
The Gut-Microbiota-Brain Axis in Autism Spectrum Disorder

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Abstract: Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by impairments in social interactions/behaviors and increased stereotypical repetitive behavior. Gastrointestinal disorders, ranging from severe constipation to diarrhea, are particularly prevalent for people on the autism spectrum, which may relate to an underlying dysbiosis (breakdown or imbalance) in the gut microbial community. Many studies have also identified changes in the gut microbiome in ASD compared to neurotypical cohorts in animal models and human populations. Microbial probiotics to help revert these gut microbial changes have been tested in animal models of ASD. Some have shown reversal of ASD behaviors and modulating the integrity of the gastrointestinal epithelial barrier. The gut-microbiota-brain axis has been described as a multidirectional communication channel between the three systems: the gut, gut microbes, and the brain, but whether these gastrointestinal microbes play a role in the context of ASD and whether they can be harnessed as a target for gastrointestinal therapies in humans is yet to be determined.

Keywords: autism spectrum disorder; animal model; gut microbiome; microbiota; zinc

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INTRODUCTION

Autism spectrum disorder (ASD) is a neurodevelopmental condition that typically includes impairments in social communication/interaction and an increase in stereotyped, repetitive behavior, as defined by the DSM-5 Manual (1). Autism was first clinically described by Kanner in 1943, with the reported prevalence of the condition increasing in recent years from ~5 in 10,000, in 1999, (2) to 1 in 54 in 2020 (3). The factors responsible for this apparent increase are unclear. However, it could be due in part to greater awareness of the condition as well as changes to the diagnostic criteria of ASD. ASD is more commonly diagnosed in males than females (3 males: 1 female) (4). While the characteristics described above are generally associated with ASD, it involves a highly heterogeneous phenotype that differs from person to person and ranges from so-called 'high-functioning' individuals of exceptionally high intellect to severely intellectually handicapped people requiring life-long care.

ASD commonly co-occurs with other conditions such as epilepsy, Down syndrome, and Fragile X syndrome, which may mask the presence of ASD due to their more prominent features (5). Based on twin studies, the concordance rate of ASD in monozygotic twins (who share all of their genes) is higher than in dizygotic twins (who share around half of their genes), indicating that there are strong genetic factors associated with ASD (2, 3). A wide range of genetic mutations has been identified in genome-wide association studies of ASD, but no single gene mutation has been linked to more than 1% of all ASD cases (4, 5). Of the vast array of genetic de novo mutations, copy number variations, and chromosomal rearrangements associated with ASD, many converge towards disrupted synapse pathways in the brain (6). For example, single protein mutations that affect any of the components involved in the neurexin-neuroigin-PSD-95-SAPAP-SHANK complex result in a similar mechanism of synaptic disruption in ASD (7).

This chapter introduces the potential role of gut microorganisms (the "gut microbiota") in ASD and discusses how these complex microbial communities may be manipulated as therapies for ASD-related challenges.

ASD AND THE GUT

The gut microbiota refers to the microorganisms (mainly bacteria and fungi) living in the gastrointestinal tract, while the microbiome describes both the organisms themselves and their collective genomic capabilities. The colonization of the gut by microbes may begin before birth, although this remains controversial (8, 9). The microbiome dynamically fluctuates as diet and environmental exposure change throughout development (10). Bacterial cells were once thought to outnumber human cells by 10: 1 (11), though recent estimates suggest that this ratio is closer to 1.3 : 1 (12). Gut commensals are essential for aiding digestion of food (13), providing resistance against pathogens (14), and stimulating the host (human) immune system (15). Although the bacterial biota of the human gastrointestinal tract is quite conserved at the broad phylum level, where members of the *Bacteroidetes* and *Firmicutes* typically dominate (13, 16), the composition of bacterial *species* varies widely among individuals (17). Despite this variation in

bacterial composition, the functional capabilities of the gut microbiota are highly conserved among individuals, suggesting the presence of a ‘core microbiome’ of microbial genes (18).

