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A Link Between Gut Microbes & Depression: Microbial Activation of the Human Kynurenine Pathway

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A Link Between Gut Microbes & Depression: Microbial Activation of the Human Kynurenine Pathway

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by

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Abstract

Our gut microbiota is involved in human development, nutrition, and the pathogenesis of gut disorders, but has more recently been implicated as a possible mechanism in the pathophysiology of several brain disorders, including disorders of mood and affect, such as depression. Researchers have referred to this dynamic, bidirectional signaling pathway between the gut and the brain as the "gut-brain axis." However, most research on this axis has been limited to rodent studies, and there has been little insight into the mechanism behind it. I propose that the kynurenine pathway, where tryptophan is converted to kynurenine, is a compelling mechanism mediating the gut microbiota's influence on depression. Kynurenine is a metabolite associated with depression, and this pathway has been shown to be manipulated through probiotic (*Lactobacillus reuteri*) consumption. I propose to study a probiotic intervention in humans, which would assess tryptophan metabolism along the kynurenine pathway by measuring metabolites downstream of this pathway. Urine, feces and blood samples would be collected from two groups, control and probiotic treatment, on day zero and day thirty. Colonic biopsies would be obtained on day thirty, and various analyses would be run to measure metabolite concentrations from the collected samples. The results from this study will help clarify a mechanistic connection between gut microbes and depression via the kynurenine pathway. Additionally, findings could indicate that a probiotic intervention has the ability to influence depressive behavior via a two-pronged approach originating from the kynurenine pathway.

Introduction

Depression and the Importance of our Gut Microbes

Our gastrointestinal tract is home to over 1×10^{14} microorganisms that are responsible for many functions throughout our body and essential for health (1). It has been estimated that at least 1×10^{4} distinct species cohabit the human gut and compose the microbiota, an ecological community of commensal, mutualistic and pathogenic microorganisms (2). This provides a glimpse into the size and complexity of the human microbiome, and the important role our gut microbiota plays in our health. Our gut microbiota is involved in the pathogenesis of gut disorders, but has more recently been implicated as a possible mechanism in the pathophysiology of several brain disorders, including disorders of mood and affect (3). There is growing evidence of bidirectional, reciprocal communication between the gut and brain (1, 3, 4) that has important implications in human health. Researchers have referred to this dynamic, bidirectional signaling pathway as the "gut-brain axis," and components of this axis have, and are currently receiving extensive study as dysfunction of this axis has broad pathophysiological consequences (1). Several experimental approaches have been used to study the effect of gut microbiota on gut-brain interactions, including gut microbial manipulation with probiotics, prebiotics, and antibiotics, fecal microbial transplantation and germ-free animal models (3). While there are many limitations of these approaches, there has been significant progress towards better understanding the role our gut microbiota plays in CNS disorders.

Depression is a mood disorder that is frequently studied in this axis, with the hope to further clarify its link to the gut. Several studies in mice have demonstrated that there is a distinct difference in the composition of gut microbiota in animal models of depression and

chronic stress (5). In addition, the high co-morbidity between psychiatric symptoms, such as depression or anxiety, with gastrointestinal disorders including irritable bowel syndrome (IBS) and inflammatory bowel disorder (IBD) is further evidence of the importance of this axis (6). Further clarifying the microbiome's link to depression is important, as depression is considered the leading cause of disability worldwide, impacting more than 350 million people (7). In addition, according to the World Health Organization, depression will displace heart disease in terms of cost of caring for patients by the year 2020 (7). Serotonin-specific reuptake inhibitor medications (SSRIs) are the primary medications prescribed for patients who are diagnosed with depression, however, these medications do not treat the root cause of depression, and instead only improve symptoms. With this in mind, it is interesting to note that antidepressants are one of the three most commonly used therapeutic drug classes in the U.S. (8). This common use of antidepressants is alarming, because the cause of depression is not entirely understood, and these antidepressants are only aiding in symptom relief, rather than curing the problem (2). SSRIs block the reabsorption of serotonin in the brain, making more serotonin available, however, this same neurotransmitter found in the brain is primarily produced in the gut (2). In fact, more than 90% of the body's serotonin is synthesized in the gut (9). Ultimately, a further understanding of the connection between the gut and depression could prove to have important implications in the medical field, and potentially reduce antidepressant use among U.S. society.

One of the major recent findings in this field was from an experiment led by Irish scientist John Cryan in 2011. His research group fed half their mice with *Lactobacillus rhamnosus* (JB-1), a lactic acid bacterium shown to be beneficial for the gut and commonly present in probiotics, then evaluated several different measures of behavior between the two

groups of mice, such as tests for anxiety and depression-related behavior, and stress-induced corticosterone levels (10). They found that the mice given *L. rhamnosus* kept swimming for longer and with more motivation in the forced swim test analysis, which is a test for depression-related behavior (10). In addition, these mice were also found to have lower stress-induced corticosterone levels, showing that *L. rhamnosus* had beneficial effects on the mouse (10). Yet when they severed the mouse's vagus nerve, there was no difference recorded between the two groups of mice, thus the vagus nerve plays a critical role in communication between the gut and the brain (10). Overall, this study highlights the important role of bacteria in the bidirectional communication of the gut-brain axis and suggests that certain organisms may prove to be useful therapeutically in stress-related disorders such as anxiety and depression. One of the first human studies assessing the psychotropic-like effects of probiotics was also in 2011 by a group of researchers led by Messaoudi. The clinical study demonstrated that in the general population, when a probiotic formulation (PF) of *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175 was taken in combination for 30 days, global scores of hospital anxiety and depression scale decreased (11). Thus oral intake of this PF showed beneficial effects on anxiety and depression related behaviors in human volunteers, just like it did in the mice. Mouse studies have begun to elucidate a link between gut microbiota and depression, and clinical trials, such as Messaoudi *et al.*'s, are working to translate these findings from mouse studies to the human body. These studies suggest a potential for positive effects of some probiotics on mood, however, there is a need for larger clinical trials, with biological and self-reported outcomes in patient populations, in order to make clear conclusions on a mechanistic connection between the gut microbiota and the brain (12). Deciphering the mechanism by

which microbiota communicate with the gut-brain axis will be crucially important for the development of any microbiota-based and microbiota-specific therapeutic strategies for CNS diseases and mood disorders.

The gut is composed of a variety of different bacterial phyla, such as *Firmicutes* (genera such as *Lactobacillus, Clostridium, Enterococcus)* and *Bacteroidetes* (genera such as *Bacteroides)*, which account for the majority (13). Other phyla are also present in the gut such as *Actinobacteria (Bifidobacteria), Proteobacteria (Escherichia coli), Fusobacteria, Verrucomicrobia* and *Cyanobacteria* (13). Not all genera of microbes are considered beneficial to mental health, and some are more than others, so it is important for future research to focus on deciphering which genera or species of bacteria are more beneficial than others, and what they specifically do that proves to be beneficial. *Lactobacillus* and *Bifidobacterium* are the main genera showing beneficial effects on mood disorders, such as anxiety and depression (12). *B. infantis* has been shown to increase plasma tryptophan levels, *L. helveticus* was shown to prevent diet-induced anxiety-like behavior and memory, and *B. longum* NCC3001 reversed colitis-induced anxiety in the mouse via the vagus nerve (5). These results have not been replicated in humans. Clinical validation of these findings is necessary in order to determine whether or not probiotics can be used therapeutically in mood disorders. In conclusion, findings from mouse studies begin to give a greater glimpse into the powerful role our gut plays in depression.

