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Characterizing A Link Between Gut Microbiome and Attention Deficit Hyperactive Disorder

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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

**CHARACTERIZING A LINK BETWEEN GUT MICROBIOME
AND ATTENTION DEFICIT HYPERACTIVE DISORDER**

Department of Biology

College of Arts and Science

By

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2017

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List of Abbreviations

ADHD	Attention Deficit Hyperactive Disorder
HMP	Human Microbiome Research
OTU	Operational Taxonomic Units
EF	Executive Function
ER	Emotional Regulation
5-HT	Serotonin
GABA	Gamma-aminobutyric acid
GF	Germ Free
SPF	Specific Pathogen Free
ASD	Autistic Spectrum Disorder
HPA	Hypothalamus- Pituitary-Adrenal
BDNF	Brain Derived Neurotropic Factor
ASA24	Automated Self-Administered 24-hour Dietary Recall
CC	Conventionally Colonized
ASRS	ADHD Self-Report Scale
CORT	Corticosterone
CRF	Corticotropin Releasing Factor
DA	Dopamine
PCR	Polymerase Chain Reaction
LefSe	Linear Discriminant Analysis

Abstract

The role of microbiome is slowly emerging in the field of mental health. Gut microbiome has been related to anxiety, autism, mood disorder and to memory impairments. However its interplay with higher cognition such as Executive Functions (EF) and Emotion Regulation (ER) still remains an open question. Attention Deficit Hyperactivity Disorder (ADHD), a highly prevalent mental disorder, is associated with EF and ER dysfunction. Given that microbiome influences the brain through the release of neurotransmitters such as, dopamine, this study aims to link microbiome with ADHD. Consequently, the proposed project compares participants with ADHD and healthy controls. Fecal samples were collected from each individual using a Gut Kit. Each sample was then analyzed using 16S MiSeq Illumina sequencing to look at the differential abundance of various bacteria. It is hypothesized that the gut microbiome composition will differ between ADHD and the healthy control. The human microbiome is quite malleable, and provided that a link is formed between ADHD and microbiome, a plausible treatment at an earlier developmental stage can be applied.

Introduction:

The gut microbiome refers to the genetic material (DNA) of the 100 trillion microbial cells, including the various types of bacteria and fungi, present in an individual's gut. These cells are ten times the total number of cells within a human being. These microbes do not occupy the human body during gestation but rather obtain it from mother when given birth or through the environment in the first two years of development (Koenig *et. al* 2010). Fundamentally, gut microbes have been known for their mutualistic relationship with humans where the gut microorganisms ferment dietary

fiber, synthesize vitamin B and K and metabolize bile acids and sterols (Clarke et al. 2014). It has been found that in an adult, two phyla, *Bacteroidetes* and *Firmicutes*, colonize 90% of the gut. Bacteroidetes have carbohydrate-degrading enzymes while Firmicutes are simple sugar fermenting bacteria that produces short fatty chains like butyrate (Fischbach and Somenburg 2011). Subsequently, these microbes have also been associated with maturation and proper functioning of the central nervous system. Studies have shown that the microbes release neuroactive molecules such as dopamine and serotonin as well as neuro-inhibitory molecules like aminobutyric acid which interact with the human nervous systems (Spencer et al. 1995). Proper regulation of these molecules ensures the proper functioning of the brain and its various regions.

This critical interaction between the gut and brain, that has also come to be known as the gut brain axis, has allowed scientists to relate the microbiome with neuropsychiatric issues. In neurobiological terms the imbalance of the neurotransmitters

is one of the reasons that individuals suffer from psychiatric disorders like autism and depression. In fact, as of

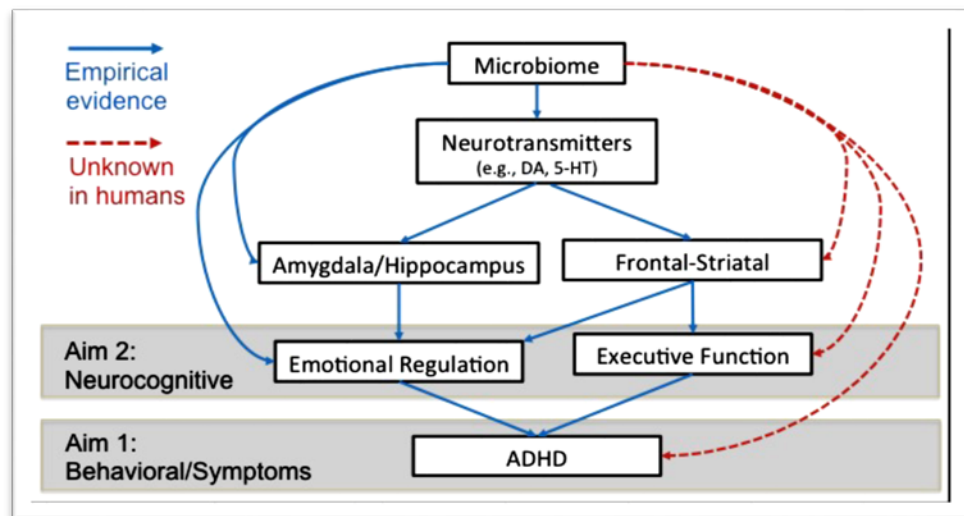


Fig 1: Currently well-known information as compared to information that will potentially be identified from the study

current research, the gut microbiome has been linked to autism and depression (Mangiola

et. al, 2016). However, the link between Attention Deficit Hyperactive Disorder (ADHD) and the microbiome is still unclear. As shown in Figure 1 certain areas where plays a significant microbiome have been identified by past research. Previous research has shown that microbiome regulates the neurogenesis of hippocampus predominantly in the dorsal area (Ogbonnaya et al. 2015). Other research also provide empirical evidence that microbiome can influence the Emotional Regulation (ER) region of the brain. Moreover, rodent models have also shown that microbiome influences neurotransmitters such as dopamine (Sampson and Mazmanian, 2015). In regards to ADHD, certain neurotransmitters like dopamine, serotonin or GABA influence various brain regions such as the hippocampus, Emotional Regulation and Executive Function. Lastly, there is also empirical evidence that individuals with ADHD have a deficit in executive function and emotional regulation areas of the brain (Vaidya and Stollstorff, 2008). However, no evidence have been collected that related microbiome to Executive function or ADHD itself.

ADHD is a major neuropsychiatric disorder that occurs at a relatively high prevalence, affecting 4-14% of adolescents and adults (Faraone & Biederman, 2005; Messaoudi et al.,2011). It is characterized by hyperactive behavior, impulsivity, and inattention. Due to these impairments in behavior, the individual can also suffer from lower academic achievements, lower employment success, and an increased risk of incarceration (Satterfield et al. 2007). Multiple factors are associated with the pathogenesis of ADHD including environment and genetics. The disease has a 70% to 80% heritability rate (Brikell, Kuja, Larsson, 2015) suggesting a very high likelihood for an offspring to have ADHD if their parents have been diagnosed with it. Among others

(such as autism and mood disorder, both known to be linked to microbiome dysbiosis), individuals with ADHD also tend to have a dysfunction in Executive function (EF). Executive function in its simplest form consists of cognitive processes, including cognitive control working memory, reasoning, problem-solving and planning, to monitor and control behavior that facilitates in attaining a desired goal (Chan et al 2008). In ADHD, the dysfunction in EF does not allow them to perform these processes properly and thus, these individuals struggle in achieving their goals. This dysfunction is often related to the imbalance in neurotransmitters such as dopamine and serotonin the striatum and prefrontal cortex regions of the brain (Vaidya & Stollstorff, 2008). Thus, the most commonly used treatment for ADHD today is methylphenidate, which blocks dopamine in the striatum, which then influences the prefrontal cortex.

Furthermore, working memory (a crucial component of EF) has also been linked to the gut microbiome using a working memory like task in rodents. According to the research, it was shown that rodents that were germ-free (GF), that is they were sterile with no microbiome, displayed impaired working performance as compared to controls (the specific pathogen free (SPF) rodents) (Gareau et al. 2011). Given these findings, as well as the studies involving rodent models that show microbiome influencing dopamine levels in the frontal cortex and striatum, a plausible relationship can be suggested between microbiome and ADHD.

Given that the microbiome releases neurotransmitters in the host, an imbalance in their relative composition can lead to a disproportion concentration of neurotransmitters in the body. Furthermore, ADHD has a high morbidity with Autism and Depression, and individuals with these disorders do have a different bacterial composition as compared to

healthy controls (Zhou *et. al* 2008) (Jiang *et. al* 2015) (Parracho *et.al* 2005). Hence, it is reasonable to suggest that the microbiome plays some role in ADHD as well. Therefore, the aim of the study is to form a link between ADHD and the gut microbiome. It is hypothesized that the microbial composition of the gut will differ between the ADHD and healthy controls. Given that a relationship is formed, there could be a new approach to treating ADHD, through altering diet and exercise or by pre- and pro-biotics.

Literature Review

The following literature review is conducted to form a theoretical link between ADHD and the gut microbiome.

The microbiome is notably known for its role in the digestion of complex carbohydrates and dietary fiber and releasing metabolites like butyrate and propionic acid in the body (Cummings *et. al* 1987). As a result, it can be assumed that an imbalance in the microbiota can lead to an imbalance in the relative concentration of these metabolites. A study conducted by MacFabe, Cain, Rodriguez-Capote *et. al* 2011 examined the impacts of propionic acid (PPA) on adolescent male rats in terms of restricted/repetitive behaviors, social behavior and cognition (behavioral traits linked to autism). PPA was administered to the rats prior to each behavioral test. It was observed that rats treated with PPA presented with restricted behavior to certain objects, impaired social behavior, and cognitive dysfunction as compared to the control. Since ADHD patients can also experience a similar form of dysfunctionality, it can be inferred that a bacterial imbalance in the gut is resulting in an imbalance in the metabolites in the blood. These metabolites then influence the individual's behavior and cognition.