Due to difficulties with sampling the gut tissue itself (e.g., via colonoscopy), many studies use fecal samples as a non-invasive proxy for the gut microbiota. Although the fecal microbiota appears to be highly representative of the colonic microbiota, it is a less useful proxy for other parts of the gastrointestinal tract (19, 20). In practice, fecal matter includes a subset of both mucosa- and lumen-inhabiting microorganisms, and sampling of feces remains the method of choice for most human gut microbiome studies. Next-generation sequencing technologies have provided a highly efficient, culture-independent approach to study gut microbiota, whereby the 16S ribosomal RNA gene is specifically amplified by PCR for taxonomic classification due to the universal presence of this gene in bacteria and archaea.

Children with ASD commonly have a more limited food repertoire and increased refusal to eat certain foods compared with neurotypical children. This could be due to hyper/hyposensitivity to sensory inputs associated with ASD (21, 22). Diet plays a large role in determining gut microbial composition and function (23, 24); thus, a selective diet could affect the gut microbial community. Some parents of children with ASD suspect that food allergies and sensitivity to dietary gluten and casein result in gastrointestinal problems and therefore implement gluten-free, casein-free (GF/CF) diets (25). However, despite the selective eating habits and specialized diets of some individuals on the autism spectrum, such as a GF/CF diet, there is no evidence of differential energy intake and growth compared to neurotypical children (26, 27). A small number of studies have found changes in ASD-related behaviors following implementation of a GF/CF diet (28), but many other studies have identified no such changes (25, 29). Overall, the effect of a GF/CF diet on ASD behaviors remains inconclusive.

The reported prevalence of gastrointestinal (GI) disorders (ranging from severe constipation to diarrhea in individuals with ASD varies widely, with estimates ranging from 2.2% to 96.8% of the ASD population (30, 31). Despite this considerable heterogeneity, overall, most studies suggest a greater prevalence of GI problems in children with ASD compared with their neurotypical counterparts (32, 33). Behavioral changes combined with communication difficulties often make it hard for clinicians to detect underlying gastrointestinal problems in individuals with ASD (34), contributing to the variable findings of gut problems in this population. A compromised intestinal barrier (so-called “leaky gut”) describes GI problems associated with the permeability and integrity of the gut epithelial barrier (35–37), which may contribute to ASD. In this situation, dietary products and/or bacterial metabolites can pass through this barrier and enter the bloodstream, where immune responses and interactions and other effects on the central nervous system/brain may occur.

ASD AND THE GUT-MICROBIOTA-BRAIN AXIS

The gut-microbiota-brain axis refers to the bidirectional communication between gut microbes and the brain (38–40). Changes in the gut microbiome have been

described in children with ASD (41–43), though this relationship varies considerably among studies, and differences reduce somewhat once ASD, and control cohorts are carefully matched (44). If changes in the gut microbiome do occur, this may also alter the synthesis of microbial products. Gut microbial metabolites include short-chain fatty acids, as well as vitamins essential for human health (24, 45, 46). Other microbial products include compounds that act as neurotransmitters that may influence synapse function and communication with the brain (47, 48). For example, gastrointestinal disorders and changes in the gut microbiota are known to affect serotonin signaling in the gut and the brain (49, 50). Serotonin and other microbial metabolites, such as gamma-aminobutyric acid (GABA), can act as neurotransmitters, whereby the vagus nerve presents a direct pathway for communication between the brain and gut (51) (Figure 1).

By inducing stress in mice, researchers have been able to measure changes in the gut microbiota (52), demonstrating how the brain can affect the gut in ways that could potentially lead to disruption of the gut barrier (53) and inflammation from gut immune responses (54). In germ-free mice that are not exposed to any bacterial sources and are kept under strictly sterile conditions, anxiety-related behaviors are common, as well as deficiencies in gut physiology and immune development (55, 56). Specific pathogen-free mice harbor gut bacteria but are