The Immune System: Mediator of Communication between the Gut and the Brain

The digestive tract does not operate independently, but instead requires functional integration with other organ systems. Communication between the gastrointestinal tract and the central nervous system (CNS) and enteric nervous system (ENS) are examples of how signals originating in one of these organ systems affect the function of the other (14). The ENS is a complex network of neurons that can function independently of the CNS, control gut motility and local blood flow, and directly communicate with cells of the intestinal barrier (14). However, mechanisms by which the ENS integrates with microbe-mediated GI immune responses are still poorly understood. For example, it is largely unknown whether the ENS senses the microbial environment and initiates an immune response, or whether the ENS senses immune responses and then modifies or propagates those signals (15).

Microbial regulation of tryptophan, serotonin and kynurenine metabolism has become a focal point in understanding the influence exerted by the gut microbiota on brain function and behavior. The kynurenine pathway, where tryptophan is converted to kynurenine (Figure 1), is of particular interest because kynurenine pathway metabolism might be regulated by microbial control of neuroendocrine function and parts of the immune system (16). Changes in the supply and availability of tryptophan has many implications for ENS and CNS functioning and consequently gut-brain axis signaling.

Tryptophan is an essential amino acid, so it must be supplied from one's diet (17). Some common sources of tryptophan are bananas, dried prunes, milk, peanuts, chocolate and white meats, such as chicken, turkey and tuna (17). The gut microbiota can also directly utilize tryptophan, which could potentially limit its availability to the host (18). Additionally, *B. subtilis* is a known producer of tryptophan, a precursor to serotonin, so there are bacterial

strains that have the ability to directly influence tryptophan levels and indirectly influence serotonin levels (19). Thus, bacterial tryptophan utilization, tryptophan synthesis and dietary supply of this essential amino acid, all play a role in the tryptophan level in the gut and circulating tryptophan availability for the individual.

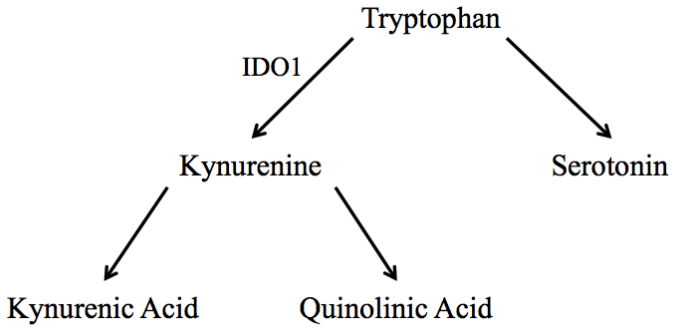

Figure 1. The kynurenine pathway, where tryptophan is either converted to kynurenine or serotonin.

While tryptophan is the precursor molecule to serotonin, around 90% of tryptophan is metabolized to kynurenine or the other downstream metabolites, kynurenic acid and quinolinic acid (Figure 1) (16). The rate of tryptophan metabolism along the kynurenine pathway is dependent on expression of indoleamine-2,3-dioxygenase (IDO1), and IDO1 expression can be induced by the action of inflammatory cytokines such as Interferon (IFN)-γ (16). As IDO1 is induced by proinflammatory cytokines, its expression has been proposed as a biomarker of GI diseases, including inflammatory bowel disease and colon cancer (16). Additionally, there are two interleukins, IL-10 and IL-4, that influence tryptophan metabolism down the kynurenine pathway and interact directly with IFNγ (20). Regulatory T cells produce IL-10, which directly inhibit T_H1 cells that produce IFNγ (Figure 2) (20). IL-4, produced by T_H2 cells, directly inhibits IFNγ, and conversely, IFNγ inhibits IL-4 (Figure 2)

(20). Thus IL-10 and IL-4 both inhibit tryptophan metabolism down the kynurenine pathway, and instead drive the pathway towards serotonin production (Figure 2).

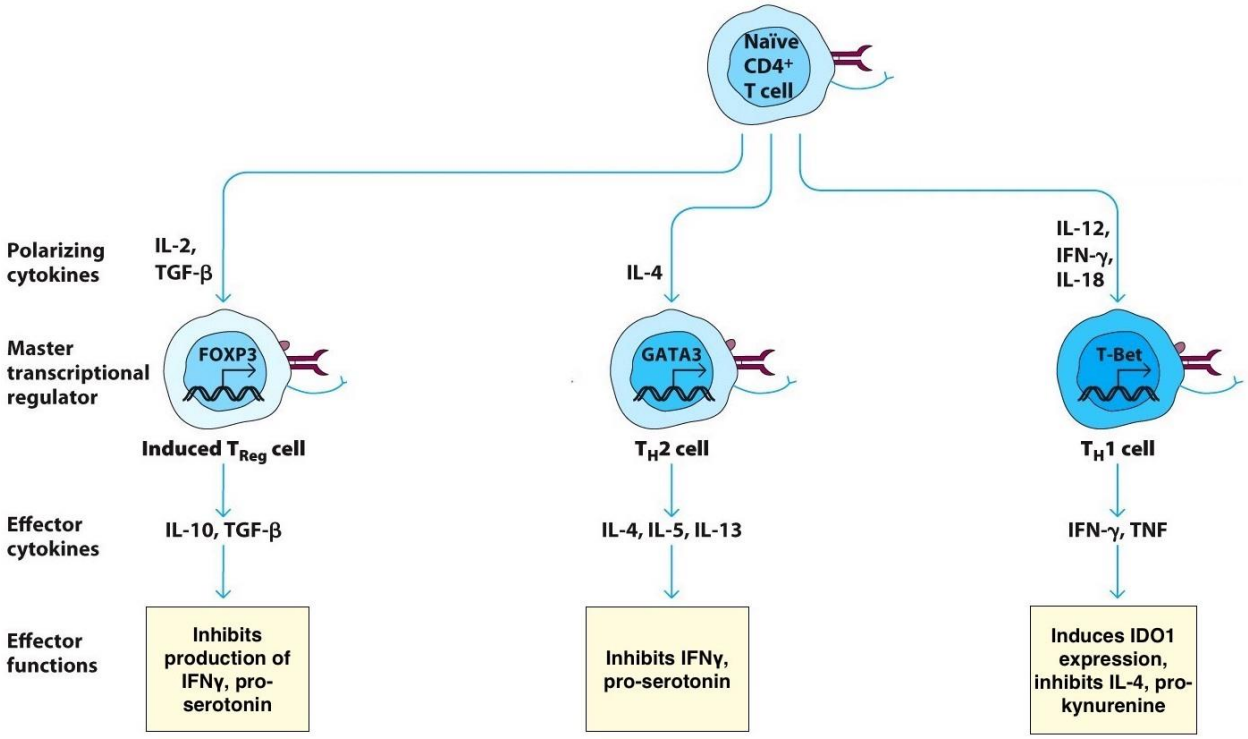

Figure 2. Immune system components involved in the kynurenine pathway. Adapted from reference 20.

IDO1, the enzyme that converts tryptophan to kynurenine, was shown to be inhibited by *Lactobacillus* in a mice study conducted by Valladares *et al*. in 2013. Valladares *et al.* found that feeding *L. johnsonii* to rats increased H_2O_2 concentrations in the ileal lumen, and correlated with decreased peripheral kynurenine concentrations and altered IDO distribution (21). Most importantly, *L. johnsonii* was found to produce reactive oxygen species (ROS), H_2O_2, that inhibits the expression of the metabolizing enzyme, IDO1, in the intestine (21). Valladares *et al.* found that this decreased IDO activity in the gut consequently reduced peripheral kynurenine levels and increased ileum and peripheral serotonin levels (21). These

findings are supported by another mice study conducted by Marin *et al.* in 2017. Marin *et al.* used *L. reuteri* instead of *L. johnsonii* for their probiotic feeding in mice, because *L. reuteri* has been shown to colonize several vertebrate hosts, and was shown to improve despair and anxiety-like behaviors in mice (22). Their results show increased *ido1* expression in the intestines after stress, which is decreased after *L. reuteri* treatment (22). Marin *et al.* found that chronic stress significantly altered intestinal microbiota composition, primarily by decreasing the abundance of *Lactobacillus*. In addition, they observed increased circulating kynurenine levels in stressed mice (22). Ultimately, Marin *et al.* found that treatment with *L. reuteri* alleviates despair behavior by directly inhibiting intestinal *ido1* expression and decreasing the circulating level of kynurenine, which is a metabolite associated with depression (23).

