Special Section on Drug Metabolism and the Microbiome—Perspective

Microbiome Disturbances and Autism Spectrum Disorders

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ABSTRACT

Autism spectrum disorders (ASDs) are considered a heterogenous set of neurobehavioral diseases, with the rates of diagnosis dramatically increasing in the past few decades. As genetics alone does not explain the underlying cause in many cases, attention has turned to environmental factors as potential etiological agents. Gastrointestinal disorders are a common comorbidity in ASD patients. It was thus hypothesized that a gut-brain link may account for some autistic cases. With the characterization of the human microbiome, this concept has been expanded to include the microbiota-gut-brain axis. There are mounting reports in animal models and human epidemiologic studies linking disruptive alterations in the gut microbiota or dysbiosis and ASD symptomology. In this review, we will explore the current evidence that gut dysbiosis in animal models and ASD patients correlates with disease risk and severity. The studies to date have surveyed how gut microbiome changes may affect these neurobehavioral disorders. However, we harbor other microbiomes in the body that might impact brain function. We will consider microbial colonies residing in the oral cavity, vagina, and the most recently discovered one in the placenta. Based on the premise that gut microbiota alterations may be causative agents in ASD, several therapeutic options have been tested, such as diet modulations, prebiotics, probiotics, synbiotics, postbiotics, antibiotics, fecal transplantation, and activated charcoal. The potential benefits of these therapies will be considered. Finally, the possible mechanisms by which changes in the gut bacterial communities may result in ASD and related neurobehavioral disorders will be examined.

Introduction

Autism spectrum disorders (ASDs) are currently estimated to affect about one in every 68 children (http://www.cdc.gov/ncbddd/autism/data.html), with the number of diagnosed boys outnumbering girls by 5:1 (Bespalova and Buxbaum, 2003; Fombonne, 2005). The recent surge in frequency may be partly attributed to increased awareness/diagnosis; however, intrinsic and extrinsic factors (including environmental chemicals, diet alterations, metabolic status, and microbiota changes) cannot be excluded (Rizzo et al., 1997; Bello, 2007; Newschaffer et al., 2007; Deth et al., 2008; Rogers, 2008; Leeming and Lucock, 2009; Currenti, 2010; Landrigan, 2010; Beard et al., 2011; LaSalle, 2011; Chaste and Leboyer, 2012; Krakowiak et al., 2012; Jones et al., 2013; Lyall et al., 2013; Gore et al., 2014; Sullivan et al., 2014). While extensive heterogeneity exists in ASD patients, this class of disorders is typified by a range of symptoms including decreased verbal communication and social skills, outright withdrawal, insistence on sameness in the daily routine, engagement in repetitive behaviors, and heightened response to external stimuli. The ASD core symptoms are classified based on the revised autism diagnostic interview (Rutter et al., 2003), autism diagnostic observation schedule (Lord et al., 2002), and social responsiveness scale (Constantino et al., 2000).

Comorbidity with gastrointestinal (GI) disturbances is often observed in ASD patients with estimates ranging from 9 to 70% (Buie et al., 2010). This diverse range likely reflects variation in sample size, self-reporting, and specialty area of the reporting clinic. Even so, it is recognized that there is a link between the GI system and brain function, thereby leading to the coinage of the term gut-brain axis. With mounting evidence indicating that the gut microbiota populations may underpin some neurobehavioral disorders, this term has been since broadened to the gut-microbiome (microbiota)-brain axis (Collins and Bercik, 2009; Rhee et al., 2009; Cryan and Dinan, 2012). In fact, 90% of the cells contained in most mammalian organisms are of prokaryotic origin. The gut microbiota population is comprised of 500–1000 denizen species representing 7000–40,000 bacterial strains spanning 1800 genera (Luckey, 1972; Ley et al., 2006; Frank and Pace, 2008; Qin et al., 2010; Clemente et al., 2012; Douglas-Escobar et al., 2013; Forsythe and Kunze, 2013; Gilbert et al., 2013). The 1×10^3 to 1×10^4 microorganism gut inhabitants possess a diverse and complex genome encompassing approximately 150 times more genes than the human genome (Gill et al., 2006; Qin et al., 2010). The gut microbiota are thus considered a forgotten organ in and of itself with the 100 trillion prokaryotic cells weighing about 1–2 kg (O'Hara and Shanahan, 2006; Forsythe and Kunze, 2013). The Human Microbiome Project was initiated with the overarching goal of understanding the comprehensive effects microbiota populations exert on host health and disease, including neurologic disorders (Human Microbiome Project

ABBREVIATIONS: ASD, autism spectrum disorder; BA, butyric acid; BBB, blood brain barrier; DA, D-arabinitol; 4-EPS, 4-ethylphenylsulfate; FMT, fecal microbiota transplantation; GF, germ free; GI, gastrointestinal; HPA, hypothalamic-pituitary axis; LPS, lipopolysaccharide; MD, mitochondrial disease; MIA, maternal immune activation; NE, norepinephrine; PPA, propionic acid; SCFA, short chain fatty acid; SPF, specific pathogen free; VPA, valproic acid.

Consortium 2012a,b). To understand this interaction, we need to examine when microbes initially inhabit the gut.

Bacterial colonization of the gut likely occurs at the time of birth when infants born via natural delivery are inoculated with a complex mixture of maternal vaginal microorganisms. Thus, concern has a risen that babies delivered via Caesarean section may receive insufficient maternal bacterial transmission (Murgas Torrazza and Neu, 2011; Walker, 2013; Mueller et al., 2015). Notwithstanding, the intestinal microbiome of most infants resembles that of their mother; however, after 1 year of age a complex and distinct microbiome profile develops (Mackie et al., 1999; Palmer et al., 2007). The perinatal data suggest that there are critical windows for gut microbiome establishment and later health and disease effects. This temporal requirement may have important ramifications for potential preventative and therapeutic remediation strategies.

Animals are dependent upon intestinal microbes for several functions. Gut microbiota synthesize various essential vitamins and cofactors, such as folate and vitamin B_{12}, which may affect DNA and histone protein methylation (Le Galliard et al., 2008; LeBlanc et al., 2013). Bacteria also metabolize complex lipids, proteins, and carbohydrates, including those that are indigestible by the host (Hooper et al., 2002; Saulnier et al., 2008; Dai et al., 2012). Bacterial fermentative processes result in the production of various short chain fatty acids (SCFAs), such as acetic, propionic, and butyric acid (BA) (Hooper et al., 2002). The bacterial-derived SCFAs may serve as a fuel source for enterocytes lining the intestinal system; however, these chemicals can also alter the intercellular spaces between the cells, resulting in a leaky gut that allows for more metabolites and bacteria to pass through the epithelial barrier, which as discussed subsequently can lead to detrimental neurologic effects. Furthermore, disturbances in the gut and other microbiomes (dysbiosis) can affect host immunity and neurobehavioral responses (Cryan and Dinan, 2012; Douglas-Escobar et al., 2013; Ding and Schloss, 2014; Galland, 2014; Stilling et al., 2014; Sherman et al., 2015). In this review, we will primarily focus on how alterations in microbiomes, especially in the gut, and their products affect the risk for ASD and related neurobehavioral disorders.

First, the evidence in animal models indicating that gut dysbiosis can lead to ASD-like behavioral disturbances will be addressed. Next, we will review the handful of human epidemiologic studies correlating microbiota and bacterial metabolite changes in ASD patients. While the majority, if not all, of the studies to date have focused on the potential role of gut microbiome alterations and ASD, it is now clear that other organs and systems, including the oral cavity (Ding and Schloss, 2014), lung (Dickson et al., 2014), placenta (Aagaard et al., 2014; Amarasekara et al., 2014; Antony et al., 2014; Doyle et al., 2014), and vagina (Stumpf et al., 2013; Ding and Schloss, 2014), possess unique microbiomes that may influence distal target systems. Therefore, we will consider whether disruptions in these other microbiomes may be contributing etiologies to ASD risk. Prebiotics, probiotics, antibiotics, and related treatments have been proposed to be useful adjuvant treatments for ASD. Thus, the evidence indicating that these factors may improve clinical signs will be explored. Finally, a sampling of the potential mechanisms by which gut dysbiosis may contribute to ASD will be considered.

ASD and Gut Microbiome Animal Model Studies

Table 1 summarizes the animal model studies to date linking alterations in the gut microbiota and neurobehavioral changes. The first evidence associating gut microbiota disturbances and neurobehavioral disorders originated from germ-free (GF), axenic mice.

They were delivered via Caesarean section and then maintained in a sterile gnobiotic environment. These mice were likely devoid of any microorganisms. Direct inferences can then be made on how the absence or presence of gut microbiota may influence behavioral patterns. The initial study demonstrated that restraint stress of adult GF mice resulted in hypersecretion of adrenocorticotropic hormone and corticosterone (two commonly associated stress hormones) relative to specific pathogen-free (SPF) controls (Sudo et al., 2004). However, reconstitution of GF mice with *Bifidobacterium infantis* ameliorated the exaggerated hypothalamic-pituitary axis (HPA) responses. Transplantation of feces from SPF to GF animals partially reversed the hormonal abnormalities but only if such intervention was performed early in life; suggestive microbes must colonize the gut at a critical postnatal period for normal neural programming to occur. This study also showed the essential neurotropin, BDNF, and NR2A proteins were suppressed in the cerebral cortex and hippocampus of GF mice.