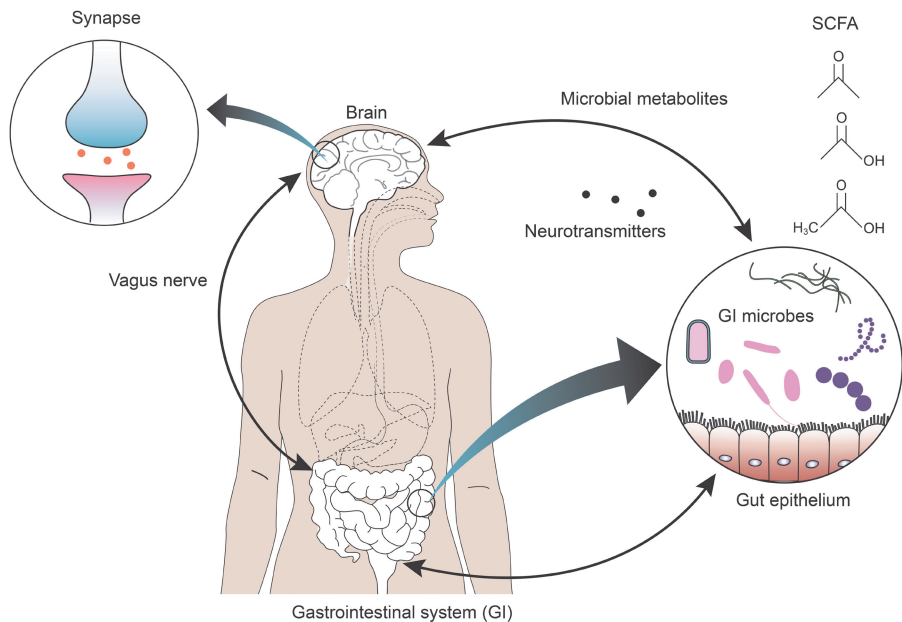


Figure 1. The bidirectional communication pathways between the gastrointestinal system, gut microbes, and the brain. Direct stimulation between the gastrointestinal system and the brain occurs via the vagus nerve and metabolites from gastrointestinal microbes such as short-chain fatty acids (SCFA), as well as neurotransmitters that can communicate with the brain via the blood.

free from a list of specific pathogens. Compared to germ-free mice with no gut microbes, these mice exhibit profound differences in behavior and brain chemical signaling (57, 58), suggesting the importance of gut microbe interactions.

GUT MICROBIAL THERAPIES

The gut microbiota offers a therapeutic target due to their manipulable nature. There exist several avenues by which the microbiota can potentially be altered, and we discuss these in the context of ASD research below.

Probiotics

Probiotics consist of live microbes that are used to benefit host health by supplementing 'healthy' gut microbiota (59). Better-known types include bacteria such as various species of *Lactobacillus* and *Bifidobacterium*, which are readily available in fermented products as well as in powder or capsule form. Probiotics have been used to treat a wide variety of conditions in humans, such as antibiotic-associated diarrhea (60, 61) and irritable bowel disease (62–64). There is much anecdotal evidence that probiotic consumption has helped individuals with ASD, both in terms of improving gut problems (such as diarrhea or constipation) and improving some ASD-related behaviors. The use of probiotic treatments for ASD-associated behavioral characteristics has also been supported in mouse models of ASD (65, 66), while trials in humans with ASD (60, 67) have sometimes been equivocal due to limitations such as small numbers of participants or lack of a placebo group. A 2020 review of probiotic supplementation for individuals with ASD concluded that the efficacy of probiotics looks promising, but there is still a need for standardized clinical trials to assess the effects of probiotic supplementation in individuals with ASD (67).

Diet alterations

It is well established that changes in diet have the potential to alter the composition of the human gut microbiota. For example, in a landmark 2014 paper, David and colleagues demonstrated significant and reproducible changes to the gut microbiota within 1–2 days of transitioning to a heavily animal protein-based diet (68). Similarly, marked differences in gut microbiota composition can be seen between members of more traditional, hunter-gatherer societies compared with urban dwellers consuming more of a contemporary, Western-type diet (69). Microbial diversity tends to be richer in the former group, likely reflecting a lesser reliance on antimicrobial products as well as consumption of a greater breadth of seasonal foods (69).

