The Pharmabiotic for Phenylketonuria: Development of a Novel Therapeutic

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THE PHARMABIOTIC FOR PHENYLKETONURIA: DEVELOPMENT OF A NOVEL THERAPEUTIC

By

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Thesis Summary

Phenylalanine hydroxylase deficiency is a rare genetic disorder in which patients are unable to metabolize the amino acid phenylalanine. Phenylalanine accumulates in the body as a consequence of the enzymatic deficiency. If the patient’s levels of phenylalanine are too high, there are many negative consequences. Pediatric patients may even experience mental abnormalities and deficiencies as a consequence of phenylalanine hydroxylase deficiency.

A pharmabiotic prototype has been created to treat phenylalanine hydroxylase deficiency in which the human enzyme phenylalanine hydroxylase is expressed by a safe, nonpathogenic bacteria. The modified bacteria will be formulated into a yogurt or capsule for oral administration. The bacteria will then express the enzyme in the body and replace the deficient phenylalanine hydroxylase enzyme, preventing the negative consequences of phenylalanine hydroxylase deficiency. This technology is patent pending.
Abstract

Phenylketonuria, now known as phenylalanine hydroxylase (PAH) deficiency, is a genetic disorder of metabolism affecting approximately one in every 15,000 infants born in the United States. Patients have nonfunctional PAH enzyme secondary to one or more genetic mutations. The enzyme deficit results in destructive supraphysiologic blood phenylalanine levels upon consumption of the essential dietary amino acid phenylalanine. Current standards of care mitigate signs and symptoms of the disorder, but do not approach a cure. The methods for creating a prototype pharmabiotic as an innovative treatment strategy for PAH deficiency are described herein.

DNA molecular cloning techniques were utilized to engineer a novel plasmid termed LiLi5, which was subsequently transformed into *Lactobacillus helveticus*. A cDNA for human PAH was amplified and inserted into a gram-neutral shuttle vector to create LiLi5, the bacterial plasmid which induces the expression of human phenylalanine hydroxylase. The LiLi5 plasmid was confirmed by restriction enzyme digest and sequencing of the cDNA insert. Successful transformation of LiLi5 into *Lactobacillus helveticus* generated a novel strain of *Lactobacillus*, which was christened “HELin”. PCR-based detection confirmed the presence of the human PAH cDNA in HELin.

Phenylalanine hydroxylase deficiency is a debilitating disorder causing somatic, psychosocial, and financial distress for patients and their families. Current treatment strategies do not adequately address the decreased quality of life experienced by the associated patient population. *Lactobacillus helveticus* is a GRAS gram-positive bacterial species commonly formulated in commercially marketed probiotics. The novel strain of *Lactobacillus* expressing human phenylalanine hydroxylase that has been named ‘HELin’ is proposed to be formulated
into a pharmabiotic for testing as a new treatment for phenylalanine hydroxylase deficiency. Patent pending.

**Introduction**

**Phenylalanine Hydroxylase Deficiency**

Phenylketonuria, now known as phenylalanine hydroxylase deficiency, is the textbook example of an incurable, lifelong metabolic disorder. Phenylalanine hydroxylase deficiency is primarily managed by strict dietary avoidance of phenylalanine. However, the psychosocial, physical, and financial ramifications of the disorder necessitate that treatments extending beyond the current standards of care be explored. Concomitantly, the human microbiome is one of the most rapidly advancing fields of research today. As academic and industrial research into the microbiome continues to produce various biome-altering formulations, it is inevitable that numerous applications will be discovered for such products. Here, the argument is made that it is plausible to both create and market a pharmabiotic for the management of phenylalanine hydroxylase deficiency.

Phenylalanine hydroxylase deficiency is an inborn genetic error of metabolism. An intrinsically hepatic enzyme, phenylalanine hydroxylase is responsible for the production of tyrosine from the essential dietary amino acid phenylalanine. This process reduces plasma levels of phenylalanine. In cases of phenylketonuria, the enzyme is either nonfunctional or partially functional, secondary to a mutation in the PAH gene. Deficient expression of phenylalanine hydroxylase results in
supraphysiologic plasma levels of phenylalanine upon consumption of foods containing phenylalanine.

Image 1 Phenylalanine hydroxylase converts the amino acid phenylalanine to tyrosine.

Phenylalanine is present in dietary sources of protein such as fish, meat, nuts and eggs. Though autosomal recessive, phenylalanine hydroxylase deficiency affects 1 of every 15,000 infants born in the United States. Diagnosis is made primarily from plasma screenings of newborns. Such screenings were made mandatory in the United States in the 1960’s.

Hyperphenylalaninemia is diagnosed when untreated blood levels of phenylalanine are greater than the population norm of 60 μmol/liter, but less than the 1.2 mmol/liter diagnostic of classical phenylketonuria. Untreated classical phenylketonuria is associated with the most severe manifestations of the metabolic disorder.¹ ²

Hyperphenylalaninemia causes significant clinical effects, including mental and psychosocial developmental delays. Psychotic symptoms, aggression, and autistic-type behaviors are reported in untreated patients. Depression and anxiety are frequently reported, even among patients who have maintained treatment for years. Lack of social awareness is also common among both treated and untreated patients. Several mechanisms for the psychiatric and neurotoxic effects of hyperphenylalaninemia have been suggested, including the induction of myelin abnormalities, the physical obstruction of amino acid transporters throughout the blood brain barrier by phenylalanine, and disturbances in neurotransmitter pathways. These biological mechanisms result in variable psychological effects, usually manifested in a direct positive, linear relationship to blood phenylalanine levels. It is also hypothesized that the stress of
maintaining a strict phenylalanine-limited diet for the entirety of the patient’s lifetime may contribute to the noted psychological disturbances.\textsuperscript{3}

Hyperphenylalaninemia causes physical as well as psychological consequence. Bone mineral density is negatively affected in patients with phenylalanine hydroxylase deficiency.\textsuperscript{4} Concerns have been also expressed for the increased incidence of overweight noted among adolescent patients with PAH deficiency.\textsuperscript{5} Interestingly, muscle mass seems to be properly maintained among patients with appropriate intake. Anecdotal reports of spastic paraplegia in unmanaged patients have been recorded. Of less clinical significance, but still of note, patients with untreated phenylalanine hydroxylase deficiency are described as having a strong “distinctive musty odor”.\textsuperscript{6}