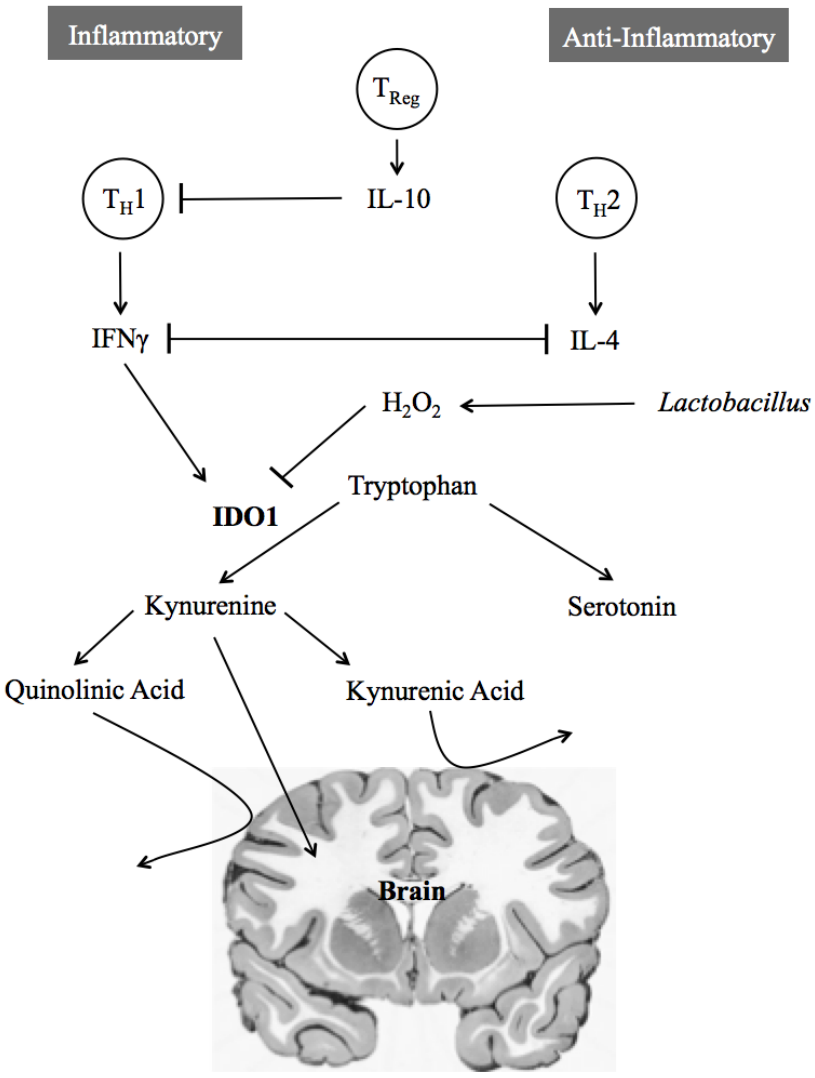

Figure 3. The kynurenine pathway & the link to inflammation and the gut-brain axis. Image of the brain adapted from reference 24.

The kynurenine pathway appears to be a compelling mechanism mediating the gut microbiota's influence on the brain (Figure 3). Our gut microbes, specifically *Lactobacillus,* are involved in the mechanism by producing H_2O_2 that directly inhibits IDO1, the enzyme that converts tryptophan to kynurenine. Because kynurenine has been associated with depression, this pathway connects to the brain (23). More specifically, kynurenine can readily cross the blood-brain barrier to drive depression in the CNS by disrupting

neurotransmitter balance and driving neuroinflammation (25, 26). Thus, disruptions in tryptophan-kynurenine metabolism are an important factor in mediating despair behavior (22). The downstream metabolites of kynurenine, kynurenic acid and quinolinic acid, cross the blood-brain barrier very poorly and must be formed locally within the brain (25, 27). Quinolinic acid has been found to promote local inflammation within the CNS and modulate neuronal excitability, thus it can influence the brain in alternate ways (28). Kynurenic acid is protective against this neuronal excitotoxic action of quinolinic acid, and is considered neuroprotective (29). Thus, the downstream metabolites of kynurenine are not directly associated with depression, however, they influence the body in different ways that are both beneficial and harmful. Another important aspect of the kynurenine pathway is that IDO can also catalyze the conversion of 5-HT (serotonin) to 5-hydroxykynuramine, meaning increased IDO activity has been correlated with increased serotonin turnover (21). This is an important consideration, as lower IDO activity alone could result in both decreased kynurenine and increased serotonin concentrations. This creates a mechanism in which depressive behavior could be influenced via a two-pronged approach that begins with gut microbiota. It is uncertain whether or not this mechanism functions in the human body as studies have only been in mice.

General Direction of Study: Proposing a Probiotic Intervention in Humans

I am proposing to study a probiotic intervention in humans, by measuring the effect of probiotics (*Lactobacillus reuteri*) on metabolite concentrations. These metabolites are involved in the kynurenine pathway, which is a pathway that has been associated with depression in mice (16, 21, 22). The majority of studies in mice have focused on total

circulating tryptophan levels with less attention given to the dynamics of tryptophan metabolism along the kynurenine pathway (16, 30). This study would look directly at tryptophan metabolism along the kynurenine pathway by measuring metabolites downstream of this pathway.

Aims

An aim of this study is to assess whether *L. reuteri* inhibits IDO expression in humans, as seen in Valladares *et al.*'s mouse study, and if this probiotic feeding influences tryptophan metabolism down the kynurenine pathway. Another goal is to further understand the impact of *L reuteri* in the human body and its role in depression.

Predictions

I predict that if individuals are fed *L. reuteri* for 30 days, then tryptophan metabolism along the kynurenine pathway will be affected. More specifically, I predict that in group 2, patients taking the *L. reuteri* supplement, there will be lower levels of IDO expression, kynurenic acid, quinolinic acid, kynurenine, c reactive protein and IFNγ, and higher levels of tryptophan, serotonin, interleukins (IL-4 and IL-10) and H_2O_2. Thus, I predict that there will be less tryptophan metabolism down the kynurenine pathway in group 2 compared to group 1 (control/placebo group). In addition, I predict there will be higher levels of serotonin and lower levels of kynurenine per each patient in group 2, which will consequently impact their mood given previous findings in this pathway, thus, there will be lower levels of depression according to the PHQ-9 among group 2. I predict that at day 30, the microbiota community structure of individuals in group 2 will be substantially different, with a higher

concentration of *Lactobacillus* in the gut in comparison to group 1. I also predict that microbiota community structure will be indistinguishable between both groups at day 0 and group 1 on day 30.