People on the autism spectrum typically have a more selective food repertoire, which can result in lower nutritional intake, ultimately leading to the development of deficiencies in fiber, calcium, and protein uptake (70, 71). Selective eating habits may involve food refusal and high-frequency selective food intake or even gluten-free/casein-free/lactose-free parental restriction of diets, often making it difficult to provide a balanced diet to children with ASD, especially those with

sensory sensitivities (72, 73). Many studies only focus on children with ASD and their eating behaviors, with a need for longitudinal studies focusing on dietary deficiencies and their association with selective food intake.

There is also increasing interest in the use of prebiotics, so-called functional foods that are not broken down by host enzymes but are instead fermented by gut microbes, in turn stimulating their growth and activity (74, 75). Prebiotics such as partially hydrolyzed guar gum (76), galactooligosaccharide (77), and bovine colostrum product (78) have contributed to a reduction in gastrointestinal symptoms: mainly abdominal pain and increased bowel movement along with changes in the membership of the gut microbiota. Suggestions to combine prebiotics with probiotics or exclusion diets (e.g., GF/CF diet) to improve efficacy have the potential to have long-term effects but to date have been demonstrated with limited effectiveness, partially due to the vast differences in treatment duration, dosage, and regimens (79).

Antibiotics

Antibiotic usage has substantially increased in the past few decades (80), accompanied by a concomitant increase in antibiotic-resistant infections (80, 81). While antibiotics can be exceptionally effective against various diseases of bacterial origin, the widespread non-targeted use of antibiotics calls for stricter guidelines on antibiotic stewardship (82). Narrow-spectrum antibiotics or even vaccines targeting specific pathogens in the gut (83) could be used to improve gut health and cure infections (84). Doing so may open up an ecological niche in the gut following the eradication of specific microbes, therefore pro- or prebiotics may be beneficial to fill this gap with commensal beneficial microbes (85, 86).

A small oral vancomycin treatment study resulting in short-term beneficial changes in ASD behaviors was one of the first few papers to suggest a link between the gut-microbiota-brain axis and ASD (87). The authors suggested that the vancomycin antibiotic treatment may temporarily deter colonization of potentially pathogenic bacteria in the gut, hinting at the promise of gastrointestinal therapies to ameliorate ASD symptoms.

Fecal microbiota transplants

Fecal transplants, whereby the gut microbes from a healthy donor(s) are introduced into the gut of an affected individual, have received considerable recent attention. To date, they are most notable for their success in treating persistent *Clostridioides difficile*-associated diarrhea (88, 89), with a highly impressive >90% rate for curing recalcitrant infections (90). This approach has now been refined to reduce the risk of conferring infection and disease and improve patient palatability (91). Careful consideration of the fecal donor sample is crucial as various unwanted effects such as new-onset obesity may occur after fecal transplant therapy (92). Currently, the euphemistically termed “microbiota transfer therapy” has been proposed for a variety of human conditions, such as obesity (93), ulcerative colitis (94), and ASD (95). However, to date, the efficacy of this approach has only been unequivocally proven for *C. difficile* infections.

Microbiota transfer therapy has shown promise to shift gut microbial composition in individuals with ASD towards neurotypical gut profiles (95). Importantly, these changes were accompanied by improvements in GI symptoms and behaviors associated with ASD, at least for eight weeks following a 10-week treatment period. Although promising, these results need to be viewed cautiously: this was an open-label study where placebo effects could not be assessed. Moreover, confounding effects such as antibiotic treatment to suppress pathogenic bacteria, and acid-pump inhibition medication to reduce stomach acidity and improve the survival rate of the transferred microbes, could also have impacted the observed effects (95). Encouragingly, however, Kang et al. subsequently reported sustained ASD behavior and GI symptom improvements for at least two years post-microbiota transfer therapy (96).

ANIMAL MODELS OF ASD

Although ASD is only known to affect humans, there are many different animal models of ASD. The use of such models can enable a better understanding of the potential causes and mechanisms underlying ASD, which could ultimately result in the development of treatments for those that require them. Existing animal models of ASD include nematodes (*Caenorhabditis elegans* (97)), monkeys (*Macaca* sp. (98)), rats (*Rattus norvegicus* (99)), and the widely used mice (*Mus musculus* (100)). While using animals that are most similar to humans, such as monkeys, would be logical, this brings wider complications of financial burden, long developmental periods, and ethical concerns.

