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Gastrointestinal issues and the role of the gut microbiota in children with autism spectrum disorder

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GASTROINTESTINAL ISSUES AND THE ROLE OF THE GUT MICROBIOTA IN CHILDREN WITH AUTISM SPECTRUM DISORDER

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ABSTRACT

Autism spectrum disorder (ASD) is characterized by deficits in social communication and interaction as well as by repetitive patterns of behavior. It is thought to affect 1 in 68 children in the United States, yet researchers do not know what causes it and treatments are primarily focused on alleviating symptoms associated with ASD rather than treating any underlying cause. Various theories have been proposed over the years regarding what causes ASD in the hopes of finding effective treatment options. One of these theories, and the topic of this work, is that the intestinal bacteria play a role in the development of autism. The idea that gut bacteria may play a role in health and disease is one that has been gaining increased interest lately, and this has spread to the field of autism research.

Reports of children with ASD suffering from gastrointestinal (GI) issues are widespread, and even the first reports of children with ASD mentioned that some of them experienced GI symptoms or had issues with feeding. While GI symptoms are uncomfortable for any child, they pose special circumstances for those with ASD because these children are likely unable to effectively communicate what they are experiencing. This thesis will first review the prevalence of GI issues in children with ASD as well as discuss studies that have examined if there is a difference between the gut bacteria of

children with ASD compared to neurotypical children. As will be shown, many studies have in fact found a significant difference, but these differences vary across studies and a consensus has not been reached. Following this, the link between the gut bacteria and the brain, as well as how this relates to ASD will be discussed. Then, an overview of various treatment studies aimed at targeting the gut bacteria in animal models of ASD as well as in children with ASD will be analyzed.

While this field of research is certainly exciting, there is still a lot of work to be done by researchers. For one, the wide range of methodologies used and populations studied introduces variables that could be skewing the results and contributing to the lack of agreement between researchers regarding what bacterial strains might be relevant to ASD. Additionally, just because there is a correlation between certain bacterial strains and ASD does not mean it can be assumed that this is causing the development of ASD in so many children. Nonetheless, the fact that some treatment studies have led to improvements in ASD-related behaviors when targeting the gut bacteria of children indicates that this field of research is worthy of attention and continued support.

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LIST OF ABBREVIATIONS

4EPS.....4-ethylphenylsulfate

ABC .....Aberrant Behavior Checklist

ACTH.....Adrenocorticotropic hormone

ADI .....Autism Diagnostic Interview

ADI-R .....Autism Diagnostic Interview-Revised

ADOS..... Autism Diagnostic Observation Schedule

ADOS-CSS .....Autism Diagnostic Observation-Schedule Calibrated Severity Score

ANS .....Autonomic nervous system

APA ..... American Psychiatric Association

ASD .....Autism spectrum disorder

ATEC .....Autism Treatment Evaluation Checklist

B-GOS .....Galactooligosaccharide

BBB .....Blood-brain barrier

BMI.....Body mass index

CARS .....Childhood Autism Rating Scale

CBCL/1.5-5.....Child Behavior Checklist for Ages 1.5-5

CDC .....Centers for Disease Control and Prevention

CNS.....Central nervous system

CRH ..... Corticotropin releasing hormone

DA.....D-arabinitol

DBC-P..... Development Behavior Checklist Parent/Primary Caregiver Report

DSM ..... Diagnostic and Statistical Manual of Mental Disorders

DSR ..... Daily stool record

EC ..... Enterochromaffin cells

FITC-dextran ..... Fluorescein isothiocyanate–dextran

FISH ..... Fluorescence *in situ* hybridization

FMT ..... Fecal microbiota transplant

GABA ..... Gamma-aminobutyric acid

GERD ..... Gastroesophageal reflux disease

GFCF ..... Gluten-free and casein-free

GI ..... Gastrointestinal

GSRS ..... Gastrointestinal Symptom Rating Scale

HFSFI ..... High-frequency single food intake

HPA ..... Hypothalamic-pituitary-adrenal

IBD ..... Inflammatory bowel disease

IL ..... Interleukin

MHFD ..... Maternal high-fat diet

MIA ..... Maternal Immune Activation

MTT ..... Microbiota Transfer Therapy

OTUs ..... Operational taxonomic units

PCR ..... Polymerase chain reaction

PGI ..... Parental Global Impressions

PVN ..... Paraventricular nucleus

RBS-R ..... Repetitive Behavior Scale-Revised

RRB ..... Restricted, repetitive behavior

rRNA ..... Ribosomal RNA

SA ..... Social affect

SCFAs ..... Short chain fatty acids

SHGM ..... Standardized human gut microbiota

SRS ..... Social Responsiveness Scale

TBPS ..... Total Behaviour Problem Score

TD ..... Typical development

TeNT ..... Tetanus neurotoxin

VABS-II ..... Vineland Adaptive Behavior Scales II

INTRODUCTION

What is ASD?

Autism was first described by Kanner in a paper published in 1943, in which he described children who lacked the "predisposition to be social" (Kanner, 1943). Since then, the defining characteristics of autism have undergone several revisions (Volkmar & McPartland, 2014). In the *Diagnostic and Statistical Manual of Mental Disorders, 3rd edition* (DSM–3) by the American Psychiatric Association (APA) (1980), ASD was divided into two groups, infantile autism and pervasive developmental disorder. Then, the APA's *Diagnostic and Statistical Manual of Mental Disorders, 4th edition* (DSM-4) (1994) described five subtypes of autism which were autistic disorder, pervasive developmental disorder, Asperger disorder, childhood disintegrative disorder, and Rett's disorder. The first three of these are now under the classification of autism spectrum disorder (ASD) in the APA's *Diagnostic and Statistical Manual of Mental Disorders, 5th edition* (DSM–5) (2013), which defines ASD as a neurodevelopmental disorder characterized by "persistent deficits in social communication and social interaction across multiple contexts" as well as the presence of "restricted and repetitive patterns of behavior, interests, or activities." Most children are diagnosed with ASD at around age two, and according to data from the Centers for Disease Control and Prevention (CDC), ASD affects 1 in 68 children aged 8 years old in the United States, with boys being four and a half times more commonly affected than girls (American Psychiatric Association, 2013; Christensen, 2016).

Currently, there are two medications (risperidone and aripiprazole) that are widely used to treat some ASD-related behaviors, such as aggression and irritability (Choueiri & Zimmerman, 2017). While these medications may be helpful for some children, they are associated with many side effects such as weight gain, drowsiness, and fatigue (Masi, DeMayo, Glozier, & Guastella, 2017). Additionally, these drugs do not actually treat the core symptoms of ASD. To target ASD-related behavioral symptoms, the "gold-standard treatment" is behavioral intervention (Masi et al., 2017). Other types of therapies are also employed, such as occupational therapy, speech therapy, physical therapy, counseling, and social skills training (Choueiri & Zimmerman, 2017).

The exact pathophysiology of ASD remains largely unknown, however there are various hypotheses that have been proposed. Per the DSM-5, there is evidence that about 15% of cases of ASD are associated with a genetic mutation, but there are also various risk factors thought to be at play, including low birth weight, fetal exposure to valproate, or having older parents (American Psychiatric Association, 2013). Other researchers have also speculated whether maternal immune factors, metabolic dysregulation, or caesarean section might play a role in the progression of ASD (Buie, 2015). More recently, there has been an increasing amount of evidence for the role of the intestinal flora in the development of ASD.

Before discussing the details of this interaction, the terms microbiota and microbiome should be defined because although these two terms are frequently used interchangeably, they are not the same. The microbiota is defined as the "microorganisms that live inside and on humans" whereas the microbiome is defined as the genomes of

these organisms (Turnbaugh et al., 2007). Many researchers used the term microbiome when referring to the actual organisms, but for the sake of accuracy, the more specific definition of "gut microbiota" will be used when referring to the organisms that reside in the gut (Knight et al., 2017).

While some review articles in recent years have discussed the role of the gut microbiota in ASD, the ultimate goal of this thesis is to provide a more detailed analysis of various studies in this field as well as to present some of the most relevant research over the years such that one can fully grasp how developments in this field have come about. Additionally, in the appendices, an overview of the various methods used across the field will be discussed so as to provide a framework for the analysis of the research done to date.

ASD and Intestinal Permeability, Food Selectivity, and GI Symptoms

While the core features of autism deal with behavior and communication, many children with ASD suffer from gastrointestinal symptoms, and even Kanner in 1943 noted that five of the eleven children he studied had issues with food. He described how one of the children vomited a lot during the first year, another had to be tube fed all throughout her first year, and three others presented "severe feeding difficulty" (Kanner, 1943). Likewise, in the DSM-5, it is noted that many children with ASD demonstrate extreme restrictions of food and that this "may be a presenting feature" of the disorder (American Psychiatric Association, 2013).

One of the earliest studies found upon searching for GI related issues and ASD dates back to 1996, when researchers in Italy conducted a study to determine if the

intestinal permeability of children with autism (without GI symptoms) is different from control subjects. Using a sugar intestinal permeability test, where the recovery of mannitol and lactulose are measured in the urine, the authors found that nine out of 21 children with autism had a high level of lactulose recovery, while none of the control subjects displayed abnormal recovery of either sugar (D'Eufemia et al., 1996). The authors state that there had been research prior to theirs in which it was found that autistic children had elevated levels of some peptides in their urine, so it was hypothesized that some peptides derived from food were able to pass through the intestinal barrier and contribute to behavioral abnormalities. It should be noted that some have called into question the reliability of using a sugar intestinal permeability test to determine the health of the intestinal mucosa (Vojdani, 2013). Nonetheless, this article by D'Eufemia et al. shows that interest in the intestinal permeability of children with ASD began over 20 years ago.

