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Autism Spectrum Disorder and the Gut Microbiota in Children: A Systematic Review

Navya Bezawada[a] Tze Hui Phang[b] Georgina L. Hold[c] Richard Hansen[d]

[a]Aberdeen Royal Infirmary, NHS Grampian, Aberdeen, UK; [b]Royal Cornhill Hospital, NHS Grampian, Aberdeen, UK; [c]Microbiome Research Centre, St George and Sutherland Clinical School, University of New South Wales, Sydney, NSW, Australia; [d]Glasgow Children's Hospital, NHS Greater Glasgow and Clyde, Glasgow, UK

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Abstract
Introduction: Differences in microbiota composition in children with autism spectrum disorder (ASD) compared to unaffected siblings and healthy controls have been reported in various studies. This study aims to systematically review the existing literature concerning the role of the gut microbiota in ASD. ***Methods:*** An extensive literature search was conducted using MEDLINE and EMBASE databases to identify studies (January 1966 through July 2019). ***Results:*** A total of 28 papers were included. The studies ranged from 12 to 104 participants who were aged between 2 and 18 years from various geographical areas. Majority of studies included faecal samples; however, 4 studies examined mucosal biopsies from different sites. The heterogeneity in ASD diagnostic methodology, gut site sampled and laboratory methods used made meta-analysis inappropriate. Species reported to be significantly higher in abundance in autistic children included *Clostridium, Sutterella, Desulfovibrio* and *Lactobacillus*. The findings are however inconsistent across studies. In addition, potential confounding effects of antimicrobial use, gastrointestinal symptoms and diet on the gut microbiota are unclear due to generally poor assessment of these factors. ***Conclusion:*** It is clear that the gut microbiota is altered in ASD, although further exploration is needed on whether this is a cause or an effect of the condition.

Introduction

Growing evidence suggests that the gut microbiota has a role in the pathophysiology of autism spectrum disorder (ASD) [1]. Differences in composition of the gastrointestinal (GI) microbiota in children with ASD compared to unaffected siblings and/or healthy unrelated controls have been reported in various studies. The rates of diagnosis of ASD have increased dramatically in the past few decades [2]. Although changes to diagnostic criteria and greater awareness of the condition may be contributing to the rise, it is thought that environmental factors are also important [3]. Non-genetic risk factors, including maternal and pregnancy-related factors such as intrauterine infections and exposure to medications have gained interest as ASD incidence has continued to increase at a rate that cannot be explained by genetics [4]. In addition, there is an increasing interest in the gut microbiota in relation to ASD [1].

karger@karger.com
www.karger.com/anm

Dr. Richard Hansen
Department of Paediatric Gastroenterology
Hepatology and Nutrition, Glasgow Children's Hospital
1345 Govan Road, Glasgow G51 4TF (UK)
E-Mail richard.hansen@nhs.net

The GI tract is home to one of the most complex ecosystems and contains around 100 trillion microbes [5]. "Microbiota" is a collective term for this microbial community which includes bacteria, archaea, eukaryotes and viruses. There is a degree of variation in the adult composition of gut microbiota and this can be influenced by diet, antibiotic use, lifestyle and genetics [5, 6]. The gut microbiota is crucial for health in humans, with several important metabolic, protective and trophic functions and has often been referred to as the "forgotten organ" in the literature [7]. With such an impressive metabolic capacity and contribution to host health, it is no surprise, that the gut microbiota has also been implicated in disease. Characterising and understanding the gut microbiota in health and disease is a promising avenue that may lead to therapeutic benefits through its manipulation via so-called microbial therapeutics.

Changes in the gut microbiota seen in ASD may have a causative role and perpetuate GI symptoms or may simply be a confounder driven by dietary restriction. Children with autism often suffer from a range of GI symptoms, including diarrhoea, abdominal pain, constipation and gastroesophageal reflux. Estimates of the prevalence of such symptoms vary from 9 to 91% across studies [8]. A meta-analysis by McElhanon et al. [9] concluded that there was a three-fold higher risk of GI symptoms in children with ASD than in those without. Recent studies have suggested that alterations in the gut microbiota composition in children with ASD may contribute to both GI and neurological symptoms. Findings appear to be inconsistent across studies. If the gut microbiota plays a role in pathophysiology, there may be scope for novel treatment through its manipulation by microbial therapeutics [10–13]. The aim of this article is to systematically review the existing literature to evaluate variations in the gut microbiota and understand its significance in ASD.

Methods

Search Strategy and Selection Criteria

An extensive literature search was conducted using MEDLINE and EMBASE databases by 2 independent researchers (N.B. and G.L.H.). All studies published between 1966 and July 2019 were included. The following Medical Subject Heading [MeSH] terms were used, which included both the root term and text word: GI; gut; microbiota; autism; and ASD. The studies were evaluated for sample size, age range of children included, study methodology and composition of the gut microbiota. Manual searching of reference lists from potentially relevant articles was also undertaken to identify additional studies.

Types of Studies

Randomised controlled trials, cohort studies and observational studies were included. Studies which reported duplicate results were excluded. Those where data could not be extracted were also excluded.

Inclusion Criteria

Studies were included if they compared the intestinal microbiota analysis of autistic children with those of healthy children and provided information on specific bacterial taxa.

Exclusion Criteria

Studies were excluded if they did not report on patterns of individual bacterial taxa differences. General reviews, studies based on adult subjects or animal models and in vitro studies were also excluded, along with conference abstracts and texts not in English. Clinical trials with an intervention were not included, except if the microbiota was assessed in both groups at baseline before intervention. Solely culture-based studies were excluded from this review. We also excluded case reports and studies of fewer than 12 patients.

Quality Assessment

The Newcastle and Ottawa scale for case-control studies was used to assess the quality of the studies and a quality score [1–9] was allocated to each [14]. Data collection and quality assessment were conducted independently by 2 researchers (N.B. and T.H.P.). Any disagreements were resolved through discussion until a consensus was reached.

Results

The initial search identified 898 records from MEDLINE and EMBASE. 595 articles remained following removal of duplicates. Of these, 61 studies were identified for full text eligibility after title and abstract screening. After full text screening, 26 studies were included in the review. Manual searching identified 2 further eligible articles, and therefore a total of 28 papers were included in the review (Fig. 1).

Quality Assessment

The quality scores of the 28 studies were assessed according to the Newcastle-Ottawa Scale and given a score (Table 1).