Methods

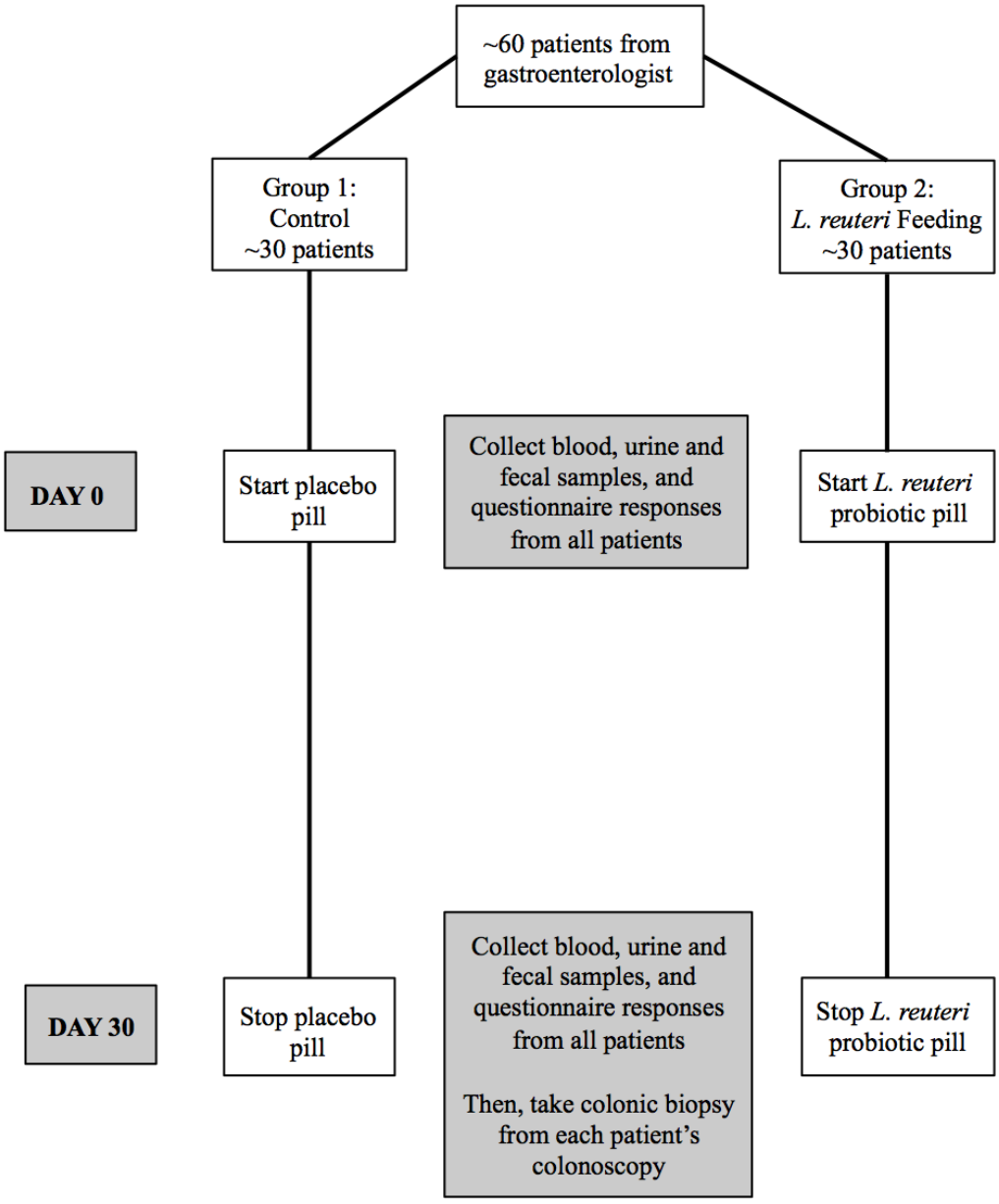

Figure 4. Clinical study timeline

I will collect urine, feces and blood samples at the beginning of my study from all patients. I would partner with a gastroenterologist who is performing colonoscopies to gather my sample for this study. I would recruit patients of the gastroenterologist who were already scheduled for colonoscopies, and who were willing to participate and donate samples. This sample would hopefully be at least 60 people, and then patients would be randomized into two separate groups. Ideally each group of 30 is a random sample of people representative of the broader population, yet with a similar makeup across the 2 groups. I would hope to have a sample size of at least 60 people total, which is based on previous mouse and human studies (11, 21, 22). Most mouse studies have a sample size of 20-30 mice total, and most human studies have a sample size of 60-70 humans total. Thus, I hope to get at least 60 people enrolled in my study, which would then allow 30 people at the minimum in each group.

Group 1: Roughly 30 people who will be taking a placebo pill = control group

Colonoscopy= Day 30

Group 2: Roughly 30 people who will be taking the *L. reuteri* probiotic

Colonoscopy= Day 30

I chose to administer a probiotic to group 2 of solely *L. reuteri*, as it has been shown to directly inhibit IDO1 in the kynurenine pathway in mice (22). In addition, *L. reuteri* is a species that colonizes several vertebrate hosts, including rodents and humans, and was shown to improve depression and anxiety-like behaviors, including the forced swim test in mice (10, 22, 31). 2×10^9 CFU/mL of *L. reuteri* will be given to group 2 in oral capsule form for 30 days. Amount of CFUs given per milliliter was determined based on previous studies in mice

and humans (11, 22). *L. reuteri* would be obtained from ATCC (23272) (22), and then encapsulated for oral consumption. Group 1, the control group, will be given a placebo of identical taste and appearance as the probiotic, for 30 days (11). All patients will be advised to take their oral pill during or just after breakfast, and will be reminded through a daily text message. Study compliance would be assessed by counting the number of pills returned by participants at the end of the study (11).

Samples would be collected again at 30 days. These samples would be urine, feces and blood samples again from both groups, and I would collect colonic biopsies from their colonoscopies after taking the other samples (Figure 4). I would then quantify metabolite concentrations using the methods described below.

Blood Serum Sample Analysis

All blood samples would be collected from the patient's vein in their arm. For analyzing whole blood, blood would be collected into glass Vacutainer Tubes containing disodium EDTA (32). Platelets would be counted with a Model S plus 4 Coulter Counter and then whole blood would be apportioned into 1 mL aliquots, stored at -20˚C and would be analyzed within 1 week after collection (32). For analyzing serum, blood would be collected into a covered test tube that is prepped for serum analysis. This sample would be centrifuged at 2000 g for 10 minutes at 4˚C, and apportioned into 0.5 mL aliquots and stored at -80˚C (21) until ready to be analyzed. These individual aliquots then allow for various serum sample analyses later on, and provide several aliquots to pull from, especially if a different analysis is desired much farther in the future.

To measure kynurenine, tryptophan and kynurenic acid levels in blood samples, I would use reverse-phase high performance liquid chromatography (HPLC), as described previously (30). In short, this involves using blood serum and a HPLC procedure with ultraviolet and fluorimetric detection to separate and quantify tryptophan, kynurenine and kynurenic acid. It has the ability to measure other kynurenine metabolites, but has not been expanded to measure quinolinic acid yet (30). Because Badawy's method is unable to measure quinolinic acid, I would use a gas chromatography/mass spectrometry (GC/MS) assay to measure quinolinic acid in whole blood as described previously by Heyes and Markey (33). This experiment used whole blood samples, rather than serum samples, as it was found to be more effective in the GS/MS assay. In short, this GS/MS assay includes sample analysis through Finnigan 3200 chemical ionization quadrupole mass filter with Extrel electronics and a Teknivent data system 1050, and quinolinic acid is analyzed by selected ion monitoring (33). In addition, I would use HPLC with amperometric detection for determination of serotonin in whole blood, as described previously (32). This experiment was also found to be most effective using whole blood samples, rather than serum samples. In short, this HPLC includes a Model 510 HPLC pump, a Model SIL 9A autosampler from Shimadzu Analytical Division and a Model 460 amperometric detector equipped with a glassy carbon electrode maintained at a potential of +0.6V (32). In addition, I would measure inflammation through c reactive protein levels in blood serum, which can be done using a Human C Reactive Protein ELISA Kit by following the manufacturer's instructions (Thermo Fischer KHA0031, Walthan, MA).

