2020

AP CollegeBoard

# AP® Research Academic Paper

## Sample Student Responses and Scoring Commentary

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**Sample B**

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| The Response… | | | | |
|---|---|---|---|---|
| **Score of 1**<br>**Report on Existing Knowledge** | **Score of 2**<br>**Report on Existing Knowledge with Simplistic Use of a Research Method** | **Score of 3**<br>**Ineffectual Argument for a New Understanding** | **Score of 4**<br>**Well-Supported, Articulate Argument Conveying a New Understanding** | **Score of 5**<br>**Rich Analysis of a New Understanding Addressing a Gap in the Research Base** |
| Presents an overly broad topic of inquiry. | Presents a topic of inquiry with narrowing scope or focus, that is NOT carried through either in the method or in the overall line of reasoning. | Carries the focus or scope of a topic of inquiry through the method **AND** overall line of reasoning, even though the focus or scope might still be narrowing. | Focuses a topic of inquiry with clear and narrow parameters, which are addressed through the method and the conclusion. | Focuses a topic of inquiry with clear and narrow parameters, which are addressed through the method and the conclusion. |
| Situates a topic of inquiry within a single perspective derived from scholarly works **OR** through a variety of perspectives derived from mostly non-scholarly works. | Situates a topic of inquiry within a single perspective derived from scholarly works **OR** through a variety of perspectives derived from mostly non-scholarly works. | Situates a topic of inquiry within relevant scholarly works of varying perspectives, although connections to some works may be unclear. | Explicitly connects a topic of inquiry to relevant scholarly works of varying perspectives **AND** logically explains how the topic of inquiry addresses a gap. | Explicitly connects a topic of inquiry to relevant scholarly works of varying perspectives **AND** logically explains how the topic of inquiry addresses a gap. |
| Describes a search and report process. | Describes a nonreplicable research method **OR** provides an oversimplified description of a method, with questionable alignment to the purpose of the inquiry. | Describes a reasonably replicable research method, with questionable alignment to the purpose of the inquiry. | Logically defends the alignment of a detailed, replicable research method to the purpose of the inquiry. | Logically defends the alignment of a detailed, replicable research method to the purpose of the inquiry. |
| Summarizes or reports existing knowledge in the field of understanding pertaining to the topic of inquiry. | Summarizes or reports existing knowledge in the field of understanding pertaining to the topic of inquiry. | Conveys a new understanding or conclusion, with an underdeveloped line of reasoning **OR** insufficient evidence. | Supports a new understanding or conclusion through a logically organized line of reasoning **AND** sufficient evidence. The limitations and/or implications, if present, of the new understanding or conclusion are oversimplified. | Justifies a new understanding or conclusion through a logical progression of inquiry choices, sufficient evidence, explanation of the limitations of the conclusion, and an explanation of the implications to the community of practice. |
| Generally communicates the student's ideas, although errors in grammar, discipline-specific style, and organization distract or confuse the reader. | Generally communicates the student's ideas, although errors in grammar, discipline-specific style, and organization distract or confuse the reader. | Competently communicates the student's ideas, although there may be some errors in grammar, discipline-specific style, and organization. | Competently communicates the student's ideas, although there may be some errors in grammar, discipline-specific style, and organization. | Enhances the communication of the student's ideas through organization, use of design elements, conventions of grammar, style, mechanics, and word precision, with few to no errors. |
| Cites **AND/OR** attributes sources (in bibliography/ works cited and/or in-text), with multiple errors and/or an inconsistent use of a discipline-specific style. | Cites **AND/OR** attributes sources (in bibliography/ works cited and/or in-text), with multiple errors and/or an inconsistent use of a discipline-specific style. | Cites **AND** attributes sources, using a discipline-specific style (in both bibliography/works cited **AND** in-text), with few errors or inconsistencies. | Cites **AND** attributes sources, with a consistent use of an appropriate discipline-specific style (in both bibliography/works cited **AND** in-text), with few to no errors. | Cites **AND** attributes sources, with a consistent use of an appropriate discipline-specific style (in both bibliography/works cited **AND** in-text), with few to no errors. |

# Academic Paper

## Overview

This performance task was intended to assess students' ability to conduct scholarly and responsible research and articulate an evidence-based argument that clearly communicates the conclusion, solution, or answer to their stated research question. More specifically, this performance task was intended to assess students' ability to:

- Generate a focused research question that is situated within or connected to a larger scholarly context or community;
- Explore relationships between and among multiple works representing multiple perspectives within the scholarly literature related to the topic of inquiry;
- Articulate what approach, method, or process they have chosen to use to address their research question, why they have chosen that approach to answering their question, and how they employed it;
- Develop and present their own argument, conclusion, or new understanding while acknowledging its limitations and discussing implications;
- Support their conclusion through the compilation, use, and synthesis of relevant and significant evidence generated by their research;
- Use organizational and design elements to effectively convey the paper's message;
- Consistently and accurately cite, attribute, and integrate the knowledge and work of others, while distinguishing between their voice and that of others; and
- Generate a paper in which word choice and syntax enhance communication by adhering to established conventions of grammar, usage, and mechanics.

# How can gut microbiota composition within SwHi and SwLo rats support or negate the Porsolt Swim Test?

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5,000 Words

## Abstract

Major Depressive Disorder (MDD) has become one of the most common mental disorders in the United States, illustrating the importance of antidepressant research. The Porsolt Swim Test (PST) is a rare assay intended to test experimental manipulations meant to stimulate or prevent depressive states for the purpose of evaluating antidepressant compounds. The volume of controversy over the reliability and interpretation of the test can be accredited to its basis on conditional stressors and lack of holistic consideration of behavior. But with its intentions and economical and noninvasive nature, it is a better model than many available, suggesting high potential and abilities for valuable reinstitution as a common assay. This research utilized the concept of the gut-brain axis and codependent relationship of the body with microorganisms by quantifying PST rat gut bacteria genera relevant to serotonin synthesis. The significant imbalances of relevant taxa identified in the gut compositions of the rats aligned with their behavioral phenotypes, validating the PST as a model for studying depression. This research provides support for reviving the PST as a common antidepressant research practice. Implicating many other applications, composition diversity and functions of microbial communities have potential to inform more personalized approaches to drug development strategies and augment treatment efficiency.


*Keywords:* Depression, Gut Microbiota, Porsolt Swim Test

# How can gut microbiota composition within SwHi and SwLo rats support or negate the Porsolt Swim Test?

## Background

In the United States, Major Depressive Disorder (MDD) has become one of the most common mental disorders. The National Institute of Mental Health defines depression according to the 5th edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5):

> A period of at least two weeks when a person experienced a depressed mood or loss of interest or pleasure in daily activities, and had a majority of specified symptoms, such as problems with sleep, eating, energy, concentration, or self-worth.