In regards to eating patterns in children with ASD, Bandini et al. (2010) looked at food selectivity in children with ASD compared to neurotypical children (those who are developing in a typical fashion). Food selectivity for the purposes of this study was categorized into either refusal of food, limited "food repertoire," or "high-frequency single food intake (HFSFI)" (Bandini et al., 2010). The researchers found that children with ASD were found to display more food refusal as well as ate fewer types of food. While they were expecting to find a high incidence of HFSFI in children with ASD based on anecdotal reports they had heard, this was not the case. They note that this could be due to their definition of HFSFI being very specific. In both groups of children, limited

"food repertoire" was significantly associated with nutrient inadequacy (Bandini et al., 2010). Since children with ASD were found to eat a smaller variety of foods, their findings about nutrient inadequacy imply that children with ASD are more likely to suffer from not receiving enough nutrients.

Knowing that children with ASD are more likely to have issues with feeding, it is not all that surprising that reports of GI symptoms in this population occur. Various studies in recent years have tried to examine the exact prevalence of GI symptoms among children with autism, with reports ranging from 9 to 90% (Vuong & Hsiao, 2017). One study that retrospectively analyzed co-morbidities in over 14,000 children at four different hospitals in the Boston area found that 11.74% of children with ASD suffered from bowel disorders compared to only 4.5% of the overall hospital population (Kohane et al., 2012). The range in the percentage of children exhibiting GI symptoms is incredibly large, but it is clear that there are children with ASD who suffer from gastrointestinal issues. While GI issues are bothersome to any population, they can present different problems for children with ASD. For example, Buie et al. (2010) state that children with ASD who suffer from GI issues such as chronic abdominal pain or gastroesophageal reflux disease (GERD) and who have trouble communicating may manifest their discomfort via atypical behaviors such as displaying sleep disturbance, being aggressive, tapping on the area which bothers them, or restricting certain foods.

Gorrindo et al. (2012) compared 40 children aged 5-18 with ASD+GI symptoms, 45 children with only ASD, and 36 children with only GI symptoms. Of the children who had ASD+GI symptoms, the researchers found that constipation was the most commonly

diagnosed gastrointestinal issue with 85% of those children receiving the diagnosis from a pediatric gastroenterologist. In terms of factors associated with constipation, they found that children with ASD who were younger, more socially impaired, or nonverbal were more likely to experience this symptom. They also examined whether GI symptoms in children with ASD were associated with a limited diet, but there were no significant differences regarding the different types of foods/quantity of each food type eaten between the different groups.

One particular study by Wang, Tancredi, and Thomas (2011) aimed at determining more definitively how prevalent GI symptoms in individuals with ASD really are. They note that population-based studies had reported rates between 9% and 19%, and that clinical-based studies showed higher rates, however, they argue that the methods used in those studies were not reliable for various reasons such as lacking a control group or not using reliable assessments in the diagnosis of ASD patients (L. W. Wang et al., 2011). For their study, they examined 589 children that came from families in which there were at least two family members on the autism spectrum and compared them to their unaffected siblings. In order to confirm a diagnosis of ASD, they used the Autism Diagnostic Observation Schedule (ADOS) as well as the Autism Diagnostic Interview-Revised (ADI-R) (see Appendix I regarding techniques for ASD evaluation). It should be noted that Wang et al. (2011) grouped the ASD children into "full autism," "almost autism," or "spectrum subgroup" based on their results from ADOS and ADI-R. Any child that met the classification of autism based on both of these tests was classified as having "full autism." Of all the children in those three categories, 42% had GI

problems and of those in the "full autism" group, 47% had GI problems. In comparison, only 12% of their unaffected siblings had any GI problems. The main issues reported for the children with ASD were constipation and chronic diarrhea, and the researchers found that children with more severe autism were more likely to have these GI problems. They note that theirs is the first study to determine if autism severity is associated with the prevalence of GI problems, although another article published in the same year also found that GI symptoms were strongly correlated with the severity of autism as measured by the Autism Treatment Evaluation Checklist (ATEC) (Adams, Johansen, Powell, Quig, & Rubin, 2011).

Chaidez, Hansen, and Hertz-Picciotto (2014) completed a study which was the "largest ethnically diverse population based case-control study to date" comparing GI issues in almost 1,000 children in the United States with ASD, developmental delay, or typical development (TD). They also used ADOS and ADI-R to confirm a diagnosis of ASD, and like Wang et al., (2011) they grouped any child who met the autism classification of both tests into an autism group, while those that were close to reaching a classification of autism into an ASD group. They found that there was no statistical difference between these two groups in terms of ASD symptoms, so they grouped these individuals into one ASD group. Compared to children with TD, children with ASD were found to be over three times more likely to experience frequent GI problems, including abdominal pain, constipation, diarrhea, and vomiting. Using the Aberrant Behavior Checklist (ABC), Chaidez et al. (2014) also found that children with ASD and frequent episodes of abdominal pain, constipation, diarrhea, or gaseousness were more likely to

display irritability, social withdrawal, stereotypy, and hyperactivity. In their discussion, the authors discuss how it is "plausible" that children who deal with chronic GI issues and who have ASD may be more irritable and be more socially withdrawn due to their difficulty in communicating.

A study by Tomova et al. (2015) also found that autism severity was positively and strongly correlated with the severity of GI dysfunction in children with ASD. The researchers used the Childhood Autism Rating Scale (CARS) (see Appendix I) and ADI to evaluate ASD symptoms, but it should be noted that this study was done in Slovakia and had a sample size of only 10 children with ASD.

In contrast to studies that indicate that GI symptoms are positively correlated with increased ASD severity, a different study by Chandler et al. (2013) did not find this same association. Another more recent study conducted in Italy found that out of 163 preschoolers, 25.8% experienced GI symptoms, with constipation and abdominal pain being the most common, however they acknowledge the fact that reports of prevalence of GI symptoms in other studies have varied greatly (Prosperi et al., 2017). They note that this variability could be due to the methods employed in collecting symptoms as well as the time frame being studied. In terms of the relationship between GI symptoms and ASD severity, Prosperi et al. (2017) found that children with ASD who suffered from GI symptoms tended to display higher autism severity as measured by ADOS Calibrated Severity Score (ADOS-CSS) (see Appendix I), but they do not declare this as being significant. They do however state that children with ASD suffering from GI issues displayed more stereotyped behavior as measured by a repetitive behavior scale.

Regardless of the exact percentage of children with ASD who experience food selectivity or GI symptoms, there is enough research to suggest that this is in fact a problem for some children with ASD, which makes it an important line of research.

Due to the fact that some children with ASD show preference for specific foods, some may argue it is not surprising that various studies have found that the gut microbiota of children with ASD is different from that of typically developing children. It has been shown that eating specific diets over a long period of time may influence the groups of bacteria present in one's gut (Wu et al., 2011). For example, Wu et al. found that increased presence of *Bacteroides* was associated with animal protein, some amino acids, and saturated fats, which the authors state is like a Western diet in which there is high meat consumption. *Prevotella* was associated with high consumption of carbohydrates and simple sugars, which they state is typical of agrarian societies. As Dinan and Cryan (2017) point out, the relationship between ASD and an altered gut microbiota is like the "chicken or egg issue." In other words, is it that the food specificity shown by children with ASD is causing their altered gut microbiota, or is their gut microbiota contributing to their behaviors?

BACTERIA IMPLICATED IN ASD

The earliest report implicating the role of a specific bacteria in ASD dates back to the late 1990s, when parents of children with regressive-onset autism found that their children exhibited changes in their behavior after being given antibiotics for otitis media which subsequently caused diarrhea (Bolte, 1998). The author hypothesized that children with this type of autism suffer from colonization of their intestinal tract by *Clostridium tetani* and that these bacteria produce a neurotoxin, called tetanus neurotoxin (TeNT), that directly causes neurological symptoms via traveling through the vagus nerve. A review of the literature regarding the mechasnism of action of TeNT provided no support for the idea that TeNT produced by *C. tetani* in the GI system might affect the CNS. Rather, the toxic effects of TeNT are produced when *C. tetani* colonize a wound and then the toxin enters the bloodstream and can bind to motor neurons at the neuromuscular junction (Surana et al., 2017). Even though Bolte's hypothesis was incorrect, the reports which he based his hypothesis on urged other researchers, such as Finegold et al. (2002), to investigate whether there are specific gut bacteria associated with ASD.

Finegold et al. (2002) provided the following reasons for why microorganisms should be considered as being involved in late-onset autism: "(1) onset of the disease often follows antimicrobial therapy, (2) gastrointestinal symptoms are common at onset and often persist, (3) other antimicrobials (e.g., oral vancomycin) may lead to a clear-cut response . . ., (4) some patients have responded to several courses of vancomycin and relapsed each time it was discontinued." In line with their hypothesis, they found, based on stool samples, that children with ASD had increased levels of *Clostridium* and

Ruminococcus spp. compared to control children and that there were specific species from these groups unique to children with ASD.

A study by Song, Liu, and Finegold (2004) used real-time polymerase chain reaction (PCR) to quantitate the levels of three clusters of *Clostridium* as well as one specific *Clostridium* species in the stool of children with ASD. The authors note that the method used previously by Finegold et al. (2002) relied on sequence data from cultures of bacteria, and they state that these methods are not as reliable as culture-independent techniques. Based on their new methods, they found that mean counts of *Clostridium boltae* as well as of *Clostridium* clusters I and XI were significantly greater in children with late-onset autism compared to control children. Counts for cluster XIVab however were not significantly different.

Since these early studies, many studies have aimed to identify possible differences in the intestinal flora of children with ASD compared to neurotypical children. Giving an in depth overview of all of these articles is beyond the scope of this thesis due to the number of published studies related to the topic. However, Ding, Taur, and Walkup (2017) compiled some of this data into a phylogenetic tree, which is a useful way of visualizing some of the differences in the bacterial species present in children with ASD (Figure 1). Additionally, a recent review article by Vuong and Hsiao (2017) summarized some of these studies into a table, which has been modified with additional information and additional studies (Table 1).

