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Evidence-based Probiotic Intervention for Behavioral and Social Deficits in Autism Spectrum Disorder

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Abstract

Autism Spectrum Disorder (ASD) refers to a heterogenous neurological condition characterized by repetitive and restrictive behaviors and social communication deficits. ASD diagnoses are at a record high, at approximately 1 in 59 children according to the US Center for Disease Control. Currently, there are no available interventions that effectively treat the core symptoms of ASD. All pharmaceutical options address comorbid side effects of ASD but not core deficits and are particularly associated with negative side effects. Additionally, there are economic and geographic barriers that can prevent families of individuals with ASD from seeking or receiving effective interventions. Many of the available interventions are extremely costly, time-consuming, and age dependent. These factors, as well as others, have led to an increase in families independently utilizing complementary and alternative interventions. Due to the large amount of misinformation available on the Internet, families have become more susceptible to trying alternative forms of interventions that have not been scientifically proven as effective, and in some cases, are significantly detrimental. Thus, the need for accessible and inexpensive evidence-based nonpharmaceutical interventions is critical and must be addressed. Fortunately, recent groundbreaking research has discovered two strains of probiotics, *Bacteroides fragilis* and *Lactobacillus reuteri*, that have been shown to ameliorate behavioral and social deficits respectively, in validated ASD mouse models in a non-age-dependent manner. Probiotic intervention with a combination of these specific strains would effectively target both repetitive behaviors and social deficits, core ASD symptoms, and provide families with an accessible and inexpensive form of intervention. The mechanisms underlying the efficacy of these probiotics are thought to be associated with the gastrointestinal (GI) system and the oxytocin pathway. This study seeks to examine the necessity of accessible nonpharmaceutical interventions and to provide an effective intervention that is neither expensive or age dependent. This study also aims to provide greater insight into the pathways and systems in which these probiotics operate.

I. Introduction

Autism spectrum disorder (ASD) is a pervasive neurodevelopmental disorder that encompasses a wide range of pathology. The most recent estimate of the incidence of autism in America is from the Center of Disease Control and Prevention (CDC, 2018) in 2014. The CDC's Autism and Developmental Disabilities Monitoring (ADDM) estimates that about 1 in 59 children has been diagnosed with ASD. Studies in Asia, Europe, and North America have estimated an average prevalence of ASD as between 1%-2% (CDC, 2018). The estimated worldwide incidence rate is about 1% (Lai, Lombardo, & Baron-Cohen, 2014). ASD diagnoses are at a historic high and with such a large population of individuals, ASD and ASD treatments have gained greater importance.

Additionally, the financial burden that accompanies a diagnosis of autism is astonishingly excessive. It is estimated that in the United States, the total costs per year for children with ASD falls between $11.5 billion- $60.9 billion (2011 US dollars) (CDC, 2018). This number includes a variety of direct and indirect costs from medical care, loss of parental economic productivity, and special education (CDC, 2018). On average, the average medical expenditures of children and adolescents with ASD exceeded those without ASD by $4,110-$6,200 per year, approximately 4.1-6.2 times greater (CDC, 2018). In addition to medical expenditures, general expenditures were 8.4-9.5 times greater for children and adolescents with ASD, in comparison to those without (CDC, 2018). In 2005, the average annual medical costs for Medicaid-enrolled children with ASD were $10,709 per child, while the average costs for children without ASD was $1,812, approximately six times less (CDC, 2018). Early intensive behavioral interventions, one of the most effective interventions available, can cost between $40,000-$60,000 annually per child (CDC, 2018). Thus, ASD places a huge

economic burden on affected families and there is a dire need for more affordable treatment options as ASD indiscriminately affects families of all socio-economic levels.

Before the widespread application of early intervention programs, studies showed that 58%-78% of adults with autism had poor or very poor outcomes in terms of independent living, educational attainment, employment, and peer relationships (Lai, Lombardo, & Baron-Cohen, 2014). Only 46% of adults with autism are employed (regular, supported, or sheltered) (Lai, Lombardo, & Baron-Cohen, 2014). Creating an accessible and inexpensive non-pharmaceutical intervention that is not time-sensitive, like previous interventions that must be applied early in life to be effective, will allow adults with ASD to increase their independence and better their life outcomes. This would also decrease the large financial responsibility associated with the life-long care of dependent adults with ASD.

Currently, there are no available treatment options for individuals with ASD that are both inexpensive and easily accessible. However, recent research and its resulting revolutionary evidence points to a promising new avenue of intervention; the use of probiotics. There are two specific probiotic strains that have been identified as having the potential to become a relatively accessible and affordable means of intervention for individuals with ASD; *Bacteroides fragilis* and *Lactobacillus reuteri*. Furthermore, evidence shows that intervention with these particular probiotic strains may improve the core symptoms of ASD, deficits in social communication and repetitive behavior, a feat that has not been accomplished by any of the current treatment options available to those with ASD (Hsiao et al., 2013; Buffington et al., 2016; Sgritta et al., 2019).

II. Background

1.Autism Spectrum Disorder

Symptoms

The hallmark symptoms of ASD are difficulties in social communication and social interaction, and restricted, repetitive behavior, interests, or activities (APA, 2013). The cognitive abilities of people with ASD can range from gifted to severely challenged, representative of the wide range of symptomology and severity in ASD (CDC, 2018). About 45% of individuals with ASD have intellectual disability and 32% experience regression (Lai, Lombardo, & Baron-Cohen, 2014). ASD is also associated with poor mental flexibility, thought to be an underlying basis of repetitive behaviors and restrictive interests (Mišić et al., 2015). Fixations tend to involve systems that operate deterministically and repeatably according to salient sets of rules (Belmonte et al., 2014). Stereotyped movements and compulsive and repetitive behavior are common and self-injurious behavior occurs in approximately 30% of children with ASD (Amihaesei & Stefanachi, 2013). There are different severities of speech impairments; some children never fully develop speech, or are limited to echolalia (Belmonte et al., 2014). Communication impairment also includes nonverbal signals such as gaze, facial expression, and gestures (Belmonte et al., 2014). Social deficits involve difficulty with processing information of other people as well as self-referential information (Lai, Lombardo, & Baron-Cohen, 2014). Other common symptoms include sensory abnormalities, motor impairment, and alimentary abnormalities (Amihaesei & Stefanachi, 2013). In addition, comorbidity is frequent in ASD populations; more than 70% of individuals with ASD have concurrent developmental, or psychiatric conditions (Lai, Lombardo, & Baron-Cohen, 2014). Commonly associated comorbidities include intellectual

delay, epilepsy, metabolic syndromes, ADHD, Tourette syndrome, and sleep issues (Amihaesei & Stefanachi, 2013). Headaches/migraines, respiratory issues, food allergies, physician visits, prescription medication, and rate of infections are also more common in children with ASD (Rodriguez & Kern, 2011). A meta-analytic study showed that the mortality risk of individuals with ASD is 2.8 times higher than those without, despite controlling for age and sex and is thought to be related to concurrent medical conditions (Lai, Lombardo, & Baron-Cohen, 2014). Approximately 25% of children with ASD develop seizures, although this is more common in girls with ASD than in boys with ASD. The prevalence of sleep problems including disturbed sleep, decreased sleep duration, and increased sleep onset delay, is higher in children with ASD than in children with intellectual impairment (Belmonte et al., 2014). GI disorders are also more 3.5 times more prevalent in children with ASD (Sharon, Sampson, Geschwind, & Mazmanian, 2016).

The numerous symptoms and comorbidities that accompany an ASD diagnosis often cause some amount of impairment in independent living and sustaining social relationships. Although impairment can range from nearly insignificant to considerably pronounced, individuals with ASD face the common challenge of creating and maintaining social relationships and managing restrictive and repetitive behavior. These core symptoms have yet to be improved by any currently available interventions.

2.Diagnostic Criteria

To be diagnosed with ASD, certain clinical requirements, provided in the Diagnostic and Statistical Manual of Mental Disorders (DSM) must be met. The most current version is the DSM 5, published by the American Psychiatric Association (APA, 2013).

The diagnostic criteria from the DSM 5 is as follows:

A. Persistent deficits in social communication and social interaction across multiple contexts, as manifested by the following, currently or by history:

    a. Deficits in social-emotional reciprocity

    b. Deficits in nonverbal communication behaviors used for social interaction

    c. Deficits in developing, maintaining, and understanding relationships

B. Restricted, repetitive patterns of behavior, interests, or activities, as manifested by at least two of the following, currently or by history:

    a. Stereotyped or repetitive motor movements, use of objects, or speech

    b. Insistence on sameness, inflexible adherence to routines, or ritualized patterns of verbal or nonverbal behavior

    c. Highly restricted, fixated interests that are abnormal in intensity or focus

    d. Hyper- or hyporeactivity to sensory input or unusual interest in sensory aspects of the environment

C. Symptoms must be present in early development (but may not become fully manifest until social demands exceed limited capacities or may be masked by learned strategies in later life).