Urine Sample Analysis

I would measure kynurenic acid and tryptophan levels in urine samples using reverse-phase HPLC as described previously by John B. Tarr (34). In short, this method includes a Model 6000A pumping system, Model 440 ultraviolet absorbance detector set at 254 nm, and Model R40l differential refractometer, and all chromatograms are obtained at ambient temperature (around 20°C) (34). Tarr's method, however, was unable to measure quinolinic acid levels in urine, because the conditions used in Tarr's analysis gave too broad of an elution for quinolinic acid for it to be of analytical use. So I would measure quinolinic acid levels through a process described by McDaniel *et al.* (35). This process involves bringing urine samples to pH 1.4 with hydrochloric acid and then adding 3 grams of Norit-A to each 50mL aliquot of urine. Norit-A is an activated carbon that is a great adsorbent and is commonly used in purification processes and the removal of dyes (36). This addition of Norit-A creates a charcoal suspension that is then filtered out and ammonium hydroxide is added. The ammonium hydroxide filtrate is evaporated to dryness over a 50°C water bath, and this dry residue is then taken up in 5 mL of glacial acetic acid, and heated at 105°C. After cooling, 1 mL of the acetic acid is then brought to pH 7.3 by the addition of sodium hydroxide. 3 mL of 1M Tris-HCl is added to the neutralized acetic acid solution and the final volume is brought to 10 mL with distilled water. 1 mL of this solution is then placed in a tube with ethanol, cyanogen bromide and *o*-toluidine. Absorbance of this solution is compared to that of the blank at 450nm using a Beckman DU or Zeiss spectrophotometer (35). Ultimately, this protocol achieves the desired chemical reaction, the decarboxylation of quinolinic acid to nicotinic acid, which is necessary to determine the quinolinic acid concentration in urine (35). Urinary serotonin would be purified by two successive cationic and anionic extraction

steps, then serotonin levels would be measured under the same chromatographic conditions as used for quantifying blood serum levels, which was described previously by Pussard *et al.* (32). All urine samples would be collected and then frozen at −20 °C and transferred to lower storage temperatures (−80 °C) within one week (37). Total protein concentration in each patient's urine sample would be measured using a Bradford assay (Bio-Rad Laboratories, Hercules, CA) following the manufacturer's instructions. Each patient's tryptophan measurement would then be compared to their total protein concentration in order to obtain the amount of tryptophan per microgram of protein in the urine. This would be done with each measurement from the urine, so kynurenic acid, quinolinic acid and serotonin as well. This ratio then allows for a more accurate comparison across patients, as it accounts for confounding variables, for example if someone just drank alcohol, their protein levels would be much higher than another patient.

Fecal Matter Sample Analysis

I would assess bacterial community structure by first performing 16S rRNA sequencing on genomic DNA isolated from fecal samples. Whole genomic DNA would be isolated via phenol-chloroform extraction as described by Marin *et al.* (22). For 16S rRNA sequencing, the V3-V4 region of the 16S rRNA gene would be amplified using polymerase chain reaction (PCR) for 25 cycles using specific primers (forward 5'-TCGTCGGCAGCGTCAGATGTGTA TAAGAGACAGCCTACGGGNGGCWGCAG- 3' and reverse 5'- GTCTCGTGGGCTCGGAGATGTGTA TAAGAGACAGGACTACHVGGGTATCTAATCC-3') as per the Illumina library preparation guide as explained in the supplemental figures by Marin *et al.* (22). DNA sequences will be processed in a data curation pipeline, such as mothur (38), that will remove

all sequences with indicators of low sequence confidence, such as those with a read quality score of under 25, sequences that contain ambiguous characters or contain more than four mismatches to the reverse primer sequence, or sequences that are less than 200 nucleotides or greater than 600 nucleotides (39). The sequences are then clustered into operational taxonomic units (OTUs), which are based on sequence identity (%ID), and various thresholds of sequence identity are used to represent different taxonomic levels (e.g., 97% ID for species, 95% for genera) (40). Using each patient's compiled list of OTUs (from fecal samples) and their relative abundance (assigned a value between 0 and 1), a similarity matrix would be created to further reduce the complexities of each patient's microbiota community structure and facilitate statistical analysis. I would then use principal coordinates analysis (PCoA) to visualize similarities and dissimilarities of data across patients (40).

I would measure H_2O_2 (reactive oxygen species, ROS) in feces using the Amplex Red hydrogen peroxide/peroxidase assay kit (Thermo Fischer) according to the manufacturer's protocol. For ROS production by individual *L. reuteri* species, fecal *L. reuteri* would be cultured, and individual colonies would be selected and dissociated as described by Marin *et al.* (22), and then quantified using the Amplex Red hydrogen peroxide/peroxidase assay kit as described above.

I would prep all fecal samples by collecting the fresh sample, weighing it, and then resuspending part of it in sterile phosphate-buffered saline (PBS) (22). After brief sedimentation of insoluble particles, part of this bacterial slurry would be incubated at 37˚C for 30 minutes (22). After bacterial culture centrifugation, the supernatant would be reacted with the Amplex Red assay kit. Otherwise, fecal matter samples will be stored at -80˚C for microbiota composition assessment.

Colonic Biopsy Analysis

Each patient will have a colonoscopy at 30 days, and during the colonoscopy, the gastroenterologist will take a tissue sample from the colon. Half of this tissue sample will then be preserved in RNA*later*, which is a tissue storage reagent that stabilizes and protects cellular RNA, in order to analyze IDO expression (21). The other half of this tissue sample will be homogenized using a Tissue Master 125 (Omni International, Kennesaw, GA) with protease inhibitors (Sigma-Aldrich, St. Louis, MO) in order to analyze levels of inflammatory cytokines and chemokines (21). All samples will be stored at -80˚C until processing. For RNA quantification, frozen tissues would be homogenized by bead beating in RNA TRI Reagent (Life Technologies, Carlsbad, CA) and RNA would be extracted according to manufacturer's protocol (22). IDO expression would then be measured using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), as described by Valladares *et al.* (21). In short, the desired RNA template would be copied into a complementary DNA (cDNA) using a retroviral reverse transcriptase, and then this cDNA is amplified exponentially by PCR. cDNA would be synthesized using iScript cDNA synthesis kit (Bio-Rad Laboratories), by following the manufacturer's instructions (21). qRT-PCR would then be performed using an iCycler detection system (Bio-Rad Laboratories) using 200nM concentrations of each primer, and following the manufacturer's instructions (21). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) would be used as an internal control. Primers used would be: human IDO, forward 5'-GGTCATGGAGATGTCCGTAA-3' and reverse 5'-ACCAATAGAGAGACCAGGAAGAA-3', and human GAPDH forward 5'-ACCACAGTCCATGCCATCAC-3' and reverse 5'-TCCACCACCCTGTTGCTGTA-3' (21). Reaction conditions would be the same as those used by Valladares *et al*. Data from the

iCycler detection system (Bio-Rad Laboratories) would be analyzed with iCycler software using the cycle threshold values (C_t) method of relative transcript analysis ($2^{-\Delta\Delta Ct}$) to determine the relative quantification (21). For determining levels of inflammatory cytokines and chemokines [IFNγ, interleukin (IL-4 and IL-10)] in tissue samples, I would use the homogenized tissue sample with protease inhibitors and a tissue lysate enzyme-linked immunosorbent assay (ELISA). I would specifically use the Human Inflammatory Cytokines Multi-Analyte ELISArray Kit from QIAGEN (Hilden, Germany), which is a kit that was similarly used by Valladares *et al.* in their mouse study (21). Total protein concentration in each patient's colonic biopsy sample would be measured using a Bradford assay (Bio-Rad Laboratories) following the manufacturer's instructions, as described above with urine samples. Each patient's IFNγ, IL-10 and IL-4 measurements would then be compared to their total protein concentration in order to obtain the amount of IFNγ, IL-10 or IL-4 per microgram of protein in the tissue sample.

