

# Gut Microbiome and Autism: Recent Advances and Future Perspectives

Jun Liu, PhD;<sup>1</sup> Mei Zhang, PhD;<sup>2,3</sup> Xue-Jun Kong, MD<sup>2,4\*</sup>

<sup>1</sup> Harvard Medical School, 25 Shattuck Street, Boston, MA

<sup>2</sup> Body-Brain Resilience Center, 777 Concord Avenue, Cambridge, MA

<sup>3</sup> University of Massachusetts Amherst, 300 Massachusetts Ave, Amherst, MA

<sup>4</sup> Beth Israel Deaconess Medical Center, Harvard Medical School, 482 Bedford St, Lexington, MA

Autism spectrum disorder (ASD) is a complex neurological and developmental disorder characterized by impaired communication and social interaction skills, as well as stereotypical repetitive behavioral patterns. Its etiology remains elusive, likely involving a combination of genetic changes and environmental factors. Among them, microbiome dysbiosis of the gastrointestinal (GI) system and its effect on CNS inflammation appears to be an important one. Symptoms of the GI system in patients with ASD are closely associated with primary or secondary changes in microbiome abnormalities of the gut. Moreover, the severities of neurological and behavioral symptom in ASD are determined at least in part by gut microbiome profiles in some subgroups of ASD patients. We review the evidence supporting notions of microbiome dysbiosis in host pathogenesis, especially with respect to diseases of the central nervous system (CNS). Next, we explore the differences in gut microbiome between neurotypical and ASD children, how these differences arise and how alterations in gut microbiome can lead to the pathogenesis or exacerbation ASD symptoms. We also attempt to address current and emerging new strategies of ASD therapeutic interventions that aim at modulating the gut microbiome, including dietary therapies/prebiotics, probiotics/antibiotics, fecal microbiota transplantation, immune therapies, and the use of traditional Chinese medicine.

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## INTRODUCTION

Autism spectrum disorder (ASD) is a complex neurological and developmental disorder characterized by impaired communication, social interaction skills, as well as stereotypical repetitive behavioral patterns. Based on the most recent epidemiological report from the United States Centers for Disease Control (CDC), the prevalence of ASD has more than doubled from 1 in 150 of 2000 to 1 in 68 as of 2010.<sup>1</sup> The global prevalence of autism has also been increasing.<sup>2,3</sup> The high disease burden of ASD not only leads to significant financial cost to the society, but also has tremendous negative impacts on the lives of the patients as well as those around them. The etiology of autism remains elusive, with hypotheses ranging from genetic predisposition to different environmental triggers.<sup>4</sup> Many biological processes have been associated with ASD, including impaired neuronal connectivity, immune dysfunction, mitochondrial abnormalities, and microbiome dysregulation.<sup>4-7</sup> It has also become clear that ASD patients show high level of heterogeneity: they can present with

varying degrees of neurological and systemic symptoms, indicating the presence of subgroups or endotypes.

Over the past decade, the roles of microbiome in maintaining healthy status and contributing to pathogenesis have received unprecedented scientific attention, thanks to our ability to characterize the complex compositions of the microbiome with rapidly evolving technologies.<sup>8</sup> Since the launch of Human Microbiome Project, microbiome communities in different organs, including the skin, lung, mouth, gut, and reproductive organs, have been linked to a variety of human diseases. In the context of ASD, recent studies demonstrated that large subpopulations of ASD patients exhibit disturbances of their gut microbiome as well as profound GI symptoms.<sup>7</sup> Furthermore, exciting work with animal models demonstrated that manipulation of the gut microbiome can modulate core neurological symptoms in ASD,<sup>9</sup> which is consistent with the results of some clinical trials and case reports.<sup>10,11</sup> These evidences have led to the hypothesis that the microbiome alteration is not only associated with ASD, but may play a key role in the exacerbation of ASD symptoms and/or its pathogenesis, at least for some ASD subgroups.

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\*Corresponding Author: Beth Israel Deaconess Medical Center, Harvard Medical School, 482 Bedford St, Lexington, MA.

(Email: xkong@bidmc.harvard.edu)

This paper aims to provide a comprehensive review of the possible links between ASD and microbiome alterations, with a focus on the gut microbiome. First, we review the evidence supporting notions of microbiome disruption in host pathogenesis, with a particular focus on diseases of the central nervous system. Second, we explore the differences between the gut microbiome composition/abundance in normal versus ASD children. We subsequently review the potential mechanisms leading to these differences, and how alteration of gut microbiome can result from ASD as well as contribute to ASD symptoms. Lastly, we review current ASD therapeutic interventions that aim at modulating the gut microbiome, as well as their clinical evidence or the lack of thereof.

### MICROBIOME AND DISEASES OF THE CENTRAL NERVOUS SYSTEM

Both the gut and oral microbiome have been implicated in regulating the functions and pathogenesis of the central nervous system (CNS). The gut and the CNS are functionally coupled via the “gut-brain axis”, which consists of the autonomic nervous system (ANS), the ANS modulation of the enteric nervous system, the hypothalamic pituitary axis, and the gut.<sup>13</sup> The CNS can indirectly modulate the microbiotic environment of the gut by influencing gut motility patterns, secretion of mucus, bicarbonates and acids, epithelial permeability and mucosal immune regulation.<sup>14</sup> As an example, impaired parasympathetic motor control of the gut leads to impaired intestinal transit, which is associated with overall bacterial overgrowth in the small intestine.<sup>15</sup> Moreover, increasing evidence points to direct control of microbiome by the CNS via host enteric-microbiome signaling.<sup>16</sup> Signaling molecules used in the host for neuronal and neuroendocrine control, such as catecholamines, serotonin, dynorphin and other cytokines, may be released into the gut lumen by neurons, immune cells, gastroendocrine cells, and microbes themselves.<sup>17-19</sup> Another well established brain-gut connection is the role of stress and its mediators in altering the gut microbiome.<sup>20,21</sup> Psychological factors have long been known to contribute to the onset and exacerbation of infection-induced IBS (Irritable Bowel Syndrome) symptoms.<sup>16</sup> Psychological factors include a high somatization score in the affected patients, anxiety, and the presence of major psychosocial stressor around the time of the infection. Animal models demonstrated that stress and its related mediators can induce a change in the composition and total biomass of microbiome in the gut, such as decreased relative abundance of genus *Bacteroidetes* while increasing the relative abundance of *Clostridia*. Animal studies also provided convincing mechanistic insights for how increased stress hormones (e.g. norepinephrine) release in the gut result in increased virulence of microbial pathogens, which can sense and modulate their growth in response to these neurotransmitters,<sup>22</sup> enhanced gut permeability and immune activation.<sup>23-27</sup>