Gareau, Rodrigues and Cho *et. al* (2011) demonstrated that the working memory is associated with gut microflora in rodent models. It showed that the germ-free (GF) mice involved in the study displayed impaired working memory in response to the novel object task and the T-maze task as compared to the specific pathogen mice (SPF) mice. This highlights that the presence of microbes is crucial for mediating non-spatial or working memory. This outcome could be translated to humans as well since the human gut is also composed of numerous bacterial taxa, fungi, and archaea. This study is particularly important because it linked working memory with microbial composition. Working memory is one of the most important cognitive process performed by executive function (EF) and its deficit is one of the characteristics of ADHD patient. Since germ-free rodents experienced low working memory it can be inferred that an imbalance of microbiota in humans can result in developing ADHD symptoms, although this has never been tested.

In order to understand the influence of the gut microbiome on the neural system, Sudo *et. al* (2004) examined the effects of post-natal microbial colonization on infant brain development. The comparison between GF and SPF revealed an increased level of CRF mRNA in the hypothalamic region of the GF mice which resulted in an enhanced Hypothalamic- Pituitary-Adrenal (HPA) stress response. However, the acute HPA stress in GF mice was partly normalized in 3 weeks after reforming the microflora at an earlier development stage. As a result, the study suggests that a commensal microbiota is important in the development of the HPA stress response. Since ADHD is a neurodevelopmental disorder, it can be theorized that an imbalance in early gut bacteria prevented the development of key brain regions. Furthermore, individuals with ADHD

also experience a low level of cortisol, which indicates a dysregulation of the HPA-axis (Ma *et. al* 2011). As the study suggests, microbiota is important for developing HPA-axis, and it is possible that individuals with ADHD experienced an imbalance in their microbiota which resulted in them having lower levels of cortisol, a hypothesis that has yet to be tested.

The influence of the microbiome on neurodevelopment was further supported by the study conducted by Williams *et al.* (2016). The study hypothesized that that increased growth of the good bacteria at an early age will lead to an optimum brain development and maturation. Twelve suckling rat pups were administered with either a solution of BGOS, a galactic-oligosaccharide prebiotic, or a control solution. It was found that the introduction of BGOS preparation significantly elevated synaptic proteins such as synaptophysin and brain-derived-neurotropic factor (BDNF) in the hippocampus of 22-day old rats. Synaptophysin is major synaptic vesicle protein that regulates the kinetics of synaptic vesicle endocytosis (Kwon and Edwin, 2011). BDNF is a growth factor that supports the existing neurons in surviving and promotes the growth and maturation of new neurons and synapses (Huang and Reichardt, 2001). Therefore, the study confirms that the manipulation of gut bacteria at an early developmental stage can have central effects on neurodevelopment which can persist until young adulthood. It was also observed that the method of action was through influencing neurotransmission and neural signaling rather than the cellular structure of the brain. Individuals with ADHD also experience abnormal levels of neurotransmitters, such as dopamine and serotonin. As a result, it can be inferred that an improper colonization by the gut bacteria in early life can

result in less neuron synapsis and less synaptic vesicle protein, which then leads to an imbalance of neurotransmitters being released in different brain regions.

The role of neurotransmitters in EF is the bridging point between microbiome and ADHD. A study conducted by Yoon, Bruce and Robyt (2009) showed that bacteria, such as *Bacillus* strain, contains an enzyme that can be used to synthesize dopamine. The study showed that dopamine was synthesized when cyclomaltohexaose, a complex carbohydrate, was reacted with an enzyme called cyclomaltodextrin glucosyltransferase or CTGase. This enzyme was retrieved from the bacteria *Bacillus macerans* and the reaction was performed in vitro. The synthesis of dopamine was further confirmed by mass spectrometry. Therefore, the results of the study suggest that bacterial species such as *bacillus* have the components to produce neurotransmitters. As a result, it is possible that they make their own contribution in maintaining a balance in neurotransmitter levels in an individual's body. Lastly, since *bacillus* is able to synthesize dopamine, and dopamine modulates EF, it is reasonable to say that microbiome is linked to executive functions and consequently ADHD as well.

Serotonin is another important neurotransmitter in determining proper cognitive functions and an imbalance of serotonin levels is characterized in ADHD individuals. The study conducted by Yano et al. (2015) suggests that certain gut microbes regulate serotonin (5-HT) levels in the colon and the individual's blood. This is in lieu to the observation that the gastrointestinal tract consists of most of the body's serotonin as either 5-hydroxytryptamine or 5-HT. The intestine consists of specialized endocrine cells called chromaffin cells that synthesize serotonin using tryptophan hydroxylase (Tph). The results from the study show that by administering para-chlorophenylalanine (PCPA),

a Tph inhibitor, to bacteria inhibits its contribution of 5-HT in the colon and the blood. Moreover, it is also observed that Germ Free (GF) mice have substantially reduced serotonin levels than specific pathogen free mice. Further observation also shows that the microbiota regulates 64% of colonic serotonin levels and 49% serum concentrations. The study also demonstrates that the indigenous spore-forming microbes within the colon substantially mediate the effects of 5-HT concentrations. These microbes release certain metabolites that influence the chromaffin cells to synthesize more 5-HT. Fecal samples of the participants show that metabolites such as α -tocopherol, tyramine and PABA are elevated suggesting that they play the major role in influencing chromaffin cells. Overall the study suggests that the indigenous bacteria produces metabolites which signal the chromaffin cells to increase Tph levels and 5-HT biosynthesis. The 5-HT is then released in the periphery and can travel to specific brain regions such as EF and ER.

Clarke, Grenham and Scully et al. (2003) examined the effects of microbiota on the hippocampal serotonergic system. The serotonergic system works in brain regions involved in stress, anxiety, and depression (Graeff *et. al* 1996). The study included first generation offspring of Germ-Free (GF) mice and Conventionally Colonized (CC) mice that were randomly allocated into groups. A group of male GF mice was placed in a conventional animal facility with bedding and fecal sample from CC mice. This was primarily because these environmental conditions have been known to restore normal microbiota in mice. The results from the study indicated a significant increase in serotonin transporter gene concentration in GF as compared to CC mice. The analysis also showed that the GF mice had increased tryptophan concentration and decreased kynurenine to tryptophan ratio. However, following the microbial colonization in the GF

mice these levels were restored to normal. The GF mice also experienced a 1.3 fold increase in serotonin levels. In all, following the colonization, plasma tryptophan leveled off and as tryptophan is a precursor of serotonin, its reduction can be related to the increase in the plasma serotonin level. This change in the plasma serotonin levels following colonization suggests that microbiota in the gut interacts using neurotransmitters like serotonin.

In order to further support a possible link between the gut microbiome and ADHD, and its effects on other neuropsychiatric disorders including autism, depression, and anxiety were examined by multiple research groups (McConachie *et. al* 2005) (Naseribafrouei *et. al* 2014) (Crumevolle-Arias 2014). The correlation of microbiome and Autism Spectrum Disorder (ASD) was shown in the study conducted by Parracho, Bingham, and Gibson et al. (2005). Individuals with (ASD) tend to suffer from severe gastrointestinal problems most of which arise from the disruption of the gut microflora. The participants involved in the study were children with ASD that were being compared with the healthy children. Following the assessment of the fecal samples, it was found that the fecal flora of ASD individuals have higher abundance of *Clostridium histolyticum* bacteria (Clostridium clusters I and II) than that of healthy children. These results were confirmed when a subsequent part of the study reported that when patients with ASD were given vancomycin orally, they experienced a short term, but significant, improvement in their ASD symptoms. Vancomycin is an antimicrobial agent that may have temporarily affected the growth of the bacteria. Although autism and ADHD are two distinct mental illnesses, both disorders share similar symptoms, such as impulsivity, social awkwardness, and inattention, and most importantly, EF and Emotional Regulation

(ER) dysfunction. Due to such similarities from the study, it can be inferred that the individual's gut microflora might play a significant role in ADHD patients.

Furthermore, the correlation of microbiome with depression was shown in the study conducted by Naseribafrouei, Hestad, and Avershina et al. (2014). Fecal samples of 37 depressed patients and 18 non-depressed controls were collected. Then, using the Illumina deep sequencing of 16S rRNA the microbial compositional data was analyzed. The results from the study showed that *Bacteroidales* were underrepresented in depressed individuals while *Alistipes* and *Oscillibacter* were over-represented. *Bacteroidetes* have previously been associated with obesity, which is also linked to depression through low-grade inflammation (Stunkard et al. 2006). Lastly, the genus *Oscillibacter* has valeric acid as its metabolic end-product. Valeric acid is structurally like Gamma-Amino Butyric acid (GABA), an inhibitory neurotransmitter, and being a homolog of GABA it can bind to GABA receptors as well (Katano et al. 2012). The over-representation of *Oscillibacter* leads to an increase in valeric acid, which can then lead to increased inhibitory neurotransmission.