Following this definition, it was found that in 2017 an estimated 17.3 million Americans had at least one depressive episode— 7.1% of the population of all adults in the U.S. (National Institute of Mental Health [NIMH], n.d.).

The high prevalence of depression makes researching and developing antidepressants critical. Relevant to preclinical research, the purpose of the Porsolt Swim Test (PST) is to test experimental manipulations intended to stimulate or prevent depressive states and use those to evaluate the efficacy of antidepressant drugs and compounds. The PST has presently founded countless research papers. Characterized by a non-invasive nature and requiring minimal stress of the rodents involved, the essential idea is that rats are placed in a tank of water, and their mobility behavior relevant to escape is measured and observed. When compared to active swimming behaviors, passive floating behavior is considered a more depressed state, categorizing rodents as either SwHi (non-depressed) or SwLo (depressed) (Can et al., 2012; Yankelevitch-Yahav et al., 2015; Whitworth University IACUC guidelines, 2019).

## Literature Review

### The Porsolt Swim Test

The PST was optimized from previous versions by its namesake, Dr. Roger D. Porsolt, as an animal behavior model that would hopefully reflect depressive states *and* be sensitive to

known effective clinical antidepressant treatments. The study demonstrated significant reduction in immobile behaviors in rats after treatment with three different antidepressants with known effectiveness, leading to the conclusion that identifiable passive behaviors could be indicative of a state of despair (Porsolt, Le Pichon, & Jalfre, 1977). The discovery that active coping strategies could be induced in animals by antidepressants of known effectiveness resulted in the popularization of the model in research (Commons et al., 2017).

However, in years since, the model has been found to be selectively effective, opening consideration for questions regarding the validity of the PST as an antidepressant evaluation method.

**Controversy**

The largest concerns surrounding the test are ethicality and quality of the data the test yields. Reardon (2019) prescribed that a variety of factors could negatively influence the results of the Porsolt Swim Test results (i.e. despondency, learnt behaviors, and water temperature), citing findings of Neuroendocrinologist Ron de Kloet in Molendijk and de Kloet (2015) that "Many scientists will admit that the tests do not show what they should." Because the test relies heavily on the observation of behavior, strict adherence to procedural details and minimizing unwarranted stress to the animals involved is critical to successful utilization of the procedure If an experiment introduces environmental variables unaccounted for, the conclusions revealed from PST data may be flawed (Can et al., 2012).

Regarding ethicality, a consequential critic of the test is the organization of People for the Ethical Treatment of Animals (PETA). PETA argues that the test does not actually yield any useful data. They have called the test inhumane, stating that, "…in university and pharmaceutical laboratories [today], animals are being dosed with drugs and then dropped into cylinders of water

so that experimenters can measure how long they struggle" (People for the Ethical Treatment of Animals [PETA], 2019).

Hatfield (2018) refutes the picture of cruelty painted by protesting parties by pointing out that rats are natural swimmers that can swim for up to three days straight and hold their breath underwater for three minutes. She argues that the Porsolt Swim Test is critical to helping scientists understand depression and efforts to combat it. But recent research continues providing new interpretation of the value of the PST.

First opposing the notion that passive swimming behavior is indicative of depressive states, West (1990) reviewed the forced swimming test, concluding that it was not a valid model of depression. When exposed to inescapable shock, coping responses of the rats were impaired; however, the details of the study led to the deduction that immobility was not a failure of coping, but rather a *successful* implementation of *energy conservation*. Agreeing with these results, scientists de Kloet and Molendijk (2016) took a different approach by examining rodent information processing throughout the PST. They focused on analyzing the implications of brain circuitry and dopamine and the glucocorticoid stress hormone during coping with the inescapable stress, interpreting that the behavioral responses of rodents to the PST were not reflective of depressive states, but adaptations of stress coping.

Strongly connecting to the work of both West (1990) and de Kloet and Molendijk (2016), Commons e al. (2017) asserted that the shift from active to passive coping strategies is typical of most rodents, also concluding that floating was a strategy adapted for energy conservation. While finding that emotional state and physical actions undeniably share an inextricable link, Commons et al. determined the PST an invalid model for depression, blaming incorrect generalizations of mental states on failure to holistically consider animal behavior.

Commons et al. (2017) also concluded that the test is not an applicable model to human MDD due to limited similarity in the clinical symptoms measured across rodents and humans. The depressive behaviors observed in the rats of their work were induced conditional stressors (being placed in an inescapable tank of water) uncharacteristic of the chronic, pathological, and emotional factors that contribute to human MDD. Hatfield (2018) would agree that testing on animals may not be the most efficient because many symptoms manifested in depression, like suicidality, are uniquely human. Yet, Hatfield found it to currently be the best available model because measurable symptoms like fatigue, weight loss, sleep abnormalities, and other behavioral changes do exhibit themselves in both humans and rodents.

With all the conflicting viewpoints, evaluation of the efficacy of the PST is imperative considering the amount of antidepressant research that has already been supported and produced with the test as a foundation. Likely attributed to the disagreement on its value, the PST is not widely used anymore. But with its expected purpose and results and economical and noninvasive nature, the PST has high abilities for valuable application. Evaluating it provides potential for promoting its reinstitution as a common assay.

**The Addition of Biological Objectivity**

Behavior can be influenced by a myriad of variables. Many studies find that it is necessary for more biologically based reasoning to be considered when categorizing depressive behaviors. Limited biological backing of the PST exists because the SwLo rats included in this research are the only ones in the United States.

Relevant to obtaining a biological perspective to the PST, gut microbiota and their host organisms have dependent relationships, exemplified by their coevolution and codependency for survival. The gastrointestinal (GI) tract of humans is home to an extensive microbial ecosystem, bursting with diversity. Many of these organisms play critical roles in host functions like food

digestion and regulation of immune functions (Rintala et al., 2017). More importantly to this research, bacterium can somewhat act as a "second brain." The influence of the gut on the brain and behavior has become a complex area of study that pedestals gut microbiota as an important regulator of the gut-brain axis (See Figure 1) (Foster et al., 2017).