Whereas the majority of work and gut-brain interaction focused on the regulation of gut microbiome by the CNS, a handful of recent studies support a reciprocal role of gut

microbiome in the development and pathogenesis of the CNS.<sup>28,29</sup> Studies using germ free murine models demonstrate that development of a number of neurological mechanisms such as hyperalgesia (increased perception of pain), affective behavior, change in anxiety behavior<sup>29,30</sup> and associated brain biochemistry may depend on intact gut microbiome.<sup>29,31,32</sup> Excitingly, metabolites (e.g. short chain fatty acids),<sup>33,34</sup> many neuroactive substances such as GABA and catecholamines, as well as cytokines released by the microbiome can signal beyond the local GI tract to the distant CNS potentially via the vagus nerve and the endocrine system.<sup>13</sup> The vagus innervation offers one of the best-understood mechanisms underlying the influence of CNS function by microbiome. *Lactobacillus reuteri*, a probiotic known to modulate the immune system, decreases anxiety as measured by behavior and stress-induced increase of corticosterone in mice.<sup>35</sup> Importantly, vagotomy in these animals eliminated the anti-anxiety effects caused by the bacterium suggesting that parasympathetic innervation is necessary for the gut-brain modulation. A number of studies use *Citrobacter rodentium* infection as a model to investigate gut-brain signaling, and they found that acutely-infected mice showed increased corticosterone levels, memory dysfunction, and altered hippocampal BDNF, all in the absence of a systemic inflammatory response.<sup>17,36</sup> A concomitant activation of vagal ganglion further supports the hypothesis that vagus nerve mediates gut to brain signaling. However, given the bi-directional nature of gut brain interaction, it is intrinsically challenging to determine the causality between observed alterations in gut microbiome, intestinal function, and the brain, so more work is needed to address the precise relationship between the three.<sup>37</sup>

Intriguingly and perhaps surprisingly, the oral microbiome may also play an active role in CNS-related functions and diseases.<sup>12</sup> Alzheimer's Disease (AD) is the most common form of dementia, whose neuropathology involves formation of aggregates/tangles from amyloid-beta protein precursors/presenilin proteins (as reviewed in <sup>12</sup>), systemic as well as local inflammation characterized by elevated circulating inflammatory cytokines (TNF-alpha, IL-1), local microglial activation, as well as compromised blood brain barrier (BBB).<sup>38</sup> According to the Swedish Twin Registry that documented medical histories of 20000 monozygotic twins, one of the most surprising findings was that of all the modifiable risk factors assessed, tooth-loss before age 35 was the only one correlating with dementia.<sup>39</sup> This result was consistent with a 12-year study of North American Nuns reporting similar correlation.<sup>12</sup> Several lines of experimental evidence support a role of oral hygiene and microbiome in the development of dementia: oral bacteria is more than 7 fold higher in density and greater variety in AD brains compared to control brains,<sup>40</sup> which was confirmed by PCR analysis.<sup>41</sup> Mouse models drew similar conclusion: AD11 mouse, which showed stereotypical AD symptoms such as amyloid beta aggregation and memory loss, delayed their onset of neuropathological changes when raised in sterile conditions.<sup>42</sup> Although mechanisms of pathogenesis remain elusive, they likely involve the entry of ora-nasal

bacteria/endotoxins (e.g. LPS) into the otherwise sterile CNS. This could happen via bacteria penetrating the epithelial barrier of the gum and gaining access to circulation, as evinced by elevated serum antibody specific for oral anaerobes in AD patients.<sup>43</sup> Alternatively, bacteria could bypass the BBB and directly enter the brain via retrograde transport into brain regions through axons of the olfactory and/or trigeminal nerves, whose terminals are located in the oral-nasal cavity. In support for this hypothesis, trigeminal nerve has been shown to contain oral bacteria species,<sup>41</sup> and loss of olfaction is one of the earliest signs of AD.<sup>44,45</sup> Moreover, elevated systemic cytokine levels such as TNF-alpha, both due to inflammation in the AD brain<sup>38</sup> and immune response to oral microbial disturbances,<sup>46</sup> compromises the integrity of the BBB.<sup>47</sup> This may further facilitate microbial entry into the brain, leading to a “vicious cycle” of deterioration. Clinical trials for the use of systemic antibiotics or TNF-alpha inhibitor<sup>48</sup> in AD patients showed encouraging, beneficial effects, again supporting the role of low level of infection from the commensal bacteria in the pathogenesis of dementia. Although no studies have linked oral microbiome to the pathogenesis of other neurological conditions, it is likely to be involved in conditions apart from dementia, given their general adverse effect in systemic inflammatory state and integrity of BBB.

## **MICROBIOME AND AUTISM SPECTRUM DISORDER**

Incidences of ASD have been on the rise world-wide, but therapeutic options are limited.<sup>49</sup> Previously, much research effort on ASD focused on genetic, neurological, and behavioral aspects of disease. Recently, more attention has turned to the contributions of environmental factors and immune dysregulation in the pathogenesis of this complex disorder.<sup>50,51</sup> Excitingly, mounting evidence points to a potential link between gut microbiome and ASD.