Study Characteristics

Among the 28 studies, 19 studied the difference between ASD and unaffected, unrelated children [15–33], 4 studies looked at the difference between ASD and unaffected siblings [34–36] or blood relatives [37] and 5 studies considered all 3 groups [38–42] (Table 2). The number of enrolled patients in a single study ranged from 12 to 104 participants and all were aged between 2 and 18 years.

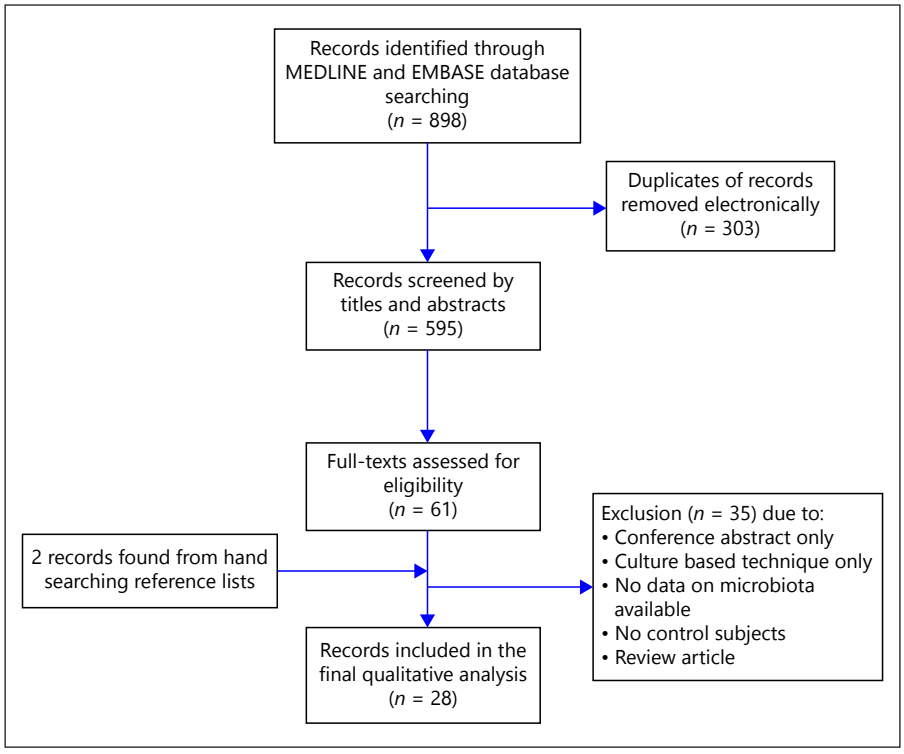

Fig. 1. PRISMA flow diagram for selection of published articles for systematic review.

Of the included studies, the majority (n = 11) were from the United States, 6 from Europe [15, 20, 22, 35, 39, 42] and 3 from Australia [34, 40, 41]. The remaining 8 studies were from Asia [30, 37]. Study design including sampling strategies and microbiome analysis differed between studies. Twenty-four studies assessed faecal samples and 4 used mucosal biopsies (Table 2). Biopsy site varied for mucosa-based studies: 2 sampled ileum and caecum [21, 31], 1 sampled duodenum [17], and 1 sampled rectum [18]. The methods of collection of faecal samples also varied: from a single sample; 3 separate samples; cumulative samples over 48 h; freshly evacuated faeces; and faecal samples following an overnight fast. In 4 studies, microbial analysis was conducted using quantitative real-time amplification of bacterial DNA (qPCR), 4 used both qPCR and 16S rRNA sequencing techniques, one study used fluorescent in-situ hybridisation and the majority (18 studies) solely used 16S rRNA sequencing. One study used shotgun metagenomic sequencing [28].

Criteria Used for the Definition of ASD

The majority of studies referred to the Diagnostic and Statistical Manual of Mental Disorders, editions IV or V [43, 44] for the diagnosis of ASD. Other diagnostic tools were also used including Autism Diagnostic Interview-Revised [45], Autistic Diagnostic Observation Schedule [46], Childhood Autism Rating Scale [47], Pervasive Developmental Disorders Autism Society Japan Rating Scale [48], Modified Checklist for Autism in Toddlers [49], Autism Treatment Evaluation Checklist [50], Pervasive Developmental Disorder Behaviour Inventory [51], INCLEN Diagnostic Tool for ASD [52], Indian Scale for Assessment of Autism [53] and ICD-10 [54]. Some studies simply stated that the formal diagnosis was made by a psychiatrist, psychologist or a multidisciplinary team through history and observation. In one study, families were recruited through a registry called the Simons Simplex Community through the Interactive Autism Network [36]. As ASD is an umbrella term, some studies specified certain subtypes of children in their ASD groups, for example, Asperger's syndrome and pervasive developmental disorder not otherwise specified. For control patients, numerous studies did not specifically exclude behavioural and developmental characteristics of ASD to ensure they were truly unaffected controls.

The heterogeneity in methodology within the included studies, specifically the diagnosis of ASD; gut site sampled; and laboratory methods used made meta-analysis inappropriate within this systematic review, and hence it has not been attempted.

Table 1. Newcastle-Ottawa Scale quality assessment of included studies, by the year of publication

Study	Year	Selection				Comparability	Exposure			Score
		Q1	Q2	Q3	Q4	Q5	Q6	Q7	Q8	
Song et al. [19]	2004	–	–	–	–	**	–	–	–	2
Parracho et al. [42]	2005	–	–	–	–	**	–	*	–	3
Finegold et al. [38]	2010	*	–	–	*	**	–	–	–	4
Wang et al. [40]	2011	*	–	*	*	–	–	*	*	5
Williams et al. [31]	2011	*	*	*	*	**	–	*	–	7
Gondalia et al. [34]	2012	–	*	*	–	**	–	*	–	5
Williams et al. [21]	2012	*	*	*	*	**	–	*	–	7
De Angelis et al. [35]	2013	*	*	*	–	**	–	*	–	6
Kang et al. [16]	2013	*	–	–	–	**	–	*	–	4
Wang et al. [41]	2013	*	–	*	*	–	–	*	*	5
Tomova et al. [39]	2014	*	*	*	–	*	–	*	–	5
Son et al. [36]	2015	–	*	–	*	**	–	*	–	5
Inoue et al. [30]	2016	*	*	–	*	**	–	–	–	5
Kushak et al. [17]	2017	*	–	–	–	–	–	–	–	1
Luna et al. [18]	2017	*	*	*	*	**	–	*	*	8
Strati et al. [20]	2017	*	*	–	–	**	–	*	–	5
Berding et al. [29]	2018	–	*	*	–	**	–	*	–	5
Coretti et al. [22]	2018	*	*	*	–	*	–	*	–	5
Kang et al. [32]	2018	*	*	–	–	*	–	*	–	4
Pulikkan et al. [37]	2018	*	–	*	*	**	–	*	–	6
Rose et al. [33]	2018	*	*	–	*	**	–	*	–	6
Zhang et al. [25]	2018	*	*	*	*	*	–	*	–	6
Li et al. [26]	2019	*	*	*	–	–	–	*	–	4
Liu et al. [23]	2019	*	*	*	*	**	–	*	–	7
Ma et al. [24]	2019	*	*	*	*	**	–	*	–	7
Plaza-Diaz et al. [15]	2019	*	*	*	*	**	–	*	–	7
Wang et al. [28]	2019	–	*	*	–	–	–	*	–	5
Zhai et al. [27]	2019	*	*	*	*	**	–	*	–	7