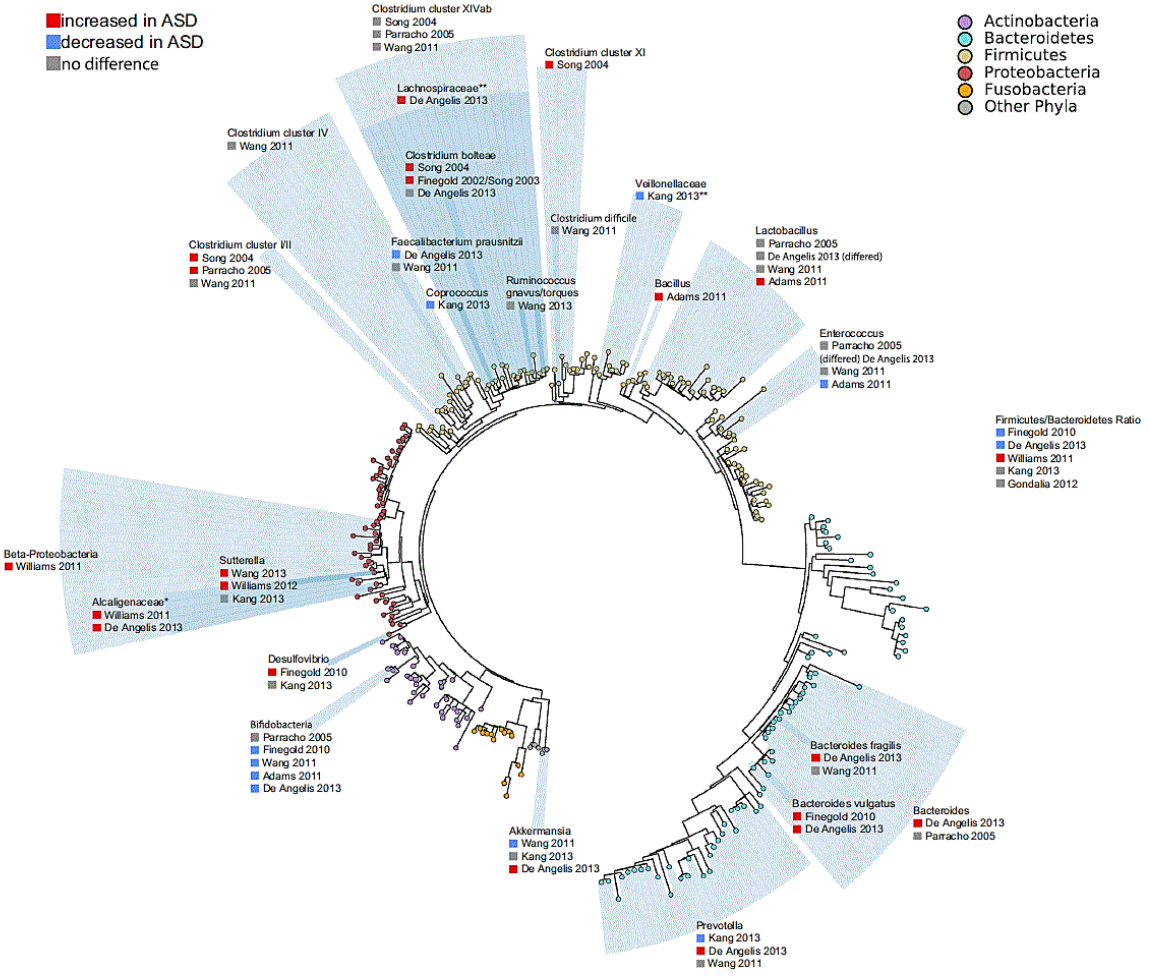

Figure 1: Alterations of the gut microbiota in ASD as determined by various studies. "Results of studies are shown on a phylogenetic tree. Tree tips (circles) represent bacterial species known to colonize humans; color denotes phylum-level classification. Bacterial taxa (blue shaded areas) studied are shown, at various levels of classification. Study findings (squares) are listed for each bacterial taxon; color denotes direction of change observed in ASD. Studies vary considerably by design, sample size, molecular approach, and analysis. *Sutterellaceae is a new family originally from Alicaligenaceae. **Correlation was with unclassified Veillonellaceae." Figure and Legend taken from Ding et al. (2017).

Table 1. Findings from various studies over the years regarding bacterial species altered in ASD. Adapted from Vuong and Hsiao (2017) with additional notes added

Description	Sample Size	Source/Location
More clostridial species in ASD group. Those in the ASD group all had GI symptoms (diarrhea or constipation).	13 ASD children; 8 controls	(Finegold et al., 2002) Chicago
ASD group had more *Clostridium boltae* and more *Clostridium* group I and XI.	15 ASD children; 8 controls	(Song et al., 2004) Chicago
ASD patients had more bacteria from *Clostridium* clusters I and II. Used FISH technique.	58 ASD patients; 22 controls	(Parracho et al., 2010) England
Pyrosequencing results showed more Bacteroidetes in ASD children as well as more *Desulfovibrio* species and *Bacteroides vulgatus*. The ASD children in this study all had GI symptoms. More Firmicutes in the control group.	33 ASD patients	(Finegold et al., 2010) Oregon and California
ASD patients with GI symptoms had less Bacteroidetes and an increase in Firmicutes/Bacteroidetes ratio.	15 ASD patients; 7 control patients	(Williams et al., 2011) New York
Children with ASD had less *Bifidobacterium* and *Enterococcus*. They also had much higher levels of *Lactobacillus*. Used Vitek 2 identification cards for gram positive and gram negative organisms as well as yeast.	58 ASD patients with GI symptoms; 39 controls	(Adams et al., 2011) Arizona
No difference in bacterial species or in bacterial diversity between groups. Note: about half of the ASD group had GI symptoms while the others did not.	51 ASD patients; 53 controls (siblings)	(Gondalia et al., 2012) Australia
Less *Bifidobacterium*, more Bacteroidetes, and more *Clostridium* in ASD. Of bacteria in fecal samples of ASD patients, *Caloramator*, *Sarcina*, and *Clostridium* were present in the highest amounts.	10 ASD patients; 10 controls (siblings)	(De Angelis et al., 2013) Italy
Increased *Sutterella* species and *Ruminococcus torques* in children with ASD.	23 ASD patients; 31 controls (22 were siblings)	(L. Wang et al., 2013) Australia
Decreased Bacteroidetes/Firmicutes ratio. Levels of *Desulfovibrio* were not significantly different between groups, but higher levels of this species were associated with autism severity.	10 ASD patients; 10 controls	(Tomova et al., 2015) Slovakia

GUT MICROBIOTA COMMUNICATION AND DEVELOPMENT

The Link Between the Gut and the Brain

The ways in which the CNS can modulate motility, secretion, and blood flow of the gut via the autonomic nervous system (ANS) is a topic that has been well researched and is taught widely. Likewise, communication in the opposite direction, from the gut to the brain, has also been studied in depth. In a review by Rhee et al. it is discussed how the vagus nerve can transmit information from the gut to the CNS and that there are nerve terminals of vagal afferents that are found close to enterochromaffin cells (EC) (Rhee, Pothoulakis, & Mayer, 2009). These cells release serotonin as well as other signaling peptides, so it was proposed that this connection between EC cells and the vagus nerve could be a path for communication between chemical stimuli in the lumen of the gut and the CNS. The role of the gut microbiota in this line of communication is a topic that has been gaining more momentum in recent years since the 2000s and there have been studies which have indicated that the vagus nerve is in fact important for this bidirectional communication between the gut microbiota and the CNS (Dinan & Cryan, 2017).

In one study, it was found that *Lactobacillus rhamnosus* was able to regulate emotional behavior in mice as well as gamma-aminobutyric acid (GABA) receptor expression via the vagus nerve (Bravo et al., 2011). The researchers determined that the vagus nerve was crucial for this relationship because mice that had undergone vagotomy did not experience the same effects. GABA is the main inhibitory neurotransmitter in the central nervous system (CNS) and it is thought to play a role in anxiety and depression (Bravo et al., 2011). Therefore, the fact that a particular strain of bacteria could modulate

the expression of the GABA receptor is very interesting. Additionally, the fact that the vagus nerve was necessary for this effect adds evidence for its role in the gut-brain axis. While this route of communication seems likely in a healthy state, it is possible that a diseased state causing increased intestinal permeability could allow bacterial products and inflammatory molecules to access nerve terminals (Clapp et al., 2017; Rhee et al., 2009).

Microbes in the gut have been found to be able to regulate important central neurotransmitters like serotonin and there have even been studies indicating that some bacteria can synthesize and release neurotransmitters (Dinan & Cryan, 2017). For example, *Lactobacillus* and *Bifidobacterium* can make GABA, and additionally, they can increase intestinal motility (Dinan & Cryan, 2017; Rhee et al., 2009). Some *Escherichia*, *Bacillus*, and *Saccharomyces* species are able to make noradrenaline, and some *Escherichia* species can also make serotonin (Dinan & Cryan, 2017). Per Dinan and Cryan, these neurotransmitters likely act directly on the enteric nervous system rather than on the CNS, because they are unable to cross the blood-brain barrier (BBB). These researchers also suggest two other ways in which the gut microbiota may influence the brain. The first is via the release of products of their metabolism, such as short chain fatty acids (SCFAs) like butyrate, propionate, and acetate. And the second is through cytokines, which can travel to the brain and may be able to communicate across the BBB. They note that two cytokines in particular, IL-1 and IL-6, are able to activate the hypothalamic-pituitary-adrenal (HPA) axis. This then leads to cortisol release, which activates the stress system (Figure 2) (Dinan & Cryan, 2017). SCFAs are produced as a

result of the fermentation of dietary fiber by anaerobic organisms and therefore, the levels are thought to be representative of the concentration of bacteria in the gut as well as the amount of soluble fiber being consumed (Adams et al., 2011).