While this pioneering study did not test behaviors in GF animals, the hormonal findings suggest GF animals may be more anxious. This prediction, however, was not borne out. Instead, GF animals were less anxious and more exploratory (Diaz Heijtz et al., 2011; Neufeld et al., 2011b; Clarke et al., 2013). Furthermore, the anxiolytic behavior was resistant to SPF fecal transplantation (Neufeld et al., 2011a). GF mice did show cognitive deficits in nonspatial and working memory tasks (Gareau et al., 2011). A subsequent study supported these original findings, especially in males, and further revealed these animals avoided social situations (Desbonnet et al., 2014). Postweaning bacterial colonization abolished the latter but not the former disturbance, in line with the critical window for gut microbiota colonization.

Two approaches have been employed to discern how gut dysbiosis may lead to behavioral abnormalities in animal models that recapitulate the clinical signs observed in ASD patients. The first approach entails administration of bacterial metabolites or virulence factors to such rodent models. Other studies instead compared the gut microbiome in affected models to control counterparts, and in some cases to whether fecal transplantation/monospecies transfer improves the symptomology.

Daily injection of pregnant rats with the bacterial-metabolite propionic acid (PPA) (500 mg/kg s.c.) or the virulence factor, lipopolysaccharide (LPS) (50 mg/kg) led to ASD-like behavioral impairments in male and female offspring, including olfactory-mediated social recognition abnormalities, persistence in examining a novel object, hyperlocomotion, and social deficits (Foley et al., 2015). Two additional studies by this same group with a similar approach showed that pre- and postnatal exposure to these substances resulted in other sex-dependent behavioral disruptions (Foley et al., 2014a, b). Males, but not females, subjected to prenatal LPS treatment were hypersensitive in acoustic startle testing. In contrast, females exposed to pre- and postnatal PPA (double hit) became sensitized in this test. In prepulse inhibition testing, animals are first exposed to a weak acoustic stimulus (prepulse), which should decrease the reflexive flinching startle response when they are then exposed to a more intense stimulus (pulse). Normal animals should be able to filter out irrelevant auditory information, whereas those with neurobehavioral deficits are not be able to do so. Females exposed to PPA during the prenatal period exhibited a lower prepulse inhibition threshold; however, similar effects were not observed in males. Males and females exposed to prenatal PPA treatment spent less time in the center of the open-field maze, suggestive of increased anxiety-like behaviors. Anxiogenic behaviors were also exhibited by females exposed to PPA pre- and postnatally when tested in the elevated plus maze, where more time spent in the closed arms suggests increased anxiety. Increased amount of time in the open arms is indicative of

TABLE 1

Animal models linking gut microbiota changes to ASD-like behavioral disruptions

Publication	Animal Model	Type of Treatment	Major Finding
Sudo et al. (2004)	GF and SPF mice	• The gut of GF mice was reconstituted with *B. infantis* via oral administration to the parents with transmission to the offspring at the neonatal stage. • GF mice received fecal transplantation from SPF animals (0.5 ml of 1×10^{-2} dilution of fresh SPF mouse feces 1 or 3 weeks prior to the stress protocol at 9 or 17 weeks of age).	• Adult GF mice subjected to restraint stress exhibited hypersecretion of ACTH and corticosterone. • Adult GF mice had suppression of neurotropin, BDNF, and NR2A in the cerebral cortex and hippocampus. • Reconstitution with *B. infantis* alleviated the exaggerated HPA responses. • Feces from SPF partially reversed the hormonal abnormalities in GF mice, but only if done early in life.
Diaz Heijtz et al. (2011); Gareau et al. (2011); Neufeld et al. (2011b); Clarke et al. (2013); Desbonnet et al. (2014)	GF and conventionally colonized mice	• No treatment in Diaz Heijtz et al. (2011), Gareau et al. (2011), Neufeld et al. (2011b), and Clarke et al. (2013). • Postweaning bacterial colonization (details not specified) in Desbonnet et al. (2014).	• Combined studies indicate GF mice demonstrate reduced anxiety-like but increased exploratory behaviors and cognitive deficits in nonspatial and working memory tasks, and males, especially, avoided social situations. • Postweaning bacterial colonization mitigated the avoidance of social situations by GF mice (Desbonnet et al., 2014).
Foley et al. (2015)	Rats	• Pregnant P_0 rats were treated with the bacterial metabolite, PPA (500 mg/ kg b.wt. s.c. on GDs 12–16) or the LPS (50 mg/ kg b.wt. s.c. on GD 15 or 16). Control pregnant P_0 rats received vehicle control on GDs 12–16 or 15 to 16.	• F_1 male and female offspring derived from the treated rats showed impairments in olfactory-mediated social recognition, persistence in examining a novel object, hyperlocomotion, and disruptions in social behaviors.
Foley et al. (2014a, b)	Rats	• Pregnant P_0 rats were treated with the bacterial metabolite, PPA (500 mg/ kg b.wt. s.c. on GDs 12–16) or the LPS (50 mg/ kg b.wt. s.c. on GD 12 or GDs 15 to 16), and these treatments were continued during the postnatal period. Control pregnant P_0 rats received vehicle control on GDs 12, 12–16, or 15 to 16.	• F_1 males subjected to prenatal LPS treatment were hypersensitive in acoustic startle testing. • F_1 females exposed to pre- and postnatal PPA (double hit) were sensitized in this test. • F_1 females exposed to PPA during the prenatal period had reduced prepulse inhibition, but similar effects were not observed in males. • F_1 males and females exposed to prenatal PPA treatment spent less time in the center of the open field maze, suggestive of increased anxiety-like behaviors. • Anxiogenic behaviors were also exhibited by F_1 females exposed to PPA pre- and postnatally when tested in the EPM. • The double-hit female group also engaged in more repetitive behaviors in the open-field tests.
Thomas et al. (2012)	Rats	• Rats (47–49 days of age) were exposed twice daily to intraventricular injection of PPA (4.0 μl of a 0.26 M solution) for an acute period (8 days).	• Treated rats exhibited hyperlocomotion and architectural changes in the brain.
Shultz et al. (2008)	Male rats	• Adult male rats were assigned to one of four treatments: 1. PPA (4 μl of 0.26 M solution) 2. SA (4 μl of 0.26 M solution) 3. PROP (4 μl of 0.26 M solution) 4. PBS vehicle control (4 μl of 0.1 M solution)	• PPA-treated rats demonstrated social behavioral deficits evidenced by increased mean distance apart and less time spent in proximity to other animals, reduced playful bouts, and altered response to playful intentions from companions. • Another SCFA, sodium acetate, induced similar impairments but no effects were detected with PROP (alcohol analog of PPA). • Reactive astrogliosis (neuroinflammation) occurred in the brains of PPA-treated rats.
Shultz et al. (2015)	Seizure-prone (FAST) and seizure-resistant (SLOW) male rats	• Nine week old male rats were assigned to one of four groups: 1. FAST + PPA (4μl, 0.26 M) 2. SLOW + PPA (n = 14) 3. FAST + PBS vehicle control (4μl) 4. SLOW + PBS	• PPA induced social abnormalities in FAST and SLOW rat strains. • PPA treatment resulted in neuroinflammation (astrogliosis) in the corpus callosum and cerebral cortex. • Neuroinflammatory effects were more prominent in FAST compared with SLOW rats.
Shultz et al. (2009)	Male rats	• Adult male rats were assigned to one of five treatment groups: 1. PPA-5 (4 μl of 0.26 M solution) 2. PPA-3 (4 μl of 0.26 M solution) 3. SA (4 μl of 0.26 M solution) 4. PROP (4 μl of 0.26 M solution) 5. PBS (4 μl)	• PPA-treated rats showed impairments in spatial acquisition and reversal training in the water maze test and beam task. • Neuroinflammation was increased in the brains of PPA-treated rats.

(*continued*)

TABLE 1—*Continued*

Publication	Animal Model	Type of Treatment	Major Finding
de Theije et al. (2014)	Mice	• Pregnant P_0 mice were treated on GD 11 with VPA (600 mg/kg b.wt. s.c.) or PBS (controls).	• F_1 VPA-treated offspring demonstrated social behavioral deficits. • The composition of SCFAs was changed in the F_1-treated offspring. • Gut dysbiosis with changes in the Bacteroidetes and Firmicutes phyla and Desulfovibrionales resulted in F_1 exposed offspring. • F_1-exposed male offspring had alterations in *Alistipes, Enterohaabdus, Mollicutes, and Erysipelotrichalis* genera. • Gut microbiome disturbances were more pronounced in F_1 male than female offspring.
Hsiao et al. (2013)	MIA mouse model and wild-type (naive) mice	• Pregnant P_0 mice were treated on GD 12.5 with saline (control) or the immunostimulant Poly I:C acid, 20 mg/kg b.wt. via i.p. injection). This latter treatment results in MIA offspring. • MIA offspring were orally treated with *B. fragilis* (ATCC 9343) or vehicle every other day for 6 days at weaning. 1×10^{10} CFUs of freshly grown *B. fragilis* or 1.5% sodium bicarbonate was administered in sugar-free applesauce. • Wild-type mice were treated with the bacterial metabolite (4-EPS, 30 mg/kg b.wt. via i.p. injection from 3 to 6 weeks of age).	• MIA offspring demonstrated disruption of the intestinal barrier. • Approximately 8% of bacterial metabolites were altered in MIA offspring with a leaky gut. • MIA offspring exhibited communication deficits, and stereotypic, anxiety-like, and sensorimotor behaviors. • Supplementation of MIA offspring with *B. fragilis* restored the intestinal barrier and mitigated the gut dysbiosis, metabolomics changes (including for 4-EPS), and behavioral changes. • Wild-type mice treated with 4-EPS exhibited anxiety-like behaviors similar to those observed in other ASD animal models.

