​​An Investigation of the Human Gut Microbiome’s Potential Influence on Tryptophan Metabolism in ADHD

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*Abstract -* Attention-deficit hyperactivity disorder (ADHD) is a common neurodevelopmental disorder [5]. However, despite extensive research, the mechanisms of ADHD are still dimly understood. While there is certainly a genetic component, research has also indicated that there are environmental factors at play [3]. Recently, gut dysbiosis has been proposed as a potential factor in the development of ADHD. Dysfunction of the serotonergic system has been previously implicated in the symptomatology of ADHD, particularly in the presentation of hyperactivity and impulsion. Serotonin is synthesized from tryptophan, an essential amino acid that must be obtained through diet [2]. Previous research has proposed that dysregulation of gut tryptophan metabolism may have a role in the presentation of ADHD, though research in this area is limited [18]. This analysis uses tryptophan pathway information, gut microbe sequences for individuals with and without ADHD, and known disease-gene associations to determine what microbes, metabolites, and genes may be involved in this. Differential abundance analysis with QIIME 2 revealed no significant differences in the microbe compositions of those with ADHD compared to a healthy control. However, the genes, microbes, and metabolites identified to be of interest may be useful targets in further investigation.

**Introduction**

​​*Attention-Deficit Hyperactivity Disorder (ADHD)*

ADHD is the most prevalent neurodevelopmental disorder. In the United States, an estimated 11% of children and 4.4% of adults have an ADHD diagnosis [5]. While ADHD is a lifelong condition, only an estimated 1/3rd of children with ADHD will have significant symptoms in adulthood. The reason for this is widely debated. Some psychiatrists have suggested that this reflects an overdiagnosis of ADHD in childhood, and others have argued that this can be attributed to an underdiagnosis of adult ADHD. Adults with ADHD tend to present with more internalized symptoms [20]. ADHD has three subtypes: a predominantly inattentive presentation, a predominantly hyperactive-impulsive presentation, and a combined presentation. Individuals with a predominantly inattentive presentation tend to struggle with organization, focus, and completion of tasks. They tend to be easily distracted and forgetful. Individuals with a predominantly hyperactive-impulsive presentation tend to fidget or talk a lot.

They may constantly feel restless and struggle with impulsivity. This greater impulsiveness confers an elevated risk for accidents or injuries. One Danish cohort study followed nearly 2 million individuals from their first birthday to 2013, for a maximum of 32 years. The cohort included more than 32,000 people diagnosed with ADHD. A review of premature deaths found that individuals with ADHD were twice as likely to die prematurely when compared to individuals without the disorder. This held true, even after adjusting for factors known to increase the risk of premature death - including age, sex, parental ages, and parental education. Most of the premature deaths in those with ADHD were from unnatural causes, primarily accidents [5]. While the overall risk of premature death is still relatively low, this highlights that ADHD is not merely an issue of poor focus. The symptoms of ADHD can be majorly disruptive in an individual’s life.

*Tryptophan Metabolism and the Gut-Brain Axis*

The gut-brain axis is a bidirectional network between the gastrointestinal tract and the central nervous system. This complex system not only maintains gastrointestinal homeostasis, but also influences mood and higher cognitive functions. The network consists of the central nervous system, the autonomic nervous system, the enteric nervous system, and the hypothalamic pituitary adrenal axis [1]. Serotonin is an essential signaling molecule in this axis. An estimated 90-95% of serotonin is produced by the gut, where it is primarily located and used by the enteric nervous system. This system consists of numerous neurons that regulate function of the gastrointestinal tract [9].

A deficiency in serotonin is associated with many psychological symptoms. These include anxiety, a depressed mood, aggression, insomnia, impulsive or compulsive behaviors, and even cognitive decline. It can also have physiological effects, such as digestive problems or persistent fatigue [16]. The only precursor for serotonin is tryptophan, an essential amino acid. Humans cannot synthesize tryptophan and must obtain it through diet. A few microbes of the gut microbiome, such as Escherichia coli, are capable of tryptophan production, however research indicates that is not significant enough to impact host physiology [17]. Current scientific evidence suggests that the gut microbiome plays a significant regulatory role in the gut-brain axis. By acting on tryptophan metabolism, microbes can directly affect the levels of serotonin in the host.