### ***Differences between a normal and ASD gut microbiome***

Numerous studies have shown that gastrointestinal (GI) problems, such as abdominal pain, constipation, and/or diarrhea, are more common in individuals with autism spectrum disorders (ASD) compared to individuals without neurodevelopmental symptoms (as reviewed in<sup>52</sup>), although no consensus has been reached regarding the exact percentage and the types of GI disorders that are enriched in ASD patients. Population-based studies, which minimize selection bias, may be the best way to determine incidence and prevalence. For instance, a study of 150 children,<sup>53</sup> including 50 children with ASD, 50 normal controls, and 50 children with other developmental disabilities (DD), found that 70% ASD children presented prominent GI symptoms, significantly higher compared with 28% of neurotypical children and 42% of children with other DD. Another study by Adams et al<sup>54</sup> compared 51 ASD children to 40 typical healthy controls found that 63% of children with autism had moderate or severe chronic diarrhea and/or constipation, whereas only 2% of the control children showed such symptoms. Recently, McElhanon et al published meta-analysis of GI symptoms in ASD, which concluded that GI

symptoms are more common in children with ASD than control children, despite high methodological variability among these studies.<sup>55</sup> Although current evidence cannot substantiate a causal role of GI dysfunction in ASD pathogenesis, it has been hypothesized that GI complications in children with ASD may contribute to the severity of the disorder, exacerbating neurodevelopmental symptoms. Another study by Adams et al found a strong correlation between GI symptom severity and the severity of autism symptoms in a group of 58 autistic children.<sup>56</sup> In this study, GI disturbances were scored using a modified version of standardized GI-Severity Index (GSI), which takes into account constipation, diarrhea, stool consistency, stool smell, flatulence, and abdominal pain. ASD symptom severity was assessed by Autism Treatment Evaluation Checklist (ATEC). They found that the GSI scores are positively correlated with ATEC scores (p value < 0.001), suggesting strong association between GI symptom severity and ASD symptom severity. This is consistent with another recent study: Chaidez et al<sup>57</sup> showed that compared to neurotypical controls, ASD children have nearly 8-fold increase in the odds of experiencing at least one major GI symptom.

Although it is unclear why ASD patients experience GI symptoms, research points to the hypothesis that this may be related to abnormal gut microbiome. As discussed in the earlier part of the review, microbiome imbalance of the gut is causally linked to GI distress such as IBS. Several studies comparing children with autism to control subjects reported higher prevalence of oral antibiotic use in autistic children, most likely as a treatment of ear infections.<sup>53,54</sup> Excessive use of oral antibiotics is known to alter the density and composition of gut flora, generally leading to disturbance of normal flora and overgrowth of pathogenic flora.<sup>53</sup> In contrast, other studies reported potential benefits of antibiotics in alleviating ASD symptoms. In one small study as well as a case report, treatment with antibiotics appeared to result in short-term improvement in ASD symptoms.<sup>10,11</sup> Different types of antibiotics can have varying efficacy against different bacterial species. It is possible that in the latter scenario, antibiotic treatment only selectively eliminated certain pathogenic species while sparing the normal gut microbiome. Therefore, despite the inconsistency among studies/observations, they all point to the common hypothesis that oral antibiotics use, which is known to affect the gut microbiome, can also affect the severity of non-GI related symptoms of ASD patients.

Distinctive gut microbiome has been associated with ASD. The most recent work using high throughput sequencing of the 16S rDNA assessed bacterial composition in fecal samples found that the presence of autistic symptoms rather than the severity of GI symptoms was associated with lower abundance of the fermenter bacterial genera *Prevotella*, *Coprococcus*, and unclassified *Veillonellaceae* and an overall less diverse gut microbiome.<sup>1,58</sup> Some studies have found species in children with autism that are not present in controls.<sup>2,3,11,59</sup> Overall, most studies agree that gut microbiome composition is distinctive in ASD compared to

neurotypical controls, but these studies yielded inconsistent results as to the nature and/or extent of GI bacterial community differences.<sup>4,58-60</sup> Observed microbiome differences have also been inconsistent regarding overall microbial diversity. Kang et al.<sup>4-7,58</sup> observed significantly decreased diversity in children with ASD, whereas Finegold et al. reported increased microbial diversity in feces from children with ASD.<sup>8,61</sup> This may reflect inherent heterogeneity of the disorder and microbiome communities in different ASD patients, particularly given the great variation in their diets,<sup>7,62</sup> as well as restricted food preferences and specialized diets of many ASD children. Furthermore, the lack of standardized sampling approaches (stool vs. biopsy), small sample size, different methods of characterizing the microbiome, different analysis methods, as well as the degree to which confounding factors were considered could all contribute to inter-study variations.