The 2014 American College of Medical Genetics clinical practice guidelines emphasize that once phenylalanine hydroxylase deficiency has been diagnosed, it is imperative for treatment to begin immediately, with an attempt to reach goal blood phenylalanine levels within two weeks of life. Disappointingly, the backbone of clinical treatment for phenylalanine hydroxylase deficiency remains dietary restriction of phenylalanine.\textsuperscript{7} For most patients, simply choosing to avoid foods rich in phenylalanine is not enough to prevent the consequences of hyperphenylalaninemia and may instead induce the negative physical consequences of inadequate protein consumption. This first tier of treatment alone may not be appropriate in such cases. Instead, medical formulas that contain sufficient levels of all other dietary amino acids, but restricted phenylalanine, must be utilized as a primary food source, or highly relied upon secondary to a strict diet, to support growth. It is acknowledged that medical foods improve the variety of dietary options for patients with phenylalanine hydroxylase deficiency, but at an increased cost. Certain medical foods are often not covered by insurance and therefore increase
the financial burden of PAH deficiency. Patients have also described the medical foods as having
the distinct taste of bile, which, while highly unpleasant to the layperson, is imagined to be
nearly unbearable in the pregnant and pediatric populations. The social and emotional
consequences of having a diet restricted to medical formulas alone must also not be overlooked.

Certain mutations in the gene encoding phenylalanine hydroxylase yield an enzymatic
deficiency that is termed tetrahydrobiopterin-responsive phenylalanine hydroxylase deficiency.
In such cases, the administration of certain pterin cofactors will increase the metabolism of
phenylalanine to tyrosine by phenylalanine hydroxylase. In 2007, Kuvan (sapropterin
dihydrochloride) tablets and oral solution were approved by the FDA for use in the United
States. Kuvan is indicated to reduce blood phenylalanine levels in conjunction with a
phenylalanine restricted diet. However, studies have shown that the medication has no effect in
patients who are non-responders. In June of 2018, the price of Kuvan was listed as $48.00 per
tablet in non-contract and non-340B pricing by McKesson, a third-party distributor of the
product. Investigations into large neutral amino acid (LNAA) supplementation as adjacent or
alternative therapy have been undertaken, however, strict treatment guidelines have not been
established and results in the scientific literature are underwhelming. LNAAs are treated as
medical foods in the United States and are not recommended in younger patients due to the lack
of evidence for safety.

The management of phenylalanine hydroxylase deficiency poses a challenge not only for
healthcare providers, but also for the families of those affected. A 2016 cross-sectional study
performed in the United Kingdom reported that a median of 19 hours per week was spent by
caretakers in activities related to the management of the disorder. According to the National
PKU Alliance of the United States, treatment of phenylalanine hydroxylase deficiency costs
approximately $15,000 per year. Third party payer support is payer-specific and variable. The alliance also states that inpatient care for a patient who has sustained neurological damage secondary to PAH deficiency may cost upwards of $200,000 per year. It can be inferred that the burden of this cost would fall upon the US healthcare system.

The temporal, emotional and financial burden of phenylalanine hydroxylase deficiency demand that a more effective or novel treatment methodology be developed to improve the quality of life for patients and their families. The current clinical practice guidelines emphasize that “any interventions, including medications, or combination of therapies that help to achieve that goal [of lowered blood phenylalanine levels] in an individual, without other negative consequences, should be considered appropriate therapy”. Leveraging the human microbiome could provide a breakthrough treatment approach to the management of phenylalanine hydroxylase deficiency.

**The Microbiome**

The National Institute of Health launched the first phase of the Human Microbiome Project (HMP1) in 2008 with the goal of characterizing the cohabitants of the human body. Not only did the project initiators attempt to create an accessible list of microorganisms acknowledged as “normal” through 16S RNA sequencing of samples from hundreds of human donors, but also to generate a foundational understanding of the metabolic interplays resulting from a human-contained ecosystem. HMP1 was birthed conceptually from the confounding deficit in human protein-generating genes sequenced by the preceding Human Genome Project. It was concluded that the portion of protein-producing genetic information sequenced during HMP1 which was determined to be inhuman did not simply materialize and was likely the result of the inhabitants of the human microbiome. Thus, intensive investigation into the metabolic
consequences of the human microbiome began. HMP1 sparked a nation-wide interest in the human microbiome, fueled by the intrinsic and often desperate desire of the scientific community to understand, prevent, and treat human disease.

Joshua Lederberg, a molecular biologist, coined the term microbiome “to signify the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space”. The community that Lederberg defined is not only comprised of bacterial cells, but “bacteria, bacteriophage, fungi, protozoa and viruses”. It is acknowledged that the number of microbial cells outnumbers those of human cells by a factor of at least 10. In 2009, only one year after the launch of the first phase of the Human Microbiome Project, a marker paper published by the HMP Working Group acknowledged investigations into the implication of the microbiome in the pathophysiology of psoriasis, pediatric febrile illness, obesity, bacterial vaginosis, and cancers of the gastrointestinal tract. By 2011, the list had expanded to include preliminary investigation into the role of the microbiome in Crohn’s disease, ulcerative colitis, pediatric inflammatory bowel syndrome, neonatal necrotizing enterocolitis, and gastroesophageal reflux disease. In 2013, Phase 2 of the Human Microbiome Project began, with intense focus on the role of the microbiome in human disease.

The behavior of the human microbiome is governed by many of the same principles as large-scale ecology. Each person’s individual microbiome may be considered a complete and separate ecosystem. The development of the human microbiome begins immediately after birth, when a neonate is colonized with pioneer bacterial colonies from the mother. These vertically transferred pioneer colonies are most frequently documented as Lactobacilli. As the neonate ages, the microbial ecosystem forms, with adult-like colonization theorized to be completed near age three. As a general principal, a diverse microbial population
is considered the most physiologically beneficial to the host, as it potentiates species resilience, which is known to promote human homeostasis. However, certain environmental factors can induce extinction or species imbalance. Dietary changes, consumption of alcohol, natural aging, the use of antimicrobial agents and pathophysiology are well known to cause species variation in the microbiome.