Questionnaire

I would use the Patient Health Questionnaire (PHQ-9) (41) to test for depression-like symptoms pre and post treatment. This questionnaire allows for a quantitative measurement of depression. I would also propose a questionnaire to test for confounding variables, such as smoking, drinking, diet, stress levels, general physical activity levels, and any prescription drugs each patient is taking. This questionnaire would be given to patients on days 0 and 30 to assess for confounding variables but also to test whether there was any variance or change in 30 days. I plan on first using this questionnaire to assess whether someone needs to be removed from the study. The criteria for a participant's removal are: daily consumption of a significant amount of pre- and probiotics, daily consumption of five or more alcoholic

beverages, use of illegal drugs, or any indication that they will not reliably take their given pill for thirty days. Any other factor indicating that a participant would skew results is also grounds for removal from the study. I would then adjust for the remaining confounding variables through stratification. Stratification is a method in which the data set is broken into a number of subsets, called strata, corresponding to the number of potential confounders (e.g., smoking and stress levels) (42). The association between treatment (group 1 or 2) and time (day 0 or 30) with metabolite concentration is evaluated within each stratum of the confounder, for each metabolite (43). This then calculates a stratum-specific estimate, and makes it evident whether a factor introduces confounding in the analysis (42). The Mantel-Haenszel method can then be used to provide an adjusted result according to strata, by calculating a weighted average of them (44). This calculated adjusted result would then be reported and discussed as a limitation of the study.

Statistical Analysis

For each group, I would perform a t-test between day 0 and day 30 for every metabolite (between column 1 and 2 in Table 1 below), not including microbiota composition and findings from the colonic biopsy. All metabolite measurements across patients in each group would be averaged and then statistically analyzed. I would perform a t-test for average findings for each metabolite between groups 1 and 2 on day 0 and on day 30. Figure 5 describes the general overview for which t-tests I would perform.

Table 1. Samples and average measurements from each group.

Average Metabolite Level on Day 0	Average Metabolite Level on Day 30	Sample
Serotonin	Serotonin	**Blood**
Kynurenine	Kynurenine	
Tryptophan	Tryptophan	
Kynurenic acid	Kynurenic acid	
C reactive protein	C reactive protein	
Tryptophan	Tryptophan	**Urine**
Kynurenic acid	Kynurenic acid	
Quinolinic acid	Quinolinic acid	
Serotonin	Serotonin	
Microbiota composition	Microbiota composition	**Feces**
H_2O_2	H_2O_2	
N/A	IDO expression	**Colonic Biopsy**
N/A	IFNγ	
N/A	Interleukins • IL-4 • IL-10	
Patient Health Questionnaire (PHQ-9)	Patient Health Questionnaire (PHQ-9)	**Questionnaire**
Confounding variables questionnaire	Confounding variables questionnaire	

Figure 5 shows expected t-test findings between the groups for each metabolite if my hypotheses were supported (see expected results section for further analysis). For example, Figure 5 shows average measurements for kynurenine across groups 1 and 2.

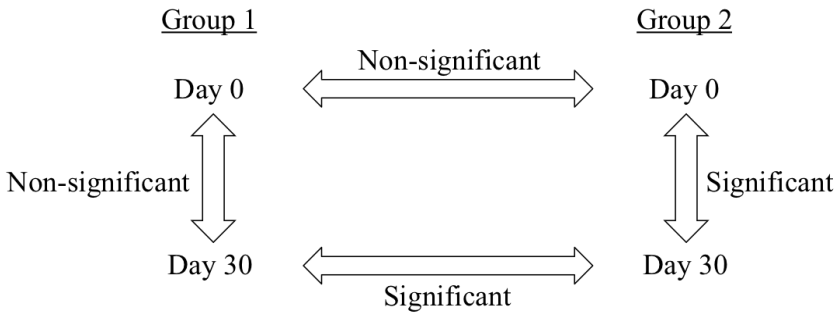

Figure 5. Statistical analysis of average findings for each metabolite. Also, the expected t-test findings for each metabolite between groups 1 and 2 if my hypotheses were supported.

A permutational multivariate analysis of variance (PERMANOVA) would be used to assess microbiota composition across individuals, which will compare the similarity scores given for each individual in the similarity matrix (as described in fecal matter sample analysis above) (45). PERMANOVA will indicate if microbiota community structure is significantly different between groups 1 and 2. More specifically, it will provide further information on whether treatment or time affects the p-value, and which is more significant. In addition, I would use principal coordinates analysis (PCoA) to visualize dissimilarities between bacterial communities across patients. I would be able to see if there is a difference between individuals on day 30 after taking *L. reuteri* and individuals on day 30 after taking the placebo. Additionally, I will be able to use the PCoA ordination to discover the species and factors that strongly correlate to the patterns seen across treatment groups by using key indicator species analysis (46). Using key indicator species analysis, it is possible to identify which treatment (group 1 vs. 2), time (day 0 vs. 30), and metabolite is driving this shift in microbiota.

Ultimately the findings from the colonic biopsies would be additional support to the other samples (feces, urine & blood), as they are more difficult to collect, so there will likely

be a higher failure rate. I would plan on correlating findings of IDO expression, IFNγ, IL-10 and IL-4, with findings from the other samples for each individual. This would be done through calculating the average level of IDO expression, IFNγ, IL-10 and IL-4 across groups, and then performing a t-test between group 1 and 2 for each finding to test for statistical significance.

Expected Results

If my hypotheses were supported, I expect certain levels of metabolites to be higher than others, and some t-tests to result in p-values that are significant and others with p-values that are non-significant. I expect a significant difference for every metabolite between groups 1 and 2 on day 30 (Figure 5 in methods). In addition, I expect a significant difference within group 2 on day 0 vs. 30 for every metabolite (Figure 5). However, I expect no significant difference between groups on day 0, and within group 1 between day 0 and 30 (Figure 5). Table 2 shows the expected average metabolite level in group 2 day 30, where I expect there to be a statistically significant difference when compared to both groups at day 0, and when compared to group 1 at day 30. Overall, I suspect metabolite levels in group 2 day 30 to be substantially different (Table 2). I chose to measure some metabolites in multiple samples throughout my study, so for these, serotonin, kynurenine, tryptophan and kynurenic acid, I have chosen to label them with varying degrees of expected increase or decrease in findings. + indicates an increase, and – indicates a decrease, in metabolite level as compared to group 1 and 2 day 0, and as compared to group 1 day 30 (Table 2). ++ indicates an even greater increase, whereas - - indicates an even greater decrease (Table 2).

Table 2. Expected average metabolite concentration level in group 2 on day 30 as compared to group 2 on day 0 and group 1 on day 0 and 30.