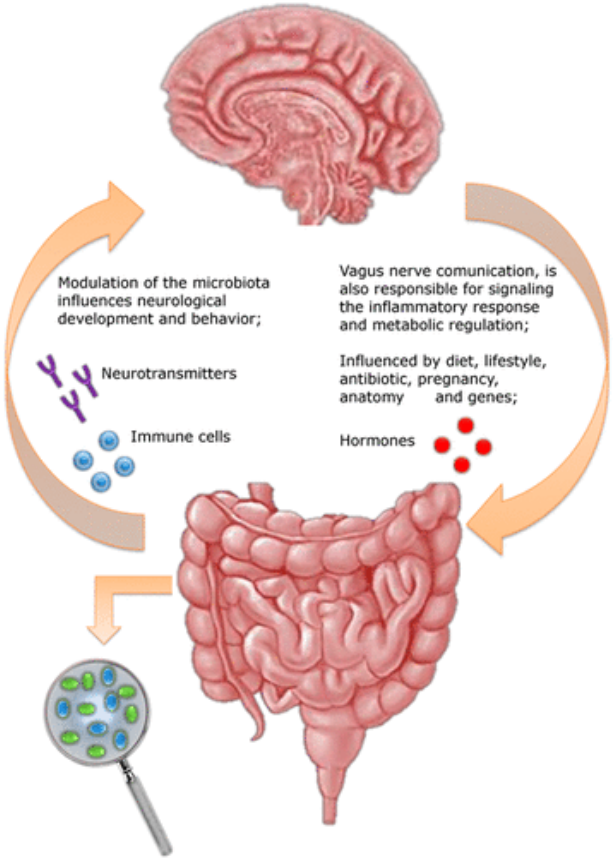


*Figure 1:* Illustration of the bidirectional communication between the gut microbiota and the brain that stimulates the synthesis of neurotransmitters, from de la Fuente-Nunez, et al. (2017).

Serotonin, or 5-HT (5-hydroxytrptamine), is a critical component of that axis. As an important neurotransmitter that influences mental health, serotonin contributes to a wide range of physiological processes within the body (Lin et al., 2014). Relevant to depression, low levels of serotonin have been associated with mood disorders (Evrensel & Ceylan, 2015; Lin et al., 2014; Yano et al., 2015). Yohn et al. (2017) also revealed through neuroimaging evidence across numerous studies an association of MDD and negative stress reactivity to low levels of 5-HT,

while higher levels were correlated with positive symptoms like reductions in anxiety-like behaviors.

Over 90% of 5-HT of the body is synthesized in the gut. Microbiota located in the GI tract of the body may play critical roles in the mechanisms involved in serotonin production, evident of data indicating that gut microbiota is largely involved in raising levels of colon and serum 5-HT through inducible and reversible manners in colonic enterochromaffin cells (Yano et al., 2015).

Ott et al. (2004) finds that gut Flora composition is critical to the status of the health of a host. Additionally, Panek et al. (2018) states that "…importance of this easily available metabolic waste comes from its microbial content found to hold the potential in diagnosis, disease prediction and therapeutic intervention" (p. 1).

The evidence that microbiomes do affect their host in some ways lead to the hypothesis that there are *specific* bacterial genera affecting synthesis pathways, interacting with the host to stimulate intestinal cells to produce serotonin, increasing active coping strategies in PST mice. Evaluating imbalances between the mice phenotypes provided the biological component to the validation of the mental state of a PST rodent. Supporting this idea, Foster et al. (2017) found that disturbances in microbial balance, particularly in early life, can lead to maladaptive behaviors (e.g. increased occurrences of stress responsiveness and other stress related disorders). Detailed as an aberrant gut microbiota composition, gut microbiota dysbiosis has shown links to several diseases and disorders (Evrensel & Ceylan, 2015; Rintala et al., 2017; Yang et al., 2015), further evidence that studying gut microbiota composition could serve as a biological form of evaluating PST rat mental health.

Modern medicine and modern diets are evolving; what humans eat today looks much different than pre-industrial revolution or even pre-agricultural revolution diets. The evolution of

medicine has introduced the need for the health effects of antibiotics and probiotics to be considered. Changes introduced by these dietary components can affect microbiome composition, which may have correlation with various conditions including depression (Hidaka, 2012). Quantifying gut bacteria of SwHi and SwLo rats relevant to serotonin synthesis should yield insight to proper interpretation of the PST. Proving differences between SwHi and SwLo rat gut microbiota compositions relevant to serotonin synthesis could lead to a revival of the PST as a common practice in antidepressant research. Composition diversity and functions of microbial communities could also potentially inform more personalized approaches to drug development strategies (Lozupone, 2012). Overall, gaining more biological knowledge of the efficacy of the PST supports the value of future antidepressant research.

## Methods

### Rationale

Studying taxonomic-level relations of gut microbiota in this sort of application is highly relevant to antidepressant research for humans. Abundant research suggests that the serotonergic functions of many GI bacterial communities are conserved between humans and rats, highlighting the fit of the model.

PST rats were perfect targets when formulating a research question regarding antidepressant research because of their categorizations of depressive and non-depressive behaviors. Their behavioral phenotypes allowed for the comparison of categories that significant imbalances in serotonin synthesis relevant bacteria could be compared between. This research took a quantitative approach, measuring the relative abundance of 16s sequences of bacteria genera pertinent to serotonin synthesis within each rat gut consortium using fluorescence yielded via high-throughput sequencing and Quantitative Polymerase Chain Reaction (qPCR) Taqman assay (see Figure 2) methods. Due to the known relationship of serotonin production to mental

health, these biologically based results could be used to quantitatively evaluate if the gut biology of PST rats aligned with their coping behavior.