D. Symptoms cause clinically significant impairment in social, occupational, or other important areas of current functioning.

E. These disturbances are not better explained by intellectual disability (intellectual developmental disorder) or global developmental delay.

Accompanying the diagnosis is an assessment of severity, from one to three, with one being the least severe and three being the most severe and is shown in Table 1 (APA, 2013).

Table 1 Severity levels for autism spectrum disorder

Severity level	Social communication	Restricted, repetitive behaviors
Level 3 "Requiring very substantial support"	Severe deficits in verbal and nonverbal social communication skills cause severe impairments in functioning, very limited initiation of social interactions, and minimal response to social overtures from others. For example, a person with few words of intelligible speech who rarely initiates interaction and, when he or she does, makes unusual approaches to meet needs only and responds to only very direct social approaches.	Inflexibility of behavior, extreme difficulty coping with change, or other restricted/ repetitive behaviors markedly interfere with functioning in all spheres. Great distress/ difficulty changing focus or action.
Level 2 "Requiring substantial support"	Marked deficits in verbal and nonverbal social communication skills; social impairments apparent even with supports in place; limited initiation of social interactions; and reduced or abnormal responses to social overtures from others. For example, a person who speaks simple sentences, whose interaction is limited to narrow special interests, and who has markedly odd nonverbal communication.	Inflexibility of behavior, difficulty coping with change, or other restricted/ repetitive behaviors appear frequently enough to be obvious to the casual observer and interfere with functioning in a variety of contexts. Distress and/ or difficulty changing focus or action.
Level 1 "Requiring support"	Without supports in place, deficits in social communication cause noticeable impairments. Difficulty initiating social interactions, and clear examples of atypical or unsuccessful responses to social overtures of others. May appear to have decreased interest in social interactions. For example, a person who is able to speak in full sentences and engages in communication but whose to-and-fro conversation with others fails, and whose attempts to make friends are odd and typically unsuccessful.	Inflexibility of behavior causes significant interference with functioning in one or more contexts. Difficulty switching between activities. Problems of organization and planning hamper independence.

An ASD diagnosis now encompasses several conditions that were formerly diagnosed separately including: Asperger syndrome, typical autism, pervasive developmental disorder not otherwise specified (PDD-NOS), Rett syndrome, and child disintegrative disorder (CDD) which all vary in severity (CDC, 2018; Amihaesei & Stefanachi, 2013). ASD is approximately four times more common amongst boys than girls, although it occurs in all racial, ethnic, and socioeconomic groups (CDC, 2018). However, it is more common in Caucasian children compared to African-American or Hispanic children (Bhat, Acharya, Adeli, Bairy, & Adeli, 2014).

Early indicators of ASD include delays in verbal and nonverbal communication, reciprocal affective behavior, joint attention and pretend play, atypical implicit perspective

taking, unusually repetitive behaviors, inflexibility in visual disengagement, and extreme variation in temperament (Amihaesei & Stefanachi, 2013; Lai, Lombardo, & Baron-Cohen, 2014). Studies have shown that parents tend to notice a developmental problem before their child's first birthday if their child has ASD (CDC, 2018). Popular instruments used to aid in diagnosis include the Autism Diagnosis Interview-Revised, the Autism Diagnosis Observation Schedule, and the Childhood Autism Rating Scale (Amihaesei & Stefanachi, 2013). ASD diagnoses by clinicians are considered very reliable by the age of 2, although most children with ASD are not diagnosed until after 4 (CDC, 2018).

There is a sizeable range of ages at which individuals receive their ASD diagnoses. The variability in age at diagnosis can affect the opportunity of individuals with ASD to receive early intensive behavioral intervention, currently the most effective intervention. Thus, there is a clear need for an intervention that remains effective at all ages.

3.Etiology

The etiology of ASD is extremely complex and relatively unexplained, despite the substantial amount of research dedicated to this topic of interest. There have been hundreds of different factors implicated in the genesis of ASD, including environmental, biological, and genetic factors (CDC, 2018). This expansive variety of known precipitators of ASD parallels the extreme heterogeneity of the disorder. The critical period for developing ASD is thought to be before, during, and immediately after birth (CDC, 2018).

Genetics

There is a strong genetic component to the genesis of autism and it has been well researched. Over 90% of ASD incidence is estimated to be attributed to genetic factors

(Amihaesei & Stefanachi, 2013). Studies have shown extraordinary heterogeneity in ASD, predicting hundreds of rare risk genes, none accounting for more than 1% of cases (Peñagarikano et al., 2015). Researchers estimate that up to 1000 genes, with a high degree of locus heterogeneity, are also implicated in ASD (Lai, Lombardo, & Baron-Cohen, 2014). Many of the risk genes regulate synaptic functions of neurons that underly learning and plasticity (Guastella and Hickie, 2016). Having a sibling with ASD increases risk of having ASD; hereditary transmission in families with individuals with ASD is approximately 30% (CDC, 2018; Amihaesei & Stefanachi, 2013). Certain genetic or chromosomal conditions such as fragile X syndrome, Down's syndrome, and tuberous sclerosis are correlated with increased incidence of ASD (CDC, 2018). Around 10% of the ASD population have comorbid genetic or chromosomal disorders (Bhat, Acharya, Adeli, Bairy, & Adeli, 2014). Rare de novo mutations (copy number variations [CNVs] in the form of microdeletion or microduplication, and nonsense, splice-site, and frame-shift mutations) have also been implicated in the genesis of ASD (Lai, Lombardo, & Baron-Cohen, 2014; Bhat, Acharya, Adeli, Bairy, & Adeli, 2014). For example, the mutation of CHD8 (chromodomain helicase DNA binding protein 8) gene is linked to the development of ASD and results in macrocephaly and wide set eyes (Bhat, Acharya, Adeli, Bairy, & Adeli, 2014). The large network of genes affected by de novo CNVs are primarily related to synaptic development, axon guidance, and neuron motility (Gilman et al., 2011; Bhat, Acharya, Adeli, Bairy, & Adeli, 2014). Both large-effect rare mutations and small-effect common variants contribute to risk of ASD (Lai, Lombardo, & Baron-Cohen, 2014). Rare mutations associated with ASD can occur in the form of Mendelian genetic syndromes (also called syndromic autism), chromosomal abnormalities, rare CNVs, and single nucleotide variants (Lai, Lombardo, &

Baron-Cohen, 2014). In simplex cases, when only one individual in the family has autism, de novo mutations are thought to be significant contributors to the genesis of ASD (Lai, Lombardo, & Baron-Cohen, 2014). Genome-wide association studies have identified many significant single nucleotide polymorphisms, but none of these have a large enough effect to be causal (Lai, Lombardo, & Baron-Cohen, 2014). Gene networks regarding neuronal function are under expressed in ASD while gene networks associated with immune function are overexpressed (Lai, Lombardo, & Baron-Cohen, 2014). Many of the genes implicated in ASD have a high degree of pleiotropy (one gene affects more than one phenotype) (Lai, Lombardo, & Baron-Cohen, 2014). The gender discrepancy in the incidence of ASD is in part explained by mutations in the X chromosome patched-related (PTCHD1) gene which result in a recessive phenotype in girls and a dominant phenotype in boys (Falco, 2014). This gene mutation has been implicated in the development of autism (Falco, 2014). Another contributor, duplications in the 15q11-13 loci are also associated with ASD (Wagner). Gene mutations can also affect the formation of cortex layers, resulting in cortical disorganization (Bhat, Acharya, Adeli, Bairy, & Adeli, 2014).

Environment

Advanced parental age, both paternal and maternal, at the time of conception is implicated in the development of ASD (Amihaesei & Stefanachi, 2013; CDC, 2018; Lai, Lombardo, & Baron-Cohen, 2014). Gestational factors, such as gestational diabetes, metabolic conditions, and obesity, also affect neurodevelopment and increase the likelihood of ASD (Amihaesei & Stefanachi, 2013; Bhat, Acharya, Adeli, Bairy, & Adeli, 2014; Buffington et al., 2016). Additional perinatal risk factors include: small birth weight, hypoxia at birth, and mercury, radiation, and diesel exhaust exposure (Amihaesei & Stefanachi, 2013;

Bhat, Acharya, Adeli, Bairy, & Adeli, 2014). The prescription drugs valproic acid and thalidomide, taken during pregnancy, also increases risk of having a child with ASD, as well as maternal viral infection (CDC, 2018; Bhat, Acharya, Adeli, Bairy, & Adeli, 2014; Belmonte et al., 2014).

Recent evidence supports the association between ASD and extensive biological systems dysregulation, the most significant being the gastrointestinal environment, immune-inflammation pathways, and nervous system (Azhari, Azizan, & Esposito, 2018; Belmonte et al., 2014).