Aside from natural processes, industrialized products have been developed and introduced to the market with claims of altering the microbiome for improved wellbeing. Probiotics are marketed as formulations of live bacteria that are administered to the patient. Prebiotics are substances that may or may not be digested which yield a favorable environment for microbes of choice. Synbiotics are commonly accepted as combinations of the two aforementioned products. As the number of such quasi-pharmaceutical products grows, so does the clinical interest and scrutiny of such treatments. Clinical reviews have acknowledged that probiotics certainly play a role in treating disorders of the gastrointestinal tract and are useful in the maintenance of general wellbeing.\textsuperscript{18} Ongoing research is validating the tentative use of microbiome-altering products for metabolic disorders and treatment of recurrent \textit{Clostridium difficile} infections.\textsuperscript{19} Recent research even hints at the possibility of the psychological benefits of probiotics, exploiting the gut-brain axis.\textsuperscript{20}

Extending beyond probiotics, prebiotics, and synbiotics is the concept of the pharmabiotic. Pharmabiotics have been defined as “bacterial cells of human origin, or their products, with a proven pharmacological role in health or disease”.\textsuperscript{21} It can be argued that genetically modified organisms isolated from the microbiota would be classified as pharmabiotics, especially in the case of a genetically modified organism producing a physiologically beneficial protein \textit{in vivo}. Recent research has explored the potential for
application of pharmabiotic treatment in cases of hyperammonemia, even going so far as to claim that the pharmabiotic approach to this disease state is ideal. If properly developed, the therapeutic applications secondary to synthetic alterations in the microbiome are limited only by the threshold of imagination. Here, in contrast to hyperammonemia, we discuss the development of a pharmabiotic for the treatment of phenylalanine hydroxylase deficiency.

The model organism for creation of a pharmabiotic should exhibit several essential characteristics. Most obviously, the organism should be non-pathogenic, clearly taxonomically identifiable, and commercially available. The organism would ideally be amenable to genetic manipulation, but retain genetic stability after insertion of a plasmid or an induced chromosomal integration event. Subsequent generational identically, the epitome of genetic stability, would be desired. In instances when a plasmid is inserted into a bacterium, it is desirable for the antibiotic resistance gene used for laboratory selection processes to be excised before in-vivo administration, especially if the resistance gene were transferable to other organisms, to avoid potential generation of a multi-drug resistant organism. There may be a place in therapy for a pharmabiotic retaining antibiotic resistance, but this concept will be further explored in the discussion. Finally, the model organism would need to retain viability in an appropriate dosage form, such as a yogurt, slurry, or capsule. Once administered, the pharmabiotic organism would need to be resistant to bile acids and proliferate near or adhere to epithelial tissues in the gastrointestinal tract. The location of adherence would also play a large role in the purported efficacy of pro- or pharmabiotics. The small intestine is the site of reabsorption for amino acids and would be preferred for the location of action of a pharmabiotic. After much comparison, it was determined that a specific strain of *Lactobacillus*, *Lactobacillus helveticus*, was extremely qualified for formulation into a pharmabiotic.
*Lactobacillus helveticus*, belonging to the family of lactic acid bacteria, has historically been utilized in the production of Swiss cheese and is present in fermented milk. The genus *Lactobacillus* has been given “Generally Recognized as Safe” status and *L. helveticus* particularly has been given the designation of a “Qualified Presumption of Safety”. It is generally accepted as a probiotic strain and is formulated into commercially available products. The full genome sequence of *L. helveticus* has been documented.\textsuperscript{24} Genetically modified *L. helveticus* has been documented to survive passage through the human gastrointestinal tract, suggesting bile resistance.\textsuperscript{25} *In vitro* studies have demonstrated the ability of *L. helveticus* to co-aggregate efficiently with *E. coli* and adhere to epithelial cells.\textsuperscript{26} Each of these qualities lend themselves to the theory that *L. helveticus* could be genetically manipulated and subsequently formulated into a pharmabiotic for the treatment of phenylalanine hydroxylase deficiency. A detailed description of the creation of such a pharmabiotic is provided in the Materials and Methods.
Model 1 - Creation of the Novel Plasmid LiLi5

Commercial plasmid PCMV6-XL4 (navy blue) was cut with Not1 restriction enzyme (scissors) to release an insert containing human PAH cDNA (orange). Primers specific to PAH and introducing Sac1 and Sal1 cut sites (blue boxed arrows) were used to PCR amplify the PAH cDNA insert, which was later purified (light blue). pTRKH2 (green), a second commercial plasmid, was cut with restriction enzymes Sac1 and Sal1 (scissors). A ligation reaction (paper clips) was performed between pre-cut pTRKH2 and purified PAH cDNA to create a novel plasmid, LiLi5 (green and light blue).
Materials and Methods

Bacterial Strains

*Escherichia coli* was utilized to amplify DNA for manipulation. Specifically, stock strains of electrocompetent *Escherichia coli* cells were obtained from Lucigen (Middleton, WI). The plasmid pCMV6-XL4 containing the cDNA for human phenylalanine hydroxylase (PAH) was obtained from Origene (Rockville, MD). pCMV6-XL4 was introduced into *E. coli* by the preset *E. coli* protocol of the BioRad Gene Pulser Xcell Electroporation System. *E. coli* containing the plasmid pTRKH2 were purchased from Addgene (Cambridge, MA) and proliferated. pTRKH2 is a well-known shuttle vector that can be propagated in both *E. coli* and gram-positive bacteria such as *Lactobacillus helveticus*. Wild-type *L. helveticus* strain number #15009 were purchased from ATCC (Manassas, VA).

*E. coli* were grown aerobically, agitated at speeds between 180 and 200 rpm, angled, and incubated overnight at 37 °C. TB broth or ‘Terrific Broth’, a commonly used bacterial media, was used for growth in most instances, although no substantial variations were noted between the growth of *E. coli* in either LB (Luria-Bertani) or TB broth. MRS broth (deMan, Rogosa and Sharpe), a bacterial growth media specifically designed to provide luxuriant growth conditions for *Lactobacillus*, was used for propagation of *L. helveticus*, which was grown in anaerobic conditions without shaking at 37 °C. *L. helveticus* was allowed 48 hours for growth on plates and 72 hours for growth in liquid culture. Liquid cultures of *L. helveticus* were morphologically distinct from those of *E. coli*, with growth appearing as settled flakes and having the gross appearance of dandruff. As evidenced by the image below, *Lactobacillus helveticus* grows and settles in the form of a pellet on the bottom of any container containing liquid media, leaving the upper media clear.
This is in contrast to *E. coli*, which often grew equally distributed throughout liquid media, creating a cloudy appearance.

![Image 2](Image2.png)

*Image 2* Early stages of *Lactobacillus helveticus* growth as indicated by the arrow.