Within the gut, tryptophan metabolism has three major pathways that can be influenced by the microbiome. There is direct metabolism of tryptophan by metabolites in the indole pathway, the kynurenine pathway in immune and epithelial cells, and serotonin production in enterochromaffin cells. Within each of these pathways, there are intermediate products called metabolites. While tryptophan is the least abundant amino acid in animal cells, it is an essential precursor for a significant number of host and microbial metabolites [16]. Some commensal bacteria can promote serotonin synthesis in intestinal enterochromaffin cells through a metabolite-dependent mechanism. Some other microbes can directly produce serotonin by expressing tryptophan synthetase to metabolize tryptophan. The kynurenine pathway is controlled by immune responsive enzymes. Microbiome composition is essential in the intestinal immune response of the host, helping shape the response with bidirectional crosstalk between the microbiota and the immune system. Dynamic interactions between the gut microbiome and the host’s immune system are critical for intestinal homeostasis. By activating Toll-like receptors, microbes can initiate tryptophan metabolism in the kynurenine pathway and induce kynurenine production [17]. Disruption of the kynurenine pathway is associated with the pathogenesis of many serious disorders. These include cancer, autoimmune system dysfunction, schizophrenia, and neurodegenerative disease [2].

Specifically, when it comes to neuropsychiatric disorders, there is evidence that an imbalance of tryptophan metabolites triggers pathophysiological mechanisms that can lead to various neuropsychiatric disorders. As an example, one proposed mechanism for the onset of depression is overactivation of the kynurenine pathway. Not only does this result in increased inflammation, but it also decreases the availability of tryptophan and neuroactive metabolites, which are essential for serotonin synthesis in the brain. Serotonin produced in the gut, referred to as peripheral serotonin, cannot cross the blood-brain barrier, but tryptophan and neuroactive metabolites can [7]. Dysfunctions in tryptophan metabolism increase the risk that an individual will develop a psychiatric disorder. Given the role of the gut microbiome in regulating tryptophan metabolism, it is speculated that dysbiosis may promote psychiatric symptoms in some disorders, primarily through disruption of the gut-brain axis [14].

*Gut Tryptophan Metabolism and ADHD*

The underlying causes of ADHD are still poorly understood. While scientists know that it’s a combination of genetic and environmental factors, the exact mechanisms are still unknown. Some researchers have explored gut microbiome dysbiosis in individuals with ADHD. While generally smaller studies, multiple papers have found that certain microbes have higher or lower abundance in ADHD when compared to controls [11].

Dysfunction of the serotonergic system has been previously implicated in ADHD. Tryptophan is the sole precursor to serotonin, and its metabolism may influence ADHD symptomatology, though the results have been unclear. Some studies have suggested that chronic tryptophan depletion may increase hyperactivity and disruptive behaviors in children with ADHD. One study found that in medication-free children with ADHD combined presentation, serum 3-hydroxykynurenine/kynurenine ratios were significantly lower than in a control group. Researchers attributed this to an abnormal balance between serotonin synthesis and tryptophan degradation [2].

This analysis seeks to determine if there are any detectable variations in human gut microbiome composition between individuals with ADHD and a healthy control that may result in abnormal tryptophan metabolism. Given previous studies, some differentially abundant microbes would be expected. Additionally, this investigation aims to identify metabolites and genes that might be implicated in microbiome dysbiosis in individuals with ADHD. This may help unravel the complicated relationship between the host gut microbiome, tryptophan metabolism, and ADHD. It will also help determine potential avenues for more targeted research.

**Materials and Methods**

***Sequencing Data***

All metagenomic data used in this analysis originated from the American Gut Project (AGP). Launched in 2012, the AGP aimed to collect massive amounts of data about the human gut microbiome to better understand its link to certain phenotypes and lifestyle variables. Samples were collected from more than 10,000 participants, primarily from the United States, United Kingdom, and Australia. Participants were citizen scientists who were sent standardized kits for fecal sample collection. Pre-processing of the data was done to correct for the effects of microbial overgrowth during room-temperature shipping [7]. The AGP used 16S rRNA amplicon sequencing for all samples. A survey on health status, medical history, and lifestyle data was given to all participants - though all questions were optional. Median response rate was 70.9% [7].