Immune System

Ongoing neuroinflammation in various brain regions has been found in children with ASD (Rodriguez & Kern, 2011). This is supported by post-mortem brain examinations that found elevated levels of activated microglia and astrocytes and irregular, proinflammatory cytokine profiles (Rodriguez & Kern, 2011). Extended microglia activation increases the production of mediators which results in a loss of synaptic connections, underconnectivity and neuronal cell death (Rodriguez & Kern, 2011). Additionally, changes in major histocompatibility complex (MHC) expression can lead to neurodevelopmental defects as neurons in developing and adult brains express these proteins (Belmonte et al., 2014). Cerebellar Purkinje cells are a site of MHC class 1 expression and they are significantly reduced in number in ASD (Belmonte et al., 2014; Rodriguez & Kern, 2011) Decreased expression of MHC class 1 impairs the pruning of inappropriate synaptic connections, which could contribute to the increased brain volume of individuals with ASD at birth (Belmonte et al., 2014). The abnormal immune-inflammation profile of individuals with ASD contributes to the irregular organization and dysfunction of the nervous system.

Neurobiology

Atypical neural development at the systems level results in the atypical cognitive profiles found in ASD, such as impaired social cognition and perception, executive dysfunction, and atypical information processing (Lai, Lombardo, & Baron-Cohen, 2014). The brain regions involved in social perception and cognition, including the medial prefrontal cortex, superior temporal sulcus, temporoparietal junction, amygdala, and fusiform gyrus, are hypoactive in ASD (Lai, Lombardo, & Baron-Cohen, 2014). However, recent evidence supports the idea that ASD is characterized by atypical neural connectivity rather than a discrete set of abnormal brain regions (Lai, Lombardo, & Baron-Cohen, 2014). The brains of people with ASD are overdeveloped at birth as evidenced by increases in neuronal count and synapses in key cortical zones, and unbalanced functioning of the neuronal excitatory, versus inhibitory, networks (Amihaesei & Stefanachi, 2013). Increased brain volume at birth is further specified as an excessive volume of cerebrum and cerebral white matter with the greatest degree of enlargement in the frontal lobes and the least in the occipital lobes (Belmonte et al., 2014). Abnormal early neurodevelopment includes early postnatal brain overgrowth and subsequently stunted growth in both white and grey matter, disorganization of cortical layers affecting both horizontal laminar compartments and vertical columnar structure, and reduced functional and anatomical connectivity (Belmonte et al., 2014, Nair et al., 2013). Aberrant organization and decreased coherence are a product of differences in white matter microstructure, in both white matter tracts and the superior temporal gyrus, in individuals with ASD (Mišić et al., 2015; Anderson et al., 2011). Abnormalities in synaptic and columnar structure and neuronal migration, are also found in the cerebral cortexes of ASD subjects (Bhat, Acharya, Adeli, Bairy, & Adeli, 2014). The

cortical areas that are essential to complex cognitive functions are the most affected and ASD also affects the temporal organization of these areas which results in impaired cognitive set shifting (Belmonte et al., 2014; Mišić et al., 2015). Cerebellar activation of ASD subjects during cognitive tasks reflects the opposite of controls (Belmonte et al., 2014). There is also increased cortical thinning in the frontal lobe, parietal lobe, occipital lobe, and cortex in ASD (Zielinski et al., 2014). The functioning of the neural network involved in social and emotional processing, including mirror neurons, is reduced in ASD and there are disruptions in cortical response to dynamic social stimuli (Amihaesei & Stefanachi, 2013; Bhat, Acharya, Adeli, Bairy, & Adeli, 2014).

Functional connectivity is significantly altered in ASD subjects and is represented in Figure 1 (Amihaesei & Stefanachi, 2013; Rodriguez & Kern, 2011; Tyszka, Adolphs, Paul, & Kennedy, 2013).
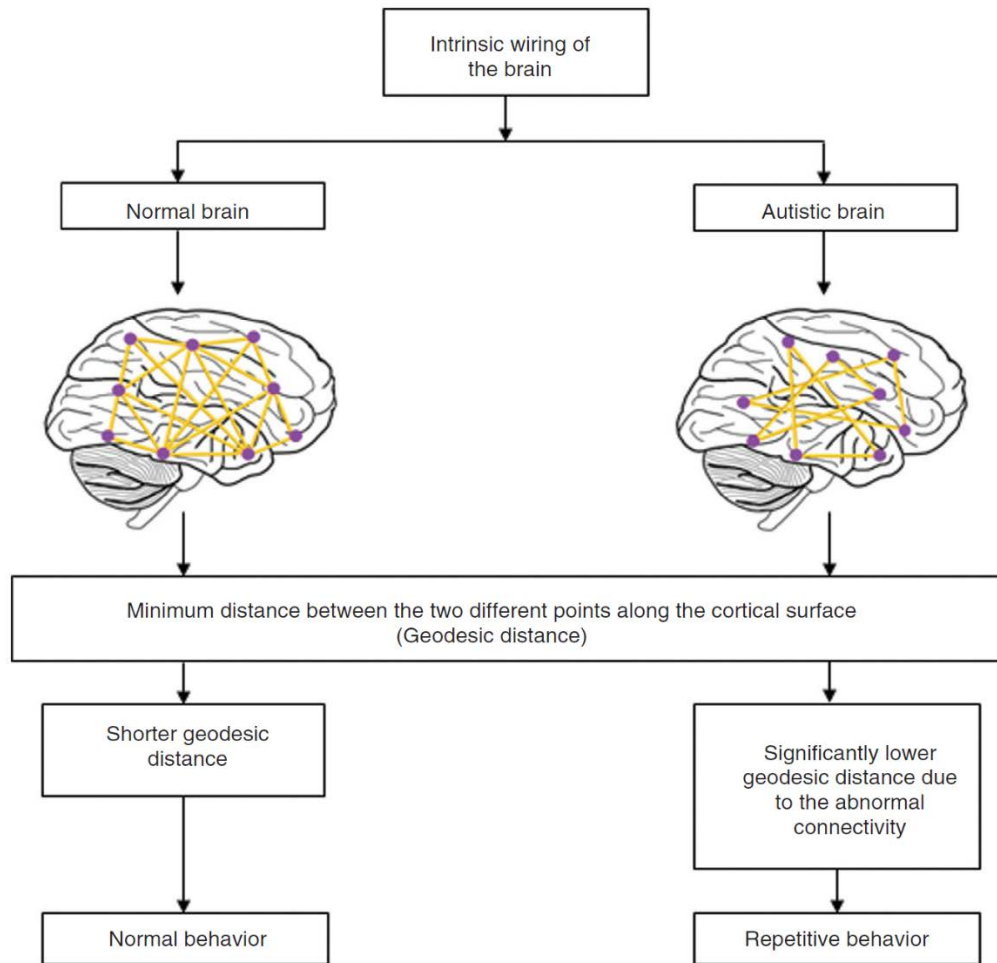
Figure 1. The atypical functional connectivity of an autistic brain compared to a neurotypical brain. (Bhat, Acharya, Adeli, Bairy, & Adeli, 2014)

The degree of connectivity abnormality is correlated with the severity of ASD symptomology (Rodriguez & Kern, 2011). Functional and anatomical connectivity between the cerebral cortex and thalamus shows bilateral impairment in ASD as well as between the frontal lobe and other cortical regions (Nair et al., 2013; Amihaesei & Stefanachi, 2013; Bhat, Acharya, Adeli, Bairy, & Adeli, 2014). Mišić et al. found that long-range functional connectivity is reduced in ASD subjects (Mišić et al., 2015). High local connectivity is another prominent feature of ASD, prominent in the cerebellum (Anderson et al., 2011;

Belmonte et al., 2014; Bhat, Acharya, Adeli, Bairy, & Adeli, 2014). Together, the high local connectivity and low long-range connectivity result in atypical information processing (Anderson et al., 2011; Bhat, Acharya, Adeli, Bairy, & Adeli, 2014; Mišić et al., 2015). Interhemispheric correlation is also significantly reduced in regions with functional relevance to ASD, but the largest difference was found in the anterior frontal insula which is a core component of social processing networks (Anderson, Rodriguez & Kern, 2011). Dinstein et al., found that toddlers with ASD had weak interhemispheric neural synchronization (Dinstein et al., 2011). Verbal ability was positively correlated with strength of synchronization while severity of ASD was negatively correlated (Dinstein et al., 2011). The corpus callosum is also a site of significant abnormality in ASD subjects as its size is decreased, resulting in decreased interhemispheric connection (Anderson et al., 2011; Rodriguez & Kern, 2011).