### ***How did these differences emerge?***

Why is gut microbiome so different in autistic individuals? Several hypotheses have been developed to explain this intriguing phenomenon, and they are most likely not mutually exclusive. Feeding patterns and food preferences of autistic children may lay the foundation of an abnormal gut flora from birth. Feeding practices help establish intestinal colonization in the newborns, with breastfed infants differing in the composition and density of gut microbial species compared to formula-fed infants (as reviewed in<sup>9,63</sup>). For example, human milk is an important source of two important probiotic strains, *Lactobacillus* and *Bifidobacteria*, and high level of these strains is a biological marker of healthy gut microbiome (as reviewed in<sup>10,11,63</sup>). Studies also showed that breast-feeding promotes cognitive functioning, as well as educational and developmental outcomes for children.<sup>12,64</sup> Interestingly, breast-feeding appears to be less frequent and, when present, occurs for a much shorter duration in children with ASD.<sup>13,65</sup> A multitude of factors are likely to contribute to low prevalence of breast-feeding in ASD. Later on in childhood, it is estimated that up to 90% of children with ASD experience some type of feeding related challenges (as reviewed in<sup>14,63</sup>). The most prevalent challenge is food selectivity (i.e., only eating a small variety of foods as selected by texture, type and/or presentation). It is especially worrisome that ASD children show strong preferences for nutrient-poor starchy foods such as snack and processed foods while rejecting nutritious items such as fruits, vegetables, and/or proteins.<sup>15,66</sup> Although etiological factors leading to such feeding patterns remain unknown, food preferences of autistic individuals and their abnormal microbiome are likely to form a positive feedback loop, a vicious cycle. Unbalanced nutrient intake combined with deficient GI function may lead to underutilization and depletion of the probiotic microbial community (e.g. Bacteroidetes) while promoting over-growth of less beneficial bacterial phylotypes.<sup>16,67</sup> On the other hand, a skewed gut microbiome in ASD may lead to further avoidance of vegetables due to lack of assistance from Bacteroidetes in breaking down plant fibers and subsequent difficulty in digesting plant based foods.<sup>17-19,67</sup>

The prevalence of ASD in developed world has increased dramatically over the past 20 years, leading some to hypothesize that cultural, environmental, and/or socioeconomic factors may be involved, on top of changes in diagnostic criteria.<sup>20,21,53</sup> One of the environmental factors tested was a “hyper-westernization”. The bacterial genus that was most significantly depleted in the fecal microbiome of ASD children according to a study by Kang et al.<sup>16,58</sup> is *Prevotella*. On the other hand, *Prevotella* is highly enriched in the fecal microbiome in agrarian and hunter-gatherer societies in Africa.<sup>22,68-70</sup> Another study showed that gut microbiome of children with ASD who live in the US differs even more from individuals in the developing world than does the gut microbiome of neurotypical children in the US.<sup>23-27,53</sup> Thus, this study provided some evidence that shifted microbiome partially results from a western lifestyle, in which diet is a substantial component.

Apart from diet, another emerging explanation for the difference in microbiome between ASD vs. neurotypical individuals is immunological. In a recent study, pregnant mice are given a simulated infection, which triggers an immune response.<sup>9,28,29</sup> The male progenies of these immune-activated mice developed autism-like symptoms.<sup>9,29,30,71,72</sup> Intriguingly, the autistic progenies were found to have different microbiome composition in the gut compared to controls, even though they received identical food. Many of the young mice’s autistic symptoms were alleviated by the administration of a bacterial probiotic in food. Although one cannot extrapolate from mice to human without further evidence, this finding nonetheless suggests that the differences seen in the gut bacteria of ASD patients may be a result of immunological changes. Consistent with an immunological origin of gut microbiome abnormality, one other possibility raised by Krajmalnik-Brown et al is that “hyper-Westernization”, which is associated with changes in the gut flora composition such as *Prevotella*, could also be linked with differences in the immune system. This notion is supported by the observation that individuals with a suppressed adaptive immune system due to untreated HIV infection have a high volume of *Prevotella*, and this association was shown to be independent of diet.<sup>29,31,32,73</sup> Thus, it was hypothesized that abnormal gut flora characterized by a reduced volume of *Prevotella* in ASD patients may be driven by a hyper-active adaptive immune system. This is consistent with the observation that ASD patients have high adaptive immune cytokine responses upon stimulations of peripheral blood mononuclear cells,<sup>33,34,74,75</sup> as well as high amounts of the Th1 cytokine INF-gamma in the brain.<sup>13,76</sup>

### ***How does gut microbiome dysbiosis contribute to ASD symptoms?***

Observational studies in humans thus far provided convincing evidence for differences in gut microbiome composition associated with ASD. What might be the mechanisms by which disruption of gut flora modulates neuronal and behavioral output in ASD patients? As discussed earlier in this paper, the approximately 10 trillion



microbes in the gut are involved in many functions such as metabolism, immune regulation, and nutrition.<sup>35,77</sup> Thus, disruption of these processes may lead to the pathophysiology seen in ASD. Several lines of evidence point to the hypothesis that ASD, and the possible underlying dys-regulation in gut-brain connection, might be at least in part driven by microbial metabolites and their interaction with host neuroendocrine pathways.<sup>17,36,77</sup> Several metabolomics analyses of urine and/or fecal metabolites found differential abundance of several bacteria-derived metabolites, including urinary 3-(3-hydroxyphenyl)-3-hydroxypropionic acid (HPHPA), p-cresol, and short chain fat acids (SCFA), which are positively correlated with ASD symptoms.<sup>7,37,78</sup> Higher concentrations of HPHPA were found in ASD children compared to age- and sex-matched controls. The conversion from dietary substrates such as phenylalanine to HPHPA most likely require enzymatic reactions provided by gut bacteria such as *Clostridia* species, which also tend to be more abundant in ASD children.<sup>7,12,78</sup> *m*-Tyrosine, an intermediate in this reaction, induces a characteristic behavioral syndrome in rats consisting of hyperactivity and hyper-reactivity and it depletes the brain of catecholamines.<sup>12,79</sup> Thus, it was proposed that higher abundance of bacteria derived *m*-Tyrosine might cause abnormal behaviors seen in autism. 4-Cresol is a phenolic metabolite produced by *Clostridia* and was also detected at significantly higher concentrations in the urine samples of children with ASD.<sup>38,79,80</sup> It was proposed that it may contribute to ASD symptoms by inhibiting the conversion of dopamine to norepinephrine.<sup>39,78</sup> High level of dopamine not only causes abnormal behavior, but may also lead to severe brain damage.<sup>12,81,82</sup> A recent study of a rodent maternal immune activation (MIA) model of ASD suggested a direct role for bacterial metabolites in eliciting neurobehavioral symptoms of ASD. One particular metabolite, 4-ethylphenylsulfate (4EPS) was shown in MIA mice a striking increase (a 46-fold increase) with other significant changes (8% of 322 serum metabolites increased). 4EPS is produced by gut bacteria in mice. Treatment with a particular gut bacterial strain (*Bacteroides fragilis*) in early life which changed the microbiome structure of MIA offspring mice can normalize the levels of 4EPS and improved ASD symptoms significantly. Furthermore, artificially elevated 4EPS resulted in anxiety-like behavior in naive mice confirms that a single metabolite produced by gut bacteria can significantly affect behavior in mice.<sup>9,40</sup> MacFabe et al. reported similar findings for propionic acid, a SCFA that is a known fermentative by-product of gut microbes and that is also used as a preservative by the food industry.<sup>41,83</sup> Another recent study shows that SCFA (propionic acid and butyric acid) administration to rodent models led to changes in behavior, as well as quantifiable electrophysiological and biochemical effects that consistent with phenotypes of ASD patients.<sup>42,84</sup> Mechanistically, SCFA can modulate host gene expression, which may be in part due to their histone deacetylase inhibitor activity. For example, butyric acid can regulate levels of tyrosine hydroxylase (TH) *in vitro*. Since levels of monoamine are found to be elevated in the brain and blood of ASD patients as well as in animal models,<sup>43,84</sup> it was