The Newcastle and Ottawa scale for case-control studies was used to assess the quality of the studies across 3 domains: selection, comparability and exposure. A quality score (0–9) is given based on the number of stars awarded; the highest quality studies are awarded up to nine stars. Selection contains 4 criteria: (Q1) is the definition adequate? (Q2) representativeness of the cases, (Q3) selection of controls, (Q4) definition of controls; comparability means comparability of cases and controls on the design or analysis (Q5, maximum 2 stars); exposure contains 3 criteria: (Q6) ascertainment of exposure, (Q7) same method ascertainment of cases and controls, (Q8) non-response rate. * Represents 1 quality score.

Factors Affecting Gut Microbiota

Antibiotic Usage across Studies

Due to the dramatic impact of antibiotics on the gut microbiota [55] antibiotic usage was specifically interrogated. Reported antibiotic use by participants varied across studies (Table 2). More than half of the studies excluded subjects who reported the use of any antibiotics prior to sample collection. The duration of not using antibiotics ranged from 15 days to 3 months. The majority used 1 month as a cut-off. Eight studies did not address antibiotic use [15, 17, 19, 21, 25, 28, 39, 41]. The 3 remaining studies did not exclude participants based on antibiotics but did collect data [31, 40, 42]. Parracho et al. [42] was the only paper to report on the effect of antibiotics, finding no relationship between exposure and the microbiota.

GI Symptoms and Microbiota in ASD

The vast majority of studies considered and collected information on GI symptoms. In most cases, parents or carers were asked to complete questionnaires (Table 2). Only a few studies specified a standardised assessment such as ROME III [56] or IV criteria [57], the 6 item GI Severity Index [58] and the CHARGE GI history survey [59]. Two studies excluded all subjects with gut problems

Table 2. Characteristics and methodology of included studies

Study by year of publication, Country	ASD	Control		Age, years	ASD diagnosis	Assessment of GI symptoms	Assessment of diet	Assessment of antibiotics
		unaffected siblings	unrelated children					
Studies using faeces samples								
Song et al. [19], 2004, USA	15	–	8	Not reported	No comment	No comment	No comment	No comment
Parracho et al. [42], 2005, UK	58	12	10	2–16	No comment	Questionnaire	Questionnaire	Questionnaire
Finegold et al. [64], 2010, USA	33	7	8	2–13	Paediatrician evaluation, validated by study author	Assessed but details not provided	Not assessed but addressed that some children had specific diets and the effect of diet could not be controlled in the study	No antibiotics for 1 month
Wang et al. [40], 2011, Australia	23	22	9	3–18	CARS and/or DSM-IV	FGID questionnaire	Dietary data presented but unclear how ascertained	Questionnaire
Gondalia et al. [34], 2012, Australia	51	53	–	2–12	Psychiatrist or psychologist evaluation using CARS	Questionnaire	No comment	No antibiotics or antifungals for 15 days
De Angelis et al. [35], 2013, Italy	20	10	–	4 – 10	DSM-IV-TR to categorise PDD-NOS and ASD ADI-R, ADOS and CARS	Excluded children with chronic diarrhoea, constipation, gas, heartburn, bloating	Not assessed	No antibiotics for 1 month
Kang et al. [16], 2013, USA	20	–	20	3–16	ADI-R, ADOS, ATEC and PDD-BI	Modified GSI questionnaire	Recording of GF/CF diet, probiotics use, seafood consumption, and usage of nutrient supplements	No antibiotics or antifungals for 1 month
Wang et al. [41], 2013, Australia	23	22	9	3–18	CARS and/or the DSM-IV	FGID questionnaire	No comment	No comment
Son et al. [36], 2015, USA	59	44	–	7–14	Recruited via a registry called the Simons Simplex Community through the Interactive Autism Network	Pediatric Rome III questionnaire	Daily dietary intake recorded	No antibiotics for 1 month
Tomova et al. [39], 2015, Slovakia	10	9	10	4–12	ICD-10 by clinical psychologist and child psychiatrist Additional CARS and ADI	Questionnaire	No comment	No comment
Inoue et al. [30], 2016, Japan	6	–	6	3–5	DSM-V, PARS and M-CHAT	No infant had a considerable gut disorder	No comment	No antibiotics for 1 month
Strati et al. [20], 2017, Italy	40	–	40	3–17	DSM-V, ADOS, CARS and Autism Behaviour Checklist	Constipation assessed	Not assessed but all o Mediterranean-based diet	No antibiotics for 3 months
Berding et al. [29], 2018, USA	26	–	32	2–7	No comment	Adapted GI Severity Index	Questionnaire; 3-day food diary; Youth and Adolescent Food Frequency Questionnaire	No antibiotics for 3 months
Coretti et al. [22], 2018, Italy	11	–	14	2–4	DSM-V, ADOS 2 and ADI-R. Griffiths Mental Development Scales, Vineland Adaptive Behaviour Scales and CARS	Rome III questionnaire, Italian Version. Excluded chronic diseases of the GI or respiratory tract	3 days diary	No antibiotics for 4 weeks

Table 2. (continued)