4.Available Treatments

Through intervention and support, an individual's functional independence and quality of life can be maximized through learning and development, improved social skills and communication, and reductions in disability and comorbidity (Lai, Lombardo, & Baron-Cohen, 2014). The most commonly used forms of therapy for ASD are Applied Behavior Analysis (ABA), developmental models, structured learning techniques, speech therapy, social skills therapy, and occupational therapy (Amihaesei & Stefanachi, 2013). Early intensive behavioral interventions that are targeted and comprehensive can improve social communication skills and reduce anxiety and aggression (Lai, Lombardo, & Baron-Cohen, 2014). Prescription medications can also be used in the treatment of ASD. The most frequently prescribed medications are antidepressants, stimulatory drugs/psychotropes, and

antipsychotics (Amihaesei & Stefanachi, 2013). However, none of these drugs target core ASD symptoms and instead treat the co-morbidities of ASD such as hyperactivity, anxiety, and self-stimulatory behaviors (Wagner & Harony-Nicolas, 2018). Antipsychotic drugs have been effective in reducing repetitive and aggressive behaviors in children with ASD (Lai, Lombardo, & Baron-Cohen, 2014). Risperidone, an atypical antipsychotic, is the most widely prescribed treatment for ASD although it only reduces hyperactivity, aggressive, self-injurious, and repetitive behaviors and does not improve social deficits (Guastella and Hickie, 2016). Risperidone has also been associated with significant side effects including weight gain, drowsiness, extrapyramidal side effects, and hormonal changes related to galactorrhea, amenorrhea, and gynecomastia (Guastella and Hickie, 2016). To date, no biomedical agents have been shown to reliably improve social deficits (Lai, Lombardo, & Baron-Cohen, 2014). Some unconventional therapies used in the treatment of ASD are: acupuncture, antifungal therapy, art therapy, the Early Start Denver model, therapy with dolphins, aerobics, interactive computer programs, facilitated communication, music therapy, contact therapy, homeopathy, neuro-feedback, rhythms therapy, and yoga (Amihaesei & Stefanachi, 2013). Despite their popularity and significant expense, these treatments have little evidence to support their efficacy and are not specified for a subtype of ASD (Guastella and Hickie, 2016). All available treatments are either lengthy, expensive, or time-sensitive and do not alleviate core symptoms of ASD, highlighting the need for effective and accessible interventions that address the core symptoms of ASD.

5.Animal Models of ASD

To establish effective evidence-based interventions for ASD, successful interventions in animal models of ASD must be examined. Two common and validated animal models of

ASD are the Maternal Immune Activation (MIA) model and the Maternal High Fat Diet (MHFD) model, used in the recent evidence to support probiotic intervention.

Maternal Immune Activation Model

The MIA mouse model is based on large epidemiological studies that linked maternal infection to increased incidence of autism in offspring (Atladóttir et al., 2010). This model is also supported by studies linking ASD risk to familial autoimmune disease and elevated levels of inflammatory factors in the maternal blood, placenta, and amniotic fluid (Atladóttir et al., 2010; Comi et al., 1999; Abdallah et al., 2013; Brown et al., 2004; Croen et al, 2008). Stimulating maternal immune activation in mice triggers global changes in the gut microbiome of offspring which is correlated with abnormal behavior, neuropathologies, immune dysfunction, and GI impairment (Vuong & Hsiao, 2017). MIA in mice is stimulated by injecting pregnant mice with the viral mimic poly (I:C) and results in offspring that express core behavioral symptoms and neuropathologies of ASD (Malkova et al., 2012). The offspring exhibit dysbiosis of gut microbiota, prominent in alterations of the bacterial classes Clostridia and Bacteroidetes (Hsiao et al., 2013). The MIA offspring display ASD-like behaviors, impaired intestinal integrity, and altered gut microbiome profiles (Hsiao et al., 2013). These symptoms are comparable to similar endophenotypes found in subsets of individuals with ASD (Hsiao et al., 2013).

Maternal High Fat Diet Model

The MHFD model is based on epidemiological studies that support a link between maternal obesity and increased risk of neurodevelopmental disorders, such as ASD in offspring (Connolly et al, 2016; Krakowiak et al., 2012; Sullivan). The MHFD triggers

abnormal behavior in offspring and is mediated by alterations in the gut microbiome of the offspring (Buffington et al., 2016). Buffington et al. found that the MHFD-induced changes in the gut microbiome of offspring block long-lasting neural adaptation in the mesolimbic dopamine reward system, specifically in the ventral tegmental area (VTA) (Buffington et al., 2016). These neuronal adaptations enhance the salience of social stimuli and the MHFD-induced alterations in these neural networks results in social behavioral deficits (Buffington et al., 2016). In comparison to offspring from mice fed a regular fat diet, MHFD offspring had fewer reciprocal social interactions, impaired sociability, and lack of preference for social novelty (Buffington et al., 2016). These offspring also exhibit repetitive behaviors and anxiety, symptoms that are also associated with ASD, as well as fewer oxytocin immunoreactive neurons in the hypothalamus (Buffington et al., 2016).

Utilizing these two validated animal models of ASD, two strains of probiotics have been shown to successfully ameliorate behavioral and social deficits in affected offspring: *Bacteroides fragilis* and *Lactobacillus reuteri*.

III. The Current State of Non-Pharmaceutical Interventions

Complementary and Alternative Medicine

Complementary and alternative medicine (CAM) is incredibly popular among families with children with ASD (Akins, Angkustsiri, & Hansen, 2010). Examining non-pharmaceutical interventions is imperative as significant adverse side effects have been associated with and increased by some conventional psychiatric medications used in children with ASD (Akins, Angkustsiri, & Hansen, 2010). Additionally, families are often inclined to

search for more progressive and less expensive interventions, especially outside of the medical field. Geographic and economic barriers can limit access to high-quality behavioral and educational interventions, leaving families to find alternative forms of intervention (Akins, Angkustsiri, & Hansen, 2010). The National Center for Complementary and Alternative Medicine (NCCAM, 2013) defines CAM as "a group of diverse medical and health care systems, practices, and products that are not generally considered part of conventional medicine" (NCCAM, 2013). Complementary medicine is typically defined as nontraditional treatments used in conjunction with conventional medicine, such using light therapy to treat seasonal affective disorder in tandem with antidepressants (Akins). Alternative medicine is defined as being used in place of conventional medicine, such as using melatonin instead of sedatives to treat insomnia (Akins, Angkustsiri, & Hansen, 2010). The American Academy of Pediatrics defines integrative medicine as "relationship-based care that combines mainstream and complementary therapies for which there is some high-quality scientific evidence of safety and effectiveness to promote health for the whole person in the context of his or her family and community" (Kemper, Vohra, & Walls, n.d.). The American Academy of Pediatrics also recommends the discussion of CAM with the family of every ASD patient (Akins, Angkustsiri, & Hansen, 2010). CAM usage in children with ASD is amongst the highest of any population with reported use between 52% and 95% in families (Akins, Angkustsiri, & Hansen, 2010). Of families who have children with ASD and use CAM treatments, approximately 50-70% choose a biologically based CAM treatment (Akins, Angkustsiri, & Hansen, 2010). Parents of children with ASD reported current use of an average of four treatment modalities and 80% reported some form of dietary intervention (Lange, Hauser, & Reissmann, 2015). Qualitative studies have found that receiving outdated

information for conventional systems of care, limited provider knowledge of their child's condition, parental frustration with discouraging prognoses, and attempts to construct an alternative identity for their children and themselves increase CAM usage by parents of children with disabilities (Akins, Angkustsiri, & Hansen, 2010). These findings highlight the desire for families to gain more control over medical decision-making (Akins, Angkustsiri, & Hansen, 2010). This desire coupled with geographic and economic barriers create a need for effective non-pharmaceutical interventions in the treatment of ASD.

With the rise of the internet, online ASD support communities have become popular, allowing parents to engage with other parents facing similar struggles and ASD-specific information (Akins, Angkustsiri, & Hansen, 2010). However, internet usage also increases families' exposure to targeted marketing, testimonials, and unproven claims that could look promising in the treatment of ASD (Akins, Angkustsiri, & Hansen, 2010). These ineffective treatments exploit desperate parents and can even be dangerous. Some examples of dubious popular treatments with negative side effects are: chelation therapy, antifungal agents, hyperbaric oxygen therapy, and immune therapies including intravenous and oral Ig (Akins, Angkustsiri, & Hansen, 2010). To date, chelation therapy, when used to treat symptoms of ASD, has been linked with several deaths (Akins, Angkustsiri, & Hansen, 2010). There are also many other purported treatments that are considered generally safe, but their efficacy is unknown or even disproven such as use of multivitamins, secretin, and various amino acid therapies (Akins, Angkustsiri, & Hansen, 2010).

Families of individuals with ASD are often searching for more effective alternatives to pharmaceuticals. The high use of CAM shows that families are eager to do all they can and desire to have more control over their situation. Additionally, some families cannot afford

expensive treatments or are unable to access them, which also leads to higher CAM usage. Instead of trying to suppress this phenomenon, researchers must try to meet the needs of these families by creating an accessible and affordable alternative to pharmaceuticals or lengthy behavioral therapy sessions. Due to increasing misinformation, the families of ASD individuals are susceptible to making poor medical choices for their child, despite their best intentions. Thus, the need for evidence-based intervention is imperative.