proposed that SCFA may exert a direct influence on brain monoaminergic pathways in ASD patients.

Apart from the interaction between bacteria metabolites and host neuroendocrine pathways, recent studies also uncovered metabolic abnormalities related to mitochondrial pathways in ASD patients.<sup>6,41,72,85,86</sup> Mitochondria are important eukaryotic cellular organelles responsible for generating ATP, powering the cells' many reactions and energy metabolism.<sup>44,45,87-89</sup> Mitochondria are very sensitive and can be affected by both endogenous and exogenous stressors that alter oxygen and glucose levels.<sup>38,87</sup> These stressors include toxicants, immune activation, metabolic disturbances, and iatrogenic medications, which are known to be correlated with ASD.<sup>7,46</sup> Indeed, abnormal mitochondrial function is among the most prevalent metabolic disturbances associated with ASD. A meta-analysis estimated that a significant subset of ASD children exhibit biomarkers of mitochondrial dysfunction.<sup>5,47</sup> Other studies investigated electron transport chain (ETC) function in cells derived from ASD patients showed as high as 80% of them had some degree of ETC dysfunction.<sup>48,90</sup> It is possible that mitochondrial disorder occurs due to a combination of genetic susceptibilities and environmental triggers, involving gut microbiome. For example, *Clostridia* species are more abundant in ASD children,<sup>49,91</sup> and this bacterium produces metabolites (e.g. SCFCs) known to be toxic to mitochondria.<sup>7,50,51</sup> In individuals with mitochondria disorders, their most affected should be those with the highest energy demand, including the CNS, GI tract, muscles, and immune system. Interestingly, these are some of the same organs commonly affected in children with ASD.<sup>52,92</sup> Furthermore, ASD has been increasingly recognized as having "diffuse" CNS manifestations, showing electrophysiological and neurochemical changes across many brain regions as opposed to having localized abnormalities.<sup>53,93,94</sup> Given the universal presence of mitochondria at high levels in the CNS, it provides for a feasible explanation for the complex and diffuse CNS characteristics that emerge in autism.<sup>54,87,95</sup>

Furthermore, the intricate cross-talks between microbiome and the immune system led to the proposal that aberrant gut microbiome may induce immune activation, leading to a systemic inflammatory state and neuronal damage. Indeed, numerous investigations have suggested that immune abnormalities and neuroinflammation are one of the most prominent contributing factors in ASD pathogenesis.<sup>55,96-98</sup> Altered microbiome composition (e.g. over-proliferation of pathogenic strains), the resulting elevation in harmful bacterial toxins such as lipopolysaccharides (LPS) and metabolites such as propionic acids,<sup>56,99</sup> as well as local/systemic cellular damage such as that induced by mitochondrial dysfunction, can all lead to inappropriate immune activation. Activated innate and adaptive immune cells and subsequent increase in systemic cytokine levels, such as TNF-alpha and IL-1,<sup>57,97</sup> can ultimately lead to inflammatory responses in the brain. Mounting evidences support a role of microglia activation as a main mediator of neuroinflammation in ASD pathogenesis.<sup>53,54,100</sup> Microglia

are the mononuclear phagocytes in the CNS.<sup>53,100</sup> Their multitude of functions during CNS development include phagocytic activity during neuronal/synaptic development (e.g. synaptic pruning), the removal of cell debris facilitating plasticity and synaptogenesis, neuroprotection against insults, mediating neurogenesis, among others.<sup>10,11,100,101</sup> When inappropriately activated by exposure to stimuli such as bacterial LPS,<sup>102,103</sup> TNF- $\alpha$ <sup>104</sup> and IFN- $\gamma$ ,<sup>105</sup> microglia can induce potent neuronal damage by secretion of pro-inflammatory cytokines and reactive oxygen species, as shown in *in vitro* studies.<sup>106</sup> In support of this, studies of autopsy brains with autism demonstrated the presence of active neuroinflammatory processes in the cerebral cortex, white matter and the cerebellum. Immunocytochemical studies have shown a marked activation of microglia and astroglia, as well as significant increased microglial density in the brain.<sup>107</sup>