ACTH, adrenocorticotropic hormone; CFU, colony forming unit; EPM, elevated plus maze; GD, gestational day; PBS, phosphate-buffered saline; Poly I:C, polyinosinic:polycytidylic; PROP, 1-propanol; SA, sodium acetate.

anxiolytic or increased exploratory behaviors. The double-hit female group engaged in more repetitive behaviors in the open-field tests. Intraventricular injection of PPA for an acute period (8 days) to rats resulted in hyperlocomotion and architectural changes in the brain reminiscent of the pathobiology observed in select ASD cases (Thomas et al., 2012).

Intracerebroventricular injection of PPA (4 ml of 0.26 M solution) to Long-Evans, seizure-resistant (SLOW), and seizure-prone (FAST) rats led to social behavioral impairments, exemplified by increased mean distance apart and less time spent in proximity to other animals, reduced playful bouts, and altered response to playful intentions from companions. Neuroinflammation (astrogliosis) was evident in these animals (Shultz et al., 2008). This same treatment also impaired cognition and sensorimotor ability in Long-Evans rats (Shultz et al., 2009; Shultz et al., 2015).

Valproic acid (VPA) is a commonly used drug to treat epilepsy and other neuropsychological disorders. A linkage exists between maternal usage of this drug while pregnant and later risk of ASD in offspring (Christensen et al., 2013). Mice exposed in utero to VPA subsequently demonstrated autistic-like social behavioral deficiencies, altered intestinal SCFAs, and gut dysbiosis (de Theije et al., 2014). VPA-exposed animals displayed changes in the operational taxonomic units for genera classified within the main phyla of Bacteroidetes and Firmicutes and the order of Desulfovibrionales, which is similar to bacterial shifts observed in human ASD patients (described subsequently). Male offspring developmentally exposed to VPA demonstrated alterations in operational taxonomic units in the Alistipes, Enterohaabdus, Mollicutes, and Erysipelotrichalis genera. In general, microbiome differences were more pronounced in VPA-exposed males than females. The gut microbiome of these males positively correlated with increasing levels of cecal butyrate and neutrophil inflammation but was negatively linked to increasing intestinal concentrations of serotonin and social behavior scores.

The most convincing study linking gut microbiota alterations and ASD-like behaviors in an animal model employed the maternal immune activation (MIA) mouse model for ASD (Hsiao et al., 2013). Gestational administration to the mother of the immunostimulant polyinosinic:polycytidylic acid recapitulates the offspring neuro-disruptive effects of a viral infection. The data revealed that this treatment significantly disturbed the offspring gut-microbiome-brain axis, including compromising the intestinal barrier, which was likely due to gut dysbiosis with ~8% of bacterial metabolites diverging in MIA offspring with a leaky gut. Notably, administration of the human commensal bacteria *Bacteroides fragilis* restored the gut permeability and microbiota population, corrected the metabolome changes, and mitigated the defects in communicative, stereotypic, anxiety-like, and sensorimotor behaviors. One metabolite restored to normal levels in the treated MIA offspring was 4-ethylphenylsulfate (4-EPS), which has been implicated as an autistic biomarker (Persico and Napolioni, 2013). Administration of 4-EPS sulfate to wild-type mice replicated the anxiety-like behaviors observed in ASD animal models (Hsiao et al., 2013). Collectively, this single study provides strong causative evidence indicating that gut dysbiosis results in a cascade of effects culminating in ASD-like behavioral disturbances. However, probiotic treatment of this animal model may reverse pathologic changes in the gut, bacterial metabolic disruptions, and mitigate the neurobehavioral abnormalities.

Human Epidemiologic Studies Linking Gut Dysbiosis and ASD

GI system disorders are a common comorbidity in ASD patients (Buie et al., 2010; Mayer et al., 2014). Disruptions in the gut microbiota may be one common thread linking these two disparate systems. For instance, intestinal colonization by the anaerobic bacteria, *Clostridium tetani*, was postulated to increase the risk and severity of ASD; however, this past work did not test for a direct linkage (Bolte, 1998). These bacteria produce a neurotoxin that may reach the brain via the vagus nerve. Once there, the chemical impacts neurotransmitter release precipitating a range of behavioral deficits. Antimicrobial treatment against this disease partially alleviates the stereotypic behaviors observed in these patients.

Polymerase chain reaction–based approaches have paved the way to isolating the microbe changes in ASD children with and without concurrent GI symptoms, as detailed in Table 2. Only one study to date has suggested that there is no association between the gut microbiota composition and ASD symptoms (Gondalia et al., 2012). Other follow-up studies showed other Clostridial groups to be signficantly elevated in ASD children, such as *Clostridium bolteae* and clusters I and XI (Song et al., 2004). Another study by this group revealed nine species of Clostridium present in ASD but not control children, where three unique species were identified. Non-spore-forming anaerobes and microaerophilic bacteria were abundant in the stool of ASD children but absent in control children (Finegold et al., 2002). *Clostridium histolyticum* (Clostrium clusters I and II) is another toxin-producing species abundant in the fecal flora of ASD children. Nonautistic siblings possessed intermediate levels of this microbe, suggesting that it can be transmitted in the home environment (Parracho et al., 2005).

Newer technologies, such as 16S rDNA sequencing, have revealed other gut microbiota imbalances in ASD children. *Desulfovibrio*, another anaerobic bacillus possessing several virulence factors and generating various metabolic by-products, was plentiful in ASD children. As with *C. histolyticum*, the fecal contents of non-ASD siblings contained intermediate amounts of *Delsulfovibrio* (Finegold, 2011). An earlier report suggested that *Delsufovibrio* spp. was elevated in the stool of severely autistic children. These children also showed high amounts of fecal *Bacteroides vulgatus*. Dybiosis was evident at the phylum level, with autistic showing greater amounts of Bacteroidetes in the stool than controls. In contrast, Firmicutes predominated in the stool of control children. The two groups also had significant differences in the phyla Actinobacterium and Proteobacterium (Finegold et al., 2010).

Slovakian children with ASD displayed significant reduction in the Bacteroidetes/Firmcutes ratio but increased amount of *Lactobacillus* spp. *Delsulfovibrio* spp. showed a trend to increase, especially with increasing autistic severity (as determined by the autism diagnostic interview and restricted/repetitive behavior subscale score). The clinical severity of GI symptoms was positively correlated with autism severity. Supplementation of these patients with a probiotic diet corrected the imbalanced Bacteroidetes/Firmicutes ratio, suppressed *Delsufovibrio* spp., and increased the amount of *Bifidobacterium* spp. present in the stool (Tomova et al., 2015).

Another study suggested ASD children exhibited suppression of transcripts encoding disaccharidases, hexose transporters, and the transcription factor CDX2. The host transcriptomic changes correlated with the degree of gut dysbiosis observed in this ASD child cohort, as revealed by a decrease in *Bacteroidetes* and ratio of *Bacteroidetes* to Firmcutes, and greater preponderance of *Betaproteobacteria* in the intestinal biopsy samples (Williams et al., 2011).

ASD children with and without GI disorders possessed greater amounts of fecal *Sutterella* spp.; whereas, *Ruminococcus torques* was elevated in the stool of children with ASD and GI symptoms compared to those without such disorders (Wang et al., 2013). Another study indicated that in addition to phylum changes in Bacteroidetes, Firmicutes, Fusbobacteria, and Verrucomicrobia in ASD compared with healthy children, other microbial disruptions were evident between these two groups. Caloramator, *Sarcina*, and Clostridium genera were greater in ASD children. Variations within the Lachnospraceae family were also observed. ASD children showed high amounts of fecal Bacteroidetes genera, select *Alistipes* and *Akkermansia* species, and Sutterellaceae; however, Enterobacteriaceae, Eubacteriaceae, and *Bifidobacterium* species were reduced in this group. Correspondingly, levels of free amino acids and volatile organic compounds within the stool were affected in the autistic group, with some increasing and others decreasing relative to controls (De Angelis et al., 2013). A separate study found similar findings with lower levels of Bifidobacteria species but greater abundance of the mucolytic bacterium, *Akkermansia muciniphilia* in ASD children (Wang et al., 2011). *Sutterella* spp. (*wadsworthensis* and *stercoicanis*) predominated in the gut microbiota of ASD children with concurrent GI dysfunction. However, these species were absent in children with GI symptoms only (Williams et al., 2012), further highlighting that such species might play an important link between the microbiota-gut-brain axis.