In addition to depression, the correlation of microbiome and anxiety has also been established. Crumeyrolle, Jaglin, and Bruneau, et al. (2014) examined the relationship between microbiota, anxiety-like behavior and Hypothalamus-Pituitary-Adrenal (HPA) axis in stress-sensitive rodents. The study aimed to assess the impact of the GF status on HPA axis reactivity and anxiety. As a result, the experiment involved subjecting the GF and SPF rats to a social interaction experience and an open-field test (OF). To account for the differences in HPA axis reactivity between the animals, the serum corticosterone (CORT) concentration, the expression of CRF gene in the hypothalamus and the

expression of glucocorticoid receptor gene in the hippocampus were measured. Moreover, monoamine concentrations in the frontal cortex, hippocampus, and striatum, anxiety related regions, were also measured. According to the results the GF rats had increased CRF mRNA levels, increased CORT, as well as impaired social behavior as compared to SPF rats. They also had a lower DA turnover rate in the frontal cortex, hippocampus, and striatum. These results suggest that the GF rats displayed greater anxiety-like behaviors than SPF rats, which can be associated with the absence of microbiota in GF. The 2-fold lower DA concentration in the frontal cortex, hippocampus, and striatum of GF mice also accounts for impaired stress regulation. Therefore, the study shows that anxiety-like behavior is effected by the absence of microbiota. The effect is the result of the intensified HPA axis reactivity and altered of monoamine concentrations. It is important to note that DA activity in the frontal cortex and striatum is critical for EF (Sawaguchi & Goldman et. al 1991). Disruption in DA in these regions leads to EF dysfunction, and ADHD (Stollstorff et al 2010).

Following the discovery of the link between the microbiome and some neuropsychiatric disorders, methods involving microbiota have been introduced to reduce the symptoms of these disorders. In a study conducted by Messaoudi, Violle, and Bisson et al. (2011) it was demonstrated that within the general population the levels of anxiety and depression decreased substantially following an intake of a probiotic formulation (PF). The PF consisted of the bacterial strains *Lactobacillus helveticus* and *Bifidobacterium longum* that have no known negative side effects or addiction. The study involved 25 individuals that had urinary cortisol levels between 10 – 50 ng/ml and identified as psychologically distressed using the scores from hospital anxiety and

depression scale (HAD) and Hopkins symptoms checklist (HSCL). Ten participants were given PF while the remaining 15 were provided with a placebo for 30 days. It was found that the administration of PF significantly increases the general scores in HAD and HSCL. Moreover, the sub-scores relating to anxiety and depression in HSLC improved substantially as well. As a result, it can be proposed that certain probiotics can help support in reducing the symptoms of neuropsychiatric disorders such as anxiety and depression.

Some other researchers have also proposed a mechanism of action taken by probiotics. A study conducted by Bravo, Forsythe and Chew et al. (2011) hypothesized that the ingestion of *Lactobacillus* strain can alter the expression of the GABA receptors through the Vagus Nerve. GABA is a main inhibitory transmitter in the central nervous system and is an important pharmacological target for antidepressant agents. The study involved identifying a difference in GABA receptor expression within the mice that were fed broth without bacteria and the mice that were fed broth with bacteria *Lactobacillus rhamnosus*. According to the results, *L. rhamnosus* fed mice showed reduced levels of GABA mRNA in the basolateral amygdala, central amygdala, locus coeruleus, dentate gyrus, cornus ammonis region 3, and cornus ammonis region 1 as compared to broth fed mice. Furthermore, it was also examined that Vagus Nerve played a central role in administering the effects of *L. rhamnosus*. It was found that the effects of *L. rhamnosus* on the GABA receptor were completely inhibited in mice with diaphragmatic vagotomy. Thus, this implies that certain probiotics may cause a direct effect upon associated behavioral and physiological responses in a manner which is reliant on the vagus nerve. Also, the reduction in the expression of GABA mRNA in areas like the amygdala,

hippocampus, and, Locus Coeruleus following *L. rhamnosus* administration is consistent with the antidepressant-like effect of GABA receptor antagonists, suggesting a plausible treatment for depression.

Even though oral intake of probiotics is a common method of altering microbiota in an individual's body, many studies have found that diet and exercise can alter the gut microbiome as well. A study conducted by Clarke, Murphy and O'Sullivan et al. (2014) explored that diet and exercise can be altered to influence the gut microbiota. The study involved two groups: a healthy control group and a professional athletes group. The compositional analysis of microbiota was performed using 16S rRNA amplicons sequencing with each participant completing a food recall questionnaire prior to fecal collection. Following the analysis, it was found that the athlete's group had a higher diversity of microorganisms representing approximately 22 distinct phyla. The diversity was positively correlated with the athletes' protein consumption and blood creatinine kinase level that were higher than the control due to their extreme diet and extensive exercise. In all, the results provide evidence that exercise and diet both can alter the gut microbiome diversity. Therefore, if an individuals' diet is altered at an early stage in life and the exercise level is increased, it is reasonable to say that the symptoms of the mental illnesses associated with microbiome can be reduced. As a result, if a link is formed between ADHD and microbiome, these treatment methods can be practiced upon children to reduce the effects of ADHD at an earlier stage.

Given that the microbiome influences brain regions, neurochemistry and behavioral symptoms associated with ADHD, it is plausible that such a link exists. It is hypothesized that the microbial composition will differ between ADHD and controls.

Research Methodology:

Participants:

The proposed study recruited 34 individuals for the pilot study comprising of 14 ADHD individuals and 20 healthy controls. Initially 156 participants were contacted from the 575 participants that participated in the Phase 1 study and completed Adult ADHD Self-Reported (ASRS) questionnaire. All individuals were students at Florida International University and varied with their age ranging from 18 years old to 28 years old ($p = 0.525$, $p > 0.05$). A total of 19 females and 15 males participated ($p = 0.306$, $p > 0.05$) but did not differ in terms of race ($p = 0.016$, $p < 0.05$). These individuals first participated in a phone interview which was conducted to exclude individuals that were not considered suitable for the study.

The selected ADHD individuals were diagnosed with ADHD based on their score on the questionnaire. A score below 16 was considered having no ADHD, a score between 16-24 was considered likely to have ADHD and a score of 24 or high was considered as most likely to have ADHD. For this study, individuals with a score of 16 or higher were considered ADHD. Also, Individuals with ADHD were further divided into a subcategory of combined (Hyperactive and impulsive), hyperactive and impulsive and were compared against a control. Controls were individuals that scored below 16 on the ASRS scale.

Data Collection

The fecal samples were collected from the participants using the OMNIgene GUT kit from DNA Genotek Inc. Individuals were given the kit to collect the sample in the privacy of their home by following all the instructions provided with the kit. It was stated

by the manufacturer that sample can remain stable at ambient room temperature for at least 5 days, for precautionary reasons it was asked that the participant return the sample the next day. These samples were stored in the lab for subsequent 16S MiSeq Illumina sequencing conducted by the Zymo Inc.

The Neurocognitive task involved in Phase II is the N-back task, with relatively advanced

competitiveness. The task involves a participant viewing a single letter that appears once on the screen. In 1-back

conditions, the participant is

instructed to respond by pressing a button when they see the same letter as before.

Therefore, if it was a n-back condition the participant had to press the button if they saw the same letter n letter before. The task is divided into nine 30s blocks of the task and alternatively eight 15s blocks of fixation. The fixation was used to prevent the participant from being primed with the conditions before. Each of the nine blocks consisted to nine serially presented consonants appearing for 500ms. Before each block is presented a screen informing the participant of the condition for 3,000 ms. The total time for the task was 6:26 approximately. The N-back load condition varies between task blocks where the condition was pseudo randomized. The task was presented on a

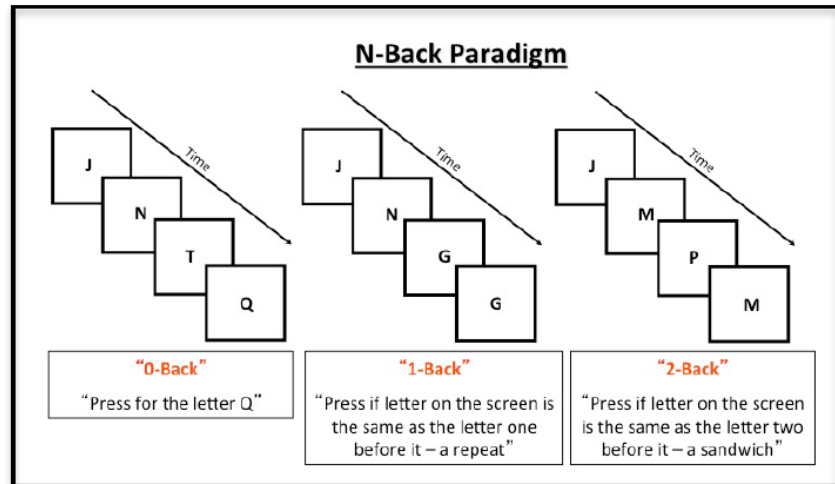


Fig 2. N-back paradigm used in the study. The participants also took part in 3-back and 4-back in this study.

computer using the E-prime software. Unlike Phase I where the task had 1-back, 2-back, and 3-back, this task has no 1-back and instead includes 4-back. The 4-back increases the difficulty level for the task subjecting the participants to perform more cognitive thinking and attention. Therefore, using this task, it will be confirmed that the data from Phase I is consistent that is individuals with ADHD who struggled with the task are still having the same if not more difficulty with performing well at the task.