Filtering of samples was done using criteria that the AGP had used in their analysis to define a “healthy adult” subset. Participants had to be between the ages of 20 and 69, have a BMI between 18.5 and 30, and the sample had to have at least 1,250 sequenced reads [7]. Additionally, sequencing of the sample had to pass quality control. Filtering was done using the web interface of GMrepo, a curated and annotated repository of human gut metagenomes. GMrepo was created to increase the usability and accessibility of metagenomic data, as well as enable phenotype and cross-project comparisons [246 While GMrepo v2 contains 353 projects, the American Gut project (EBI accession: PRJEB11419) was the only one to contain the ADHD phenotype (MeSH ID: D001289). Other publicly available repositories of human gut metagenomic data had very few samples associated with the ADHD phenotype. For the sake of standardization, only AGP samples with a Health phenotype (MeSH ID: D006262) were used for comparative analysis. Phenotype designations were made by the AGP. A Health phenotype indicates that the sample originated from an individual who did not have current or chronic health issues. An ADHD phenotype indicates that the sample originated from someone who had been previously diagnosed with ADHD by a medical professional [7].

Three groups of samples were defined for this analysis: those with a Health phenotype, those with an ADHD phenotype only (ADHD-single), and those with an ADHD phenotype and at least one additional phenotype (ADHD-multi). ADHD is known to have comorbidity with mental health conditions and autoimmune conditions [12]. The definition of two ADHD groups was done to capture how the presence of co-morbid conditions might affect observed microbial composition differences. GMrepo does not actually contain any raw sequencing data; only run IDs, metadata, and relative abundance information. For each of the three groups of samples, the run ID of each sample was used to retrieve the raw sequencing data from NCBI’s Sequence Read Archive (SRA). A provided SRA toolkit allows for retrieval to be done programmatically. Raw sampling data was retrieved as FASTQ files and sequences had already been demultiplexed.

***QIIME 2 Analysis***

From GMrepo, 990 runs had been identified for Health phenotype, 29 runs had been identified for the ADHD-single phenotype, and 99 runs had been identified for the ADHD-multi phenotype. Analysis of the collected raw sequencing data was done using QIIME 2, an open-source microbiome analysis platform [3]. The command line interface and default QIIME 2 environment were used.

Given that QIIME 2 analysis can be computationally intensive, and to make testing between phenotypes even, random sampling was used to select subsets of runs for analysis. Two sets of QIIME 2 analysis were conducted, one for each individual group and one for all three groups together. For each individual group, four trials were done, each with five randomly selected runs. For the combined group analysis, ten trials were done, with each group having five randomly selected runs, for a total of fifteen runs in each trial. Metadata for each randomly selected run was compiled into a single metadata table for QIIME 2 analysis. Deblur, which is implemented in QIIME 2, was used for denoising and filtering. Default parameters were used, though trim length was set to 125 nucleotides, as that was the trimming used by AGP in their processing of 16S sequence data [7]. QIIME 2 was used to perform alpha and beta diversity analysis, as well as taxonomic analysis and differential abundance testing with ANCOM. Permutational analysis of variance (PERMANOVA) was used to further assess dissimilarity between phenotypes. The gg-13-8-99-515-806-nb classifier was used in taxonomic analysis. Maximum sampling depth and even sampling depth were manually chosen for each trial by reviewing the “Frequency per sample” and “Interactive Sample Detail” to maximize the number of sequences retained per sample. For the individual groups, ANCOM was used to assess sequence variation by sex.

***Microbe and Metabolite Extraction***

GMrepo’s provided API was utilized to programmatically retrieve relative abundance data for each run used in this analysis. Relative abundance data was retrieved separately for each phenotype. Microbes that were not present in at least two runs were discarded, as were any microbes that had a median relative abundance below 0.01%. Additionally, only the microbes present in both the ADHD and health phenotypes were utilized. Two sample t-tests were conducted to determine which microbes had significant differences in relative abundance between phenotypes. The microbes with significant variations in relative abundance were then used in TrpNet to predict involved metabolites. TrpNet is a resource for researchers studying tryptophan metabolism in human or mouse gut microbiomes. Tools on TrpNet include an annotated tryptophan metabolism database, network creation from a set of selected microbes or metabolites, high-resolution mapping of tryptophan metabolism across more than 5,270 microbes, and prediction of production potential of ​​tryptophan metabolites [24].

DisGeNet was used to retrieve gene-disease associations for ADHD. DisGeNet is an extensive database of human disease-gene and disease-variant associations, integrating data from a variety of sources: GWAS catalogs, animal models, scientific literature, and expert-curated repositories [8]. The phenotype definitions used in DisGeNet can be very specific. Due to the broad definition of the ADHD phenotype used by the AGP, a total of nine ADHD phenotypes were used to query DisGeNet for disease-gene associations. Table 1 summarizes the phenotypes used in the query. Results were filtered to gene-disease associations with at least one supporting curated source.