The presence of ASD rather than GI symptoms may be a better predictor of a less diverse gut microbiota composition (Kang et al., 2013). This past study also disclosed that the genera *Prevotella*, *Coprococcus*, and unclassified *Veillonellaceae* are reduced in the stool of ASD children. However, it is not clear whether the microbial changes were due to variation in dietary habits or directly correlated with ASD symptomology.

Associations exist between bacterial metabolites in the feces or urine and behavioral impairments in ASD, including children receiving antibiotic or probiotic treatment. Adams et al. (2011) reported a strong positive correlation between GI symptoms and ASD clinical severity. Furthermore, especially in those individuals on a probiotic treatment, decreased levels of fecal SCFAs (specifically acetate, propionate, and valerate) were noted. The stool of these children contained less *Bifidobacter* but greater amounts of *Lactobacillus*. Lysozyme was suppressed in children with autism, which may partly be attributed to the probiotic treatment. A separate study treated 11 children who had regressive-onset autism with a poorly absorbed oral antibiotic, vancomycin, and their disease symptomology was monitored. The treatment improved the behavioral performance of these children; however, the beneficial effects were transient, with the clinical signs recurring upon discontinuation of the antibiotic (Sandler et al., 2000).

In contrast to Adams et al. (2011), another study found fecal SCFAs were significantly higher in children with ASD. Acetic, butyric, isobutyric, valeric, and isovaleric acids were elevated in the stool of ASD children, whereas caproic acid was reduced. The ASD patients had greater concentrations of ammonia in the stool than controls (Wang et al., 2012). In the urine, the free amino acids glutamate and taurine were increased in ASD children, possibly suggesting disruptions in sulfur and amino acid metabolism. Disturbances in the patterns of the bacterial metabolites dimethylamine, hippurate, and phenylacetylglutamine were also identified in ASD patients (Yap et al., 2010). The concentration of D-arabinitol (DA) in urine was higher in affected children compared with controls before and after probiotic supplementation. However, the probiotic therapy appeared to partially alleviate the elevated urinary concentrations of DA and the DA/L-arabinitol ratio, and there was noticeable improvement in the behaviors of the cohort ASD children, particularly in their ability to concentrate and follow orders (Kałużna-Czaplińska and Blaszczyk, 2012).

The conclusions that can be drawn from the collective studies to date suggest that *Clostridia*, *Desulfovibrio*, *Sutterella*, and *Bacteroidetes* are elevated in the stool of ASD children. In contrast, *Firmicutes*, *Prevotella*, and *Bifidobacter* tend to be reduced in these patients. The gut microbe changes are associated with alterations in fecal concentrations of SCFAs and urinary concentrations of amino acids and ammonia. Conflicting reports exist as to whether antibiotics and probiotics are useful in treating bacterial imbalances observed in ASD patients (discussed in more detail subsequently). Further studies are needed with larger data sets to perform side-by-side comparisons of gut microbiota, fecal and urinary analysis for bacterial SCFAs, amino acids, and ammonia in ASD patients with and without GI symptoms and those who have and

TABLE 2

Human epidemiologic studies linking gut microbiota changes to ASD symptomology

Publication	Cohort Population	Type of Analysis and/or Treatment	Major Finding
Gondalia et al. (2012)	ASD children without GI dysfunction ($n = 23$); ASD children with GI dysfunction ($n = 28$); neurotypical siblings ($n = 53$)	• bEFAP was performed on the stool samples from all three groups.	• Firmicutes (70%), Bacteroidetes (20%), and Proteobacterias (4%) comprised the major microbiota present in the stool regardless of disease state and sociodemographic features. • No evidence was found linking gut disease, dysbiosis, and ASD symptoms.
Song et al. (2004)	ASD children ($n = 15$); nonrelated controls ($n = 8$)	• Group and species-specific primers were designed to target the 16S rRNA genes for qRT-PCR analysis on the stool samples.	• *C. bolteae* and clusters I and XI were elevated in the stool of ASD children.
Finegold et al. (2002)	ASD children ($n = 13$); nonrelated controls ($n = 8$)	• Bacterial cultures were performed on the stool samples.	• Nine species of *Clostridium* were present in ASD but not the control children, where three unique species were identified. • Non-spore-forming anaerobes and microaerophilic bacteria were abudant in ASD but lacking in the control children.
Parracho et al. 2005)	ASD children ($n = 58$); control siblings ($n = 12$); nonrelated controls ($n = 10$)	• FISH analysis was performed on the stool samples.	• *C. histolyticum* (*Clostrium* clusters I and II) was abundant in ASD children. • Nonautistic siblings possess intermediate levels of this intestinal microbe.
Finegold et al. (2010)	ASD children ($n = 33$); control siblings ($n = 7$); nonrelated controls ($n = 8$)	• bEFAP procedure was used to analyze the stool samples.	• Phyla changes: Bacteroidetes and Protebacteria were increased in ASD children; whereas Firmcutes and Actinobacteria were less abundant in the stool of this group. • Genus changes: *Delsufovibrio, Bacteroides, Alkaliflexus, Acetanaerobacterium,* and *Parabacteroides* were elevated in ASD children, whereas *Clostridum, Weissella, Turicibacter, Anaerofilum, Pseudoramibacter, Ruminococcus,* and *Streptococcus* were decreased in this group.
Tomova et al. (2015)	ASD children ($n = 10$); control siblings ($n = 9$); nonrelated controls ($n = 10$)	• qRT-PCR analysis was performed on the stool samples. • Probiotic supplementation of one Children Dophilus capsule, which contains three strains of *Lactobacillus* (60%), two strains of *Bifidumbacteria* (25%), and one strain of *Streptococcus* (15%) was provided orally to the ASD group 3 times a day for 4 months.	• ASD children showed significant reduction in the Bacteroidetes/Firmcutes ratio but increased amount of *Lactobacillus* spp. • *Delsulfovibrio* spp. showed a trend to be increased in this group, especially with increasing autistic severity. • Clinical severity of GI symptoms was positively correlated with autism severity. • Probiotic supplementation of the autistic children corrected the imbalanced Bacteroidetes/Firmicutes ratio, suppressed *Delsufovibrio* spp., and increased the amount of *Bifidobacterium* spp. present in the stool.
Williams et al. (2011)	ASD children with GI dysfunction ($n = 15$); nonrelated controls with GI symptoms only ($n = 7$)	• qRT-PCR with human mRNA samples for SI, MGAM, LCT, SGLT1, GLUT2, Vilin, and CDX2. • Pyrosequencing of intestinal microbiota. • qRT-PCR of Bacteroidete and Firmicute 16s rRNA genes from intestinal biopsies.	• ASD children exhibited decreased expression of disaccharidases, hexose transporters, and the transcription factor CDX2. • The host transcriptomic changes correlated with the degree of gut dysbiosis observed in this ASD child cohort. • ASD children showed decrease amounts of *Bacteroidetes* and ratio of *Bacteroidetes* to Firmcutes, and greater preponderance of *Betaproteobacteria* in the intestinal biopsy samples.
Wang et al. (2013)	ASD children ($n = 23$); control siblings ($n = 22$); nonrelated controls ($n = 9$)	• qPCR on the stool samples.	• ASD children with and without GI disorders showed high amounts of fecal *Sutterella* spp. • *Ruminococcus torques* was elevated in the stool of children with ASD and GI symptoms compared with those without any such disorders.
De Angelis et al. (2013)	ASD children ($n = 10$); nonrelated controls ($n = 10$)	• bEFAP procedure was used to analyze the stool samples. • Measurement of free amino acid and volatile organic compounds in the stool samples.	• Phylum changes were evident in Bacteroidetes, Firmicutes, Fusbobacteria, and Verrucomicrobia in ASD compared with health children. • Caloramator, *Sarcina*, and Clostridium genera were greater in ASD children. • Variations within the Lachnospracease family were observed.

(continued)