Study by year of publication, Country	ASD	Control		Age, years	ASD diagnosis	Assessment of GI symptoms	Assessment of diet	Assessment of antibiotics
		unaffected siblings	unrelated children					
Kang et al. [32], 2018, USA	23	–	21	4–17	ATEC and PDD-BI	Questionnaire and 6-GSI	Questionnaire	No antibiotics for 1 month
Pulikkan et al. [37], 2018, India	30	24	–	3–16	DSM-V, CARS, ISAA, INDT-ASD and paediatric neurologists, psychologists and specialist nurses	No comment	Assessed in 7 days and addressed that all on omnivore native diet	No antibiotics for 1 month
Rose et al. [33], 2018, USA	50	–	41	3–12	DSM IV, ADI-R and ADOS	GIH survey and Rome III questionnaire	Questionnaire	No antibiotics for 1 month
Zhang et al. [25], 2018, China	35	–	6	3–8	DSM-V	No comment	No comment	No comment
Li et al. [26], 2019, China	59	–	30	2–10	DSM-V, ADOS, Autism Behaviour Checklist	Assessed but details not provided	Not assessed but all on hospital cafeteria Chinese-based diet	No antibiotics for 3 months
Liu et al. [23], 2019, China	30	–	20	2.5–18	DSM-V ICD-10	Modified 6-GSI	No comment	No antibiotics for 3 months
Ma et al. [24], 2019, China	45	–	45	6–9	DSM-V, CARS	No comment	No comment	No antibiotics for 3 months
Plaza-Diaz et al. [15], 2019 Spain	57	–	57	2–6	DSM-V, ADI-R	Noted only 2 ASD children reported abdominal pain, no other GI symptoms	24-h dietary record Validated semiquantitative food frequency questionnaire used	No comment
Wang et al. [28], 2019, China	92	–	42	ASD 4.51 ± 2.23 Control 3.14±1.73	DSM-V	Rome IV criteria for FGIDs	Questionnaire	No comment
Zhai et al. [27], 2019, China	78	–	58	ASD 4.96±1.01 Control 4.90±0.97	DSM-V, ICD-10, ATEC	No comment	No comment	No antibiotics for 1 month
Studies using intestinal biopsy samples								
Williams et al. [31], 2011, USA	15	–	7	3–5	DSM-IV-TR. Confirmed by ADI-R	Questionnaires and standardised data collection forms	No comment	Questionnaires and standardized data collection forms on medication use
Williams et al. [21], 2012, USA	15	–	7	3–5	DSM-IV-TR. Confirmed by ADI-R	Questionnaires and standardised data collection forms	No comment	No comment
Kushak et al. [17], 2017, USA	21	–	19	ASD 14.43±1.07 Control 16.05±1.25	DSM-IV	All had GI symptoms and data presented but unclear how ascertained	Dietary data presented but unclear how ascertained	No comment
Luna et al. [18], 2017, USA	14	–	21	3–18	ADOS	Rome III questionnaire	No comment	No antibiotics for 3 months

ADI-R, Autism Diagnostic Interview-Revised; ADOS, Autistic Diagnostic Observation Schedule; ASD, autistic spectrum disorder; ATEC, Autism Treatment Evaluation Checklist; CARS, Childhood Autism Rating Scale; CF, casein free; DSM-IV-TR/V, Diagnostic and Statistical Manual of Mental Disorders, editions IV Text Revision/V; FGID, functional gastrointestinal disorder; GI, gastrointestinal; GIH, gastrointestinal history; GF, gluten free; ICD-10, International Classification of Diseases, 10th revision; INDT-ASD, INCLEN Diagnostic Tool for autism spectrum disorder; ISAA, Indian Scale for Assessment of Autism; M-CHAT, Modified Checklist for Autism in Toddlers; PARS, Pervasive Developmental Disorders Autism Society Japan Rating Scale; PDD-BI, Pervasive Developmental Disorder Behaviour Inventory; PDD-NOS, pervasive developmental disorder not otherwise specified; 6-GSI, 6-item gastrointestinal severity index.

Table 3. Differences in composition of microbiota by the type of bacterial detection method