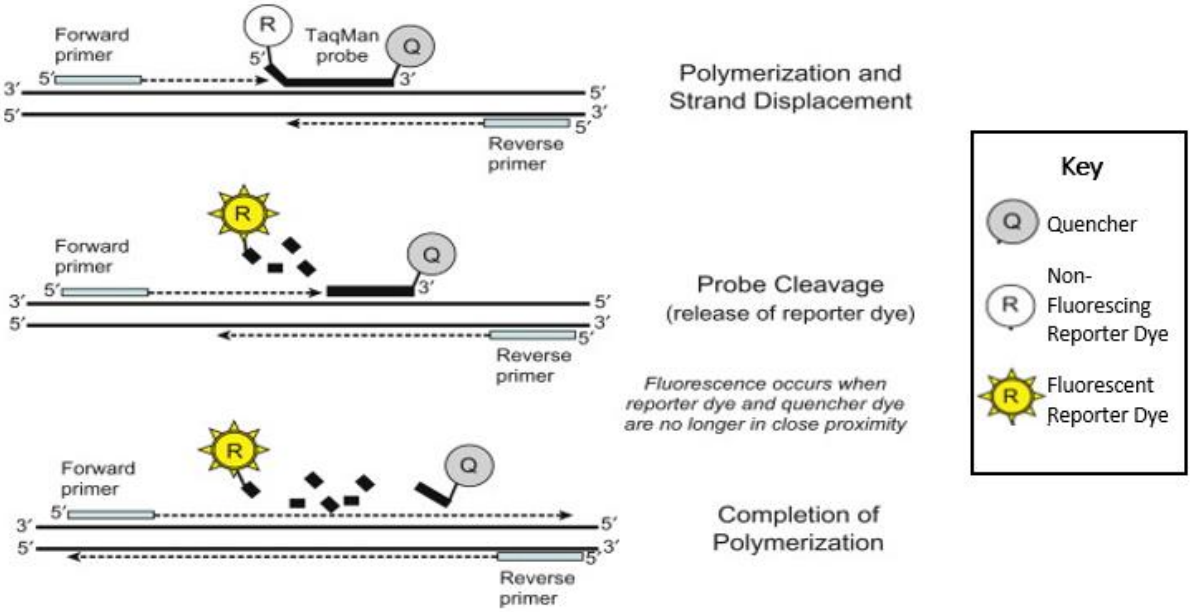


*Figure 2:* The Mechanisms invloved in a Taqman qPCR assay, from John M Butler's Forensic DNA Typing: Methodology (2012)

An anticipated change in methodology would have been the genera selected for primer design and experiment focus. Because the target genera were selected based on Next Generation Sequencing service results from only one SwHi and one SwLo rats each (due to service cost), it is quite possible that the imbalances identified among various gut microbiota taxons that provided targets could have been anomalies within a single rat. Thus, verification from a larger sample set prior to forming conclusions was necessary in this research.

**Sample Collection**

Bacterial samples were collected from the fecal samples of rats from the Whitworth University vivarium and classified according to their performance in the PST (conducted by the university) as either SwHi or SwLo rats. The sample pool was comprised of 22 total rats (SwHiM (6), SwHiF (4), SwLoM (8), SwLoF (4); where M and F indicate gender). Becker et al. (2016) found that in neuroscience research, while behavioral data between sexes can show

intrinsic variability, male and female rats did not demonstrate significant enough disparity in research, and therefore are no more variable than males. Thus, gender was excluded from consideration in analysis because it would not have made a large enough impact.

In order to preserve genomic material as best possible, feces were frozen following collection as soon as possible between -20℃ and -80℃ and kept on ice as much as possible when aliquoting. An excess amount of feces has been shown to have negative effects on extraction outputs, especially when sensitive techniques like qPCR are utilized (Ferrand et al., 2014). Keeping that in mind, all fecal samples were in 180mg-220mg aliquots to achieve optimal DNA elution.

**DNA Extraction**

DNA extraction was done following the QIAamp DNA Stool Kit Procedure. Its intended purpose for DNA extraction from feces was perfectly suited for this research, and its reagents were readily available through the laboratory utilized. Prior to beginning protocol, all reagents were ensured to have been prepped according to manufacturer specifications and heat blocks preheated to 70°C. Feces pellets were kept on ice and remained frozen until it was ready to be broken up into a 2ml microcentrifuge tube with 1600µl Buffer ASL added. The mixture was vortexed until thoroughly homogenized, then centrifuged at 21,000 RCF (all centrifugation steps were performed at this speed) for two minutes to pellet the stool. Into a new 2ml microcentrifuge tube, 1400µl of supernatant was transferred and the pellet discarded. One inhibitEX Tablet was added to each sample, vortexed until completely suspended, then sample incubated for one minute at room temperature to allow extraction inhibitors to absorb into the inhibitEX matrix. Following, a six-minute centrifugation step pelleted unwanted stool particles and inhibitors bound to the matrix. A longer centrifugation in that step from the protocol was an optimization made because a large source of contaminates were precipitated in this step, and loose precipitate

particles in the supernatant heavily diminished extraction quality. Additionally, it is important to note that no more than twelve samples were extracted at a time to ensure that no diffusion of extraction inhibitors occurred after critical separation steps. Immediately following centrifugation, all supernatant (~550µl) was pipetted into a new 1.5ml microcentrifuge tube, pellet discarded, and centrifuged for three minutes. The lysate and 500µl Buffer AL were added to new 2ml microcentrifuge tubes previously prepared containing 25µl ProteinaseK. The new tube was vortexed for 15s and incubated in preheated block for ten minutes. After incubation, 600µl of the lysate was added to a QiaAmp Spin Column and centrifuged for one minute, discarding the filtrate afterwards and collection tube replaced (discarding of the filtrate occurred after each filtration step except for the final one); this step was repeated until all lysate was filtered. Next, 500µl of Buffer AW1 was added to the column and centrifuged for one minute, followed by filtration of 500µl Buffer AW2 added to column and centrifuge for three minutes. Spin column was then transferred to a clean 1.5ml tube. For the final elution step, another optimization unique to this experiment was substituting 200µl of Buffer AE and a one-minute centrifugation for 100µl of Buffer with two one-minute passes through the column. The purpose was to augment DNA concentration, as the initial bacterial extractions did not yield enough template.

Following DNA elution, assessment of nucleic acid quantity and purity was done on a Nanodrop 2000c with a 2µl blank of Buffer AE. Once adequate quantities and limited concentrations of contaminates and inhibitors (such as residual reagents) were achieved for a sample, the isolated DNA was ready for downstream application.

**Target Genera Identification**

To efficiently identify which bacterial species were present in each relevant consortium, one sample each of a SwHi and a SwLo rat were sent to GENEWIZ after prepping them for a

16sEZ Metagenome analysis according to Next Generation Sequencing (NGS) service specifications for purified mixed DNA. Species level data sequenced from the V3 and V4 regions on the 16s RNA gene provided the taxonomic classifications of the bacteria within the fecal samples. After receiving results, due to the time frame of the project and resources available, it was decided that looking at the 5-HT relevant bacterial genera would be more efficient than looking at each individual species, especially because multiple species of each genera were relevant to 5-HT processes and exhibited significant ratio imbalances between the rat phenotypes.

**Primer Design**

After target genera were identified, a Taqman Minor Groove Binding (MGB) probe primer was identified for Bifidobacterium spp. from existing research (see Table 1). For the other two, primer and known MGB probe sequences were identified for each genus using NCBI Nucleotide BLAST to align the genomic sequence of each selected genus with a region-specific primer and sent to ThermoFischer for final design and ordering. MGB probes were opted for due to the high efficiency they demonstrate in qPCR by forming extremely stable duplexes with DNA targets (Ott et al., 2004).