Lastly, this part will also include a 24-hour recall for all individuals to control for the diet. The recall will provide a detailed information about the food and/or beverages consumed by the participants in the previous 24-hour. The ASA24 collects data on portion sizes and frequency of consumption of common food items. This inventory will be administered twice through the Automated Self-Administered 24-hour Dietary Recall (ASA24). The first one will be done in the lab before the sample is collected and the other one will do in after the sample has been collected. This data will then be assessed using the Food and Nutrition Dietary Studies (FNDDS) to provide the nutritional value of the food consumed which will then be incorporated into the abundance matrix. This will help minimize or control the effects of diet on gut microbiome composition. We will use this if necessary as a covariate in the microbiome analysis.

Exclusion Criteria

The criteria for exclusion included, use of antibiotic or antifungal or probiotic in the last month, experiencing any stomach or gut problems e-g chronic diarrhea, constipation IBS etc., history of neuropsychiatric Disorder (any DSM-5 diagnosis) other than ADHD for ADHD group, history of neurological disease such as epilepsy, traumatic

brain injury etc. and use of any psychotropic medications which they cannot discontinue during the sessions such as antidepressants.

Metagenomics:

Fecal samples were sent to Zymo Research for microbiome analysis. For each sample, 200 µL of vortexed fecal sample solution was taken from the OMNIgene Gut Collection Tube and extracted with the Zymo BIOMICS DNA Mini kit (Zymo Research, CA, US) according to the manufacturer's protocol. A negative control of DNA extraction process was performed together with the actual samples. Real time PCR quantification showed that the negative control has a high Ct value (>37 cycles), indicating that the DNA extraction process carries low bioburden.

Bacterial 16S ribosomal RNA gene targeted sequencing was performed. The general bacterial 16S primers used were 341f (CCTACGGGNGGCWGCAG) and 805r (GACTACHVGGGTATCTAATCC), which amplified the v3-4 region of the 16S rRNA gene. The sequencing library was prepared by following a published protocol (Kozich et al., 2013) with some modifications to prevent PCR chimera formation: the number of PCR cycles was carefully controlled for each sample so that each individual reaction did not overrun. The amplicon libraries were cleaned up with Zymo's Select-a-Size DNA Clean & Concentrator™ (>200 fragments were kept), quantified with Tape Station, normalized and pooled together. The final library was quantified with quantitative PCR and sequenced on Illumina MiSeq with v2 reagent kit (500 cycles). The sequencing was performed with >10% PhiX mix and in paired-end mode.

Raw sequence reads were trimmed with Trimmomatic-0.33 (Bolger et al., 2014). The two paired-end reads in each pair were assembled to construct a complete amplicon

sequence with SeqPrep. Chimeric amplicon sequences were identified and removed with Usearch (v. 6.1) (Edgar et al., 2011) in ref mode against a curated database. Amplicon sequences smaller than 320 bp were removed. For each sample, up to 50,000 sequences were randomly sampled to reduce potential bias caused by uneven sampling. These amplicon sequences were compiled, clustered and analyzed with Qiime 1.9.1 (Caporaso et al., 2010). OTUs were picked by the workflow of pick_open_reference_otus.py using GreenGene database (gg_13_8) as reference database. Singleton OTUs were removed. Composition bar charts were generated with the workflow of summarize_taxa_through_plots.py. Alpha- and beta-diversity analyses were performed with the workflow of core_diversity_analyses.py with default settings. Taxa abundance heat maps with hierarchical clustering of taxa and samples were prepared with an in-house python script. Taxa that have an abundance significantly different among groups were identified by LEfSe (Segata et al., 2011) with default settings ($p > 0.05$ and LDA effect size > 2) if applicable.

This sequencing data was then analyzed using the software tools to assess for the differential abundance of taxa. The differential in abundance can then be used as a biomarker for one group of samples as compared to others.

Results

Metagenomic Analysis

The project involves the collection of the fecal sample of two groups to conduct a qualitative and quantitative data analysis. Group 1 were healthy individuals and Group 2 were Individuals with ADHD. The sequence generated abundance level at various

taxonomic levels including, species, genus, family, order, class and phyla. For each taxonomic level graph charts were generated with relative abundance on y-axis and sample number and cohort group (Group1 and 2) on x-axis. Fig 3 shows that all individuals for the most part had similar phyla of bacteria regardless of ADHD or the control group. However, the abundance of each phyla differs within everyone as well as the group suggesting that the link between ADHD and microbiome is not due to different phyla but because of their differential abundance. Fig. 4 shows the relative abundance by order levels in each sample. The bars show that the different order of bacteria is consistent in all individuals. However, each order is present in a different abundance in each individual and the difference is significant between the ADHD group and the control. Therefore, the cognitive difference in control and ADHD can be associated with differential of various bacterial orders.

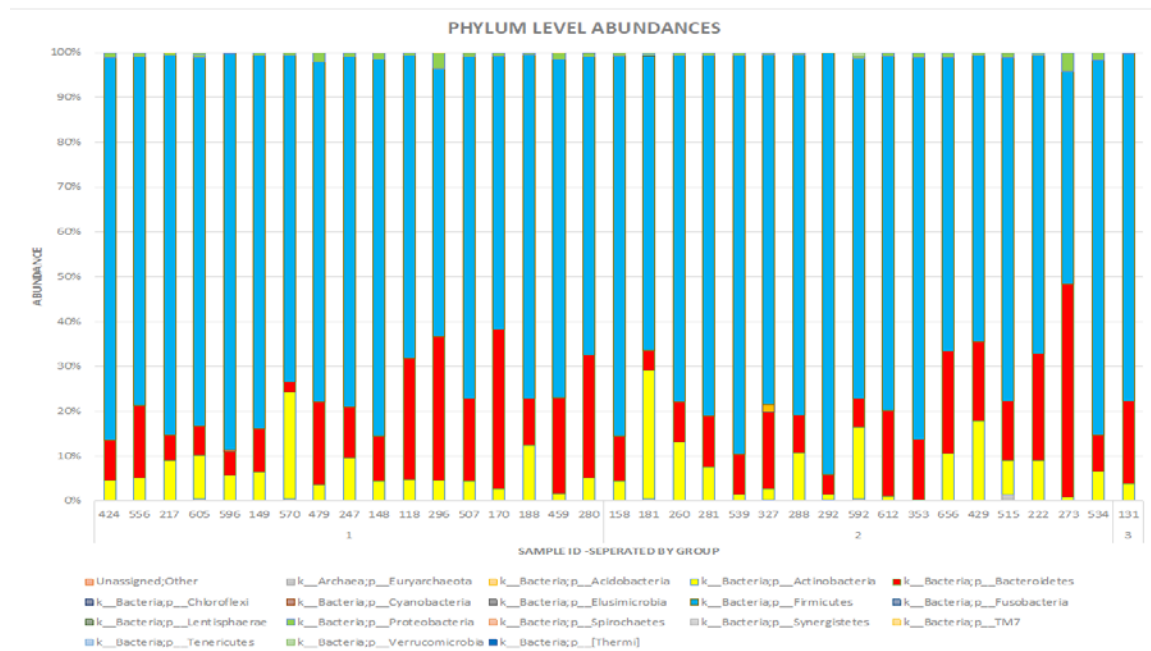


Fig. 3: Relative bacterial phylum abundance in each sample

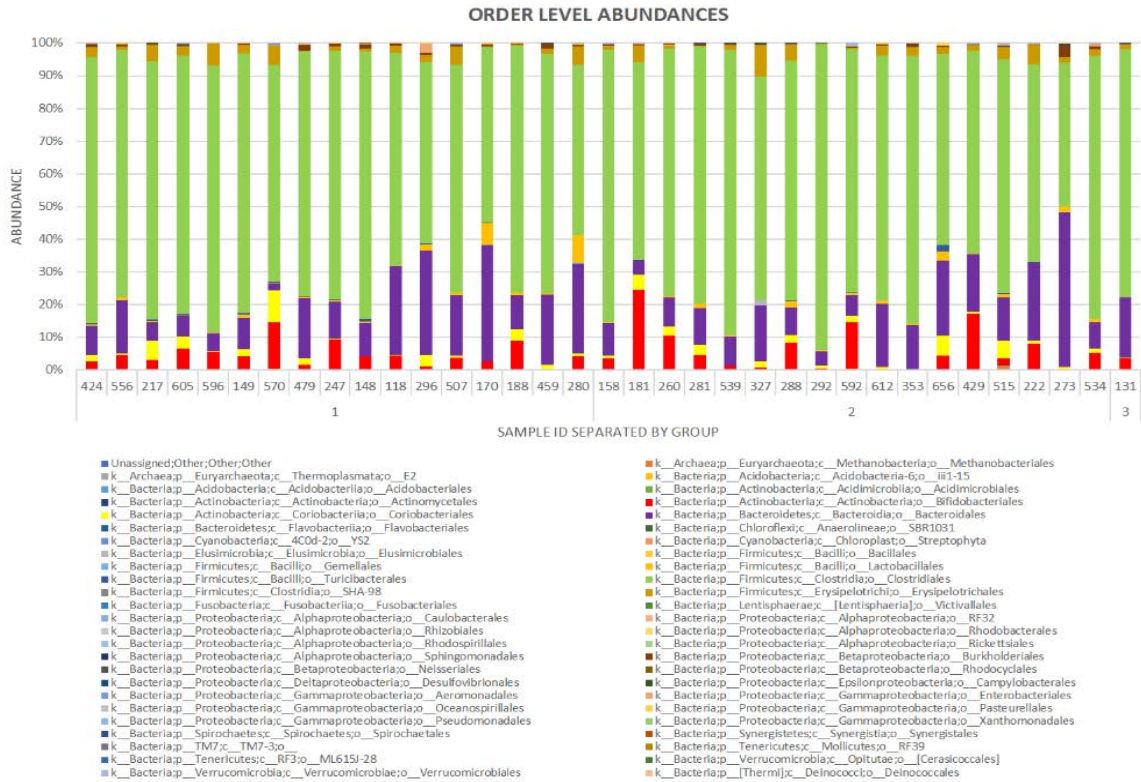


Fig. 4: The relative abundance by order levels in each sample

Fig 5 represents the abundance at family level in each sample. As shown in the bars, all had approximately same bacterial families however the abundance in each sample differed. The difference is found to be significant between the ADHD and the control group. Fig 6 represent abundance at genus level and Fig. 7 shows species abundance in each sample. each individual had approximately same bacterial abundance however the abundance in each sample differed. The difference is found to be significant between the ADHD and the control group.