**Plasmid Production**

The pCMV6-XL4 and pTRKH2 plasmids were amplified by growing *E. coli* harboring the respective plasmids in TB plus appropriate antibiotic for selection. *E. coli* containing pCMV6-XL4 were propagated in 1-3 milliliters of liquid culture after loop inoculation of TB media containing 50 μg/ml ampicillin and incubated overnight at 37 °C with angled shaking at 180 rpm. Liquid cultures were grown for no less than sixteen hours. The gram-positive shuttle vector pTRKH2 was amplified by propagating *E. coli* as above but with the use of 150 μg/mL erythromycin for selection. Both plasmids were isolated from *E. coli* using a Qiagen MiniPrep plasmid extraction kit and following the manufacturer’s protocol.
**Determination of Sample DNA Concentration**

All sample DNA concentrations were determined in 2 μL aliquots via use of a NanoDrop Spectrophotometer by ThermoFisher Scientific, model 2000. Measurements were performed per manufacturer standards, including the use of appropriate blank solutions.

**Gel Electrophoresis, Restriction Enzyme Digests, Gel-Based Isolation of DNA Fragments**

All gel electrophoresis was carried out on 0.8% agarose gels for identification of correct plasmid size and estimation of quality and purity. Restriction enzymes NotI, SacI (High Fidelity) and SalI were obtained from New England Biolabs (Ipswich, MA). Digests were carried out per manufacturer instructions. To prepare for ligation of the PAH cDNA into the pTRKH2 backbone, the DNA samples were each incubated with both SacI and SalI, followed by separate gel electrophoresis. The plugs of agarose gel containing each DNA fragment were excised from the gel with a razor blade. Digested DNA was purified from the agarose using a Thermo Scientific GeneJET Gel Extraction kit #K0691.

**Polymerase Chain Reaction (PCR)**

PCR of the cDNA for phenylalanine hydroxylase was performed with 12.5 μL of Mastermix, 1.25 μL of forward and reverse primers at 10 μM each, 3 μL of the isolated NotI fragment from pCMV6-XL4 at a concentration of 3 ng/μL and 7 μL of sterile DNase and RNase-free water. Standard PCR reactions are performed in a thermocycler and commonly involve an initial activation step, followed by three-step cycling of denaturation at 94 °C, annealing at primer-specific temperatures, and extension at 72 °C for Taq polymerase. An annealing temperature of 57 °C was used for the primers designed for human PAH. The forward primer was:
5’-ATGTCCACTGCGTCTGGGAAGCCAGGCTTG.

The reverse primer was:

5’-TTACTTTATTTTCTGGAGGGCACTGCAAAGGATTCC.

A second PCR reaction was performed as above to introduce \textit{Sac1} and \textit{Sal1} cut sites on the PAH isolate with a concentration of 3 ng/μL. The forward primer, including the additional sequence of the \textit{Sac1} cut site, was:

5’-CGCGGAGCTCATGTCCACTGCGTCTGGGAAGCCAGGCTTG

and the reverse primer, containing the \textit{Sal1} cut site was:

5’-GCGCGGTCGACTTACTTTATTTTCTGGAGGGCACTGCAAAGGATTCC.

\textbf{Ligation}

DNA ligase was obtained from Lucigen (Middleton, WI). The ligation was performed per manufacturer instructions between the double-digested products of the shuttle vector pTRKH2 and the PAH cDNA fragment which was amplified by PCR using primers to introduce appropriate the enzyme cut sites. 4 μL of digested pTRKH2, 7 μL of the digested PAH fragment, 1 μL of DNA ligase, and 1.5 μL each of 10x ligation buffer and DNase and RNase-free water were placed together in a microcentrifuge tube and incubated at room temperature for five minutes. The tube was then incubated in a water bath at 70 °C for 15 minutes and then centrifuged for one minute at 10,000 rpm.

\textbf{Electroporation of \textit{E. coli}}
Electroporation of *E. coli* was performed with a BioRad Gene Pulser Xcell Electroporation System and a preset protocol for *E. coli*. The pre-set protocol included 1800 V, 25 μF, 200 Ω, and a 1 mm cuvette.

**Electroporation of Lactobacillus helveticus**

Preparation of electrocompetent *Lactobacillus helveticus* followed the protocol described by Welker (doi: 10.1093/femsle/fnu033). For the electroporation of the prepared *Lactobacillus helveticus*, 100 μL of electrocompetent cell suspension was loaded into electroporation microcuvettes followed by 200 ng of sample plasmid DNA. In separate cuvettes, pTRKH2, LiLi5, and a control with no plasmid were subjected to an electroporation in a BioRAD GenePulser XCell under the following conditions: 25 μF capacitance, 400 Ω resistance, and 2000 V. After retrieval from the cuvette, 100 μL of each cell suspension was then placed in 900 μL of MRS recovery media and incubated for 4 hours at 37 °C. The entire volume of the incubated cell suspensions was then transferred into 10 mL of MRS broth containing 0.5 μ/mL erythromycin for antibiotic selection in liquid culture. A control of wild type *Lactobacillus helveticus* lacking plasmid was also incubated with 0.5 μg/mL erythromycin to confirm positive selection in the presence of plasmids containing antibiotic resistance.

**Colorimetric Analysis of Media Tyrosine Content**

It was hypothesized that if fully functional phenylalanine hydroxylase enzyme were expressed from the LiLi5 plasmid in *Lactobacillus*, the PAH enzyme would metabolize the phenylalanine in the media to tyrosine and levels of tyrosine in the MRS broth in which *Lactobacillus* were growing would rise in a detectable fashion. It was also hypothesized that this increase in tyrosine levels in the media would be detectable by colorimetric tyrosine assay kit.
1.5 mL aliquots of MRS broth with 0.5 μg/mL erythromycin for selection were loop inoculated separately with *Lactobacillus helveticus* expressing pTRKH2 and *Lactobacillus helveticus* expressing novel plasmid LiLI5. A 1.5 mL aliquot of plain MRS broth was carried through the experiment as a control for baseline levels of tyrosine media in MRS broth. Samples were incubated statically at 37 °C for 48 hours and then the optical density (OD<sub>600</sub>) was taken for the samples containing *Lactobacillus*. The samples were adjusted for optical density via a dilution factor and three additional aliquots of 1.5 mL of MRS broth with 0.5 μg/mL erythromycin were inoculated separately with HELin and *L. helveticus* containing pTRKH2. Samples were again incubated statically at 37 °C for 36, 48, and 60 hours, respectively. The Tyrosine Colorimetric Assay Kit #K573-100 was purchased from BioVision (Milpitas, CA) and performed per manufacturer instructions. MRS broth at each timepoint was substituted for traditional serum or urine sampling. Prior to removing the supernatant for sampling, samples were centrifuged for sufficient time to produce a bacterial pellet. ~100 μL of supernatant was then deproteinized using a 10 kDa spin column and centrifuged again at 10,000 x g for 10 minutes at 4°C.