Efficacy of Gluten Free and Casein Free Diets

A product of misinformation, the gluten free and casein free diet (GFCF) has become one of the most popular CAM treatments in children with ASD (Akins, Angkustsiri, & Hansen, 2010). The GFCF diet is utilized in the treatment of ASD in 29% of families that use dietary interventions; approximately 80% of families with ASD individuals (Lange, Hauser, & Reissmann, 2015). The rationale for this diet remains unproven and is based on the "opioid excess" theory which claims that individuals with ASD have impaired ability to break down dietary proteins in gluten and casein, and that this results in the formation of opioid-like peptides that cross the blood brain barrier (BBB) and contribute to the neurobehavioral symptoms of autism (Akins, Angkustsiri, & Hansen, 2010). Parents have claimed that their child's GI symptoms (54%), concentration and attention (42%), communication (29%), and social interaction (25%) improved after implementation of the GFCF diet (Lange, Hauser, & Reissmann, 2015). However, it is important to address the measurement bias within these findings as the parents were not blinded to the treatment and are likely hoping for their child's symptoms to improve, thus influencing their observations. The GFCF diet can be difficult to implement as families face challenges such as increased food preparation time, increased food-related expenses, and children refusing to eat the dietary selections (Akins,

Angkustsiri, & Hansen, 2010). Additionally, further dietary restriction in a child with an already limited food repertoire can induce negative behavioral and biological side effects (Akins, Angkustsiri, & Hansen, 2010). In a study by Ghalichi et al., they showed that there may be some potential of the GF diet to alleviate stereotyped behaviors and improve social communication and interaction in some children with ASD (Ghalichi, Ghaemmaghami, Malek, & Ostadrahimi, 2016). However, it is important to note that, once again, the parents of the patients, who rated their child's symptoms, were not blinded to the intervention and thus could have been influenced by their awareness (Ghalichi, Ghaemmaghami, Malek, & Ostadrahimi, 2016). Lange et al. did an in-depth meta-analysis of studies about the intervention of GFCF diets on ASD symptoms (Lange, Hauser, & Reissmann, 2015). They evaluated eight published case studies that included anecdotal case reports that attempted to establish a casual role of gluten and casein in the pathology of ASD (Lange, Hauser, & Reissmann, 2015). Lange et al. found that none of these studies used an appropriate experimental control (Lange, Hauser, & Reissmann, 2015). It was also noted that all but two studies found evidence of positive dietary effects of the GFCF diet for at least some of the measures assessed and the two studies with null results were the case studies that met the largest number of quality indicators of experimental validity (Lange, Hauser, & Reissmann, 2015). Lange et al, states that none of the case studies were conduced with adequate scientific rigor and thus, the results of the studies can only be regarded as weak evidence at best (Lange, Hauser, & Reissmann, 2015). Common issues included measurement bias, as a result of relying on the subjective ratings of parents not blinded to the treatment, and lack of appropriate control groups (Lange, Hauser, & Reissmann, 2015). Although the GFCF diet is popular and there are a multitude of positive reviews from parents regarding its effects, most

scientific studies have failed to confirm significant therapeutic effects (Lange, Hauser, & Reissmann, 2015). It has been shown that there is an association between the casein-free diet and bone loss in children, an alarming side effect that must be acknowledged with regard to parental desire to implement the GFCF diet (Hediger et al., 2008). However, there may be a possibility that there are specific subtypes of autism (possibly due to different genesis) that may be sensitive and responsive to such dietary elements (Whiteley, 2017). As a concluding thought, Lange et al., suggests that a GFCF diet should only be administered if an allergy or intolerance to nutritional gluten or casein is diagnosed and present in the child with ASD (Lange, Hauser, & Reissmann, 2015).

With the widespread prevalence of misinformation and the susceptibility of desperate parents who want to help their children with ASD, evidence-based non-pharmaceutical intervention is an incredibly important area of interest as it may help parents overcome geographic and economic barriers and satisfy their desire for control and self-determination. Additionally, an intervention based on strong evidence may prevent families from enduring the financial and biological repercussions of implementing popular dietary interventions, such as the GFCF diet, that have yet to be scientifically proven as effective.

IV. Probiotics of Interest

The two probiotic strains of interest in this proposed treatment of ASD are *Bacteroides fragilis* and *Lactobacillus reuteri*.

1.*Bacteroides Fragilis*

Previously shown to correct colitis in infants, *B. fragilis* gained interest as a potential probiotic intervention for individuals with ASD (Hsiao et al., 2013). In a groundbreaking

experiment conducted by Hsiao et al, *B. fragilis* was discovered to correct gut permeability,

alter microbial composition, and ameliorate deficits in communicative, stereotypic, anxiety-

like, and sensorimotor behaviors in the offspring of MIA mice, a reliable animal model of

ASD (Hsiao et al., 2013). The MIA offspring exhibited altered serum metabolites of gut

origin that were normalized by *B. fragilis* treatment (Hsiao et al., 2013). Two of these

metabolites, 4EPS and indolepyruvate, are potentially associated with ASD (Hsiao et al.,

2013). Intestinal epithelial hyperpermeability, as well as altered levels of tight junction

proteins and cytokines, were also corrected by *B. fragilis* treatment. Hsiao et al. suggests that

B. fragilis is able to correct leaky gut by directly targeting tight junction expression, cytokine

production, and/or microbiome composition (Hsiao et al., 2013). This is consistent with the

role of gut microbiota in regulating metabolic homeostasis and intestinal permeability (Hsiao

et al., 2013). However, despite the improvements in communicative, repetitive, anxiety-like,

and sensorimotor behaviors, *B. fragilis* treatment fails to ameliorate deficits in sociability and

social preference (Hsiao et al., 2013).

2.*Lactobacillus Reuteri*

In a revolutionary and replicated study, Buffington et al. and Sgritta et al. found that

oral treatment with *L. reuteri* corrected oxytocin levels and synaptic dysfunction in the VTA

of MHFD offspring, a validated animal model for ASD (Buffington et al., 2016; Sgritta et al.,

2019). Buffington et al. was the first to complete this study and Sgritta et al. replicated this

study and examined possible mechanisms. Treatment with *L. reuteri* selectively ameliorates

social deficits in genetic, environmental, and idiopathic ASD models (Sgritta et al., 2019).

MHFD offspring who received *L. reuteri* treatment showed increases in reciprocal social

interactions, sociability, and preference for social novelty (Buffington et al., 2016). Only

social deficits are ameliorated with *L. reuteri* treatment, no other behavioral endophenotypes associated with ASD were affected (Buffington et al., 2016). Sgritta et al. also found that treatment with *L. reuteri* corrects social deficits in several ASD mouse models (Sgritta et al., 2019). The method in which *L. reuteri* alleviates social deficits was discovered to be via the vagus nerve (Sgritta et al., 2019). A vagotomy prevented amelioration of social deficits despite *L. reuteri* treatment (Sgritta et al., 2019). Vagal nerve fibers project to the paraventricular nuclei (PVN), where oxytocin is produced, and subdiaphragmatic vagotomy blocks this neural activity in the PVN (Sgritta et al., 2019). Additionally, Sgritta et al. revealed that the effects of *L. reuteri* are not mediated by the restoration of the gut microbiome as monocolonization in germ-free mice still successfully restored social behaviors (Sgritta et al., 2019). Thus, *L. reuteri* treatment ameliorates social deficits and related changes in synaptic function within the social reward neural circuits, in a vagus nerve and oxytocin dependent manner (Sgritta et al., 2019).

The success of these probiotic interventions is contributable to two biological systems linked to ASD pathology; the gastrointestinal system and the oxytocin system. To understand the efficacy of *B. fragilis* in reducing repetitive behaviors, the relationship between ASD and the GI system needs to be analyzed. Similarly, the oxytocin system must be addressed to understand the efficacy of *L. reuteri* in ameliorating social deficits.

V. ASD and the GI system

An extensive study of over 14,000 individuals with ASD showed a higher prevalence of irritable bowel syndrome (IBD) and other GI disorders in ASD subjects compared to

controls (Kohane et al., 2012). The most reported GI symptoms in children with ASD are "any GI symptom/aggregate of symptoms" (46.8%), constipation (22.0%), chronic diarrhea (16.2%), and abdominal pain (14.0%) (Holingue et al., 2018). A study from the Childhood Autism Risks from Genetics and Environment (CHARGE) revealed that frequency of GI symptoms was associated with greater social withdrawal, stereotypy, irritability, and hyperactivity (Vuong & Hsiao, 2017). Additional GI abnormalities associated with ASD include altered gut microbiome composition, overproduction of bacterial metabolites, and increased GI mucosa permeability (Azhari, Azizan, & Esposito, 2018). Adams et al. found that ASD symptom severity is positively correlated with severity of GI dysfunction, a link supported by various studies (Adams et al., 2011; Tomova et al., 2015; Yang et al., 2018).