TABLE 2—*Continued*

Publication	Cohort Population	Type of Analysis and/or Treatment	Major Finding
			• ASD children possessed greater amounts of fecal Bacteroidetes genera, select *Alistipes* and *Akkermansia* species, but Sutterellaceae, Enterobacteriaceae, Eubacteriaceae and *Bifidobacterium* species were reduced in this group. • Levels of free amino acids and volatile organic compounds within the stool were affected in this group.
Wang et al. (2011)	ASD children ($n = 23$); control siblings ($n = 22$); nonrelated controls ($n = 9$)	• qPCR on the stool samples.	• Bifidobacteria species was reduced and mucolytic bacterium, *Akkermansia muciniphilia*, was increased in the stool of ASD children.
Williams et al. (2012)	ASD children with GI dysfunction ($n = 15$); nonrelated controls with GI symptoms only ($n = 7$)	• Pyrosequencing and qPCR of ileal and cecal biopsies.	• *Sutterella* spp. (*wadsworthensis* and *stercoicanis*) predominated in the gut microbiota of ASD children with concurrent GI dysfunction, but these species were absent in children with GI symptoms only.
Kang et al. (2013)	ASD children ($n = 20$); nonrelated controls ($n = 20$)	• Pyrosequencing of the stool samples.	• The presence of ASD rather than GI symptoms was a better predictor of a less diverse gut microbiota composition. • The genera *Prevotella*, *Coprococcus*, and unclassified *Veillonellaceae* were reduced in the stool of ASD children.
Adams et al. (2011)	ASD children (not on probiotic supplement and those taking a daily probiotic, $n = 58$); nonrelated controls ($n = 39$)	• Bacterial culture of the stool samples. • Concentrations of Lysozyme, lactoferrin, secretory IgA, elastase, SCFAs were measured in the stool.	• There was a positive correlation with GI symptoms and ASD clinical severity. • Decreased number of SCFAs, specifically acetate, propionate, and valerate were identified in ASD children, especially those consuming a daily probiotic. • The stool of ASD children contained less *Bifidobacter* but greater amounts of *Lactobacillus*. • Lysozyme was suppressed in ASD children.
Sandler et al. (2000)	Regressive-onset autistic children ($n = 11$)	• The children received a 12-week treatment of oral vancomycin (125 mg 4 times a day for a daily dose of 500 mg). • Once antibiotic treatment was discontinued, the children received probiotic supplementation with a mixture of *L. acidophilus*, *L. bulgaricus*, and *B. bifidum* (40×10^9 CFU/ml). • Psychologic evaluations were performed before and after the antibiotic treatment.	• The vancomycin treatment improved the behavioral scores of ASD children, but the beneficial effects were transient with the behavioral symptoms recurring upon discontinuation of the antibiotic.
Wang et al. (2012)	ASD children ($n = 23$); nonrelated controls ($n = 31$)	• Concentrations of SCFAs, phenols, and ammonia were measured in the stool samples.	• Fecal SCFAs were significantly higher in ASD children. • Acetic, butyric, isobutryic, valeric, and isovaleric acids were elevated in the stool of ASD children, whereas caproic acid was reduced. • The ASD group had greater ammonia concentration in the stool.
Yap et al. (2010)	ASD children ($n = 39$ with 35 males and 4 females); control siblings ($n = 28$ with 14 males and 14 females); nonrelated controls ($n = 34$ with 17 males and 17 females)	• H NMR spectroscopy and pattern recognition methods were used to measure the concentration of free amino acids and bacterial metabolites in the urine.	• The free amino acids glutamate and taurine were elevated in the urine of ASD children. • Disturbances in the patterns of bacterial metabolites dimethylamine, hippurate, and phenylacetylglutamine were also observed in this group.
Kaluźna-Czaplińska and Blaszczyk (2012)	ASD children with GI dysfunction ($n = 22$)	• Concentrations of DA, LA, and the ratio of DA/LA in the urine were determined by capillary gas chromatography/mass spectrometry before and after probiotic therapy. • The children were provided an oral probiotic capsule containing *L. acidophilus* twice daily for 2 months (strain Rosell-11, containing 5×10^9 CFU/gram).	• The concentration of DA in the urine was higher in ASD children compared with controls before and after probiotic supplementation. • The probiotic therapy appeared to partially improve the elevated urinary concentrations of DA and ratio of DA/LA, and there was noticeable improvement in the behaviors of ASD children, particularly in concentration ability and carrying out orders.

bEFAP, bacterial tag encoded FLX amplicon pyrosequencing; CFU, colony forming unit; FISH, fluorescent in situ hybridization; LA, L-arabinitol; qRT-PCR, quantitative real-time polymerase chain reaction.

have not received antibiotic or probiotic treatment. It is also important that repeated analyses are done over time for each patient to monitor how these microbiome/metabolic parameters correlate with disease symptomology. Based on reports suggesting environmental transmission, siblings should also be assayed. To determine the temporal order of events, and to possibly establish causation, it would be useful if fecal and urinary samples were obtained from all babies (including those who will develop ASD and healthy individuals) at the time of birth.

Other Microbiomes

The emphasis of current animal model and human studies has been to link gut microbiota changes and ASD. For the most part, the

neurologic effects of microbiomes inhabiting other systems have been overlooked. Microbial communities in other systems can undergo dynamic fluctuations in response to various intrinsic and extrinsic factors. These may in turn affect brain function (Human Microbiome Project Consortium 2012b; Ding and Schloss, 2014). Other body regions known to harbor unique microbes include the skin, oral cavity and associated structures, respiratory system, and vagina.

There are isolated reports detailing the effects that the oral cavity microbiome exerts on neural function. Oral cavity microbial shifts are associated with seizure severity in epileptic patients (Costa et al., 2014) and Alzheimer's disease (Noble et al., 2014; Shaik et al., 2014; Shoemark and Allen, 2015). Vaginal dysbiosis, such as may occur through maternal stress, might affect offspring neurobehavioral development (Jašarević et al., 2015); however, sufficient data to support this claim is currently lacking. Even so, disruptions in these and other microbiomes might hold promise in relation to understanding and developing therapeutic intervention strategies against various neurologic diseases, including ASD.

Intriguingly, a novel microbiome was discovered in the placenta (Aagaard et al., 2014), a region previously considered sterile (Wassenaar and Panigrahi, 2014). It is also thought of as the primary organ to buffer the fetus against environmental insults (Rosenfeld, 2011). While initial doubts were cast on whether the microbiome identified truly originated from the fetal placenta (Kliman, 2014), subsequent work by the original and other groups supports this pioneering finding. Moreover, these additional studies suggest that microbial communities within the placenta may vary according to maternal weight and gestational state, and in women afflicted with preeclampsia (Amarasekara et al., 2014; Antony et al., 2014; Doyle et al., 2014). A floodgate of further questions is raised by this discovery. First and foremost, how do alterations in the placenta microbiome contribute to later disease or health effects, i.e., developmental origins of health and disease? Are there potential sex differences in the placental microbiome that might also impact offspring health? What other intrinsic and extrinsic maternal factors might influence the placental microbiome? In relation to the last two questions, prior work determined maternal diet interacting with offspring sex yields unique placental epimutations and transcriptomic changes, especially for genes regulating metabolic pathways (Gallou-Kabani et al., 2010; Mao et al., 2010; Gabory et al., 2012, 2013). Therefore, it is highly plausible that the placental microbiome might also be vulnerable in a sex-dependent manner to in utero changes. It is too early to ascertain the effects of placenta dysbiosis on neurobehavioral programming and central nervous system diseases. However, other placental disruptions have been linked with neurobehavioral disorders, especially in males (Mueller and Bale, 2008). Future work is needed to determine how alterations in these other microbiomes might contribute to neurologic diseases, including ASD. Work in this area should also be directed at looking for microbiota inhabiting other host systems and tissues, including those possessing abundant nutrients and environments hospitable to colonization by anaerobic or aerobic bacteria.

Therapeutic Modulation of Gut Dysbiosis and ASD

Based on the premise that the microbiome-gut-brain axis is a potentiating risk factor in ASD, several interventionary measures may be conceived. As shown in Fig. 1, these include diets that facilitate the growth of good bacteria, prebiotics, probiotics, synbiotics, postbiotics, antibiotics, fecal transplantation, and activated charcoal (Critchfield et al. 2011; Gilbert et al., 2013; Fond et al., 2014). The benefits of each of these adjuvant therapies will be considered.

Diet. Considerable evidence links contrasting diets to gut microbiota changes and subsequent health effects (reviewed in (Fond et al., 2014; Luna and Foster, 2015; Salazar et al., 2014; Voreades et al., 2014). Diets containing noncaloric artificial sweeteners have been linked to gut dysbiosis and glucose intolerance (Suez et al., 2014). The gut microbe complexity and behavioral patterns of mice provisioned for 3 months with either a rodent chow or beef diet were examined (Li et al., 2009). Beef-fed animals possessed greater gut microbial diversity and displayed superior working and reference memory, and reduced anxiety compared with those fed the chow-based diet. Another study tested the effects of transplanting the microbiota from mice maintained on a high fat diet to those reared on a control diet (Bruce-Keller et al., 2015). Recolonization of control mice with microbiota from the high fat diet animals shifted the microbiota diversity and taxonomies, impaired gut barrier function, elevated circulating endotoxin levels, increased lymphocyte markers and neuroinflammation, and disturbed cerebrovascular homeostasis. A gluten-free and casein-free diet might be used to treat core and peripheral behavioral symptoms in certain ASD patients. This diet appears to favorably influence gut microbiota populations and intestinal barrier function (Whiteley et al., 2012; Pedersen et al., 2014; Whiteley, 2014). As with other proposed treatments, this potential remedy needs to undergo rigorous testing.

Prebiotics. Prebiotics include carbohydrates, such as inulin and various oligosaccharides, and other food ingredients indigestible by the host. The compounds may preferentially promote bacterial colonies capable of fermenting them into SCFAs that then affect the gut and distal target organs (Saulnier et al., 2008; Fond et al., 2014). No studies to date have considered whether prebiotic treatment alone improves ASD clinical signs. One study administered two prebiotic treatments (fructo-oligosaccharids and Bimuno galacto-oligosaccharides) to healthy volunteers and then monitored their hormonal and behavioral responses (Schmidt et al., 2014). The findings revealed that the latter prebiotic formulation reduced salivary cortisol secretion, which is indicative of a suppressed neuroendocrine stress response, and increased the subject's attention span. These are two behavioral domains impacted in patients with ASD.

Probiotics/Synbiotics. The use of probiotics to treat various aliments dates back to almost a century ago when Dr. Elie Metchinkoff developed a milk-based diet fermented with bacterium he termed *Bulgarian bacillus*. His premise was that such bacteria may stimulate the growth of advantageous bacteria at the expense of harmful microbes, resulting in the long-term effect of promoting an overall healthy lifespan (reviewed in Fond et al., 2014).