A 96 well plate was loaded with samples of each sample MRS supernatant at each timepoint following kit instructions. A plate reader was utilized to report out sample absorbance values. The Colorimetric Assay Kit utilizes the enzymatic oxidation of tyrosine, which produces a stable signal of OD 492 nm. A standard tyrosine curve was produced per kit instructions (see Figure 10). The equation \( y = 0.0048x + 0.0154 \) (where \( Y \) represents absorbance at OD 492 nm and \( X \) represents nanomolar tyrosine) was generated from the tyrosine standard curve. This equation was used to approximate nanomolar tyrosine content in the various MRS samples and
graphed as to display the change over time. Results from the plate reading are displayed in Figure 11.

**Western Blotting**

Western blots were performed by Jacob Massey, PhD candidate, using standard laboratory protocols and anti-PAH goat antibody purchased from Fisher Scientific (Pittsburg, PA). Samples were run at 140 V for 50 minutes in a 4-20% gradient pre-cast gel (Bio-rad 4561093) in homemade SDS-Page buffer. The gel was transferred onto a PVDF membrane at 100 V for 100 minutes at 4 °C. The membrane was blocked in 5% blocking milk in PBST for one hour prior to overnight incubation with goat anti-PAH primary antibody (Novus NBP1-52084, 1:500). After primary incubation, the secondary rabbit anti-goat-HRP antibody (Fisher PI-31402 1:2000) was incubated at room temperature for 1 hour. The HRP was activated using the ECL western blotting substrate (ThermoFisher 32106) prior to being imaged using a Bio-Rad ChemiDoc Imaging System.
Results

The intent of the project was to create a modified strain of *Lactobacillus helveticus*, producing the human enzyme phenylalanine hydroxylase. The first step in the process was to isolate the plasmid containing the cDNA for the PAH enzyme, pCMV6-XL4. The far-left lane of Figure 1 shows 5 μL of pCMV6-XL4 DNA loaded onto a 0.8% agarose gel. The plasmid was identified by size comparison to a BioRad Log 2 Ladder, lane 3 of Figure 1. This figure demonstrates that pCMV6-XL4 was successfully propagated in and subsequently isolated from *E. coli*. The appearance of three bands shows that the plasmid was visualized in three predominant forms: open circular, linear and supercoiled.

![Figure 1](image_url) Commercial plasmid pCMV6-XL4 containing human PAH cDNA was isolated from *E. coli* following standard plasmid mini-prep protocol. The left lane labeled ‘pCMV6’ shows the plasmid DNA isolated from *E. coli*. The right lane labeled ‘ladder’ shows the BioRad Log2 ladder with different molecular weight bands labeled by their size.
The pCMV6-XL4 plasmid was next digested with the restriction enzyme *NotI*. The manufacturer’s stated size of pCMV6-XL4 backbone was 4.7 kb with a 2.3 kb insert containing the PAH cDNA. *NotI* sites flank the PAH cDNA, thus *NotI* digest removes the cDNA from the backbone. Figure 2 shows an agarose gel loaded with 40 μL of pCMV6-XL4 plasmid cut twice with *NotI* in comparison to a log 2 ladder. This result confirms that a DNA fragment corresponding to the 2.3 kb fragment that contains the human PAH cDNA was successfully excised from the pCMV6-XL4 plasmid after *NotI* digest. The 3 ng/μL yield of PAH cDNA necessitated that a polymerase chain reaction be performed to amplify the product.

![Image](image.png)

**Figure 2** The PAH cDNA fragment was freed from plasmid pCMV6-XL4 via *NotI* restriction enzyme digest, as demonstrated by the left lane labeled ‘PAH cDNA’. The right lane labeled ‘ladder’ shows the BioRad Log2 ladder with different molecular weight bands labeled by their size. The arrow indicates the fragment removed from the backbone.
Next, PCR was used to specifically amplify the human PAH cDNA from the 2.3 kb fragment excised from the pCMV6-XL4 plasmid. Lane 1 of Figure 3 shows the 2.3 kb fragment (labeled excerpt) released from digesting the PCMV6-XL4 plasmid with \textit{NotI} and purified by gel electrophoresis. Lane 2 demonstrates the result of PCR performed on the \textit{NotI}-excised fragment in lane 1 using primers specific to human PAH cDNA, yielding a 1.3 kb product. This result confirms that the primers specific to human PAH cDNA amplified a 1.3 kb portion of cDNA within the 2.3 kb insert removed from the pCVM6-XL4 plasmid. No amplification was seen with the control PCR, confirming that the reaction was specific to amplification of human PAH cDNA with the primers used. An additional PCR was performed using the same conditions as above, but with the substitution of primers also containing \textit{SacI} and \textit{SalI} enzyme cut sites. Primers with additional flanking cut sites were used to provide compatible ends for subsequent ligation of the PAH cDNA into the pTRKH2 shuttle vector backbone.

![Image](image-url)

\textbf{Figure 3} The human PAH cDNA fragment was amplified via PCR. The far-left lane labeled ‘Excerpt’ shows the 2.3 kb PAH cDNA fragment removed from PCMV6-XL4 with \textit{NotI}. The left lane labeled ‘PAH’ shows the 1.3 kb PAH cDNA amplified via PCR. The ‘control’ lane demonstrates that the primers were specific to PAH as no superfluous amplification took place. The right ‘ladder’ lane exhibits the BioRad Log2 ladder with different molecular weight bands labeled by their size.

Figure 4 demonstrates a gel run with 3 \(\mu\)L of
PAH cDNA after PCR amplification in the far-right lane using primers with the flanking enzyme cut sites.

**Figure 4** The PCR of the PAH cDNA was repeated with the use of primers to introduce *Sac1* and *Sal1* cut sites, the result of which is pictured in the right lane labeled ‘PAH (Cut Sites)’. The left lane is another visualization of the BioRad Log2 ladder with different molecular weight bands labeled by their size.
The PCR product of PAH containing enzyme cut sites at the 5’- and 3’- ends was then subjected to a double restriction enzyme digest and loaded onto a gel to separate and purify the DNA fragment with ligatable ends. After electrophoresis, a small plug of agarose containing the DNA of interest was excised from the gel, and the DNA subsequently purified away from the agarose as described in Materials and Methods.

pTRKH2 was also isolated from *E. coli* and subjected to gel electrophoresis to confirm successful extraction. The right lane of Figure 5 shows 5 μL of pTRKH2 DNA in comparison to a log 2 ladder. In preparation for ligation with the PAH cDNA fragment, pTRKH2 was double digested by *SacI* and *SalI* restriction enzymes, separated on a gel, and purified from the agarose plug as described above.