The bidirectional relationship between the brain and the gut, also referred to as the gut-brain axis, can influence development, neurochemistry, gene expression, and brain function (Tomova et al., 2015). The gut microbiome, the most significant part of the GI system, is comprised of 500-1000 denizen species representing 7,000-40,000 different strains spanning 1800 genera and total to approximately $1x10^{13}$-$1x10^{14}$ microorganisms (Rosenfeld, 2015). The gut microbiota is essential for digestion as it synthesizes various vitamins and cofactors, and metabolizes complex lipids, proteins, and carbohydrates, even those that are indigestible by the host (Rosenfeld, 2015).

Subjects with ASD have been repeatedly reported to have significant differences in species richness and diversity, across phylum and species with a marked decrease in bacterial diversity (Sharon, Sampson, Geschwind, & Mazmanian, 2016; Tomova et al., 2015; Kang et al., 2017). A proposed explanation for this phenomenon is that many children with ASD undergo increased oral antibiotic treatment during the first 3 years of life, which could

destabilize their gut microbiome and create opportunities for competitive potential pathogens

to contribute to ASD severity (Kang et al., 2017). The gut microbiome becomes stable

between 6 and 36 months of life, thus the use of antibiotics could disrupt this critical process

(Mangiola et al., 2016). Children with ASD exhibit alteration in the Bacteroidetes/Firmicutes

ratio, even in comparison to neurotypical children with GI problems (Tomova et al., 2015).

Elevated levels of Clostridia, Desulfovibrio, Sutterella, Bacteriodetes, Lactobacillus,

Prevotella, Ruminococcus, and Alcaligenaceae have been found in the gut microbiomes of

ASD subjects (Tomova et al., 2015; Sharon, Sampson, Geschwind, & Mazmanian, 2016;

Rosenfeld, 2015; Mangiola et al., 2016). There is a very strong correlation between levels of

Desulfovibrio with the severity of ASD symptoms (Tomova et al., 2015). High levels of

Clostridia correlated with increased GI problems, within the ASD group (Ding, Taur, &

Walkup, 2017).

Gut dysbiosis can impact host immunity and neurobehavioral responses (Rosenfeld,

2015; Tomova et al., 2015). The gut microbiome directly and indirectly affects the intestinal

epithelium which, through the local mucosal immune system and enteric nervous system,

affects neuronal pathways from the gut to the brain (Tomova et al., 2015). Neuro-active

neurotransmitters, such as GABA, and short chain fatty acids (SCFA) are synthesized by the

gut microbiome and, through interactions upon the HPA (hypothalamic-pituitary-adrenal)

axis, can alter cognition and mood (Tomova et al., 2015). It has been suggested that

alterations in the gut microbiome could influence long-lasting changes in synaptic efficacy in

the mesolimbic dopamine reward system underlying social behaviors (Sgritta et al., 2019).

The epithelial gut barrier is critical to proper function as it controls the flow of

molecules between the GI tract and bloodstream and is maintained by tight junctions (Ding,

Taur, & Walkup, 2017). These junctions can be affected by gut microbiota and their ligands and can result in compromised integrity of the epithelial barrier, termed "leaky gut" (Ding, Taur, & Walkup, 2017). Increased intestinal permeability can be problematic for the host as it may allow for passage of bacteria, toxins, and metabolites into the bloodstream (Ding, Taur, & Walkup, 2017). Increased bacterial translocation and direct measurements show increased intestinal permeability in individuals with ASD (Whiteley, 2017; Hsiao et al., 2013). In addition, altered levels of SCFAs, produced by gut microbiota, are capable of passing through the BBB and have been noted in individuals with ASD (Azhari, Azizan, & Esposito, 2018). Increased levels of SCFAs are notable because they can impact CNS function via changes in neurotransmitter synthesis and release, mitochondrial function, immune activation, lipid metabolism, and gene expression (Ding, Taur, & Walkup, 2017). These neuroactive compounds have the ability to alter behavior once they pass through the BBB and are in part, responsible for the abnormal behaviors prevalent in ASD. The bidirectional relationship between the gut microbiome and the brain that allows for GI impairments to result in abnormal behavior is depicted in Figure 2.
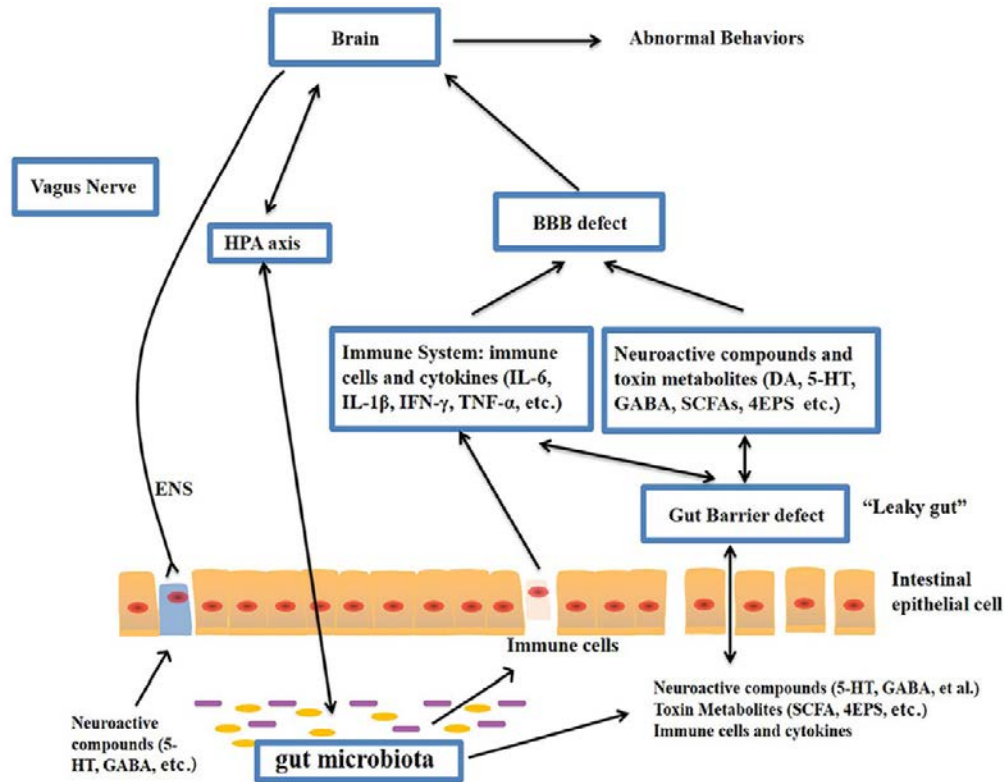
Figure 2. The bidirectional relationship between the gut microbiome and the brain in the pathology of ASD (Li, Han, Dy, & Hagerman, 2017).

VI. The Role of Oxytocin in ASD

Oxytocin is a nine-amino-acid neuropeptide produced in the hypothalamus with well-established neuroendocrine functions and remarkable influence over prosocial behavior (Young & Barrett, 2015; Jones et al., 2017; Parker et al., 2017; Peñagarikano et al., 2015). Oxytocin binds to four receptors: OXTR, V1AR, V1BR, and V2R though its prosocial effects are associated with OXTR and V1AR (Parker et al., 2017). Oxytocin influences social cognition, social behavior, fear conditioning, social attachment, pair bonding, and aggression (Jones et al., 2017; Yamasue & Domes, 2018). Oxytocin expressing neurons in the PVN

project to brain regions including the amygdala, hippocampus, and frontal cortex, that are important in behaviors such as fear, memory, sociability, and attention (Peñagarikano et al., 2015). Oxytocin increases the salience of social stimuli by activating VTA neurons and has anxiolytic effects (Young & Barrett, 2015; Buffington et al., 2016; Jones et al., 2017). Altered genotypes in oxytocin receptor genes have been associated with ASD symptomology (Yamasue & Domes, 2018). In neurotypical subjects, there is a gradual shift of GABA action from excitatory to inhibitory during development, termed "the GABA switch," that when interrupted, leads to ASD symptomology in animal models (Wagner & Harony-Nicolas, 2018).

Numerous studies regarding intranasal oxytocin intervention in ASD have been completed. A replicated finding, intranasal administration of oxytocin temporarily enhances social cognition, empathy, and reciprocity in individuals with ASD and increase social behaviors such as eye gaze, feelings of rest, and recognition of affective speech (Young & Barrett, 2015; Jones et al., 2017). Response to oxytocin treatment could be predicted by pretreatment blood oxytocin levels, which suggests that a specific subset of the ASD population could be more susceptible to improvements (Parker et al., 2017). The prosocial effects of oxytocin have also been well-documented in animal models of ASD (Teng et al., 2016; Peñagarikano et al., 2015; Wagner & Harony-Nicolas, 2018).