However, the term probiotic was not employed until 1965 (Lilly and Stillwell, 1965). The widely accepted definition of probiotics today is that they are living nonpathogenic organisms, which when consumed in adequate amounts may confer various health benefits to the host organism (Critchfield et al. 2011; Caselli et al., 2013). Lactic acid producing bacteria, such as lactobacilli, lactococci, and bifidobacteria or yeast (e.g., *Saccharomyces boulardii*) comprise the majority of currently available probiotic products. Probiotics are deemed safe food products for human consumption by the U.S. Food and Drug Administration and its European counterparts.

While not defined at the time, the first case of using such bacteria to treat neurologic diseases dates to 1910, when Dr. George Porter reported that a gelatin-whey formula with living lactic acid producing bacteria alleviated depression in at-risk individuals (Phillips, 1910; reviewed in Fond et al., 2014). Approximately one-fifth of physicians today encourage the use of probiotics in ASD children, especially those plagued with GI symptoms (Golnik and Ireland, 2009). Examples of direct benefits conferred on the gut by probiotics include
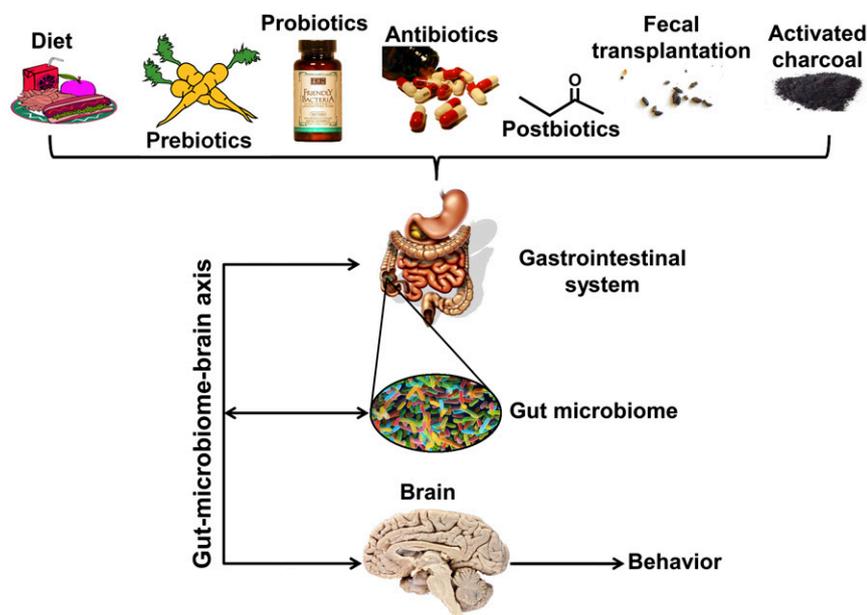
Fig. 1. Diagram of dietary and potential therapeutic factors that can affect the gut-microbiome-brain axis. Diets, including those enriched with legumes can affect nutrient availability and prebiotics available to the gut microbiota. Probiotics may colonize the gut and shift the bacterial populations within this system to the so-called good bacteria. The data to date though on the potential therapeutic benefit of probiotics for ASD are mixed. Antibiotics have also been proposed as a potential treatment of ASD, but they may indiscriminately destroy both good and bad bacteria. Antiobiotics are also unlikely to be a viable long-term remedy for autism and other neurobehavioral disorders because the effects on gut microbe populations are generally transient upon discontinuation. Postbiotics include supplementation of bacterial-produced amino acids and SCFAs. While some of these metabolites might be beneficial, others may induce behavioral disturbances. Fecal microbial transplantation has been proposed and tested for improving neurobehavioral impairments and may be a long-term option. The downside is that this approach might also introduce pathogenic bacteria into the host intestinal system. Activated charcoal may be a useful adjuvant in treating gut-microbiome-brain disorders under the premise that it will bind bacterial toxins and products. This treatment is unlikely to be a sustainable long-term treatment of ASD and chronic neurologic disorders.

alteration of host immunity (in particular, stimulating secretory IgA); restoration of normal commensal bacterial colonies and simultaneous suppression of microbial pathogens; stabilization of the intestinal mucosal barrier predominantly due to stimulation of mucin production (which reduces the likelihood of bacterial spread and absorption of harmful bacterial metabolites); and promoting the synthesis of antioxidant substances (Lutgendorff et al., 2008).

The term psychobiotics refers to living organisms with beneficial effects on mental health (Dinan et al., 2013). While a handful of reports speculate on the use of potential probiotics/psychobiotics as adjuvant treatments for ASD (Critchfield et al.; Linday, 2001; Garvey, 2002; Levy and Hyman, 2005; Finegold, 2011; Borre et al., 2014; Fond et al., 2014; Ianiro et al., 2014; Reardon, 2014), definitive evidence that this complementary alternative therapy improves behavioral symptomology in ASD patients is for the most part lacking. Probiotic treatment of 19 ASD children restored the Bacteriodetes-to-Firmicutes ratio, *Desulfovibrio* spp., and the amount of *Bifidobacterium* spp. (Tomova et al., 2015). However, the behavioral patterns of the children pre- and postprobiotic supplementation were not assessed. Another report showed that urinary concentrations of DA and the ratio of DA/L-arabinitol were elevated in autistic children but that probiotic treatment reversed the metabolic disruptions and improved behavioral performance (as discussed previously) (Kalużna-Czaplińska and Błaszczyk, 2012).

There are limited animal model studies in this area. Wild-type mice fed a chow-based diet and provided the probiotic *Lactobacillus helveticus* exhibited a decrease in anxiety-like behaviors (Ohland et al., 2013). As discussed previously, treatment of the MIA mouse model for ASD with the probiotic *B. fragilis* improved the mucosal barrier and gut dysbiosis, mitigated elevations in several metabolites associated with ASD, and abolished ASD-like behavioral disruptions (Hsiao et al., 2013). While much work remains to be done in order to determine whether probiotics should be promoted as an adjunct treatment of ASD patients, another idea that is gaining interest is the combined treatment of pre- and postbiotics, which is referred to as synbiotic therapy (Kaur et al., 2009; Firmansyah et al., 2011; Szajewska and Makrides, 2011; Fond et al., 2014). The efficacy of synbiotics in alleviating neurologic diseases merits further pursuit.

Postbiotics. Another potential therapeutic approach against gut dybiosis-induced neurobehavioral disorders is to identify the specific metabolites or molecules altered by microbial changes and then supplement such nutrients or their precursors in the diet. Examples of metabolites affected by the gut microbiota populations include amino acid derivatives and SCFAs (Fond et al., 2014; Klemashevich et al., 2014). On the other hand, certain metabolites are elevated in ASD animal models or children. Examples of such biomolecules include 4-EPS, *p*-cresol, indolepyruvate, indolyl-3-acrylolylglycine, *n*-acetylserine, PPA, and urinary concentrations of dimethylamine, hippurate, and phenyacetylglutamine (Yap et al., 2010; Gilbert et al., 2013; Hsiao et al., 2013; Persico and Napolioni, 2013). Therefore, tailored pre- and postbiotic diets might be conceived to prevent the bacterial synthesis of harmful metabolites and simultaneously supplement those that may be beneficial.

Antibiotics. Antimicrobiotics (antibiotics) may either be bacteriolytic or bacteriostatic for susceptible intestinal bacteria; however, such compounds may also suppress the growth of commensal and beneficial populations of bacteria. Moreover, pathogenic bacteria may rebound once the antibiotic is discontinued. For these reasons, antibiotics are generally not given much credence as a long-term therapy for ASD. One small-scale study treated 11 ASD children for 8 weeks with vancomycin, an antibiotic commonly used in the treatment of *Clostridium difficile* colitis, and found the children's communication and other behavioral scores improved significantly during the treatment period (Sandler et al., 2000). However, the beneficial effects were ephemeral, with behavioral impairments recurring upon termination of the antibiotic treatment.

Fecal Microbiota Transplantation (FMT). FMT is a therapeutic remediation strategy employed to restore normal intestinal flora balance (Aroniadis and Brandt, 2013; Ianiro et al., 2014). It has been particularly successful in the case of recurrent *C. difficile* infection (Bakken et al., 2011; Borody and Khoruts, 2012). However, this shotgun approach may inadvertently introduce opportunistic infections into the recipient's GI system. Nonetheless, neurologic improvement was reported in a single Parkinson's patient after FMT (Anathaswamy, 2011), and there is an anecdotal report of beneficial effects in two ASD children receiving FMT (Aroniadis and Brandt, 2013).

Activated Charcoal (Carbon). Activated charcoal or carbon is a standard treatment in many acute oral toxicity cases because the

compound binds to toxins present in the upper GI system and thereby prevents absorption across the intestinal wall. Toxins produced by gut microbiota may also lead to injurious effects in the brain. Consequently, activated charcoal may be useful in microbe-induced neurobehavioral disorders, including ASD. A few studies suggest activated charcoal suppresses the growth of antibiotic-resistant intestinal bacteria (Khoder et al., 2010; Grall et al., 2013). However, similar to antibiotic treatments, it is likely that any beneficial effects of this intervention will be short-lived, and disease symptomology will return upon cessation of this therapy.