Metabolite	**Expected Average Change in Metabolite Concentration (+/-)**
Serotonin from blood sample	+
Serotonin from urine sample	++
Kynurenine from blood sample	-
Kynurenine from urine sample	- -
Tryptophan from blood sample	+
Tryptophan from urine sample	++
Kynurenic acid from blood sample	-
Kynurenic acid from urine sample	- -
C reactive protein	-
Quinolinic acid	-
H_2O_2	++
IDO expression	-
IFNγ	-
Interleukins • IL-4 • IL-10	 + +
Patient Health Questionnaire (PHQ-9) numerical results	-

In general, I expect kynurenine pathway metabolite levels to be higher in urine than in blood (47). A critical aspect of Table 2 is that I expect lower IDO expression and lower levels of kynurenine, and higher tryptophan and serotonin levels in the group of patients that have been taking *L. reuteri* as compared to the placebo group (21, 22). This would imply that the *L. reuteri* feeding was effective in influencing tryptophan metabolism down the

kynurenine pathway, and a potential reason for this is that *L. reuteri* produces H_2O_2, which inhibits IDO and consequently results in higher levels of tryptophan and serotonin, and lower levels of kynurenine.

I expect to see a reduction in depression attributable to a certain treatment. My expected results consistent with this would be from the PHQ-9 responses. I expect to see lower levels of depression in group 2 day 30 as indicated through the numerical value from the questionnaire. I would then correlate this finding to serotonin and kynurenine levels. I would correlate these findings using a linear regression to obtain slope, r^2 and r, the Pearson product-moment correlation coefficient. I would then use Vassar stats (http://vassarstats.net/) to test for the significance of the Pearson product-moment correlation coefficient. I expect to see significance between low questionnaire values and low levels of kynurenine, and low questionnaire values with high levels of serotonin.

I expect to see a significant change in community structure due to probiotic feeding. My expected results consistent with this would be a significant shift in community structure on day 30 for group 2, as compared to group 2 day 0, and group 1 day 0 and 30. This expected shift is depicted in Figure 6. I would expect to see more *L. reuteri* present in the stool samples of individuals from group 2 on day 30, than individuals from group 1 on day 0 and 30, and from group 2 on day 0.

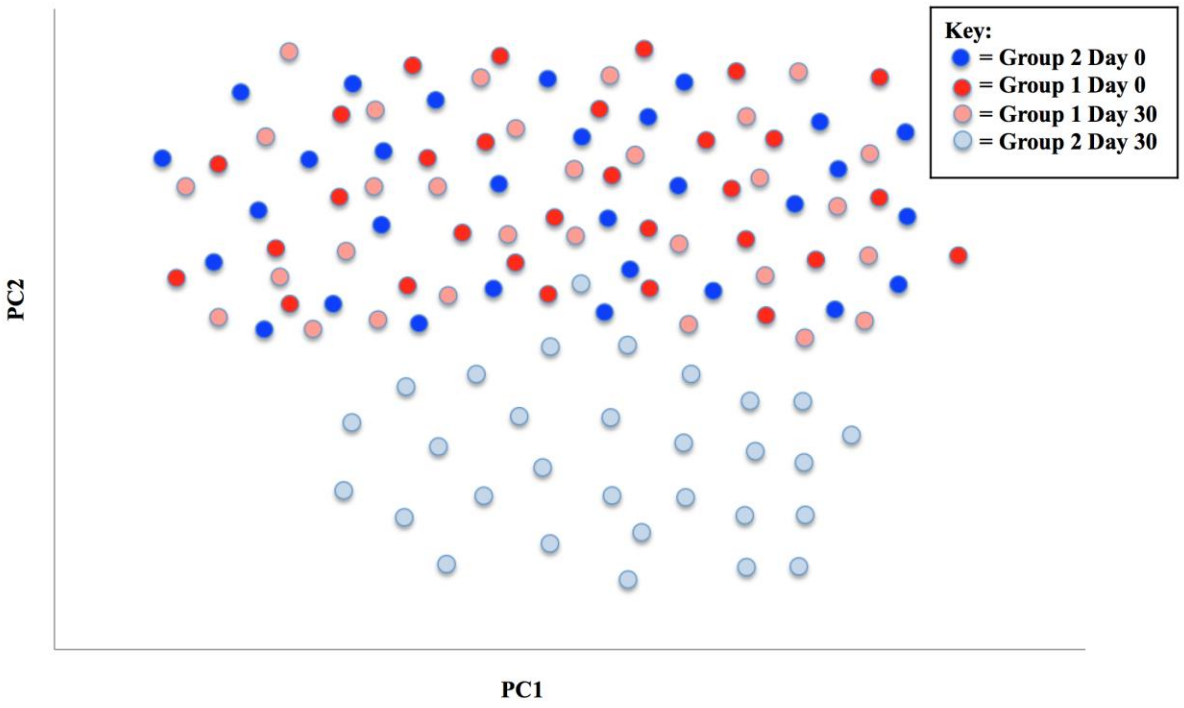

Figure 6. Expected experimental ordination of microbial communities for both treatments on day 0 and day 30 (n=30). Community structure is expected to shift in group 2 on day 30 after probiotic consumption.

Discussion

If my hypotheses were supported, then this would indicate that the mouse findings from Valladares *et al*., and Marin *et al.* could be translated to humans. This study would give a greater glimpse into a mechanism behind this gut-brain axis in humans. If findings support my hypotheses, then the kynurenine pathway proves to be a significant pathway in understanding how our gut microbes influence depression. In addition, *L. reuteri* would prove to be influential in this axis and play a role in depression. This could broaden the view on probiotics as one that is not just focused on the gut, but also on the brain.

This study contributes measurements of specific markers using several samples, such as H_2O_2 in feces or kynurenine levels in urine or blood, which could be valuable for other purposes, such as prognosis or monitoring changes throughout drug or probiotic intervention. The results from this study would be valuable in establishing a mechanistic connection between the gut and the brain. Additionally, the kynurenine pathway could serve as a target for drugs or other therapies in treating depression. This would especially be true if the link to depression is supported through the PHQ-9 questionnaire responses, because this would open the opportunity to view depression in an entirely different way than the current view in the medical field and society. Antidepressants would no longer be the main way to ease depression symptoms, and this pathway could serve as a way to truly cure and treat depression. If gut microbes and tryptophan metabolism along the kynurenine pathway play significant roles in depression, then the emphasis previously placed on the brain would lessen. Depression would no longer be viewed as a chemical imbalance in the brain, but instead an imbalance in gut microbiota.

Complicating Factors

This study contains a few complicating factors that are uncontrolled. First, compliance with taking the probiotic treatment will be facilitated through counting pills returned at the end of the study, but this could allow patients to throw out or keep pills in order to make it seem like they took it every day, when they really did not. Another important consideration in my study is the role of confounding variables on my results. I plan on assessing for confounding variables through the questionnaire given at the beginning and end of my study, however, these confounding variables could influence my data. For

example, the diet of each patient will be not be controlled or restricted, meaning that the occasional consumption of prebiotic and probiotic rich foods could influence metabolite levels and PHQ-9 findings. It would be detrimental to my study if a patient in group 1 consumed prebiotic and probiotic rich foods, because their microbiota and metabolite levels may look similar to a patient in group 2. Other variables such as physical activity and moderate alcohol consumption could influence my data as well. Another influencing factor is the level of depression among patients in group 1 and 2 on day 0. I plan to randomize my sample into two groups, however, this does not account for people who are depressed. Major depressive disorder (MDD) affects about 6.7% of the U.S. population, and is more prevalent in women than in men (48). 6.7% of my 60-person sample size is 4 people, so I calculated the binomial probability of my sample to contain 4 people with MDD. This resulted in the mere chance probability of 20.21% for exactly 4 people in my study with MDD. In addition, there is 42.27% probability of having 4 or fewer people with MDD, and 37.52% probability of having 4 or more people in my study with MDD. I also calculated the binomial probability of my sample to contain one person with MDD, because having only one person with MDD in my study would most significantly impact my results, as one of the two groups would have zero people with MDD. This binomial probability resulted in the mere chance probability of 6.7% for exactly 1 person in my study with MDD. In addition, there is 1.5% probability of having 1 or fewer people with MDD, and 91.8% probability of having 1 or more people in my study with MDD. Thus I would expect between 1-4 people in my study to have MDD, so upon randomizing people into two groups, this randomization could potentially lead to an uneven distribution of people with depression across the two groups, which would influence my results, especially when looking at PHQ-9 outcomes and metabolite levels. Ultimately, I

expect my sample to contain people with major depressive disorder, which is an important factor that will not be controlled for. Finally, another important influencing factor is that people coming in to get colonoscopies and regularly checking in with a gastroenterologist may not be representative of the general population. This is because people who are coming in to get colonoscopies are likely of a certain age, health status and socioeconomic status. These patients have the money and time to access a gastroenterologist, and are typically around 50 years old. Additionally, women are more likely than men to have a colonoscopy (49), so there may not be an even split among males and females in my sample. If my sample group is not representative of the general population, then my data can not necessarily be extrapolated for broader medical purposes.