Study (by year of publication)	Analysis method	Differences in gut microbiota
Studies of the gut microbiota and ASD using pyrosequencing only		
Finegold et al. [38], 2010	bTEFAP	Higher microbial diversity in ASD Bacteroidetes phyla at high levels in severely autistic group Firmicutes phyla predominant in control group *Desulfovibrio* species and *Bacteroides vulgatus* species higher in ASD Reduced populations of the *Bifidobacterium* genus in severely autistic group
Gondalia et al. [34], 2012	bTEFAP	No significant difference in the bacterial composition
De Angelis et al. [35], 2013	bTEFAP 16S rRNA (active bacteria) and 16S rDNA (total bacteria)	Higher microbial diversity in ASD Total and active Firmicutes lower in ASD Bacteroidetes highest in ASD *Faecalibacterium* and *Ruminococcus* genera highest in PDD-NOS and controls *Caloramator, Sarcina, Clostridium* genera highest in ASD *Bacteroidetes* genera, *Alistipes* and *Akkermansia* species highest in PDD-NOS or ASD Sutterellaceae and Enterobacteriaceae family higher in ASD *Bifidobacterium* species decreased in ASD
Kang et al. [16], 2013	bTEFAP V2 and V3 regions of 16S rDNA	Lower microbial diversity in ASD Significantly lower *Prevotella, Coprococcus*, and unclassified *Veillonellaceae* genera in ASD
Inoue et al. [30], 2016	Illumina Miseq desktop sequencer V3 and V4 regions of 16S rRNA	Significantly higher *Faecalibacterium* and lower abundance of *Blautia* genera in ASD
Kushak et al. [17], 2017	bTEFAP	No differences in microbial diversity *Burkholderia* genus more abundant and *Neisseria* genus less abundant in ASD Decrease in abundance of 2 Bacteroides species (*Bacteroides vulgatus* and an unknown *Bacteroides species*) and *Escherichia coli* in ASD *Oscillospira, Actinomyces, Neisseria, Peptostreptococcus*, and *Ralstonia* over-represented in ASD *Devosia, Prevotella, Bacteroides*, and *Streptococcus* depleted in ASD
Luna et al. [18], 2016	Illumina MiSeq pyrosequencing V1, V3 and V4 regions of 16S rRNA	Increase in several mucosa-associated Clostridiales in ASD, except for decreases in *Dorea* and *Blautia* Decrease in *Sutterella* in ASD *Terrisporobacter* species associated significantly with the ASD-FGID group Statistically significant increases in *Faecalibacterium prausnitzii, Roseburia intestinalis, Oscillospira valericigenes, and Bilophila wadsworthia* in NT-FGID group
Strati et al. [20], 2017	454 pyrosequencing V3-V5 of 16S rRNA Amplicon-based sequencing of fungal ITS1 region	Bacterial microbiota of ASD clusters apart from that of controls Firmicutes/Bacteroidetes ratio increased, reduction of the Bacteroidetes in ASD Reduced *Prevotella* in ASD, not supported by statistical analysis Decrease in relative abundance of the taxa *Alistipes, Bilophila, Dialister, Parabacteroides,* and *Veillonella* in ASD Increase in the taxa *Collinsella, Corynebacterium, Dorea*, and *Lactobacillus* in ASD High levels of bacterial taxa belonging to *Escherichia/Shigella* and *Clostridium* cluster XVIII in ASD children with constipation Relative abundance of the fungal genus Candida in ASD
Coretti et al. [22], 2018	Illumina MiSeq pyrosequencing V3-V4 regions of the 16S rRNA	Reduction of *Actinobacteria* and significant increase in *Bacteroidetes* and *Proteobacteria* in ASD Bacteroidetes/Firmicutes ratio significantly higher in ASD At family level *Actinomycetaceae, Coriobacteriaceae, Bifidobacteriaceae, Gemellaceae* and *Streptococcaceae* were significantly reduced in ASD
Kang et al. [32], 2018	454 16S rRNA pyrosequencing	Lower microbial diversity and a significantly different gut microbiota in ASD *Prevotella* genus significantly reduced and *Coprococcus* genus had marginally lower relative abundance in ASD Relative abundance of phylotypes most closely related to *Faecalibacterium (F. prausnitzii)* and *Haemophilus (H. parainfluenzae)* were significantly lower in ASD
Pulikkan et al. [37], 2018	NextSeq500 Illumina pyrosequencing V3 region of 16S rRNA	Microbial diversity of the ASD group was similar to controls Increased proportion of Firmicutes in ASD Differences seen in the microbiome composition of children with ASD irrespective of GI symptoms Higher relative abundance of *Prevotellaceae* in controls, higher abundance of *Veillonelleaceae* in the ASD Significantly higher relative abundance of *Lactobacillaceae, Bifidobacteraceae* and *Veillonellaceae* in ASD Higher relative abundance of genera *Bifidobacterium, Lactobacillus, Megasphaera* and *Mitsuokella* in ASD The most prominent variation was shown by *Lactobacillus* genus, which was observed to be ~32-fold higher in ASD Phylogenetic plot of the taxa of discriminatory OTUs displayed an abundance of *Prevotella* from family Prevotellaceae, *Faecalibacterium* from family *Clostridiaceae*, and *Roseburia* from family *Lachnospiraceae* in healthy children *Ruminococcus* from family *Ruminococcaceae, Coprococcus*, and *Butyrivibrio* from family *Lachnospiraceae*, and *Klebsiella* from family *Enterococcaceae* were found abundant in ASD
Rose et al. [33], 2018	Illumina MiSeq pyrosequencing V3–V4 regions of the 16S rRNA	Increased *Bacteriodaceae, Lachnospiraceae, Ruminococcaceae* and *Prevocellaceae* in ASD children with GI symptoms compared to control children with GI symptoms No difference between the groups in those without GI symptoms

Table 3. (continued)