**Figure 5** The commercial plasmid pTRKH2 was isolated from *E. coli* via standard plasmid mini-prep protocol, as shown in the right lane labeled ‘pTRKH2 (Uncut)’. The middle lane labeled “Ladder” is a visualization of the BioRad Log2 ladder with different molecular weight bands labeled by their size.
A ligation was then performed between the purified double-digest products of the shuttle vector pTRKH2 and the PAH cDNA PCR product with flanking enzyme cut sites.

The products of the ligation reactions were termed the “LiLi” series of plasmids. The LiLi plasmids were introduced into stock strains of *E. coli* via electroporation as described above. Original pTRKH2 plasmid and vehicle (no plasmid) also went through the electroporation protocol as controls. After electroporation, 15 and 150 μL aliquots of cells were plated on LB plates containing 150 μg/ml erythromycin and incubated for 15 hours at 37 °C. *E. coli* colony formation was noted for cells containing ligation products or pTRKH2, but not for *E. coli* lacking plasmid. Individual colonies from the pTRKH2-PAH ligation reactions were isolated and streaked out on a secondary plate and for overnight incubation. Colonies containing the LiLi series of plasmids were chosen to inoculate four tubes of 2 mL liquid LB media with 150 μg/ml erythromycin. A plasmid extraction was performed using the Qiagen MiniPrep plasmid extraction kit, yielding approximately 30 ng/μL of DNA per colony. Figure 6 compares the sizes of several extracted LiLi plasmids to a ladder and pTRKH2.
Figure 6 The LiLi series of plasmids were generated via ligation between commercial plasmid pTRKH2 cut with SacI and SalI and human PAH cDNA amplified with added enzyme cut sites. Each individual colony in the series was given a name beginning with LiLi and then a sequential number. pTRKH2, as pictured in the far-right lane labeled ‘pTRKH2’ was used as a size reference to infer the success or failure of the ligation reaction. The LiLi series of plasmids are visualized separately in the lanes labeled ‘LiLi5, LiLi6’, etc. after extraction from *E. coli*. The lane labeled ‘Ladder’ is a visualization of the BioRad Log2 ladder with different molecular weight bands labeled by their size.
As demonstrated by Figure 6, LiLi5, LiLi7, and LiLi3 are larger plasmids than pTRKH2, suggesting that they may have been the product of a successful ligation reaction between the pTRKH2 backbone and the human PAH cDNA. LiLi6 appeared to be simply a reconstitution of the original pTRKH2 backbone. LiLi8 was an uncharacterized plasmid. Subsequent digests revealed that LiLi3 lacked appropriate enzyme cut sites and was thus excluded from further experimentation.

The plasmids LiLi5 and LiLi7 were then each digested in two separate reactions. A single digest with \textit{SalI} and a double digest with \textit{SalI} and \textit{SacI} were conducted in the manner described above. The single digest linearized the plasmids to determine approximate size and the presence of appropriate cut sites in the plasmid. The left lanes of Figure 7 show the results of the single and double digests, respectively. Double digestion of LiLi5 and LiLi7 revealed that the 1.3 kb PAH insert was removed from pTRKH2 backbone, validating that LiLi5 and LiLi7 contained a DNA fragment of the correct size and which was excised from plasmid as expected with the restriction enzymes corresponding to the ligation sites.

Additionally of note, LiLi5 and LiLi7 appear to be larger plasmids upon single digest with \textit{SalI} when compared to the pTRKH2 single digest with \textit{SalI}. This is suggestive that a successful ligation had taken place, making the plasmids generated from the ligation larger. LiLi5 and LiLi7 were then sent for Sanger sequencing. The read length for LiLi7 was deemed too short to be acceptable and it was not further analyzed. As demonstrated by Figure 8, the LiLi5 plasmid sequence was aligned with the NCBI sequence for the PAH (NM_000277.2) and determined to be 100% identical across the first 716 bases aligned and 99.8% identical across the
first 994 bases aligned, which is typically the upper limit of accuracy for standard Sanger sequencing.

**Figure 7** A double restriction enzyme digest was performed to further elucidate the genetic contents of the LiLi series of plasmids. The LiLi5 plasmid was subjected to a single digest by Sal1 (high fidelity) and is pictured in the lane marked ‘L5 (Sal1HF Digest)’. LiLi5 was also subjected to a double digest with Sal1 (high fidelity) and Sac1, the results of which are shown in the lane marked ‘L5 (Double Digest)’. As shown in the lane marked ‘L5 (Double Digest)’, a fragment is released which is later demonstrated to be the PAH insert via Sanger sequencing. LiLi7 and LiLi3 underwent similar digests, as depicted in the ‘L7’ and ‘L3’ lanes. pTRHK2 was subjected to a single Sal1 (high fidelity) digest and is shown here in the lane marked ‘pTRKH2 (Sal1HF Digest)’. The lane marked “Ladder” is a visualization of the BioRad log2 ladder.
Figure 8 The LiLi5 plasmid was Sanger sequenced and the results were compared to the NCBI reference genetic sequence for human PAH (red box). The blue box demonstrates the overall 98% identicality between the two sequences.
Next, the LiLi5 and pTRKH2 plasmids were transfected separately into *Lactobacillus helveticus* in the manner described in Materials and Methods. pTRKH2 was carried through the electroporation as a control to demonstrate positive selection for *Lactobacillus helveticus* containing plasmids yielding erythromycin resistance in liquid culture with antibiotic selection. The successful transfection with pTRKH2 and LiLi5 further validated electroporation protocols and characterized the behavior of *Lactobacillus helveticus* post-transfection with plasmid. After 96 hours, growth in liquid media was noted for cultures undergoing electroporation to introduce LiLi5 and pTRKH2 plasmids, suggesting that a plasmid conferring erythromycin resistance had successfully been transferred into *Lactobacillus helveticus*.

DNA extraction was performed using the Zymo Plasmid MiniPrep kit, per manufacturer protocol to further characterize the genetic information of the *Lactobacillus helveticus* strains which presented with erythromycin resistance. A standard PCR reaction was then performed on the eluted DNA using primers specific to the PAH cDNA.