**Table 1**

*Primers and Probes for qPCR*

| Target | Primers and MGB Probes | Tm (°C) | Reference |
|---|---|---|---|
| Bifidobacterium spp. | F- CGCGTCYGGTGTGAAAG<br>R- CCCCACATCCAGCATCCA<br>MGB- AACAGGATTAGATACCC | | Delroisse et al. (2008) |
| Ruminococcus spp. | F- CGGTGGAGCATGTGGTTTAA<br>R- GGGATGTCAAGAGCAGGTAAGG<br>MGB- TCGAAGCAACGCGAAGA | | Hastie, Mitchell, and Murray (2008) |
| Sutterella spp. | F- CGAAAAACCTTACCTAGCCTTGAC<br>R- CGGGCACCCCCGAAT<br>MGB- TGCCAGGAACCCTGAA | | Williams, Hornig, Parekh, and Lipkin (2012) |

**Quantitative PCR for Absolute Quantification**

To quantify the levels of bacteria within each rat, custom TaqMan assays were performed via qPCR using custom genera-specific primers in each corresponding mastermix (see Table 2 for solutions). A Taqman assay was most appropriate for acquiring data with its purpose for measuring gene expression. Running a triplicate no-template-control containing only mastermix against triplicates of template dilutions (beginning at 20ng/µl in a dilution sequence of 1:1, 1:2, 1:4, 1:8, and 1:16) established a standard curve to assess primer efficacy and amplification efficiency. An ideal statistical value near R=1 determined from the line of regression would indicate method consistency and experiment reproductivity (Larionov et al., 2005).

**Table 2**

*Reaction Contents Per Individual qPCR Reaction*

| Component | Volume |
|---|---|
| Master Mix (2X) | 5.00µl |
| Custom Taqman Gene Expression Assay (20X) | 0.50µl |
| dH2O | 3.50µl |
| DNA Template | 1.00µl |
| Total Volume | 10.00µl |

Once primer effectiveness was confirmed, the bacterial communities of the remaining samples were measured using a comparative quantification method to provide relative genera proportions between the phenotypes. A Comparative Cycle Threshold (CCt) Value yielded the cycle number when the fluorescence of the PCR product could be differentiated from background signals and was associated with the amount of product present in the reaction.

**Data Analysis**

The quantitative nature of this methodology enabled proportions of bacteria genera known to be involved in the process of serotonin synthesis to be observed and compared between the categories of rats. The biological results allowed for adequate conclusions to be drawn about

the efficacy of the PST as an assay for testing antidepressants. It would have been most efficient to run all samples through the NGS services; however, that would have taken away from self-conducting the experiments and significantly increased dollar expenditure.

Another potential approach to biologically validating the state of a PST rat would have been to simply measure the amount of serotonin present in the brain, but the laboratory utilized lacked access to that equipment. That approach was also not the most relevant to the research question because 5-HT levels fluctuate in the brain, and this research was more interested in the biological tools available for serotonin synthesis than merely a snapshot of serotonin presence.

The method chosen was the most efficient and optimal choice when compared to sequencing- based approaches. It cut the number of procedural steps by eliminating the guess and check nature of identifying genes relevant to serotonin synthesis, opting for analysis of a simpler serotonin synthesis pathway rather than getting lost in the extensive gene activity involved in 5-HT synthesis.

## Results

The DNA extraction data of the 22 samples yielded purity values approvable for qPCR. Originally, there were 26 samples, but even after multiple extraction attempts, four were not able to move forward in the experiment. A possible reason could have been that the fecal pellets were too old, and the DNA they contained too degraded.

The NGS results identified significant microbial imbalances between the SwHi and SwLo rats (see Table 3). From those, it was determined that the most important genera to focus on were Bifidobacterium, Ruminococcus, and Sutterella because of the significant imbalances in the abundance of those taxa that the NGS sequencing results displayed. Sutterella spp. was three times more abundant in the SwLo rat than the SwHi. Additionally, existing research implicates involvement of the other two in 5-HT production through their various relationships to

symptoms of depression. Probiotics of Bifidobacterium have demonstrated positive correlation with reducing depressive behaviors (Pinto-Sanchez et al., 2017; Tian et al., 2019). Lukić et al. 2019 found that Ruminococcus flavefaciens was also able to significantly reduce depressive behaviors result of gut dysbiosis.

When evaluating selected primers, the standard curves demonstrated that the Bifidobacterium and Ruminococcus assays had adequate efficiency to move forward in the experiment and enable reliable data collection. The Sutterella assay had such poor efficiency ($R < 0.8$) that it was removed from the measured target genera.

**Table 3**

*Results from 16s metagenome analysis by genus*

| Taxon | Abundance in SwHi Consortium | Abundance in SwLo Consortium |
|---|---|---|
| Unclassified | 67.63 | 64.64 |
| Anaeroplasma | 0.01 | 1.05 |
| Bifidobacterium | 1.09 | 0.34 |
| CF231 | 2.80 | 3.07 |
| Coprococcus | 3.02 | 2.16 |
| Mucispirillum | 0.37 | 0.96 |
| Oscillospira | 1.30 | 0.65 |
| Prevotella | 6.77 | 6.08 |
| Ruminococcus | 7.78 | 8.40 |
| Sutterella | 2.03 | 6.23 |
| Totals | 100.00 | 100.00 |

For both Ruminococcus spp. and Bifidobacterium spp. the mean CCt values measured in qPCR were on average lower in SwHi rats than SwLo. Lower CCt values indicated greater quantification (see Table 4). For the Ruminococcus assay, the standard deviation was small, indicating lower variance in the samples than the Bifidobacterium assay. The larger deviation within the Bifidobacterium data set can be attributed to a few prominent outliers in the data pool. It is highly possible the greater quantity variance can be attributed to the fact that the

Bifidobacterium genera is comprised of sixty known taxa (Mattarelli & Biavati, 2018), whereas Ruminococcus spp. encompasses only six (La Reau et al., 2016), leaving wider room for variability.

**Table 4**

*Average CCt Value Yielded from qPCR*

| Taxon | SwHi | Standard Dev. (σ) | SwLo | Standard Dev. (σ) |
|---|---|---|---|---|
| Bifidobacterium spp. | 32.89 | 4.48 | 33.25 | 4.85 |
| Ruminococcus spp. | 19.91 | 1.09 | 21.02 | 1.08 |

## Conclusion

The hypothesis that deficiencies of specific microbial genera would correlate with the mental state of a rate was supported by the data quantified. In existing research, the Ruminococcus and Bifidobacterium genera in lower abundances have demonstrated strong links to serotonin deficiencies and more depressive symptoms (Cao et al., 2018; Singhal et al., 2019; Zhang et al., 2019). Based on the data collected, this research was able to conclude that the PST is a valid assay for qualifying antidepressants. The imbalances in microbiota demonstrated that the SwLo rats were deficient in both Ruminococcus spp. and Bifidobacterium spp. Their behavior was thus correctly qualified by the PST, indicating more depressive states than their counterparts as the PST is meant to demonstrate.