Potential Mechanism Microbiota Alterations Lead to ASD and Related Neurologic Disorders

The following sections will delve into some of the mechanisms by which microbiomes (especially in the gut) may impact brain function. Because we are still at the nascence of understanding the interactions between microbiota and ASD, this section will, for the most part, employ a holistic approach in order to examine how dysbiosis in general may result in neurological diseases.

Breaking Down Barriers. The intestinal epithelial (mucosal) barrier is the primary site of contact for microorganisms, antigens, and immunogenic proteins. In this sense, it serves as the homeland security for the host to process and permit entry of essential extrinsic factors, while blocking transmission of detrimental microorganisms and antigens. This barrier processes over 100 tons in food-borne factors in an individual's lifetime (Alonso et al., 2014). Disruptions in this region are likely pivotal to gut-microbiota-brain comorbid disorders (Ait-Belgnaoui et al., 2012; Alonso et al., 2014). Leakiness of the barrier provides a portal for bacterial spread, along with increasing the potential for systemic transmission of antigens, virulence factors, other pathogens, and bacterial metabolites. Such factors may then impact brain function and inflammatory processes (Alonso et al., 2014). One study suggests that probiotic treatment with *Lactobacillus farciminis* restores the intestinal barrier and reduces the concentration of circulating LPS levels culminating in a blunting of the HPA response and neuroinflammation (Ait-Belgnaoui et al., 2012). Probiotics may restore the mucosal barrier by directly competing and preventing translocation of pathogenic bacteria, stimulating mucosal immunity (secretory IgA), and upregulating mucin and antioxidant expression (van Minnen et al., 2007; Lutgendorff et al., 2008, 2009).

The blood brain barrier (BBB) serves as a gatekeeper to minimize the chance of pathogens and foreign particles transferring across the vasculature to reach the brain parenchyma. The integrity of this barrier is essential for normal brain development and function. It has recently been shown that the development of the BBB is contingent upon the presence of commensal gut flora. Beginning in utero, the permeability of the BBB of GF mice is greater than that of SPF mice, and these differences persist through adulthood. The tight junction proteins (occludin and claudin-5), which govern the endothelial portion of this barrier, are reduced in GF animals. However, there is the potential to rescue these animals even later in life as inoculation of adult GF mice with nonpathogenic gut microbiota reverses these abnormalities (Braniste et al., 2014).

Enteric Nervous System and Vagal Nerve. The enteric nervous system and vagal nerve provide a bidirectional communication route between the gut and brain, resulting in the expansion of the concept to the brain-gut-enteric microbiota axis. This association between the brain and gut was first conceived to explain how the central nervous system may impact GI function (reviewed in Mayer, 2011). However, the current focus has turned to understanding the other direction of this pathway, including transmission of gut microbes via the enteric nervous system and vagus nerve to the brain (reviewed in Douglas-Escobar et al., 2013; Stilling et al., 2014).

As detailed previously, behavioral patterns of GF relative to SPF mice provide clear evidence that the gut microbiota can exert neural effects. Proteins regulating synaptogenesis (synaptophysin and PSD-95) are markedly reduced in the striatum of the former mice (Diaz Heijtz et al., 2011). Vagotomy or chemical sypathectomy abolishes the behavioral differences between the two groups (Bercik et al., 2011). Mice provided chronic supplementation with a probiotic (*Lactobacillus rhamnosus*) demonstrate less anxiety-like and depressive behaviors and corresponding expression changes in brain neurotransmitters. However, vagotomy ablates these neurobehavioral responses, supporting the presumption that the vagus nerve is an essential route of transmission between the gut microbiota and brain (Bravo et al., 2011).

Infection of mice with the pathogenic gut bacteria *Campylobacter jejuni* results in a rapid increase in anxiety-like behaviors (Lyte et al., 1998). After *C. jejuni* infection, c-Fos expression is acutely up-regulated in the brainstem of visceral sensory nuclei, particularly in the nucleus tractus solitarius (the termination site of the vagus nerve) (Gaykema et al., 2004). A follow-up study verified that the bacterial-induced neurobehavioral responses are attributed to *C. jejuni* activation of vagal ascending pathways (Goehler et al., 2005). The intestinal pathogen, *Salmonella enterica* subspecies enterica serovar Typhimurium (*S. typhi*), also can disseminate to the brain via the vagus nerve (Wang et al., 2002).

Bacterial Metabolites. Several bacterial-derived metabolites modulate neurobehavioral responses. Spermidine is one such example, and it may suppress aging and age-related memory impairment (Eisenberg et al., 2009; Gupta et al., 2013). Other bacterial metabolites are attributed to potential encephalopathic effects. Two such well-characterized bacterial metabolites are D-lactic acid and ammonia. D-lactate results from an excessive carbohydrate load. A surge in fecal D-lactate producing bacteria is associated with chronic fatigue syndrome (Sheedy et al., 2009). While some probiotics reduce generation of D-lactic acid by gut microbiota, others increase this bacterial metabolite and may exacerbate cognitive disorders (Mack, 2004; Munakata et al., 2010).

Ammonia results from bacterial urease cleavage of urea, whereupon it circulates to the liver in the portal vein and is further metabolized via the urea cycle. Ammonia does not pose a threat in individuals possessing a normal portal system and functional liver. However, extra- or intrahepatic shunts, where the blood from the intestinal system bypasses the liver and enters the systemic circulation, result in delivery and concentration of ammonia in the brain. Here, ammonia induces neurotoxic effects, otherwise termed hepatic encephalopathy (Qureshi et al., 2014). Other pathologic changes include disruption of the BBB, suppression of serotonin and dopamine synthesis, and stimulation of octopamine, an atypical neurotransmitter (Skowrońska and Albrecht, 2012). Increased urease-producing gut bacteria and cirrhosis of the liver raise the potential for hepatic encephalopathy/cognitive dysfunction (Zhang et al., 2013). Synbiotic treatment of cirrhotic patients appears to improve cognitive function (Malaguarnera et al., 2007).

Bacteria produce several volatile fatty acids comprised of two to four carbon atom chain lengths; hence, they are called SCFAs. The SCFAs, acetate, propionate-PPA, and butyrate-BA, result from bacterial fermentation of indigestible carbohydrates in the large intestine. Health benefits ascribed to these compounds include energy supplementation for colonic epithelium, anti-inflammatory activity, and improved insulin regulation (Segain et al., 2000; Al-Lahham et al., 2010; De Preter et al., 2011). On the other hand, animal model and human epidemiologic studies suggest SCFAs may also induce neurotoxic

effects, which might contribute to ASD development (MacFabe, 2013). Some of these detrimental effects are likely due to mitochondrial and epigenetic disruptions (discussed subsequently).

In animal models, offspring exposed during development to PPA, and to a lesser extent BA, exhibit behavioral disturbances resembling clinical signs observed in ASD patients (Thomas et al., 2012; Foley et al., 2014a, b, Foley et al., 2015). For this reason, this approach is widely used to model ASD in animals, which otherwise do not develop such disorders. Elevated SCFAs and PPA are found in the stool of ASD children (Wang, 2010; Wang et al., 2012, 2014).

The bacterial metabolite, 4-EPS was markedly elevated in the MIA offspring model of ASD (discussed previously). Administration of this metabolite to wild-type mice recapitulated the anxiety-like phenotype observed in MIA offspring (Hsiao et al., 2013). Moreover, 4-EPS is related to p-cresol, a putative biomarker metabolite identified in high amounts in ASD children (Persico and Napolioni, 2013). However, the involvement of 4-EPS in human ASD remains to be elucidated.

Mitochondrial Dysfunction. Mitochondrial disease (MD) is often diagnosed in conjuction with ASD. Thirty percent of patients show biomarkers linked with this former disease (Rossignol and Frye, 2012). The MD observed in autistic cases is thought to be acquired rather than genetic in origin. The SCFAs, especially PPA, which are increased in ASD-associated gut microbes (*Clostridia*, *Desulfovibrio*, *Sutterella*, and *Bacteroidetes*), might contribute to mitochondrial dysfunction (MacFabe, 2012, 2013; Frye et al., 2013). One mechanism by which the bacterial metabolite PPA may disrupt mitochondria is through alteration of the tricarboxylic acid cycle via conversion of PPA to propionyl-CoA.

PPA can sequester and hinder the metabolism of carnitine, which is a quaternary ammonium cofactor required to transport long-chain fatty acids into the inner mitochondria membrane for β-oxidation and energy production (Jones et al., 2010). Chronic antibiotic administration inhibits carnitine absorption and reabsorption by the intestines and kidney, respectively (Pochini et al., 2008). The limited circulating carnitine may eventually be depleted if bound for prolonged periods to unprocessed fatty acids, as would occur when mitochondrial fatty-acid beta oxidation is suppressed (Haas et al., 2008). Abnormal mitochondrial fatty acid oxidation manifests as increased circulating concentrations of acyl-carnitine, a potential biomarker for this disorder. Carnitine is reduced in ASD children (Mostafa, 2005). In contrast, long-chain and very long-chain fatty acids and acyl-carnitine are increased in these patients (Pastural et al., 2009; Frye, 2012). Similar metabolite changes are evident in animal models of ASD treated with PPA and butyrate (Thomas et al., 2010, 2012).