Future Directions

The gut-brain axis and its link to depression is an increasingly popular topic of study and conversation. However, research on this link is limited, especially in humans. Most studies are limited to manipulation of rodents, which makes it difficult to apply these findings to humans, and consequently impact the medical field. My study proposes a probiotic intervention in humans, with the hope of getting a greater glimpse into the role the kynurenine pathway plays in humans and the link between gut microbes and depression. There are many areas that future researchers could explore in order to learn about and utilize this relationship further.

A future direction is to look further into the kynurenine pathway and probiotics in younger populations, as my sample will primarily contain people over 50 years old. These

studies would not need to be so comprehensive, and could instead analyze feces or urine samples in different populations to look at tryptophan metabolism along the kynurenine pathway. In addition, I anticipate that IDO expression may not be statistically significant with a small group of people, thus if this proves to be true, then a future direction for this study would be to add more people to obtain a greater sample size to analyze IDO expression in order to see statistical significance. Probiotic feeding of a larger sample size is recommended, and also to use other strains of bacteria besides *L. reuteri* for probiotic consumption. There is a lot of uncertainty surrounding which genera or species of bacteria are most beneficial in this axis' link to depression, and what they specifically do that proves to be beneficial. Future research must focus on deciphering which bacteria influence the kynurenine pathway, and if a probiotic formulation of such bacteria can serve as a therapeutic strategy for mood disorders.

To conclude, this study holds the potential to demonstrate that the kynurenine pathway is a critical link between the gut microbiome and depression in humans. Additionally, this study could find that a thirty-day *L. reuteri* probiotic intervention can influence tryptophan metabolism down the kynurenine pathway to increase circulating levels of serotonin and tryptophan in the body, and decrease levels of kynurenine, which all contribute to decrease levels of depression in an individual. An emphasis on the microbiome in the treatment of depression could drastically alter the use of antidepressants and work towards curing depression, rather than simply alleviating symptoms.

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Appendix

Patient Health Questionnaire (PHQ-9) (41)

STABLE RESOURCE TOOLKIT

The Patient Health Questionnaire (PHQ-9) - Overview

The PHQ-9 is a multipurpose instrument for screening, diagnosing, monitoring and measuring the severity of depression:

- The PHQ-9 incorporates DSM-IV depression diagnostic criteria with other leading major depressive symptoms into a brief self-report tool.
- The tool rates the frequency of the symptoms which factors into the scoring severity index.
- Question 9 on the PHQ-9 screens for the presence and duration of suicide ideation.
- A follow up, non-scored question on the PHQ-9 screens and assigns weight to the degree to which depressive problems have affected the patient's level of function.

Clinical Utility

The PHQ-9 is brief and useful in clinical practice. The PHQ-9 is completed by the patient in minutes and is rapidly scored by the clinician. The PHQ-9 can also be administered repeatedly, which can reflect improvement or worsening of depression in response to treatment.

Scoring

See PHQ-9 Scoring on next page.

Psychometric Properties

- The diagnostic validity of the PHQ-9 was established in studies involving 8 primary care and 7 obstetrical clinics.
- PHQ scores ≥ 10 had a sensitivity of 88% and a specificity of 88% for major depression.
- PHQ-9 scores of 5, 10, 15, and 20 represents mild, moderate, moderately severe and severe depression.[1]

The Patient Health Questionnaire (PHQ-9) Scoring

Use of the PHQ-9 to Make a Tentative Depression Diagnosis:
The clinician should rule out physical causes of depression, normal bereavement and a history of a manic/hypomanic episode

Step 1: Questions 1 and 2
Need one or both of the first two questions endorsed as a "2" or a "3"
(2 = "More than half the days" or 3 = "Nearly every day")

Step 2: Questions 1 through 9
Need a total of five or more boxes endorsed within the shaded area of the form to arrive at the total symptom count. (Questions 1-8 must be endorsed as a "2" or a "3"; Question 9 must be endorsed as "1" a "2' or a "3")

Step 3: Question 10
This question must be endorsed as "Somewhat difficult" or "Very difficult" or "Extremely difficult"

Use of the PHQ-9 for Treatment Selection and Monitoring
Step 1
A depression diagnosis that warrants treatment or a treatment change, needs at least one of the first two questions endorsed as positive ("more than half the days" or "nearly every day") in the past two weeks. In addition, the tenth question, about difficulty at work or home or getting along with others should be answered at least "somewhat difficult"

Step 2
Add the total points for each of the columns 2-4 separately
(Column 1 = Several days; Column 2 = More than half the days; Column 3 = Nearly every day. Add the totals for each of the three columns together. This is the Total Score
The Total Score = the Severity Score

Step 3
Review the Severity Score using the following TABLE.

PHQ-9 Score	Provisional Diagnosis	Treatment Recommendation ***Patient Preferences should be considered***
5-9	Minimal Symptoms*	Support, educate to call if worse, return in one month
10-14	Minor depression ++ Dysthymia* Major Depression, mild	Support, watchful waiting Antidepressant or psychotherapy Antidepressant or psychotherapy
15-19	Major depression, moderately severe	Antidepressant or psychotherapy
>20	Major Depression, severe	Antidepressant and psychotherapy (especially if not improved on monotherapy)

** If symptoms present ≥ two years, then probable chronic depression which warrants antidepressants or psychotherapy (ask "In the past 2 years have you felt depressed or sad most days, even if you felt okay sometimes?")*

++ If symptoms present ≥ one month or severe functional impairment, consider active treatment

The Patient Health Questionnaire (PHQ-9)

Patient Name _____ Date of Visit _____

Over the past 2 weeks, how often have you been bothered by any of the following problems?	Not At all	Several Days	More Than Half the Days	Nearly Every Day
1. Little interest or pleasure in doing things	0	1	2	3
2. Feeling down, depressed or hopeless	0	1	2	3
3. Trouble falling asleep, staying asleep, or sleeping too much	0	1	2	3
4. Feeling tired or having little energy	0	1	2	3
5. Poor appetite or overeating	0	1	2	3
6. Feeling bad about yourself - or that you're a failure or have let yourself or your family down	0	1	2	3
7. Trouble concentrating on things, such as reading the newspaper or watching television	0	1	2	3
8. Moving or speaking so slowly that other people could have noticed. Or, the opposite - being so fidgety or restless that you have been moving around a lot more than usual	0	1	2	3
9. Thoughts that you would be better off dead or of hurting yourself in some way	0	1	2	3

Column Totals _____ + _____ + _____

Add Totals Together _____

10. If you checked off any problems, how difficult have those problems made it for you to Do your work, take care of things at home, or get along with other people?

☐ Not difficult at all ☐ Somewhat difficult ☐ Very difficult ☐ Extremely difficult