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Impact of gut microbiota on neurogenesis and neurological diseases during early life

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Impact of gut microbiota on neurogenesis and neurological diseases during early life

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Editorial: Impact of gut microbiota on neurogenesis and neurological diseases during early life

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Editorial on the Research Topic
Impact of gut microbiota on neurogenesis and neurological diseases during early life

In this Research Topic several studies have analyzed the ability of nutritional microbial environment during perinatal and early life to reduce the risk of non-communicable and mental diseases later in life. Furthermore, the effect of the early gut microbiome development in the congenital alteration of brain functions has been studied, as well as the effect of prebiotic, probiotic, parabiotics and postbiotics supplementation on neurodevelopment, and neurodegenerative disorders; in addition, a study about the how neonatal infection influences on early brain development through the gut-brain axis is published within this Research Topic.

In the case of the gut microbiome and early environmental relationships with neurodevelopment, three studies have explored the link between early dysbiosis and long-term infant health. Beghetti et al., carried out a review delving into the relationship between both dynamic patterns and static features of the gut microbiota during preterm infants' early life and brain maturation, as well as neurodevelopmental outcomes in early childhood. Ozorio Dutra et al., explored associations between the infants' gut microbiome and early childhood behavior at 4 years of age in 19 children who were previously born with very low birth weight. They identified the bacterial taxa through a multivariate analysis by linear models, where *Veillonella dispar*, *Enterococcus*, *Escherichia coli*, and *Rumincococcus* were statistically significantly associated with later behavior at 4 years. Bezerra et al., wrote an opinion article which discussed how the double burden of malnutrition compounded with the environmental enteric dysfunction in growing children under adverse environments may negatively influence the intestinal microbiota homeostasis and hence the gastrointestinal tract-related melatonin function.

In the case of the association between gut microbiome and neurological disorders, Bao et al., characterized the gut microbial profiles in 32 children with Tourette syndrome (TS) and 29 healthy controls (HC), indicating a different gut microbial composition in children with TS with respect to HC, with multiple Gut-Brain Microbiota (GBM) neurotransmitter

modules (Histamine degradation, Dopamine degradation, and 3,4-dihydroxyphenylacetic acid (DOPAC) synthesis) significantly increased. Moreover, combined physiotherapy (CES therapy and biofeedback training) was associated with a lower abundance of several genera and significant decreases in GBM neurotransmitter modules in patients following this treatment, indicating a possible improvement of clinical symptoms. Mendive Dubourdieu and Guerendiain, carried out a descriptive cross-sectional study analyzing the dietary intake and the gut composition of 30 children with autism spectrum disorder vs. 28 children with typical development, classified by their body mass index. Children with excess weight and ASD had lower *Roseburia* and *Faecalibacterium prausnitzii* and higher *Eubacterium ventricosum* and *Flavonifractor plautii* than the TD group with the same nutritional status. Moreover, they found positive and negative associations between the bacteria genus and species, and the nutrition in adjusted models, ASD/TD.

The effect of nutritional supplementation (prebiotic, probiotic, parabiotics and postbiotics) on early neurodevelopment was also explored by Rahim et al., by using 3,393 electronic databases with a total of 720 individuals between the ages of 2 and 17, as well as 112 adults ranging from 5 to 55 years old, all of whom had received a diagnosis of ASD. They observed that although there was no significant effect of such therapy on autism-related behavioral symptoms, psychobiotics had a significant effect on the brain connectivity through frontopolar power in beta and gamma bands mediated by chemicals and cytokines, such as TNF-α. In addition, Campbell et al., studied the influence of *in-utero* vitamins and minerals (BSM) exposure on infant temperament antenatally and for 12 months postpartum, in a cohort of 114 mother-infant dyads (45 infants exposed to BSM during pregnancy and 69 non-exposed). Results showed that BSM exposure did not significantly predict infant temperament, however, it may mitigate risks associated with antenatal depression. Furthermore, BSM-exposed infants displayed temperamental characteristics on par with typical pregnancies, supporting the safety of BSM treatment for antenatal depression.

Lastly, two studies in this Research Topic evaluated the function of the gut-brain axis in neurodegenerative disorders and neonatal infection. Vaia et al., carried out a mini-review that explored the intricate bidirectional relationship between gastrointestinal disorders and neurodegeneration in leukodystrophy infantile population, a disease relatively frequent in childhood causing neuro-motor disability, to affect the white matter of the brain. Tagi et al., performed a narrative review analyzing the state of the link between post-streptococcal autoimmune neuropsychiatric disorders (PANDAS) and gut microbiota composition in children. Notable changes included reduced microbial diversity and shifts in bacterial populations, which affect metabolic functions crucial for neuroinflammation. Moreover, elevated serum levels of sNOX2-dp and isoprostanes seem to indicate oxidative stress, while the presence of lipopolysaccharides (LPS) may contribute to neuroinflammation.

Overall, these findings might be important for developing gut microbiota-based therapeutic strategies for the treatment and/or prevention of behaviors or brain pathologies. These nine articles try to understand molecular mechanisms and pathways involved in microbiota-brain connections, elucidate some of the numerous sources of conflicting evidence and answer unanswered questions about the influence of intestinal dysbiosis on neurogenesis and neurological diseases during early life. However, it is important to emphasize that more studies are required to overcome the considerable gaps in transferring the results obtained in reductionist animal models to human clinical practice.

Author contributions

TC: Conceptualization, Supervision, Writing – original draft, Writing – review & editing. CC: Conceptualization, Supervision, Writing – original draft, Writing – review & editing.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Understanding the link between gut microbiota, dietary intake, and nutritional status in children with autism and typical development

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Background: Gut microbiota plays a potential role in human health and different disorders such as autism spectrum disorder (ASD). Therefore, we analyzed gut bacteria composition in children with ASD and typical development (TD), and its relationship with nutritional status and dietary intake.

Methods: A descriptive cross-sectional study was carried out in 3- to 12-year-old children (ASD = 30, TD = 28). Dietary intake (applying food frequency questionnaires) and body mass index-for-age (expressed in z-score) were determined. Children were divided into normal weight and excess weight (risk of overweight + overweight + obesity), and the ASD group was categorized into gluten- and casein-free diet (ASD-diet) or no diet (ASD-no diet). The relative abundance of gut bacteria was analyzed in fecal samples by 16S rRNA sequencing.

Results: Children with excess weight had lower *Roseburia* than normal weight. Fewer *Bifidobacterium longum* and higher *Clostridium glycolicum* were found in the ASD group compared with TD one. Participants with excess weight and ASD had lower *Roseburia* and *Faecalibacterium prausnitzii* and higher *Eubacterium ventricosum* and *Flavonifractor plautii* than the TD group with the same nutritional status. Positive and negative associations were found between the bacteria genus and species, and the intake of dairy, vegetable drinks, cereals with and without gluten, food source of proteins, fish, food source of fat, and coconut oil, in unadjusted models and after adjustment for age, diet/no diet, ASD/TD.

Conclusion: Significant differences in microbial community composition were found between children with ASD and TD, considering their nutritional status and dietary intake.

KEYWORDS

autism spectrum disorder, gut microbiota, nutritional status, gluten- and casein-free diet, dietary intake, children, adolescents

Introduction

Autism spectrum disorder (ASD) is a neurodevelopment condition that has had a rapidly increasing prevalence. However, there is no standard treatment due to its complex ethology, involving genetic and environmental factors (1, 2). In the last few decades, it has been recognized that gut microbiota plays a major role in human health and different disorders such as autism (3). Multiple cohort studies indicate that several inflammation-related disorders and neurodevelopmental diseases have been associated with alterations in the gut ecosystem, a condition known as dysbiosis (4, 5). For example, a greater relative abundance of certain bacteria such as *Clostridium* and *Sutterella* has been observed in

children with ASD as opposed to typical development (TD) ones, but the findings from different investigations are still controversial (6).

Robust literature data show that there is a two-way communication between gut and the brain, in which microbiota, the enteric nervous system, autonomic nervous system, endocrine system, immune system, and central nervous system are involved (4). Bacterial metabolites have been shown to be implicated in the secretion of neurotransmitters that are part of memory, learning, and behavioral processes (7). Diet plays an important role in gut microbiota homeostasis and metabolism, and children with ASD have difficulties in maintaining a balanced diet due to multiple factors such as highly selective food preference and gastrointestinal problems (8). In addition, many families with children with ASD have chosen to follow a gluten- and casein-free diet (GCFD) under the unproven hypothesis that these proteins are metabolized into gluteomorphin and casomorphin and that, via a leaky gut, they bind to opiate receptors in the central nervous system causing autism symptoms (9). There is still no consensus on the use and effectiveness of this type of diet for treating autism, and additional studies are needed to describe the effects of the diet on gut microbiota (9–11).

Dietary intake can modulate gut microbiota throughout life, and this action would depend on the type and amount of foods chosen, which can inflect up to 60% of the microbiome composition since it provides countless substrates for microbial metabolism (12, 13). Furthermore, there are bacteria with specific enzymes that convert certain nutrients into different metabolites that influence brain function (14). For example, from the metabolism of tryptophan, it is possible to obtain indole which has a positive effect on mental health, but another metabolite such as indoxyl sulfate has been linked to the development of ASD.

In relation to food intake and its association with bacterial taxonomy, a study of European children (fed with a Western diet rich in animal protein) and African children (fed with local vegetables and whole grains) showed that children in Burkina Faso have higher levels of *Prevotella* and lower levels of *Bacteroides* and *Enterobacteriaceae* than children from Italy (15). This diet and intestinal microbiota in rural African children have been linked to lower inflammatory conditions and infectious colonic diseases (11, 15). On the contrary, it has been shown that diets with a high intake of red meat, refined carbohydrates and fat, and a lower consumption of fish and vegetables could cause dysbiosis (13).

Moreover, scientific evidence has shown that nutritional status is related to gut microbiota (16). There is a lack of consensus as regards a healthy-type taxonomic microbiome composition, but recent studies show that there is a difference in gut bacteria between obese and lean children and adolescents (17). Bervoets et al. (18) observed that children with obesity had a higher level of *Lactobacillus* spp. and a lower level of *Bifidobacterium vulgatus* than the lean ones. A prospective study showed that obesity in children was associated with an increase in *Bacteroidaceae* and a lower relative abundance of *Prevotellaceae* compared with children with normal weight (16). The relation between gut bacteria and weight gain is still unclear (12).

It has been shown that early intervention in children's gut microbiota can help prevent health disorders, but it is necessary to elucidate the link between diet and intestinal microbe composition to define a strategy to improve their health (19, 20). The symptoms and comorbidities of ASD could be improved with dietary interventions carried out after a deeper understanding of how foods relate to the intestinal microbiota (14). Therefore, the purpose of this study was to analyze gut bacteria composition in children with ASD and TD, and its relationship with nutritional status and dietary intake.

Materials and methods

Participants and ethics statement

From February to March 2020, we recruited a total of 65 children and adolescents aged 3 to 12 years at the nutritionist's office in Montevideo, Uruguay, through an open call (8); in this gut microbiota study, 30 with ASD and 28 neurotypicals were included (Figure 1). Diagnoses of ASD by a psychiatrist or a pediatric neurology specialist met the criteria of the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) (21). Participants had not been taking medication, antibiotics, or probiotics for at least 1 month prior to enrollment in the study, and no children in the TD group were on a restricted diet. In addition, those diagnosed with attention deficit and hyperactivity disorder, diabetes mellitus, genetic diseases, inborn errors of metabolism, inflammatory bowel disease, celiac disease, and motor disability were excluded from both groups. This research was performed in accordance with the Helsinki Declaration 2000, approved by the Research Ethics Committee of the School of Nutrition, University of the Republic, and registered with the Ministry of Health of Uruguay (No. 282599). The study was explained to the participants' parents by telephone and discussed personally during their first visit to the research clinic, where informed written consent was obtained from every parent.

Sample collection, gut microbiota sequencing, and taxonomic classification

Children's parents were given a fecal microbiota kit (tube with transport media and specimen collection swab) and thorough instructions as to how they should collect the stool samples from their children at home. They collected a single fecal sample that was refrigerated until delivery to the clinic within 48 h. Once received, the samples were transferred to the laboratory with a cold pack and stored in an ultra-freezer at −80°C until analysis.

Extraction of bacterial DNA was performed at Enteria SRL laboratory, which followed the protocol recommended by Quick–DNA Fecal/Soil Microbe Miniprep Kit (Zymo Research—Catalog No. D6010). The quantity and quality of DNA were assessed by measuring absorbance at 260 and 280 nm using the Tecan Infinite M200 Pro (absorbance range 1.8–2 OD280/260). The extracted genomic DNA was sent to Genia laboratory to amplify hypervariable V1-V9 regions of the 16S rDNA gene from bacteria with Ion 16S™ Metagenomics Kit in PCR cycler using the Ion Torrent™ semiconductor sequencing workflow. Amplified

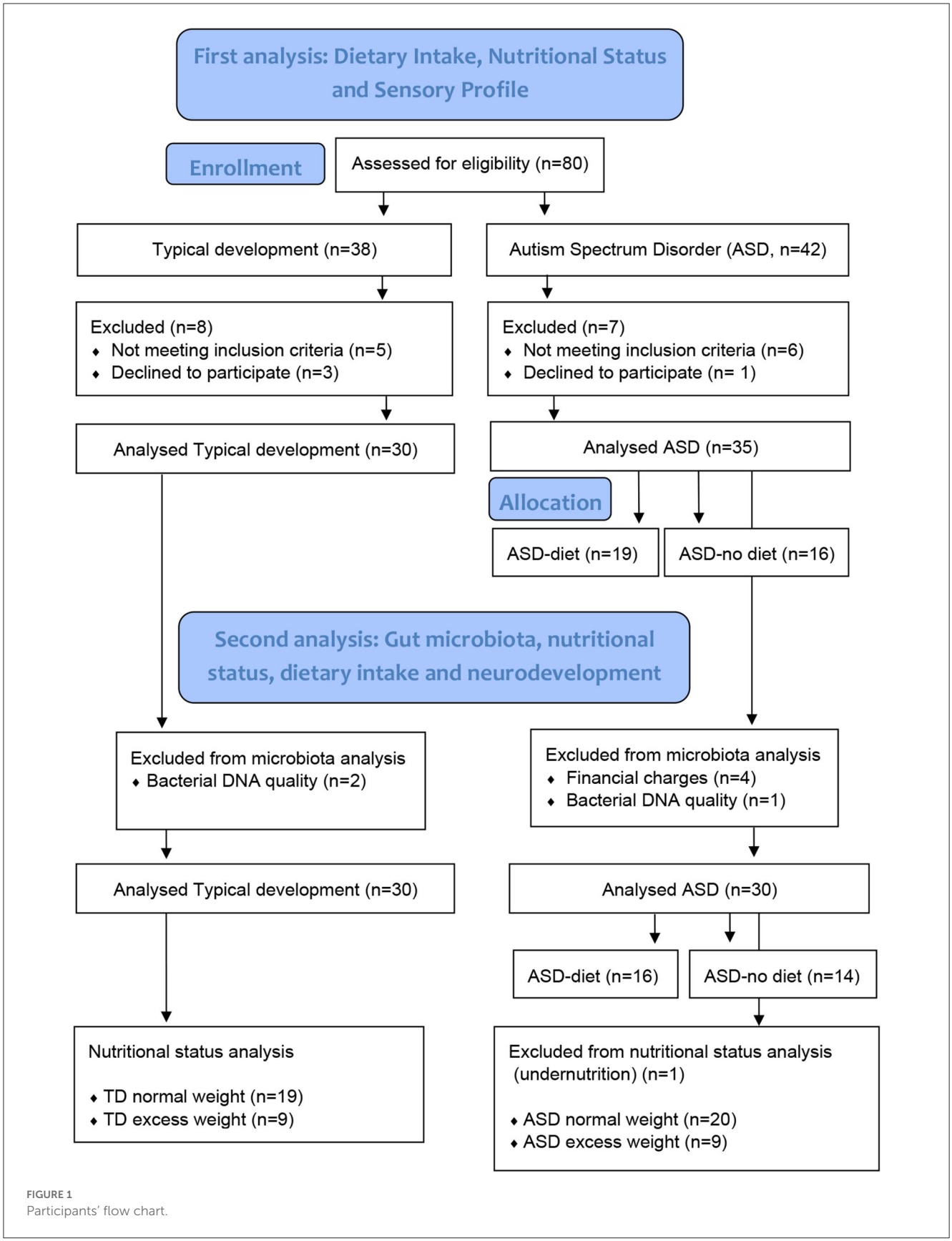

FIGURE 1
Participants' flow chart.

fragments were sequenced using the Ion PGM™ Sequencing 400 Kit on the Ion PGM™ platform and analyzed using the Ion 16S™ metagenomics analysis module within Ion Reporter™ software.

Stool samples were studied to determine the relative abundance of gut bacteria. Operational taxonomic units (OTUs) were defined at 97% sequence homology, and the abundances of these bacterial genera or species were normalized. Taxonomic classification was performed using the SILVA 128 reference database up to the species level.

Anthropometric measures and dietary intake

All protocols including anthropometric measures and dietary intake were performed as previously described by Mendive Dubourdieu et al. (8). Participants' height and weight were measured by the same nutritionist researcher. Height was determined by using a portable height rod (208 Seca) with a 810–2,060 mm range and a 1 mm precision, and weight was measured using a portable electronic scale (Seca 813, Hamburg, Germany) with a 100 g accuracy, while subjects were barefoot and wearing light clothing according to techniques standardized by Frisancho (22) and the World Health Organization (WHO) (22, 23). Data were analyzed in Anthro (for children aged 3 to 5 years) and Anthro plus (for children and adolescents aged 5 to 12 years) software (WHO v.1.0.2. 2007), which apply WHO child growth curves (22). Body mass index-for-age (BMI/A), expressed in z-score (z), was the indicator studied to classify children into one of the following categories: normal weight (NW) and excess weight (EW, risk of overweight + overweight + obesity). Cutoff points used for children aged 2–5 years were >3SD, obesity; >2SD, overweight; >1SD, risk of overweight; between <1SD and >-1SD, normal weight; ≤-1SD, risk of wasting; ≤-2SD, emaciation; and ≤-3SD, severe emaciation and in those over 5 years old were ≥2SD, obesity; ≥1SD, overweight; between <1SD and >-2SD, normal weight; ≤-2SD, wasting; and ≤-3SD, severe emaciation. Undernutrition was dismissed because the sample size was small, and therefore, those participants were not considered for anthropometric analysis.

Children with ASD were divided into two groups depending on whether they followed a GCFD (ASD-diet, $n = 16$) or did not have a restricted diet (ASD-no diet, $n = 14$). Food intake over the past 3 months was estimated based on data obtained through the SAYCARE study food frequency questionnaire (FFQ) (24), which was adapted to this study to gather information on the consumption of gluten-free and casein-free foods. Children's parents were asked to indicate the consumption frequency and portion size of each food item according to a food photo booklet as a reference. The average daily consumption of each food (g/day or ml/day) was calculated and organized into different groups as follows: (1) "dairy": milk, yogurt, chocolate milk, dairy desserts, and cheese; (2) "vegetable drinks": birdseed, chestnut, almond, oat, rice, and coconut drinks; (3) "cereals with gluten": pasta, bread, cookies, bakery products, breakfast cereals, pizza, and *empanadas* (dough stuffed with meat, fish, vegetables, etc., baked or fried), "cereals without gluten": the same foods in the previous group without gluten and rice; (4) "food source of proteins": meat, minced meat, chicken, pork, eggs, fresh and canned fish, and *milanesa* steak with and without gluten (a thin slice of beef dipped in beaten eggs and breaded; the fact that 25% of its weight is due to cereal has been taken into account); (5) "food source of fat": butter, ghee (fat obtained by heating cow milk butter), and oil. Dairy, cereals with gluten, cereals without gluten, food source of fat (for children with typical development and ASD without diet), vegetable drinks, and food source of proteins (for children with ASD and a GCFD) were divided into two subgroups, considering the 50th percentile of the intake (intake ≤ p50 and >p50).

Statistical analysis

For statistical analyses, IBM SPSS Statistics 22.0 (IBM Corp, Armonk, NY, USA) was used. We performed the Kolmogorov–Smirnov test to verify variable normal distribution. Data that were not normally distributed were log10 transformed. A p-value of lower than 0.05 was accepted as significant (two-tailed).

To compare genus and species relative abundance according to neurodevelopment (ASD vs. TD) and between autistic groups (ASD-diet vs. ASD-no diet), independent sample t-test was assessed. To study bacteria relative abundance according to nutritional status (NW and EW) in ASD and TD children, the two-way ANCOVA was applied (adjusted for age, birth weight, and GCFD/not restricted diet). Pair comparisons between the different groups were adjusted by the Bonferroni *post-hoc* test. Comparison between NW and EW in all children was carried out using one-way ANCOVA and correcting for the same potential confounders.

To evaluate associations between gut microbiota and dietary intake, univariate linear regression was used and adjusted for the following potential confounders: age, GCFD/not restricted diet, and TD/ASD (when all the children were analyzed); age (in the TD group), and age and GCFD/not restricted diet (in the ASD). In children with TD and ASD-no diet, differences in bacterial abundances among food intake p50 groups were examined using two-way ANOVA. Finally, the Student t-test and Mann–Whitney test were used to determine whether there were differences in gut bacteria relative abundances between those with higher or lower dairy intake in the ASD-diet group.

Results

Anthropometric characteristics, dietary intake, and age of children with autism spectrum disorder and typical development have been previously published (8). In Table 1, we compared the mean relative abundance of 20 selected genera and 16 species, according to ASD-diet vs. ASD-no diet and ASD vs. TD groups. No significant differences were found in the relative abundances of bacterial genera between neurotypical and ASD children. However, there is a significant difference in the mean relative abundance of *Bifidobacterium* ($p = 0.008$), *Roseburia* ($p = 0.002$), and *Sutterella* ($p = 0.015$) between the ASD-diet and ASD-no diet groups. At the species level, the ASD-diet group showed fewer *Bifidobacterium adolescentis* ($p = 0.046$) and *Bifidobacterium longum* ($p = 0.002$) but higher *Roseburia hominis* ($p = 0.002$) than the ASD-no diet group. Additionally, the ASD group had lower *Bifidobacterium*

TABLE 1 Relative abundance of gut bacteria genus and species in children with autism spectrum disorder and typical development.

Gut bacteria	ASD group			ASD group ($n = 30$)	TD group ($n = 28$)	p^{**}
	ASD-diet ($n = 16$)	ASD-no diet ($n = 14$)	p^{*}			
Genus						
Akkermansia	1.39 ± 1.90	1.84 ± 2.74	0.618[a]	1.60 ± 2.30	1.45 ± 2.17	0.938[a]
Alistipes	3.61 ± 2.82	4.42 ± 3.61	0.494[b]	3.98 ± 3.18	4.30 ± 2.92	0.565[b]
Bacteroides	23.98 ± 11.66	21.36 ± 7.82	0.482[b]	22.75 ± 9.98	24.45 ± 10.14	0.554[b]
Bifidobacterium	1.44 ± 1.80	4.22 ± 3.62	**0.008**[a]	2.73 ± 3.09	3.53 ± 2.35	0.054[a]
Blautia	2.84 ± 1.11	2.34 ± 1.18	0.114[a]	2.60 ± 1.15	2.65 ± 1.23	0.803[a]
Clostridium	6.47 ± 3.16	7.60 ± 3.65	0.280[a]	6.99 ± 3.39	6.90 ± 3.35	0.901[a]
Coprococcus	0.85 ± 0.84	0.63 ± 0.48	0.760[a]	0.74 ± 0.63	0.77 ± 0.69	0.962[a]
Dialister	0.60 ± 1.10	1.36 ± 1.65	0.105[a]	0.94 ± 1.40	0.67 ± 0.78	0.871[a]
Enterococcus	0.56 ± 2.03	0.10 ± 0.10	0.427[a]	0.34 ± 1.48	0.33 ± 0.83	0.122[a]
Eubacterium	3.66 ± 2.38	2.54 ± 1.54	0.114[a]	3.13 ± 2.08	3.07 ± 2.05	0.988[a]
Faecalibacterium	16.34 ± 7.68	11.72 ± 4.62	0.060[b]	14.18 ± 6.75	15.13 ± 5.09	0.312[b]
Lachnoclostridium	1.18 ± 0.81	0.96 ± 1.09	0.561[a]	1.07 ± 0.96	0.82 ± 0.71	0.586[a]
Lactobacillus	0.64 ± 0.65	1.03 ± 2.28	0.786[a]	0.82 ± 1.61	0.67 ± 1.26	0.697[a]
Prevotella	12.93 ± 12.52	10.87 ± 11.72	0.739[a]	11.96 ± 11.99	6.89 ± 9.16	0.093[a]
Pseudomonas	0.65 ± 2.46	0.05 ± 0.05	0.683[a]	0.37 ± 1.79	0.06 ± 0.06	0.534[a]
Roseburia	3.47 ± 1.98	1.32 ± 0.99	**0.002**[a]	2.47 ± 1.92	2.89 ± 1.92	0.304[a]
Ruminococcus	2.55 ± 1.72	2.54 ± 1.23	0.618[a]	2.55 ± 1.48	2.81 ± 1.34	0.350[a]
Streptococcus	0.49 ± 0.33	2.27 ± 5.60	0.394[a]	1.32 ± 3.86	1.53 ± 1.71	0.058[a]
Sutterella	0.62 ± 1.22	2.13 ± 1.98	**0.015**[a]	1.32 ± 1.77	1.78 ± 1.76	0.119[a]
Turicibacter	0.80 ± 1.21	0.62 ± 0.65	0.868[a]	0.72 ± 0.98	0.56 ± 0.60	0.749[a]
Species						
Akkermansia muciniphila	1.19 ± 1.40	2.27 ± 3.30	0.269[a]	1.69 ± 2.49	1.75 ± 2.60	0.935[a]
Bacteroides frágilis	0.45 ± 0.47	1.21 ± 2.85	0.301[a]	0.80 ± 1.97	1.21 ± 1.32	0.075[a]
Bacteroides intestinalis	0.02 ± 0.03	0.13 ± 0.33	0.944[a]	0.07 ± 0.23	0.55 ± 2.43	0.453[a]
Bifidobacterium adolescentis	0.30 ± 0.47	1.25 ± 2.10	**0.046**[a]	0.74 ± 1.52	0.65 ± 1.12	0.463[a]
Bifidobacterium longum	0.23 ± 0.49	1.11 ± 2.67	**0.002**[a]	0.64 ± 1.87	1.21 ± 1.80	**0.002**[a]
Clostridium bartletti	0.42 ± 0.39	0.63 ± 0.63	0.647[a]	0.52 ± 0.09	0.22 ± 0.04	0.335[a]
Clostriduim glycolicum	0.42 ± 0.28	0.55 ± 0.49	0.678[a]	0.48 ± 0.39	0.31 ± 0.30	**0.028**[a]
Coprococcus comes	0.19 ± 0.20	0.37 ± 0.36	0.156[a]	0.28 ± 0.29	0.27 ± 0.25	0.767[a]
Eubacterium eligens	1.76 ± 1.83	1.48 ± 1.53	0.561[a]	1.68 ± 0.30	1.01 ± 1.01	0.171[a]
Eubacterium ventriosum	10.77 ± 9.20	8.60 ± 8.14	0.618[a]	9.76 ± 8.64	12.58 ± 8.89	0.101[a]
Faecalibacterium prausnitzii	6.60 ± 11.80	3.95 ± 5.60	0.982[a]	5.36 ± 9.37	3.99 ± 7.69	0.735[a]
Flavonifactor plautii	0.95 ± 2.00	0.76 ± 1.03	0.802[a]	0.86 ± 1.60	0.89 ± 0.89	0.111[a]
Lactobacillus reuteri	0.29 ± 0.44	0.18 ± 0.42	0.498[a]	0.24 ± 0.43	0.30 ± 0.41	0.350[a]
Lactobacillus salivarius	0.04 ± 0.07	0.52 ± 1.71	0.386[a]	0.27 ± 1.17	0.03 ± 0.08	0.182[a]
Roseburia hominis	0.37 ± 0.40	0.10 ± 0.17	**0.002**[a]	0.24 ± 0.34	0.49 ± 1.37	0.331[a]
Trabulsiella odonototermitis	0.63 ± 1.33	0.34 ± 0.55	0.483[a]	0.49 ± 1.03	0.50 ± 0.65	0.366[a]

The results are expressed as means ± SD. Statistically significant differences (indicated in bold): $p < 0.05$ ([a]Mann–Whitney test, [b]independent sample t-test); comparison between *ASD-diet and ASD-no diet, **ASD group and TD group. ASD, autism spectrum disorder; ASD-diet, ASD children with gluten- and casein-free diet; ASD-no diet, ASD children without restricted diet; TD, typical development.

longum ($p = 0.002$) and higher *Clostridium glycolicum* ($p = 0.028$) than TD children.

In Table 2, the comparison of genus and species relative abundance between nutritional status (normal weight vs. excess weight) and neurodevelopment (ASD vs. TD) groups was studied by two-way ANCOVA. Children in the All group (ASD + TD) with excess weight had a lower relative abundance of *Roseburia* than normal weight ($p = 0.012$). In addition, children with excess weight in the ASD group had a lower relative abundance of *Roseburia* ($p = 0.005$) and *Faecalibacterium prausnitzii* ($p = 0.038$) and a higher relative abundance of *Eubacterium ventriosum* ($p = 0.019$) and *Flavonifractor plautii* ($p = 0.043$) than those with excess weight in the TD group.