**Table 1.** Phenotypes used in DisGeNet Query

| **Name** | **UMLS CUI (Concept Unique Identifier)** |
| --- | --- |
| Attention Deficit Disorder | C0041671 |
| Attention Deficit Hyperactivity Disorder | C1263846 |
| Attention Deficit and Disruptive Behavior Disorders | C0236964 |
| Attention-Deficit/Hyperactivity Disorder Predominantly Inattentive Type | C0339002 |
| Attention deficit hyperactivity disorder: combined type | C2945552 |
| Undifferentiated attention deficit disorder | C3665679 |
| Attention Deficit Hyperactivity | C3844818 |
| Child attention deficit disorder | C0004269 |
| ATTENTION DEFICIT-HYPERACTIVITY DISORDER SUSCEPTIBILITY TO 7 | C2751802 |

KEGG was used to determine which of these genes were implicated in tryptophan metabolism. The Kyoto Encyclopedia of Genes and Genomes (KEGG) is a collection of databases with biological pathways, genomes, diseases, drugs, and chemical substances. The KEGG PATHWAY database contains pathway maps, networks of molecular interactions and reactions [14].These can be used to map genes to gene products in the pathway. Human tryptophan metabolism has 45 associated genes in KEGG. These genes were compared to the gene-disease associated extracted from DisGeNet to determine which tryptophan metabolism genes are potentially involved in ADHD. Using the KEGG pathway, the metabolites of each identified gene’s reactions in tryptophan metabolism were determined. This was given as input into TrpNet to predict involved microbes. The lists of extracted and predicted microbes were compared to each other, as were the lists of extracted and predicted metabolites.

**Results**

***QIIME 2 Analysis***

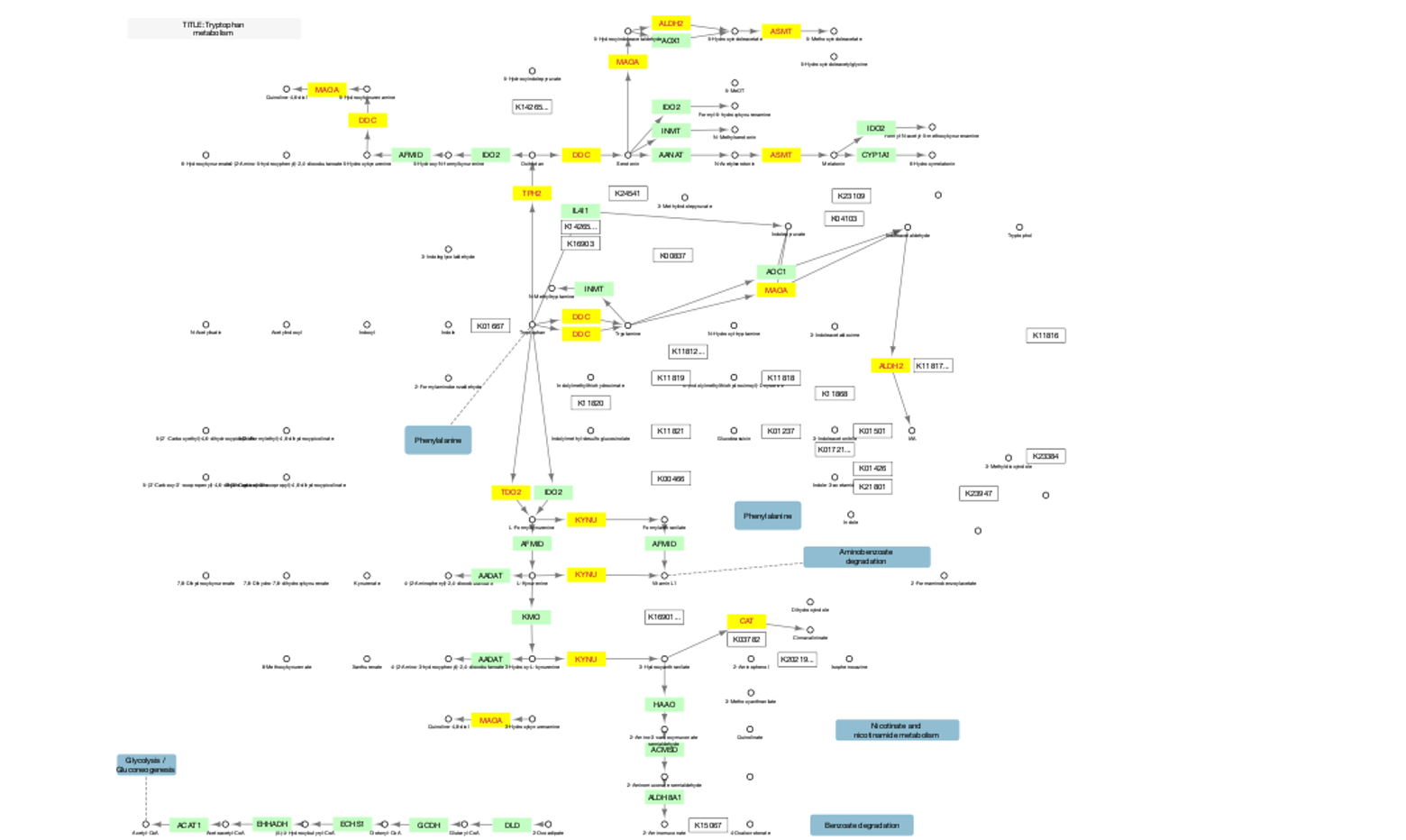
In microbial diversity analysis with QIIME 2, four trials were run for each group (health, ADHD single phenotype, ADHD multiple phenotypes). None of these trials had significant differences in Shannon’s diversity index or Faith’s phylogenetic diversity; two measures of alpha diversity. All p-values were greater than 0.05. Bray-Curtis distance and Jaccard distance revealed that each phenotype’s selected samples had no significant dissimilarities. Differential abundance testing with ANCOM (Analysis of composition of microbes) found no significant features for sex.