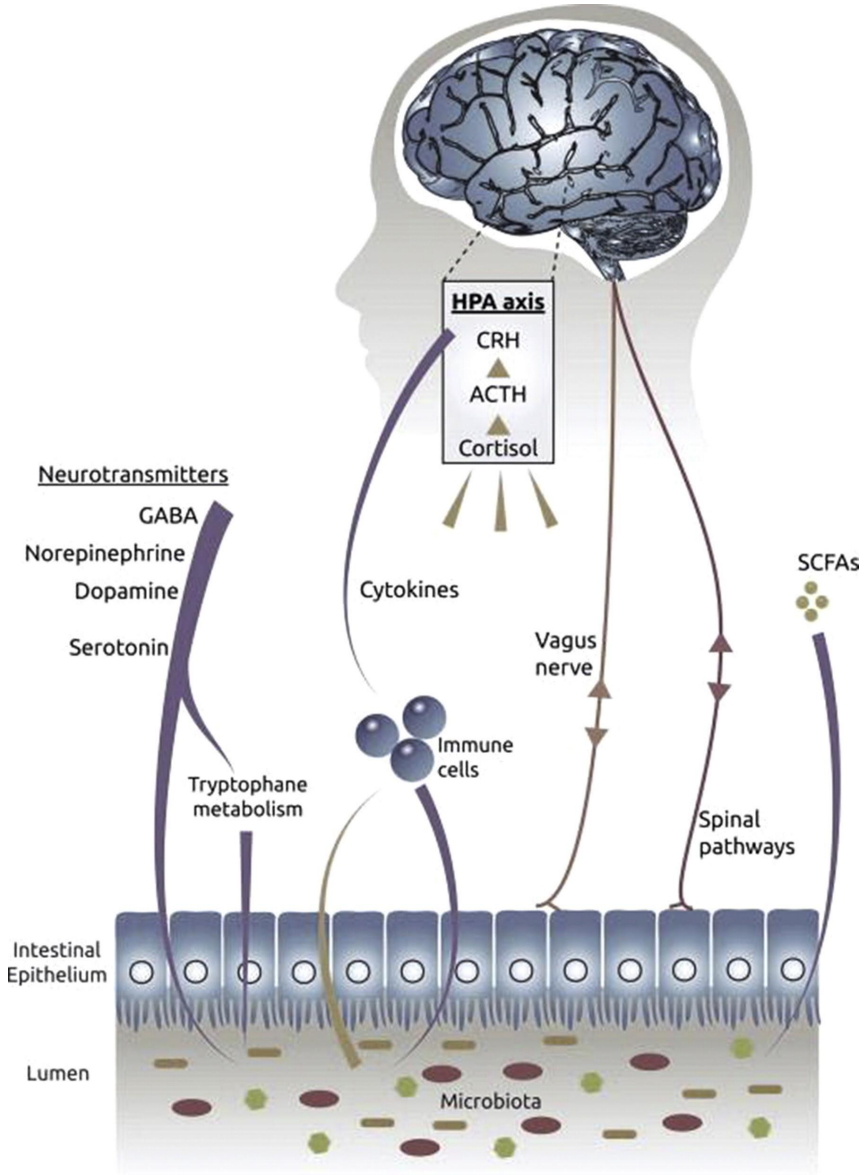

Figure 2: Communication between gut microbes and the brain. "These include the vagus nerve, SCFAs (butyrate, propionate, and acetate), cytokines, and tryptophan. ACTH, adrenocorticotropic hormone; CRH, corticotropin releasing hormone." Figure and legend taken from Dinan and Cryan (2017).

The microbiota plays a role in health and is able to influence the development of many organ systems including the immune system (Rook, Bäckhed, Levin, McFall-Ngai, & McLean, 2017). A healthy state has been associated with high gut bacterial diversity due to "resilience afforded by higher functional redundancy" (Kang et al., 2017). Sommer, Anderson, Bharti, Raes, and Rosenstiel (2017) note that this stability is important for maintaining the integrity of the intestinal mucosal barrier and that when the state of equilibrium is disrupted, dysbiosis occurs and disease can follow. Changes in the gut microbiota have been found to be associated with several diseases that affect children including allergies, inflammatory bowel disease (IBD), and type I diabetes mellitus (Slattery, MacFabe, & Frye, 2016). While Slattery et al. seem optimistic that targeting the gut microbiota as a way to treat certain conditions has promise, they do caution that there is not enough information about the long term effects of this type of treatment. They worry that treating the initial condition through changing the gut microbiota may then predispose children to other conditions. As an example, they note that while antimicrobial treatment for *Heliobacter pylori* reduces the likelihood of a patient developing peptic and duodenal ulcers, it can also increase the risk of esophageal cancer. Additionally, they argue that because children are unable to give consent, it would not be appropriate to initiate these types of treatments without first knowing what the long term effects are.

While many of the exact mechanisms by which bacteria modulate health and disease are not fully known, this relationship has been studied by many in recent years and in regards to autism, researchers have tried targeting the gut microbiota in hopes of improving both ASD-related symptoms and GI symptoms. Before discussing these

studies, the way in which the gut microbiota develops in humans will be discussed briefly.

Development of the Gut Microbiota

Previously, it was thought that the gut of neonates was colonized at the time of birth, when an infant passes through the birth canal, however, more recent research suggests it begins even earlier (Slattery et al., 2016; Walker, Clemente, Peter, & Loos, 2017). Studies have found that the placenta and meconium both have distinct microbiomes, so it is now believed that the development of the gut microbiome begins in utero (Slattery et al., 2016). Its continued development is thought to be affected by delivery mode, diet, breastfeeding, how old the child's mother is at the time of birth, body mass index (BMI), antibiotic use, and maternal stress (Munyaka, Khafipour, & Ghia, 2014; Walker et al., 2017). Maximum diversity is thought to be reached during adolescence, and then it declines in older adults (Ho & Ross, 2017).

One study examining the development of the gut microbiota in children during the first year of life found that vaginally delivered children had a more similar gut microbiota to their mothers compared to children delivered by C-section (Bäckhed et al., 2015). These researchers also determined that nutrition played a role in the development of the gut microbiota, with children who were breast-fed up until one year having different bacteria present in their gut compared to those who had stopped breast-feeding. Diet can also have a profound impact on the organisms present in an individual's gut microbiota, so it is important to take this into account when trying to compare different populations (Wu et al., 2011). Another factor that can affect the gut microbiota is antibiotic use.

Broad-spectrum antibiotics reduce the diversity of fecal microbiota and their use has been tied to the presence of GI symptoms (Cryan & O'Mahony, 2011).

Over time, the gut microbiota changes greatly, and the species it contains play an important role in human processes including nutrient metabolism, synthesis of vitamin K, metabolism of xenobiotics and drugs, and protection from pathogenic organisms (Jandhyala et al., 2015; Rook et al., 2017). The vast majority of bacteria in the gut of healthy adults belong to one of two phyla, Firmicutes or Bacteroidetes (Figure 3) (Knight et al., 2017; Rook et al., 2017; Turnbaugh et al., 2007).

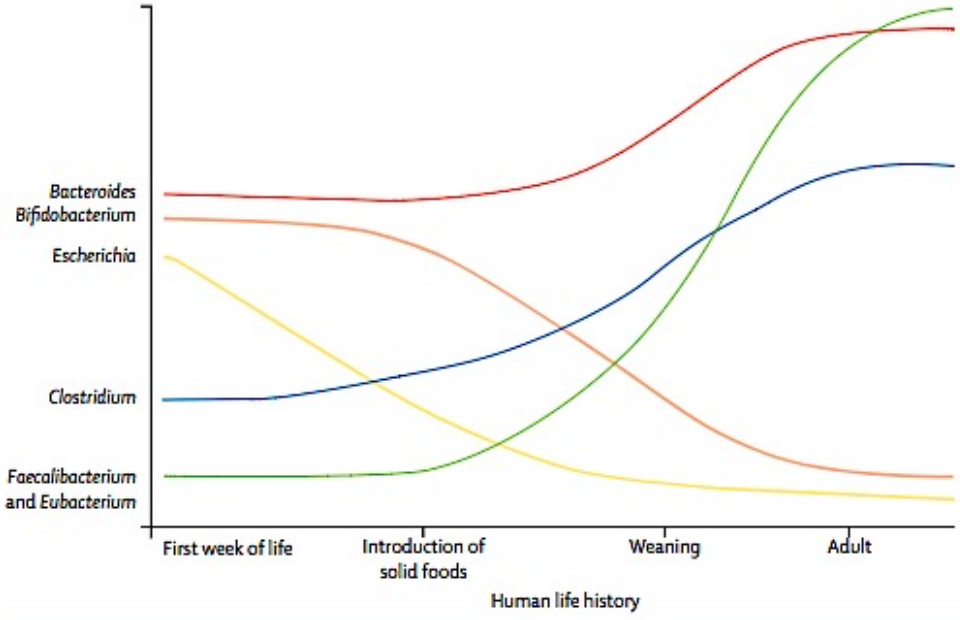

Figure 3: Development of the human gut microbiota. "In the adult, this development is dominated by two phyla: Bacteroidetes (*Bacteroides*) and Firmicutes (*Clostridium*, *Faecalibacterium*, *Eubacterium*), which replace the early dominance of Actinobacteria (*Bifidobacterium*) and Proteobacteria (*Escherichia*). The windows of opportunity for the correct establishment of the immune system, metabolic system, gut–brain axis, and stress responses could occur during the periods of complex change between birth and adulthood. These crucial windows are documented in animal models, but not yet in human beings." Figure and legend taken from Rook et al. (2017).

Compared to the number of human genes we have (about 20,000), our bodies are associated with 2-20 million microbial genes, which is at least 100 times more than the number of our own genes (Knight et al., 2017). In terms of cells, the most commonly cited estimate for the number of microbial cells in humans is 10^{14}, which was thought to be 10 times the amount of human cells present (Sender, Fuchs, & Milo, 2016). However, after revisiting those calculations, Sender et al. found that the ratio is much closer to 1:1. Regardless of which is correct, microbes constitute a significant part of our bodies, so it is not surprising that these microbes can play a role in disease processes.