FAMILY LEVEL ABUNDANCES

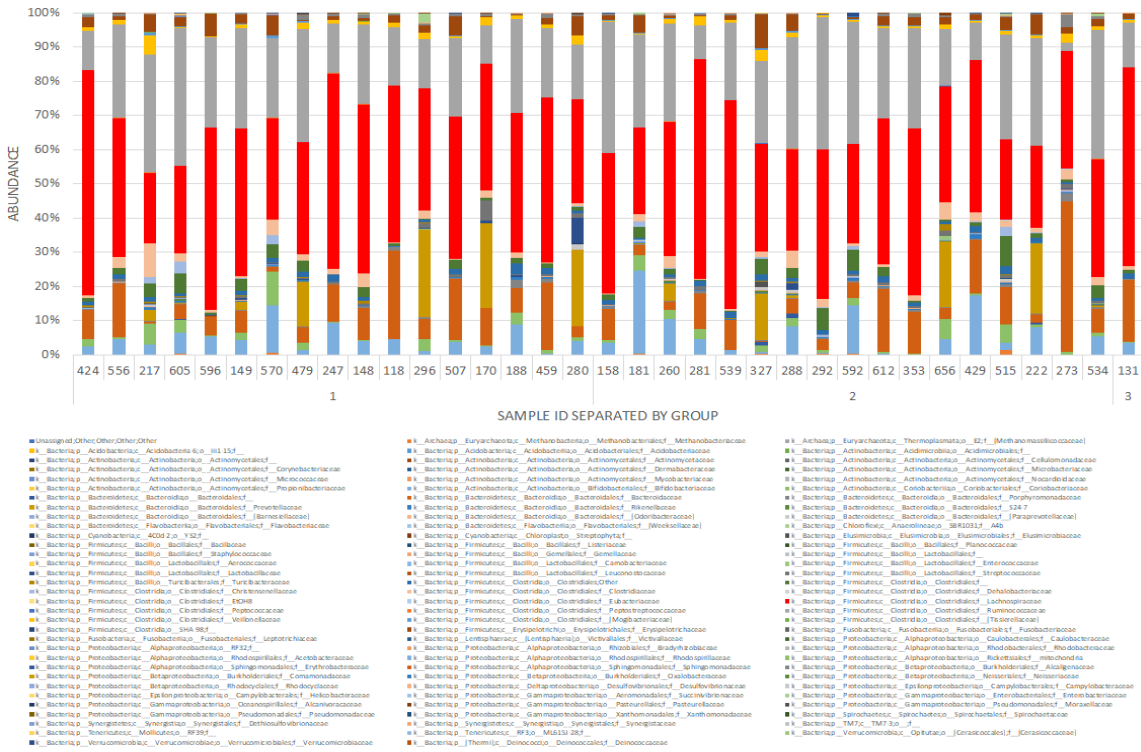


Fig 5: The relative abundance at family level in each sample.

GENUS LEVEL ABUNDANCES

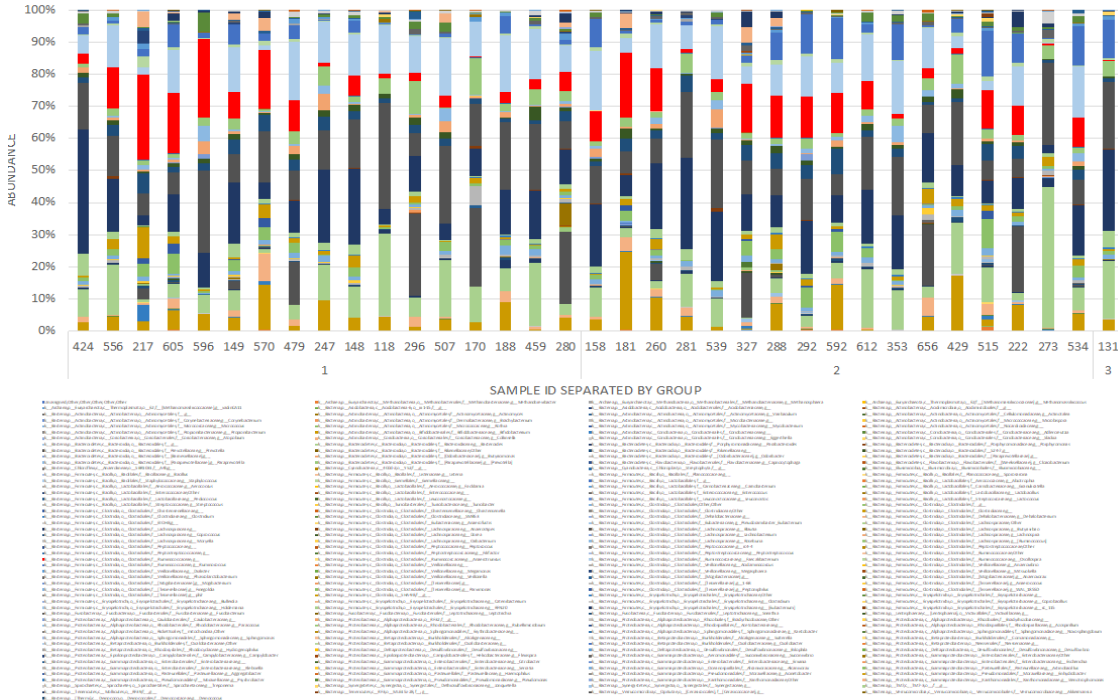


Fig 5: The relative abundance at genus level in each sample.

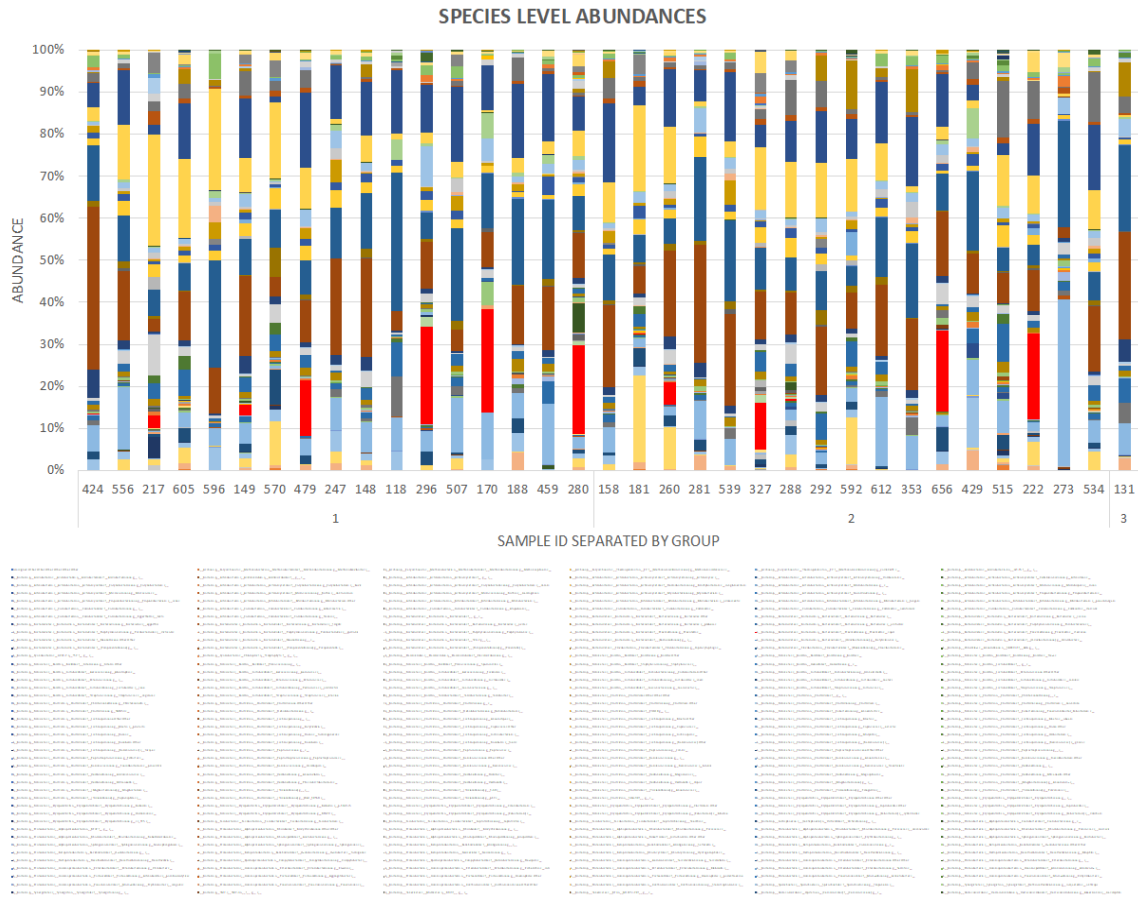


Fig. 7: The relative abundance at species level in each sample

Furthermore, a comparison was performed between the healthy students in the study and the Human Microbiome Project data which is shown in Fig. 8. Taxa that have an abundance significantly different among groups were identified by LEfSe analysis. Fig. 9 shows a chart displaying the 7 different bacteria that differed between the healthy controls and the individuals with ADHD. These include *Phascolarctobacterium*, Burkholderiales, *Paraprevotella*, Alcaligenaceae, Erysipelotrichaceae, *Veillonella*, and *Odoribacter*.