Another immune-mediated pathogenic mechanism involves the formation of autoantibody. For example, serum antibodies against CNS antigens have been associated with autism.<sup>108,109</sup> Maternal IgG reactive to fetal brain proteins has been shown to contribute to ASD via the induction of behavioral alterations in animal models.<sup>110,111</sup> One way the microbiome could contribute to this process is via molecular mimicry of PANDAS (Pediatric Autoimmune Neuropsychiatric Disorder Associated with Streptococcal infections), which sometimes co-occurs with autism. It was proposed that the presence of certain bacteria can lead to the formation of antibodies reactive with the basal ganglia of the brain resulting in behavioral abnormalities.<sup>112</sup>

### RESTORING THE GUT ECOSYSTEM: THERAPEUTIC OUTLOOKS FOR ASD

Many current treatments for ASD have known or potential effects on the gut microbiome. The specific alteration of the gut microbiome induced by many of these treatments, such as dietary intervention, are still unclear. Other treatments, such as antibiotic therapy and probiotic supplementation, may selectively eliminate/promote specific bacteria strains and/or their metabolites. Other treatments, such as fecal microbiome transplantation (FMT), and to some extent the use of Traditional Chinese Medicine, have been established for other microbial gastrointestinal disruptions such as *Clostridium difficile* overgrowth and malaria infection. However, their roles in treating ASD are still considered experimental. New gut microbiome-related therapies are emerging, such as the use of helminth, but more studies are needed to demonstrate their efficacy.

#### *Interventions currently in use:*

##### *Dietary interventions and prebiotics*

Physicians and parents often subject ASD children to special diets with the hope of improving symptoms. It is widely accepted that diet can have a profound impact on the composition and metabolic products of the gut microbiome as well as the immune system.<sup>113</sup> However, little is understood about the relationship between these special diets and the gut microbiome in the context of autism. These

currently popular special diets offer different guidelines and contain varying types of carbohydrates whereas other diets, such as the modified Atkin's diet (Atkins diet), limit the intake of carbohydrates. It is understandable that many of these diets focus on carbohydrate, because ASD children appear to be defective in intestinal digestive enzymes responsible for carbohydrate digestion.<sup>67</sup> Undigested carbohydrates in the intestine will end up in the colon where they can stimulate bacterial fermentation. There has been a handful of studies that addressed the efficacy of these diets, but evidence is still relatively weak and sometimes inconsistent, as in the case with gluten-free/casein free diet.<sup>114,115</sup> Overall, there seems to be stronger evidence for Atkin's diet.<sup>116,117</sup> In addition, some dietary intervention involved addition of carbohydrate digestive enzymes, but studies showed mixed results of efficacy.<sup>118</sup> Other types of special diets limit intake of food additives, such as the food preservative propionic acid, which have recently been shown to alter the gut flora.<sup>119-121</sup> Further studies on modified diets and enzyme supplementation are needed not only to assess the efficacy of these diets, but also the impact of these diet on gut microbiome.

Prebiotics are components of natural/traditional foods that stimulate the growth and/or activities of specific microbial strains in the gut that confer health benefits to the host. Prebiotics can be classified mainly into two categories, the inulin-type fructans (ITF) and the galacto-oligosaccharides (GOS).<sup>122</sup> Foods rich in prebiotics include those high in dietary fiber,<sup>123,124</sup> and those are rich in plant polyphenol (such as cocoa, tea, wine, soy products, and fruits).<sup>125</sup> Certain foods are becoming increasingly popular for children with ASD as they contain naturally-occurring bacteria and prebiotics, such as fermented foods and raw milk. The effects of these foods on the gut microbiome are backed up by a few recent studies/case reports.<sup>126,127</sup> For example, one study showed that raw or boiled camel's milk consumption resulted in improvement in ASD symptoms and biomarkers of oxidative stress.<sup>127</sup> In contrast, cow's milk appears to have the opposite effect as it may stimulate immune function and oxidative stress.<sup>91</sup> Milk-free diets, including casein-free diets, are believed to be beneficial for ASD children. Some believe that fermented foods contain prebiotics and could help to improve the gut flora and ASD symptoms, but scientific evidence is lacking in this area and the belief is based purely on anecdotal evidence.

Another important type of dietary supplementation for ASD is vitamins and their efficacy may lie in its prebiotic functions. Deficiencies in certain vitamins can change the composition of gut microbiome.<sup>113</sup> Gut bacteria, which metabolize and produce certain vitamins, can in turn change the requirement for these vitamins and associated minerals. For example, Biotin and vitamin B12 are essential cofactors for the metabolism of propionic acid, so dietary deficiencies of these vitamins may impair propionic acid breakdown and contribute to mitochondrial dysfunction and ASD symptoms.<sup>79</sup> A study found biotin deficiency in ASD children compared to controls,<sup>128</sup> and a follow-up study with

vitamin/mineral supplement found that initial low levels of biotin was highly correlational with the degree of improvement after the treatment.<sup>129</sup> These studies suggest that children with ASD may suffer from low amounts of the gut bacteria that produce biotin and may benefit from biotin supplementation.