## Discussion

The NGS results of this research agree with Singhal et al. (2019), which found that serotonin deficient mice had lower abundances of Bifidobacterium spp. and Ruminococcus spp. SwLo rats hypothetically, based on behavior, have lower levels of serotonin. When further validated with qPCR, in general, it was demonstrated based on serotonin production influencing

bacteria proportions, the SwLo rats were in more depressive states than their SwHi counterparts based on the disproportions of serotonin-relevant microbiota quantified.

**Limitations**

This research was only partially conclusive for a couple reasons. First, the conclusion reached was based on average CCt values, rather than absolute quantification. The CCt values enabled a generalization of each category to be made, finding within the sample pool that *on average*, SwHi rats had higher levels of the Bifidobacterium and Ruminococcus genera abundance measured within their consortiums than SwLo rats did. Absolute quantification would have yielded more accurate values but was not pursued due to cost limitations. Each individual sample would have required its own standard curve of dilutions for 16s against each of the three other custom Taqman Assays. With the triplicate nature of qPCR, each individual sample would effectively be required to be ran sixty times, consuming a massive number of reagents.

Secondly, a considerable limitation is that the design of this experiment depended on identifiable components to design the primers for qPCR. Gut microbiota research is still young, evident of roughly two-thirds of the bacteria present in the NGS results quantifying as unknown. It is highly possible that there are unknown bacterial genera involved in serotonin synthesis. Therefore, while strong support is demonstrated, it is difficult to provide a certain answer as to whether deficiencies in taxa abundance are *the* cause of depressive behaviors. As previously stated, behavior can be influenced by a multitudinous number of variables.

Similarly, Singhal et al. (2019) found it difficult in their study with serotonin transport knockout (KO) mice to attribute serotonin deficiencies to gut dysbiosis or that dysbiosis was the fault of KO genes. Likewise, this research did not contain the authority to state that gut dysbiosis

is responsible for serotonin deficiency; however, it did allow the conclusion that studying PST rats was an accurate way to study depressive behaviors.

**Further Research**

In continuation with evaluating various genera imbalances between the phenotypes via qPCR, one taxon of special interest that did not get quantified was Campylobacter spp. It was omitted from this research because it did not show up in the NGS results. Mice with higher levels have previously demonstrated correlation with more depressive behaviors (Foster et al., 2017). Looking into how that genus affects PST rats would be another factor of evaluation.

Conducting this same research with the absolute quantification method would be a potential next step in evaluating the value of the PST based on gut flora compositions. Even more so, putting every sample through NGS would be the most efficient way to determine gut flora composition, and would measure beyond the few chosen assays of this experiment.

A substantial portion of future research would involve the complete genomic sequencing and identification of all bacterial genera and species present within PST rat consortiums. A large part of this experiment was limited by only being able to work with genera already identified and with known links to serotonin synthesis.

Finally, a major extension to this research would be to focus on serotonin transport. Tph1 is a serotonin transport gene that catalyzes the first-rate limiting step in serotonin biosynthesis. Mutations of the gene are associated with an elevated risk of anxiety disorders anger-related traits, bipolar disorder, and suicidal symptoms (Yano et al., 2015). A direct relationship of gut microbiome diversity and serotonin transporter content has been observed (Kwon et al., 2019; Yano et al., 2015). The transport molecule for a neurotransmitter is just as important as the

neurotransmitter itself. An abundance of serotonin in the body would be useless if there was no molecule to enable its processing.

**Applications**

The area of gut microbiota research arena continues to grow with every new addition of information. One potential application that this research can lead to is foundations for a biological marker of depression. This would involve quantifying gut bacteria relevant to serotonin synthesis and correlating deficits with depressive behaviors. Quantifying taxa that demonstrate negative effects in high abundance (e.g. *Campylobacter jejuni*) could also contribute to diagnosis.

Another highly important way microbiome research is applicable is with personalized drug approaches. Once specific variables within a case of gut dysbiosis is identified, the bacteria lacking can be cultured in a lab and revitalized via fecal transplants. It is possible to treat and reverse depressive symptoms via fecal transplants to revive GI tract microbiome abundance (Zhang et al., 2019). Likewise, for taxa that negatively affect a host when overrepresented, beneficial amounts of a more competitive and favorable taxa can be introduced. Hibbing et al. (2010) described how bacteria have diverse mechanisms by which they can coexist or dominate a microbial community that competes for the same resources. Researching benign taxa that can suppress detrimental communities is another direction for further research for clinical applications of gut microbiota research.

**Validity**

Much of the controversy over the ability of the PST to demonstrate what it should is founded on reasonable hesitations. Yet, the quantitative data yielded in this experiment supported the PST by demonstrating that the gut microbiome compositions of the SwHi and SwLo rats

regarding serotonin-relevant genera aligned with their behavior. Thus, for now it can be concluded that the PST satisfies an adequate degree of antidepressant research. There is still much to be learned about the complex mechanisms of microbiota and the gut-brain axis, let alone gut microbiome relationships to serotonin production and processing. Currently, gut microbiota compositions based on NGS data and qPCR techniques have so far demonstrated support for the PST. Therefore, reviving the PST as a common research practice would be highly beneficial to antidepressant research.

5,000 Words

## References

Becker, J. B., Prendergast, B. J., & Liang, J. W. (2016). Female rats are not more variable than male rats: a meta-analysis of neuroscience studies. *Biology of sex differences*, *7*(1), 34. https://dx.doi.org/10.1186%2Fs13293-016-0087-5

Butler, J. M. (2012). *Advanced topics in forensic DNA typing: methodology*. London: Academic Press. https://doi.org/10.1016/C2011-0-04189-3

Cao, Y. N., Feng, L. J., Wang, B. M., Jiang, K., Li, S., Xu, X., ... & Wang, Y. M. (2018). Lactobacillus acidophilus and Bifidobacterium longum supernatants upregulate the serotonin transporter expression in intestinal epithelial cells. *Saudi journal of gastroenterology: official journal of the Saudi Gastroenterology Association*, *24*(1), 59. https://dx.doi.org/10.4103%2Fsjg.SJG_333_17

Carabotti, M., Scirocco, A., Maselli, M. A., & Severi, C. (2015). The gut-brain axis: interactions between enteric microbiota, central and enteric nervous systems. *Annals of gastroenterology: quarterly publication of the Hellenic Society of Gastroenterology*, *28*(2), 203. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/25830558

Commons, K. G., Cholanians, A. B., Babb, J. A., & Ehlinger, D. G. (2017, May 17). The rodent forced swim test measures stress-coping Strategy, not depression-like behavior. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/28287253.