In regards to autism, one study, which will be discussed in further detail later, noted that in the individuals they studied, compared to neurotypical children, more children with ASD were delivered by C-section (61% vs. 15%) and more were fed non-standard formula as infants (39% vs. 5%) (Kang et al., 2017). The children with ASD had been breast-fed for an average of 3.0 months while the neurotypical children had been breast-fed for an average of 8.8 months (Kang et al., 2017). Additionally, the mothers of children with ASD had consumed significantly less fiber than those of neurotypical children, something that could have been true during their pregnancy and could have affected the gut microbiota of their children. Given that mode of birth and diet have been found to affect the gut microbiota, it is interesting that there was a difference in these two regards when comparing an ASD group to a neurotypical group.

A Note About the Role of Yeast and Phage Populations in the Gut

Research on the gut microbiota has been largely focused on bacterial populations. For this reason, the focus of this work is on the role of bacteria in ASD. However, it is

important to note that this collection of organisms also encompasses fungi, protozoa, and viruses including phages (Rook et al., 2017).

Some studies have implicated yeast in the development of ASD. For example, Burrus (2012) hypothesized that *Candida albicans* could play a role in ASD for a subset of patients. He said that this particular yeast species produces ammonia, and that when this combines with propionic acid, beta-alanine may be synthesized, which is structurally similar to GABA, the main inhibitory neurotransmitter. He notes that other studies found that beta-alanine is higher in children with ASD and that prior studies had shown it can cross the BBB. However, a search for articles regarding whether beta-alanine does in fact cross the BBB and if it is able to mimic the actions of GABA did not provide any results, but maybe future studies will examine this possible link. Plasma levels of GABA have been found to be elevated in children with ASD compared to controls, but GABA is unable to cross the BBB, so it is not clear what role increased plasma levels of GABA or beta-alanine in children with ASD would have (Al-Otaish et al., 2018; Coghlan et al., 2012). Studies examining GABA levels in the brains of children with ASD have actually found low GABA levels. For example, Puts et al. (2017), found that children with ASD have low levels of GABA in the sensorimotor cortex and that this is associated with impairments in the processing of tactile information seen in this group. Some researchers believe that low levels of GABA in some areas of the brain, such as in the motor cortex, the visual cortex, and the auditory cortex, may be responsible for a hyperexcitable state in children with ASD (Takarae & Sweeney, 2017).

In terms of the yeast species present, Kantarcioglu et al. (2016) found that *Candida albicans* was the most common species isolated from the stool samples of children with ASD. This is important because while this species is normally present in the GI system, unusually high levels can decrease absorption of carbohydrates and minerals (Kantarcioglu et al., 2016). Another study which aimed to examine the phage population in an ASD group did not see any changes after a particular treatment plan (Kang et al., 2017). However, they point out that changes in phage diversity can occur more slowly than for bacteria, so it is possible they their study did not span enough time to see these changes.

Now that it has been shown that GI problems in children with ASD is a recurring theme in the literature and that many studies have found a difference in the gut microbiota of children with ASD, the remainder of this thesis will focus on treatment studies that have been done in recent years. First, a few studies done in animal models will be reviewed, and then those done in humans will be analyzed.

TREATMENT STUDIES TARGETING THE GUT MICROBIOTA IN MICE

One mouse study, by Hsiao et al., (2013) used a maternal immune activation (MIA) model, which displays features common to ASD including "communicative, social, and stereotyped impairments," to study the GI barrier and gut microbiota in mice. Compared to controls, adult MIA offspring were found to have a significant decrease in the integrity of their intestinal barrier as measured by increased movement of fluorescein isothiocyanate–dextran (FITC-dextran) from the gut into the circulation as well as decreased gene expression in the colon of tight junction proteins, occludin, and claudin 8, which are responsible for regulating membrane permeability. Gene expression of claudin 15 was actually increased in this population, but the authors do not propose an explanation for this. They also found that compared to control mice, colons of MIA offspring had higher levels of interleukin-6 (IL-6) mRNA and protein. They state that this is in line with the idea that gut permeability is associated with alterations in the immune response. In terms of gut microbiota, the authors found that based on unweighted UniFrac analysis (see Appendix II), there was a significant difference between the species present in MIA offspring compared to controls. Specifically, they found that this difference was primarily driven by changes in the Clostridia and Bacteroida classes.

Hsiao et al. (2013) then treated MIA offspring with *Bacteroides fragilis* at weaning and found that this treatment was able to correct the increased intestinal permeability in MIA offspring, reduce their colon IL-6 mRNA and protein levels, and also increase the relative abundance of six bacterial species that were significantly decreased in this group prior to treatment. This treatment also improved certain ASD-

related behaviors including anxiety-like behavior, issues with communicating, and repetitive behavior. The authors note that there was no evidence to indicate that *B. fragilis* had colonized the gut of these mice, so they suggest that the effects seen could be due to the treatment causing "persistent shifts in the compositions of resident microbiota." Interestingly, they saw an improvement in these behaviors after treatment with a different species of the Bacteroida class, *Bacteroides thetaiotaomicron*.

Hsiao et al. (2013) argue that the way in which probiotic treatment is able to correct gut permeability and improve behavioral symptoms is through its effect on metabolites. Prior to treatment, MIA offspring displayed about an 8% difference, that was statistically significant, in serum metabolites present compared to control offspring. MIA offspring showed levels of 4-ethylphenylsulfate (4EPS) that were 46-fold higher than in controls as well as significantly higher levels of serum indolepyruvate and serotonin. Interestingly, adding 4EPS to wild type mice caused anxiety-like behavior, similar to what they saw in MIA offspring. Indolepyruvate is a product of tryptophan catabolism and is thought to be produced by gut microbes. Treatment with *B. fragilis* was able to correct the levels of both 4EPS and indolepyruvate to resemble levels found in control offspring. It should be noted that 4EPS and indolepyruvate have not been confirmed as being implicated in ASD. However, given the fact that treatment with *B. fragilis* ultimately led to improvements in ASD-related behavioral symtpoms, it is interesting that this improvement was accompanied by a change in the levels of these two metabolites.

Another mouse study, by Buffington et al. (2016), looked at the effects of maternal high-fat diet (MHFD) on behavior and the gut microbiota of mice. Offspring

born to mothers receiving MHFD were found to have social deficits in line with the deficient social interactions seen in ASD, such as impaired sociability and lack of interest in social novelty. In addition, and in support of the authors' hypothesis that lack of certain bacterial species can cause social deficits, they found that germ-free mice showed impaired social behavior. These mice reverted to having normal behavior if they received fecal microbiota from offspring of mothers eating a regular diet at 4 weeks, but not at 8 weeks. In comparing the feces of offspring born to MHFD mothers with those born to mothers having a regular diet, the authors found that there was a significant difference between the bacterial community structures when using unweighted UniFrac analysis but not when using weighted UniFrac analysis (see Appendix II for differences between these methods).

Buffington et al. (2016) used metagenomic shotgun sequencing to determine if there were any particular species that were missing in the fecal samples from MHFD offspring and found that *L. reuteri* was decreased more than 9-fold compared to offspring born to mothers fed a regular diet. The authors note that *L. reuteri* has been found to promote oxytocin levels, which plays a role in social behaviors, so they hypothesized that the decrease in *L. reuteri* seen in MHFD offspring could be causing their social deficits. To test this, they supplemented the water of MHFD offspring with *L. reuteri* and found that this led to a significant improvement in sociability and preference for social novelty. However, they do note that some of the behavioral changes seen in MHFD offspring, such as anxiety and engaging in repetitive behavior, were unchanged with *L. reuteri* treatment. Since this bacteria is known to promote oxytocin levels, the authors examined

whether MHFD offspring showed any difference in the number of oxytocin-expressing cells in the paraventricular nucleus (PVN) of the hypothalamus, which is the main site of synthesis for oxytocin. They found that MHFD offspring did in fact have less oxytocin immunoreactive neurons, and that this decrease was restored with *L. reuteri* treatment. In regards to how *L. reuteri* might be able to increase oxytocin levels in the brain, Buffington et al. (2016) say they "favor the idea that the vagus nerve could be the main pathway of communication between the gut/*L. Reuteri* and changes in oxytocin in the PVN." The ability of *L. reuteri* to increase levels of oxytocin via the vagus nerve has in fact been proven. In a mouse model for wound healing, Poutahidis et al. (2013) found that mice who received *L. reuteri* treatment showed increased levels of oxytocin and that this effect was abolished following vagotomy. Interestingly, treatment with nasal oxytocin has previously been studied in children with ASD. For example, one study found that 5 weeks of twice daily administration of a nasal spray containing oxytocin led to improvements in the social responsiveness of children with ASD (Yatawara, Einfeld, Hickie, Davenport, & Guastella, 2016).

While the aforementioned mice studies show promising results, it is important to keep in mind that these findings are not directly relatable to humans because, as Cryan and O'Mahoney (2011) point out, mouse studies "do not represent any true situation in the human population."

TREATMENT STUDIES TARGETING THE GUT MICROBIOTA IN HUMANS

Several studies in recent years have attempted to target the gut microbiota of children with ASD in the hopes of improving some of their ASD symptoms. These studies have used various techniques including modified diets, prebiotics, probiotics, antibiotics, and microbiota transfer. Below, some of the most promising studies will be summarized and analyzed by type of treatment to get a sense of how the field has evolved in recent years.