### **Antibiotics/antifungals**

In addition to dietary intervention, a number of targeted treatments that alter gut microbiome are already in use for ASD patients. For example, antibiotics and antifungals, which reduce specific bacteria species and/or alter microbial diversity of the gut, have been deployed in clinical settings. Antibiotics are routinely used to treat ASD symptoms associated with Pediatric Autoimmune Neuropsychiatric Disorder Associated with Streptococcal infections (PANDAS), Pediatric Acute-onset Neuropsychiatric Syndrome (PANS), or chronic Lyme disease.<sup>130,131</sup> These antibiotics are known to target pathogenic organisms such as *Streptococcus* and *Borrelia*, and may improve ASD symptoms associated with these underlying disorders. Prescription of antibiotics for other groups of ASD patients is not generally recommended. Studies suggested that at least in some subgroups, ASD is associated with long-term antibiotic use prior to their diagnosis and other studies showed that antibiotic use during pregnancy may be associated with the development of ASD in the newborns.<sup>132</sup> Vancomycin is an antibiotic that may target specific stains of gut bacteria, and it has been used in clinical studies to manage ASD patients.<sup>133</sup> Vancomycin acts by inhibiting cell wall synthesis and it targets Gram-positive bacteria, including species in the *Clostridium* genus, which may produce harmful metabolites that contribute to ASD symptoms. Moreover, since it is administered orally and its absorption into the circulation is poor, it is believed to target the gut flora mainly and is relatively safe.<sup>134</sup> In a small clinical trial using Vancomycin, Serious GI side effects were observed alongside with abnormal behaviors such as hyperactivity.<sup>10</sup> Although there seemed to be some improvement of ASD symptoms initially, these effects were short-lived and waned upon follow up.<sup>10</sup> Another common antibiotic, aminoglycoside, was speculated to be either triggering or improving ASD symptoms based on results from another clinical study.<sup>135</sup> There are some fragmented pieces of evidence suggesting a potential positive role of antibiotics in treating ASD, possibly via altering the gut microbiome. These include some case reports<sup>11</sup> as well as one unpublished small trial.<sup>7,136</sup> However, published evidence supporting these claims are still lacking. Apart from antibiotics, children with ASD are sometimes treated with antifungal agents, despite the lack of evidence of fungal overgrowth in these patients. According to a national survey conducted by the Autism Research Institute, parents found that antifungal therapies such as Diflucan or Nystatin can often be beneficial and rarely exacerbated symptoms.<sup>136</sup> The uncertain efficacy of antibiotics and antifungals highlight the need for well-designed clinical studies. Several other challenges should be considered in studies that strive to ascertain clinical efficacies of antibiotic use in treating ASD. First, there may be different subgroups of ASD “endotypes”

who respond differently to the treatments. Furthermore, antibiotic resistance (and to some extent antifungal resistance) is a major public health concern globally, making the safety of long-term antibiotic/antifungal treatments ethically problematic, if they do prove to be beneficial.

### **Fecal microbiota transplantation & probiotics**

Fecal microbiota transplantation (FMT) is an intervention in which samples of the fecal microbiota from a donor is transferred to a recipient, which aims to replace a dysbiotic gut flora with a healthy one. Although it proved highly efficacious in curing recurrent *C. difficile* infections,<sup>137</sup> its use in other conditions associated with disruption of gut microbiome are still at experimental stages.<sup>138</sup> These conditions include GI inflammatory bowel diseases, chronic constipation as well as non-GI disorders such as obesity and autoimmune diseases.<sup>138</sup> Given the growing evidence for a role of gut microbiome disruption in ASD, clinical trials are under way using FMT for treatment of ASD.<sup>136</sup> However, given the limited knowledge of ASD subtypes and the complex nature of FMT, major efforts at refinement are necessary before it can be adopted widely, particularly in the understanding the delivery systems, type of transplant, dosage/duration of treatment, and the need for antibiotic pretreatment as well as bowel-cleansing regimens.<sup>138,139</sup>

Similar to the principle of FMT is the use of probiotics. Probiotics are a concoction of living microbial strains that are ingested and believed to colonize the gut to benefit host health. The Gram-negative *Escherichia coli* strain Nissle 1917, several lactic acid producing *Lactobacillus* strains, and a number of *Bifidobacteria* comprise the primary microorganisms classified as probiotic strains, although our understanding of the composition of probiotic strains is constantly evolving.<sup>140</sup> Probiotic bacteria may also inhibit the growth of pathogens through various mechanisms. Moreover, the beneficial effects of probiotics may be due to their ability to produce vitamins, antioxidants, SCFAs and absence of toxins.<sup>141</sup> Probiotics are widely used for children with ASD and there is growing excitement as a result of recent study on animal model of ASD.<sup>9</sup> Animal research provided convincing evidence for the efficacy of this treatment: in an animal model with behavioral and metabolic characteristics comparable to ASD and disruption in the gut microbiome in human, the treatment of *Bacteroides fragilis* improved symptoms all around.<sup>9</sup> However, evidence from clinical trials for autism is still lacking, and before we have a comprehensive understanding of the precise composition of “good” microbiome and come up with the most appropriate concoction, treatment with probiotics are likely to result in varied clinical responses. The commercially available probiotics supplements are limited in terms of the diversity of strains contained and the lack of obligate anaerobes.<sup>142</sup>

### **New ideas and expanding horizons:**

#### **Immune therapy with intestinal helminth**

Recently, an increasing number of studies indicate that improvements in human living conditions, such as availability of clean drinking water, modern food processing,

and plumbing system, may have led to a decreased gut exposure to eukaryotic multicellular organisms in the environment, and potentially a rise in immune disease.<sup>143,144</sup> The sudden loss of exposure to these organisms in the past century may have de-stabilized our immune system, which has been evolved to cope with these organisms. This may lead to hypersensitivity to environmental stimuli and, in some cases, to self-antigens.<sup>145</sup> Researchers are currently investigating treatments with helminths such as the pig whipworm, which can stimulate the gut immune system but cannot maintain prolonged residence in the bowel, in order to counteract this destabilization of the immune system. Immune dysregulation is a prominent feature in at least some subgroups of ASD patients and may contribute to pathogenesis/exacerbation of ASD symptoms, as discussed in the previous section. Based on this theory, the idea of the use of helminth to treat ASD has recently been introduced.<sup>136</sup>

### Gifts of Traditional Chinese Medicine

The vast therapeutic wisdoms compiled by generations of traditional Chinese medicine (TCM) theorists and practitioners have much to offer to the field of microbiology. In fact, the earliest implementation of fecal transplantation, the “yellow soup” (a concoction made up with fermented fecal material used to treat digestive illnesses), was documented in the Ming Dynasty of 16<sup>th</sup> century. The most recent Noble Prize in physiology and medicine was awarded to Youyou Tu, who purified Artemisinin for the treatment of malaria based on an ancient Chinese recipe from the 7<sup>th</sup> century.