Study (by year of publication)	Analysis method	Differences in gut microbiota
Zhang et al. [25], 2018	Illumina HiSeq Sequencing V3-V4 regions of 16S rRNA	Bacteroidetes/Firmicutes ratio was significantly higher in ASD *Streptococcus, Veillonella* and *Escherichia* significantly less abundant in ASD
Li et al. [26], 2019	Illumina HiSeq pyrosequencing V1–V2 regions of the 16S rRNA	Firmicutes/Bacteroidetes ratio similar in ASD and controls Significant increase in the relative abundance of Proteobacteria in ASD Significant increases in the relative abundance of *Enhydrobacter, Chryseobacterium, Streptococcus*, and *Acinetobacter* (at the genus level), as well as *Acinetobacter rhizosphaerae* and *Acinetobacter johnsonii* (at the species level), in addition to a significant reduction in *Prevotella melaninogenica* (at the species level) in ASD
Lui et al. [23], 2019	Illumina MiSeq Sequencing V3–V4 regions of 16S rRNA	Composition of the ASD gut microbiota was different from controls *Firmicutes* significantly decreased and *Acidobacteria* considerably increased in ASD *Veillonellaceae* and *Enterobacteriaceae* increased in ASD, *Ruminococcaceae, Streptococcaceae, Peptostreptococcaceae* and *Erysipelotrichaceae* significantly decreased Significant increase of taxa *Veillonellaceae* and significant decrease of *Erysipelotrichaceae* in ASD *Megamonas* was increased in ASD, *Eubacterium* and *Lachnospiraceae-NC2004-group* genera increased in controls
Ma et al. [24], 2019	Illumina HiSeq Sequencing V3–V4 regions of 16S rRNA	Firmicutes/Bacteroidetes ratio not significantly different between ASD and controls Differences at the family level did not reach statistical significance except for *Acidaminococcaceae* No significant group difference was found in the relative abundance of microbiota at the class and order level At the genera level, *Bacteroides* constituted the most abundant genus in both ASD and controls, but with no significant difference. *Lachnoclostridium, Tyzzerella subgroup 4, Flavonifractor* and unidentified *Lachnospiraceae* less abundant in ASD At the species level, *Clostridium clostridioforme* more abundant in ASD
Plaza-Diaz et al. [15], 2019	Illumina MiSeq Sequencing V3–V4 regions of 16S rRNA	*Actinobacteria* and *Proteobacteria* phyla higher in ASD At class level, *Actinobacteria, Bacilli, Erysipelotrichi* and *Gammaproteobacteria* were higher in ASD *Bacillaceae, Bifidobacteriaceae, Corynebacteriaceae, Desulfohalobiaceae, Enterobacteriaceae, Enterococcaceae, Erysipelotrichaceae, Fusobacteriaceae, Microbacteriaceae* and *Thermoactinomycetaceae* were significantly higher in ASD, *Lachnospiraceae* was lower in ASD At genus and species levels, *Bacillus, Bifidobacterium, Butyrivibrio, Enterococcus, Hespellia, Prevotella, Clostridium bolteae*, and *Clostridium difficile* significantly higher in ASD
Wang et al. [28], 2019	Illumina Hiseq4000 sequencer, shotgun metagenome sequencing	Significantly lower species richness in ASD children Significantly higher levels of the phylum *Actinobacteria* in ASD 11 taxons were significantly higher ASD: including 3 *Clostridium taxons*, 2 *Eggerthella* taxons, and 2 *Klebsiella* taxons 8 taxons were significantly lower ASD: *Bacteroides vulgatus, Betaproteobacteria, Campylobacter jejuni subsp. jejuni 81-176, Campylobacter jejuni subsp. jejuni ICDCCJ07001, "Candidatus Chloracidobacterium thermophilum B", Coraliomargarita akajimensis DSM 45221, Proteus mirabilis, and HI4320 Spirochaeta thermophila DSM 6192*
Zhai et al. [27], 2019	Illumina MiSeq Sequencing V3–V4 regions of 16S rRNA	The gut microbiota diversity of ASD children was significantly higher The ratio of Bacteroidetes to Firmicutes showed a significant increase in ASD ASD children had a significant increase in nine genera: *Bacteroides, Parabacteroides, Sutterella, Lachnospira, Bacillus, Bilophila, Lactococcus, Lachnobacterium* and *Oscillospira*
Studies of the gut microbiota and ASD using pyrosequencing and qPCR		
Williams et al. [31], 2011	qPCR and 454 pyrosequencing V2 of 16S rRNA	Lower abundance of Bacteroidetes in autistic children Increased Firmicutes/Bacteroidetes ratio in autistic children Increased *Clostridiales* levels in ASD, largely attributable to increases in *Lachnospiraceae and Ruminococcaceae* families Elevated levels of Betaproteobacteria, primarily due to presence of *Alcaligenaceae* family
Williams et al. [21], 2012	qPCR and 454 pyrosequencing V2 region of the 16S rRNA	*Sutterella* species (*S. stercoricanis* and *S. wadsworthensis*) present in ASD children with gastrointestinal symptoms but completely absent in controls with gastrointestinal symptoms
Son et al. [36], 2015	qPCR and Illumina Miseq pyrosequencing V1V2 and V1V3 regions of 16S rRNA	No significant difference in diversity or overall microbial composition Increased relative abundance of Chloroplast genus in ASD No differences in *Sutterella, Prevotella* and total Bacteroidetes groups in qPCR analysis
Berding et al. [29], 2018	qPCR and Illumina Miseq pyrosequencing V3-V4 regions of 16S rRNA	Lower abundance of *Bacteroidetes* but higher abundance of *Firmicutes* in ASD The abundance of *Clostridiales* higher and abundance of *Streptophyta* lower in ASD At a family level, significantly higher abundance of *Coriobacteriaceae, Clostridiaceae* and *Peptostreptococcaceae* but lower abundance of *Rikenellaceae* in ASD At a genera level, increased abundances of *Clostridiaceae Clostridium, SMB53, Blautia*, and *Roseburia* and decreased abundances of *Butyricimonas, Butyrivibrio, Faecalibacterium, Dialister*, and *Bilophila* in ASD *Bifidobacterium* and *C. perfingens* determined by qPCR higher in controls
Studies of the gut microbiota and ASD using qPCR only		
Song et al. [19], 2004	qPCR	*Clostridium bolteae, Clostridium* clusters I and XI significantly elevated in ASD
Wang et al. [40], 2011	qPCR	Lower relative abundances of *Bifidobacterium* and *Akkermansia muciniphila* genus in ASD Elevated relative numbers of *B. fragilis* group in ASD children experiencing FGIDs

Table 3. (continued)

Study (by year of publication)	Analysis method	Differences in gut microbiota
Wang et al. [41], 2013		*Sutterella* elevated in ASD
	qPCR	*Ruminococcus torques* higher in ASD children with FGID
Tomova et al. [39], 2015		Bacteroidetes/Firmicutes ratio decreased in ASD
	qPCR	*Lactobacillus, Desulfovibrio* and *Clostridium* cluster l elevated in ASD
Studies of the gut microbiota and ASD using FISH		
Parracho et al. [42], 2005	FISH	*Clostridium histolyticum* (*Clostridium* clusters I and II) higher in ASD Sibling group had an intermediate level of *C. histolyticum*

ASD, autistic spectrum disorder; bTEFAP, bacterial tag-encoded FLX amplicon pyrosequencing; FGID, functional gastrointestinal disorder; FISH, fluorescence in situ hybridization; GI, gastrointestinal; NT, neurotypical; OTUs, operational taxonomic units; PDD-NOS, pervasive developmental disorder not otherwise specified; qPCR, quantitative PCR.

[30, 35]. In contrast, in 4 studies, all participants had GI symptoms and all used mucosa-based analyses [17, 18, 21, 31]. This is unsurprising as it would be unethical to perform an endoscopy without any clinical indication. The studies that assessed GI symptoms consistently reported a significantly increased burden in autistic children. The analyses of the gut microbiota did not however always take symptoms into account. Five studies described distinct microbiota profiles in ASD with and without GI symptoms [20, 23, 33, 40, 41]. Two studies found no association [26, 28]. The presence of GI symptoms is an important confounding factor in the study of gut microbiota and ASD.

Dietary Impact on the Microbiota in ASD

Dietary restriction is a fundamental component of ASD throughout life, and diet is also a potent driver of the individual microbiota [29, 60, 61]. Therefore, good dietary data is fundamental in understanding microbial diversity in ASD. Despite this, data on dietary patterns were collected in less than half the studies (Table 2). Most used individual questionnaires or non-validated surveys to acquire information about diet and primarily collected information on whether participants were on restricted diets (Table 2). Son et al. [36] used the Stony Brook University Medical Centre Department of Paediatrics Food Diary/Calorie Count Sheet and asked parents to record intake for 7 days prior to stool collection. They also used nutrient analysis as well as reporting the number of children on special diets [36]. Kang et al. [16] gathered information on diet patterns such as gluten-free/casein-free diets as well as information on the use of probiotics, vitamins and seafood consumption. Rose et al. [33] asked parents to report allergies, if patients were on restricted diets and if they had any specific food dislikes and foods that made symptoms worse.