The relationship between the relative abundances of bacterial genus and species and children's dietary intake is shown in Figure 2. For the analysis, we applied unadjusted and adjusted (for age, GCFD/no diet and ASD/TD) models. Vegetable drink intake had an association with *Enterococcus* ($n = 10$; unadjusted model: $r = 0.640$, $p = 0.046$), *Pseudomonas* ($n = 9$; unadjusted: $r = 0.671$, $p = 0.048$), and *Sutterella* (unadjusted: $n = 12$, $r = -0.692$, $p = 0.013$), which disappeared after adjustment for covariates; and a relationship with *Lactobacillus* was independent of age, GCFD/no diet, and ASD/TD ($n = 13$; unadjusted: $r = -0.683$, $p = 0.010$; adjusted: $r = -0.906$, $p = 0.009$). Dairy intake ($n = 43$) had an association with *Bacteroides* (unadjusted: $r = 0.353$, $p = 0.020$; adjusted: $r = 0.435$, $p = 0.048$) and *Bifidobacterium longum* (unadjusted: $r = 0.503$, $p = 0.001$; adjusted: $r = 0.552$, $p = 0.009$) that remained when the covariates were applied; it also had a relationship with *Bifidobacterium* (unadjusted: $n = 43$, $r = 0.320$, $p = 0.036$) and *Prevotella* (unadjusted: $n = 43$, $r = -0.380$, $p = 0.012$) which did not hold after adjustment. Cereals with gluten did not show a significant correlation with bacteria genus or species. However, cereals without gluten ($n = 58$) had a relationship with *Alistipes* (unadjusted: $r = -0.336$, $p = 0.015$; adjusted: $r = -0.373$, $p = 0.024$), *Bifidobacterium longum* (unadjusted: $r = -0.418$, $p = 0.003$; adjusted: $r = -0.537$, $p = 0.006$), and *Clostridium glycolicum* ($n = 58$; unadjusted: $r = 0.364$, $p = 0.009$; adjusted: $r = 0.374$, $p = 0.021$), independent of confounding factors. They also had an association with *Bifidobacterium* ($n = 56$; unadjusted: $r = -0.290$, $p = 0.041$), which disappeared after adjustment for covariates. In addition, the association between cereals with and without gluten intake and *Eubacterium eligens* ($n = 58$; unadjusted: $r = -0.290$, $p = 0.027$) and coconut oil intake with *Ruminococcus* ($n = 58$; unadjusted: $r = -0.532$, $p = 0.011$) disappeared after adjustment. The relationship between coconut oil intake and *Bacteroides intestinalis* ($n = 58$ unadjusted: $r = -0.891$, $p = 0.001$; adjusted: $r = -0.776$, $p = 0.021$) and food source of fat ($n = 58$) with *Clostridium glycolicum* (unadjusted: $r = 0.442$, $p = 0.001$; adjusted: $r = -0.430$, $p = 0.001$), *Eubacterium ventriosum* (unadjusted: $r = 0.356$, $p = 0.008$; adjusted: $r = 0.518$, $p = 0.004$), *and Flavonifractor plautii* (unadjusted: $r = 0.325$, $p = 0.026$; adjusted: $r = 0.337$, $p = 0.026$) was independent of confounding factors. Fish intake was associated with *Bacteroides intestinalis* ($n = 28$; unadjusted: $r = 0.582$, $p = 0.023$) only in the model without adjustment. Food source of proteins had a relationship with *Faecalibacterium* ($n = 58$; unadjusted: $r = -0.358$, $p = 0.006$; adjusted: $r = -0.349$, $p = 0.007$), *Lactobacillus* ($n = 49$; unadjusted: $r = -0.365$, $p = 0.010$; adjusted: $r = -0.398$, $p = 0.005$), and *Lactobacillus reuteri* ($n = 58$; unadjusted: $r = -0.363$, $p = 0.044$; adjusted: $r = -0.413$, $p = 0.027$) in unadjusted and adjusted models, but the association with *Streptococcus* disappeared after considering potential confounders ($n = 56$; unadjusted: $r = -0.287$, $p = 0.032$).

The association between bacteria genus and species and dietary intake in children with typical development is presented in Figure 3, where unadjusted and age-adjusted models were used. Dairy intake ($n = 43$) had an association with *Alistipes* (unadjusted: $r = 0.427$, $p = 0.026$; adjusted: $r = 0.509$, $p = 0.022$), *Bacteroides* (unadjusted: $r = 0.498$, $p = 0.008$; adjusted: $r = 0.584$, $p = 0.007$), *Bifidobacterium ventrosum* (unadjusted: $r = 0.388$, $p = 0.046$; adjusted: $r = 0.545$, $p = 0.013$), *Bifidobacterium longum* (unadjusted: $r = 0.544$, $p = 0.004$; adjusted: $r = 0.621$, $p = 0.007$), and *Eubacterium ventrosum* (unadjusted: $r = 0.541$, $p = 0.004$; adjusted: $r = 0.426$, $p = 0.021$), independent of the age, but the association with *Prevotella* (unadjusted: $r = -0.547$, $p = 0.003$) disappeared after adjustment, and the relationship with *Sutterella* (adjusted: $r = -0.459$, $p = 0.045$) only appeared when the covariate was considered. Cereals with gluten had a relationship with *Lactobacillus* (unadjusted: $n = 49$, $r = 0.525$, $p = 0.007$) that disappeared after adjusting, and with *Lactobacillus reuteri,* it was remained independent (unadjusted: $n = 58$, $r = 0.579$, $p = 0.015$, and adjusted: $r = 0.532$, $p = 0.047$). Cereals without gluten intake had an association with *Alistipes,* independent of age (unadjusted: $n = 58$, $r = -0.439$, $p = 0.028$; adjusted: $r = -0.456$, $p = 0.028$), but the association with *Clostridium glycolicum* disappeared when considering the confounder (unadjusted: $n = 58$, $r = 0.402$, $p = 0.047$). Food source of fat had an association with *Lactobacillus* ($n = 49$, unadjusted: $r = -0.563$, $p = 0.003$; adjusted: $r = -0.508$, $p = 0.004$), *Clostridium glycolicum* ($n = 58$; unadjusted: $r = 0.546$, $p = 0.003$; adjusted: $r = 0.539$, $p = 0.004$), *Eubacterium ventriosum* ($n = 58$; unadjusted: $r = 0.407$, $p = 0.035$; adjusted: $r = 0.351$, $p = 0.050$), *and Flavonifractor plautii* ($n = 58$; unadjusted: $r = 0.487$, $p = 0.016$; adjusted: $r = 0.456$, $p = 0.021$), which remained after adjusting. Fish had an association with *Bacteroides intestinalis* (unadjusted: $n = 58$, $r = 0.555$, $p = 0.039$), but it disappeared after considering the age. No significant associations were found between bacteria genera or species and cereals with and without gluten, coconut oil, and food source of proteins.

The association between dietary intake and relative abundance of bacteria genus and species in children with ASD is shown in Figure 4. The analysis was unadjusted and adjusted for age and gluten- and casein-free diet/no diet. Vegetable drinks were associated with *Lactobacillus* in both models ($n = 49$, unadjusted: $r = -0.620$, $p = 0.042$; adjusted: $r = -0.919$, $p = 0.034$), but they were associated with *Sutterella* only without considering confounding factors ($n = 49$; unadjusted: $r = -0.645$, $p = 0.032$). Intake of dairy, cereals with gluten, coconut oil, and food source of fat did not show a significant correlation with gut bacteria. Cereals without gluten had a correlation with *Faecalibacterium* which was kept after adjustment ($n = 58$; unadjusted: $r = 0.482$, $p = 0.011$; adjusted: $r = 0.470$, $p = 0.040$). Cereals with and without gluten had an association with *Ruminococcus* ($n = 58$; unadjusted: $r = -0.388$, $p = 0.034$), but it disappeared when

TABLE 2 Comparison of gut bacterial genus and species between nutritional status groups (normal weight vs. excess weight) and neurodevelopment groups (autism spectrum disorder vs. typical development) by two-way ANCOVA.

Gut bacteria	ASD group		TD group		All (ASD + TD)		Pair comparisons				
	Normal weight ($n = 20$)	Excess weight (OW + OB) ($n = 9$)	Normal weight ($n = 19$)	Excess weight (OW + OB) ($n = 9$)	Normal weight ($n = 39$)	Excess weight (OW + OB) ($n = 18$)	P^a	P^b	P^c	P^d	P^e
Genus											
Akkermansia	1.12 ± 1.76	2.52 ± 3.17	1.73 ± 2.54	0.86 ± 0.90	1.42 ± 2.17	1.69 ± 2.42	0.282	0.812	0.518	0.956	0.295
Alistipes	3.69 ± 2.85	4.35 ± 4.03	4.84 ± 3.12	3.16 ± 2.17	4.25 ± 3.00	3.76 ± 3.20	0.215	0.172	0.997	0.905	0.051
Bacteroides	21.71 ± 10.19	24.86 ± 10.30	25.72 ± 10.75	21.78 ± 8.68	23.66 ± 10.52	23.32 ± 9.38	0.325	0.369	0.902	0.140	0.697
Bifidobacterium	2.36 ± 2.59	3.71 ± 4.13	3.73 ± 2.63	3.11 ± 1.66	3.03 ± 2.67	3.41 ± 3.07	0.565	0.986	0.655	0.668	0.871
Blautia	2.62 ± 1.36	2.40 ± 0.31	2.86 ± 1.42	2.21 ± 0.54	2.74 ± 1.37	2.30 ± 0.44	0.892	0.406	0.646	0.209	0.841
Clostridium	7.21 ± 3.68	6.29 ± 2.88	7.22 ± 2.99	6.22 ± 4.11	7.22 ± 3.32	6.25 ± 3.44	0.287	0.275	0.131	0.671	0.798
Coprococcus	0.88 ± 0.67	0.52 ± 0.49	0.76 ± 0.72	0.79 ± 0.65	0.82 ± 0.69	0.66 ± 0.58	0.188	0.509	0.585	0.645	0.211
Dialister	0.90 ± 1.49	1.01 ± 1.36	0.63 ± 0.72	0.77 ± 0.95	0.76 ± 1.16	0.89 ± 1.14	0.205	0.438	0.549	0.571	0.141
Enterococcus	0.46 ± 1.81	0.10 ± 0.12	0.43 ± 0.99	0.10 ± 0.13	0.45 ± 1.45	0.10 ± 0.12	0.927	0.235	0.462	0.251	0.901
Eubacterium	3.47 ± 2.32	2.49 ± 1.41	2.99 ± 1.89	3.24 ± 2.47	3.24 ± 2.10	2.87 ± 1.99	0.526	0.882	0.712	0.625	0.318
Faecalibacterium	14.84 ± 7.58	12.11 ± 4.38	15.14 ± 5.27	15.11 ± 5.01	14.99 ± 6.48	13.61 ± 4.82	0.596	0.983	0.706	0.260	0.197
Lachnoclostridium	0.86 ± 0.84	1.53 ± 1.15	0.83 ± 0.79	0.80 ± 0.52	0.84 ± 0.81	1.17 ± 0.94	0.192	0.913	0.371	0.420	0.567
Lactobacillus	0.94 ± 1.94	0.60 ± 0.58	0.46 ± 0.59	1.10 ± 2.07	0.71 ± 1.45	0.85 ± 1.50	0.456	0.461	0.928	0.172	0.913
Prevotella	11.52 ± 12.36	14.11 ± 11.79	6.67 ± 10.05	7.36 ± 7.45	9.16 ± 11.41	10.74 ± 10.18	0.402	0.505	0.287	0.372	0.392
Pseudomonas	0.52 ± 2.20	0.07 ± 0.04	0.07 ± 0.06	0.04 ± 0.03	0.30 ± 1.57	0.06 ± 0.04	0.658	0.367	0.353	0.654	0.924
Roseburia	2.73 ± 2.21	1.99 ± 1.09	2.82 ± 2.05	3.03 ± 1.69	2.77 ± 2.11	2.51 ± 1.48	0.965	0.560	0.671	**0.005**	**0.012**
Ruminococcus	2.37 ± 1.44	2.87 ± 1.68	3.01 ± 1.46	2.38 ± 1.00	2.68 ± 1.47	2.62 ± 1.36	0.550	0.434	0.932	0.193	0.912
Streptococcus	1.56 ± 4.72	0.83 ± 0.90	1.76 ± 1.94	1.04 ± 1.00	1.66 ± 3.60	0.94 ± 0.93	0.773	0.831	0.721	0.708	0.709
Sutterella	1.27 ± 1.48	1.57 ± 2.41	1.37 ± 1.61	2.66 ± 1.84	1.32 ± 1.53	2.12 ± 2.15	0.732	0.306	0.750	0.333	0.745
Turicibacter	0.81 ± 1.09	0.51 ± 0.74	0.61 ± 0.66	0.44 ± 0.46	0.71 ± 0.90	0.48 ± 0.60	0.593	0.799	0.568	0.422	0.750

(Continued)

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TABLE 2 (Continued)

Gut bacteria	ASD group		TD group		All (ASD + TD)		Pair comparisons				
	Normal weight ($n = 20$)	Excess weight (OW + OB) ($n = 9$)	Normal weight ($n = 19$)	Excess weight (OW + OB) ($n = 9$)	Normal weight ($n = 39$)	Excess weight (OW + OB) ($n = 18$)	P^a	P^b	P^c	P^d	P^e
Species											
Akkermansia muciniphila	0.97 ± 1.30	3.08 ± 3.82	2.09 ± 3.02	1.03 ± 1.14	1.51 ± 2.34	2.06 ± 2.93	0.184	0.619	0.517	0.634	0.261
Bacteroides frágilis	0.96 ± 2.38	0.45 ± 0.64	1.37 ±1.48	0.86 ± 0.85	1.16 ± 1.98	0.65 ± 0.76	0.804	0.727	0.681	0.530	0.646
Bacteroides intestinalis	0.02 ± 0.02	0.19 ± 0.41	0.80 ± 2.94	0.03 ± 0.03	0.34 ± 0.28	0.26 ± 0.42	0.435	0.274	0.421	0.381	0.872
Bifidobacterium adolescentis	0.64 ± 1.15	1.07 ± 2.25	0.54 ± 1.21	0.89 ± 0.90	0.59 ± 1.16	0.98 ± 1.66	0.612	0.719	0.545	0.575	0.539
Bifidobacterium longum	0.32 ± 0.45	1.34 ± 3.39	1.45 ± 2.13	0.71 ± 0.52	0.87 ± 1.60	1.02 ± 2.37	0.640	0.574	0.473	0.222	0.350
Clostridium bartletti	0.50 ± 0.57	0.56 ± 0.46	0.34 ± 0.26	0.26 ± 0.10	0.42 ± 0.44	0.411 ± 0.35	0.641	0.793	0.867	0.696	0.382
Clostriduim glycolicum	0.42 ± 0.34	0.63 ± 0.49	0.33 ± 0.34	0.26 ± 0.22	0.38 ± 0.34	0.45 ± 0.41	0.546	0.748	0.817	0.319	0.140
Coprococcus comes	0.33 ± 0.33	0.18 ± 0.18	0.28 ± 0.28	0.27 ± 0.19	0.311 ± 0.30	0.22 ± 0.19	0.511	0.786	0.496	0.304	0.699
Eubacterium eligens	1.76 ± 1.93	1.47 ± 1.06	1.09 ± 1.13	0.86 ± 0.70	1.43 ± 1.61	1.17 ± 0.93	0.410	0.658	0.755	0.780	0.418
Eubacterium ventriosum	7.91 ± 9.51	12.74 ± 4.95	13.60 ± 9.06	10.42 ± 8.61	10.68 ± 9.61	11.58 ± 6.92	0.064	0.415	0.414	**0.019**	0.719
Faecalibacterium prausnitzii	7.98 ± 10.60	0.13 ± 0.34	3.07 ± 6.55	5.93 ± 9.38	5.59 ± 9.10	3.03 ± 7.38	0.294	0.994	0.395	**0.038**	0.774
Flavonifactor plautii	0.32 ± 0.49	1.26 ± 1.18	0.89 ± 0.79	0.89 ± 1.14	0.60 ± 0.70	1.07 ± 1.14	0.223	0.295	0.846	**0.043**	0.915
Lactobacillus reuteri	0.11 ± 0.27	0.50 ± 0.59	0.30 ± 0.45	0.30 ± 0.35	0.20 ± 0.38	0.40 ± 0.48	0.696	0.773	0.624	0.464	0.502
Lactobacillus salivarius	0.39 ± 1.43	0.01 ± 0.04	0.02 ± 0.79	0.06 ± 0.09	0.21 ± 1.03	0.03 ± 0.07	0.637	0.422	0.966	0.218	0.847
Roseburia hominis	0.27 ± 0.38	0.21 ± 0.26	0.51 ± 1.62	0.45 ± 0.62	0.38 ± 1.16	0.33 ± 0.48	0.944	0.756	0.867	0.144	0.141
Trabulsiella odonototermitis	0.50 ± 1.21	0.45 ± 0.63	0.60 ± 0.71	0.28 ± 0.46	0.55 ± 0.99	0.36 ± 0.54	0.541	0.355	0.287	0.864	0.723

The results are expressed as means ± SD. Statistically significant differences (indicated in bold, $p < 0.05$; adjusted for age, birth weight, GCFD/not restricted diet): (a) Two-way ANCOVA, comparisons between [a]normal and excess weight in the ASD group, [b]normal and excess weight in the TD group, [c]normal weight in ASD and TD, [d]excess weight in ASD and TD; (b) one-way ANCOVA, comparison between [e]normal weight and excess weight in all participants. ASD, autism spectrum disorder; TD, typical development; OW, overweight; OB, obesity.

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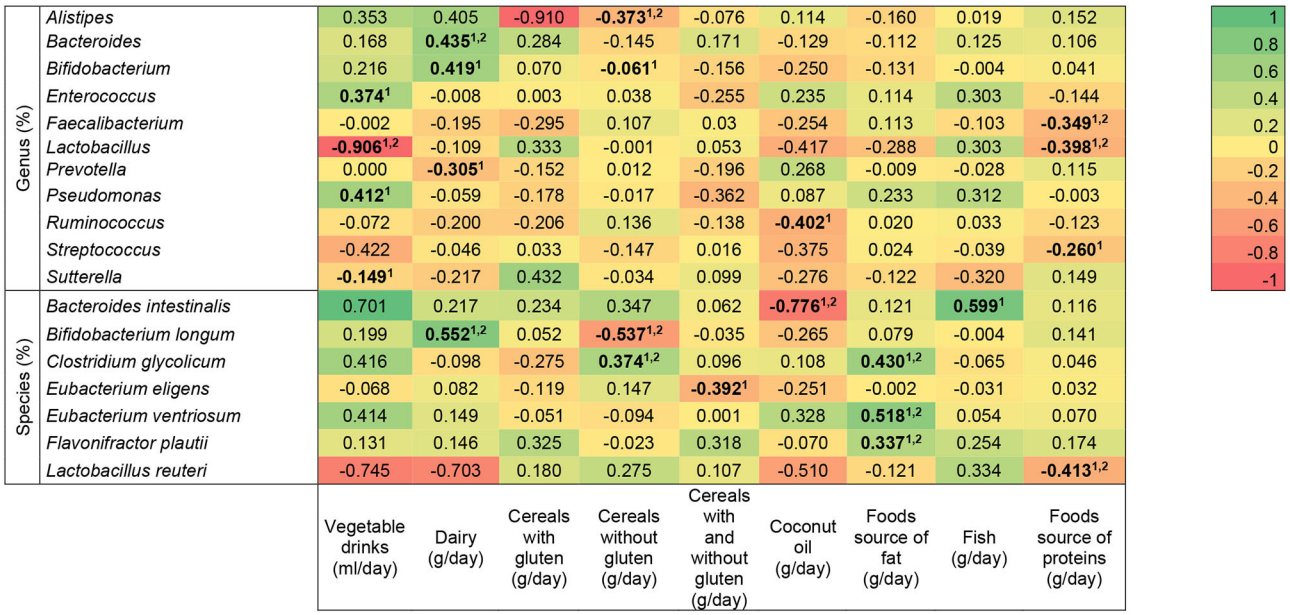

FIGURE 2
Heat map of the association between bacterial genera and species and dietary intake in children with autism spectrum disorder and neurotypical. Statistically significant differences (indicated in bold): $p < 0.05$ (linear regression model [1]unadjusted, and [2]adjusted for age, GCFD/not restricted diet, autism spectrum disorder/typical development). The R-values presented correspond to the adjusted models.

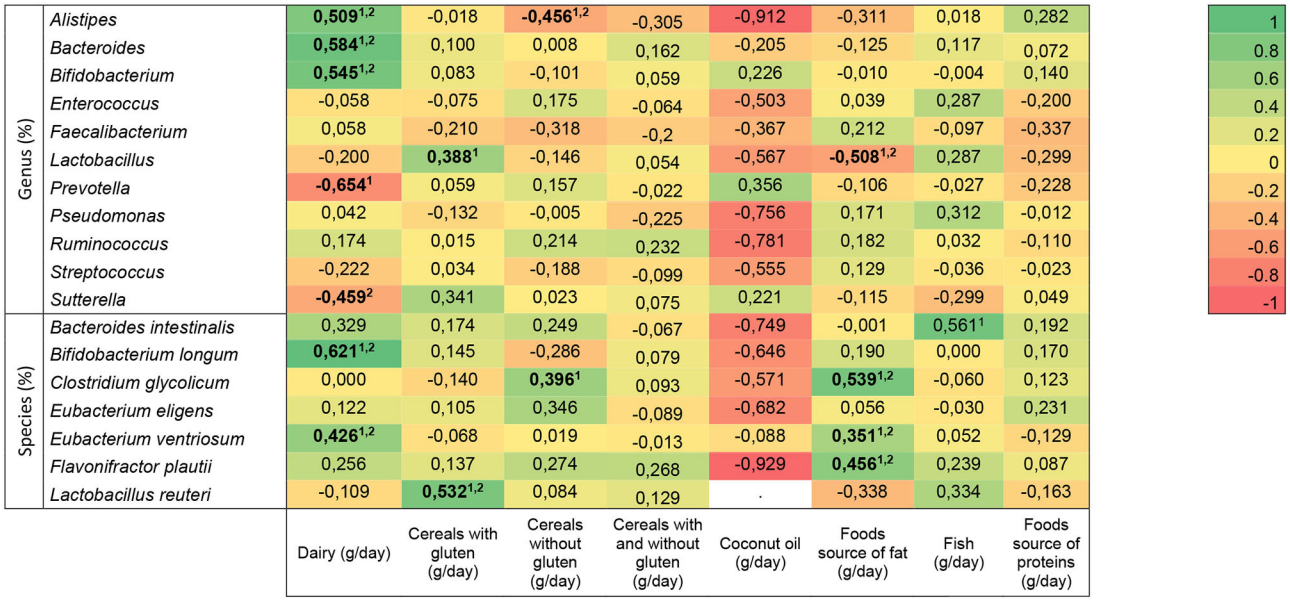

FIGURE 3
Heat map of the association between bacterial genera and species and dietary intake in neurotypical children. Statistically significant differences (indicated in bold): $p < 0.05$ (linear regression model [1]unadjusted, and [2]djusted for age). The R-values presented correspond to the adjusted models.

potential confounders were considered. The association between food source of proteins and *Faecalibacterium* was observed only when considering the covariates ($n = 58$; adjusted: $r = -0.356$, $p = 0.049$); with *Streptococcus*, the relationship was found in unadjusted and adjusted models ($n = 58$; unadjusted: $r = -0.437$, $p = 0.020$; adjusted: $r = -0.435$, $p = 0.023$).

Table 3 shows the comparison of gut bacterial genus and species between dietary intake groups, categorized by the 50th percentile, in children with TD and ASD without a GCFD by means of two-way ANOVA. Children with ASD-no diet and a dairy intake of >334.3 g/day had a higher relative abundance of *Lactobacillus* ($p = 0.025$) and *Streptococcus* ($p = 0.003$) than those with an intake

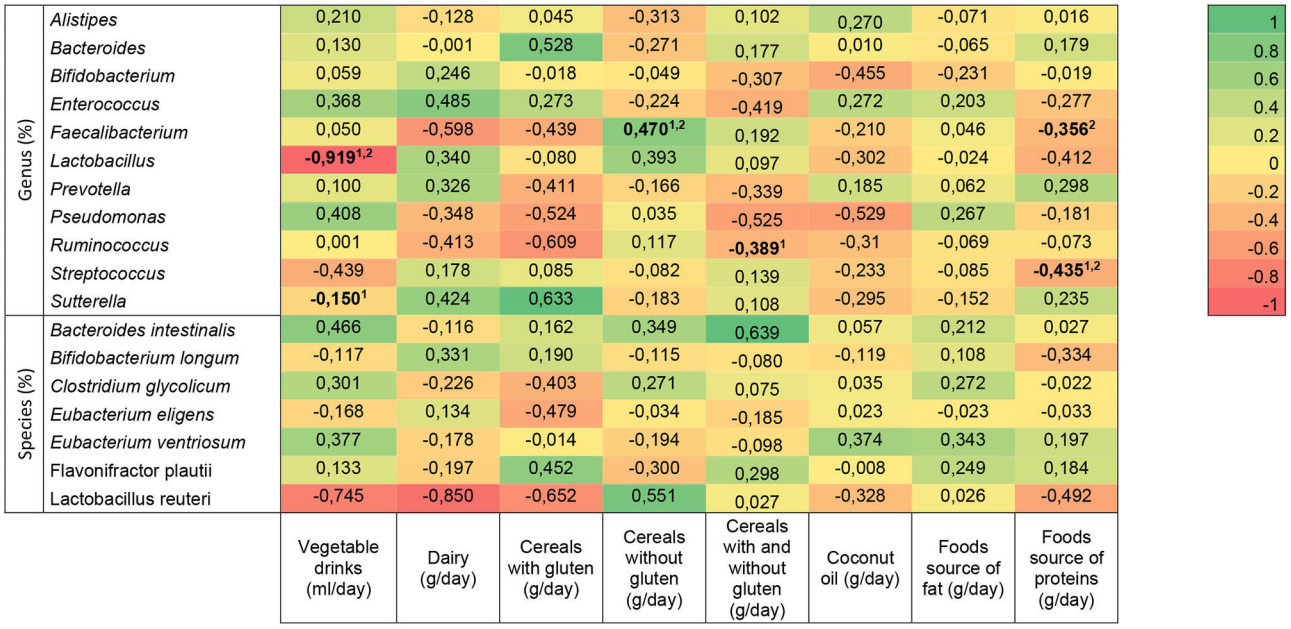

FIGURE 4
Heat map of the association between bacterial genera and species and dietary intake in autism spectrum disorder children. Statistically significant differences (indicated in bold): $p < 0.05$ (linear regression model [1]unadjusted, and [2]adjusted for age, gluten- and casein-free diet/no diet). The *R*-values presented correspond to the adjusted models.

of ≤334.3 g/day. In the TD group with a dairy intake of >334.3 g/day, a greater amount of *Alistipes* ($p = 0.013$) and *Eubacterium ventriosum* ($p = 0.024$) was found as compared with children with a lower dairy intake. When gut bacteria were compared between TD and ASD-no diet children in the dairy intake of >334.3 g/day group, we observed a lower relative abundance of *Faecalibacterium* ($p = 0.005$) and a higher one of *Lactobacillus* ($p = 0.012$) and *Streptococcus* ($p = 0.009$) in ASD-no diet children. In relation to cereals with gluten, *Faecalibacterium* was higher ($p = 0.042$) in ASD-no diet children with an intake of >198.35 g/day than TD with the same intake. ASD-no diet children with an intake of cereals without gluten ≤7.12 g/day had higher *Flavonifractor plautii* ($p = 0.013$) than those with an intake of >7.12 g/day. In addition, in the group with an intake of cereal without gluten >7.12 g/day, *Flavonifractor plautii* ($p = 0.014$) and *Eubacterium ventrosum* ($p = 0.017$) were higher in TD children than in ASD-no diet. Regarding food source of fat, when the intake was >25 g/day, the abundance of *Faecalibacterium* was higher in TD children than in ASD-no diet ($p = 0.031$).

Table 4 shows the comparison of gut bacterial genus and species between dietary intake groups categorized by the 50th percentile in children with ASD and a GCFD. *Alistipes* was higher ($p = 0.002$), and *Lactobacillus* was lower ($p = 0.004$) in the >309.5 ml/day vegetable drink intake group than in children with an intake of ≤309.5 ml/day. Higher levels of *Faecalibacterium* ($p = 0.045$) were found in individuals who consume >102.46 g/day of cereals without gluten compared with those with lower intake. No significant difference was found when comparing gut bacteria between the higher and the lower intake of food source of proteins.

Discussion

In this study, differences were observed in the composition of fecal bacteria as per nutritional status and dietary intake in ASD and TD children. Several studies have shown different gut bacteria compositions in children with ASD as compared with TD (25). In contrast, we have not found significant differences at the genus level, but at the species level, we observed lower abundances of *Bifidobacterium longum* and higher abundances of *Clostridium glycolicum* in ASD than in the TD group. Wang et al. (26) reported lower levels of *Bifidobacterium spp.* and *Akkermansia muciniphila* in children with autism compared with TD ones, but in our study, no significant difference was observed in the abundance of this mucolytic bacterium. Our findings are in line with previous studies that found decreased *Bifidobacterium* spp. and elevated *Clostridium* spp. in ASD as compared with controls and suggested that the latter bacterium is a determinant of the risk of autism (12, 27, 28). Research conducted in 1998 hypothesized that ASD could be due to a dysbiosis context with colonization by *Clostridium tetani* and to its neurotoxic effects in neurons producing gamma-aminobutyric acid (inhibitory neurotransmitter of the central nervous system) (29, 30). Another study shows a positive correlation between *Clostridium* cluster XVIII and gastrointestinal symptoms such as constipation in autistic and neurotypical subjects (27, 31).

Interestingly, in the ASD-diet group, *Bifidobacterium* (*B.*) were significantly lower compared with the ASD-no diet group, and this bacteria had a positive association with dairy intake and a negative association with cereals without gluten in the All (ASD + TD) group. A greater abundance of *Bifidobacterium* has been described as having beneficial effects on health since it inhibits pathogen

TABLE 3 Comparison of gut bacterial genus and species between dietary intake groups (categorized by the 50th percentile) in children with typical development and autism spectrum disorder without diet by means of two-way ANOVA.