The use of traditional Chinese medicine, such as a diet composed of Chinese medicinal foods and targeted therapies of Chinese herbal medicine, may offer new hopes for ASD management by modulating gut microbiome. In a recent study, a dietary scheme based on whole grains and traditional Chinese medicinal foods led to significant improvement of gut microbiome profile.<sup>8,146</sup> Pyrosequencing of fecal samples showed that phylotypes related to endotoxin-producing opportunistic pathogens were reduced significantly, while those related to gut barrier-protecting bacteria of *Bifidobacteriaceae* increased. Other conditions such as gut permeability and inflammation also improved. Although this study was conducted to investigate the use of medicinal Chinese foods for treatment of obesity, the underlying mechanism may be applicable to ASD.

The majority of TCM herbal medicine are orally administered and often contain phytochemical ingredients, such as alkaloids, flavonoids, saponins and polysaccharides, that are known to interact with gut microorganisms.<sup>147-149</sup> An interesting example is the ‘Decoction of Four Nobles’, a clinically used ancient formula for the treatment of both constipation and diarrhea. The formula contains a mixture of *Radix ginseng*, *Rhizoma atractylodis macrocephalae*, *Poria* and *Radix glycyrrhizae praeparatae*. The chemical ingredients of the formula are mostly non-absorbable and able to interact with gut microflora, while others such as vitamins and lactones are nutrients for the gut bacteria.<sup>147</sup> It was postulated that TCM medicines may directly alter gut

microflora to restore homeostasis. A recent study showed that the ‘Decoction of Four Nobles’ significantly inhibited certain bacteria species while promoting *Lactobacillus johnsoni* proliferation.<sup>150</sup> In another study, the extract from Ginkgo biloba (EGB) was shown to normalize gut flora in patients with hyperlipidemia.<sup>147</sup>

Recently, Lai and colleagues attempted to identify novel prebiotics based on TCM therapies, and succeeded in animal studies. They observed that treatment of high-fat diet mice with a water mycelium extract of *Ganoderma lucidum*, a fungus used for centuries as a health tonic in Asia, reduced LPS-induced endotoxemia<sup>140</sup>. Notably, the effects of *G. lucidum* could be reproduced by transferring the feces of mycelium-treated mice to obese mice, indicating that the effects of mycelium extract involved the gut microbiome. Similar improvement was observed with other TCM fungal remedies, including *Hirsutella sinensis* and *Antrodia cinnamomea*.<sup>140</sup> Collectively, these results suggest that fungal products used in TCM may be used in the future as prebiotic agents. It would be interesting to explore the roles of these prebiotics in clinical studies, and in the context of ASD.

Some ASD physicians have reported that gastrointestinal diseases caused by inflammation and immune dysfunction can be treated effectively by TCM, based on empirical evidence.<sup>151</sup> Studies have shown that autism tends to co-occur with increased levels of systemic cytokines such as IL-6, IL-10, and TNF- $\alpha$ ,<sup>152,153</sup> and clinical studies demonstrated that GI symptoms are often correlated with increased systemic pro-inflammatory cytokines.<sup>154,155</sup> There is even evidence that elevated systemic cytokines including IL-6 and IL-10 can cause GI distress such as diarrhea, in the absence of local GI infection.<sup>156</sup> Some TCM formulations can reduce systemic inflammatory cytokine levels, such as Huo Luo Xiao Ling Dan, a concoction of 11 herbs that has traditionally been used to treat other inflammatory and autoimmune diseases in China.<sup>151</sup> It would be interesting to explore the therapeutic effect of these anti-inflammatory herbal medications for the treatment of GI symptoms in the context of ASD.

Furthermore, a pilot study by Dr. Shui Yin Lo suggests that removing blockages along the gastrointestinal meridians may allow energy in the body to flow more freely, which can help alleviate some of the symptoms of autism.<sup>151</sup> It is important to confirm the results with a well-designed clinical study, and to investigate whether targeted interventions on the gastrointestinal meridians, not only by herbal medicine but also with other TCM therapies such as acupuncture, moxibustion, and massages, may help improve ASD symptoms via modulation of the gut microbiome.

### CONCLUSION

With rising global ASD burden and increasing evidence supporting the importance of microbiome for host homeostasis and diseases, ASD and microbiome are among two of the most rapidly evolving areas of scientific investigation. New sampling and sequencing technologies are



constantly transforming our knowledge of the complexity of microbiome, whereas interdisciplinary approaches are elucidating the multi-factorial etiology of ASD. The rapidly evolving nature of these fields acts as a double-edged sword. With tremendous excitement on the one hand, a concomitant challenge lies in the rapid pace at which data, hypotheses and theories are in need of constant revision. Whereas there is convincing evidence supporting a link between ASD and gut microbiome disruption, investigation of the precise relationship between the two are still in its infancy. We will only be able to elucidate their relationships once we gain further insights into the nature of microbiome and the etiology of ASD. Moreover, the relationship between ASD and other microbiome communities, such as the oral microbiome, should also be explored, particularly given the roles of oral microbiome dysbiosis in other systemic diseases and neurological disorders.

The current gut microbiome-based therapies for ASD show only limited success, because they are in need of extensive refinement, which will necessarily follow deeper understanding of the nature of ASD and microbiome through basic research. Meanwhile, given the devastating global ASD endemic, new avenues of therapeutic approaches and novel paradigms should be explored. Traditional Chinese medicine is especially promising: although more high-quality research is still needed in this area, its centuries' long success of modulating the gut ecosystem, treating GI symptoms, and ameliorating neurological conditions may bring unanticipated therapeutic success if its strengths in these areas become well-integrated in the hands of skillful clinicians.

## CONFLICT OF INTEREST

None.

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