ASD is generally diagnosed in a clinical setting, whereas autism-like behaviors in animals are assayed through a series of behavioral tests. To determine if the animal model accurately reflects the human disorder, determining construct, face, and predictive validity is important (101). Construct validity refers to how well the causative mechanism of the disorder in the animal model represents the human condition; this can be difficult to achieve for ASD models as there is no single mutation that represents all ASD cases. Therefore, there is an intention to mimic environmental and/or genetic aspects of ASD in humans in various ASD animal models. Face validity is the extent to which the animal model phenotype represents the human disorder on a behavioral and cellular level. Behavioral tests are well established in mouse models of ASD (102). However, ASD behaviors such as deficits in social-emotional reciprocity and non-verbal communicative behaviors are challenging to model in mice. Predictive validity relates to how well the treatments for reversing or preventing some of the symptoms of the human disorder are reflected in the animal model. Currently, there are no reliable “treatments” for ASD, making it difficult to achieve predictive validity in animal models of ASD.

Mouse models of ASD can be created using varying approaches. For example, the maternal immune activation (MIA) mouse model is created by infecting a pregnant mouse with either live viruses or immunogenic substances (e.g., the bacterial endotoxin Lipopolysaccharide or the synthetic double-stranded RNA analogue polyinosinic–polycytidilic acid) to stimulate an immune reaction, resulting in offspring that display ASD-like behaviors, as well as autism-related cellular

pathological changes (103). This model is based on data for humans that have correlated viral infection in the first trimester of pregnancy with a significantly increased likelihood of the offspring developing ASD (104). Administering valproic acid in utero is another method used to create an ASD mouse model that exhibits ASD-associated lesions in the motor nuclei of the brain (105). Buffington et al. (66) identified a specific bacterium (*Lactobacillus reuteri*) as being absent from mice in the maternal high-fat diet (MHFD) mouse model of autism spectrum disorder. After introducing *L. reuteri* into the MHFD mice, the social behavior deficits were restored with further effects on synapses and oxytocin levels in the brain, providing further evidence of a brain-gut connection (66) (Figure 2).

Inoculation of the probiotic bacterium *Bacteroides fragilis* in the MIA mouse model (Figure 2) led to a reversal of some ASD-like behaviors, restored blood serum bacterial metabolite levels, and improved gut epithelial barrier function (65) (Figure 2). This study illustrated a possible mechanism of ASD, as well as suggesting a potential therapy. However, translating results from animal models into humans requires caution. In animal models of other disorders, such as schizophrenia, the treatments seem promising, but the same beneficial effects are not always observed when treating humans (106). However, by using animal models of ASD, one can have more control over environmental variables, which are valuable for understanding this complex disorder.

Genomic animal models are developed using ASD-related genetic mutations/variants detected in humans (107), which generally also display face and construct validity. Although these models do not embody all aspects of ASD, they do encapsulate certain features of ASD that are useful to study. Animal models are invaluable for understanding the histopathological and pathophysiological mechanisms of ASD in the brain, as well as for testing treatments that may aid people carrying the same genetic/histopathological changes as the animal model.