Gut bacteria	ASD-no diet group		TD group		ASD-no diet group		TD group		ASD-no diet group		TD group		ASD-no diet group		TD group	
	Dairy (g/day)				Cereals with gluten (g/day)				Cereals without gluten (g/day)				Foods source of fat (g/day)			
	≤334.3 (*n* = 11)	>334.3 (*n* = 3)	≤334.3 (*n* = 10)	>334.3 (*n* = 18)	≤198.3 (*n* = 9)	>198.3 (*n* = 5)	≤198.3 (*n* = 13)	>198.3 (*n* = 15)	≤7.1 (*n* = 8)	>7.1 (*n* = 6)	≤7.1 (*n* = 13)	>7.1 (*n* = 15)	≤25.0 (*n*=7)	>25.0 (*n* = 7)	≤25.0 (*n* = 14)	>25.0 (*n* = 14)
Genus																
Alistipes	4.07 ± 2.81	5.67 ± 6.52	**2.70 ± 1.87**[b]	**5.19 ± 3.05**[b]	4.19 ± 3.00	4.81 ± 4.91	4.63 ± 2.50	4.01 ± 3.30	4.28 ± 4.00	4.65 ± 3.22	5.06 ± 3.13	2.93 ± 1.96	4.98 ± 4.11	3.85 ± 3.26	4.85 ± 3.00	3.75 ± 2.84
Bacteroides	21.57 ± 8.88	20.56 ± 1.23	22.24 ± 11.41	25.68 ± 9.48	20.74 ± 7.52	22.45 ± 9.12	22.97 ± 6.37	25.74 ± 12.64	3.91 ± 3.40	4.79 ± 3.95	5.06 ± 3.07	3.64 ± 2.71	21.34 ± 6.80	21.36 ± 9.28	26.76 ± 11.47	22.14 ± 8.40
Bifidobacterium	4.58 ± 3.99	2.87 ± 1.48	2.26 ± 1.59	4.24 ± 2.44	5.34 ± 4.04	2.19 ± 1.46	4.06 ± 2.15	3.08 ± 2.49	3.82 ± 3.60	4.74 ± 3.92	4.16 ± 2.57	2.99 ± 2.08	3.66 ± 3.21	4.77 ± 4.18	3.36 ± 2.16	3.71 ± 2.59
Faecalibacterium	13.12 ± 4.04	**6.55 ± 2.55**[c]	14.14 ± 5.02	**15.68 ± 5.19**[c]	12.88 ± 4.42	**9.60 ± 4.67**[c]	15.58 ± 4.12	**14.74 ± 5.93**[c]	12.27 ± 4.83	10.96 ± 4.67	16.20 ± 6.15	14.20 ± 3.95	12.08 ± 4.85	**11.34 ± 4.74**[c]	13.97 ± 5.57	**16.29 ± 4.46**[c]
Lactobacillus	**0.50 ± 0.41**[a]	**2.96 ± 5.10**[a,c]	1.24 ± 2.00	**0.35 ± 0.34**[c]	0.51 ± 0.44	1.95 ± 3.86	0.78 ± 1.76	0.57 ± 0.63	0.40 ± 0.41	1.86 ± 3.45	0.94 ± 1.74	0.43 ± 0.61	0.44 ± 0.43	1.61 ± 3.21	0.99 ± 1.73	0.35 ± 0.35
Streptococcus	**0.86 ± 0.89**[a]	**7.43 ± 12.22**[a,c]	0.78 ± 0.53	**1.95 ± 2.00**[c]	1.02 ± 0.92	4.53 ± 9.51	2.18 ± 2.07	0.97 ± 1.13	4.26 ± 8.50	0.78 ± 0.91	1.97 ± 2.19	1.15 ± 1.10	0.81 ± 0.96	3.73 ± 7.88	1.13 ± 1.53	1.93 ± 1.85
Suterella	2.33 ± 2.14	1.38 ± 1.29	2.36 ± 1.73	1.46 ± 1.75	1.73 ± 1.72	2.85 ± 2.43	1.88 ± 2.05	1.69 ± 1.54	1.92 ± 2.25	2.40 ± 1.73	1.63 ± 1.72	1.91 ± 1.85	1.43 ± 1.36	2.82 ± 2.36	1.47 ± 1.79	2.09 ± 1.74
Species																
Bifidobacterium longum	1.29 ± 3.01	0.47 ± 0.15	0.47 ± 0.50	1.62 ± 2.12	1.51 ± 3.32	0.39 ± 0.23	1.22 ± 1.93	1.20 ± 1.75	0.34 ± 0.26	2.14 ± 4.02	1.37 ± 1.81	1.07 ± 1.84	0.45 ± 0.25	1.78 ± 3.78	0.68 ± 0.52	1.74 ± 2.42
Clostridium glycolicum	0.49 ± 0.50	0.78 ± 0.45	0.25 ± 0.21	0.34 ± 0.35	0.63 ± 0.56	0.41 ± 0.31	0.37 ± 0.38	0.26 ± 0.22	0.43 ± 0.26	0.71 ± 0.68	0.22 ± 0.21	0.39 ± 0.36	0.47 ± 0.37	0.64 ± 0.60	0.23 ± 0.22	0.39 ± 0.36
Eubacterium ventriosum	9.57 ± 8.76	5.04 ± 4.80	**5.76 ± 7.79**[b]	**16.36 ± 7.13**[b]	9.60 ± 8.82	6.82 ± 7.31	14.95 ± 8.35	10.52 ± 9.10	10.76 ± 8.64	**5.73 ± 7.11**[c]	11.50 ± 10.21	**13.51 ± 7.81**[c]	8.20 ± 9.63	9.01 ± 7.10	10.83 ± 9.08	14.33 ± 8.66
Lactobacillus reuteri	0.22 ± 0.47	0.01 ± 0.03	0.14 ± 0.33	0.38 ± 0.43	0.25 ± 0.52	0.04 ± 0.08	0.27 ± 0.32	0.32 ± 0.49	0.31 ± 0.53	0.00 ± 0.01	0.35 ± 0.50	0.25 ± 0.33	0.24 ± 0.57	0.12 ± 0.23	0.35 ± 0.45	0.24 ± 0.38
Flavonifactor plautii	0.73 ± 1.03	0.86 ± 1.24	0.38 ± 0.49	1.18 ± 0.95	0.67 ± 1.10	0.91 ± 0.97	1.11 ± 0.91	0.70 ± 0.86	**1.27 ± 1.12**[a]	**0.08 ± 0.11**[a]	0.71 ± 0.89	1.05 ± 0.90	0.68 ± 0.84	0.83 ± 1.25	0.69 ± 0.56	1.10 ± 1.12

The results expressed as means ± SD. Statistically significant differences (indicated in bold, $p < 0.05$): Two-way ANOVA, comparisons between [a]lower and higher dietary intake groups in the ASD-no diet children; [b]lower and higher dietary intake groups in TD children; [c]ASD-no diet and TD children in the higher dietary intake group. ASD-no diet, children with autism spectrum disorder without a restricted diet; TD, typical development.

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TABLE 4 Comparison of gut bacterial genus and species between dietary intake groups (categorized by the 50th percentile) in children with autism spectrum disorder and a gluten- and casein-free diet.

Gut bacteria	Vegetable drinks (ml/day)			Cereals without gluten (g/day)			Foods source of proteins (g/day)		
	≤309.5 ($n = 8$)	>309.5 ($n = 8$)	*p*	≤102.4 ($n = 8$)	>102.4 ($n = 8$)	*p*	≤183.6 ($n = 8$)	>183.6 ($n = 8$)	*p*
Genus									
Alistipes	1.56 ± 1.11	5.64 ± 2.51	**0.002**[a]	2.69 ± 2.46	4.51 ± 3.01	0.327[a]	3.60 ± 3.41	3.60 ± 2.32	0.860[a]
Bacteroides	22.72 ± 8.34	25.24 ± 14.77	0.111[a]	24.13 ± 12.24	23.82 ± 11.91	0.559[a]	22.44 ± 5.66	25.51 ± 15.94	0.826[a]
Bifidobacterium	2.15 ± 2.28	0.73 ± 0.79	0.156[b]	2.01 ± 2.35	0.86 ± 0.84	0.275[b]	1.36 ± 0.91	1.52 ± 2.48	0.936[b]
Faecalibacterium	14.90 ± 4.60	17.77 ± 10.02	0.458[a]	12.59 ± 6.12	20.08 ± 7.54	**0.045**[a]	16.72 ± 3.84	15.95 ± 10.55	0.862[b]
Lactobacillus	1.06 ± 0.67	0.22 ± 0.22	**0.004**[b]	0.64 ± 0.75	0.64 ± 0.58	0.925[b]	0.94 ± 0.56	0.34 ± 0.62	0.062[b]
Streptococcus	0.60 ± 0.31	0.38 ± 0.33	0.476[b]	0.47 ± 0.38	0.51 ± 0.30	0.476[b]	0.60 ± 0.32	0.38 ± 0.32	0.090[b]
Suterella	1.12 ± 1.61	0.11 ± 0.14	0.108[b]	1.11 ± 1.61	0.12 ± 0.15	0.124[b]	0.89 ± 1.60	0.34 ± 0.68	0.372[b]
Species									
Bifidobacterium longum	0.31 ± 0.67	0.15 ± 0.23	0.923[a]	0.37 ± 0.68	0.09 ± 0.12	0.511[a]	0.16 ± 0.23	0.30 ± 0.68	0.772[a]
Clostridium glycolicum	0.39 ± 0.27	0.45 ± 0.30	0.937[b]	0.30 ± 0.23	0.54 ± 0.28	0.147[b]	0.51 ± 0.29	0.34 ± 0.26	0.310[b]
Eubacterium ventriosum	9.08 ± 8.10	12.46 ± 10.46	0.330[b]	9.10 ± 7.70	12.44 ± 10.76	0.401[b]	10.42 ± 8.91	11.11 ± 10.09	0.943[b]
Lactobacillus reuteri	0.45 ± 0.56	0.13 ± 0.20	0.169[b]	0.21 ± 0.27	0.37 ± 0.57	0.430[b]	0.49 ± 0.54	0.09 ± 0.18	0.059[b]
Flavonifactor plautii	0.72 ± 0.88	1.18 ± 2.78	0.311[b]	1.44 ± 2.72	0.46 ± 0.82	0.511[b]	1.45 ± 2.78	0.46 ± 0.59	0.251[b]

The results are expressed as means ± SD. Statistically significant differences (indicated in bold): $p < 0.05$ ([a]t-test, [b]Mann–Whitney test).

growth by releasing bacteriocins (32, 33), and an increase in *B. longum* can mitigate depression in patients with irritable bowel syndrome through changes in the brain areas involved in mood regulation (34).

In line with our results, a study in adults shows a significant association between gluten-free diet and a reduced relative abundance of *Bifidobacterium* and *Lactobacillus (L.)* (35). Importantly, both genera synthesize short-chain fatty acids, that interact with receptors in the gut mucosa and contribute to mucus maintenance, have an antimicrobial effect on pathogens and can reverse leaky gut disorders (36). Therefore, the assumption is that greater abundances of *Lactobacillus* in the gut are associated with a higher intake of dairy (37). In the present study, we also observed that children with ASD-no diet and a dairy intake of >334.3 g/day had a higher mean of *Lactobacillus* than those with a lower intake. On the contrary, the ASD-diet group with an intake of >309.5 ml/day of vegetable drinks had a lower abundance of this genus than children with an intake of ≤309.5 ml/day. Considering all participants, the intake of vegetable drinks and food source of proteins had a negative association with *Lactobacillus* and *L. reuteri,* respectively. This last bacterial species was found to reverse social deficits in experimental animals with ASD (38).

Apart from that, vegetable drink intake also had a positive association with the facultative anaerobe *Enterococcus* and a negative association with *Sutterella* in all children only in the unadjusted model. In relation to this, Mangiola et al. (39) have reported a positive association between *Sutterella* genus and the development of autism in children. Nevertheless, an increase in *Enterococcus* has been detected in fecal samples from patients with diarrhea (40). In addition, a pro-inflammatory bacteria named *Alistipes* (41) was higher in ASD-diet children with an intake of >309.5 ml/day of vegetable drinks than those with a lower intake, but it was also higher in children with TD and a dairy intake of >334.3 g/day than in those with a dairy intake of ≤334.3 g/day, and a positive association between *Alistipes* and dairy intake was observed in this group. Additionally, in All and TD groups, this bacterium was inversely related to cereals without gluten. Another bacterium with anti-inflammatory properties called *Prevotella* (42) had a negative association (only in the unadjusted model) with dairy intake considering All and TD children.

In relation to nutritional status, participants in the All group with excess weight had a lower relative abundance of a beneficial butyrate-producing bacterium called *Roseburia (R.)* (43) than the normal weight ones, and *Roseburia* and *R. hominis* were higher in the ASD-diet as compared with the ASD-no diet group. In addition, children with excess weight in the ASD group had a significantly lower relative abundance of *Roseburia* and *Faecalibacterium prausnitzii* and a higher relative abundance of *Eubacterium ventriosum* and *Flavonifractor plautii* than those with excess weight in the TD group. A study performed in Japan showed that *E. ventriosum* was significantly associated with obese subjects (44). Moreover, a significantly reduced abundance of this

species was described in people with colorectal cancer and could be considered a risk biomarker for the illness (45). On the other hand, *Faecalibacterium prausnitzii* is responsible for degrading mucin-producing butyrate and peptides that inhibit the NF-kB pathway in intestinal epithelial cells with an anti-inflammatory effect, and it has been associated with a reduced abundance in obesity (46, 47).

It has recently been reported that eating bread made from transgenic low-gliadin wheat produces a significantly higher abundance of *Faecalibacterium* and *Roseburia* genera with potentially beneficial changes in the composition of the intestinal microbiota, due to the increase in butyrate, which maintains good gut permeability (48). In our study, considering the ASD group, cereals without gluten also had a positive correlation with *Faecalibacterium*. Additionally, we observed that the ASD-diet group had a significantly higher level of *Faecalibacterium* in individuals who consume >102.46 g/day of cereals without gluten as compared with those with lower intake. It is worth mentioning that Jiang et al. (49) observed a negative correlation between *Faecalibacterium* abundances and the severity of depressive manifestation and overexpression of *Alistipes* in this psychiatric disorder.

In relation to cereal intake, other studies have shown that a high carbohydrate intake was associated with higher abundances of *Bifidobacterium* and *Lactobacillus* in fecal samples (50). In our study, the TD group precisely had a positive relationship between the intake of cereals with gluten and the abundances of *Lactobacillus* and *L. reuteri*. Apart from that, by comparison, children with TD and an intake of >7.12 g/day of cereals without gluten had higher abundances of *Flavonifractor (F.) plautii* than the ASD-no diet group with the same food intake, and in this last group, those with an intake of ≤7.12 g/day had more *F. plautii* than children with an intake of >7.12 g/day. Mikami et al. (51) have recently reported that an increased abundance of this bacterium has a beneficial effect as a modulator of gut inflammation, mediating IL-17 suppression in animals.

A number of studies have reported that an animal-based diet with high protein and fat intake seems to increase bile-tolerant bacteria called *Bacteroides* and could boost intestinal bowel disease risk (15, 52). Consistent with the said finding, we found that fish intake was positively associated with *Bacteroides intestinalis* in All and TD children (without adjustment for potential confounders). Even so, an investigation with mice fed with fish oil for 11 weeks described that, due to the interaction with the gut microbiota, there was less white adipose tissue inflammation and Toll-like receptor activation as compared with the lard diet (53). However, another research shows that in humans, salmon consumption has no effect on gut microbiota of pregnant women (54).

Regarding the intake of food source of fat, a comparison study showed that rats on a diet rich in coconut oil for 2 weeks had a lower abundance of *Ruminococcus flavecaciens* than those fed with soy oil (55). Along those lines, our study has found a negative association between coconut oil intake and *Ruminococcus* (unadjusted model) and *Bacteroides intestinalis* in the All group. In addition, a meta-analysis shows 11 trials with a higher abundance of *Ruminococcus* (involved in the fermentation of dietary fibers) in ASD children than in TD ones (25), but we have not found a significant difference in those groups.

In short, advances in the study of intestinal microbiota have led to new research, but the results have been disparate due to the complexity of the subject. A number of investigations show that gut microbiota may modulate brain function via metabolic and signaling pathways in charge of social cognition and emotional regulation (56). Our results show how gut microbiota composition is related to food consumption, nutritional status, and neurodevelopment. Based on these results and the possibility of further investigating the interaction between diet, gut microbiota, and autism through intervention studies, it would be possible to establish a clear relationship between specific bacteria profiles, food intake, and neurodevelopment. This could help establish preventive and treatment strategies for autism. Further studies focusing on an associated analysis of these topics in a large sample of children are needed to improve recommendations for this population.

Study limitations

Research limitation is related to the small size of the sample, and the fact that the amount of dietary intake was estimated by the food frequency questionnaire instead of being accurately measured. However, these questionnaires are the most economical and validated method used worldwide, and we consider this study as an important input to the knowledge on this topic and for other types of research, since there are few studies that address the relationship of the intestinal microbiota with dietary intake and nutritional status in children with ASD.

Conclusion

In this study, we observed differences in the composition of gut bacteria in children with autism spectrum disorder and typical development in only two species (*Bifidobacterium longum* and *Clostridium glycolicum*), but when we analyzed these two populations taking into account dietary intake and nutritional status, we were able to observe more differences. We found positive and negative associations between the intake of dairy, vegetable drinks, cereals with gluten and without gluten, food source of proteins, fish, food source of fat, and coconut oil, with the gut microbiota, independent of potential confounder variables such as age, being on a gluten- and casein-free diet, and neurodevelopment. Moreover, analyzing and comparing the higher and lower intake of these food groups allowed us to observe in greater depth how intake quantities are associated with higher or lower abundances of gut bacteria. Pending further studies, these results might be considered as a starting point for the nutritional treatment of ASD children.

Data availability statement

The data presented in the study are deposited in the NCBI with links to BioProject accession number PRJNA988151 in the NCBI BioProject database (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA988151).

Ethics statement

The studies involving human participants were reviewed and approved by the Research Ethics Committee of the Universidad de la República's School of Nurition (CEIN). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

PM: designed and conducted research, analyzed data, performed statistical analysis, wrote the manuscript, and had primary responsibility for final content. MG: designed research, analyzed data, performed statistical analysis, had primary responsibility for final content, and supervision and review. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Early-life gut microbiota and neurodevelopment in preterm infants: a narrative review

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Infants born preterm are at a high risk of both gut microbiota (GM) dysbiosis and neurodevelopmental impairment. While the link between early dysbiosis and short-term clinical outcomes is well established, the relationship with long-term infant health has only recently gained interest. Notably, there is a significant overlap in the developmental windows of GM and the nervous system in early life. The connection between GM and neurodevelopment was first described in animal models, but over the last decade a growing body of research has also identified GM features as one of the potential mediators for human neurodevelopmental and neuropsychiatric disorders. In this narrative review, we provide an overview of the developing GM in early life and its prospective relationship with neurodevelopment, with a focus on preterm infants. Animal models have provided evidence for emerging pathways linking early-life GM with brain development. Furthermore, a relationship between both dynamic patterns and static features of the GM during preterm infants' early life and brain maturation, as well as neurodevelopmental outcomes in early childhood, was documented. Future human studies in larger cohorts, integrated with studies on animal models, may provide additional evidence and help to identify predictive biomarkers and potential therapeutic targets for healthy neurodevelopment in preterm infants.

KEYWORDS

preterm infants, gut microbiota, *Bifidobacterium*, gut-brain axis, neurodevelopment, brain maturation, developmental windows

1. Introduction

Over the last two decades, the impact of the gut microbiota (GM) on host health and physiological processes, including neurodevelopment, has been the subject of increasing research (1–4). However, only few studies have explored the relationship between GM assembly, brain growth, and neurodevelopment in preterm infants (5–11). As a result of continuous improvements in neonatal intensive care, the mortality rate of extremely preterm infants [i.e., those with a gestational age (GA) of less than 28 weeks] has dramatically decreased over time. However, the improved survival of these infants is associated with a substantially elevated risk of severe morbidities and life-long neurodevelopmental impairment (including cerebral palsy, autism-spectrum disorders, anxiety, antisocial behaviors, and learning disabilities) (12). The

third trimester of pregnancy is a critical period for brain growth and function, during which the brain increases significantly in volume, and cognitive function gains complexity (13, 14). Preterm birth interrupts the physiological growth and development of the brain that would have occurred during the third trimester of pregnancy. Furthermore, preterm brain development is hampered postnatally by a variety of noxious environmental stimuli and insults that are closely linked to neonatal immaturity and the neonatal intensive care unit (NICU) environment (15), including early microbial colonization. Accumulating evidence suggests that, during early-life, GM is involved in bidirectional signaling between the gut and the brain, forming the so-called microbiota-gut-brain axis (MGBA) (4). However, in premature infants, the GM-host relationship is likely to be severely impaired, predisposing preterm infants to adverse outcomes, such as necrotizing enterocolitis (NEC) and late-onset sepsis (LOS), ultimately interfering with the MGBA (16).

In this narrative review, we first provide an overview of the developing GM in early life, then discuss the emerging pathways linking GM and brain development, including current animal models, and the potential prospective relationship with neurodevelopment. Finally, we aim to provide an up-to-date review of available studies that have specifically explored the relationship between early-life GM and neurodevelopmental outcomes in preterm infants.

2. Gut microbiota in early life: assembly and influencers

Newborns born at term, vaginally, exclusively breastfed and not exposed to antibiotics have the ideal characteristics of a healthy early-life GM (17). One of the most important factors influencing microbial colonization patterns in newborns is the vertical transmission of bacteria from mother to child (18). At the time of birth, the passage of the baby through the birth canal represents the first event of exposure, first to microbes present in the vagina, on the mother's skin and in feces, and subsequently to microbes present in the surrounding environment (19). This event represents early maternal imprinting, which plays a pivotal role in the assembly and maturation of the GM in early childhood. Consequently, any event potentially capable of preventing the vertical transmission of the mother microbiota may potentially alter the primary colonization in the newborn. The assembly of the GM is also influenced by the mode of delivery. In particular, Cesarean delivery has an enormous perturbing influence in the context of term deliveries during the perinatal period (20–22), even independent of antibiotic exposure (22–25). The early colonizer community in Cesarean-born infants borders in composition on the microbial community associated with the mother's skin, as well as that present in the operating room, and is characterized by a depletion of Bacteroidetes compared to vaginally delivered infants (24, 26). Disruption of maternal microbiota transmission has been associated with a greater representation of opportunistic pathogens, even those resistant to antimicrobials, which is a risk factor for compromising neonatal health (23, 27). During early development, any disruption of GM-host interactions could irreversibly damage the infant priming process, thus hindering the establishment of a healthy homeostasis, and the existence of a critical period has been proposed (24, 28). Such disruptions are a major contributor to developmental issues, predisposing infants to develop impaired intestinal barrier function, inflammatory and metabolic diseases (29, 30), as well as alterations in communication with the brain *via* the MGBA, reflected in an increased risk of developing neurological diseases (31).

3. Emerging pathways linking gut microbiota to brain development: lessons from animal models

Accumulating evidence suggests that GM plays a role in several aspects of the host central nervous system (from development to function) through direct and indirect communication with the brain along the MGBA (32–34). However, the underlying mechanisms are far from being fully elucidated. Below, we discuss the emerging pathways linking GM to healthy or impaired neurodevelopment, and the major microbial intermediates involved [i.e., short-chain fatty acids (SCFAs), histamine, and tryptophan derivatives].

Such information has been derived from murine models [including germ-free (GF) mice, specific pathogen-free (SPF) mice, and other specific models], which, despite obvious limitations mainly due to differences in brain structure and physiology compared to humans, provide a powerful tool for mechanistic insights. Over the past 10 years, behavioral and cognitive assessments in juvenile GF mice have demonstrated the potential role of GM in influencing host neurodevelopment (35). Similarly, the comparative evaluation of motor activity and anxiety-related behaviors in GF mice vs. SPF mice allowed the researchers to highlight the potential involvement of intestinal microorganisms in the MGBA (36). In particular, GF mice showed increased motor activity and decreased anxiety, suggesting that microbial colonization may be an integral part of brain developmental programming, initiating signaling mechanisms that influence neuronal circuits related to motor control and anxiety-like behavior. Regarding the impact on brain maturation in early life, in a recent study, Lu et al., evaluated the effects on postnatal brain development in GF mice colonized with the GM of preterm infants known to induce high- or low-rate growth phenotypes (37). The GM configuration associated with the stunted phenotype was linked to an increase in neuroinflammation and a decrease in circulating insulin-like growth factor-1 (IGF-1), suggesting an unfavorable impact of particularly dysbiotic GM layouts on the early development of neurons and oligodendrocytes (37). In addition, Zhou et al. (38) demonstrated in a murine model of NEC that the presence of gut-released interferon-γ-producing CD4+ T cells in mice was associated with features of brain injury that are also observed in human infants with NEC, such as microglial activation, inflammation, and myelin loss (39, 40).

Several studies have also demonstrated impaired working memory functioning in GF mice related to decreased hippocampal levels of 5-hydroxytryptamine receptor 1A (5-HT1A) and brain-derived neurotrophic factor (BDNF) (41, 42). Increases in dopamine, serotonin (5-HT), and synaptic vesicle proteins were also observed in the striatum of GF mice, affecting motor and emotional responses in a brain region closely related to the basal ganglia and motor limbic, and causing anxiety-like behavior (36). In addition, lower levels of N-methyl-D-aspartic acid receptor (NMDAR), 5-HT1 receptor, and BDNF were found in the amygdala, which is part of the "emotional brain" limbic system, leading to increased risk-taking behavior (41, 43). Finally, GF mice exhibited an exaggerated hypothalamic–pituitary–adrenal (HPA) stress response, suggesting that the presence

of GM from early developmental stages is required for the HPA system to become fully susceptible to inhibitory neural regulation (43).

Interestingly, a differential role for host genetics and GM features on neurodevelopmental outcomes has been documented in a specific mouse model, Cntnap2$^{-/-}$, in which the hyperactive phenotype was linked to host genetics, whereas the social behavior phenotype was mediated by GM features (44). In this murine model, social deficits were restored by specific microbial interventions (i.e., administration of *Lactobacillus reuteri*), with the upregulation of metabolites involved in the synthesis pathway of tetrahydrobiopterin, a coenzyme relevant for the alleviation of symptoms related to social behavior in individuals with autism spectrum disorders (45). Finally, the maternal immune activation (MIA) murine model allowed the identification of potential probiotic therapies to alleviate gastrointestinal and behavioral symptoms associated with neurodevelopmental disorders (3). Specifically, Hsiao et al., demonstrated that administration of the human commensal *Bacteroides fragilis* to MIA offspring altered GM composition, positively modulated intestinal permeability, and ameliorated specific behaviors associated with autism spectrum disorders (3).

The MGBA is composed of several bidirectional pathways, involving neural, hormonal, and immunological signaling (46). Several microbial metabolites, such as SCFAs, histamine, and tryptophan derivatives, are essential mediators along this axis (47–50). SCFAs (derived from microbial fermentation of complex polysaccharides) play a pivotal role in promoting the maturation and proper functioning of microglia (51), which is in turn involved in early neurodevelopment and is responsible for antigen presentation, phagocytosis, and inflammatory regulation (52, 53). *In vitro* tests on organotypic slice cultures also showed that butyrate may act directly on oligodendrocytes to suppress demyelination, enhance remyelination, and promote oligodendrocyte differentiation, all critical factors in the pathogenesis of multiple sclerosis (54). Murine models deficient in the SCFA receptor FFAR2 exhibited microglial defects commonly associated with GF conditions, such as alterations in cell number and phenotype, resulting in an impaired innate immune response (51). Histamine, primarily produced in the gastrointestinal tract by *Escherichia coli* and *Morganella morganii* (55), is also important for microglial signaling involved in the regulation of host behavior and cognition, and contributes to microglia-mediated inflammation in the brain (56, 57). Finally, an important role in the regulation of MGBA has been hypothesized for tryptophan derivatives of GM origin. These microbial metabolites have the potential to affect neuroinflammation, nerve signal transduction, and blood–brain barrier maintenance by activating aryl hydrocarbon receptors on astrocytes and microglia, resulting in an overall suppression of inflammation (58). Derived from 5-hydroxytryptophan, serotonin is produced by several clostridial species (49) and also plays a key role in neurodevelopment, influencing neuronal differentiation and migration, axon growth, myelination, and synaptogenesis (46, 60).

4. The case study of preterm infants

4.1. Gut microbiota in preterm infants

The structural and immunological immaturity of the gut, which is distinctive of preterm infants, coupled with specific environmental conditions (delivery mode, NICU procedures and environment, drug administration, feeding), can severely interfere with a healthy microbial colonization (61). Indeed, lower GM diversity, wide inter-individual variation and increased proportions of potential pathogens are typically observed. For example, antibiotic exposure is known to reduce GM diversity and influence its composition, with an overabundance of Proteobacteria, to the detriment of Clostridia and *Bifidobacterium* (62). Colonization by the latter microbial genus is delayed and much less abundant in preterm than in term infants (63). The type of feeding has also a strong influence on preterm GM (64). Mother's own milk feeding, compared to donor human milk and formula, induces higher GM diversity (65, 66) and *Bifidobacterium* abundance (67), potentially mitigating the detrimental effect of low birth weight/low GA.

The role of other microorganisms, such as fungi and archaea, that can colonize the infant gastrointestinal tract, is far from being fully understood (68–70), but the need to explore the inter-kingdom interactions that influence the assembly and maturation dynamics of the GM ecosystem is recognized. In a landmark study, Rao and colleagues have delved into the interplay between different kingdoms and showed that a single fungal species–*Candida albicans*–inhibited several dominant gut bacterial genera (71). The authors highlighted the centrality of simple microbe-microbe interactions in shaping the host-associated microbiota, which is critical for fully exploiting potential microbiota-based solutions to address altered microbiota configurations as well as impaired brain maturation and health outcomes in preterm infants.

4.2. Microbiota-gut-brain axis and signaling in preterm infants

Brain development begins *in utero* during the first month of pregnancy and involves a predefined sequence of events, many of which continue into postnatal life (72). Shortly before birth, approximately half of all neurons are cleared through apoptosis, with a second wave of synaptic pruning and elimination occurring during the peri-adolescent period (73). Numerous windows of vulnerability have been identified during prenatal and postnatal brain development. Within these windows, adverse events can significantly alter developmental trajectories and increase the risk of disease (74). For these reasons, infants born prematurely at the verge of the second and third trimesters represent a particularly vulnerable population (Figure 1), as they are at increased risk of perinatal white matter injury (PWMI), which may present with intraventricular hemorrhage, periventricular leukomalacia, or diffuse white matter injury (75). Perinatal inflammation and infections have been implicated in the pathogenesis of PWMI and may further worsen the neurological outcome (39). Interestingly, NEC, which is featured by GM dysbiosis (i.e., increased Proteobacteria levels and Toll-like receptor 4 activity) (76, 77) is associated with a significant risk of neurodevelopmental impairment (78, 79). Studies modeling neonatal infections have described the characteristics of neuroinflammation and documented the production of proinflammatory cytokines in the brain similar to those observed in the gut (40).