Son et al. [36] reported no significant differences in microbial composition with respect to daily intake of micronutrients. No relationship was found between diet and microbial populations in the study by Parracho et al. [42]. Kang et al. [16] performed multivariate analysis and reported no significant associations between dietary intake and genus abundances. Five studies highlighted the issue of diet but did not specifically collect data [20, 22, 26, 35, 37]. De Angelis et al. [35] suggested their subjects came from the same families and so differences in diet could be excluded. Similarly, Strati et al. [20] and Li et al. [26] stated their participants were consuming a Mediterranean and Chinese diet respectively and did not comment further [22]. Pulikkan et al. [37] reported all subjects were consuming an omnivore native diet but did not provide more information. Berding et al. [29] investigated the microbiota composition in relation to feeding behaviour, nutrient and food group intake as well as dietary patterns. They found that dietary fibre negatively correlated with abundance of *Clostridiales*. *Faecalibacterium* abundance was positively correlated with fried food and negatively with fruit. They also identified that 2 distinct dietary patterns were associated with unique microbial profiles in children with ASD [29]. Similarly, differential patterns in food and microbiota were also seen in children with ASD in the study by Plaza-Diaz et al. [15]. The vast majority of studies did not collect details on diet or assess dietary impact on the gut microbiota. As diet is an important confounding factor, the extent to which diet affects the microbial composition in the ASD population remains unclear.

Changes to the Microbiota in ASD

When comparing microbial diversity of the gut microbiota in children with ASD against controls, 3 studies reported an increase in ASD [27, 35, 38], 2 reported a reduc-

tion [16, 32], and 3 described no difference [17, 36, 37] (Table 3). Firmicutes, Bacteroidetes and Proteobacteria were the most abundant phyla reported in all studies (Table 3). Bacteroidetes were increased in ASD children in 5 studies [22, 25, 27, 35, 38]. Three studies noted a significant increase in the Firmicutes/Bacteroides ratio due to a decrease in Bacteroidetes in ASD children [20, 31, 37] and 2 studies showed no difference in Bacteroidetes and Firmicutes levels [24, 26].

Clostridia and ASD

Within the Firmicutes phylum, increased Clostridiales levels have been reported in ASD patients in several 16S rRNA pyrosequencing studies [15, 18, 24, 28, 29, 31, 35]. This has also been seen with qPCR studies: 3 *Clostridium* clusters (I, XI, and XIVab) and one specific *Clostridium* species, *C. bolteae*, were statistically significantly higher in ASD in Song et al. [19]. A further qPCR study reported increased *Clostridium* cluster I in ASD [39]. Using fluorescent in-situ hybridisation, Parracho et al. [42] identified an increase in *Clostridium histolyticum* in stools from ASD compared to healthy controls, although unaffected siblings of ASD children also had higher levels of *C. histolyticum* than healthy controls. Strati et al. [20] showed that GI problems in ASD children were associated with high levels of *Clostridia*. Williams et al. [31] found that the increase in Clostridiales was largely attributable to increases in *Lachnospiraceae* and *Ruminococcaceae*. Increased *Lachnospiraceae* and *Ruminococcaceae* were also seen in ASD children with GI symptoms when compared to control children with similar symptoms in Rose et al. [33]. Pulikkan et al. [37] also noted an increase in *Ruminococcaceae* in their analysis whereas Liu et al. [23] found *Ruminococcaceae* to be significantly reduced in ASD.

Sutterella and ASD

Greater abundance of *Sutterella* has been reported in ASD children [21, 27, 35, 41]. Williams et al. [31] found elevated levels of Betaproteobacteria in ASD, reflecting the presence of *Alcaligenaceae*, and later described these as *Sutterella* species [21]. They confirmed *Sutterella* species in over 50% of ASD children and their complete absence in control children. *Sutterella* species have been identified in individuals with conditions such as IBD but also in healthy individuals [62, 63]. It is currently unclear whether they are normal commensals or pathobionts. Two other studies observed *Sutterella* was less abundant in ASD [16, 18].

Other Bacteria Implicated in ASD

Desulfovibrio species were shown to be higher in ASD in 2 studies [38, 39]. Moreover, a strong correlation of *Desulfovibrio* and the severity of autism manifestations were noted [39]. *Desulfovibrio* species are associated with increased propionic acid (PPA) production, thought to be associated with ASD pathogenesis [64] *Lactobacillus* were also reported to be significantly higher in ASD in 3 studies [20, 37, 39]. Most notably, *Lactobacillus* genus was observed to be 32-fold higher in ASD children compared to healthy children in Pulikkan et al. [37].

Wang et al. [40] reported a lower relative abundance of *Bifidobacterium* in ASD compared to unrelated controls and unaffected siblings. Decreased *Bifidobacterium* in ASD children was also reported in 3 other studies [29, 35, 38], with 2 reporting a significant increase [15, 37]. Both *Lactobacillus* and *Bifidobacterium* species are commonly used in probiotic supplements [65]. Probiotic use could therefore have contributed to increased numbers of these bacteria; however, the use of probiotics at baseline was not specified in the studies concerned.

Abundance of *Akkermansia muciniphila* was reported decreased in ASD relative to controls in Wang et al. [40]. *A. muciniphila* is a mucin-degrading bacterium, usually present in large amounts in the healthy gut, thus its absence may contribute to altered mucus barrier function. In contrast, increased levels of *Akkermansia* have also been reported in ASD [35]. Lower levels of *Prevotella* genera were also noted in ASD in 4 studies [16, 17, 20, 32], although this was significant only in 3 [16, 17, 32]. A significant reduction in *Prevotella melaninogenica* species was seen in the study by Li et al. [26]. Plaza-Diaz et al. [15] reported significantly increased levels of *Prevotella* in ASD.

Discussion

The Brain-Gut-Microbiome axis is a concept that refers to the complex interactions between the central nervous system, GI system and the microorganisms of the GI tract. The significance of GI issues in ASD children has led to the investigation of gut microbial involvement in the disease. In this paper, we have systematically reviewed the literature and identified 28 studies assessing the microbiota in individuals with ASD compared to healthy individuals. Unfortunately, we were not able to complete a meta-analysis due to the heterogeneity of studies.

Gut microbiome alterations are implicated in ASD; species which have been reported to change include *Clostridium, Desulfovibrio, Lactobacillus* and *Sutterella*. Altered gut microbial composition may cause disruption of the gut barrier, potentially allowing translocation of bacteria and their antigens, toxins and metabolites [12]. Bacterial fermentation of dietary carbohydrates normally leads to the production of short chain fatty acids (SCFAs). These are signalling molecules with a variety of functions: the most abundant SCFAs are acetic acid, butyric acid, and PPA. MacFabe [66] showed that when PPA or other SCFAs were injected into the cerebral ventricles of rats, the rats demonstrated biological, chemical, and pathologic changes that were characteristic of autism. Of note, many of the bacteria implicated in ASD, such as *Clostridium* and *Desulfovibrio* are PPA producers. Additionally, specific strains produce harmful toxins such as lipopolysaccharides, which can theoretically lead to the production of inflammatory cytokines and impair neurodevelopment [38, 67]. Increased circulating levels of pro-inflammatory cytokines have been reported in ASD [68].