Ten trials were run that had five samples of each group. For Shannon’s diversity index or Faith’s phylogenetic diversity, only three of the ten trials had significant differences. However, for each trial, the identified significant features were different. This indicates that these significant features were likely unique to the subset of samples being tested. To directly test variations between phenotypes, PERMANOVA (Permutational analysis of variance) was utilized. The p-values and q-values were above 0.05 for all ten trials. These results indicate that for the samples used in this investigation, there were no statistically significant variations in microbiome composition detected.

***Tryptophan Metabolism Genes***

From the nine phenotypes used to query DisGeNet, a total of 1,640 gene-disease associations were identified with at least one curated source with evidence for the association. KEGG was used to determine which of these genes were implicated in tryptophan metabolism. A total of nine genes were identified: ACAT1, ALDH2, ASMT, CAT, DDC, KYNU, MAOA, TDO2, and TPH2. Figure 1 shows a visualization of the human tryptophan metabolism pathways. Tryptophan is indicated by a red circle. The implicated genes are highlighted in yellow. These nine genes are dispersed throughout the pathways and are involved in several key regulatory steps, such as the transformation of tryptophan into formyl-kynurenine, which is the initial step of the kynurenine pathway [1].

**Figure 1**. Tryptophan Metabolism Pathway with Identified Genes Highlighted



A literature review revealed that five of these identified genes (MAOA, CAT, DDC, and AMST) have previous research strongly implicating them in ADHD. DDC and ASMTpolymorphic variants confer greater risk of certain neuropsychiatric disorders, includingADHD. The exact relationship between these variants and ADHD is yet to be determined [8]. Another study found that decreased CAT activity resulted in reduced resilience to oxidative stress in individuals with ADHD, though the evidence was somewhat inconclusive [2]. MAOA promoter polymorphisms were significantly greater in ADHD patients when compared to a cohort of control patients [12].

Three genes (TPH2, ALDH2, KYNU) have not been directly implicated in ADHD, but have effects that may contribute to the symptoms of ADHD. Various TPH2 polymorphisms are known to influence serotonergic function. These polymorphisms are associated with mood disorders and altered responses to antidepressant medication [9]. Having one inactive copy of ALDH2 is known to reduce the risk of alcoholism in males. However, in male alcoholics with an inactive copy of ALDH2, there was a significantly higher prevalence of ADHD in comparison to the male alcoholics without an inactive copy [13]. KYNU overexpression can result in neuroinflammation and the pathogenesis of some autoimmune conditions, including psoriasis [15].

