monitor the presence of EcN in the gut. One way to do this is by real-time qPCR using DNA from fecal samples to assess the amplification of certain targeted DNA sequences. If the DNA is present and then stays at a stable level through the period of administration without decreasing, this would show that it is staying colonized in the gut. Looking at Figures 6, 7, and 8, which were examples of the controls run for every real time- qPCR run, it is proven that the samples, primers, and technique were all successful. Standard curves, like figure 6, were run to analyze the efficiency of the primers, which are critical for accurate qPCR. Most standards gave R^2 values of 0.98-0.99. Melt plot curves, like in figure 7, were used to measure purity. All the samples should have a single melting point due to having the same sequence from the *HpaB* gene.

Amplification plots, such as that in figure 8, were used to make sure that the qPCR was successful without having background noise from impurities or from primer dimerization.

Since all three curves using the plasmid selecting primer N2 showed strong amplification and the controls, melt plot curve and standard curve, were appropriate for the original PCR process, it was determined that the technique, primers, and DNA targets were shown to be successful. Since all three curves showed appropriate curves, the 1.11% primer amount was chosen in order to prevent primer dimerization and background noise in the future. After successfully targeting the plasmid, it was desired to target specifically for the *HpaBC* gene itself. By doing so, it was possible to select for the *HpaB* (Set 4 primers) gene or *HpaC* (Set 2 primers) protein. Since the gene itself shows an increase in L-DOPA production, *HpaB* (Set 4) was then chosen to target due to its oxidation properties that allow for the conversion of L-DOPA from glucose. (Munoz et al, 2011) (Wei) (Grazdanov et al, 2004) Starting in mice, EcN engineered with the extra gene were administered via oral gavage. Seen in AA-LDOPA-19, after administration, the level of EcN remains relatively constant over the period of 24 hours. This would prove that providing the EcN give a steady administration of L-DOPA throughout the day, this would ease the symptoms of dyskinesia and dystonia in the three phases of treatment using L-DOPA. The EcN L-DOPA treatment was given first to MitoPark mice in order to assess gut colonization. In figure 5, AA-LDOPA-19 showed the gut colonization of gut bacteria in mice post oral gave of EcN. The DNA isolated from fecal samples showed an average administration state of L-DOPA for the 24 hours post administration. This proves the hypothesis that causing an upregulation of L-DOPA from an administered bacterium will cause a steadier administration than the current Levodopa treatment. Then in AA-LDOPA-22, mice were given the treatment and colonization was measured over the course of several day through fecal samples. Colonization in figure 5 showed until day 6 post oral gavage of EcN. Then there was a lack of colonization after day 6, which is preferential because long term colonization has the potential to cause dysbiosis with commensal bacteria. Showing that the EcN bacteria can maintain a steady gut concentration throughout treatment while still being able to be washed out after proves that this would be an effective way to provide an L-DOPA supplement that can give a steadier dose throughout the day as opposed to current Parkinson's Disease treatment methods. In conclusion, we are successfully establishing a q-PCR based method for monitoring gut colonization of a genetically modified microbiome therapy for Parkinson's disease.

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