Much focus has been given to *Clostridium* groups in ASD, although the causal relationship remains to be proven. Bolte et al. [69] first postulated that *Clostridium tetani* could induce autism in 1998, although offered no mechanistic insight. Clostridiaceae synthesise metabolic products (e.g., phenols, p-cresol, certain indole derivatives) that are potentially toxic for humans. One of the first studies to speculate that disruption of the gut microbiota might contribute to autistic symptomatology was published in 2000 by Sandler et al. [70]. This small study showed improvement of autistic symptoms after oral vancomycin. Symptoms relapsed following cessation and it has been suggested this may be because *Clostridium* are spore-forming organisms, promoting recurrence [70]. However, of note, this study used non-validated measures to assess symptoms of autism. These findings are also yet to be replicated in further studies.

Although not a target of this review, an altered gut microbiota has also been seen in rodent models of ASD [71]. Prenatal exposure to the anticonvulsant valproate (VPA) is a risk factor for ASD and exposure to VPA in rodent models results in behavioural impairments. Studies have been reported showing altered gut microbiota, altered GI morphology and CNS inflammation following prenatal VPA treatment [71]. A study by de Theije et al. [72] showed that VPA-treated 28-day offspring had decreased abundance of *Bacteroidetes* phyla, mainly consisting of *Bacteroidales*, and increased *Firmicutes* microbial taxa, mainly consisting of *Clostridiales*. This is in keeping with findings from some of the studies discussed in this review.

There are numerous challenges to studying how the gut microbiota may be implicated in ASD and this is evident from the conflicting and complex results of studies conducted so far. Importantly, there is a lack of a uniform definition of ASD and the diagnostic criteria have continued to evolve over the last 15 years. Moreover, autistic spectrum disorders encompass a heterogeneous range of conditions with varying severity. The numbers of participants in the studies are often small; therefore, the chances of type 2 statistical errors cannot be disregarded. There is significant variation in the sampling methods, sites and laboratory techniques used across studies which invariably makes comparison difficult. The gut microbiota ecosystem in mucosal surfaces is fundamentally different to that of faeces; hence, the 2 are not comparable [73]. The majority of studies reported here focused on faeces. Mucosal samples are understandably difficult to obtain in this age group due to logistic and ethical limitations; however, this is an important sampling issue to keep in mind when drawing conclusions from studies. Bacteria at the mucosal surface are in close contact with the host and may have a more potent pathophysiologic role than luminal bacteria. The studies discussed used various techniques to study microbial communities. Differences may arise from various aspects: sample handling; DNA extraction; sequencing of different regions of DNA; varying coverage achieved by PCR primers; sequencing depth; and bioinformatic/statistical methods.

Additionally, not all the studies have shared important baseline information about the participants. All results need to be considered with caution, especially given the potential impact of diet, antimicrobial use and GI symptoms on the gut microbiota. Individuals with ASD are known to have specific dietary interests: they are commonly restrictive with food choices by texture, smell and taste [74]. Some individuals with ASD also follow restrictions, such as gluten-free diet, in an attempt to improve symptoms. Although popular with parents and clinicians, there is little evidence to support the use of dietary therapies for the treatment of core symptoms of ASD [75]. It has been established that diet has a significant role in the modelling of the gut microbiota [76]. Protein, fats, digestible and non-digestible carbohydrates, probiotic and polyphenols can change the microbiome [60]. Several studies have shown that a Western diet (high in animal protein and fat, low in fibre) leads to a marked decrease

in beneficial bacteria such as *Bifidobacterium* and *Eubacterium* species [60]. In contrast, the Mediterranean diet is highly regarded as a healthy balanced diet with positive effects on inflammation and lipids, mediated by increases in *Lactobacillus, Bifidobacterium* and *Prevotella* and decreases in *Clostridium* [60]. Furthermore, it has been shown that dietary alterations can induce large microbial shifts, even within 24 h of a change [77]. To clarify the relationship between diet, gut bacteria and autism, future studies need to be carefully designed with the inclusion of validated and objective food diaries and detailed dietary histories.

Studies have shown a higher incidence of antibiotic usage in individuals with ASD, particularly because of susceptibility to infections such as otitis media [78]. In a pyrosequencing study, healthy humans were exposed to ciprofloxacin and their microbiota was assessed before and after treatment. Ciprofloxacin was found to lower the diversity of bacteria [55]. The microbial composition largely returned to pre-antibiotic state after 4 weeks; however, a few species did not return to original numbers within 6 months [55]. It is clear that antibiotics can have a significant influence on the composition of the gut microbiota and therefore may be a confounding factor in studies assessing the gut microbiota in ASD.

It is widely accepted that children with ASD have an increased prevalence of GI symptoms. Although an altered gut microbiota has been seen in children with ASD with GI symptoms, whether these changes are also seen in ASD children without GI symptoms is not clear from studies thus far. Not all studies have addressed the presence and effect of GI symptoms on ASD and microbial profiles and very few have considered them in statistical analyses, adding to the complexity of interpreting microbial outcomes.

In conclusion, ASD is an increasingly common condition that affects millions of families across the world. Studies to date have been inconsistent in their findings; however, this in part can be explained by heterogeneous populations and methods. Future studies of the gut microbiota in autism need more objective clinical assessment, consistency of case ascertainment and description. Furthermore, details regarding antibiotic use, diet and GI symptoms are key to unravelling the link between autism and an altered gut microbiota. There are several noteworthy reasons to consider that the gut microbiota may be involved in autism: the significance of GI symptoms in ASD; the apparently distinct gut microbiome of these children; and the mechanistic plausibility of bacterial products causing neurobehavioral effects. Gaining a better understanding of this important gut-brain connection may offer insights into potential diagnostic or therapeutic options and help alleviate the rising burden of ASD.

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Statement of Ethics

Ethical approval was not required for this work. Research was conducted ethically in accordance with the World Medical Association Declaration of Helsinki.

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Author Contributions

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