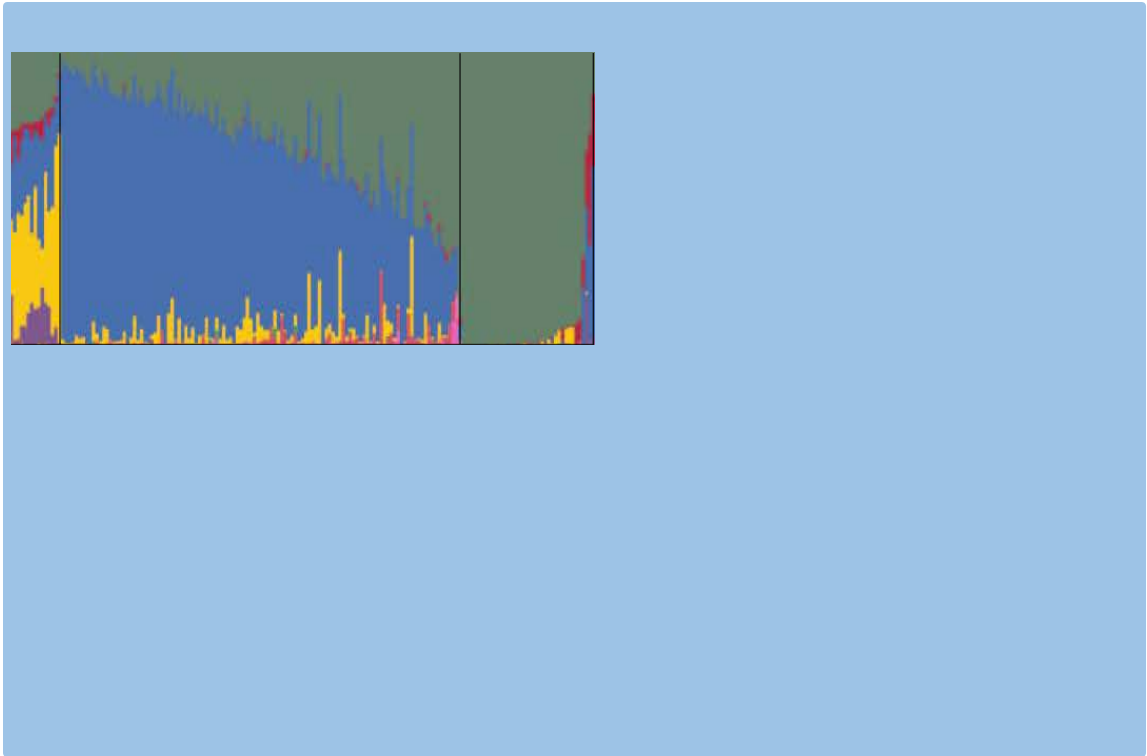


Fig. 8: Bacterial composition in Human Microbiome Project (Turnbaugh et al. 2007)

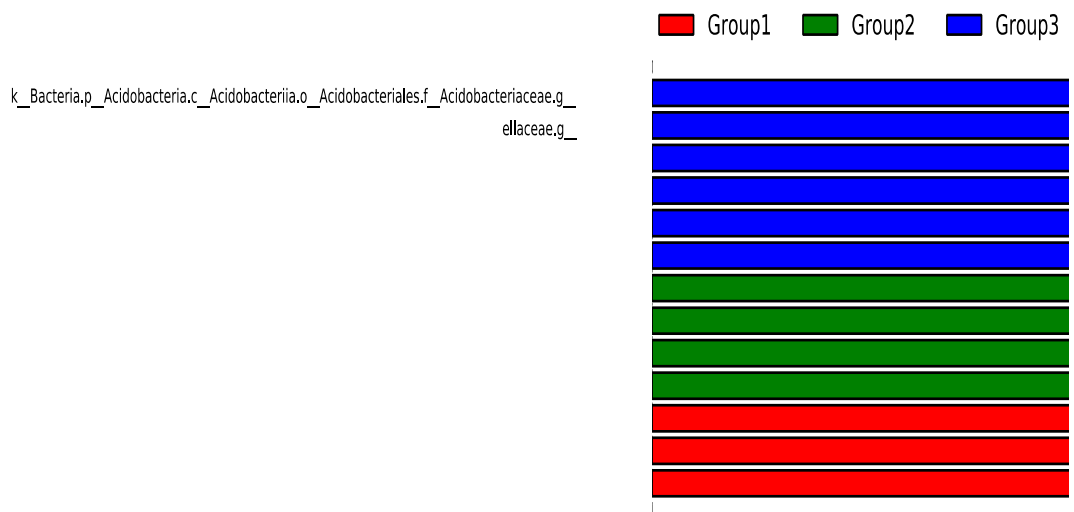


Fig. 9: Seven different bacteria that differed between the healthy controls and ADHD individuals

Lastly, the networks of co-occurrence were also plotted for both groups to determine the level of interaction between the bacterial clusters. Fig. 10 shows the co-occurrence in the healthy individuals. The plot signifies the relative interaction between different bacteria taxa. Fig. 11 shows the co-occurrence in ADHD individuals. A thorough analysis of both plots suggests that there is a significant difference between in the healthy and ADHD group. The degree to which the plot is extended and the number of lines that connects the dots are considered in the analysis. The dots on the plot are various bacterial taxa and can include family, order, genus or species. The green lines that are connecting the dots shows the relative interaction and the red line indicate no form of interaction. The majority of the bacteria are contained in the cluster and it is that cluster which interacts with other bacteria.