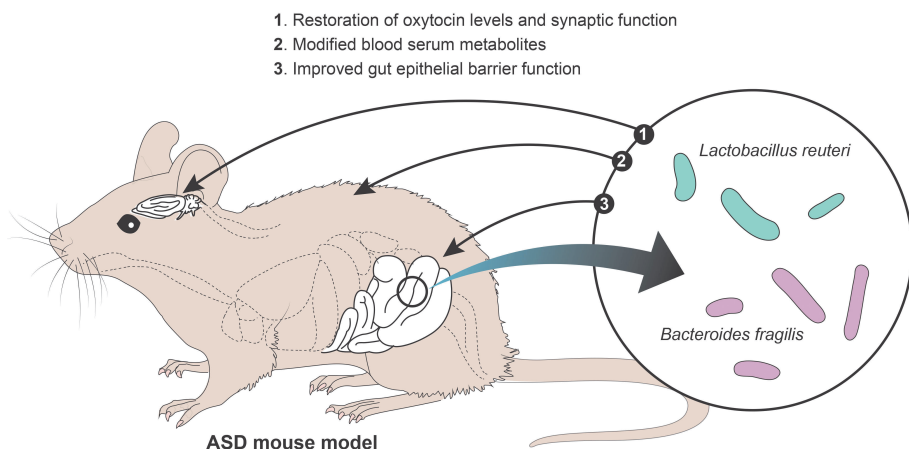


Figure 2. The effects of microbial-based therapies on mouse models of ASD (65, 66). Introduction of bacteria such as *Lactobacillus reuteri* and *Bacteroides fragilis* in mouse models of ASD have been shown to restore oxytocin levels and synaptic function in the brain, modify and restore blood serum metabolites and improve the gut epithelial barrier function.

The marked heterogeneity of ASD genetics makes it hard to model the exact deletions, frameshift mutations, or translocation mutations in different individuals with ASD. The SHANK family of synaptic proteins (SHANK1-3) is highly linked to ASD, with point, deletion, and frameshift mutations observed in people affected by ASD. In mice, the *Shank3* gene is found on chromosome 15qE3 (108), and many *Shank3* mutant mouse lines with different *Shank3* gene mutations have been created (109–114). A major ASD-*Shank3* mouse model that has been widely utilized in ASD research is the *Shank3B*^{-/-} ex13–16 mouse model, in which exons 13–16 of the *Shank3B*^{-/-} gene are deleted (7); this maintains the overall effect of knocking out the gene, resulting in SHANK3 protein loss of function as seen in human ASD cases as well as Phelan-McDermid Syndrome. Most *Shank3* mouse model studies use homozygous *Shank3* mutant mice, whereas heterozygous *Shank3* mutations are also found in individuals with ASD. The *Shank3B*^{-/-} mutant mouse model (ex13–16) displays ASD-like behaviors, including repetitive behaviors (grooming) and deficits in social interaction (7). In addition to this, the loss of several proteins in the post-synaptic structure is observed in the mouse model, which is also seen in individuals with ASD (7). These behavioral and cellular mechanisms observed in these mice reflect those that occur in people with ASD, supporting the face validity of the *Shank3B*^{-/-} (ex13–16) mouse model. In relation to the gut-brain axis, differences in *Shank3* mouse gut microbiota compared to wild-type mice have been observed, and subsequent *Lactobacillus reuteri* probiotic treatment was beneficial towards ASD behaviors; this provides further evidence that the gut-microbiota-brain axis in ASD can be manipulated (115). Overall, the use of ASD mouse models is crucial towards understanding how microbes can affect a neurodevelopmental condition (ASD) and how we can target this towards treatments for gastrointestinal disorders and behaviors associated with ASD.

DIETARY ZINC AND THE GASTROINTESTINAL TRACT IN ASD

Zinc is a key micronutrient required for many processes in the body, including enzyme functionality (116), immune system functioning (117), hormone production (118), DNA repair (119), and protection against oxidative stress (120). Dietary zinc is absorbed mainly in the small intestine, where intestinal mucosa metallothionein proteins, ZIP proteins, and zinc transporters control zinc homeostasis (121–123). Zinc deficiency is widespread in individuals with ASD and is also seen with attention deficit hyperactivity disorder (ADHD) and depression (124). Zinc deficiency can impair gastrointestinal function, conceivably resulting in further deficiencies of other micronutrients (125). ADHD and depression co-occur in some individuals with ASD, but while the role of zinc in the pathology of these conditions is unknown, zinc treatment has been trialed for these conditions (126, 127). Some of the gastrointestinal problems reported in ASD reflect increases in intestinal permeability (128, 129). This could affect the absorption of zinc, which primarily occurs in the gut, leading to the zinc deficiencies that have been described in individuals with ASD (130). On the other hand, zinc deficiencies from the diet could also affect the integrity of the intestinal epithelial membrane as zinc is required as a cofactor/catalyst for many membrane transporters and structural membrane proteins (131, 132).