It is widely accepted that oxytocin administration is well-tolerated in humans and in ASD populations specifically (Cai, Feng, & Yap, 2018; Parker et al., 2017). However, possible adverse effects have been noted, such as altered sexual development, anaphylactic shock, arrhythmia, nausea, and vomiting (Bales et al., 2013; Cai, Feng, & Yap, 2018). The preferred method of targeting peptides to the brain is through intranasal administration, as it

is less invasive of a procedure (Peñagarikano et al., 2015). Guastella and Hickie, noted that

children with poor verbal communication have difficulty tolerating nasal sprays, in their

unpublished data (Guastella and Hickie, 2016). Nasal discomfort, tiredness, irritability,

diarrhea, and skin irritation were the most common adverse reactions in children with ASD

using the intranasal method of oxytocin administration (Cai, Feng, & Yap, 2018). Still, there

are many discrepancies that need to be dealt with such as optimization of administration

route, dose, and treatment duration (Yamasue & Domes, 2018).

VII. The Current Study

Both *B. fragilis* and *L. reuteri*, bacterial strains present in the gut microbiome,

improve core symptoms of ASD (Hsiao et al., 2013; Buffington et al., 2016; Sgritta et al.,

2019). *B. fragilis* improves only restrictive and repetitive behaviors while *L. reuteri* improves

only social deficits (Hsiao et al., 2013; Buffington et al., 2016; Sgritta et al., 2019). With a

combination of the two probiotic strains, both repetitive behaviors and social deficits could

be targeted. I propose that probiotic intervention with *B. fragilis* will decrease restrictive and

repetitive behaviors in individuals with ASD. Similarly, I also expect that probiotic

intervention with *L. reuteri* will improve social deficits in individuals with ASD. I

extrapolate that, due to additive interaction effects, probiotic intervention with these two

bacterial strains will ameliorate both restrictive and repetitive behaviors and social deficits.

VIII. Method

Participants

The participants will be children from the ages of 5 to 13 that have been clinically diagnosed with ASD and have no other neurological comorbidities.

Materials

Bacteroides fragilis in capsule form, *Lactobacillus reuteri* in capsule form, placebo capsules

Measures

Repetitive and Restrictive Behavior

Repetitive and restrictive behavior will be measured by the Repetitive Behavior Scale – Revised (RBS-R). This scale measures the severity of repetitive behaviors in individuals with ASD. The 43 items are organized into six subscales: stereotyped behavior, self-injurious behavior, compulsive behavior, ritualistic behavior, sameness behavior, and restricted behavior (Scahill et al., 2015). The items are scored on a four-point scale from zero to three (0=never, 1=mild, 2=moderate, 3=severe) (Scahill et al., 2015). I propose to use the RBS-R total score, out of 129, to measure repetitive and restricted behavior.

Social Deficits

Social ability, and consequently social deficits, will be measured by the Social Responsiveness Scale (SRS-2). This scale assesses the individual's ability to engage in appropriate reciprocal social interaction and communication (Frazier et al., 2014). The SRS-2 is comprised of 5 subscales: social awareness (assesses an individual's ability to recognize

social cues of others), social cognition (assesses interpretation of social behavior), social

communication (assesses reciprocal communication in social situations), and autistic

mannerisms (assesses stereotypy and restrictive interests) (Frazier et al., 2014; Bruni, 2014).

Items are scored on a scale from one (never) to four (almost always). The results of the SRS-

2 are reported in T-score format (M=50, SD=10) (Bruni, 2014). The SRS-2 total score is the

most reliable form of assessing social deficits in individuals with autism (Bruni, 2014). Thus,

I will use this scale to quantify social deficit in ASD individuals in the current study.

Procedures

There will be four experimental groups. The first is the control group who will take

two placebo capsules. The second group will take one capsule of *B. fragilis* and one placebo

capsule. The third group will take one capsule of *L. reuteri* and one placebo capsule. The

fourth group will take one capsule of *B. fragilis* and one capsule of *L. reuteri*. All groups will

take the capsules orally, once a day with breakfast. Caretakers will assess the participants

using the RBS-R and the SRS-2 scales three times a week. The children will also participate

in an academic medical center-based program for families with ASD that focuses on

integrations of interventions, clinical trials, and childcare. The children will visit the center

weekly, allowing blinded researchers to assess the participants using the RBS-R and SRS-2

scales. The study will proceed for the duration of a year, allowing for short term and long-

term observations to be made.

Ethics

As this study involves human participants, the study must be approved by the

Institutional Review Board at Scripps College in accordance with the currently applicable

U.S. Public Health Service Guidelines. Additionally, this study involves children with cognitive impairment and they are considered a protected and vulnerable population. Thus, informed consent will need to be provided by the legal guardians of the children. The participants must be children with ASD as that is the target population of this intervention and its efficacy must be examined in the ASD population. Furthermore, most treatment interventions for ASD occur in childhood, underscoring the importance of evaluating the efficacy of this probiotic intervention in children with ASD.

IX. Expected Results

Those in the control group will see no improvement in repetitive behavior or social deficits. Those receiving only *B. fragilis* will see improvements in repetitive behavior but not in social deficits. Those receiving only *L. reuteri* will see improvements in social deficits but not in repetitive behavior. Those taking both strains will see improvements in both repetitive behavior and social deficits.

The mean RBS-R score for individuals with autism is 33.14 (Lam). All experimental groups will begin the intervention with mean RBS-R scores of 33.14. Those receiving the placebo or *L. reuteri* interventions will not see an improvement in repetitive behaviors and their RBS-R scores will not be significantly altered. The placebo intervention group will end with a mean RBS-R score of 33.67 and the *L. reuteri* intervention group will end with a mean RBS-R score of 34.59. Those receiving the *B. fragilis* or both probiotic strains intervention will see a reduction of repetitive behaviors, manifesting in lower mean RBS-R scores. The *B. fragilis* intervention group will end with a mean RBS-R score of 15.47 and the intervention

with both probiotic strains will end with a mean RBS-R score of 16.13. These expected
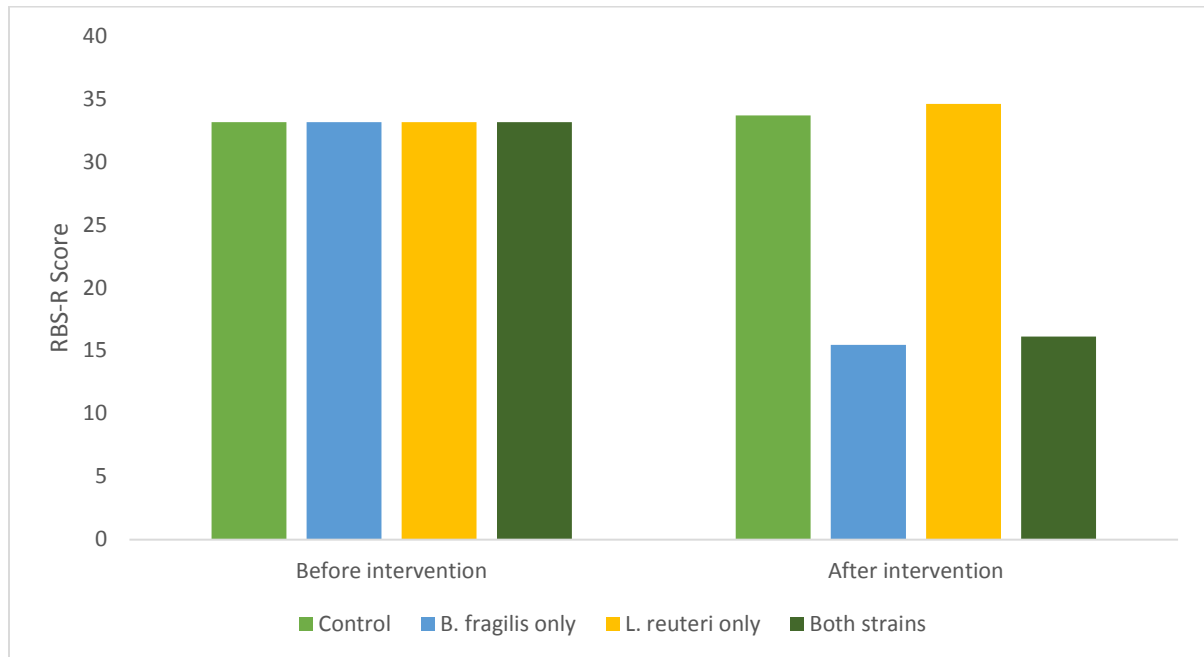
scores are represented in Figure 3.