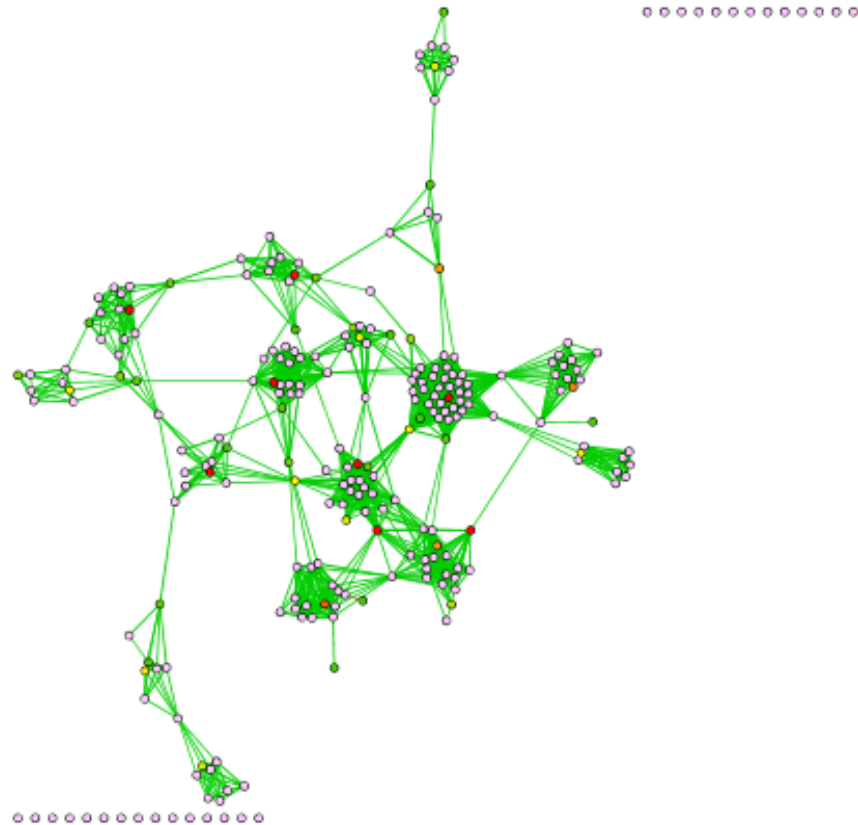


Fig. 10: The co-occurrence plot of control individuals

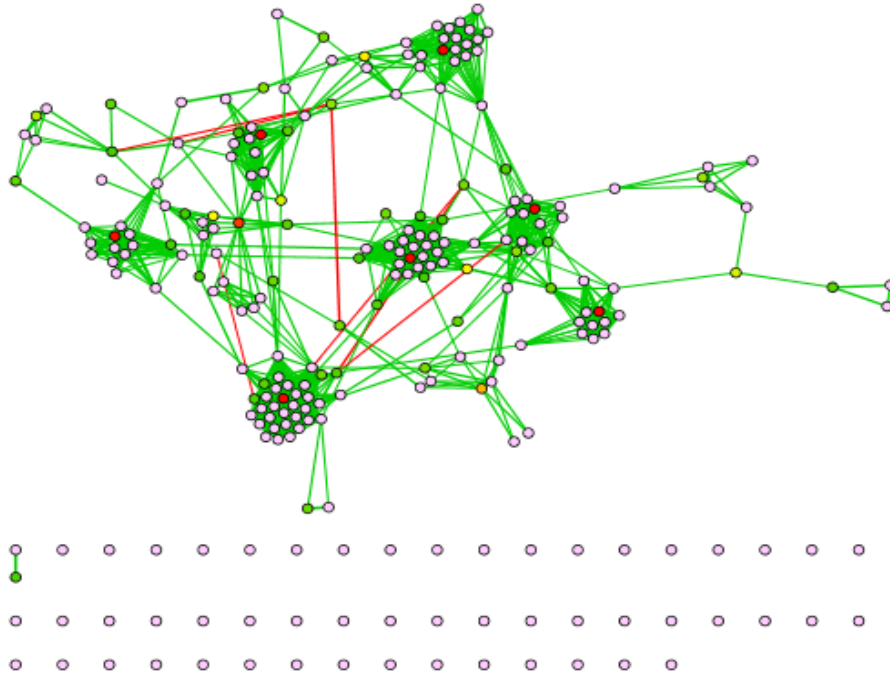


Fig. 11: The co-occurrence plot of ADHD individuals

Statistical Tests

T-tests are performed to determine if each of the factor such as age, sex or the race of the participant played a role in the individual bacterial abundance. It is determined that the neither the age nor the sex were significantly different within the participants ($n = 34$). The p value for age was 0.525 and the p value for the sex was $p = 0.306$. However, it was observed that individuals varied significantly in terms of race with a p value of 0.016, suggesting that race can play a role in individuals to have differential abundance of gut bacteria.

Discussion and Conclusion.

Attention Deficit Hyperactivity Disorder (ADHD) is considered a major neuropsychiatric disorder due to its high prevalence of 2% – 9% in children and young adults (Faranon and Biederman, 2005). It is characterized by hyperactivity, impulsivity,

and inattention (Yanez-Tellez et al. 2012). ADHD has high comorbidity with other disorders as well and thus individuals with this disorder are often under treated (Faraone and Biederman, 2005). Several candidate measures are present to screen for ADHD in adults including interviews with family, relatives or teachers in a clinical setting (Belendiuk et al., 2007). Among these, the most promising one is the screening scale used in survey known as the WHO Adult ADHD Self-Report Scale (ASRS) (Kessler et al., 2007). The significance of this scale is that it is relatively very short with only 6 questions that can be self-administered. The scale is designed based on the DSM-IV criteria for ADHD, but it is more directed towards children than adults. Adults with ADHD do not display the same symptoms as children, which creates few restriction for the using the scale in diagnosing adult ADHD (Barklay, 1995). Another limitation for the ASRS is that it is based on the self-reports. For children with ADHD, it is usually the parents or teachers that complete the questionnaire rather than the child himself. Thus, a better understanding of the behavior as both parents and teachers can observe and analyze the behavior than the child himself. In adults, however, the responses are most of the time self-reported which can result in a large degree of variation of how an individual may consider his or her behavior (Jenson et al. 1999). Nevertheless, methodological studies have shown that the ASRS underestimates the true prevalence of adult ADHD (Murphy and Schacher, 2000). Therefore, even if the individual scores close to the cut off, there is a higher chance that the individuals fall further on the ADHD spectrum. Furthermore, it has also been noted that the ASRS scale has the adequate sensitivity, specificity and classification accuracy for the general US population (Kessler et al., 2007). By taking these limitations into consideration it is reasonable to suggest that ASRS scale is a very

useful tool to diagnose individuals in non-clinical settings as in the current study. The questionnaire has a total of 18 of which the first 6 questions are directed towards understanding the presence of ADHD symptoms within the individuals. The remaining 12 questions assess the impairments that the individual is experiencing and their relevant history. Participants involved in the study completed this questionnaire to be diagnosed non-clinically with ADHD.

The N-back task that was part of Phase 1 and Phase 2 of the study was used to determine the cognitive control, especially the working memory, of the participants. Cognitive control is the process that involves selecting and successfully monitoring behaviors that facilitate the attainment of the chosen goal while at the same time ignoring and stimuli that is not relevant for the task at hand (Basak and O'Connell, 2016). For instance, an individual is standing at a traffic signal waiting for it to turn green. There are two possibilities where the light will turn green, one for the people that can turn left and the other for the people that can go straight. The goal is to press the gas pedal as soon as the light for the people going straight turns green which is often seen. However, in certain circumstances, individuals may press the gas pedal when the left turn light turns green indicating a low cognitive control. Cognitive control of behavior has been associated with working memory such that the working memory holds the information available for processing that can then be used to control the behavior of the individual (Engle, 2010). Using this relationship, it can be inferred that dysfunction of working memory may prevent an individual's cognitive control of the behavior. Furthermore, working memory is also one of the processes that is performed by the Executive Function along with cognitive control (Carpenter, Just and Reichle, 2000). Therefore, a dysfunction is the EF

may inhibit proper cognitive control and negatively affect the working memory. As seen from the scans conducted on Individuals with ADHD and control, the ADHD individuals experience a dysfunction in the Executive Function and the Emotional regulation regions of the brain. This dysfunction therefore suggests that ADHD individuals also have low cognitive control and low working memory capacity.

The N-back task measures cognitive control under emotionally neutral conditions as previous research has shown that emotionally positive or negative stimuli can influence the cognitive skills. Also, using a neutral stimulus allows to control for emotional dysregulation that is observed in ADHD individuals. The results that were obtained from the N-back task do suggest that ADHD individuals have trouble with cognitive control. The ADHD individuals, as compared to the control, had less accuracy and responded at significantly more time. The task engages crucial aspects of EF namely working memory and inhibitory control, and evokes the activation of network regions including bilateral dorsolateral prefrontal, parietal cortex/precuneus, dorsal striatum. Previous application of this task has shown that 0-back conditions have the highest accuracy whereas the 3-back has the lowest accuracy in control groups. For this study, another set of condition was added where the participants had to remember the letters presented 4 letters back. This increased the competitiveness of the task and suggested that ADHD individuals will have much lower accuracy rate than the control. The less accuracy shows the dysfunction of Executive Function mainly working memory. Since ADHD individuals have diminished working memory it does not allow them to process the stimuli and so prevent proper reasoning and decision making that is being tested in the task. The greater response time indicates that the individuals experienced a struggle in

inhibiting the irrelevant stimuli, that is the letters being presented between the conditions of the task.

An exclusion criteria were designed to pick the most suitable participants prior to fecal sample collection. The exclusion criteria involved individuals that have been using antibiotic, antifungal or probiotic in the last few months. This is because such preparations can alter the microflora of the participants and make it difficult to make a knowledgeable comparison with the control group. The antibiotics can reduce the overall microflora depending on how strong its effects are while probiotic may increase the abundance of a bacterial strain that otherwise would have been at normal levels (Preidis and Versalovic, 2009). Moreover, participants experiencing any stomach or gut problems e-g chronic diarrhea, constipation, IBS etc. are also not selected for the study. Prior research has shown that gut inflammation has been linked to abundance toxin releasing bacteria such as *Clostridium* (Tana et al. 2010). As these individuals already have an altered microflora, it would be difficult to link the microbiome to ADHD symptoms. Also, participants with a history of neuropsychiatric Disorder (any DSM-5 diagnosis) other than ADHD are not included in the study. Since microbiome is not inclusive to one single neuropsychiatric disorder and because this is a recent field in mental health, it is important to rule out any other neurological disorders. By selecting participants with only ADHD, the research will remain focused on the two variables microbiome and ADHD. Furthermore, a history of neurological disease such as epilepsy, traumatic brain injury etc. can result in reduced cognitive abilities as well (Cristofri and Levin, 2015). As these symptoms are also present in ADHD individuals, this will prevent linking microbiome to a brain injury than to ADHD. Lastly, participants that have been using some form of

psychotropic medications which they cannot discontinue during the sessions, such as antidepressants, are not selected for the study as well. These medications can mask the symptoms of ADHD and therefore will make it difficult to diagnose individuals with ADHD. It may also result in incorrect placement in the control group even though the participant has ADHD and thereby altering data. These precautions are taken to prevent erroneous results in the study.

The 24-hour recall was used to control for the diet that the participants may have before taking the neurocognitive task and before collecting the sample in privacy. Since diet of the individual can heavily disrupt the composition of the bacteria in the gut, it is reasonable to gather information about it. Previous research has shown that *Bacteroides* spp is related to high fat and protein diets, while the bacteria *Prevotella* spp is related to high carbohydrate diets (Scott et. al, 2013). Therefore, depending on what diet the individual has, the bacteria recovered from the sample can be controlled.

The metagenomic analysis consisted for LEfSe analysis which is an algorithm for the discovery and explanation of biomarkers using genomic features such as gene, pathways, characterizing the differences between two or more biological conditions. The analysis determines the OTUs that are most likely responsible for differences between the control and ADHD groups. OTU refers to the groups of microorganism that are organized using similar DNA sequence of taxonomic marker gene to be placed at a certain taxonomic level (Blaxter et al. 2005).