In these animal models, the bacterial metabolite PPA results in a number of other changes that may directly or indirectly result in MD, including neuroinflammation, increased oxidative stress, glutathione depletion, and changes in phosophlipid/acylcarnitine profiles in the brain (MacFabe et al., 2007; Thomas et al., 2010, 2012). Analogous findings have been reported in ASD patients (Chauhan and Chauhan, 2006; James et al., 2006; Al-Gadani et al., 2009; El-Ansary et al., 2010; Wegiel et al., 2010).

Mitochondria are vital for all animal cells, especially in the brain. Microbiome-induced MD can result in ASD and other neurobehavioral deficits via several mechanisms. A few examples will be considered. Neural synapses require mitochondria for ATP production, calcium maintenance, and plasticity (Mattson and Liu, 2002). Neurons with high firing rates, e.g., GABAergic interneurons, are especially vulnerable to MD (Anderson, 2008). GABA neurons are required for cerebral cortex processing of sensory information, which is affected in ASD children (Anderson, 2008). The reactive oxygen species generated by MD may induce necrosis of nervous tissue and impede synaptic transmission (Frye, 2014). A link between reactive oxygen species, mitochondrial disturbances in brain tissue, and ASD has been reported (Rose et al., 2012).

Neuroendocrine Mechanisms. Microbes have evolved the ability to eavesdrop on host neurotransmitter and hormonal conversations and even exploit such host factors to their advantage. Gut bacteria may also interject into the conversation by producing a range of their own neurotransmitters, which mimic those of the host and thus alter neural pathways, such as GABA, norepinephrine (NE), serotonin, and dopamine (al Mardini et al., 1991; Li and Cao, 2010; Barrett et al., 2012; Cryan and Dinan, 2012). This interkingdom communication is extraordinary; however, it may be more parasitic than mutualistic on the part of the bacteria.

Bacterial colonies generally self-regulate their own growth through quorum-sensing molecules. Stress-related neurochemicals produced by the host including catecholamines, NE, epinephrine, and adrenaline act upon these bacterial signaling pathways, which increases the proliferative rate of microbial colonies (Lyte, 2004, 2014; Karavolos et al., 2011). *Helicobacter pylori* utilizes L-DOPA for growth promotion but at the host's expense. Antibiotic elimination of the pathogen increases the availability of L-DOPA and may thus be beneficial in neurocognitive disorders (Lyte, 2010). A pilot study with stressed college students preparing for a final examination provides additional evidence that the neuroendocrine state of the host can impact gut microbe composition because reduced numbers of fecal lactic acid bacteria were present in these individuals (Knowles et al., 2008).

Host neuroendocrine stress responses alter an assortment of bacterial virulence factors. Under culture conditions, NE and dopamine increase motility of various *Vibrio* strains (Pande et al., 2014). However, co-treatment with catecholamine receptor antagonists mitigated this effect. *Vibrio hareyi* treated with NE or DOPA produce more harmful siderophores, exhibit enhanced swimming motility, and upregulation of genes mediating flagellar activity, biofilm formation, and exopolysaccharide production. Co-administration with α-adrenergic bacterial-derived catecholamine receptor antagonists and dopaminergic antagonists neutralized these effects (Yang et al., 2014). GABA affects the virulence of *Pseudomonas aeruginosa* (Dagorn et al., 2013). Increased production of neuroendocrine stress hormones promotes hemolysis and release of hemolysin E by *S. typhi* (Karavolos et al., 2011).

Gut microbiota can directly influence the host HPA axis (Sudo et al., 2004). Elevated serum concentrations of adrenocorticotropic hormone and corticosterone were detected in GF mice. Early exposure to SPF feces abolished the hormonal abnormalities; however, transplantation at later stages was unable to reverse these effects. Hippocampal concentrations of 5-hydroxytryptamine and its primary metabolite 5-hydroxyindoleacetic acid were also increased in GF animals (Clarke et al., 2013). Circulating concentrations of tryptophan (a serotonin precursor) is upregulated in these animals. Besides behavioral abnormalities, GF rats have upregulated hypothalamic mRNA expression of *Crf* and decreased *Gr* mRNA in the hippocampus and reduced dopaminergic turnover rate in the frontal cortex, hippocampus, and striatum (Crumeyrolle-Arias et al., 2014). Mice provided the probiotic *L. rhamosus* (JB-1) demonstrated decreased anxiety- and desperation-like behaviors, blunted stress-induced response to rising corticosterone concentrations, and altered expression of $Gaba_ar$ and $Gaba_br$ in several brain regions (Bravo et al., 2011). Another study suggests that bacterial virulence factors alone can modulate the HPA axis with LPS from *S. typhi* directly activating the host HPA axis and noradrenergic and indoleaminergic systems (Dunn et al., 2003).

Epigenetic Alterations. The term epigenetics has become vastly overused to define broadly any transcriptomic change occurring independent of DNA mutation. The field of neuroepigenetics was born under these auspices and is currently invoked as the genesis of many neurologic disorders not explained by genetics alone, including ASD (Wilkinson and Campbell, 2013; Berko et al., 2014; Ladd-Acosta et al., 2014; Lesseur et al., 2014; Tordjman et al., 2014; Wong et al., 2014). Alterations in the gut microbiome might trigger epigenetic changes leading to downstream behavioral manifestations (Mischke and Plösch, 2013; Kumar et al., 2014; Stilling et al., 2014).

Microbiota may shape the epigenome in several ways. Bacterial-derived SCFAs, including BA, PPA, and acetic acid govern key epigenetic-regulating enzymes. Of these, BA is considered one of the most potent SCFA inhibitors of histone deacetylases (Candido et al., 1978; Davie, 2003), which remove the acetyl group from histone proteins, allowing the proteins to reassociate with DNA and block DNA transcription. Other SCFAs, including PPA, lactate, and pyruvate, also act as weak histone deacetylase inhibitors (Thangaraju et al., 2006; Waldecker et al., 2008; Latham et al., 2012). In contrast, acetate upregulates the histone acetyl transferase substrate availability (Stilling et al., 2014). Inflammatory responses within the intestinal mucosa are homeostatically regulated by gut microbiota in a histone deacetylases 3–dependent manner (Alenghat et al., 2013).

Commensal gut microbiota synthesize folate and vitamin B_{12}, which are vital for methylation of DNA and histone proteins (LeBlanc et al., 2013). A recent study with pregnant women linked gut microbiota profiles, especially for Firmicutes and Bacteroidetes, and leukocyte DNA methylation patterns with genes regulating lipid metabolism and obesity (Kumar et al., 2014). In mice, a maternal methyl supplemented diet resulted in gut dysbiosis, associated changes in colonic mucosal DNA methylation and transcriptomic patterns, and colitis in the offspring (Schaible et al., 2011). Microbiota appear to orchestrate other host epimutations, such as chromatin rearrangements, alterations in noncoding RNAs, and RNA splicing factors (Bierne et al., 2012). One study suggests that pathogenic bacteria might usurp control of host gene expression by broadly suppressing RNA polymerase II, an enzyme required for synthesis of coding and noncoding RNAs (Lutay et al., 2013). Intriguingly, there has been recent evidence that some endosymbiotic bacteria produce small noncoding RNAs with the potential to exert cross-kingdom communication and affect host processes (Mayoral et al., 2014). There are presumably more such examples awaiting discovery, especially in commensal gut and other organ microbes, which have so closely evolved and adapted to their host environments. It is provocative to contemplate how such bacterial noncoding RNAs might impact host health and disease. We are at the infancy of understanding how host microbiota and their products influence the host epigenome. Mechanistic insight into these two revolutionary fields may yield key biomarkers for early diagnosis and preventative/therapeutic remediation strategies for various diseases, including ASD.

Discussion

Microbiota populations contained within various mammalian host systems, especially the GI region, may affect host health and disease. There are various mechanisms by which the microbiota or their products impact the brain. Some such methods involve commandeering control of host hormonal and epigenetic systems to the advantage of the bacteria. However, we are dependent upon microbes for certain key nutrients and promotion of normal gut development, neurobehavioral patterns, and immunologic function. Whether the scale is tipped in favor of health or disease likely depends upon the diversity of microbes present,

select populations, and absolute amount of bacteria residing within the gut and other systems. These variations may alter metabolite profiles, virulence factors, immunologic responses, and other factors impacting neurologic function.

While there are select animal model and human studies implicating gut microbiota alterations and development of ASD, it is still premature to render definitive conclusions and establish causation. More work with larger cohort studies and other animal models is needed to confirm the initial findings and ascertain the potential underlying mechanisms. Other studies should also explore whether shifts in other organ system's microbiomes is associated with this heterogeneous class of diseases. If dysbiosis is shown to be a precipitating factor in ASD, then several potential therapeutic approaches ranging from prebiotics, postbiotics, synbiotics, fecal transplantation, and other strategies used to alter the microbiomes or products may be useful adjuvant treatments in these patients. Preliminary data provide initial support for their usage; however, all of these potential therapies need to undergo rigorous testing before such huge claims can be made regarding their efficacy. In summary, to date, the data provide some evidence linking the microbiota-gut-brain axis to ASD, but the field is still in its primordial stages.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: Rosenfeld.

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