Peculiar GM compositions in the first months of life have also been associated with later neurodevelopmental outcomes. For example, Carlson et al. first performed GM 16S rRNA gene sequencing

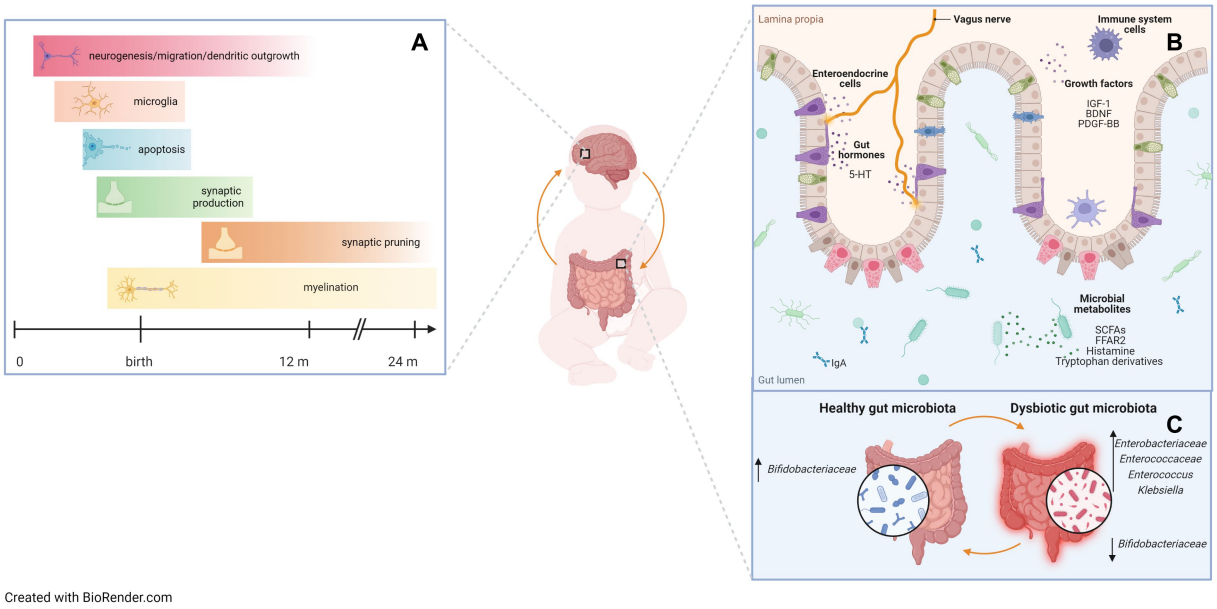

FIGURE 1
A critical window in early life for gut microbiota assembly and neurodevelopment. Preterm infants are at high risk of both gastrointestinal and neurodevelopmental impairment due to a peculiar developmental environment, with impaired gut microbiota assembly. **(A)** Brain developmental events during prenatal and early postnatal life that correspond to windows of vulnerability. Developmental processes occur in phases, setting the stage for potential periods of susceptibility to stimuli and insults that may affect brain growth and function. **(B)** Bidirectional gut-brain communication pathways. Evidence from animal studies suggests that gut hormones, growth factors, microbial metabolites, and receptors are involved in the microbiota-gut-brain axis. 5-HT: 5-hydroxytryptamine or serotonin; IGF-1: insulin-like growth factor-1; BDNF: brain-derived neurotrophic factor; PDGF-BB: platelet-derived growth factor-BB; SCFAs: short-chain fatty acids; FFAR2: free fatty acids receptor 2. **(C)** Dysbiotic gut microbiota profiles negatively affect gut-brain communication. Some specific bacterial taxa have been shown to be associated with neurodevelopmental outcomes in preterm infants. Up arrows indicate an increase in relative abundance of taxa, down arrows indicate a decrease in relative abundance of taxa.

in 89 healthy term infants at 1 year of age and correlated GM features with Mullen Scale of Early Learning (MSEL) scores and brain imaging at 1 and 2 years of age (80). The authors grouped infants according to GM features at 1 year of age: infants in the GM group characterized by a high abundance of Bacteroidetes had better MSEL scores at 2 years of age, especially in receptive and expressive language, with breastfeeding and vaginal birth acting as covariates predicting a better outcome. One-year GM alpha diversity was inversely correlated with 2-year MSEL score. On the other hand, minimal effects of 1-year GM features on brain volume at 2 years of age were found.

More recently, in a study on 309 healthy term infants exploring the relationship between GM at 3–6 months of age and the Age and Stage Questionnaire (ASQ) score at 3 years of age (81), the authors documented specific associations between early GM composition and neurodevelopmental outcomes: infants with a high abundance of *Lachnospiraceae* and Clostridiales and a low abundance of Bacteroidetes in their GM performed worse in communication and personal social skills, while infants with an early GM dominated by Bacteroidetes and low in *E. coli* and *Bifidobacterium* had lower fine motor skill scores.

Aatinski et al., investigated the relationship between GM composition in 301 infants, aged 2.5 months, from the FinnBrain Birth Cohort Study, and infant temperamental traits, by administering the Infant Behavior Questionnaire-Revised (IBQ-R) 6 months after birth (82). The composition of the GM was grouped into three different community types, each characterized by specific microbial features. For example, infants in the high *Bifidobacterium*/*Enterobacteriaceae* abundance group scored the highest on the regulation trait, while infants in the *Bacteroides* group scored the lowest. In addition, some temperamental traits were associated with GM diversity and genus-level composition, even after adjusting for potential confounders such as mode of delivery and breastfeeding.

Given the early-life window of vulnerability of preterm infants for both GM assembly and neurodevelopment, recent studies, summarized in Table 1, have explored the relationship between early-life GM layout and neurodevelopmental outcomes in this specific population.

Seki et al., described the relationship between the microbiota-immune-gut-brain axis and early neurodevelopment in 60 extremely preterm (GA < 28 weeks) and extremely low birth weight (BW < 1,000 g) infants (9). The authors described the characteristics of brain development over time in early life, assessed at multiple timepoints by cranial ultrasound and amplitude-integrated electroencephalography (aEEG) and at term-equivalent age by cerebral MRI, and identified a number of potential biomarkers of brain damage in this vulnerable population, including specific features of GM and immune function. Specifically, three distinct stages of brain development, from birth to term-equivalent age, were detailed in extremely preterm infants: first a quiescent phase, followed by a period of neurophysiological maturation, and then a term-equivalent phase. In infants with PWMI, specific microbial and immune features during the quiescent phase can trigger an inflammatory cascade, characterized by T-cell polarization and secretion of proinflammatory cytokines. Inflammation continues during the neurophysiological

TABLE 1 Human studies exploring the relationship between early life gut microbiota and neurodevelopment outcomes in preterm infants.

Author, year (Reference)	Study details	Study population	Intervention	Gut microbiota assessment timing method	Neurodevelopment assessment timing method	Results
Beghetti et al. (2021) Italy (6)	O P M	Preterm infants <32 weeks GA [*n* = 27, median GA 30.6 (IQR 28.6–33.6) weeks]	NA	1, 4, 7, and 30 days of life 16S rRNA Illumina sequencing	24-month CA Griffiths Mental Development Scale (GMDS-R) and General Development Quotient (GQ) performed by psychologist	Early-life GM of infants with normal vs. impaired neurodevelopment followed distinct temporal trajectories with peculiar compositional rearrangements. Early *Bifidobacterium* deficiency appeared to be a negative biomarker f adverse neurological outcomes.
Oliphant et al. (2021) USA (8)	O P M	Preterm infants <34 weeks GA (*n* = 58)	NA	Weekly during NICU hospitalization until discharge or 36 weeks PMA 16S rRNA Illumina sequencing	Head Circumference Growth (HCG) weekly during NICU hospitalization until discharge or 36 weeks PMA	Preterm infants with suboptimal HCG trajectories had a depletion in the abundance/prevalence of Bacteroidota and *Lachnospiraceae*, independent of morbidity and caloric restriction.
Rozé et al. (2020) France (5)	C P Multic.	Preterm newborns born at 24 to 31 weeks GA [*n* = 577, mean GA 28.3 (SD 2.0) weeks]	NA	Week 4 after birth 16S rRNA Illumina sequencing	2 years CA Survey assessing cerebral palsy completed by the referring physician and parent assessed 24-month Ages and Stages questionnaire (ASQ)	GM cluster driven by *Enterococcus* and cluster driven by *Staphyloccoccus*, were significantly associated with 2-year non optimal outcome.
Sarkar et al. (2022) United States (7)	O P M	Preterm infants with birth weight < 1,500 g [*n* = 24, mean GA 27.95 (SD 1.81) weeks]	NA	Weekly for 6 weeks after NICU admission and at 2 and 4 years of age	2 and 4 years of age Battelle Development Inventory-2Screening Test (BDI-2ST) administered by researcher team scored by psychologist	Both NICU infant stool diversity and particular microbial ASVs were associated with BDI-2 ST cognition, adaptive, and communication subscales. Network analysis of the NICU infant stool microbial ecology showed differences in children needing neurodevelopmental referral.
Seki et al. (2021) Austria (9)	O P M	Extremely preterm infants [*n* = 60, mean GA 25.5 (SD 1.2) weeks]	NA	Days 3, 7, and 14, followed by biweekly sampling until discharge	Brain injuries identification by cUS and neurophysiological development assessment by aEEG (days 3, 7, and 14, then biweekly until discharge); cMRI at term-equivalent age	*Klebsiella* overgrowth in the gut was highly predictive for brain damage and was associated with a pro-inflammatory immunological tone.

(Continued)

TABLE 1 (Continued)

Author, year (Reference)	Study details	Study population	Intervention	Gut microbiota assessment timing method	Neurodevelopment assessment timing method	Results
Sun et al. (2020) United States (10)	O P M	Preterm infants [*n* = 34, mean BW 1451. (SD 479.3) g]	NA	Daily from 5 to 28 days of life 16S rRNA Illumina sequencing	36–38 weeks of post-menstrual age or prior to hospital discharge NICU Network Neurobehavioral Scale (NNNS)	A functional log-contrast regression model identified microbiota components at order (Clostridiales, Lactobacillales, Enterobacteriales) and genus level (*Veillonella*, *Enteroccoccus*, *Shigella*) that were associated with the neurobehavioral outcome of infant assessed by Stress/Abstinence subscale (NSTRESS)
Van den Berg et al. (2016) Netherlands (11)	RCT DB M	Very preterm infants GA < 32 weeks and/ or BW < 1,500 g [*n* = 77 mean GA 29.9 (SD 1.7) weeks]	scGOS/lcFOS/ pAOS or placebo supplemented to breast milk or to preterm formula days 3–30 of life	days 1, 7, 14 and 30 fluorescent *in situ* hybridisation (FISH) analysis	24 months CA Bayley Scales of Infant and Toddler Development (BSID) administered by blinded psychologist	Lower percentages of bifidobacteria at days 7 and 14 were associated with lower mental developmental index. Total bacterial count did not influence mental and psychological developmental index scores.

RCT, randomized controlled trial; P, prospective; B, blinded; DB, double-blinded; C, cohort; O, observational; M, monocentric; Multic, multicentric. BW, birth weight; GA, gestational age; PMA, postmenstrual age; GM, gut microbiota; ASVs, amplicon sequence variants. cUS, cranial ultrasound; aEEG, amplitude-integrated electroencephalography; cMRI, cranial magnetic resonance imaging. NA, not applicable.

maturation period, which has a delayed onset and specific pathological features, such as alterations in brain electrical activity, cranial oxygen saturation, and neuroprotective secretion (i.e., platelet-derived growth factor-BB [PDGF-BB] and BDNF). As for GM, *Klebsiella* overgrowth 6 weeks after birth was associated with severe brain injury and inflammatory markers, such as γδ T cells and proinflammatory cytokine secretion, while it was inversely related to neuroprotective secretion.

The relationship between a validated early marker of neurodevelopment, specifically head circumference (HC) growth, and GM establishment from the first week of life was investigated in the prospective study conducted by Oliphant et al. (8). Fecal samples were collected weekly from 58 preterm infants born before 34 weeks of GA during their NICU stay. The poor growth of HC was related to the low abundance of two bacterial taxa that are dominant in adult GMs, Bacteroidetes and *Lachnospiraceae*. Interestingly, the postmenstrual age of 30 weeks was identified as a common timepoint at which both HC growth trajectories and GM composition began to diverge between groups.

Sun et al. (10) characterized the GM of 34 preterm infants in the first month of life during NICU admission and assessed neurodevelopmental outcomes at 36–38 weeks of postmenstrual age or prior to NICU discharge using the Network Neurobehavioral Scale (NNNS) and its Stress/Abstinence subscale (NSTRESS). A functional log-contrast regression model identified GM components at order (Clostridiales, Lactobacillales, Enterobacteriales) and genus (*Veillonella*, *Enteroccoccus*, *Shigella*) level, whose relative abundance variations during the sampling time were associated with the infants' neurobehavioral outcome as assessed by NSTRESS subscale (10).

The relationship between early GM and neurodevelopment assessed in early childhood was explored in 4 of the studies included in this narrative review. The French national prospective observational cohort study EPIFLORE investigated the association between GM dysbiosis in 577 very preterm infants and long-term outcomes (5). Analysis of GM at 4 weeks after birth identified 6 GM groups influenced by infant characteristics, treatments, and specific NICU clinical strategies, such as ventilation, sedation, feeding, use of antibiotics, and skin-to-skin practice. Notably, after adjustment for confounders, such as GA, absence of assisted ventilation on day 1 was associated with a reduced risk of cluster 5 (driven by *Staphylococcus*) or cluster 6 (including non-amplifiable samples due to low bacterial load), while sedation and low-volume enteral nutrition were associated with increased risk. Skin-to-skin practice was associated with a reduced risk of cluster 5. After adjusting for the above confounder, the authors documented that infants in cluster 4 (driven by *Enterococcus*), 5 and 6 had the highest risk of a 2-year non-optimal outcome, defined as the occurrence of death or neurodevelopmental delay, as assessed by the ASQ at 2 years of age.

In a prospective observational pilot study, we explored the link between GM in the first month of life and neurodevelopment at the correct age of 24 months in 27 very low birth weight (VLBW) infants (6). Neurodevelopmental outcomes, assessed using the revised Griffiths Mental Development Scale (GMDS-R) administered by a psychologist blinded to the GM analysis, were associated with GM features at defined timepoints (taxon abundance) and over time (beta diversity trajectories). Notably, the establishment of GM over time differed based on both the presence and degree of neurodevelopmental impairment. Early GM in neurodevelopmentally impaired infants was rich in *Enterococcaceae* at days 7 and 30, showing a significantly lower abundance of *Bifidobacteriaceae* at day 30 than in neurodevelopmentally normal infants. The abundance of *Bifidobacterium* at 30 days of life was directly related to the GMDS-R General Quotient at 24 months. Neither *Bifidobacterium longum* nor *Bifidobacterium breve* were found in the GM of neurodevelopmentally impaired infants.

The relevance of *Bifidobacterium* in the neurodevelopment of preterm infants was also suggested in the study by Sarkar et al. (7). Stool samples from 24 VLBW infants were collected weekly during their NICU stay, and then at 2 and 4 years of age, to assess the GM establishment in the first years of life. The GM of VLBW infants showed dysbiotic features in the neonatal period, likely related to the NICU environment, and subsequently transitioned to an adult-like GM at 4 years of age. GM features, including diversity and abundance of specific taxa, correlated with several items of the Battelle Development Inventory-2 Screening Test (BDI-2 ST) administered at 2 and 4 years of age. Notably, at 2 years of age, children who did not require neurodevelopmental referral had a *Bifidobacterium*-dominated GM, while *E. coli*, *Citrobacter*, and *Enterobacteriaceae* were highly prevalent in children who required referral. Finally, a randomized clinical trial (11) evaluated neurodevelopmental outcome measured by the Bayley Scales of Infant and Toddler Development (BSID - III) at the corrected age of 2 years in very preterm infants after supplementation with short-chain galacto-oligosaccharides, long-chain fructo-oligosaccharides and pectin-derived acidic oligosaccharides, and possible associations with cytokine levels and stool bacterial counts during the neonatal period. Enteral supplementation with a prebiotic blend during day 3–30 of life did not improve neurodevelopmental outcomes in 77 infants evaluated at 24-month corrected age. However, higher proportions of Bifidobacteria in the GM analyzes at day 7 and day 14 of life were associated with higher BSID Mental Development Index (MDI) scores, while total fecal bacterial counts did not influence the MDI or Psychomotor Development Index (PDI) scores.

5. Discussion

In the present narrative review, we considered the existing literature exploring the relationship between early-life GM and neurodevelopment in preterm infants. According to the available evidence, which so far includes only a limited number of clinical studies, monitoring GM dynamics in preterm infants during the first months of life could reveal a possible relationship with later neurodevelopmental outcomes. A relationship has been suggested between both dynamic patterns (i.e., beta diversity trajectories, relative abundance of taxa over time) and static features (i.e., relative taxon abundance or taxonomic clusters at defined timepoints) of GM during the first month of life and brain maturation, as well as neurodevelopmental outcomes in early childhood. Furthermore, some studies have pointed out the potential role of early colonization with specific bacterial taxa, particularly *Bifidobacterium*, on neurodevelopment in early childhood. Specifically, the absence or low relative abundance of *Bifidobacterium* could constitute a biomarker of vulnerability and immaturity, and this observation could potentially lead to early intervention strategies aimed at promoting optimal neurodevelopment in preterm infants during NICU admission and after discharge. Furthermore, *Bifidobacterium* spp. are known to play a pioneering role in the healthy development of the infant GM, contributing to the fine-tuning of the immune system and potentially exerting neuroprotective effects, mainly by modulating the production and release of neuroactive metabolites (83, 84).

However, some limitations of the available evidence need to be recognized. The main limitations relate to the paucity of human studies addressing this topic. Additionally, the small number of subjects included in most published clinical studies has hindered the chance to further explore the impact of various clinical variables (i.e., NEC, LOS, feeding type) on both GM assembly and neurodevelopmental outcome. Another limitation is the time window of GM analysis, as stool samples were mainly collected during the first 30 days of life, and microbial changes after this time window were not investigated. Furthermore, the primary studies were heterogeneous in terms of sample size, clinical evaluations, and methods used to assess neurodevelopmental outcomes. Finally, yet importantly, a major limitation of the GM field is that most studies have focused on the impact of bacterial communities on brain development and subsequent health outcomes in preterm infants, while the potential critical contributions of non-bacterial populations are far from being fully characterized. The importance of considering multi-kingdom interactions when assessing microbiota-mediated effects on human health, particularly in brain development and in the prevention of future neurological disorders, becomes critical as members of microbial communities share the same niches. Consequently, perturbations in one microbial kingdom may also affect the composition and community function of the other kingdoms. Encouraging future studies that delve into this line of research will be essential to realize the full potential of microbiota-targeted solutions to combat the altered microbiota configurations, impaired brain maturation and related health problems that characterize preterm infants.

Evidence from preclinical models has demonstrated that specific bacteria with probiotic properties that confer mental health benefits, also called psychobiotics, can modulate brain function (84, 85). Underlying mechanisms include the production of neuroactive metabolites involved in MGBA, such as gamma-aminobutyric acid and 5-HT, the reduction of proinflammatory cytokines and hypothalamic–pituitary–adrenal activity, as well as GM modulation (86, 87). In the context of the potential psychobiotics effect in early life, it has been suggested that administration of *Lactobacillus acidophilus* and *Bifidobacterium infantis* to pregnant mice promotes brain development and protects the offspring brain from postnatal inflammatory insults (88). More recently, Cowan et al. (89) have demonstrated that early neural maturation in stressed newborn rats was prevented by probiotic administration. Specifically, male Sprague–Dawley rats were reared under standard conditions or exposed to

stress induced by maternal separation. The latter animals showed adult-like engagement of the medial prefrontal cortex during fear regulation. However, this response was prevented by the administration of a probiotic blend composed of *Lactobacillus rhamnosus* and *Lactobacillus helveticus*.

Moving from animal model findings to a possible role in humans, prophylactic probiotics have been suggested to reduce the incidence of several clinical outcomes, including NEC, LOS, and mortality in very preterm infants (90), while their potential efficacy as modulators of MGBA and therefore neurodevelopmental outcomes in early childhood is still debated (91, 92). Recent meta-analyzes summarizing the limited literature available on this topic showed no differences in neurodevelopment in infants treated with probiotics or prebiotics compared to controls, while a potential effect of probiotics on short-term growth has been suggested (93, 94).

6. Conclusion

Currently available human studies suggest an association between early-life GM, brain development in preterm infants, and neurodevelopmental outcomes. Although a clear mechanistic pathway linking the brain and GM in preterm infants has not yet been elucidated, it could be assumed that specific GM profiles could be the hallmark of neurodevelopmental vulnerability. This observation could pave the way for timely identification of high-risk infants and early intervention strategies aimed at promoting optimal neurodevelopment in preterm infants during the NICU stay and after discharge. Further clinical studies in larger cohorts, possibly integrating multi-omics techniques (e.g., metagenomics, metatranscriptomics, and metabolomics) and animal models, are needed to provide further evidence and mechanistic insights. Besides, studying the MGBA in the context of long-term follow-up of neurodevelopmental outcomes in preterm infants beyond NICU admission is needed to provide insight into potential therapeutic targets and predictive biomarkers for healthy development in preterm infants.

Author contributions

ST and AA: conceptualization. ST, AA, MB, and IB: methodology. PB, LC, and AS: validation. AA and ST: formal analysis. IB and MB: investigation and writing–original draft preparation. IB: resources and data curation. ST, AA, PB, LC, and AS: writing–review and editing. All authors have read and agreed to the published version of the manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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A preliminary study for the clinical effect of one combinational physiotherapy and its potential influence on gut microbial composition in children with Tourette syndrome

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Introduction: Tourette syndrome (TS) is a chronic neuropsychiatric disorder with unknown causes and inadequate therapies. Inspired by the important roles of gut microbiota in some mental illnesses, the interactions between gut microbiota and TS via the gut-brain axis have gained more and more attention. This study aimed to characterize the gut microbial profiles in children with TS, and explore the clinical effects of one combinational physiotherapy and its potential influence on gut microbial composition.

Methods: The gut microbial profiles were depicted based on the sequence data of 32 patients and 29 matched health children by 16S rDNA amplicon pyrosequencing. Thirty of thirty-two patients underwent uninterrupted two 10-day courses of combinational physiotherapy, which included a 60-minute cranial electrotherapy stimulation (CES) training followed by a 30-minute biofeedback training per session, 2 sessions a day.

Results: Our results indicated that the gut microbial composition in children with TS was different from that in healthy controls. Multiple GBM neurotransmitter modules obtained through Picrust2 functional predictive analysis were significantly increased in patients, including Histamine degradation, Dopamine degradation, and DOPAC synthesis. Moreover, this combinational physiotherapy could significantly diminish tic activity, whose positive effects were first reported in children with TS. Lastly, different gut microbial compositions and predictive metabolic pathways were also observed between patients before and after this treatment, with lower abundances of the genera (e.g., *Dorea*) and significant decreases of GBM neurotransmitter modules (e.g. dopamine degradation) in patients after this treatment, indicating that improved clinical symptoms might be accompanied by an improvement of intestinal microenvironment.

Discussion: Children with TS showed a cognizable gut microbial profile, and certain enriched bacteria with pro-inflammatory potential might induce neuroinflammatory responses. This combinational physiotherapy could significantly diminish tic activity, and the gut microbial compositions in patients after this treatment were different from those without any treatment, indicating the existence of bidirectional communication of the gut-brain axis in TS. But studies on the gut microbial characteristics in TS patients, the influences of gut microbiota on tic severity, the efficacy and safety of this treatment, and the bidirectional regulatory mechanism between brain signals and gut microbiota in TS still need to be explored.

KEYWORDS

Tourette syndrome, gut microbiota, cranial electrotherapy stimulation, electrodermal biofeedback training, combinational physiotherapy

Introduction

Tourette syndrome (TS) is a chronic neurological disease, whose symptoms include persisting multiple motor tics and alternating vocal tics (1). Population studies indicated that the prevalence of TS in children is about 1.7% in China and 0.8% worldwide (2, 3). Despite most patients can recover by themselves before the age of 18, approximately 33% of patients will continue their symptoms to adulthood factually (4).

Currently, although the cause of TS is unknown, genetic and environmental factors are regarded as substantial factors associated with its intrinsic etiologies. Many studies indicated that the development and regulation of the brain are affected by the gut microbiota through the microbiota-gut-brain axis (5, 6). In patients with tic disorders (TDs), the abundances of *Bacteroides plebeius* and *Ruminococcus lactaris* were increased compared to healthy children, while *Prevotella stercorea* and *Streptococcus lutetiensis* were decreased (7). Among these patients, a significant enrichment of *Ruminococcus lactaris* was identified in patients with TS than that in patients with other TDs (7). What's more, *Klebsiella pneumonia*, as a GABA-degrading bacterium, was positively associated with the symptomatic deterioration in TDs (8). Meanwhile, *Eubacterium* spp., *Bifidobacterium* spp., and *Akkermansia muciniphila* were related to the production of GABA, which showed a negative correlation with the Yale Global Tic Severity Scale (YGTSS) score (8, 9). However, some inconsistencies also existed. For example, as GABA-producing bacteria, *Bacteroides thetaiotaomicron* and *Bacteroides eggerthii* displayed a positive correlation with the YGTSS score (7). Thus, what's kind of abnormal alterations of gut microbiota in TS and whether these abnormal alterations were the key pathogenic factors still need to be explored.

Treatments of TS mainly include drug treatment and psychological treatment. However, both therapies could not completely relieve the clinical symptom and had their limitations, such as side effects from medication and a long treatment period for psychological treatment (10, 11). In addition, although some noninvasive techniques (e.g., transcranial magnetic stimulation or transcranial direct current stimulation) presented positive results for TS, their wide clinical applications were limited due to the high price (12, 13).

As a noninvasive transcutaneous therapeutic device, cranial electrotherapy stimulation (CES) adopted pulsed, alternating microcurrent (<1,000 μA) to the brain depending on its electrodes placed on the earlobes, mastoid processes, zygomatic arches, or the maxillo-occipital junction. CES has been approved by Food and Drug Administration (FDA) to treat patients with depression, anxiety, insomnia or pain, and it is relatively inexpensive and convenient compared with other noninvasive stimulation means (14–16). Although several studies have reported the efficacy and safety of CES in children with TS (17–19), its effectiveness using alone was less and slower in comparison with aripiprazole for children and adolescents with TS (20). Meanwhile, as a noninvasive psychophysiological intervention, biofeedback can regulate patients' physiological responses by allowing patients to perceive visual and auditory signals. The tic frequency in patients with TS was significantly decreased during relaxation biofeedback compared to arousal biofeedback, and it was positively correlated with sympathetic arousal during the sessions of arousal biofeedback (21). However, another study found that maintaining longstanding relaxation biofeedback was difficult because of the concomitant occurrence of tics, even though the tic frequency was reduced during 5 min of relaxation biofeedback (22).

Until now, the brain and the gut communicate with each other via various routes, such as the immune system, tryptophan metabolism, vagus nerve and enteric nervous system (6, 23). Many earlier studies regarding gut-brain communication focused on digestive function and satiety (24–26), whereas recently growing works have concentrated on higher-order cognitive and psychological effects of this bidirectional communication (27–29). As a chronic neurological disease, certain gut microbial alterations have been observed in TS. However, little information about the gut microbial composition after the clinical improvement in TS patients was reported. Zhao et al. have reported that tic symptoms ameliorated notably after 8 weeks of fecal microbiota transplantation (FMT) for patients with TS, and the gut microbial composition was significantly altered, especially with the restoration of *Bacteroides coprocola* (30, 31). In the TS mice model, the symptoms were dramatically improved after receiving feces from healthy mice for 3 weeks, and the abundances of *Turicibacteraceae* and *Ruminococcaceae* were significantly increased in their feces (32). In addition, the treatment of acupuncture and massage was effective for children with TS, whose gut microbial characteristics after the treatment was close to those in healthy children (33). Noteworthy, in addition to diet and drugs, other undiscovered mechanisms also might have potential influences on the distribution of gut microbiota. On the other hand, the occurrence of gut microbial alterations is reasonable when the clinical symptoms associated with emotion ameliorated dramatically, partly due to the bidirectional communication between the gut and the brain.

The aims of this study were as follows: (1) to characterize the gut microbial distribution in children with TS; (2) to explore the clinical effects of one combinational physiotherapy on children with TS; (3) to explore the potential impact of the combinational physiotherapy on gut microbial composition.

Materials and methods

Ethical approval

This study was approved by the Ethics Committee of the Xiangyang No. 1 People's Hospital, Hubei University of Medicine (Xiangyang, China). The corresponding Institutional Review Board (IRB) number was No.2020KYLL. All written informed consents were obtained from all subjects' guardians.

Subjects

Thirty-two children with TS who visited the Department of Child Healthcare, Xiangyang No. 1 People's Hospital, Hubei University of Medicine from June 2021 to July 2022 were recruited for this study. The patients were aged 2.92–13 years, with a median age with IQR being 7.00 (5.27–9.75) years (Table 1). Twenty-six of thirty-two children were male and six were female. The inclusion criteria were as follows: (1) diagnosed as TS by a comprehensive evaluation according to the Expert Consensus on the Diagnosis and Treatment of Tic Disorders in Children (2017 Practical Edition) and the 5th edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5); (2) diagnosed for the first time not receiving any treatments. The exclusion criteria consisted of the following: (1) the presence of intellectual disability, autism, tristimania, manic-depressive psychosis, epilepsy, Parkinson's disease, schizophrenia, chorea, athetosis, and other nervous system diseases; (2) a history of hepatolenticular degeneration and other extra vertebral diseases; (3) a history of obesity, gastrointestinal disease, gastrointestinal injury, head injury, and other physical illnesses; (4) other diseases which were unsuitable for this study evaluated by our investigators; (5) the experience of glucocorticoids, immune-suppressants, antihistamines, and other drugs for neurological diseases within the last year; (6) the experience of antibiotics, probiotics, or prebiotics within 3 months before sampling. The diagnosis was made by three independent neurological clinicians. Twenty-nine children were recruited as healthy controls during the same period, including 24 males and 5 females. All healthy controls underwent health checkups in our hospital to exclude any physical illnesses, mental diseases, or the above-mentioned experiences. The healthy children were aged 2.75–14 years, with a median age with IQR being 6.42 (4.75–8.59) years (Table 1). In addition, all recruited subjects' mothers did not take probiotics or prebiotics during pregnancy.