***Metabolite and Microbe Extraction***

The nine identified genes had 22 metabolites associated with their reactions in the tryptophan metabolism pathway. For this, TrpNet prediction generated a list of 1,789 potential microbes. Predicted microbes either produce or interact with one of the given metabolites. Given that many microbes can have common metabolites, microbe prediction from metabolites is generally less useful than metabolite prediction from microbes [6].

In GMrepo, for the Health phenotype runs, relative abundance data was available for 343 species and 113 genera. For the ADHD phenotype runs, 354 species and 99 genera had relative abundance data. Mean, median, and standard deviation was calculated for the relative abundance values of each species/genus. A two-sample t-test was used to analyze the statistical significance of variations in relative abundance between phenotypes. A total of 76 species and 29 genera had a p-value less than 0.05 and a total of 54 species and 22 genera had a p-value less than 0.01. TrpNet prediction is less accurate on the species-level, so only the genera were used for prediction. The 29 genera that had a p-value greater than 0.05 were used for metabolite prediction in TrpNet. The microbe and metabolite lists generated from associated genes were compared to microbe and metabolite lists generated from relative abundance data obtained from GMrepo for the tested samples. From GMrepo and t-testing, 37 metabolites were predicted from the 29 microbes.

In a comparison of the predicted and extracted metabolite lists, there were only 10 shared metabolites. While the overlap is unexpectedly low, the 10 shared metabolites are integral to tryptophan metabolism. Figure 2 shows the metabolites in the TrpNet tryptophan metabolism network.

**Figure 2**. Tryptophan Metabolism Network for Shared Metabolites



All the metabolites have both microbe and host origin. For the microbe lists, there was an overlap of 27 microbes. Only two of the differentially abundant microbes, Victivallis and Candidatus Methanomethylophilus, were not in the TrpNet predicted microbe list. Table 2 presents a summary of the overlapping genus with statistically significant differences in relative abundance between the ADHD and Health phenotype. Green indicates that the microbe had higher relative abundance in the ADHD phenotype than in the Health phenotype. Red indicated that the relative abundance was lower in the ADHD phenotype compared to the Health phenotype.

**Table 2. Genus with significant differences in relative abundance between the ADHD and Health phenotypes**

| **Genus** | **Prevalence in Health Runs (%)** | **Prevalence in ADHD Runs (%)** | **p-value** | **Δ Mean Relative Abundance (Health - ADHD)** |
| --- | --- | --- | --- | --- |
| **Parabacteroides** | 98.48 | 96.88 | 0.02388984 | -0.544 |
| **Holdemanella** | 45.25 | 32.81 | 0.0014329 | -0.420 |
| **Erysipelatoclostridium** | 97.78 | 96.09 | 0.02713935 | -0.204 |
| **Peptococcus** | 26.57 | 22.66 | 7.91E-06 | -0.087 |
| **Dorea** | 93.13 | 91.41 | 0.0255287 | -0.034 |
| **Bosea** | 2.12 | 3.13 | 1.49E-06 | -0.033 |
| **Citrobacter** | 14.14 | 16.41 | 7.76E-07 | -0.032 |
| **Thermus** | 3.64 | 3.91 | 1.61E-21 | -0.029 |
| **Holdemania** | 81.62 | 78.13 | 0.00912956 | -0.012 |
| **Slackia** | 39.39 | 37.50 | 0.03581417 | -0.012 |
| **Methanomassiliicoccus** | 2.02 | 1.56 | 3.88E-08 | 0.009 |
| **Dickeya** | 8.79 | 5.47 | 0.00221279 | 0.024 |
| **Butyricimonas** | 10.81 | 7.03 | 0.02779029 | 0.033 |
| Victivallis | 37.68 | 21.09 | 3.65E-06 | 0.044 |
| **Odoribacter** | 86.46 | 76.56 | 0.0013326 | 0.063 |
| **Saccharibacillus** | 1.72 | 1.56 | 1.53E-46 | 0.067 |
| **Christensenella** | 76.57 | 67.19 | 0.00340604 | 0.073 |
| **Granulicatella** | 33.43 | 30.47 | 4.88E-14 | 0.095 |
| **Sporobacter** | 92.42 | 89.84 | 0.0015079 | 0.104 |
| **Mitsuokella** | 16.97 | 17.97 | 0.00011372 | 0.123 |
| **Clostridium** | 98.59 | 96.88 | 0.00024506 | 0.126 |
| Candidatus Methanomethylophilus | 2.93 | 4.69 | 9.00E-30 | 0.151 |
| **Acidaminococcus** | 77.17 | 82.03 | 0.00162168 | 0.197 |
| **Coprococcus** | 95.76 | 92.97 | 0.00040057 | 0.209 |
| **Oscillibacter** | 97.47 | 97.66 | 0.0071201 | 0.290 |
| **Barnesiella** | 89.29 | 78.13 | 0.01704705 | 0.310 |
| **Dialister** | 87.17 | 80.47 | 0.01863108 | 0.374 |
| **Ruminococcus** | 98.38 | 96.88 | 4.98E-06 | 0.468 |
| **Alistipes** | 98.69 | 96.88 | 0.00454 | 0.604 |