Diet Modifications

Targeting the gut microbiota of children with ASD by changing their diet is arguably one of the less invasive methods that researchers have employed over the years. The main elimination diet that has been used in children with ASD is a gluten-free and casein-free (GFCF) diet (Ly et al., 2017). Many studies have looked at the effects of this diet on ASD, so many that a review of all of them would not be possible here. The reason this type of diet has been of interest to researchers is because there was a theory, first proposed by Panksepp (1979), that "opioid excess" might be linked to ASD. He proposed that the opiate system of children with ASD was overactive. Later, researchers postulated that the metabolism of gluten and casein peptides creates opioid peptides that can cross the intestinal barrier due to the high gut permeability seen in ASD and then bind to opioid receptors and lead to behavioral abnormalities (D'Eufemia et al., 1996). However, many studies have failed to find abnormally high levels of opioid peptides in children with ASD, so this theory is not well supported (Cass et al., 2008; Ly et al., 2017). Nontheless, the idea that a GFCF diet may improve ASD-related symtpoms persists.

One systematic review of studies examining the effect of a GFCF diet analyzed the six randomized controlled trials that had been published between 2007 and August of 2016 (Piwowarczyk, Horvath, Łukasik, Pisula, & Szajewska, 2017). The studies that Piwowarczyz et al. (2017) analyzed had a total of 214 participants, with half in a GFCF group and half in a control group, and the children in all but one study had their diagnosis of ASD confirmed by ADI-R and/or ADOS. After their thorough analyses of the six studies available, the researchers determined that there is a lack of consistent evidence in support of using a GFCF diet in children with ASD. They showed that some studies did show statistically significant improvements in certain scores, however these were not measured by standardized scales and some were subject to parental bias since the parents of the children were rating their child's symptoms. Ly et al. (2017) noted in a recent review article regarding elimination diets in ASD that there is not enough evidence to conclude that a GFCF diet is beneficial for treating ASD symptoms.

Prebiotics

One study by Grimaldi et al. (2017) examined the effects of a prebiotic on the fecal bacteria present as well as the metabolic activity in children with ASD. Prebiotics are "food ingredients that contain non-digestible oligosaccharides" (Jandhyala et al., 2015). Grimaldi et al. (2017) administered galactooligosaccharide (B-GOS) to three children with mild autism and three control children, none of whom had any GI complaints. The methods they used were somewhat unique to other studies presented here in that they made a "gut model system" composed of three compartments to model the proximal, transverse, and distal colon. Prior to treatment, the children with ASD had

lower levels of *Bifidobacterium* compared to the control children and after treatment, these levels increased. They also reported a significant increase in *Clostridium* cluster XI following B-GOS treatment. Samples from children with ASD had lower levels of butyrate and propionate compared to those from the controls, and they found that B-GOS increased the amount of acetate and butyrate detected in the compartment modeling the proximal and transverse colon. Also after B-GOS treatment, propionate was decreased in samples from children with ASD in the compartment representing the distal colon. If the theory that propionate can lead to harmful effects in the brain is correct, this study shows that B-GOS could help with this. Obvious limitations with this study include its small sample size. Additionally, some of the conclusions they make do not seem to be directly supported by their graphical data. However, this study was included here to give an example of a study targeting the gut microbiota through a prebiotic.

Probiotics

There are several studies that have looked at the effects of probiotics on groups of children with ASD as well as one case study. Probiotics are commonly used in the treatment of GI disorders, and in recent years have been used in children with ASD to see if they might be able to regulate dysbiosis of the gut microbiota and thereby improve ASD symptoms. By definition, probiotics are live microorganisms that are thought to stabilize the mucosal barrier in the gut (Santocchi et al., 2016). They have been found to have a balancing role on the bacteria in the gut and are able to decrease proliferation of *Clostridium* species (Santocchi et al., 2016). The group studies done to date on probiotics as well as a case study will now be summarized and analyzed.

The first study found, which was conducted by Parracho et al., (2010) was a placebo-controlled blinded study in which 17 children with ASD from the UK were treated with a probiotic (*Lactobacillus plantarum* WCSF1) for 12 weeks. Based on fecal sampling, they found that children who received probiotic had significantly higher counts of bacteria in the lactobacilli/enterococci group and lower counts of bacteria in *Clostridium* Cluster XIVa compared to those who received placebo. While many other studies have relied on sequencing methods to determine the prevalence of different bacterial strains, the authors of this study chose to use fluorescence *in situ* hybridization (FISH). This entailed using six specific probes known to allow the visualization of particular bacterial groups. The authors do mention that less than 50% of the total bacterial count was covered by the probes used, so it is possible that there may have been differences between the two groups in regards to other bacterial groups. There was no difference between the two groups in terms of GI symptoms, but it should be noted that the children recruited for this study did not have GI symptoms, so it would not be expected for GI symptoms to be improved. In terms of behavior, children who received the probiotic had improved scores on some of the sub-scales in the Total Behaviour Problem Score (TBPS) (see Appendix I). While this study showed some significant results, the authors state that they had originally recruited 62 children but that many dropped out due to the study design. They note that collecting six fecal samples as well as keeping a daily diary "may have been over-ambitious for this subject group" (Parracho et al., 2010). No other studies reviewed have pointed this out as clearly as this group did,

but it is something that researchers must keep in mind when working with patients who have ASD.

The next study that mentioned the effects of probiotics on ASD was not a treatment study, but rather the researchers asked whether or not each child was already taking a probiotic at the beginning of the study and they included this as a variable in their analysis (Adams et al., 2011). They studied 58 children with ASD and 39 control children, and found that 33% of the children with ASD were taking probiotics while only 5% of the control children were taking probiotics. When comparing the entire ASD group to the non-ASD control group, they found that children with ASD had lower levels of SCFAs in their stool. In interpreting these results, the authors suggest that the decreased SCFA levels in children with ASD compared to the controls could be due to lower amounts of good bacteria that make SCFAs, decreased soluble fiber intake, longer transit time, and/or increased intestinal permeability. Since the authors did not measure SCFA levels in the bloodstream, they were not able to determine which of these possibilities was most likely. However, they suggest that they favor the idea that children with ASD exhibit increased intestinal permeability, which would lead to more SCFAs in the bloodstream. Their reason for favoring this hypothesis is based on findings by MacFabe et al. (2007), in which researchers found that propionic acid (an SCFA) can lead to ASD symptoms when injected into rats. Regardless of why SCFA levels were lower in children with ASD, all of the possibilities presented by Adams et al. (2011) imply that increased levels of SCFAs in the stool would be good. However, when comparing the ASD group taking probiotics to the ASD group not taking probiotics, the ASD probiotic group

actually had lower levels of SCFAs. This would indicate that probiotics are not beneficial, although the authors of this study do not make this connection. Overall, this study does not provide very strong support for the use of probiotics, especially since there was no mention of the specific probiotics being taken by the children.

The third probiotic study was aimed at monitoring the changes in D-arabinitol (DA) in 22 children with ASD in Poland following probiotic treatment (Kałużna-Czaplińska & Błaszczyk, 2012). They were interested in this metabolite because it comes from many pathogenic *Candida* species and other studies had shown it was elevated in children with ASD. While the focus of this thesis has been on bacteria of the gut microbiota, a review of this study is being included here because of the results they found regarding ASD symptoms following probiotic treatment with *Lactobacillus acidophilus* (strain Rosell-11) twice daily for 2 months. They found that children with ASD who received the probiotic showed significantly increased ability to concentrate and carry out orders. While these results are in favor of probiotic use for improving ASD symptoms, the sample size was very small and the authors did not mention what measurements they used to determine ASD symptoms.

The fourth study examining the effects of probiotics in children with ASD studied 10 children with ASD in Slovakia and compared them to 9 siblings and 10 healthy children (Tomova et al., 2015). The main measure these researchers were interested in was whether probiotic treatment could correct the dysbiosis seen in children with ASD. The probiotic used was one capsule three times per day for 4 months of "Children Dophilus," which has *Lactobacillus* (three strains), *Bifidumbacteria* (two strains), and

Streptococcus (one strain). The researchers found that following this treatment, there was a significant decrease in Firmicutes levels such that the Bacteroidetes/Firmicutes level resembled that of the healthy children. They also found the *Desulfovibrio* levels decreased significantly after probiotic treatment. While children with ASD did not show significant differences in the levels of this species compared to controls, higher levels were found to be associated with increased autism severity, so this decrease seen with probiotics could be relevant.

Santocchi et al. (2016) are currently in the process of completing a study to examine the effects of using a probiotic mixture called Vivomixx® in children with ASD. The authors believe a "well-controlled" clinical trial is needed and that other studies done to date have not adequately examined the relationship between probiotic use and ASD. Santocchi et al. are studying 100 preschoolers in Italy with ASD and either with GI symptoms or without and are placing them into four treatment arms, with an equal ratio of children in each group. The total length of treatment will be 6 months and the researchers conducting this study state they are interested in the effects on GI symptoms, core ASD symptoms, and brain function and connectivity. They will be assessing ASD symptoms via several methods including CARS, the Child Behavior Checklist for Ages 1.5-5 (CBCL/1.5-5), the Repetitive Behavior Scale-Revised (RBS-R), and the Vineland Adaptive Behavior Sclaes II (VABS-II) (see Appendix I). Per clinicaltrials.gov, the estimated study completion date is November 2018, so the results will unfortunately not be available prior to the completion of this thesis.

One recent case study by Grossi et al. examined the effects that probiotic treatment had on a boy with ASD in Italy (Grossi, Melli, Dunca, & Terruzzi, 2016). The probiotic treatment for this child entailed VSL#3, which is a mixture of three *Bifidobacteria* species, five *Lactobacilli* species, and two *Streptococci* species and he received this treatment for 4 weeks. They found that this treatment not only alleviated his GI symptoms, but also led to an improvement in core ASD symptoms as measured by ADOS (see Appendix I). It is important to note that this boy had also been diagnosed with irritable bowel disease, so that could have played a role in the results they found.