Collection of clinical data

Information about the children's general condition and pregnancy-related condition was collected, including the date of TS onset, first symptoms, current symptoms, symptom frequency, daily activities, learning and social situations, feeding option, gestational stress, gestational infectious history, and others. The Yale Global Tic Severity Scale (YGTSS) is a clinician-rated evaluation of patients' tic severity over the past 7–10 days. Briefly, a clinician directly interviews each child and their guardian to produce the Total Motor and Phonic Tic score [range 0–50, including the separate Total Motor Tic score (range 0–25) and the separate Total Phonic Tic score (range 0–25)] and the separate tic-related impairment score (range 0–50). Specifically, 46 tic disorder symptoms are involved in the YGTSS, including 12 simple motor tics (e.g., eye blinking), 19 complex motor tics (e.g., facial expressions), seven simple vocal tics (e.g., coughing), and eight complex vocal tics (e.g., words), with unmentioned symptoms labeled as "other" symptoms in patients' medical records. The motor and phonic domains are evaluated separately on a 0–5 scale across 5 dimensions (number, frequency, intensity, complexity, and interference), but the same anchor point descriptions are adopted to guide their scoring. For categorizing the severity degree of TS, the scores of 0–25, 26–50, and 51 or above are suitable for the determinations of mold, moderate, and severe stages, respectively.

Combinational physiotherapy intervention

CES Therapy---As our selected CES device, the fifth generation alpha-stim stress control system was purchased from Electromedical Products International (Mineral Wells, Tex), and its 510(K) Number was K903014 approved by FDA. This alpha-stim stress control system could generate bipolar, asymmetric rectangular waves with a frequency of 0.5 Hz and a current intensity ranging from 0 μA to 500 μA. Before treatment, two ear clip electrodes were placed on the child's right and left earlobes. The current intensity was adjusted until the child felt a mild tingling sensation and/or dizziness, and then the selected current intensity was reduced to slightly below the threshold of sensation. For one patient, the established current intensity will be consistently used throughout the whole course of the twenty-day treatment.

Biofeedback training---The multichannel biofeedback apparatus SPIRIT-2/4/8 was provided by the manufacturer, SPIRIT (Chengdu, China), and its Registration Certificate Number was Sichuan machine registration 20,182,260,085. The procedure of biofeedback training followed the product specification. Five dry nickel-plated electrodes (about $33 \times 46\,mm^2$ area per nickel-plated electrode) were placed on the child's tic sites. The sampling rate was 256–1,024 times per second. Biofeedback was adopted as a form of computer-generated animation and displayed on the computer screen. Every child lay on a comfortable chair in front of the computer monitor to receive treatment. When the child felt relaxed and his/her skin conductance was lower than the set threshold, the videos were continuously playing; if the patients' skin conductance increased and exceeded the set threshold, the videos would get smaller until to disappear. Thus, our biofeedback therapy "rewarded" children for controlling or even reducing their electrodermal sympathetic activity by displaying more animations.

During the uninterrupted two 10-day course of treatment, the children attended a 60-min CES training followed by a 30-min biofeedback training per session, 2 sessions a day, a total of 40 sessions. Notably, the interval between two sessions per day was 4 h. During each session, a nurse instructed the children to participate in the process, and adjust the animation by encouraging them to relax both mentally and physically. Children could not fall asleep during treatment.

The therapeutic effect was evaluated at the end of each course for every patient. The Y-GTSS was used to evaluate patients' tic severity by their attending physicians, and the side effects were also recorded in patients' electronic medical records.

Collection of fecal specimens

All recruited patients were encouraged to collect their feces within 2 days before (TS-pre) and after (TS-post) the combinational physiotherapy. Fecal specimens (about soybean grain size, two pieces per time) from every child were collected by themselves or their guardians at home or hospital within 3 min after defecating. After sampling, the fecal samplers (Biotecan, Shanghai, China) were sealed,

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TABLE 1 Demographic and clinical characteristics of children with and without Tourette syndrome (TS).

Clinical characteristics	No. of patients	Groups		*p* value
		Healthy controls	TS	
Total Sample	61	29	32	
Gender				
Male	50	24	26	0.878
Female	11	5	6	
Premature birth				
Yes	4	1	3	0.350
No	57	28	29	
Natural labour				
Yes	19	10	9	0.592
No	42	19	23	
Breast feeding				
Yes	48	23	25	0.910
No	13	6	7	
Gestational stress				
Yes	7	4	3	0.589
No	54	25	29	
The history gestational infections				
Yes	0	0	0	1.000
No	61	29	32	
Taking antiemetic drugs during pregnancy				
Yes	5	5	0	0.014*
No	56	24	32	
Smoking history during pregnancy				
Yes	0	0	0	1.000
No	61	29	32	
Drinking history during pregnancy				
Yes	0	0	0	1.000
No	61	29	32	
First baby for mother				
Yes	45	23	22	0.349
No	16	6	10	
Singleton pregnancy				
Yes	57	26	31	0.255
No	4	3	1	
The history of TS				
Yes	3	0	3	0.096
No	57	28	29	
Unknown	1	1	0	
Age/year (median with IQR)	7.00 (5.00–9.00)	6.42 (4.75–8.59)	7.00 (5.27–9.75)	0.495
Height/m (median with IQR)	1.28 (1.18–1.40)	1.23 (1.10–1.40)	1.30 (1.20–1.40)	0.365
Weight/kg (median with IQR)	27.00 (20.50–32.00)	25.00 (19.50–32.00)	27.00 (21.25–33.50)	0.448
BMI/(kg/m^2) (median with IQR)	16.12 (14.73–17.78)	16.23 (14.72–18.64)	15.99 (14.74–17.51)	0.471

*$p < 0.05$.

labeled, and transferred (<18°C) to Biotecan Laboratories within 2 days and stored at −80°C.

16S rRNA gene sequencing and data processing

A total of 78 fresh feces samples (HC vs. TS-pre vs. TS-post, 29 vs. 32 vs. 17) were collected in fecal samplers and stored at −80°C until being used to perform high-throughput sequencing. The QIAamp PowerFecal Pro DNA Kit (QIAGEN, Germany) was adopted to extract bacterial genomic DNA, which was amplified by Phusion High-Fidelity PCR Master Mix (New England Biolabs, Massachusetts State, United States) targeting the V3V4 region of 16S rRNA genes (Forward primer: 341F 5′-CCTACGGGNGGCWGCAG-3′, Reverse primer: 805R 5′-GACTACHVGGGTATCTAATCC-3′). The PCR products were purified by the TransStart® FastPfu DNA Polymerase kit (TransGen, Beijing, China). The DNA quantification of the purified product was finished by the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Massachusetts State, United States). A library Quant Kit Illumina GA revised primer-SYBR Fast Universal (KAPA Biosystems, Massachusetts, United States) was adopted to perform library quantification, and then a Novaseq6000 500 cycle (Illumina, California, United States) was used to perform pair-end 2 × 250 bp sequencing.

To cope with these sequencing data, the Quantitative Insights Into Microbial Ecology 2 (QIIME 2, v2017.6.0) pipeline and previous criteria were adopted (34, 35). Vsearch V2.4.4 was used to assemble the paired-end reads (36). 16S rRNA gene sequences were assigned to operational taxonomic units (OTUs) according to a similarity cutoff value of 97%, which was against the Greengenes database by Vsearch V2.4.4. Notable, OTUs with <0.001% entire sequences were dumped. The final OTU table was averaged, rounded, and rarefied, whose generation was based on averaging 100 evenly resampled OTU subsets under 90% of the minimum sequencing depth. Abundance curves were arranged by OTU level. The sequencing depth was evaluated and determined via rarefaction analysis.

Bioinformatics and statistical analyses

The chi-square test was adopted to analyze the statistical differences in categorical variables between healthy controls and patients with TS in SPSS 23.0 (IBM, Chicago, IL, United States). Continuous variables were presented as median with interquartile range (IQR) and compared by Mann–Whitney ***U***-test between groups in GraphPad Prism version 7.0 (GraphPad, San Diego, CA, United States). Y-GTSS scores between patients before and after the combinational physiotherapy were displayed as mean with SD and compared by Wilcoxon matched-pairs signed rank test in GraphPad Prism version 7.0. Venn diagram, heat-map analysis, and correlation analysis were performed by R software (v3.6.3). GraPhlAn[1] was used to depict the phylogenetic tree.

Alpha diversity analysis, including the Chao1 index, Simpson index, and Shannon index, was constructed by QIIME 2, whose comparison adopted the Pairwise Wilcox test. Bray-Curtis distance metrics and visualized via principal coordinate analysis (PCoA) were used to carry out the beta diversity analysis. Gut microbial composition and structure were compared between groups using Permutational multivariate analysis of variance (PERMANOVA) via the Kruskal test in R software. The comparability between these two groups was assessed by a one-way analysis of similarities (Anosim) analysis. Linear discriminant analysis effect size (LEfSe) was used to identify abundant taxa with significant differences across groups based on the default parameters (logarithmic LDA score = 2) (37). Phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt, PICRUSt2 v2.3.0-b) was adopted to forecast gut microbial functions by annotating the gene catalog according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) modules, carbohydrate-active enzymes (CAZY) database, MetaCyc database, GMM metabolic modularization, and GBM neurotransmitter modules enrichment analysis (38), whose statistical differences between groups were analyzed by Univar Test.

Results

Characteristics of subjects

TS-pre group consisted of 26 males and 6 females with a median age of 7.00 years (range: 2.92–13; interquartile range, IQR: 5.27–9.75) and a median BMI of 15.99 kg/m^2 (range: 13.19–24.49; interquartile range, IQR: 14.74–17.51) (Table 1). According to clinical evaluation, all patients were diagnosed with mild severity with mean YGTSS scores of 34.97 (SD 5; range 28–45). Except for taking antiemetic drugs during pregnancy (p = 0.014), no demographic differences were observed between the TS-pre and HC groups in age, Height, Wight, BMI, gender, premature birth, natural labor, breastfeeding, gestational stress, gestational infections history, smoking history, drinking history, first baby for mother, singleton pregnancy, and the family history of TS (Table 1).

Diversity and composition of gut microbiota in children with TS

In the Venn diagram, the shared OTUs were 23,465 between the HC and TS-Pre groups, and the HC group had more unique OTUs with 3,883 than the TS-Pre group with 2,395 (Figure 1A). In α-diversity, no differences in gut microbial richness and evenness were identified between groups (Chao1 p = 0.2908, Simpson p = 0.1176, and Shannon p = 0.3394) (Figures 1B–D). In β-diversity, the difference between groups was more significant than within one group (R = 0.066, p < 0.05) (Figures 1E,F). Figure 1G and Supplementary Figure S1 displayed microbial composition at the genus level. *Bacteroides, Faecalibacterium, and Roseburia* were the major components in the TS-Pre group, whereas *Bacteroides, Faecalibacterium,* and *Bifidobacterium* in the HC group. *Faecalibacterium, Roseburia,* and *Agathobacter* were present in both groups, but were more predominant in the TS-Pre group. In contrast, although *Bifidobacterium* and *Prevotella* were also observed in these two groups, they were more prominent in the HC group.

1 http://huttenhower.sph.harvard.edu/GraPhlAn

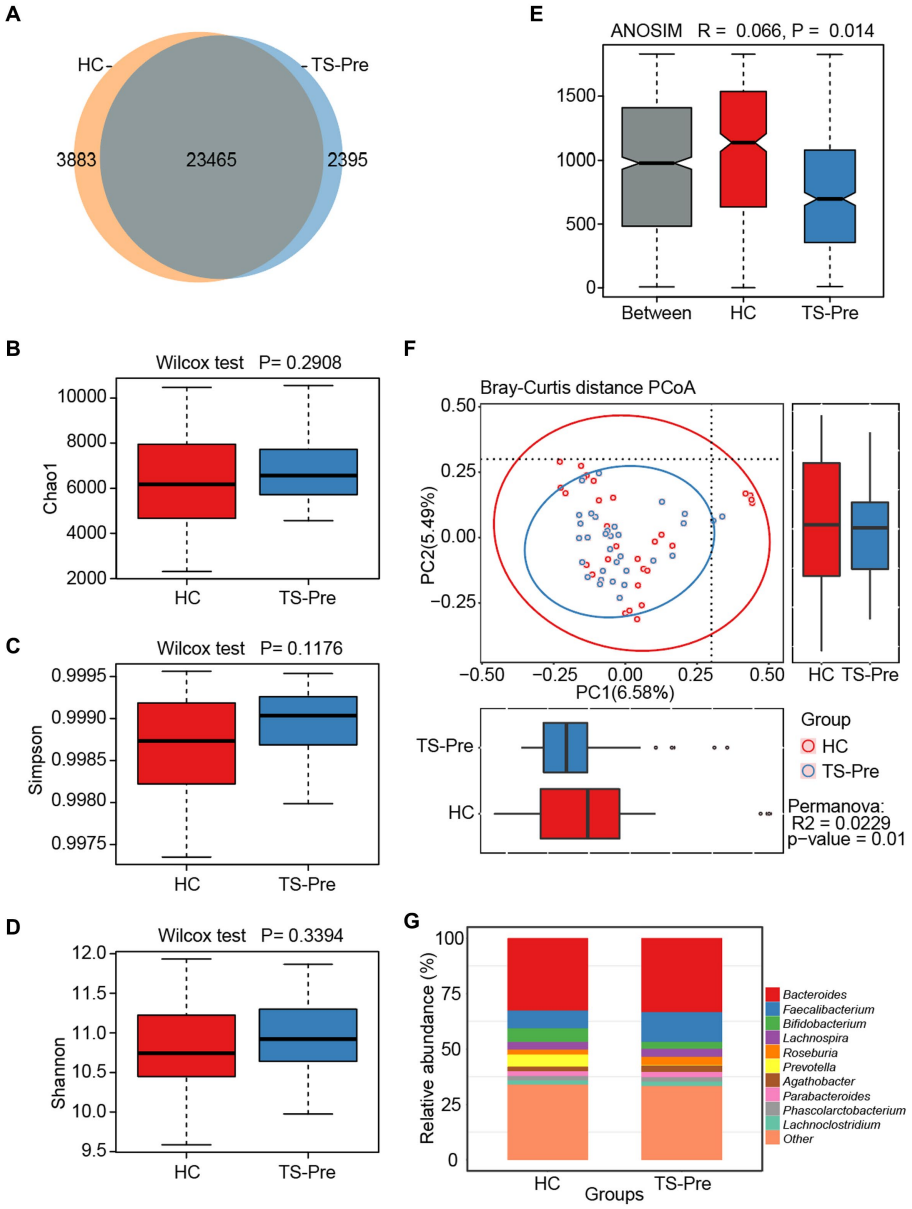

FIGURE 1
Diversity and composition of gut microbiota in healthy controls (HC, n = 29) and treatment-naive children with TS (TS-Pre, n = 32). **(A)** 23,465 OTUs were shared between groups. The HC group had the unique OTUs with 3,883, while the TS-Pre group had 2,395. Chao1 **(B)**, Simpson **(C)**, and Shannon **(D)** were used to analyze the alpha diversity between the HC group and the TS-Pre group. **(E)** Analysis of similarity (ANOSIM) presented significant differences between groups ($p < 0.05$). **(F)** The beta diversity between both groups was analyzed by principal coordinate analysis (PCoA) of the weighted UniFrac distance. **(G)** The relative abundance histograms of all genera in the HC and TS-Pre groups. The top 10 shared genera with high relative abundance were displayed by different colors and the remaining genera with less relative abundance were classified as other.

Marker genera in children with TS

Due to the limitation of 16S rDNA amplicon pyrosequencing, we mainly focused on the downstream analysis at the genus level. The Wilcoxon rank sum test (LEfSe) ($p < 0.05$, LDA > 2) was used to identify the marker genera for different groups, and the TS-Pre group had a greater number of marker bacteria than the HC group (Figure 2). *Faecalibacterium, Hungatella, Oscillibacter, Flavonifractor, Fusicatenibacter, Anaerostipes, Anaerotruncus,* and *Eisenbergiella* were the marker genera for the TS-Pre group, whereas only Clostridia_UCG_014 was the marker genus for HC group (Figure 2B). In addition, we also acquired statistical significances of the differential genera, and the p values of *Faecalibacterium, Oscillibacter, Flavonifractor, Fusicatenibacter, Anaerostipes,* and *Clostridia_UCG_014* were 0.0004, 0.0029, 0.0052, 0.0097, 0.0156, and 0.0374, respectively (Figure 2C).

Alterations of potential metabolic pathways in children with TS

To explore potential functional alterations associated with the gut microbial changes in patients with TS, KEGG, CAZY, METACYC,

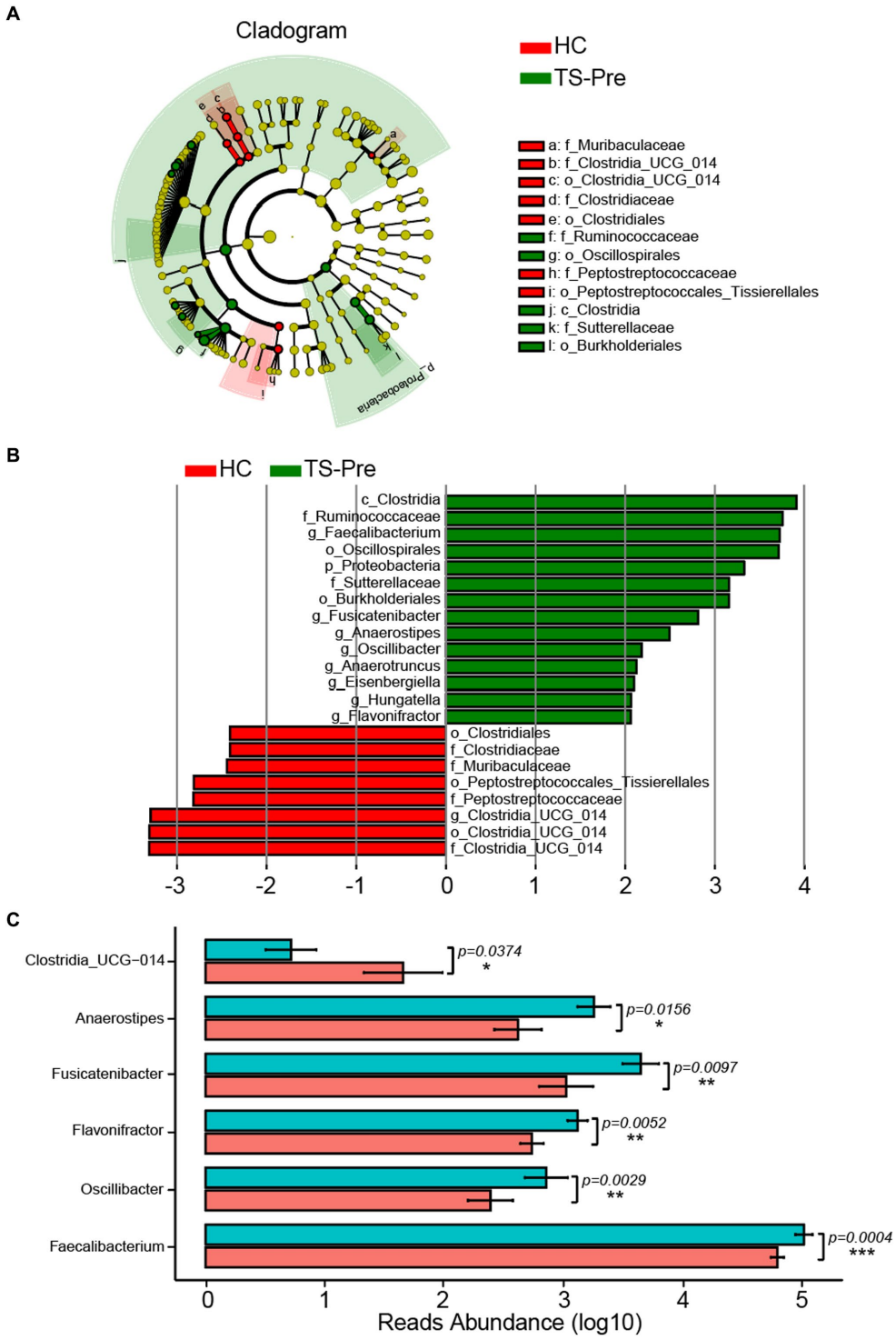

FIGURE 2
Marker Genera of gut microbiota between the HC group and the TS-Pre group. **(A)** The phylogenetic tree displayed the marker taxa according to subordinate relationship from phylum to species levels for these two groups. **(B)** Key altered phylotypes between both groups at different levels. **(C)** Significant differences in genera between the HC and the TS-Pre groups.

GMM, and GBM module enrichment analysis were predicted by Picrust2 between the HC group and the TS-Pre group. The top 10 significant differences in KEGG, CAZY, METACYC, GMM, and GBM module enrichments between groups were shown in Figure 3. Noteworthy, as shown in Figure 3E, multiple GBM neurotransmitter modules related to neurodevelopmental disorders were abnormal in the TS-Pre group, such as histamine degradation, dopamine degradation, and DOPAC synthesis.

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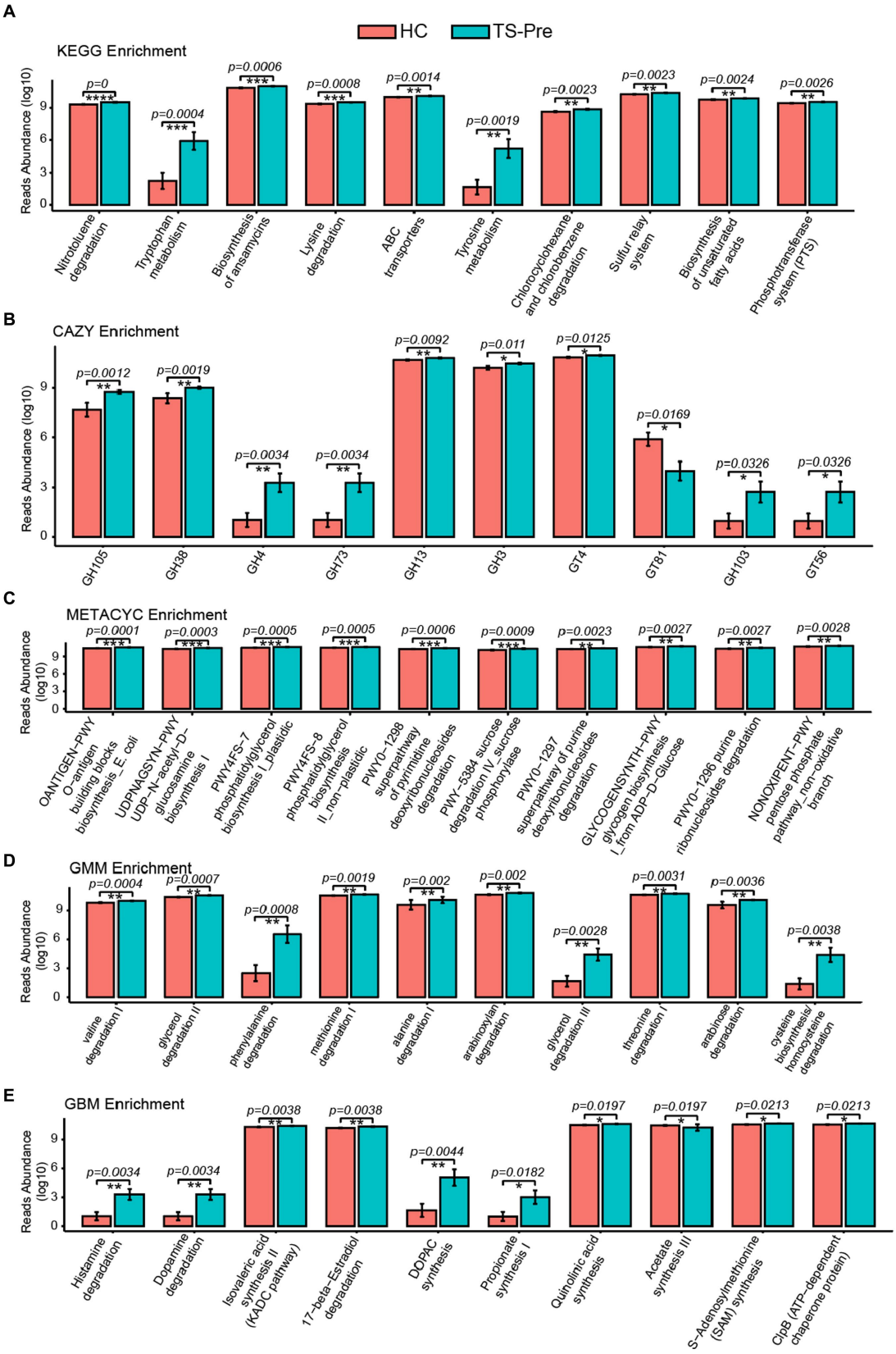

FIGURE 3
KEGG, CAZY, METACYC, GMM, and GBM were enriched by Picrust2 functional predictive analysis in the HC group and the TS-Pre group. The top 10 differential pathways in KEGG **(A)**, CAZY **(B)**, METACYC **(C)**, GMM **(D)**, and GBM **(E)** between both groups, respectively. Statistical analysis was performed by Univar Test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

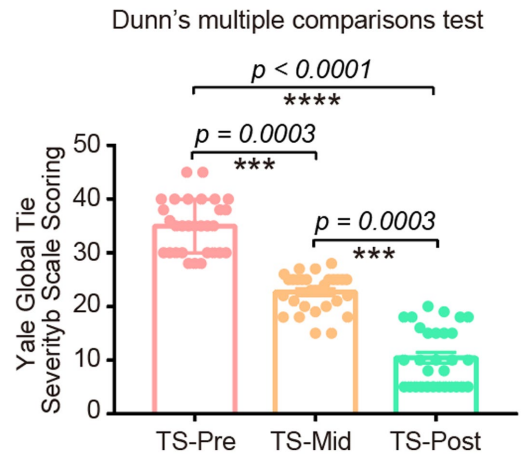

FIGURE 4
The YGTSS scores in TS patients before, after one course of, and after two courses of this combinational physiotherapy consisting of cranial electrotherapy stimulation plus electrodermal biofeedback training. These scores were significantly decreased after this treatment.

Significant improvement was found in children with TS after the combinational physiotherapy

Among 32 children with TS, 2 were treatment-naïve, and 30 were treated with this combinational physiotherapy. As the YGTSS score is an important indicator to evaluate patients' condition, we recorded the YGTSS scores for every patient before, after the first 10-day course, and after two 10-day courses of this treatment. Compared with patients before the combinational physiotherapy, patients after this treatment presented much lower YGTSS scores (Figure 4). These patients' conditions improved dramatically, including the alleviation of motor tics and vocal tics, which was confirmed by both their guardians and primary physicians. During the treatment process and follow-up period, no distinct adverse reactions were observed in any of the children.

Gut microbial composition in patient after the combinational physiotherapy as different from that in patients without any treatment

Due to the bidirectional communication between the gut and the brain, we wanted to explore the diversity and composition of gut microbiota in children with TS when their clinical symptoms were dramatically alleviated. 16S rDNA amplicon pyrosequencing was performed on fecal specimens, and no significant differences were observed in gut microbial diversity between patients before and after the combinational physiotherapy (Supplementary Figure S2). *Bacteroides, Faecalibacterium,* and *Roseburia* were the major components in the TS-Pre group, whereas *Bacteroides, Faecalibacterium,* and *Parabacteroides* in the TS-Post group (Figure 5A and Supplementary Figure S3). Five differential genera were identified between both groups by Kruskal–Wallis test and the physiotherapy-treated children had lower abundances of *Agathobacter, Dorea, Anaerostipes, Butyricicoccus,* and *Bifidobacterium* (Figure 5B).

In addition, we also explored the marker bacteria in both groups via LEfSe ($p < 0.05$, LDA > 2). At the genus level, *Agathobacter, Dorea, Anaerostipes, Butyricicoccus,* and *Bifidobacterium* were the marker genera in the TS-Pre group, whereas no marker genus was found in the TS-Post group, partly due to the relatively small sample size with 17 (Figures 5C,D).

Potential metabolic pathways in patient after this combinational physiotherapy was different from that in patients before this treatment

To explore the potential metabolic pathways associated with the gut microbial alterations in patients after this combinational physiotherapy, KEGG, CAZY, METACYC, GMM, and GBM modules enrichment analysis were also predicted by Picrust2 between patients before and after this treatment. One KEGG pathway was found between the TS-Pre group and the TS-Post group, which was Proteasome ($p = 0.0097$) (Figure 6A). Meanwhile, five, ten, and two different modules were observed in CAZY, METACYC, and GMM modules enrichment analysis, respectively (Figures 6B–D). Noteworthy, as shown in Figure 6E, three different GBM neurotransmitter modules between both groups were identified, which were Histamine degradation, Dopamine degradation, and DOPAC synthesis.

Discussion

This preliminary study not only aimed to depict the gut microbial profiles of children with TS, but also aimed to explore the potential clinical effects of this combinational physiotherapy and its influences on the gut microbiome in children with TS. Our results indicated that the gut microbial composition in patients was different from that in healthy controls, with much higher abundances of the genera *Faecalibacterium, Hungatella, Oscillibacter, Flavonifractor, Fusicatenibacter, Anaerostipes, Anaerotruncus,* and *Eisenbergiella*. Moreover, we also found that this treatment could significantly diminish tic activity in patients, whose potential positive effects were first reported in TS. Lastly, different gut microbial composition was also observed between TS patients before and after the combinational physiotherapy, with lower abundances of the genera *Agathobacter, Dorea, Anaerostipes, Butyricicoccus,* and *Bifidobacterium* in patients after this treatment, partly due to the bidirectional communication of the gut-brain axis (39, 40). However, our results should be treated with caution due to the rudimentariness of this study.