**Discussion**

This analysis failed to find any microbes that were differentially expressed between the ADHD and Health phenotypes. In analysis of microbiome composition, no significant features were found. For each individual group (Health, ADHD-Single, ADHD-Multi), no trials had significant alpha or beta diversity metrics. In testing of the three groups together, significant features were found for three of the ten trials. However, these were disregarded, as the significant features found were different in each trial. PERMANOVA testing revealed that there were no significant differences in microbiome composition between phenotypes. ANCOM results also indicated that there were no notable variations in microbiome composition between the sexes for any individual phenotype.

However, it can also not be concluded that no notable variations in microbiome compositions exist between individuals with ADHD and healthy individuals. As previously mentioned, the AGP assigned the ADHD phenotype to participants with a previous diagnosis of ADHD by a medical professional. No information was gathered on the age of diagnosis, symptoms, severity, or treatment. Each of these factors can affect/be affected by microbiome composition, resulting in greater overall variability within the ADHD phenotype. For example, methylphenidate, a common stimulant used in the treatment of ADHD, has a modulatory effect on the kynurenine pathway [14]. In one study of children with ADHD that measured the daily fluctuations of several kynurenine products through urine collection, lower excretion of certain neurotoxic metabolites was observed after treatment with methylphenidate. The researchers hypothesized that this may have been the result of decreased activation in the kynurenine pathway [12].

Without the ability to examine various subsets of the ADHD phenotype, properly exploring the relationship between the gut microbiome’s influence on tryptophan metabolism and ADHD symptomatology is impossible. It would be useful to repeat this analysis with more robust dataset(s). There is more robust data available for human gut microbiome data associated with the ADHD phenotype. However, access requires membership in a consortium and is therefore out of the scope of this analysis.

Exploring the effect of sex on results was also outside of the scope of this analysis. However, given that ADHD tends to present differently in males and females, and that roughly 3 males are diagnosed for every 1 female, it may be worthwhile to examine [3]. While cursory testing with QIIME 2 revealed no significant differential abundance between sex for any trials, many studies have found consistent differences in male and female presentations of ADHD [5]. MAOA, one of the identified genes, is an X-chromosome gene which codes for an enzyme that catalyzes the oxidative deamination of amines, including dopamine, norepinephrine, and serotonin. Multiple studies have previously implicated MAOA polymorphisms in psychiatric disorders, including ADHD. In one study examining the relationship between MAOA and sex, associations were found between ADHD and gene polymorphisms for both sexes [12]. In a future investigation, exploring the genes, microbes, and metabolites implicated in ADHD by sex may yield some additional novel targets.

While none of the differential abundant microbes cannot be confirmed with QIIME 2 analysis, they may be useful targets for a more focused investigation of gut tryptophan metabolism and ADHD. The same is true for the ten metabolites and nine genes determined to be of interest. While this investigation has not resulted in any concrete results, it has successfully established targets within the complex relationship between host, microbiome, and disease that warrant further exploration.

**Data Availability Statement**

All data and code utilized in this analysis have been deposited in a repository at <https://github.com/ecarl-glitch/SeniorCapstone>.

All microbiome samples were retrieved from the American Gut Project [7]: [PREJEB11419](https://www.ebi.ac.uk/ena/browser/view/PRJEB11419?show=publications)**.**

Pathway information for tryptophan metabolism was retrieved from KEGG pathway [hsa00380](https://www.genome.jp/entry/hsa00380).

Microbe and metabolite predictions were done with TrpNet [24]. The TrpNet database is available at <https://www.trpnet.ca/home.xhtml>.

Gene-disease associations were extracted from DisGeNet [8], available at <https://www.disgenet.org/>.

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