More recently, the results of a study examining the effects of a probiotic formula on children with ASD in Egypt were published (Shaaban et al., 2017). In this study, 30 children with ASD were administered a probiotic formula containing *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, and *Bifidobacteria longum* for 3 months. Autism severity was assessed by ATEC (see Appendix I), and the researchers found that treatment with this probiotic formula led to a significant improvement in ATEC scores as well as an improvement in GI symptoms. The results of some of these aforementioned probiotic studies do provide support for the use of probiotics in children with ASD. However, studies with large sample sizes as well as extended follow up periods and a randomized placebo control group are needed before probiotic use can be validated as a treatment option.

Antibiotics

Upon review of the literature aimed at targeting the gut microbiota in children with ASD, the first study found using methods other than diet changes or

probiotics/prebiotics used vancomycin in 11 children with regressive-onset autism (Sandler et al., 2000). In this study, the authors hypothesized "that repeated antimicrobial use might [disrupt] a protective effect of indigenous intestinal organisms and [allow] colonization by one or more neurotoxin-producing species." They developed this hypothesis after hearing reports from some parents that their children experienced an onset of autistic symptoms following chronic diarrhea after repeated broad-spectrum antimicrobial use (Sandler et al., 2000). They chose vancomycin because of its efficacy as well the fact that it is non-absorbable and stays in the GI tract.

Sandler et al. (2000) describe an "index case" of a 4.5 year old boy who developed autistic symptoms at around 19 months of age after having been treated three times over a period of 6 weeks with a 10-day course of broad-spectrum antibiotics for chronic otitis media. He then experienced chronic diarrhea, after which his ASD symptoms began. The authors note that he had normal motor, cognitive, and social development prior to this. They then treated him with oral vancomycin four times per day over the course of 12 weeks. After the sixth day, he started showing improvements including better behavior, ability to follow tasks, ability to achieve toilet training, increased vocabulary, and a decrease in repetitive behaviors. While the results were significant, his behavior quickly declined shortly after the vancomycin treatment was discontinued. These researchers then tried a similar treatment in 11 other children followed by 4 weeks of probiotic therapy (including *Lactobacillus acidophilus*, *L bulgaricus*, and *Bifidobacterium bifidum*). Improvements were seen in this group of children as well, but the results did not last, and most of them deteriorated once again

within 2 weeks after the vancomycin treatment was discontinued. Additionally, the authors note that many of the children did not take the probiotic because of unappealing taste, but this did not have any effect on the outcome of the treatment.

One of the papers discovered in researching this topic was written by the father of a child with autism after he discovered that his son showed improvement in regard to his ASD symptoms following a 10 day course of amoxicillin for strep throat. According to his report, published in Microbial Ecology, his son began making eye contact, showed improved speech, became less insistent on following a specific routine, and had more energy, all things that had posed problems for him before (Rodakis, 2015). This father tracked his son's behavior based on 20 parameters, and he notes that his son's therapists made comments about his improvement not knowing that his son had taken antibiotics. He also makes a comment that he had seen some mild improvements in his son's behavior previously when his son had a fever, although it is not clear if he is implying an infection related to the fever may have caused these changes or if it was medication given for the fever. Additionally, he states that other parents he talked to had seen improvements in their children's ASD symptoms following antibiotic therapy, and some even said they gave their children antibiotics to help, however he does not provide any concrete examples. While he attributed positive benefits to antibiotic use in regards to his son's ASD, he does also note that some parents said they believed their child's ASD developed after antibiotic therapy, which is a theory that some have favored. In his report, this father provides results from some of the studies discussed here, and he argues that increased funding on this topic is needed. It is important to recognize that a sample

size of one child obviously has its limitiations. However, the fact that antibiotic therapy was able to seemingly improve a child's ASD-related symptoms is interesting given that antibiotics are known to alter the gut microbiota.

Microbiota Transfer Therapy

One recent study by Kang et al. (2017), which arguably shows some of the most promising results, treated 18 children aged 7-17 years old with ASD with what they termed Microbiota Transfer Therapy (MTT). This treatment included 2 weeks of oral antibiotic treatment with vancomycin followed by a bowel cleanse and then fecal microbiota transplant (FMT) with an initial high dose via either an oral or rectal route followed by a 7-8 week maintenance dose via the oral route as well as treatment with Prilosec (a proton pump inhibitor) to help the newly introduced bacteria survive. For the FMT part of the treatment, the researchers used standardized human gut microbiota (SHGM) in liquid form that is made from the stool of healthy individuals. It contains more than 99% bacteria and has been shown to be effective in the treatment of *C. difficile* infections, with 95% of individuals with recurrent *C. difficile* in one study of 43 individuals being successfully treated after either one or two doses (Hamilton, Weingarden, Sadowsky, & Khoruts, 2012).

Kang et al. (2017) monitored GI symptoms by having the parents of the children fill out the Gastrointestinal Symptom Rating Scale (GSRS) as well as a daily stool record (DSR). They used ADI-R to confirm a diagnosis of ASD and then used the Parent Global Impressions-III (PGI-III) to monitor changes in each child. To rate the severity of ASD and related symptoms, they used CARS, ABC, the Social Responsiveness Scale (SRS),

and VABS-II (see Appendix I). For analyzing the gut microbiota, they clustered sequences into operational taxonomic units (OTUs) (see Appendix II). The researchers of this study found that there was a significant decrease in the GSRS score for children in the ASD group, with the average drop in score compared to the baseline being 82%, and this improvement was seen even 8 weeks after MTT was discontinued. They also found that ASD-related behaviors significantly improved as rated by PGI-III, CARS, SRS, and ABC. Additionally, the average developmental age of the children with ASD increased by 1.4 years as measured by VABS-II. These improvements in GI symptoms and ASD-related behaviors provide very promising results in support of the idea that targeting the gut microbiota can help with ASD symptoms, especially due to the numerous ASD assessments the researchers used to monitor changes.

Kang et al. (2017) note that the treatment they employed led to temporary adverse effects such as tantrums, aggression, and hyperactivity, but this went away and was thought to be caused by vancomycin. This finding is contradictory to the beneficial effects of vancomycin reported by Sandler et al. (2000) and Rodakis (2015). Therefore, further research regarding the use of vancomycin in children with ASD is needed.

In regards to differences in the gut microbiota, Kang et al. (2017) found that samples from children with ASD + GI symptoms were significantly less diverse compared to those from neurotypical children without GI symptoms. After MTT, the diversity in children with ASD + GI symptoms increased significantly and this increase persisted 8 weeks after treatment ended, with diversity appearing similar to that seen in the neurotypical group. They also note that the bacterial diversity seen in the ASD + GI

symptoms group after treatment was similar to that of the donor bacterial community. Prior to treatment, children in the ASD group had significantly less *Bifidobacterium* compared to the neurotypical controls, but after treatment, its relative abundance increased. Additionally, the abundance of *Prevotella* and *Desulfovibrio* significantly increased after MTT. The authors note that the role of *Desulfovibrio* has been controversial, with some sources claiming its relationship to the human gut is commensal while others state it is detrimental (Kang et al., 2017).

The children in this study were only followed for 8 weeks following the completion of MTT, so it possible that long-term effects could appear, but given the current information, MTT appears to be generally safe. Overall, Kang et al. (2017) make a compelling argument for the beneficial effects of MTT in treating children with ASD and GI symptoms, as it improved not only the GI symptoms of most of the children, but also some of their ASD-related behaviors. They also note that the fact that the gut microbiota of children with ASD following MTT became more like that of the neurotypical children is in line with the hypothesis that the gut microbiota may be "at least partially responsible for GI and ASD symptoms." Despite the compelling evidence Kang et al. present, there are some downfalls to their research. One of these, which they acknowledge in their discussion, is that they did not include a control group, so the placebo effect may have been at play. They also note that having GI symptoms reported by the parents of the children instead of by a pediatric gastroenterologist could have led to unreliable results, since they claim it has been shown that parental reports and those by a specialist are sometimes in disagreement in regards to the specific GI

symptoms/diagnoses that a child is experiencing. Also, in terms of their study design, it would have been helpful if they had included a group of children with GI symptoms but without ASD and if they had treated those children with MTT in order to compare them to children with ASD. The children in the neurotypical group they studied did not suffer from any GI disorders, so it is not surprising that their gut microbiota would be different from that of children with GI symptoms who also have ASD.

DISCUSSION

While there seems to be a correlation between the gut microbiota and ASD, the results are not conclusive enough to say that an altered gut microbiota causes ASD. However, the studies discussed here do provide results that indicate further research into this field is warranted. One of the biggest issues found regarding this field is that there is tremendous variation in the methodology used, something that Rodakis (2015) noted in his report as well: there are wide variations in samples used (stool vs. biopsy), how they are collected, and how these samples are analyzed. Additionally, the fact that ASD is by definition a spectrum of disorders makes it probably unlikely that one single factor can be responsible for all cases. As some authors have hypothesized, it seems more likely that there is perhaps a subset of individuals with ASD who also experience GI symptoms, and that in this population, targeting the gut microbiota as a means of treatment could be beneficial. For example, Santocchi et al. (2016) believe it is possible that there could be a particular endophenotype of ASD, in which children with ASD suffer from GI symptoms.

Many of the studies looking at the role of the gut microbiota in ASD employ various methods for determining changes in GI symptoms. While having a pediatric gastroenterologist evaluate any child with GI symptoms in order to most accurately diagnose a child's symptoms would be ideal, some of the studies presented here used parent questionnaires to evaluate GI symptoms. One study found that parents of children with ASD were able to identify that their child had some type of GI issue (92.1% correctly determined their child had a GI issue), but they were unable to accurately determine the specific type of GI issue. For example, 85% of children in the ASD+GI

symptom group were diagnosed as having functional constipation, but only 44.7% of parents in this group reported that their child suffered from constipation (Gorrindo et al., 2012). While this can certainly affect the statistics that researchers present in terms of what GI issues are most common in children with ASD, it does not undermine the fact that children with ASD do in fact suffer from GI issues.