Cussotto, S., Sandhu, K. V., Dinan, T. G., & Cryan, J. F. (2018). The neuroendocrinology of the microbiota-gut-brain axis: a behavioral perspective. *Frontiers in neuroendocrinology*, *51*, 80-101. DOI: 10.1016/j.yfrne.2018.04.002

de la Fuente-Nunez, C., Meneguetti, B. T., Franco, O. L., & Lu, T. K. (2018). Neuromicrobiology: how microbes influence the brain. *ACS chemical neuroscience, 9*(2), 141-150. https://doi.org/10.1021/acschemneuro.7b00373

De Kloet, E. R., & Molendijk, M. L. (2016). Coping with the forced swim stressor: towards understanding an adaptive mechanism. *Neural plasticity*, 2016. DOI: 10.1155/2016/6503162

Delroisse, J. M., Boulvin, A. L., Parmentier, I., Dauphin, R. D., Vandenbol, M., & Portetelle, D. (2008). Quantification of Bifidobacterium spp. and Lactobacillus spp. in rat fecal samples by real-time PCR. *Microbiological research, 163*(6), 663-670. https://doi.org/10.1016/j.micres.2006.09.004

Dinan, T. G., & Cryan, J. F. (2013). Melancholic microbes: a link between gut microbiota and depression? *Neurogastroenterology & Motility*, *25*(9), 713-719. DOI: 10.1111/nmo.12198

Evrensel, A., & Ceylan, M. E. (2015). The gut-brain axis: the missing link in depression. *Clinical Psychopharmacology and Neuroscience*, *13*(3), 239. DOI: 10.9758/cpn.2015.13.3.239

Ferrand, J., Patron, K., Legrand-Frossi, C., Frippiat, J. P., Merlin, C., Alauzet, C., & Lozniewski, A. (2014). Comparison of seven methods for extraction of bacterial DNA from fecal and cecal samples of mice. *Journal of microbiological methods, 105*, 180-185. DOI: 10.1016/j.mimet.2014.07.029

Foster, J. A., Rinaman, L., & Cryan, J. F. (2017). Stress & the gut-brain axis: regulation by the microbiome. *Neurobiology of stress, 7*, 124-136. DOI: 10.1016/j.ynstr.2017.03.001

Hastie, P. M., Mitchell, K., & Murray, J. A. M. (2008). Semi-quantitative analysis of Ruminococcus flavefaciens, Fibrobacter succinogenes and Streptococcus bovis in the equine large intestine using real-time polymerase chain reaction. *British Journal of Nutrition*, *100*(3), 561-568. https://doi.org/10.1017/S0007114508968227

Hatfield, A. (2018, December 18). Animal rights groups say the Porsolt Swim Test is unnecessary. But is it? Retrieved from https://fbresearch.org/forced-swim-test/

Hibbing, M. E., Fuqua, C., Parsek, M. R., & Peterson, S. B. (2010). Bacterial competition: surviving and thriving in the microbial jungle. *Nature Reviews Microbiology*, *8*(1), 15-25. https://dx.doi.org/10.1038%2Fnrmicro2259

Hidaka, B. H. (2012). Depression as a disease of modernity: explanations for increasing prevalence. *Journal of affective disorders*, *140*(3), 205-214. DOI: 10.1016/j.jad.2011.12.036

Kwon, Y. H., Wang, H., Denou, E., Ghia, J. E., Rossi, L., Fontes, M. E., ... & Surette, M. G. (2019). Modulation of gut microbiota composition by serotonin signaling influences intestinal immune response and susceptibility to colitis. *Cellular and molecular gastroenterology and hepatology*, *7*(4), 709-728. https://doi.org/10.1016/j.jcmgh.2019.01.004

La Reau, A. J., Meier-Kolthoff, J. P., & Suen, G. (2016). Sequence-based analysis of the genus Ruminococcus resolves its phylogeny and reveals strong host association. *Microbial genomics*, *2*(12). https://dx.doi.org/10.1099%2Fmgen.0.000099

Larionov, A., Krause, A., & Miller, W. (2005). A standard curve-based method for relative real time PCR data processing. *BMC bioinformatics*, *6*(1), 62. https://doi.org/10.1186/1471-2105-6-62

Lin, S. H., Lee, L. T., & Yang, Y. K. (2014). Serotonin and mental disorders: a concise review on molecular neuroimaging evidence. *Clinical Psychopharmacology and Neuroscience*, *12*(3), 196. DOI: 10.9758/cpn.2014.12.3.196

Lozupone, C. A., Stombaugh, J. I., Gordon, J. I., Jansson, J. K., & Knight, R. (2012). Diversity, stability and resilience of the human gut microbiota. *Nature, 489*(7415), 220-230. https://doi.org/10.1038/nature11550

Lukić, I., Getselter, D., Ziv, O., Oron, O., Reuveni, E., Koren, O., & Elliott, E. (2019). Antidepressants affect gut microbiota and Ruminococcus flavefaciens is able to abolish their effects on depressive-like behavior. *Translational psychiatry*, *9*(1), 1-16. https://doi.org/10.1038/s41398-019-0466-x

Mattarelli, P., & Biavati, B. (2018). Species in the genus Bifidobacterium. In *The Bifidobacteria and Related Organisms* (pp. 9-48). Academic Press. https://doi.org/10.1016/B978-0-12-805060-6.00002-8

Molendijk, M. L., & de Kloet, E. R. (2015). Immobility in the forced swim test is adaptive and does not reflect depression. *Psychoneuroendocrinology*, *62*, 389-391. https://doi.org/10.1016/j.psyneuen.2015.08.028

National Institute of Mental Health. (n.d.). Major Depression. Retrieved from https://www.nimh.nih.gov/health/statistics/major-depression.shtml.

Ott, S. J., Musfeldt, M., Ullmann, U., Hampe, J., & Schreiber, S. (2004). Quantification of intestinal bacterial populations by real-time PCR with a universal primer set and minor groove binder probes: a global approach to the enteric flora. *Journal of clinical microbiology*, *42*(6), 2566-2572. DOI: 10.1128/JCM.42.6.2566-2572.2004

Panek, M., Paljetak, H. Č., Barešić, A., Perić, M., Matijašić, M., Lojkić, I., ... & Verbanac, D. (2018). Methodology challenges in studying human gut microbiota–effects of collection, storage, DNA extraction and next generation sequencing technologies. *Scientific reports*, *8*(1), 5143. DOI: 10.1038/s41598-018-23296-4

People for the Ethical Treatment of Animals. Experimenters trap small animals in beakers of water until they stop swimming. (2019). Retrieved from https://support.peta.org/page/7704/action/1?locale=en-US.