Earlier research has shown that microbiome influence the concentration of neurotransmitters in an individual. These neurotransmitters then affect different brain

regions and resulting in a change of behavior. The rodent model where microbiome influence the levels of dopamine is the basis of the study because it is the imbalance of neurotransmitters that causes a dysfunction in the executive function and emotional regulation (Gareau et al. 2010). This dysfunction is also the characterizing factor of ADHD (Swanson et. al 2000). Regardless the influence of microbiome on ADHD is still unknown. Taking this into consider the results that were collected show that there is some form of link between the gut microbiome and ADHD. Most bacteria that was observed belonged to the two phyla Bacteroidetes and Firmucutis as predicted from the past literature. As hypothesized there was a difference in bacterial abundance between the control and the ADHD group. Seven bacteria were found to be in different abundance including, the genus *Phascolarctobacterium*, *Paraprevotella*, *Veillonella*, *Odoribacter* and the order Burkholderiales, Alcaligenaceae, Erysipelotrichaceae. Although the exact abundance of each bacteria still needs to be determined, the differential relationship between the ADHD and the control does suggest some form of relationship. There is a possibility that the bacteria may be found in high abundance which could increase the number of neurotransmitters in the body which can then influence EF and ER. The imbalance than can be related to symptoms such as hyperactivity or impulsivity. On the other hand, these can be observed in less abundance which could lower the number of neurotransmitters and result in symptoms like in attention etc. There is also a possibility that each of these may vary in their own way leading to a different characteristic of ADHD.

Research has also shown the abundance of *Odoribacter* was highest among the individuals with autism. Further research has shown that the bacteria *Prevotella* and

Veillonellaceae are found in lower abundance in individuals with Autistic Syndrome Disorder (Kang et. al, 2013). In ADHD individuals, it is found that *Paraprevotella* and *Veillonella* are present in different abundance as compared to control. Given that ADHD and Autism share so many characteristics including trouble focusing, being impulsive and difficulty in communicating, it is reasonable to suggest that the bacteria, which belonged to same genera, have similar effects in the individuals (Craig et. al 2015). In another research conducted on Germ Free mice and SPF mice it was shown that the absence of gut microbiome can increase the corticosterone levels in the individual leading to anxiety (Crumevolle-Arias et.al 2014). As the abundance of these seven bacteria has changed in ADHD, low levels of any one of them can contribute to the hyperactive characteristic of ADHD. Other bacteria such as *Phascolarctobacterium* has previously been seen in high abundance within individuals with Major Depressive Disorder (MDD) (Jiang et. al, 2015). ADHD and Depression have an overlap in symptoms such that in both neuropsychiatric disorders individuals experience physical agitation (or hyperactivity) and poor concentration. This overlap, as well as the presence of similar bacteria in the participants, supports the idea of microbiome influencing brain regions associated with ADHD. As of current literature little is known about the order Burkholderiales, Alcaligenaceae, Erysipelotrichaceae. Bacteria in these orders have been seen within some individuals with anxiety, depression and autism but they do not have as significant correlation as other bacteria. This lack information allows one to predict that bacteria in these orders can potentially influences brain exclusively in ADHD individuals. Further analysis of the genus and the species that varied in the ADHD needs to be conducted to support it. Lastly, the bacteria that is present in ADHD individuals is not the same as

found in other disorders, but still belongs to same genus. This difference suggests that even though similar symptoms may appear the influence that these bacteria have on brain regions (EF and ER) may differ. Further research on the presence of specific species can better help in differentiating individuals with ADHD from ASD, ADD or anxiety disorder.

The other result that was obtained from plotting the co-occurrence plot displays the relative interaction between the various bacterial taxa. The graph suggests that there is a potential relationship between the dots plotted on it. The relationship is shown by connecting these dots. Each dot represents a different bacterial taxon and consist of order, family, genus or species. The green lines that connect the dots show the level of interaction between the bacteria where the shorter the line is the closer the interaction is and vice versa. The dots are clustered together on the plot suggesting that bacteria interact as a group. The isolated dots on the bottom of the plot of the top corner of the plot are bacteria that do not have any interaction with other bacteria. It is observed that the plot for the control group has a lot more bacterial clusters interacting with one another as oppose to the ADHD group where most of the bacteria do not interact. This relationship can be seen in the number of isolated bacteria in each graph where the control group has very low bacteria not interacting while in ADHD a fairly large number of bacteria are not interacting with one another. The graph itself indicates that the level of bacterial interaction differs between the group. This difference is seen from the size of the plots where the plot for control group is bigger and narrow suggesting a wide and intimate relationship. Whereas, in the ADHD group the plot is smaller and a little scattered suggesting a lesser interaction within the bacteria. The greater the relationship

between the bacteria, the greater it is likely that individuals will have stable gut-brain interaction. As seen from the co-occurrence plot the relationship between the bacteria is broken as compared to the control where it is intact. This difference suggests that there is a link between the gut microbiome and ADHD.

Overall the results do show that there is some form of link between ADHD and gut microbiome. The results do agree with the works of other studies conducted in the field of mental health. The results also support the literature where it has been presented that a neuropsychiatric disorder may be linked to a differential abundance of gut microbiome which supports the hypothesis that the gut microbiome influences human brain as seen studies conducted on Autistic Spectrum Disorder, Major Depressive Disorder, mood and anxiety. Precautions were taken at every step of the way during the studies to prevent any issues. The fecal kit that was used had a guarantee that the specimen will not get affected even at room temperature but still, individuals were asked to return the sample within 24 hours to avoid any problems. The 24-hour food count information was used as a control but at some point, it became problematic because participants varied with the culture. The difference was identified from the T-test conducted on the participants that showed that they differed significantly. Therefore, if the experiment were to be repeated then the race and ethnicity need to be controlled for as well. Our race influences our diet which then influences the bacterial composition.

This study sets the ground for much future research in this field. As suggested, future research can be conducted to know the difference abundance of each of the seven bacteria to create a causal relationship between the gut microbiome and ADHD. Given

that the casual relationship is formed, the abundance of certain bacteria can be used as a biomarker to identify the disorder at an earlier stage in life. Certain biomarkers have been established by a study conducted on children with and without Autistic Spectrum Disorder (ASD). The results show that fecal samples of children with ASD tend to have elevated short-chain fatty acids (SCFA) including acetic acid, propionic acid, butyric acid and valeric acids (Wang et. al 2012). Moreover, fecal ammonia levels in ASD individuals were also significantly higher than the control group. These products are the outcome of fermentation carried out by the microbiota and therefore the altered microbial profiles can be linked to it. Since, a significant difference in SCFA exists between the ASD and the control, these acids can be used as a biomarker to identify potential ASD individuals at an earlier age. Previous research has shown that of the bacteria that composes an individual's gut is determined within 2 years of birth and remains consistent after that (Koenig *et. al* 2010). Taking this into consideration a biomarker can be used to identify ADHD at an early age and therefore the treatment can be introduced at an early stage as well. ADHD is a developmental disorder and therefore treatment at an earlier stage can prevent greater consequences later in life. It has been known that probiotics and exercise can manipulate the bacterial composition in an individual. Currently, the most common probiotic solutions have been designed to target lactic acids bacteria including *Lactobacillus*, *Streptococcus* and *Bifidobacteria* which recently have been associated with anxiety and mood disorders (Anal and Singh, 1993). However, a probiotic preparation may consist of more than one bacteria depending on the microflora of an individual. The exact mechanism of probiotics is not known however studies from microecology suggest that probiotics can produce antimicrobial substances, compete for

adhesion receptors in intestines, stimulate the immune system and compete for nutrients (Sanders et al. 2007). Probiotics may reduce the number of toxin producing bacteria such as *Pseudomonas* and *Clostridium* are found in individuals with irritable bowel syndrome and ASD while increasing the beneficial bacteria such as lactobacillus. Moreover, selecting probiotic strains with strong adhesions to the intestinal wall can prevent other bacteria from adhering to the wall and introducing their metabolites to the system. Some probiotic strains can be more competitive in terms of nutrients than any other bacterial strain allowing them to outcompete them and reduce their effect on the individual. Lastly, probiotics can stimulate the immune system so that it can effectively target the bacteria that are resulting in neurological disorders. Although, probiotics may be the less invasive method of treating ADHD there are some drawbacks as well for example, some strains when outcompete others can influence the intestine and the brain and if these strains are toxin producing, then the impact could be detrimental. Therefore, a specific probiotic strain must be developed that specifically allows the growth of bacteria which are found in less abundance in ADHD individuals and at the same time reduce the abundance of those that are negatively impacting the neuronal system.

An Inactive lifestyle is often linked with poor health, suggesting that exercising regularly may prevent such conditions. Data collected from elderly individuals shows that they have low fecal microbial diversity. This reduction in diversity may be correlated with their low capacity to perform exercise or their poor dietary diversity (Claesson et al. 2012). Furthermore, a study conducted on athletes and their equal BMI controls shows that the professional athletes had increased fecal microbial diversity. A total of 22 different phyla are collected from the athlete microbiota in contrast to the control that had

9 to 11 phyla (Clark et al. 2014). Although a direct causal relationship has not been established yet, it is proposed that exercise can produce certain metabolites or inflammatory markers that can mediate microbial growth. The study on athletes shows that the professional players had lower levels of inflammatory cytokines suggesting that individuals may modify their immune system through regular and less strenuous exercise. The change in the immunity may provide a condition in the gastric regions that increases certain microbes to grow or reduce abundance of harmful bacteria. Other research has also shown that regular physical activity can help alleviate symptoms of mood disorders. Since mood influences cognitive abilities, ameliorating mood can help prevent cognitive decline.

So, a healthy diet and a regular exercise can help alleviate the symptoms of ADHD. Moreover, current probiotics tend to increase the abundance overall, but after determining specific bacteria, future research can direct specific probiotic to increase its abundance. Microbiome is comparatively new field especially in terms of mental health and so there is a potential of vast research. The current study establishes the path for all the future studies that may relate microbiome with a neuropsychiatric disorder.

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