Chronic zinc deficiency can affect the diversity, composition, and functional capacity of the gut microbiota (133), but to date, such analyses with the gut microbiota of humans or mice with ASD have not been undertaken. Short-chain fatty acids produced by gut microbes may contribute to lowering intestinal pH, in turn increasing zinc solubility and thus allowing increased dietary zinc absorption (134–136). Additionally, the gut microbiota may change the availability of zinc, which is required for many membrane transport systems essential for known pathogens such as *Campylobacter jejuni* (137). As a result, zinc can become a limiting factor, leading to competition for this micronutrient among microbes. On the other hand, excess dietary zinc may negatively affect the gut microbiota, as the host immune system uses zinc to protect against pathogens (138). Thus, regulation of zinc homeostasis is important for both the health of the host and the gut microbiota.

The human immune system requires zinc to provide an innate and adaptive humoral response towards pathogens (139). Perturbations of the immune system can occur in ASD (140–142), but whether zinc plays a role in mediating this is unknown. Complex pathways connecting the central nervous system (CNS) and the immune system are essential, especially during the early stages of neurodevelopment (143). The findings of an altered immune system in some individuals with ASD (141, 144–146), together with the presence of a bidirectional immune system–CNS system, contribute to another model of ASD pathogenesis in which autoimmune reactivity could play a role.

Nutrient acquisition from the diet is partially compromised in zinc deficiency due to a reduction of functional zinc metalloenzymes and disaccharidases for digestion (147). Diarrhea and malnutrition can result from zinc deficiencies as the integrity and permeability of the gut epithelial barrier require zinc to maintain its function (148). These alterations make the gut more susceptible to pathogen establishment and growth, which can further reshape gut microbiota composition.

Some of the genes implicated in ASD interact with zinc: in particular, zinc interacts with the SHANK3 protein, the master regulator of synapses, to provide the essential post-synaptic density scaffolding network required for synaptic transmission in the brain (149). The SAM domain of SHANK3 proteins contains a zinc-binding site that requires zinc to create the scaffolding structure at the post-synaptic density (150). Zinc deficiency and the loss-of-function *Shank* gene mutations have implications for assembly, maintenance and neurotransmission function at the synapse, leading to loss of synaptic control mechanisms (151). Zinc uptake transporters (ZIP4 and ZIP2) co-localize with SHANK3 proteins at the plasma membrane: in *Shank3* $\alpha\beta$ knockout mice and *Shank3* knockdown human cell lines, there is reduced co-localization of zinc uptake transporters (ZIP4 in particular) and SHANK3 proteins at the membrane (152).

Brain electrophysiology studies have shown that zinc is responsible for stabilizing the SHANK3 post-synaptic density by supporting the association between several SHANK3 proteins (153). Arons and colleagues (149) found that zinc is important in regulating SHANK3 activation in the brain, as SHANK3 proteins are central to regulating synaptic transmission via a zinc-sensitive signaling system. Disruption of this zinc-sensitive signaling system has been observed in *Shank3* mutations related to ASD, which may have impacted the functionality and plasticity of synapses in the brain, possibly resulting in ASD behaviors (149, 154).

Increasing dietary zinc in *Shank3* mouse models of ASD reverses ASD-related behaviors in young mice and offspring of mice fed a zinc-supplemented diet (155, 156). Whether these dietary zinc effects partially or fully stem from changes in the gut, including gut permeability or the gut microbiome, is of significant interest.

CONCLUSION

There is increasing evidence for the gut-microbiota-brain axis and how the modulation of these systems may play a role in gastrointestinal health and modification of ASD-related behaviors. While many individuals on the autism spectrum may present with different gastrointestinal microbial communities from neurotypical people and experience gut disorders, the currently available treatments are limited. Supplemented dietary zinc treatment has shown potential in an ASD-mouse model with supported plasticity changes in the brain. Fecal microbial transfer therapy also shows promise towards changing the gut microbial community and has positively sustained long-term effects towards gastrointestinal issues in ASD. Understanding how these potential treatments involve the gut-microbiota-brain axis and what we can use to harness it with reproducibility in the future effectively is key.

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