Pinto-Sanchez, M. I., Hall, G. B., Ghajar, K., Nardelli, A., Bolino, C., Lau, J. T., ... & Traynor, J. (2017). Probiotic Bifidobacterium longum NCC3001 reduces depression scores and alters brain activity: a pilot study in patients with irritable bowel syndrome. *Gastroenterology*, *153*(2), 448-459. https://doi.org/10.1053/j.gastro.2017.05.003

Porsolt, R. D., Bertin, A., & Jalfre, M. J. A. I. P. (1977). Behavioral despair in mice: a primary screening test for antidepressants. *Archives internationales de pharmacodynamie et de therapie*, *229*(2), 327-336. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/596982

Porsolt, R. D., Le Pichon, M., & Jalfre, M. L. (1977). Depression: a new animal model sensitive to antidepressant treatments. Nature, 266(5604), 730. https://doi.org/10.1038/266730a0

Reardon, S. (2019, July 18). Depression researchers rethink popular mouse swim tests. *Nature*, (571), 456-457. DOI: 10.1038/d41586-019-02133-2

Rintala, A., Pietilä, S., Munukka, E., Eerola, E., Pursiheimo, J. P., Laiho, A., ... & Huovinen, P. (2017). Gut microbiota analysis results are highly dependent on the 16S rRNA gene target region, whereas the impact of DNA extraction is minor. *Journal of biomolecular techniques: JBT*, *28*(1), 19. DOI: 10.7171/jbt.17-2801-003

Rinttilä, T., Kassinen, A., Malinen, E., Krogius, L., & Palva, A. J. (2004). Development of an extensive set of 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by real-time PCR. *Journal of applied microbiology*, *97*(6), 1166-1177. DOI: 10.1111/j.1365-2672.2004.02409.x

Rogers, G. B., Keating, D. J., Young, R. L., Wong, M. L., Licinio, J., & Wesselingh, S. (2016). From gut dysbiosis to altered brain function and mental illness: mechanisms and pathways. *Molecular psychiatry*, *21*(6), 738. DOI: 10.1038/mp.2016.50

Singhal, M., Turturice, B. A., Manzella, C. R., Ranjan, R., Metwally, A. A., Theorell, J., ... & Perkins, D. L. (2019). Serotonin transporter deficiency is associated with dysbiosis and changes in metabolic function of the mouse intestinal microbiome. *Scientific reports*, *9*(1), 1-11. https://doi.org/10.1038/s41598-019-38489-8

Tian, P., Wang, G., Zhao, J., Zhang, H., & Chen, W. (2019). Bifidobacterium with the role of 5-hydroxytryptophan synthesis regulation alleviates the symptom of depression and related microbiota dysbiosis. *The Journal of nutritional biochemistry*, *66*, 43-51. https://doi.org/10.1016/j.jnutbio.2019.01.007

West, A. P. (1990). Neurobehavioral studies of forced swimming: The role of learning and memory in the forced swim test. *Progress in Neuro-Psychopharmacology & Biological Psychiatry, 14*(6), 863–877. https://doi.org/10.1016/0278-5846(90)90073-P

Whitworth University. (2019). Porsolt swim test.

Williams, B. L., Hornig, M., Parekh, T., & Lipkin, W. I. (2012). Application of novel PCR-based methods for detection, quantitation, and phylogenetic characterization of Sutterella species in intestinal biopsy samples from children with autism and gastrointestinal disturbances. *MBio, 3*(1), e00261-11. DOI: 10.1128/mBio.00261-11

Yankelevitch-Yahav, R., Franko, M., Huly, A., & Doron, R. (2015). The forced swim test as a model of depressive-like behavior. *Journal of visualized experiments: JoVE*, (97), 52587. DOI:10.3791/52587

Yano, J. M., Yu, K., Donaldson, G. P., Shastri, G. G., Ann, P., Ma, L., ... & Hsiao, E. Y. (2015). Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis. *Cell*, *161*(2), 264-276. https://doi.org/10.1016/j.cell.2015.02.047

Yohn, C. N., Gergues, M. M., & Samuels, B. A. (2017). The role of 5-HT receptors in depression. *Molecular brain*, *10*(1), 28. https://doi.org/10.1186/s13041-017-0306-y

Zhang, Y., Huang, R., Cheng, M., Wang, L., Chao, J., Li, J., ... & Yao, H. (2019). Gut microbiota from NLRP3-deficient mice ameliorates depressive-like behaviors by regulating astrocyte dysfunction via circHIPK2. *Microbiome*, *7*(1), 1-16. https://doi.org/10.1186/s40168-019-0733-3

# Academic Paper

**Note:** Student samples are quoted verbatim and may contain spelling and grammatical errors.

**Sample: B**
**Score: 5**

**How can gut microbiota composition within SwHi and SwLo rats support or negate the Porsolt Swim Test?**

This paper earned a score of 5. It provides a rich discussion of the professional conversation found on pages 3-9 and situates its research within a clear need in the field. This is highlighted on page 9: "gaining more biological knowledge in the efficacy of the PST supports the value of future antidepressant research." Additionally, the paper provides a logical rationale for a detailed, replicable method for examining the genetic makeup of gut bacteria taken from feces in SwimLo and SwimHi rats. This is articulated on page 14: "The quantitative nature of this methodology enabled proportions of bacteria genera known to be involved in the process of serotonin synthesis to be observed and compared between the categories of rats." Additionally, the paper's well-aligned method and conclusions are connected to existing research in the field. This is illustrated on page 17, when the paper states, "The hypothesis that deficiencies of specific microbial genera would correlate with the mental state of a rate was supported by the data quantified. In existing research, the Ruminococcus and Bifidobacterium genera in lower abundances have demonstrated strong links to serotonin deficiencies and more depressive symptoms (Cao et al., 2018; Singhal et al., 2019; Zhang et al., 2019). Based on the data collected, this research was able to conclude that the PST is a valid assay for qualifying antidepressants. The imbalances in microbiota demonstrated that the SwLo rats were deficient in both Ruminococcus spp. and Bifidobacterium spp. Their behavior was thus correctly qualified by the PST, indicating more depressive states than their counterparts as the PST is meant to demonstrate." The paper's ability to place its findings back into the professional community provides clear justification for its new understanding. Some might argue that the paper's use of overly scientific language and jargon hinder communication, as there are places when the paper's language is opaque and its communication too disciple specific. However, the elegant design of the paper's subheadings, figures, and tables help to offset the effects and enhance the communication of the paper's ideas.

Finally, the paper didn't earn a score of 4 as it provides substantial discussion of limitations on pages 18-19 and links these limitations to the methods used; the paper also provides a number of directions for future research on pages 19-20.