Flavonifractor degrades flavonoid quercetin (a flavonoid with antioxidant and anti-inflammatory properties), increasing oxidative stress and inflammation (41). Interestingly, our study first reported the enrichment of *Flavonifractor* in patients with TS, which was consistent with the existing assumption that pro-inflammatory pathogenesis existed in the occurrence and progression of TS (7). Last year, Eicher and Mohajeri reported that patients with seven brain-related diseases (attention deficit hyperactivity disorder, autism spectrum disorder, schizophrenia, Alzheimer's disease, Parkinson's disease, major depressive

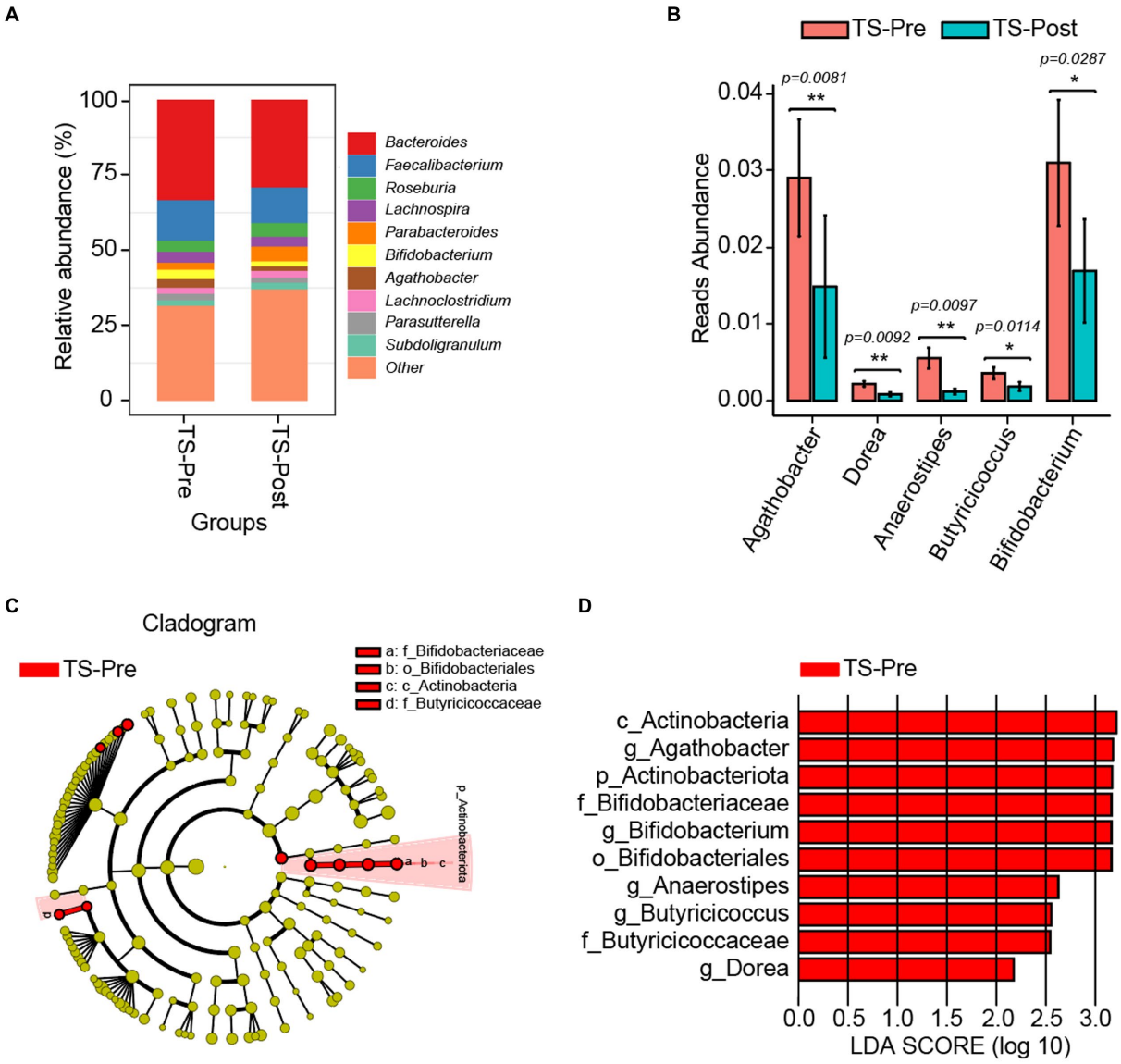

FIGURE 5
Gut microbial composition in TS patients after two courses of this combinational physiotherapy (TS-Post, n = 17) was different from that in patients before this treatment (TS-Pre, n = 32). **(A)** The relative abundance histograms of all genera in the TS-Pre and the TS-Post groups. Different colors represented the top 10 shared genera, and "other" represented the remaining genera with less relative abundance. **(B)** Significant differences in genera between the TS-Pre group and the TS-Post group. **(C)** The phylogenetic tree displayed the marker taxa according to subordinate relationship from phylum to species levels for both groups. **(D)** Key altered phylotypes between both groups in generic levels. *$p < 0.05$, **$p < 0.01$.

disorder, and bipolar disorder) presented higher abundances of pro-inflammatory bacteria (*Alistipes, Eggerthella, Flavonifractor*) in comparison with healthy controls (42). However, the abundance of *Flavonifractor* did not show a significant decrease between patients before and after the combinational physiotherapy in our study. Partly because it is less rigorous to conclude that *Flavonifractor* induced oxidative stress and inflammation in the host, and further researches about the direct associations between *Flavonifractor* and oxidative stress are essential. In addition, although several marker genera were identified in the TS-Pre group, no marker genera were found in the TS-Post group, partly due to the relatively small sample size. Moreover, the characteristic bacteria have not been established because of the short therapeutic period of 20 days. However, no researchers have reported the gut microbial alterations related to this treatment in patients with TS. Thus, these results should be treated cautiously and needed to be verified by large and multi-centric samples.

It is interesting to explore the functional changes associated with gut microbial alterations between healthy controls and patients with TS. In this study, multiple GBM neurotransmitter modules related to neurodevelopmental disorders were significantly increased in patients with TS, including Histamine degradation, Dopamine degradation, and DOPAC synthesis. Until now, several neurotransmitters produced by gut bacteria were associated with behavioral states and involved in the pathophysiology of TDs, such as dopamine and histamine (43, 44). Moreover, the abnormal dopamine pathway was studied well in TDs, and dopamine receptor antagonist (DRA) was used to suppress tics (45, 46). Published studies also have reported that DRA could alter the diversity and composition of gut microbiota in children with or without TDs

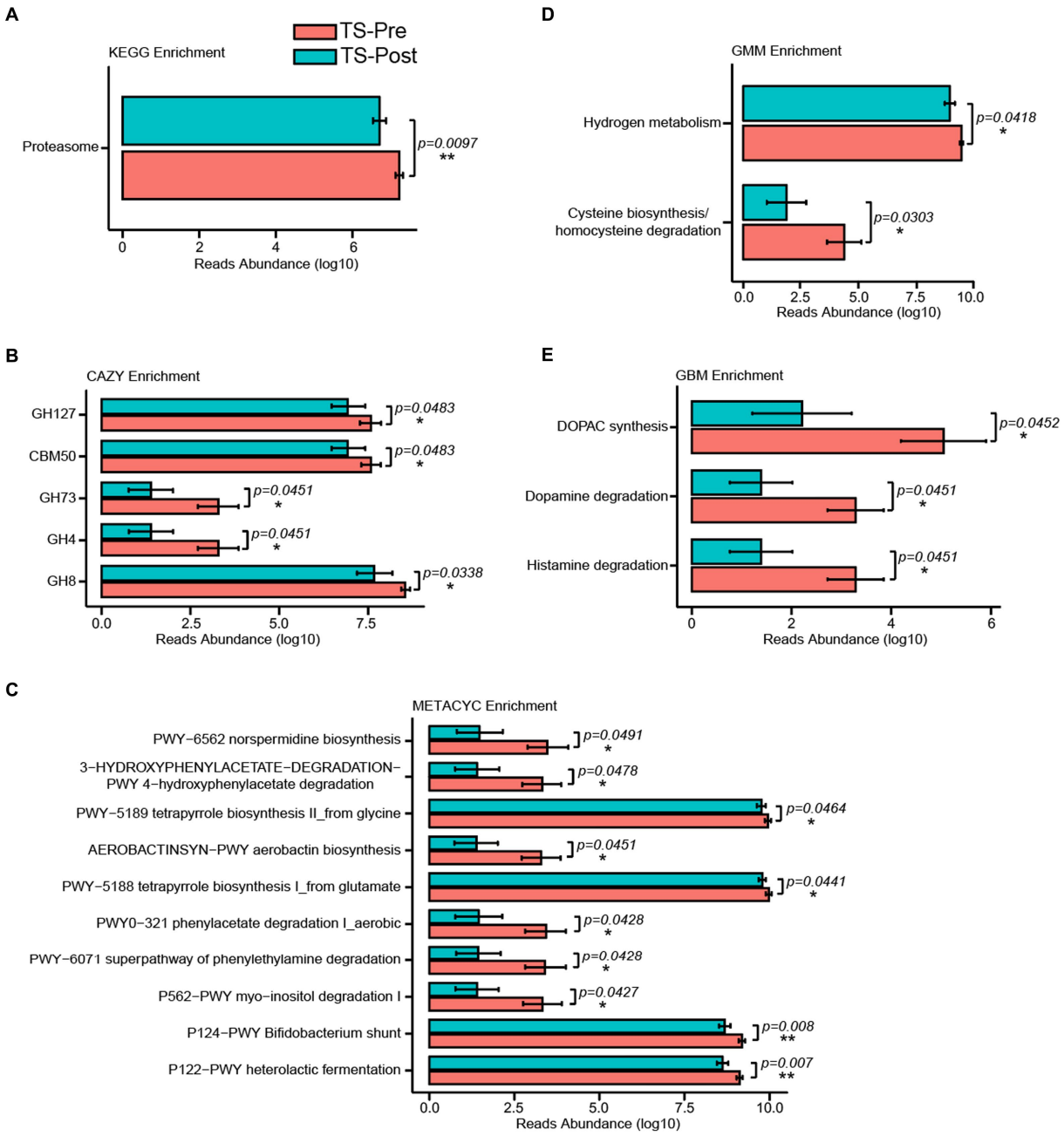

FIGURE 6
KEGG, CAZY, METACYC, GMM, and GBM were enriched by Picrust2 functional predictive analysis in the TS-Pre group and the TS-Post group. The KEGG **(A)**, CAZY **(B)**, METACYC **(C)**, GMM **(D)**, and GBM **(E)** pathways presented 1, 5, 10, 2, and 3 significant differences between both groups, respectively. Statistical analysis was performed by Univar Test. *$p < 0.05$, **$p < 0.01$.

(7, 47). Expectedly, the metabolic pathways of DOPAC synthesis, dopamine degradation, and histamine degradation were all decreased in patients after the combinational physiotherapy compared with patients before the treatment in this study, indicating that improved clinical symptoms associated with emotion might be accompanied by an improvement of the intestinal microenvironment. Signals from the brain could affect sensory, motor and secretory modalities of the gastrointestinal tract, and many studies have identified the influence of stress on gut microbiota, including physical and psychological stressors (48). A decreased abundance of *Turicibacter* spp. and an increased abundance of *Ruminococcus gnavus* were observed by exercise-induced stress in mouse cecum, and both of them play important roles in enteral mucus degradation and immune function (49). Exposure to a prolonged restraint stressor gave rise to an overgrowth of facultatively anaerobic microbiota, and a decreased diversity and abundance of cecal microbiota in mice, which could be partly explained by intestinal inflammation related to the bacterial abundance variation in the family Porphyromonadaceae (50). Exposure to a social stressor (i.e., social disruption) also could significantly change gut microbial compositions, and three changed bacterial genera (i.e., Coprococcus, Pseudobutyrivibrio, and Dorea) were dramatically positively correlated with the circulating levels of IL-6 and MCP-1 (51). Interestingly, in

comparison with patients before the combinational physiotherapy, patients after this treatment also presented a significant reduction in the abundance of genera Dorea, indicating potential decreases in circulating cytokine.

Except for overcoming the side effects of medication and the long treatment period for psychological treatment, our combinational physiotherapy also costs less than other noninvasive techniques (e.g., transcranial magnetic stimulation) (10, 11). In this study, we did observe a significant reduction in tics according to children's YGTSS scores and guardians' positive reports. As far as we know, this is the first study to combine CES with electrodermal biofeedback training to treat TS and acquire a positive effect. In the aspect of neurophysiology, the intervention of CES could improve neural dysfunction and relieve tic symptoms for TS patients by regulating their brain activity. In comparison with TS adolescents before CES, adolescents after CES presented a suppression in the functional activity and connectivity of motor pathways and an increase in the control portions within the cortico-striato-thalamo-cortical (CSTC) circuit, indicating the recovery of TS might benefit from the normalization of intrinsic neural circuits (17). In addition, both the activity of the medial orbitofrontal cortex and the amplitude of slow cortical potentials can be regulated by electrodermal biofeedback (52, 53). Since the dysfunctions of the orbitofrontal cortex and the low preparatory motor potentials were observed in TS (54–57), it was reasonable to infer that the electrodermal biofeedback could affect neural circuits related to the motor tics by regulating cortical excitability. However, Nagai et al. found that electrodermal biofeedback could briefly decrease TS patients' sympathetic activity, but not reduce their tics (53). Partly because of the high dropout rate occurring in their active-biofeedback group (53). On the other hand, in our study, a 60-min CES treatment was followed by a 30-min duration of electrodermal biofeedback training twice a day lasting for 20 days, whose training approaches and frequencies were different from the above study only including a 30-min duration of the biofeedback session once a week. However, the functional brain imaging data was not included in this study due to a lack of funds, and we have applied for another funding to perform a multicenter study with more participants and more comprehensive measures.

Several limitations should be noted in this study. Firstly, it is difficult to generalize our findings to popularity due to the relatively small sample size with homogeneous children. Ideally, data acquired from larger and multi-centric training samples are required to verify our findings. Second, the gut microbial composition was affected by fecal consistency (58), which was ignored by us in this study. However, according to our knowledge, all participants defecated smoothly and had no diarrhea during the study. Thirdly, CES treatment is regarded as a safe neuro-medical treatment for TS, which is mostly based on studies focused on anxiety, depression, or insomnia (59). Finally, functional brain imaging of patients before and after this combinational physiotherapy was not recorded, which might contribute to revealing functional activity and connectivity among different brain areas in TS (60–62). Therefore, we recommend that readers interpreted our findings with caution due to these above limitations.

Conclusion

In short, children with TS showed a cognizable gut microbial profile, and certain enriched bacteria (e.g., Flavonifractor) with pro-inflammatory potentials might induce neuroinflammatory responses. CES plus electrodermal biofeedback training could significantly diminish tic symptoms, and different gut microbial compositions were also observed between TS patients before and after this combinational physiotherapy, indicating the existence of bidirectional communication of the gut-brain axis in TS. But studies on the gut microbial characteristics in TS patients, the influences of gut microbiota on tic severity, the efficacy and safety of this treatment, and the bidirectional regulatory mechanism between brain signals and gut microbiota in TS still need to be explored.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: NCBI BioProject database PRJNA909064.

Ethics statement

The studies involving humans were approved by the Ethics Committee of the Xiangyang No. 1 People's Hospital, Hubei University of Medicine (Xiangyang, China). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

HJ and CB designed, funded, and supervised the study, revised this manuscript, approved the final version of the manuscript on behalf of MeW, HP, MiW, ZL, and YX. CB, MeW, and HP recruited eligible subjects, collected their samples, and conducted YGTSS scores. MiW, ZL, YX, and HJ worked on the analysis and interpretation of data. HJ drafted the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

MiW, ZL, YX, and HJ were employed by Shanghai Biotecan Pharmaceuticals Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1184311/full#supplementary-material

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The double burden of malnutrition and environmental enteric dysfunction as potential factors affecting gut-derived melatonin in children under adverse environments

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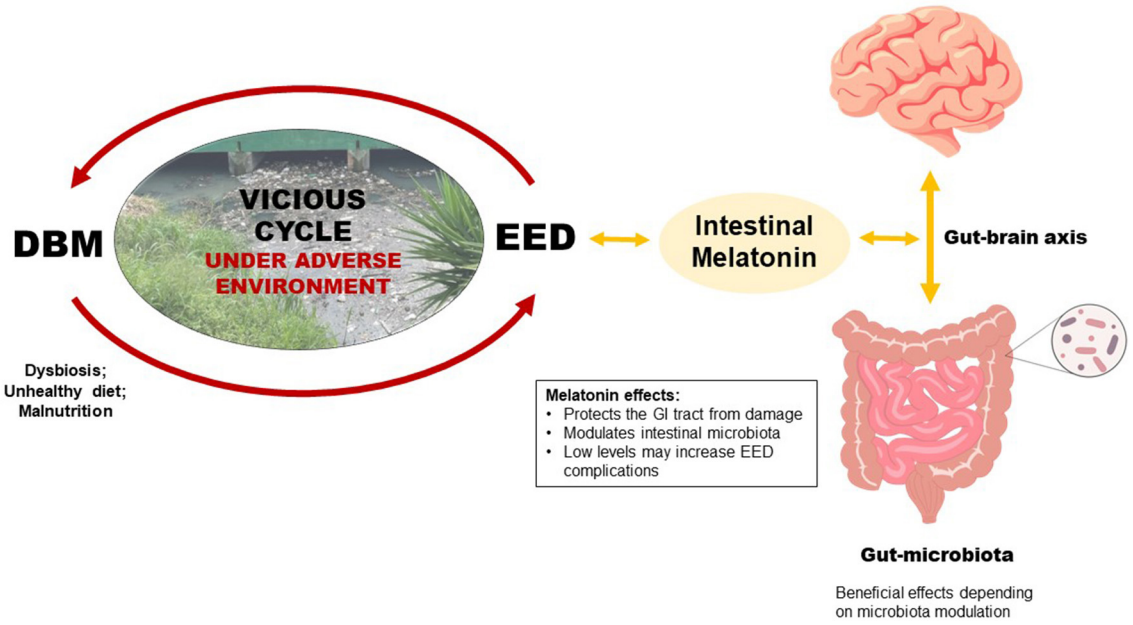
Introduction

Proper nutrition is a cross-cutting component for ensuring a population's health and economic and social development. Due to accelerated nutritional, epidemiological, and demographic transition in certain regions of the developing world, especially in overpopulated urban areas, a double burden of malnutrition (DBM) has been a growing health concern, which may lead to important metabolic disorders during the lifespan with costly health care (1).

The double burden of malnutrition in children is increasing in developing countries and may occur in settings of poverty and inadequate sanitation (2). DBM is defined as a simultaneous occurrence of overweight/obesity and undernutrition afflicting countries at an individual or societal level, frequently associated with micronutrient deficiency (1). DBM may affect children living in developing countries in poverty conditions when a low-density nutrition intake is shifted to a high-caloric and high-fat Westernized diet, increasing the risk for non-communicable chronic diseases (2).

The COVID-19 pandemic may have fueled the prevalence of DBM in emerging economic countries, such as Brazil and India, which may lead to unprecedented and escalating increases in obesity rates (3). Under adverse environments and unhealthy diets, DBM may coexist with and favor environmental enteric dysfunction (EED), with underlying chronic intestinal inflammation and intestinal microbiota imbalances (4, 5).

Children from low-income families are often exposed to poor hygiene, unsanitary conditions, and difficult access to health care (6). When poor environmental conditions collide with continuous unhealthy and unsafe diets, such a combination may substantially increase the risk of a vicious cycle of enteric infections and malnutrition in children, disturbing their developmental trajectories (7). DBM and EED may be a cause and consequence of this vicious cycle, and if persistent, can lead to intestinal microbiota disturbances allowing more pathogenic microbial communities to thrive, with impaired intestinal barrier function and disrupted immune activation, with mucosal and systemic inflammatory effects (8).



GRAPHICAL ABSTRACT
Poor environmental conditions combined with continuous unhealthy and unsafe diets may substantially increase the risk of a vicious cycle of enteric infections (EED—environmental enteric dysfunction) and malnutrition (DBM—double burden of malnutrition) in children. Gut melatonin, mainly produced by the intestinal microbiota, can modulate the composition, variety, and dynamics of the microbiota itself and may affect and be affected by intestinal microbiota alterations due to DBM and EED.

Melatonin is a critical pineal gland-derived hormone regulating the circadian rhythm; nonetheless, it has been associated with immunoinflammatory functions in different body systems (9). Melatonin is also significantly produced by the gastrointestinal (GI) tract, which harbors highly expressed melatonin receptors, and regulates the intestinal barrier function (10). This opinion paper brings to attention that DBM compounded with the EED in growing children under adverse environments may negatively influence the intestinal microbiota homeostasis and hence the GI tract-related melatonin function.

Gut derived-melatonin

Melatonin, N-acetyl-5-methoxy tryptamine, is a tryptophan-derived hormone synthesized mainly by the pineal gland but also by the retina, platelets, skin, and intestinal mucosa (9, 11). In the GI tract, melatonin is produced by enteroendocrine, endothelial, natural killer cells, and intestinal bacteria (10). In Wistar rats, gut melatonin levels are markedly high, reaching about 4–100 ng/g of wet organ weight (12). Intestinal melatonin is produced even during daylight hours when its synthesis by the pineal gland is low. Notably, animals lacking the pineal gland show stable amounts of melatonin in the GI tract (13).

Melatonin receptors (MT) are widely distributed at various sites within the Gl tract, including the intestinal mucosa. MT1 and MT2 receptors are found in blood vessels, epithelium, submucosa, and myenteric plexus. In the large intestine, MT1 and MT2 are more expressed in the epithelium (14). In addition, the enzymes necessary for melatonin synthesis are highly expressed in the GI tract (15). When intestinal inflammation prevails, changes in gene expression can lead to lower amounts of melatonin (14).

The rat intestinal mucosa undergoes morphological changes, with increased inflammatory responses, when endogenous melatonin suppression occurs following acute inhibition of MT1 and MT2 receptors by luzindole (16). Conversely, an association between bacteria that produce short-chain fatty acids (*Alistipes sp* and *Blautia sp*) with increased expression of melatonin has been found in the colon (17). Melatonin supplementation seems to have a protective action on the intestinal mucosa, improving pathogenic microbial composition in the colon, helping to prevent or treat intestinal infections (18).

Several factors, including diet and intestinal microbiota, influence intestinal melatonin levels. In absolute values, the amount of gut melatonin is 400 times higher than the pineal gland. Reductions in endogenous melatonin affect the intestinal microbiota and intriguingly trigger Alzheimer's disease-like phenotypes, including hippocampal Iba-1 activation, Aβ protein deposition, with impaired spatial memory ability in mice (11). Another source of intestinal melatonin is the intestinal microbiota, which can also induce colonic melatonin receptor expression by a mechanism of action involving short-chain fatty acids (17). The exogenous use of melatonin causes changes in the intestinal microbiota, which help the melatonergic system's function with increased intestinal epithelial regeneration (19).

Melatonin exogenous administration improves already installed intestinal damage, such as mucosal disruption and neutrophil infiltration, favoring antioxidative processes, reducing

the generation of oxygen free radicals, and protecting the integrity of intestinal mucosal cells (20). Melatonin supplementation influences appetite, improving satiety and affecting plasma leptin levels, which are higher in supplemented individuals (21), suggesting a role for melatonin in regulating food intake.

Melatonin protects the intestinal barrier function, mainly due to its anti-inflammatory and antioxidant actions, and increases the abundance of bacterial populations (22). The gut microbiota is important in modulating the metabolism of tryptophan, an essential amino acid precursor to melatonin. Tryptophan metabolization pathways also exist in some members of the human intestinal microbiota, such as *Clostridium sporogenes* and *Ruminococcus gnavus,* which can decarboxylate tryptophan into the neurotransmitter tryptamine in the large intestine (23).

Gut-derived melatonin may be affected by intestinal microbiota alterations due to the double burden of malnutrition and EED

Some factors can interfere with the gut microbiome, such as diet, genetics, age, gender, lifestyle, infections, diseases, and exposure to maternal and environmental microbiota (24). Genetics can explain changes in this microbiota by up to 12%. The dietary pattern modifies the microbiota's composition, changing the proportion between the phyla and the variety of microorganisms and explaining this variation by up to 57% (25). The gut microbiota is important in the gut-brain axis as it regulates the secretion of brain hormones, such as brain-gut peptides from intestinal endocrine cells, and bacterial compounds can cross the blood-brain barrier, regulating brain functions (26). We do not know whether altered microbiota and endogenous intestinal melatonin crosstalk to affect brain functions in children. This is an important gap in knowledge that should be addressed by innovative research.

Microbiota imbalance toward reductions in commensal bacteria, with alterations in the composition and quantity of intestinal microorganisms, is a key factor affecting gut nutrient bioavailability (24). Intestinal microbiota dysregulation facilitates and is facilitated by the luminal-to-blood translocation of pathogenic bacteria, with adverse effects on the intestinal epithelial barrier homeostasis, compromising its modulation by commensal bacteria (27).

A dietary pattern characterized by a high-fat content induces lipogenesis and causes intestinal microbiota imbalance. Oral melatonin supplementation in mice challenged with high fat intake leads to a greater diversity of the intestinal microbiome, characterized by a relative abundance of *Bacteroides*, *Alistipes*, and *Parasutterella* and reduced numbers of *Lactobacilli*. Notably, melatonin effects on the intestinal microbiota were reversed in animals treated with antibiotics (28). Melatonin supplementation alters the intestinal microbiota constitution, reduces the Firmicutes against Bacteroidetes, increases *Akkermansia*, and adjusts the abundance of *Alistipes*, *Anaerotruncus*, and *Desulfovibrionaceae* to previous levels, with beneficial effects against obesity, insulin resistance, hepatic steatosis, and low-grade inflammation (29). The impact of antibiotics use and melatonin supplementation (4 mg/kg in drinking water for 2 weeks) on high-fat diet-induced intestinal inflammation and gut dysbiosis has been investigated in rats. The findings reveal that even a brief exposure to a high-fat diet leads to a state of hepato-intestinal inflammation and shifts in bacterial populations that can be exacerbated through antibiotic administration but ameliorated by melatonin supplementation (30). Melatonin signaling may be a communication link between the intestine and the central nervous system, as it modulates the circadian rhythm, intestinal microbial metabolism, and intestinal immune system, activating the release of cytokines (10).

Children afflicted with EED often live in poor settings of the developing world, especially in tropical areas with relatively yearly constant daylight, thus affecting circulating melatonin levels (31). Lifestyle habits, high-caloric Western diets, and other factors influence melatonin synthesis and intestinal inflammation (28). High-stress levels can impact the pineal production and release of melatonin. The characteristics of ambient light also affect this production and directly impact physiological and immune functions (32).

Data on melatonin levels and intestinal barrier function biomarkers are still scarce in the literature, and such a paucity of studies with EED experimental models hamper findings from being applied in clinical settings. In addition to its antioxidant function, melatonin may contribute to increasing mucosal blood flow, strengthening the GI and immune system, controlling fecal moisture, reducing intestinal peristalsis, prolonging intestinal transit time, and protecting the GI tract from damage caused by digestive enzymes and hydrochloric acid, altering intestinal secretions (22). This favors epithelial regeneration and increases local microcirculation, promoting lower intestinal permeability.

A gut microbial community with a reduced relative abundance of *Bacteroides* and increased *Lactobacillus* and *Firmicutes* was found to be associated with marked intestinal permeability and systemic and local inflammation in an endogenous melatonin reduction mouse model (33). In addition, there was less resistance to stress when subjected to high-fat consumption, influencing weight gain and the development of hepatic steatosis. Fecal microbiota transplantation improves systemic inflammation and intestinal permeability by modulating the gut microbiota.

A healthy intestinal microbiota and reduced circulating LPS/endotoxemia would facilitate melatonin-protective antioxidant functions and improve chronic inflammation (24). Of note, maternal melatonin supplementation had a significant effect on the intestinal microbiota and decreased inflammatory mediators in the offspring following LPS injection (34). Accumulating evidence supports endogenous melatonin's influence on the intestinal microbiome, homeostasis, and stress resistance (33), suggesting that its reduction is a risk factor for EED complications.

The gut microbiome can directly influence children's growth. In a model of chronic malnutrition induced by diet, without intestinal inflammation, the mouse microbiota enriched by *Lactiplantibacillus plantarum* (strain LpWJL) provided greater growth and metabolic and hormonal alterations, with higher levels of IGF-1 and insulin. This bacterium promotes the signaling of NOD2, an innate immunological receptor in the crypts that is

inhibited due to malnutrition, with improvements in intestinal cell proliferation and nutritional absorption, increasing mouse growth (35). Melatonin found in the breastmilk can influence the composition, variety, and dynamics of the intestinal microbiota over time, as well as modulating absorption of molecules by the intestinal epithelia (36). This effect may regulate the intestinal microbiota and influence the short and long-term malnutrition states.

Intestinal pathogenic microbial populations may impair the beneficial effects of melatonin. Melatonin supplementation to mice challenged by a colitis model led to increased intestinal inflammation and permeability with augmented tissue levels of TNF and circulating mononuclear cells and neutrophils. The pro-inflammatory effect of melatonin was associated with reduced *Bacteroidetes* and abundance in the *Actinobacteria* and *Verrucomicrobia* phyla, and when the dysbiosis was corrected, this effect was not observed (37).

As far as we know the scientific literature on melatonin and EED/DBM is still missing, therefore it is difficult to distinct the underlying effects and mechanisms of melatonin's efficacy in such conditions (EED/DBM) comparing to other well-recognized gastrointestinal diseases. Up to date, beneficial effects of melatonin supplementation has been found in animal models of obesity and metabolic syndrome (38), intestinal bowel disease (39) and irritable bowel syndrome (40), mostly by antioxidant, anti-inflammatory and regulatory intestinal microbiota's effects. We expect that some of the underlying mechanisms of melatonin's protective mechanisms on these conditions also happen to EED/DBM.