In terms of methods used to study the relationship between the gut microbiota and ASD, using a mouse model can be very useful and cost effective, but results using mice are not directly translatable to humans. The gut microbiota of humans and that of the mouse are similar in that they both are dominated by Firmicutes and Bacteroidetes, but there are significant differences when looking at the genus level (Knight et al., 2017). Because of these underlying differences, Knight et al. point out that using a mouse model is suitable to study mechanisms but not for identifying specific species implicated in the biological processes that occur in humans. Therefore, those studies that monitor the effects of a particular treatment in a mouse model of ASD are not all that relevant to the study of ASD in humans.

Another issue with this field is that because ASD encompasses a spectrum of disorders and because the classification for ASD has changed over the years, it is impossible to directly compare results from time periods in which the classification for ASD was different. Also, due to advancements in the field of microbiology, there are many options available for identifying different bacterial species. In an ideal world, all researchers studying the role of the gut microbiota in ASD, or any disease, would use the

same methods for detecting different species. However, this is not the case, so it is important to note these differences when trying to draw broad conclusions.

Another factor to consider with this field of research is that the gut microbiota is dependent on many factors, such that comparing results of studies from different regions and in different age groups is exceedingly difficult. For example, Tomova et al. (2015) point out that individuals in Europe tend to have a dominance of Firmicutes in their stool and that younger people tend to have less Bacteroidetes in their fecal samples. These differences make it difficult to draw broad conclusions from multiple studies. Additionally, because the gut microbiota is so complex, finding one treatment option for all children with ASD and GI symptoms would be nearly impossible. As Staley, Khoruts, and Sadowsky (2017) point out in a review article about the use of fecal microbiota transplantation in treating GI diseases, it is "unlikely that a single microbial species will serve as a sole therapeutic lynchpin." Additionally, they also note that nobody has yet defined what makes up the "normal" microbiota of a healthy person. If we do not know what constitutes a healthy gut microbiota, it seems impossible to definitively say that a certain group of bacteria is to be blamed in ASD.

Of all the studies discussed here, the MTT study by Kang et al. (2017) seems the most promising. That being said, proposing that fecal transplants be used widely would likely draw skepticism from many. However, it should be noted fecal microbiota transplantation is now considered "well established" as a treatment for recurrent *C. difficile* infections in the United States and Europe (Staley et al., 2017). Therefore, just because it is unusual, it should not be ruled out as a potential line of treatment for

children with ASD. More studies on this as a treatment option are needed and a placebo group should definitely be included in order to verify that the placebo effect is not taking place.

There have been many developments over the years in terms of linking the gut microbiota to ASD. While many of these studies have their limitations, the research they have contributed is nonetheless important. Further research targeting the gut microbiota in children with ASD as well as examining the various ways in which these bacteria can interact with the brain of children will help further advance this field. If these studies continue to show that there is a relationship and if causation, rather than just correlation can be proven, advancements in this field may lead to treatment options for ASD. Given the prevalence of ASD and the burden that it places on families and caregivers of children with ASD, it is important that research into this field continue.

APPENDIX I: Tests Used to Diagnose and Assess ASD

Aberrant Behavior Checklist (ABC):

- The ABC "assesses problem behaviors in five areas common in children with ASD, including irritability, lethargy, stereotypy, hyperactivity, and inappropriate speech" (Kang et al., 2017).

Autism Diagnostic Interview revised (ADI-R):

- ADI-R is a structured interview which takes 2 hours. "It is one of the primary tools used for clinical diagnosis of autism and autism spectrum disorders. It is not designed to be a measure of autism severity but higher scores are generally consistent with more severe symptoms" (Kang et al., 2017).

Autism Diagnostic Observation Schedule (ADOS):

- ADOS-CSS (calibrated severity score) was found to be the "best measure of ore features of ASD in pre-school children" (Wiggins et al., 2017). Wiggins et al. note the need for calibrated scores because the chronological age and language aptitude affects the total ADOS scores, which makes it an unreliable measure of ASD severity when looking at core symptoms.
- Per a review article discussing assessment methods for ASD, ADOS is the "gold standard measure in clinics that evaluated children for autism" (Choueiri & Zimmerman, 2017).

- ADOS-2 is an updated version of ADOS. It "examines behaviors in two distinct domains. One takes into account the social affect (SA) and the other the restricted, repetitive behaviors (RRB). Both provide for the ADOS-2 comparison score, a metric for gauging overall autism severity" (Grossi et al., 2016).
- Per Grossi et al. (2016), "it is well known tha ADOS score does not fluctuate spontaneously along time in ASD and is absolutely stable."

Autism Treatment Evaluation Checklist (ATEC):

- This is "designed to provide a quantitative assessment of autism severity" and is made up of 4 subscales which are "1) speech/language/communication, 2) sociability, 3) sensory/cognitive awareness, and 4) health/physical behavior" (Adams et al., 2011). The scores are added up to provide an ATEC score.
- Scores are provided by parents, but it has been shown that this score correlates well with those obtained using CARS (Geier, Kern, & Geier, 2013).

Child Behavior Checklist for Ages 1.5-5 (CBCL/1.5-5):

- This tool is a "parent-completed measure of emotional, behavioral, and social problems in children aged 1.5-5 years. It was developed to assess a range or problem behaviors rather than ASD in particular" (Havdahl, von Tetzchner, Huerta, Lord, & Bishop, 2016). Per Havdahl et al., it is not adequate as a diagnostic tool due to the high number of false positives,

although it is useful for gathering information regarding behavioral problems.

Childhood Autism Rating Scale (CARS):

- This "can be used to both diagnose autism and ASD and asses the overall severity of the symptoms" (Kang et al., 2017).
- This scale is a "well-established, professional-rated measure in that it is widely used and well validated" (Geier et al., 2013).

Development Behavior Checklist Parent/Primary Caregiver Report (DBC-P):

- This "is a ranking score test which consists of 96 items and can be scored using a three-point scale" (Parracho et al., 2010).

Parent Global Impressions (PGI-III):

- This tool is used to determine changes in 17 autism related symptoms. It is completed by parents and it was "found to be more reliable to ask parents directly about observed changes than to have them estimate symptoms severity at the beginning and end and then compute a difference" (Kang et al., 2017).

Repetitive Behavior Scale-Revised (RBS-R):

- This questionnaire is completed by parents and is used to assess repetitive and restrictive behaviors. It was found to be a "cost effective parent-report measure" that is reliable and a "viable means of assessing repetitive behavior in toddlers with autism" (Schertz, Odom, Baggett, & Sideris, 2016).

Social Responsiveness Scale (SRS):

- This "assesses social impairments, a core issue in autism, including social awareness, social information processing, capacity for reciprocal social communication, social anxiety/avoidance, and autistic preoccupations and traits" (Kang et al., 2017).

Total Behaviour Problem Score (TBPS):

- This score is "an overall indicator of behavioural/emotional disturbances and is calculated by summation of the scores for all 96 items [on the DBC-P]. In addition, the individual items are categorized into five sub-scales that measure disturbance in five dimensions: disruptive/antisocial behavior, self-absorbed behaviour, communication, anxiety problems, and social-relating problems" (Parracho et al., 2010).

Vineland Adaptive Behavior Scales II (VABS-II):

- This test is neither diagnostic nor specific to a diagnosis of ASD because it also measures adaptive behavior (like living skills and motor skills), which makes it such that other co-occuring conditions that some children with ASD have could affect the scores (Wiggins et al., 2017).
- It is a "measure of the functioning level in four different domains: communication, daily living skills, socialization, and motor skills" (Kang et al., 2017).

APPENDIX II: Methods Used to Classify Bacteria

Below, a brief overview of advancements made in the field of classifying bacteria has been provided based on the summary given in a review article about the human microbiome (Knight et al., 2017). This is relevant seeing as the foundation of this field of study is based on classifying differences in the gut microbiota of children with ASD.

Before the advent of genetic sequencing, researchers in the field of microbiology were limited in terms of the techniques available to them to classify bacteria. They had to rely on morphology-based methods as well as cell staining characteristics and the ability of a bacterial population to carry out biochemical reactions in order to group different bacteria. This of course did not allow scientists to classify organisms into phylogenetic groups accurately. Then, in the 1970s, Carl Woese revolutionized the way in which organisms were classified by discovering that ribosomal RNA (rRNA) evolved much more slowly which allowed researchers to group bacteria based on a phylogenetic framework in which 11 major microbial phyla were identified. Following this discovery, it was determined that there are certain regions of the 16S rRNA sequence in microbial organisms that are highly conserved in each phyla across even long evolutionary periods. Based on these conserved regions, primers were developed for reverse-transcriptase-mediated direct sequencing which allowed researchers to sequence RNA genes and to group a particular bacteria into a phyla.

As more developments in the field of sequencing were made, including using PCR and automated DNA sequencing machines, it was determined that more than 1,000 bacterial phyla exist. Now, there are next-generation sequencing techniques, which allow

scientists to obtain “millions of reads for less than once cent per read.” With so many sequences, researchers began grouping them into OTUs and defined sequences that were 97% identical as being equivalent to belonging to the same species. Tools were then developed to more effectively analyze similarity between sequences such that now researchers can cluster sequences based on various parameters. In the mid 2000s, UniFrac was developed which takes phylogeny into account when comparing different microbial communities. As time goes on, it is likely that even faster and more specific methods of analyzing microbial groups will be developed, however, for now, the current techniques being used “have been very useful for functional investigations” (Knight et al., 2017).

Researchers employing a UniFrac analysis have two measures that they can use to determine the diversity of a bacterial community. One of these is unweighted UniFrac, which is a qualitative measure that uses the presence or absence of data for its analysis and ignores relative abundance. The other method, weighted UniFrac, is a quantitative measure which takes into account the relative abundance of each organism (Lozupone, Hamady, Kelley, & Knight, 2007). Per Lozupone et al., using a qualitative analysis is better at detecting the effects of different founding populations. As an example, they say it would be good at determining the source of bacteria that initially colonize the gut of newborn mice. In contrast, they say that a quantitative analysis is better at detecting the effects of factors like nutrient availability.

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