Figure 9 shows the results of the PCR amplification used to confirm the presence of LiLi5 in *Lactobacillus helveticus*. The presence of a band at ~1.3 kb, the identical size to the original cDNA isolated from pCMV6-XL4, confirms that the sequence encoding the human PAH enzyme is present in the modified *Lactobacillus helveticus* cells and is not in the pTRKH2 cells. The new strain of modified *Lactobacillus helveticus* containing LiLi5 plasmid has been christened HELin.
Figure 9 The lane marked ‘LiLi5 (LH)’ shows the result of PCR using primers specific to human PAH cDNA. This reveals the presence of human PAH cDNA in the genome of L. helveticus. The novel species of Lactobacillus that has been created was named HELin. E. coli transfected with the LiLi5 plasmid also contained human PAH cDNA as revealed in the lane marked ‘LiLi5 (E. coli)’ after a PCR reaction was performed with primers specific to PAH cDNA. As shown in the lanes marked ‘PTRKH2 (LH)’ and PTRKH2 (E. coli)’, PCR using primers specific to human PAH cDNA did not yield any DNA amplification when performed on the pTRKH2 plasmid in either species.

Additional Experimentation

Several analyses were performed with the purpose of validating the expression and functionality of the full human phenylalanine hydroxylase enzyme in Lactobacillus helveticus (HELin). Primarily, a tyrosine assay kit was purchased with the intent to demonstrate that MRS media containing HELin had a statistically significant increase in the levels of tyrosine in the media. Figure 10 demonstrates the standard curve of tyrosine absorbance across all experiments performed.
It was hoped that an increase in tyrosine content in MRS media inoculated with HELin would be detectable secondary to the production of an exogenous protein metabolizing phenylalanine into tyrosine. Unfortunately, despite media deproteinization and concentration adjustments, colorimetric analysis of media tyrosine content showed strong inconsistencies in the measurement of endogenous levels of tyrosine in control samples, as demonstrated by Figures 11, 12, and 13. The inconsistencies in the levels of tyrosine in control samples rendered the data inconsequential. As the tyrosine content was “trended” over time with timepoint samples at 36, 48, and 60 hours, there was a confusing inconsistency in both the media inoculated with Lactobacillus and the sterile MRS media. The most likely explanation for the failure of the kit to provide useful data was interference of the endogenous levels of tyrosine in the MRS broth with colorimetric reads of the sample.

**Figure 10** A tyrosine standard curve was generated and used throughout all tyrosine assay experiments. The enzymatic oxidation of tyrosine produces a stable signal (OD 492 nm), which is directly proportional to the amount of Tyr. As the amount of tyrosine in the samples increases, so does the absorbance read.
Figure 11 A tyrosine assay was performed three times in order to determine the effect of HELin on the local environment. Graphs “A”, “B”, and “C” represent three separate replicates of the tyrosine assay kit. Aliquots of the same samples of MRS supernatant were compared in each assay replicate. The blue line on the graphs “pTRKH2 (LH)” represents the nM of tyrosine detected in of MRS media inoculated with Lactobacillus helveticus transfected with the pTKRH2 plasmid, trended over a period of 60 hours. The orange line represents represents the nM of tyrosine detected in of MRS media inoculated with Lactobacillus helveticus transfected with the LiLi5 plasmid (HELin), trended over a period of 60 hours. The grey line represents represents the nM of tyrosine detected in sterile MRS media trended over a period of 60 hours. Interference from endogenous tyrosine levels within MRS broth with no bacteria (control media) rendered the data inconsequential. This is demonstrated by the fluctuations in the gray lines on the chart. MRS was incubated as a control. As this media was not inoculated with any bacteria, the tyrosine levels should have remained constant over time.
Subsequently, Western blotting was attempted to detect the human PAH protein. Although an appropriately sized blur is noted for the LiLi5 cell pellet and is absent from the pTRKH2 cell pellet, it is not entirely convincing, robust evidence of the presence human phenylalanine hydroxylase.

**Figure 12** A Western blot using anti-PAH goat antibody was performed in an attempt to detect the presence of the PAH enzyme in *Lactobacillus helveticus* bacterial pellet or local supernatant. The expected size of the PAH protein is ~50 kDa. An appropriately sized blur is seen in the lane marked ‘LiLi5 Cell Pellet’, which is suggestive of the fact that the PAH protein may be produced in *L. helveticus* transfected with LiLi5 (named HELin). However, further testing must be done to validate the presence of the fully functional protein. Nonspecific binding is also seen across all lanes.
The Western blot was repeated with the inclusion of human HCT116 cells as an additional control. Again, an appropriately sized blur is seen in the LiLi5 cell pellet at 36 hours, but is not noted in the same cell line at 60 hours, as seen in Figure 13. The lack of a convincing, clearly detectable protein at 50 kDa in all LiLi5 samples provided inconclusive evidence of the production of the PAH protein.

![Western Blot Image]

**Figure 13** A second Western blot was performed on samples of *Lactobacillus helveticus* transfected with various plasmids, although it was also inconclusive. Again, an appropriately sized blur was noted at 50 kDa in the lane marked ‘LiLi5 Cell Pellet 36h’. However, nonspecific binding was, again, also present across all lanes. The most nonspecific binding is noted in the additional control lane ‘HCT116 Cas9-FLAG’. Further investigation into the production of the fully functional PAH protein is warranted, perhaps with a more specific alternate antibody.

**Discussion and Future Experimental Directions**

The project successfully inserted human cDNA for phenylalanine hydroxylase into a GRAS probiotic-type bacterial strain, *Lactobacillus helveticus*, via the integration of a novel plasmid, LiLi5. The creation of HELin represents the first steps toward a pharmabiotic for the treatment and mitigation of the metabolic disorder phenylalanine hydroxylase deficiency.
Frustratingly, convincing evidence of successful expression of enzymatically active PAH remains lacking. As stated above, the high endogenous levels of tyrosine in the MRS broth likely interfered with colorimetric reads of the sample during the experiments involving the tyrosine assay kits. However, it is also possible that PAH was expressed so inefficiently that, had the enzyme been successfully hydroxylating phenylalanine, it was doing so to such a small degree as to be undetectable. It may be possible to perform more productive experimentation utilizing samples with an alternate medium. Experimentation using an alternate medium would also be enhanced with additional reading of a phenylalanine level via colorimetric assay.

Western blotting procedures could be improved for future experimentation with the selection of an alternate, more selective antibody and the inclusion of samples from a wider variety of time points. A literature evaluation would need to be performed when choosing a new antibody to ensure that the antibody had been used in other successful experiments with bacteria.

However, problems with enzymatic expression may be deeper, and resistant to changes in technical laboratory procedures. Issues with functional expression may, in fact, be secondary to differences in codon preferences between species. This problem may be rectified by codon optimization. Although it is too early to make definitive conclusions, it is possible that the protein was visualized in the *Lactobacillus* cell pellet at 36 hours because the protein is currently expressed intracellularly and sequestered. The phenylalanine hydroxylase may not be visualized after 36 hours because the human protein was recognized as foreign and destroyed by intracellular proteinases. This provides another opportunity for product optimization.