One gap of knowledge is that most of the melatonin studies come from experimental models and more clinical studies are needed to address the effects of melatonin on the double burden of malnutrition, especially in children under adverse environments.

Conclusion

This opinion article raises awareness that GI-tract-related melatonin function may be altered by DBM and EED (both conditions may interfere with intestinal microbiota), negatively affecting children living in adverse environments. More studies are needed to assess further the gut microbiome's modulatory effects under DBM and EED, and their crosstalk with melatonin function. Improvements in this knowledge may favor breakthrough nutritional interventions to ameliorate nutrient deficiency and healthier intestinal microbiota to halt short and late-onset overweight/obesity and its long-term risks. Further research is warranted to address whether melatonin supplementation can help to improve pathogenic gut microbiota and intestinal inflammation in experimental models of DBM and EED, possibly guiding future clinical studies in pediatric populations.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Probiotics, prebiotics, and synbiotics for patients with autism spectrum disorder: a meta-analysis and umbrella review

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Background and objective: The potential impact of gut health on general physical and mental well-being, particularly in relation to brain function, has led to a growing interest in the potential health advantages of prebiotics, probiotics, and synbiotics for the management of ASD. A comprehensive meta-analysis and systematic review was conducted in order to evaluate the effectiveness and protection of many drugs targeted at manipulating the microbiota in the treatment of ASD.

Methods: The present study employed a comprehensive examination of various electronic databases yielded a total of 3,393 records that were deemed possibly pertinent to the study. RCTs encompassed a total of 720 individuals between the ages of 2 and 17, as well as 112 adults and participants ranging from 5 to 55 years old, all of whom had received a diagnosis of ASD.

Results: Overall, 10 studies reported Autism-Related Behavioral Symptoms (ARBS). Regarding the enhancement of autism-related behavioral symptoms, there wasn't a statistically significant difference between the intervention groups (combined standardized mean difference = −0.07, 95% confidence interval: −0.39 to 0.24, $Z = 0.46$, $p = 0.65$). We observed that in the patients with ASD treated with probiotic frontopolar's power decreased significantly from baseline to endpoints in beta band (Baseline: 13.09 ± 3.46, vs. endpoint: 10.75 ± 2.42, $p = 0.043$, respectively) and gamma band (Baseline: 5.80 ± 2.42, vs. endpoint: 4.63 ± 1.39, $p = 0.033$, respectively). Among all tested biochemical measures, a significant negative correlation was found between frontopolar coherence in the gamma band and *TNF*-α ($r = -0.30$, $p = 0.04$).

Conclusion: The existing body of research provides a comprehensive analysis of the developing evidence that indicates the potential of probiotics, prebiotics, and synbiotics as therapeutic therapies for ASD. Our findings revealed that those there was no significant effect of such therapy on autism-related behavioral symptoms, it has significant effect on the brain connectivity through frontopolar power in beta and gamma bands mediated by chemicals and cytokines, such as *TNF*-α. The psychobiotics showed no serious side-effects.

KEYWORDS

autism spectrum disorder, probiotics, prebiotics, synbiotics, randomized controlled trials

Introduction

Autism Spectrum Condition (ASD), a developmental condition, significantly influences people's social interactions, behavior, and learning (1). While the diagnosis of this condition is possible at any age, its symptoms often become apparent within the first 2 years of life due to its inherent developmental characteristics. ASD has been seen to impact individuals from diverse ethnic, racial, and socioeconomic backgrounds. It is still unclear what causes autism spectrum disorder, most likely arising from a complex interplay of genetic and environmental influences (2–5). Parents and families have significant challenges when dealing with a kid who has been diagnosed with ASD since the disorder's profound and wide-ranging deficits give rise to many complexities in providing care (6). In the last three decades, there has been a notable increase in the condition's occurrence, leading to substantial research efforts to comprehend its biochemical and genetic markers (7). Nevertheless, there is a scarcity of research examining the intricate relationship between the symptoms of the condition and the dynamics within the family unit. While a considerable body of research has been dedicated to examining the difficulties encountered by these children, there has been minimal exploration of the particularities surrounding their caregiving contexts.

Unfortunately, there is no known remedy for ASD; however, various therapies have been devised and examined, primarily focusing on young children. The primary objective of these therapies is to mitigate symptoms, improve cognitive capabilities, strengthen daily life skills, and maximize social functioning among persons (8). The current body of knowledge about effective treatment approaches for individuals with ASD who are older children and adults is constrained. Although some study has been conducted on social skills groups for older children, the available data supporting their effectiveness still needs to be improved (9).

Treatment techniques with the potential to enhance outcomes throughout adulthood need to be evaluated, and this can only be done with further research. It is essential to provide services that support persons with ASD in their pursuit of education, vocational training, employment, housing, transportation, healthcare, daily functioning, and active participation in the community (10). The prompt identification and timely intervention of ASD in youngsters might provide substantial advantages, facilitating their ability to surmount several obstacles. Hence, it is essential for parents to proactively seek assistance from rehabilitation facilities upon detecting any signs of developmental delays or to meet with professionals in pediatric neurology and child and adolescent psychiatry. According to reference (11), the timely implementation of interventions may effectively minimize a significant proportion (ranging from 90 to 95%) of these concerns.

A range of therapeutic alternatives is accessible, including applied behavior analysis, social skills training, occupational therapy, physical therapy, sensory integration therapy, and the employment of assistive technologies (12). The treatments discussed in this context may be broadly classified into behavioral and communication techniques, dietary measures, medicine, and complementary and alternative therapies (13). Probiotics have garnered considerable interest within the field of nutrition. Live microorganisms provide several health advantages, a few of which will be further examined in subsequent sections of this article. In contrast, prebiotics, produced from indigestible carbohydrates, particularly fiber, function as a source of sustenance for the advantageous gut bacteria, specifically probiotics (14).

Moreover, a complete evaluation of the existing literature via an umbrella review reveals a scarcity of comprehensive meta-analyses investigating the simultaneous efficacy of probiotics, prebiotics, and synbiotics for patients diagnosed with ASD. Despite a few meta-analyses, the scope of these analyses is restricted due to the inclusion of only a small number of papers for pooled analyses (15–22). For this reason, it's crucial to expand the scope of the literature review to incorporate additional studies on the advantages of combining probiotics, prebiotics, and synbiotics for children with ASD. This study aims to collecting evidence on the efficacy of probiotic, prebiotic, and synbiotic therapy plans. It will aid in formulating well-informed guidelines and procedures for implementing these therapies within the framework of ASD care. The task at hand also necessitates investigating essential implementation details.

Methods

The standards for the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement were used to make this systematic review and meta-analysis (23).

Search strategy

We conducted a comprehensive search across widely recognized indexing databases, which included CNKI, PubMed/MEDLINE, Embase, Web of Sciences, Scopus, and the Cochran library. Our search strategy employed broad search terms encompassing various expressions including Search: ((((((autistic traits[Title/Abstract]) OR (Asperger disorder[Title/Abstract])) OR (Asperger syndrome[Title/Abstract])) OR (autistic disorder[Title/Abstract])) OR (autism[Title/Abstract])) OR (autism spectrum disorder[Title/Abstract])) AND ((((probiotics[Title/Abstract]) OR (prebiotics[Title/Abstract])) OR (synbiotics[Title/Abstract])) OR (psychobiotics[Title/Abstract])). This search covered the period from January 1, 1980, to August 15, 2023, with no language restrictions applied. Furthermore, we extended our search by screening the references of selected studies and pertinent review articles. This extra check was done to find relevant studies that did not come up in the primary database searches. To facilitate efficient organization and management of the retrieved references, we established a bibliographical database using EndNote X7. To ensure accuracy and consistency, two authors (FR and KD) independently assessed each paper for eligibility. Any discrepancies were resolved through consultation with third author (KT).

Study selection

Our study encompassed trials characterized by the following attributes:

Study Type: disciplinary trials involving the diagnosis of autism spectrum disorder were scrutinized exclusively, Asperger disorder, Asperger syndrome, or autistic disorder utilizing the widely accepted Randomized Controlled Trial (RCT) design.

Participants: our research was limited to individuals between the ages of 1–60 who were diagnosed with autism spectrum disorder (ASD), Asperger disorder, autistic disorder, or autism spectrum condition.

Intervention: we scrutinized interventions involving probiotics, prebiotics, and symbiotics alone or in conjunction with other nutritional supplements, contrasting against a placebo.

Outcomes: the outcome measures include primary outcome as Effects of Probiotics, prebiotics, and synbiotics on Autism-Related Behavioral Symptoms of Children with ASD. To assess Autism-Related Behavioral Symptoms, included studies mostly used the Aberrant Behavior Checklist. The Aberrant Behavior Checklist (ABC) (24, 25) consists of 58 questions asked of parents on a 0–3 scale, broken down as follows: (1) irritability (15 questions covering agitation, aggression, and self-injury); (2) social withdrawal; (3) stereotypes; (4) hyperactivity; and (5) improper speech (4 items) (26). The ABC is commonly used in ASD RCTs (27). The included studies' mean and standard deviation (SD) for the transformation in outcome measures from pre- to post-intervention for ASD-related conduct disorder (henceforth referred to as "change in score").

Secondary outcomes were biochemical and clinical parameters, as well as change in electroencephalogram (EEG). Neurological and psychiatric examinations were included in the clinical evaluation, in addition to a standardized assessment of gastrointestinal symptoms using the GSI (28); autism severity through ADOS-2 (29), Childhood Autism Rating Scale (CARS) (30), and Social Communication Questionnaire (SCQ) (31); limited and repetitive actions utilizing the Revised Repetitive Behavior Scale (RBSR) (32); screening for emotional, behavioral, and social issues with the Child Behavior Checklist (CBCL) (33); improvements in one's mental faculties as measured by means of the Griffiths Mental Development Scales-Extended Revised (GMDS-R) (34); improvement in adaptive skills as measured by the Vineland Adaptive Behavior Scales-II (35); language abilities can be assessed using the McArthur-Bates Communicative Development Inventories (CDI) (36).

Excluded from our analysis were trials meeting the following criteria:

- Studies lacking precise and distinct inclusion and exclusion criteria.
- Outcomes that needed to be explicitly defined or elucidated.
- Trials lacking a controlled study design.
- Pregnant or breastfeeding women participants.
- Preclinical investigations using experimental animals.

In instances where several papers presented identical or overlapping data, preference was given to articles with lengthier intervention durations or larger sample sizes, incorporating them into our study.

Gastrointestinal and autism-related symptoms

We used a 7-point Likert scale to collect information about GI symptoms by administering a customized form of the Gastrointestinal Symptom Rating Scale (GSRS) (37) in the five areas of tummy trouble (ache, reflux, indigestion, loose stools, and bowel obstruction). Using the Bristol Stool Form scale, we also collected Daily Stool Records (DSR) for a total of 14 days (1 = very hard, 7 = liquid). Parent Global Impressions-III (PGI-III), Childhood Autism Rating Scale (CARS), Aberrant Behavior Checklist (ABC), Social Responsiveness Scale (SRS), and Vineland Adaptive Behavior Scale II were all used to evaluate symptoms associated with autism, as they had been in the previous study (VABS-II). About 2 years after treatment ended, parents evaluated their child using the GSRS, DSR, PGI-III, ABC, SRS, and VABS-II, and the evaluation was conducted by the same professional evaluator who had previously conducted the CARS evaluation.

Data extraction

At the outset, a pair of researchers (referred to as FR and KD in this study) conducted an initial screening of the gathered literature. This sifting involved evaluating the abstracts and titles to determine which works met our predetermined criteria. Subsequently, these selected works underwent a thorough assessment by the same researchers. They individually reviewed the full-text articles and extracted a range of data points, encompassing fundamental participant characteristics, sample sizes, particulars of interventions, comparative measures, intervention durations, evaluations of behavioral symptoms associated with autism, scores of GI symptoms, and other relevant details.

Any disparities between the assessments conducted by these two researchers were resolved either by double check or discussion. Alternatively, a third reviewer (referred to as KT) was consulted.

Study quality assessment

Following the guidelines specified in the PRISMA statement, the evaluation of discrimination hazards in randomized controlled trials, also known as RCTs, and crossover trials involved a thorough assessment of seven crucial factors: (1) the generation of random sequences; (2) the concealment of allocation; (3) the blinding of participants and personnel; (4) the blinding of outcome assessment; (5) the handling of incomplete outcome data; (6) the elimination of chosen reporting; and (7) the identification of any additional potential sources of bias. Each of the bias-related characteristics was classified into one of three categories: low risk, uncertain risk, or high risk.

Umbrella review

We conducted an umbrella analysis by conducting systematic searches in databases such as MEDLINE, Embase, the Cochrane Central Register of Controlled Trials, and more than 30 other sources. This review followed the JBI systematic review methodology. The Grading of Recommendations, Assessment, Development, and Evaluation approach was used to assess the certainty of evidence. Covidence was used to carry out the selection process (Melbourne, Australia).

For eligibility determination, two independent reviewers evaluated titles and abstracts. The inclusion of studies was confirmed through a full-text review to ensure alignment with the selection

criteria. All screening decisions were meticulously documented and are outlined in this report, accompanied by a comprehensive list of studies that were excluded. Eligible studies underwent a thorough appraisal by one reviewer and were cross-verified by a second reviewer. The AMSTAR-2 was used to evaluate systematic reviews and meta-analyses (38).

Data analysis

The assessment of potential bias in randomized controlled trials (RCTs) and crossover studies was conducted using Review Manager 5.3. Review Manager 5.3 was used for conducting all meta-analyses and generating visual representations. Furthermore, the study used STATA/SE software (version 15.1) and the "Meta-Analysis" package. To assess the changes in scores based on behavioral associated symptoms with autism spectrum disorder (ASD) between the first assessment and the final evaluation (referred to as "change in score" afterward), the average values and standard deviations (SDs) were obtained from both the intervention and control groups in the studies included in the analysis.

When the original sources or the writers failed to include direct standard deviations (SDs) for score changes, SDs were approximated by using the baseline and endpoint score SDs, in conjunction with the correlation value of 0.5, as recommended in the Cochran handbook's recommended formula. The researchers then used Hedges' technique to compute the standardized mean difference (SMD) and 95% confidence intervals (CIs) to assess the magnitude of the impact.

To assess the heterogeneity across studies, we used the I^2 statistical and the value of p derived from Cochran's Q test. In this study, I^2 values less than 25% were indicative of low heterogeneity, while values ranging from 25 to 50% were considered as moderate heterogeneity. On the other hand, values beyond 50% were classified as high heterogeneity. Utilizing a significance threshold of $p < 0.05$, found evidence of statistically significant heterogeneity. A fixed-effects model was selected if the I^2 value was below 50%, whereas the random-effects model was utilized if the I^2 value had been equal to or higher than 50%.

The Egger and Begg tests were performed in order to assess publication bias. The investigation of causes of heterogeneity included the examination of subgroups, taking into account several characteristics such as the country in which the research was conducted, the scales that were employed, the methods of intervention, the length of the intervention, and the kinds of studies, all of which were considered as possible criteria for subgroup classification.

To ensure the robustness of the results, sensitivity analyses were conducted by excluding one research and then redoing the meta-analysis. For all analyses conducted, a significance threshold of $p < 0.05$ was used for two-sided testing.

Results

Features shared by included studies

The PRISMA flowchart guided the study selection procedure, which included several stages (Figure 1). At first, 3,393 results were found after searching multiple databases online. Among these, 1,154 records were identified as duplicates and subsequently removed. Following this, a thorough evaluation of the titles and abstracts of the remaining 2,239 unduplicated articles led to the exclusion of 1,607 articles that did not align with the criteria. Consequently, 46 reports remained for a comprehensive full manuscript review. Upon conducting a detailed review of the full articles, 16 trials met our predefined inclusion criteria. These 16 trials were consequently selected for incorporation into the present systematic review and subsequent meta-analysis. For a comprehensive overview of the characteristics of these 16 randomized controlled trials (RCTs), please refer to Table 1 (7, 39–52, 54–56). Overall, 720 children with mean ages 2 to 17 years (7, 39, 41–52, 54, 56), 112 adults and participants aged 5 to 55 years with ASD. Of 16 included studies, 15 used probiotics and one used prebiotics. Out of 16, seven were from the USA (7, 40–42, 47, 50, 52), four from China (43–45, 49), two from the UK (51, 54), one from each Italy, Taiwan, and Egypt (39, 46, 48).

Evaluation of bias and quality in individual study assessments

The analysis of 16 cases revealed that 93.75% (15/16) showing the investigations provided sufficient documentation of randomized sequence creation. However, the other two studies exhibited ambiguity in this particular area. All of the studies yielded data about the concealment of allocation. Out of the total number of trials examined, 68.75% (11 out of 16) were found to have successfully adopted double-blinding for outcome assessors. However, it is worth noting that blinding procedures were not conducted in four particular studies, namely trials (7, 44, 52, 54). The findings from most studies indicated a little risk of bias when it came to the blinding of participants and key research employees. However, it should be noted that two experiments demonstrated a significant potential for bias about this matter. Moreover, it was observed that all studies had a minimal likelihood of bias about inadequate outcomes knowledge and selective result reporting. The data is graphically presented in Figure 2, which includes a graph (A) illustrating the risk of bias and a summary (B) outlining the risk of bias for the RCTs (randomized controlled trials) that were included in the study.

Primary outcome evaluation of probiotic, prebiotic, and synbiotic effects on autism spectrum disorder-related behavioral symptoms

Overall, 10 studies reported Autism-Related Behavioral Symptoms (ARBS) (7, 39, 41, 43–45, 47, 48, 50, 51). We used the random-effects model due to high heterogeneity between studies ($p = 0.007$, $I^2 = 62\%$). Regarding the enhancement of autism-related behavioral symptoms, the results of the intervention group were not significantly different from the control group (combined standardized mean difference = −0.07, 95% confidence interval: −0.39 to 0.24, $Z = 0.46$, $p = 0.65$) (Figure 3).

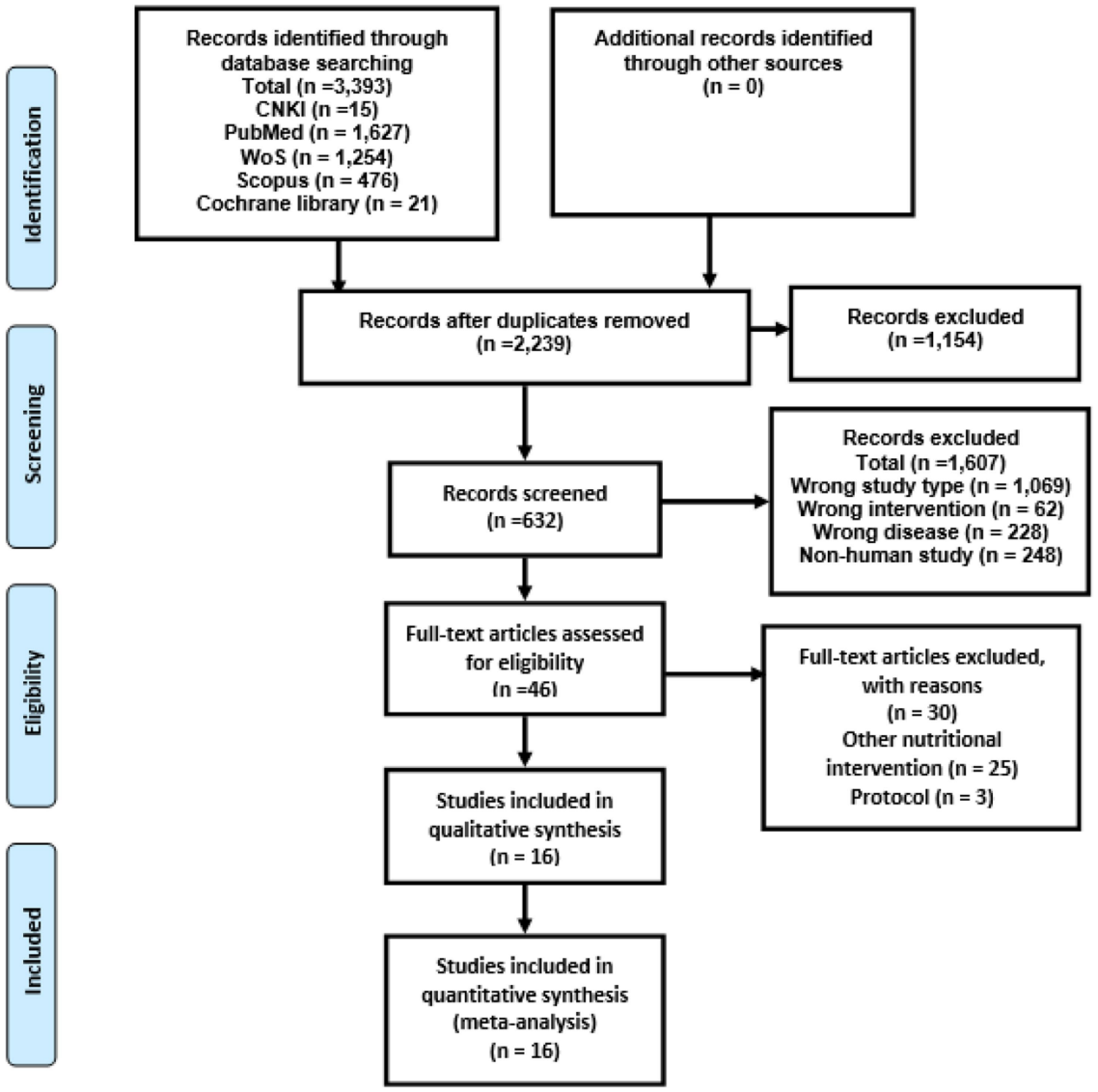

FIGURE 1
PRISMA flowchart for included studies.

Assessing secondary outcomes: effects of probiotics, prebiotics, and synbiotics on EEG, and biochemical and clinical parameters

Patients with ASD who took probiotics had a statistically significant reduction in beta band semantic similarity power between baseline and follow-up (Baseline: 13.09 ± 3.46, vs. endpoint: 10.75 ± 2.42, $p = 0.043$, respectively) also gamma spectrum (Baseline: 5.80 ± 2.42, vs. endpoint: 4.63 ± 1.39, $p = 0.033$, respectively) compared with no significant change in placebo group (39). Frontal asymmetry in individuals with ASD who were given probiotics showed a significant decrease between baseline and endpoints in delta band (Baseline: 0.029 ± 0.053, vs. endpoint: −0.024 ± 0.047, $p = 0.032$); while those on the placebo group saw a significant increase from baseline to endpoints in frontopolar asymmetry in the alpha band (Baseline: 0.022 ± 0.043, vs. endpoint: 0.077 ± 0.043, $p = 0.03$). The gamma-band power of frontopolar regions was positively correlated with the total number of RBS-R endorsements ($r = 0.28$, $p = 0.04$), which means that after taking probiotics, young children who had a lower RBS-R overall number had a lower frontopolar power in the gamma group. The beta and gamma frontopolar coherence results from VABS-II were positively correlated with one another ($r = 0.37$, $p = 0.012$ and $r = 0.40$, $p = 0.007$, respectively), so, those with ASD who scored lower on the VABS-II after taking probiotics exhibit greater beta and gamma frontopolar coherence. Frontopolar gamma coherence was found to have the strongest inverse correlation with *TNF*-α of any biochemical indicator tested. ($r = -0.30$, $p = 0.04$), resulted in greater frontopolar coherence in the gamma band after probiotic administration in ASD subjects with lower *TNF*-α levels at post-test.

TABLE 1 Qualities of included controlled experiments.

Study, year (ref.)	Country	Total sample	Intervention of experimental group (dose)	Target population	Male/ Female	Duration in weeks	Mean age (Rang)	Outcomes
Billeci et al. (39)	Italy	63	De Simone's probiotics included in the mix are: *Lactobacillus casei*, *Bifidobacterium breve*, *Bifidobacterium longum*, *Bifidobacterium infantis*, and *Streptococcus thermophilus*.	ASD	35/11	6	46.56 months ± 13.92 (18–72 months)	ADOS CSS, ADI-R, SCQ, RBS-R, General Quotient, Developmental ret., VABS-II, Linguistic Level, CBCL 1, PSI, GSI Severity Index, CARS, TNF-α, CCL2, Leptin, Resistin, PAI-1
Schmitt et al. (40)	USA	15	Probiotics: SB-121, a combination of *L. reuteri*, Sephadex® (dextran microparticles), and maltose	ASD	15/0	4	20.0 ± 3.05 (15–45 years)	Vineland factors, Oxytocin, TNF-α, and HS-CRP
Simmons et al. (41)	USA	69	Probiotics: Vivomixx	ASD	57/12	12	7.8 ± 2.6 years	ATEC GHI ABC
Kong et al. (42)	USA	35	Probiotics: *Lactobacillus plantarum*	ASD	26/9	6	10.3 (3–20 years)	serum OXT, MBP, GFAP, S100B, IL-1β, GSI, CGI
Li et al. (43)	China	41	Probiotics: Lactobacillus and Enterococcus Powder	ASD	–	3	3–6 years	Applied behavior analysis (ABA)
Santocchi et al. (44)	China	85	Probiotic supplement, DSF	ASD	71/14	24	4.13 ± 1.0 years	ADOS-CSS, VABS-II, GMDS-ER, 6-GSI, ATEC
Wang et al. (45)	China	50	Prebiotics: *Lactobacillus plantarum* + FOS	ASD	–	3	3–9 years	Dopamine metabolism disorder, hyperserotonergic state (increased serotonin), and the presence of acetic acid, propionic acid, and butyric acid
El-Alfy et al. (46)	Egypt	100	Probiotics: Lacteol Fort	ASD	–	12	2–10 years	ATEC, 6-GSI
Arnold et al. (47)	USA	13	The eight probiotic species found in VISBIOME are primarily Lactobacillus and Bifidobacterium	ASD	9/4	19	3–12 years	ADOS2, PedsQL GI, PRAS-ASD, ABC, SRS, CSHQ, PSI
Kang et al. (7)	USA	18	Probiotics: *Lactobacillus plantarum*	ASD	–	18	7–17 years	Vineland factors, ADOS2, PedsQL GI, PRAS-ASD, ABC, SRS, CSHQ, PSI
Liu et al. (48)	Taiwan	80	Probiotics: *Lactobacillus plantarum* PS128	ASD	–	4	10.01 ± 2.34 years	CGI-I, SRS, CBCL, SNAP-IV
Niu et al. (49)	China	37	Applied behavior analysis (ABA) training in combination with probiotics	ASD	25/12	4	4 (3–8 years)	ATEC, GI score
Sanctuary et al. (50)	USA	16	Probiotics: *Bifidobacterium infantis* + BCP	ASD	11/5	20	6.8 ± 2.4 (2–11 years)	ABC, GIH
Grimaldi et al. (51)	UK	41	Prebiotic: Bimuno® galactooligosaccharide (B-GOS®) prebiotic intervention	ASD	31/10	6	7 (4–11 years)	ATEC, EQ-SQ, SCAS-P
Kang et al. (52)	USA	18	Probiotics: *Lactobacillus plantarum*	ASD	–	8	7–16 years	Vineland factors, ADOS2, PedsQL GI, PRAS-ASD, ABC, SRS, CSHQ, PSI
Kałużna-Czaplińska and Błaszczyk (53)	Poland	22	Probiotics: *Lactobacillus acidophilus*	ASD	20/2	8	5.6 ± 1.6	Changes in DA/LA
Parracho et al. (54)	UK	17	Probiotics: *Lactobacillus plantarum* WCFS1	ASD	–	12	3–16 years	DBC

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FIGURE 2
Risk of bias assessment in included randomized controlled trials (RCTs).

TABLE 2 Subgroup analysis of autism-related behavioral symptoms by geographic region, intervention type.

Sub-grouped by	No. of trials	No. of participants	SMD	95% CI	*p*	I^2 (%)	*p* for heterogeneity
Geographic region							
America	4	184	−0.11	−0.6, 0.39	0.67	45%	0.14
Europe	2	131	0.33	−0.58, 1.25	0.47	82%	0.002
Asia	4	225	−0.82	−1.71, 0.06	0.07	90%	<0.00001
Intervention type							
Probiotics	8	405	−0.19	−0.42, 0.33	0.09	17%	0.30
Prebiotics	2	135	−0.80	−3.93, 2.34	0.62	98%	<0.00001

Subgroup analyses

It was found through country-specific subgroup analyses that no region showed statistically significant differences in the improved performance of assessments of behavioral symptoms related to ASD between the therapy and placebo groups (Table 2). There was also no statistically significant difference between the groups who received intervention and the groups who received a placebo when it came to the improvements in behavioral symptom severity affiliated to autism spectrum disorder (ASD) (Table 2).

Analyzing the impact of publication bias and variables

The total number of papers used in this meta-analysis was 10. Evidence of publication bias was sought using the methods established by Begg and Egger's experimental studies and visual check of funnel plots for symmetry (Figure 4). These statistical tests indicated a little chance of editorial prejudice ($p > 0.05$). To test the robustness of the results, the seven publications include in the meta-analysis were subjected to a sensitivity analysis. Importantly, when individual

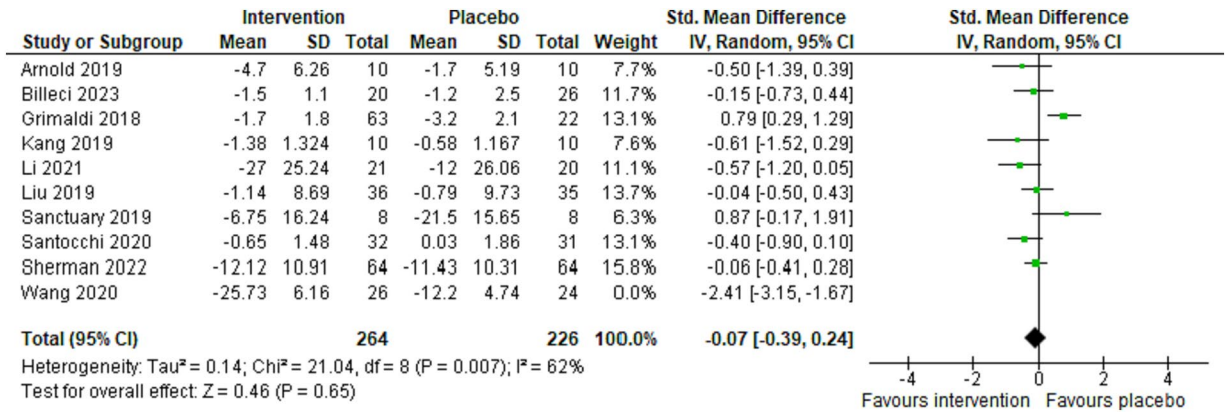

FIGURE 3
Forest plot illustrating the impact of psychobiotics on enhancing autism-related behavioral symptoms in the intervention vs. placebo groups.

research studies were removed, there was still little heterogeneity in the aggregate impact size. This further demonstrates the validity of the results of this meta-analysis.