Figure 3. Change in RBS-R scores after differing interventions.

SRS-2 T-scores are correlated with severe, moderate, and mild social deficits. Severe

and clinically significant social deficits are indicated by T-scores 76 or higher (Bruni, 2014).

The moderate range of T-scores falls between 66 and 75 and demonstrates some clinically

significant social deficits (Bruni, 2014). Scores between 60 to 65 indicate mild social

impairments (Bruni, 2014).

The mean SRS-2 T-score of all participants before intervention will be 77. Those

receiving the placebo intervention will have no significant reduction in mean SRS-2 T-scores

and will end the intervention with a T-score of 76. Those receiving the *B. fragilis*

intervention will also see no improvement in social deficits, manifesting in a final SRS-2 T-

score of 77. The individuals receiving the *L. reuteri* intervention will see significant

improvement in social deficits and end with a significantly lower SRS-2 T-score of 62. The

individuals receiving the intervention with both probiotic strains will also see a significantly

lower mean SRS-2 T-score of 63 as well as improvements in social deficits. Thus, the

placebo and *B. fragilis* interventions will not affect social deficits but the *L. reuteri* and

combined strains intervention will improve social deficits. These expected scores are
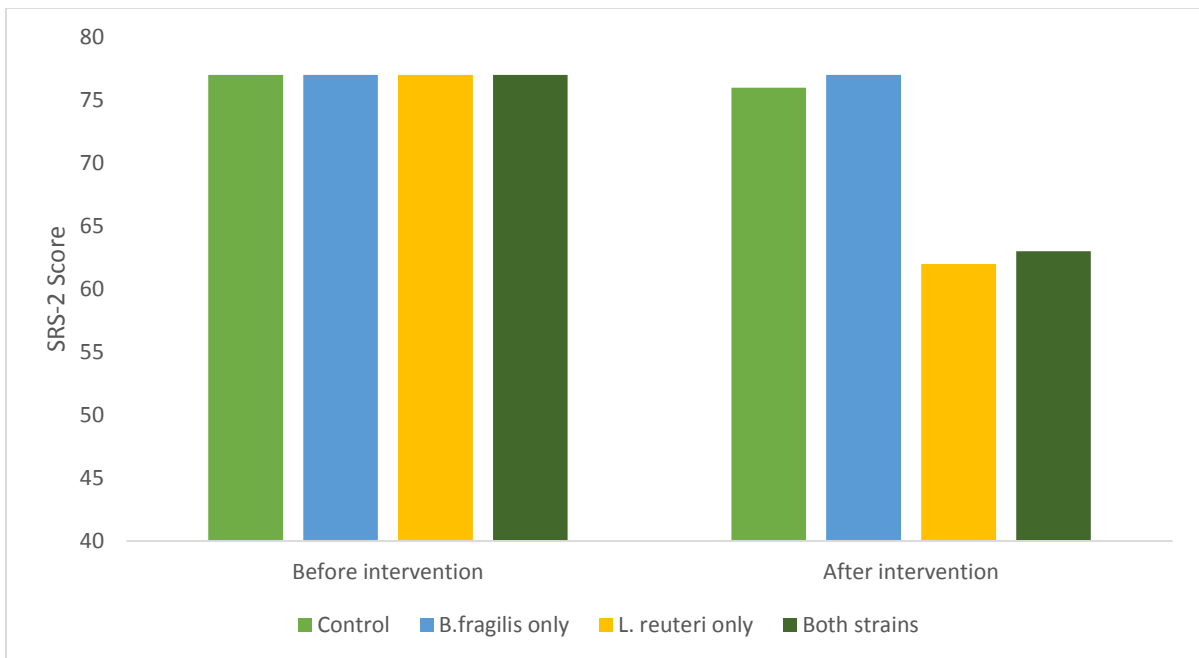
represented in Figure 4.

Figure 4. Change in SRS-2 scores after differing interventions.

X. Discussion

Implications

If probiotic intervention with *B. fragilis* and *L. reuteri* is found to be viable and

effective, it would significantly affect the lives of individuals with ASD and their families.

This would become the first intervention to target the core symptoms of ASD, repetitive behavior and social deficits. Furthermore, it could substantially decrease the economic burden associated with ASD. The estimated total costs per year for children with ASD falls between $11.5 - $60.9 billion (2011 US dollars) which includes indirect and direct expenses such as, medical care, loss of parental economic productivity, and special education (CDC, 2018). The most effective intervention to date, early intensive behavioral therapy, can cost nearly $40,000 - $60,000 per year (CDC, 2018). If this probiotic intervention is found to be effective, it could provide a more economically accessible avenue of intervention for individuals with ASD, as probiotic production costs are notably lower than pharmaceutical production costs and behavioral therapy sessions. Additionally, daily oral administration would take less time than in-person therapy sessions which could prevent the loss of parental economic productivity. Although probiotic intervention would not necessarily or directly improve the general health of an individual with ASD, it could improve behaviors that are indirectly detrimental to health, such as maintaining a narrow food repertoire and uncontrolled motor stereotypies. This reduction in restrictive and repetitive behavior would reduce the need for individuals to resort to costly medications associated with negative side effects, such as risperidone, which is used to treat irritability in individuals with ASD in the hopes of preventing injurious and self-injurious motor stereotypies. Another pertinent issue that has yet to be addressed by current interventions is the treatment of adults with ASD. Most of the available treatments for ASD are targeted towards children, resulting in a significant decline in effectiveness with increasing age. Teng et al., found that oxytocin administration improved social deficits in both young and adult mice, in an age-independent manner (Teng et al., 2016). Since *L. reuteri* administration stimulates oxytocin production, it

is comparable to and less invasive than intranasal oxytocin administration. Thus, it could be expected that the prosocial effect of *L. reuteri* would also be age-independent. If this were the case, this would allow adults with ASD to receive effective treatment for their social communication deficits which could potentially lead to an increase in independence, both economically and physically. However, if this combined probiotic treatment of *B. fragilis* and *L. reuteri* were to be established as ineffective in humans, the biological, economical, and social state of individuals with ASD would remain the same and there would be no interventions targeted towards the core symptoms of ASD.

Possible Complications

A few different factors could complicate the application of this probiotic intervention in the treatment of ASD. Most importantly, the most effective dosages of *B. fragilis* and *L. reuteri* have yet to be determined. There is potential that the most effective dose could vary among specific populations of individuals with ASD. It has been determined that there are specific subsets of individuals with ASD, attributed to different geneses, with predispositions to comorbidities and intervention effectiveness (Parker et al., 2017). It is possible that a specific endotype of individuals with ASD will be more susceptible to improvements through probiotic intervention, although biological markers differentiating these subsets have not yet been determined. Additionally, it is possible that there is optimum dosage ratio of *B. fragilis* to *L. reuteri* as both strains, to be effective, must colonize the gut microbiome and little is known about whether there would be competition between the two strains during the colonizing process. The optimal length of intervention could differ between the two strains and it is not yet known if improvements in behavioral and social deficits are dependent upon continued oral administration.

Future Studies

It is imperative that studies ascertain the optimal clinical dosage of *B. fragilis* and *L. reuteri*, both independently and concurrently. These findings would lay the foundation for future improvements and adjustments of this intervention method. In addition, future studies must examine differing lengths of probiotic intervention and the following period after discontinuation to explore the temporal dependency of improvements upon daily administration. Furthermore, Hsiao et al., found that improvements in repetitive and restrictive behavior could be induced by other probiotic strains and was not singularly dependent upon *B. fragilis* intervention (Hsiao et al., 2013). The probiotic strain *Bacteroides thetatiotacomicron* also significantly improved restrictive and repetitive behaviors in MIA offspring although the administration of the probiotic strain *Enterococcus faecalis* did not have any effect on behavior, signifying that there is some level of specificity to efficacy of probiotic intervention.

XI. Conclusions

Treatment with *B. fragilis* decreases restrictive and repetitive behaviors by correcting intestinal epithelial hyperpermeability, cytokine production, and gut microbiome composition (Hsiao et al., 2013). *B. fragilis* treatment targets the tight junction protein expression of the intestinal epithelium, and improves intestinal barrier integrity (Hsiao et al., 2013). This decreases leakage of neuro-active molecules into the blood that can lead to abnormal behaviors (Hsiao et al., 2013). Treatment with *L. reuteri* ameliorates social deficits by stimulating oxytocin production via the vagus nerve which increases oxytocin levels and

restores social interaction-induced synaptic plasticity (Sgritta et al., 2019). The increase in

oxytocin levels promotes prosocial behavior and increases reciprocal social interactions,

sociability, and preference for social novelty (Sgritta et al., 2019). It is hypothesized that

treatment with both probiotic strains will decrease behavioral and social deficits, the core

symptoms of ASD that have not yet been addressed by available interventions to date.

XII. Acknowledgements

XIII. Literature Cited

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