Further funding for optimization, characterization, and validation of the functionality of HELin has been sought and experimentation is ongoing.
Formulation and Administration Considerations

Having demonstrated the proof of principle that *Lactobacillus helveticus* can be genetically modified to express the genetic information for human phenylalanine hydroxylase, a more completely optimized product would easily lend itself to therapeutic applications. Modified *L. helveticus* can be formulated into a yogurt or capsule and marketed as a medical food or as a drug in the United States. Medical foods bypass many of the rigorous examinations conducted by the Food and Drug Administration. Although the pathway to market for drugs is more vigorous, a pharmabiotic may more closely match the FDA’s definition of a drug as “a substance intended for use in the diagnosis, cure, mitigation, treatment, or prevention of disease.” Concentrated care would need to be taken by manufacturers to ensure the identity, quality and efficacy of the product. The product would likely need to be dispensed on a prescription-only basis, ensuring that only appropriately diagnosed patients would receive the product. An anticipated adverse effect of inappropriate administration of this product may be muscle weakness due to lack of appropriate amounts of phenylalanine used to build proteins.

Daily dosages of the medical yogurt or capsule would promote stability of plasma phenylalanine levels and, depending on the degree of colonization of the gastrointestinal tract, may even allow for some dietary variation. The ability to consume foods consistent with the general population, even occasionally would be greatly welcomed by the patient population. Stability of plasma phenylalanine levels secondary to administration of an optimized HELin strain would theoretically reduce the instance of all consequences that appear directly as a result of elevated plasma levels or, alternately, as a consequence of therapy-- developmental delays, psychosocial deviations, behavioral issues, strange body odor, overweight, and decreased bone density. It is likely that multiple administrations per day would be necessary to achieve this type
of efficacious bacterial colonization and stability. However, the pill burden would be a small grievance compared to the desperation felt by this patient population for a cure, or at least a greater sense of normalcy, and is not expected to provide a barrier to market entry.

The formulation could also be modified into a slurry for inpatient nasogastric administration. Nasogastric administration would be ideal for the neonatal phenylketonuric population, as use of the entire gastrointestinal tract is certainly considered best practice and this method of administration would also allow for immediate colonization after birth. Potentially, immediate post-natal administration of such a product would disallow massive fluctuations in blood phenylalanine levels, the occurrences most detrimental to psychological development. Additionally, nasogastric administration of *L. helveticus* would be ideal for use in the pregnant phenylketonuric population in cases where rapid lowering of blood phenylalanine levels is urgent. It has been documented that spikes in the maternal plasma levels of phenylalanine damage the developing fetus, with reports of spontaneous abortion, congenital heart failure, and psychological developmental delay. Much stress is felt by the pregnant patient population, where intense focus must be kept on maintaining low blood phenylalanine levels.\(^8\)

Of note, there may be a place in therapy for a pharmabiotic bacterial species maintaining antibiotic resistance. Ostensibly, a loading dose of the antibiotic to which the organism is resistant could be administered to the patient to, in effect, “clear the way” for the rapid proliferation and colonization of the pharmabiotic species. Due to the alarming increase in rate of antibiotic resistance in the United States, this strategy should be limited to a one-time loading dose and severe cases in which blood phenylalanine levels need to be immediately lowered. As with all chemotherapeutics, risks and benefits of treatment strategies must be weighed and considered with the holistic clinical picture of the individual patient.
Safety concerns surrounding the product would largely be limited to the immune-suppressed population or those whose inpatient therapy involves a central venous catheter. There have been case reports of *Lactobacillus* bacteremia.\(^{27}\) It is unfortunately expected that these case reports will become more frequent as the national focus on the microbiome continues to intensify and probiotics become more clinically integrated at the point of care. Intentional external administration of bacteria to an immunocompromised patient is clearly contraindicated. Extreme caution should be used in the cases of an immunocompetent patient with a central venous catheter in place. Additional consideration should be used prior to administration if the patient presents with leaky gut syndrome. However, excluding these patient populations, no other safety concerns are expected to arise from the administration of such a pharmabiotic.

**Intellectual Property Rights**

Intellectual property rights were sought for the invention of a pharmabiotic for the treatment of phenylalanine hydroxylase deficiency. The provisional patent application was filed on December 20, 2018 as ATTY. DOCKET NO.:USC-601-P (1317).

**Forward Projections**

Phenylalanine hydroxylase deficiency is not the only metabolic disorder that can and should be addressed by modification of the microbiome. I predict that the microbiome will provide a vast host of resources for the treatment and mitigation of a multitude of disease states. Pharmabiotics are the next generation of supportive care and provide a targeted therapeutic with minimal risk to the patient. As scientific understanding of the interplay between microbe, host, and health expands, exponentially so does the opportunity for ingenuity.
Acknowledgements

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Funding for ongoing experiments will be provided by the Department of Clinical Outcomes Sciences, University of South Carolina College of Pharmacy.
My deepest gratitude extends to Dr. Michael Wyatt, PhD for his continuous oversight of and insight into the project. Thank you, Dr. Wyatt for your endless patience and encouragement. Jacob Massey, PhD candidate, provided not only technical advice and instruction, but a selfless friendship. Thank you, Jake, for teaching me. Dr. Douglas Pittman, PhD, provided detailed review and support for the project. Together we have created a new possibility.

It’s fine.
## Abbreviations

<table>
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>Ery</td>
<td>erythromycin</td>
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<tr>
<td>HELin</td>
<td><em>L. helveticus</em> containing LiLi5 plasmid</td>
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<td>kb</td>
<td>kilobase</td>
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<td>LiLi5</td>
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Works Cited

10. Dewees, B. ‘HIGHLIGHTS OF KUVAN PRESCRIBING INFORMATION. 22


Sequences Referenced

Primer Sequences for PCR of PAH cDNA
Forward: ATGTCCACTGCGGTCCTGGGAAAACCCAGGCTTG
Reverse: TTACTTTATTTTCTGGAGGGAAGGACATGGAAGAAGAG

Primer Sequences for PCR of PAH cDNA for the Addition of SacI and SalI Cut Sites
Forward (SacI): CGCGGAGCTCATGTCCACTGCGGTCCTGGGAAAACCCAGGCTTG
Reverse (SalI): GCGCGTCGACTTATTTTTTCTGGAGGGAAGGACATGGAAGAAGAG

pTRKH2 with Phenylalanine Hydroxylase:
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Phenylalanine Hydroxylase Insert with Enzyme Cut Sites:

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