Harmlessness

There were no unexpectedly serious AEs, which was expected. Neither treatment-attributable nor gastrointestinal AEs were more common in the probiotic preparation than in the placebo group (47, 51). This further verifies the formulation's proven safety profile.

Umbrella review

Finally, we located eight small-sample systematic reviews and meta-analyses on the probiotics, prebiotics, and synbiotics for ASD. Results from 125 randomized controlled trials were analyzed for 41 pharmaceuticals and 17 dietary supplements. ($n = 7{,}450$ participants) teenagers and kids and 18 RCTs ($n = 1{,}104$) in adults that were conducted in several worldwide databases by Siafis et al. (20). He et al. (17) did a similar meta-analysis to examine if probiotics might ameliorate behavioral indicators in children with ASD. They found seven papers that supported this hypothesis. When investigating whether probiotics and prebiotics may reduce the intensity of symptoms of ASD in young ones, the complexity of gastrointestinal (GI) disorders, and the concomitant psychopathology in ASD, Song et al. (21) did a meta-analysis using just 3 clinical controlled trials. Only Ng et al. (19) analyzed eight clinical studies to determine the impact of prebiotics/probiotics on ASD. When it comes to treating core and co-occurring behavioral problems in people with ASD, 14 papers satisfied the inclusion criteria for a recent review by Tan et al. (22), in which they critically examine the available data on the effectiveness and efficacy of probiotics, prebiotic, synbiotic, and transplantation of feces microbiota treatments. Barbosa and Vieira-Coelho (16) tried to identify the functioning clinical proof that could possibly defend the use of probiotics or prebiotics in neurological patients and included 11 studies; Ligezka et al. (18) completed a literature review on the effects of the gut microbiota on the mental health of children and adolescents; 7 studies, along with RCTs and cohort studies, met eligibility requirements. Finally, Alanazi (15) conducted a meta-analysis of randomized, controlled studies to determine whether or not probiotics and vitamins are beneficial for people with ASD. Table 3 lists the specifics and features of these evaluations.

Risk of bias in included systematic reviews

We evaluated the potential bias in all the studies that were included in the analysis. The outcomes of this bias assessment are presented in Table 4. To ensure that all relevant studies were included, systematic reviews should ask specific questions, develop thorough search strategies, and employ a variety of resources. The methods used to standardize the extraction of data and pool findings from multiple studies were also solid.

However, upon closer examination, we identified certain biases in all the systematic reviews that were included. Recurring worries included the use of predominant studies that compared all patients to the same standard test of nutritional intervention. This approach raised questions about potential bias.

Discussion

This systematic review and meta-analysis was aimed to assess the efficacy and safety of psychobiotics in ASD subjects, and show that those there was no significant effect of such therapy on autism-related behavioral symptoms, it has significant effect on the brain connectivity through frontopolar power in beta and gamma bands mediated by chemicals and cytokines, such as *TNF*-α. The psychobiotics showed no serious side-effects.

ASD represents a neurodevelopmental condition marked by enduring deficits in social interaction and communication. Alongside these challenges are repetitive and restricted behavior patterns, interests, or activities. The complexities and obstacles associated with ASD result from a combination of factors and manifest through a wide

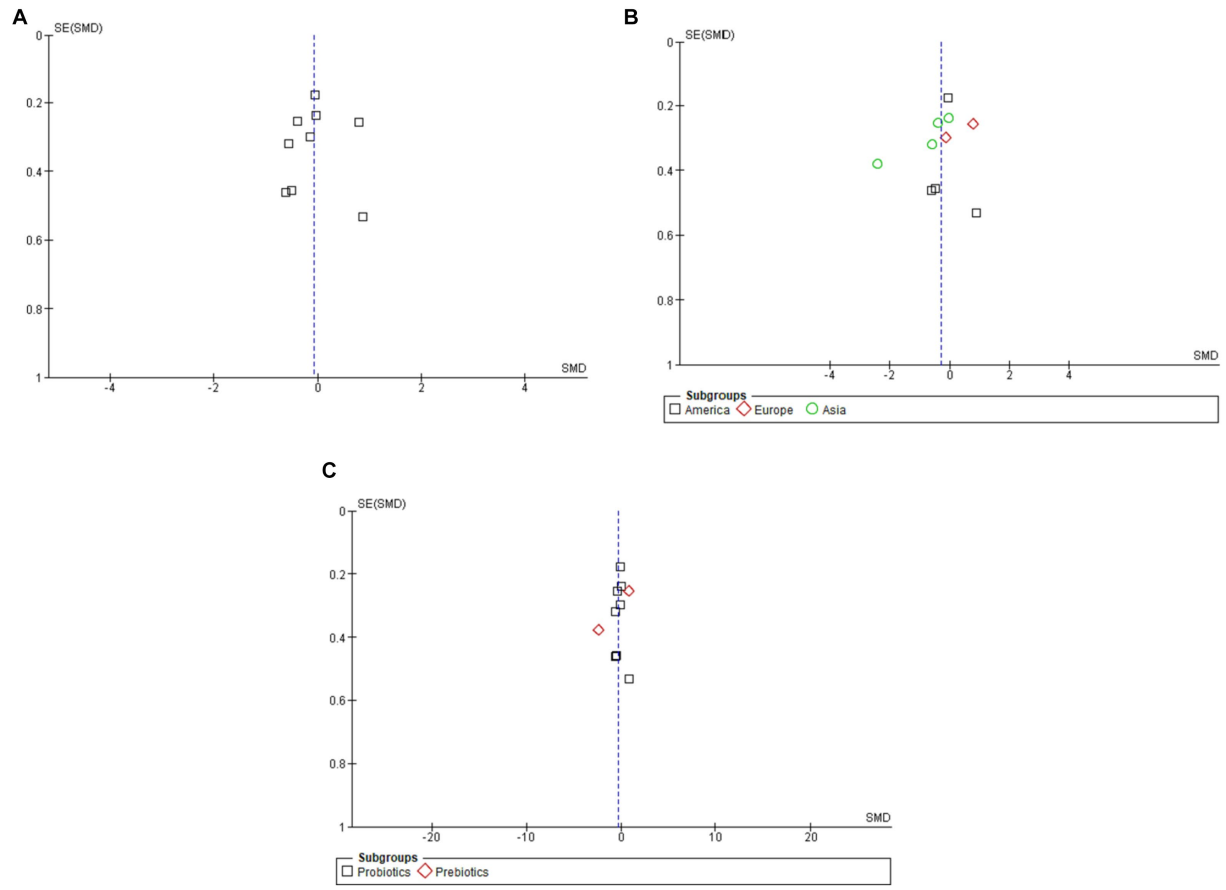

FIGURE 4
Funnel plots of overall **(A)**, subgroup analysis by geographic region **(B)**, and subgroup analysis by type of intervention **(C)**.

range of symptoms, encompassing issues like impaired social interactions, communication difficulties, and repetitive behaviors. The increasing prevalence of autism spectrum disorder highlights the urgent need to implement effective therapies all over the world.

The current understanding is that ASD arises from a complex interplay between environmental and genetic influences. Several variables have been identified as contributing to developing problems with the immune system and genetic structure (4, 5, 57–59). The study conducted by Malkova et al. (5) observed an increase in the risk of autism spectrum disorder in children whose mothers experienced immunological activation during pregnancy. The examination conducted in this context is noteworthy because it investigates the possible use of probiotics, prebiotics, and synbiotics as therapies. The intricate relationship between gut wellness and neurological problems is the focus of the article.

The study's results are supported by reputable sources, including Schmitt et al. (40) and Kang et al. (7), which enhances the study's credibility and strengthens its overall validity. The present paper includes a comprehensive meta-analysis of randomized controlled trials (RCTs) examining the effect of probiotics, prebiotics, and synbiotics on symptoms associated with ASD. The results of these studies involve improvements in actions, gastrointestinal function, and general quality of life. Nevertheless, it is important to acknowledge that the findings are influenced by the intrinsic diversity in the research, which arises from differences in the protocols of the interventions and the characteristics of the participants.

According to our data, the behavioral symptoms associated with ASD do not improve between the beginning and end of treatment.

Children who were given probiotics had reduced frontopolar power, according to the study, than that of children who did not receive probiotics, while frontopolar power was higher. Subjects with their eyes open produce beta waves, which are linked to physiological activation, attention, concentration, analytical thought, and states of focused attention, deep thought, and full mental or motor engagement (60). Gamma waves are linked to early sensory reactions and working-memory tasks (61). The resting electroencephalogram (EEG) of people with autism spectrum disorder typically displays elevated activity in the delta, theta, beta, and gamma frequency bands (62–64). When it comes to distinguishing autistic disorder from other conditions, beta power is regarded as one of the finest indices, with a 95.2% accuracy rate (65).

Coherence increases after probiotic supplementation, and this is correlated with reduced levels of cytokines like *TNF*-α, according to an analysis of the relationships between EEG and biochemical measures. Levels of *TNF*-α, an inflammatory biomarker found in the brain and CSF of many autistic people, have been found to be positively correlated with the severity of autism spectrum disorders (66). Considering the importance of *TNF*-α in controlling highly

TABLE 3 Characteristics of included systematic review and meta-analyses.

Study, year (ref.)	Country	Total included	Intervention of experimental group (dose)	Study design	Duration in weeks	Target group	Outcomes
Siafis et al. (20)	Germany	18	Pharmacological and dietary-supplement	SR and MA	8–13 weeks	Children adolescents and adults	Medication for the primary symptoms should not be prescribed on a regular basis
He et al. (17)	China	10	Probiotics	SR and MA	4–12 weeks	Children	The influence of probiotics on children with ASD need to be studied in randomized controlled trials (RCTs) that adhere to rigorous trial guidelines
Song et al. (21)	China	3	Prebiotics and probiotics	SR and MA	4–24 weeks	Children	Future, more randomized controlled studies are needed
Ng et al. (19)	Singapore	8	Prebiotics and probiotics	SR	3–12 weeks	Children	Despite promising preclinical findings, prebiotics and probiotics have limited efficacy in ASD
Tan et al. (22)	Canada	14	Probiotics, prebiotics, synbiotics	SR	1–18 weeks	Children	Beneficial effects of probiotic, prebiotic in ASD
Ligezka et al. (18)	USA	7	Prebiotics	SR	3–12 weeks	Children adolescents	Research is needed to confirm whether or not gut dysbiosis
Barbosa and Vieira-Coelho (16)	Portugal	11	Prebiotics and probiotics	SR	4–18 weeks	Children	Findings in specific psychiatric disorders are encouraging
Alanazi (15)	Saudi Arabia	11	Prebiotics and supplements	SR	–	Children adolescents	Still lacks stronger evidence
Present study	Iraq	18	Probiotics, prebiotics, synbiotics	SR, MA, and UR	4–28 weeks	Children adolescents and adults	No beneficial effects of probiotic, prebiotic in ASD

functional and plasticity, it is clear that this protein has an effect on EEG patterns (67). This suggests that the chemicals, cytokines, and hormones secreted by the gut microbiota and influenced by probiotic administration may be mediating the alterations in brain connectivity that we described.

The incorporation of several age cohorts in the research contributes an enhanced level of complexity to its results. The research recognizes the dynamic character of autism spectrum disorder (ASD) and the possible variations in intervention outcomes depending on age, taking into account both preschool-aged children (39) and people across multiple stages of development (45). Because of the well-known connection between gut health and brain health, this article centers on the microbiome of the digestive tract (13). Many neurological and psychiatric disorders, particularly ASD, have been linked to this symbiotic interaction between the brain and the digestive system. The major goal of this study is to investigate therapies that affect this axis, highlighting its possible importance in delivering comprehensive care to people with ASD.

By conducting a meta-analysis and systematic review of the relevant literature, the paper provides a substantial contribution to our understanding of the potential benefits of probiotics, prebiotics, and synbiotics as additional therapy for people with ASD. In order to

TABLE 4 Methodological quality evaluation of the included systematic reviews.

Study ID	Q1	Q2	Q3	Q4	Q5	Q6	Q7	Q8	Q9	Q10	Q11
Siafis et al. (20)	Y	Y	Y	N	Y	Y	Y	Y	N	N/A	Y
He et al. (17)	Y	N	Y	N	Y	Y	Y	Y	N	Y	N
Song et al. (21)	Y	u	Y	N	Y	Y	Y	Y	Y	N/A	Y
Ng et al. (19)	Y	Y	Y	N	N/A	Y	Y	Y	N	Y	Y
Tan et al. (22)	Y	Y	Y	N	Y	Y	Y	Y	Y	Y	Y
Ligezka et al. (18)	Y	Y	Y	U	N	Y	Y	Y	N	Y	Y
Barbosa and Vieira-Coelho (16)	Y	Y	Y	N	Y	Y	N/A	Y	U	Y	Y
Alanazi (15)	Y	Y	U	N	N/A	Y	Y	Y	N	N/A	Y
Present study	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	Y

Q1, Is the evaluation question unambiguous?; Q2, Were there sufficient inclusion criteria to answer the research question?; Q3, Was the search strategy appropriate?; Q4, Were there insufficient means or sources used to find studies?; Q5, Were the study-evaluation standards adequate?; Q6, Did at least two separate reviewers each make their own critical judgments?; Q7, Was there a way to reduce human error during data collection?; Q8, Were the strategies for combining studies adequate?; Q9, Was the potential for bias in the publication process evaluated?; Q10, Were the reported data sufficient to back up the suggested changes to policy and/or practice?; Q11, Were the detailed instructions for new studies adequate?

properly address the many complexities of ASD, the research offers a critical evaluation of the present state of affairs and highlights the need for more centralized research methodology to be used. As our knowledge of the microbiome-gut-brain axis expands, we anticipate that medicines supported by evidence that improve gut health will play an increasingly significant role in the management of ASD.

Limitations

Strict eligibility requirements imposed by the study's sponsor contributed to a relatively small sample size. Potentially illuminating splits by sex and GI dysfunction type were not possible due to the small sample size. Another is though successful blinding in double-blind RCTs is *crucial for minimizing bias*, however studies rarely report information about blinding. In double-blind RCTs of therapies in ASD, *blinding can be broken due to the apparent side effects*. It would appear that adequate allocation concealment is the more crucial indicator. Furthermore, many trials, especially those involving children, cannot be double-blinded. A standard premised on double blinding is not applicable, so those trials must be evaluated on their own merits. A third factor is the use of an insensitive anxiety scale that was chosen because it was thought to be ASD-specific.

Conclusion

The published studies on psychobiotics in patients with ASD provide encouraging insights into the potential benefits of modulating the gut microbiota for symptom improvement. The results of this review shows that psychobiotics impose a medium effect on ASD-related symptoms. These interventions may hold promise as complementary or adjunct therapies for individuals with these neurodevelopmental disorders. Our results lend credence to the use of psychobiotics in a sizable population of people with ASD. The results of this pilot study also pave the way for future studies to use EEG activity as a quantitative objective marker of efficacy of treatment in children with ASD. However, further research, including larger and more controlled clinical trials, is necessary to better understand the mechanisms at play and to elaborate clear guidelines for their use in clinical practice.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

FR: Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Validation, Writing – original draft, Writing – review & editing. KT: Data curation, Investigation, Project administration, Resources, Writing – review & editing. NQ: Data curation, Investigation, Resources, Software, Writing – review & editing. KD: Conceptualization, Methodology, Project administration, Visualization, Writing – original draft, Writing – review & editing. AZ: Data curation, Formal analysis, Investigation, Software, Writing – original draft. RK: Data curation, Investigation, Validation, Visualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Premature Infant Gut Microbiome relationships with childhood behavioral scales: preliminary insights

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Introduction: Very Low Birth Weight (VLBW) infants, born weighing less than 1,500 grams, are at risk for both gut dysbiosis and later neuropsychological developmental deficits. Behavioral effects, while related to neurodevelopment, are often more subtle and difficult to measure. The extent of later neurobehavioral consequences associated with such microbial dysbiosis has yet to be determined. We explored associations between the infants' gut microbiome and early childhood behavior at 4 years of age and identified the bacterial taxa through a multivariate analysis by linear models.

Methods: Parents completed the Child Behavior Checklist (CBCL) focused on different DSM diagnostic categories: affective, anxiety, pervasive developmental, attention deficit/hyperactivity, and oppositional defiant. All the CBCL scores were corrected for gender, delivery method, gestational age, infant birth weight, occurrence of sepsis, and days on antibiotics prior statistical analyses. Canonical correlation analysis (CCA) was performed to determine the relationship between early life gut microbiome and the adjusted CBCL scores. The association of bacterial Amplicon sequence Variants (ASVs) to the CBCL scores were tested with multivariate analysis by linear models (MaAsLin).

Results: Nineteen children who were previously born with very low birth weight and studied while hospitalized in the Neonatal Intensive Care Unit (NICU) were included in this study. Statistically significant associations were observed between early life gut bacteria such as *Veillonella dispar, Enterococcus, Escherichia coli*, and *Rumincococcus* to later behavior at 4 years. No significant association could be observed with early-life gut microbiome alpha diversity and behavioral measures at 4 years.

Discussion: These preliminary observational data provide insight into the relationships between VLBW gut microbiome dysbiosis and childhood behavior. This study contributes to the literature on gut microbiome analysis by examining various behavioral domains using a standardized tool linked to the Diagnostic and Statistical Manual of Mental Disorders (DSM).

KEYWORDS

gut microbiome, gut-brain axis, childhood, behavior, gastrointestinal microbiome, microbiota, CBCL scores

1 Introduction

Very Low Birth Weight (VLBW) infants confront unique developmental challenges and a heightened risk of experiencing behavioral issues in their future. Their premature birth means that their organ systems, including the brain, might not have fully matured, leading to potential long-term consequences for neurodevelopment (1, 2). Furthermore, many VLBW infants undergo extended stays in the neonatal intensive care unit (NICU), which can result in sensory deprivation and emotional stress during a critical phase of brain development. Additionally, VLBW infants are more prone to medical complications, such as respiratory distress syndrome, intraventricular hemorrhage, and infections, all of which can have lasting impacts on both their brain, behavior, and growth (3–5). Therefore, it is crucial to identify potential biomarkers or other biological patterns to recognize these challenges early in life.

Numerous studies have illuminated the gut-brain axis, involving bidirectional communication between the gut and the brain, which directly and indirectly impacts the host's growth, development, and behavioral functions (6–8). Consequently, comprehending the role of gut microbiota in infants could be pivotal for averting the future risk of behavioral issues by enabling the provision of suitable nutrition and early intervention.

Our previous study demonstrated a relationship between the gut microbiota of Very Low Birth Weight (VLBW) and neurodevelopment, assessed using the Battelle Development Inventory-2 Screening Test (BDI-2ST) (2). Behavioral effects, while related to neurodevelopment, are often more subtle and difficult to measure. The use of a parent qualitative scale to describe child behavior is a nuanced approach used in the current study. The Child Behavior Checklist (CBCL) is a standardized tool with six scales related to Diagnostic and Statistical Manual of Mental Disorders (DSM) diagnostic categories. The CBCL has been widely for many years and has shown a high accuracy of diagnostic efficiency (9).

The VLBW infants in this study experienced significant gut microbial dysbiosis during the first 6 weeks of life in the Neonatal Intensive Care Unit (NICU) which was characterized by a dominant abundance of Gammaproteobacteria (10). They are more likely to suffer various brain insults and injuries in their early life. Immaturity, coupled with the intensive care that is necessary, predisposes these infants to gut dysbiosis, a disequilibrium of the gut microbial community (11, 12). The roles of the dysbiotic infant gut microbiome in later childhood neurodevelopment and behavior are understudied. Pathogens present during sensitive developmental periods are associated with later anxiety-like behavior and cognitive impairment (13–16). This may happen because proinflammatory bacterial metabolites from the gut can alter the blood brain barrier or cross into the brain, altering microglia, and contributing to the development of neurological injury (17) which then translates into later neurodevelopmental and behavioral problems. Intestinal dysbiosis often includes reduced microbial alpha diversity and increased intestinal barrier permeability (18). Lower alpha diversity is often correlated to poorer health status (19, 20). The stability, diversity, and developmental succession of the early life gut microbiome may be associated with long-term health consequences (21).

This study explored associations between the VLBW infant's gut microbiome and scales related to the Diagnostic and Statistical Manual of Mental Disorders (DSM) from the Child Behavior Checklist (CBCL) at 4 years old. We also identified the bacterial Amplicon Sequence Variants (ASVs) related to the DSM-related scores. This study adds to the gut microbiome analysis literature by including analysis related to different behavioral DSM-related scales using a standardized tool.

2 Materials and methods

2.1 Study design and participants

Upon approval by the university Institutional Review Board (IRB), parents of VLBW infants admitted to the NICU of a large Florida tertiary care hospital were invited to be in the initial cohort (IRB#Pro00003468, R21 NR013094). Parents gave written informed consent to participate in the study and in additional follow up studies. Eighty-three VLBW infants were measured during the first 6 weeks of their NICU admission. Parents who consented were contacted for the follow-up study (IRB#Pro00019955, NIH grant R01NR015446) that explored relationships between the gut microbiome and later health, growth, and development. A total of 25 VLBW infants were followed from birth to 4 years of age. Home visits were done, and multiple types of data were collected. In the current paper, we report on data collected in the NICU, including stool microbiome data, and later behavioral outcomes at 4 years of age. In 19 cases, there were complete data from the 5 and 6 weeks of life for the microbiome analysis, adjustments, and behavioral follow-ups at 4 years of age. **Supplementary Figure 1** shows a flow diagram describing clearly the longitudinal follow up design.

2.2 Sample processing for measurement of infant and childhood follow up of stool microbiome

Infant stool samples were collected weekly from diapers during the first 6 weeks of life and aliquots were stored at −80 C prior to sequencing. At the 4 year home visit the investigators collected stool samples from the children and their mothers. Stool was collected from the diaper or from the toilet using the ALPCO Easy Sampler® Stool Collection kit. The stool was delivered to the lab and immediately frozen at −80 C until processing for DNA extraction. Microbial genomic DNA was extracted using the PowerSoil DNA Isolation Kit (MoBio) (22). The microbial content was profiled by one contiguous region of 16S rRNA V3-V4 sequencing on an Illumina MiSeq that generated ∼100,000 250 bp paired-end reads per sample. Sequencing quality was assessed, errors corrected, Amplicon Sequence Variants (ASVs) were generated, and their taxonomic annotations were obtained against Silva v138 using the DADA2 pipeline (23). ASVs were used to calculate the alpha diversity, which measures the bacterial diversity as a function of richness and evenness within each sample. For all statistical analyses, only the most abundant ASVs in the dataset were utilized, wherein all ASVs with less than 0.01% abundance in all samples, and ASVs observed in less than 10% of the samples were discarded

employing the filter_taxa command implemented in OTU table R package (24).

2.3 Behavioral measures

Parents completed the Child Behavior Checklist (CBCL) at home visits. The CBCL is a standardized instrument used to assess behavioral problems in children between 18 and 71 months old (25). It contains 99 items, and each is rated on a three-point Likert scale. This study focused on the six-DSM scales consistent with DSM diagnostic categories: affective, anxiety, pervasive developmental, attention deficit/hyperactivity, and oppositional defiant. The scale is a first level screening, reporting symptoms aligned with diagnostic areas such as autism spectrum disorder (ASD), attention deficit disorder (ADHD), depression, and oppositional defiant disorder (26, 27). Notably, depression symptoms in this age group are manifested mostly as emotional irritability and dysregulation, often differing from manifestations later in development (anhedonia, hopelessness, persistent sadness) (28). The results are age-normed into T-scores with a mean of 50 and standard deviation of 10. Consequently, ranges between 65–69 are considered borderline and scores of 70 or higher are indicative of clinical-range problem (29). A previous publication provides the CBCL descriptive statistics in this sample with means and standard deviations (28).

2.4 Statistical analysis

IBM SPSS Statistics version 25 [IBM, (30)] was used to calculate descriptive and frequency statistics for demographic and clinical data. Scores from the CBCL were analyzed using Spearman correlations because of non-normal bivariate distributions.

The microbiome measures were corrected for potential confounding factors by calculating the residual values for each CBCL score after correcting for gender, delivery method, gestational age, infant birth weight, occurrence of sepsis, and days on antibiotics. For all downstream statistical analyses, the residual values of the CBCL scores were utilized. The microbiome data is already available in NCBI database of Genotypes and Phenotypes (dbGaP) with study accession phs001578.v1.p1 (https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs001578.v1.p1). The data generated towards achieving the aims of the study are shared through tables and supplementary data described in this study.

2.5 Canonical correlation analysis

Canonical correlation analyses (CCA) were performed to identify correlations between infant microbiomes and later behavior. This analysis models correlations between two multivariate sets of data (31). For this purpose, the alpha diversity indices at the infant stage were considered to represent the microbiome, while the residual CBCL values represented the behavioral measures. The R package CCA (version 1.2.1) was utilized for this purpose.

2.6 Association of microbiome to later behavior

We examined the associations between early-life microbiome and later behavior (at 4 years) by employing multivariate analysis by linear models (MaAsLin) (32) implemented in the galaxy server (33). The abundant ASVs obtained previously were considered as predictors while the corrected CBCL scores were the outcome. The bacterial counts were converted to relative abundances which were subsequently utilized as input for MaAsLin (34, 35). Apart from ASVs, we further conducted association testing employing MaAsLin for different taxonomic levels including phylum, class, order, family and genus. Associations were considered to be significant if $p < 0.05$.

3 Results

3.1 Demographics

The 19 children were born early and at very low birth weight (**Table 1**-sample characteristics and S1-newborn and NICU stay characteristics). They were followed by home visits at 50.3 ± 1.7 months of age. Most were born by Cesarean section (63%), received courses of antibiotics for 15.8 ± 14.8 days, were fed varying amounts of mothers' own milk and experienced multiple illnesses associated with prematurity. **Table 1** provides sample, NICU stay characteristics, and CBCL t-scores at pre-school age.

3.2 Association between alpha diversity and residual CBCL values

Our previous publications (3–5) have summarized the microbiome features obtained in the cohort. For the samples included in this study, we have obtained 103 unique ASVs whose classifications are listed in **Supplementary Table 1**. The correlations between the alpha diversity and the adjusted CBCL scores are shown in **Figure 1**. Strong positive correlations were evident among the different CBCL scores but the associations between the alpha diversity measures and CBCL scores were not statistically significant although weak negative correlations were observed between them. The Spearman rank correlation between the adjusted CBCL scores and the alpha diversity measures are listed in **Supplementary Table 1**, while the corresponding *p*-values are listed in **Supplementary Table 2**.

3.3 Canonical correlation analysis

The relationship between the gut microbiome parameters in early life was compared with the adjusted CBCL scores at 4 years using canonical correlation analysis (CCA). As shown in **Figure 2** the adjusted CBCL measures were highly related to each other but not with the alpha diversity at early life.

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TABLE 1 Population characteristics, neonatal intensive care unit clinical data and DSM-related childhood behavior checklist t-scores.

Family Demographics	Frequency
Maternal education	46.3% high school or less
Marital status	62.5% married
Income	37.9% under 25,000/year
Ethnicity	
Caucasian	43.8%
African American	18.8%
Hispanic White	31.3%
Asian	6.3%
Gender	
Male	42%
Female	58%
Delivery method	
Vaginal	37%
Caesarean section	63%
NICU events	
Apgar at 5 min	7.72 ± 1.07
Gestational age (weeks)	27.8 ± 1.7
Birth weight (Gms)	1068.2 ± 215.5
Hemoglobin (Gms/dl)	9.11 ± 1.88
Days on antibiotics	15.8 ± 14.8
Seizures	1 infant
Necrotizing enterocolitis	1 infant
Bronchopulmonary dysplasia	1 infant
Sepsis	1 infant
Intraventricular hemorrhage	1 infant
Retinopathy	2 infants
Days on oxygen	24 ± 26.6
Discharge weight (Gms)	2913.12 ± 1069.1
At preschool age (months)	50.3 ± 1.7
Weight (Kg)	18.3 ± 7.9
Height (cm)	100.25 ± 4.49
Hemoglobin (Gms/dl)	12.09 ± 1.8
Head circumference	
CBCL scores	
CBCL1 (depression t score)	56.2 ± 9.2
CBCL2 (anxiety t score)	55.2 ± 8.8
CBCL3 (autism t score)	55.6 ± 7.8
CBCL4 (attention deficit hyperactivity t score)	55.6 ± 8.5
CBCL5 (oppositional/defiant t score)	54.3 ± 8.8

However, upon investigating the CCA between CBCL scores and prominent ASVs, close relationships were discovered as shown in Figure 3. For example, ASV_1 (family *Enterobacteriaceae*), ASV_5 (*Streptococcus*), ASV_7 (*Staphylococcus*) and ASV_13 (*Enterococcus*) were all highly associated with CBCL_ADHD adjusted values. Similarly, ASV_10 (*Citrobacter*) was related to CBCL_oppositional. ASV_4 (*Enterobacteriaceae*) was associated with both CBCL autism and CBCL ADHD adjusted scores.

3.4 Association of early-stage microbiome to behavior at 4 years

Several significant associations were observed between different ASVs and the CBCL scores. In the domain of depression, there was a positive association with *Veillonella dispar* ($p = 0.0007$) and *Escherichia coli* ($p = 0.02$), whereas *Enterococcus* ($p = 0.03$) and *Ruminococcus* ($p = 0.04$) exhibited negative associations. Conversely, in the domain of anxiety, a positive association was observed with *Enterococcus* ($p = 0.04$), while *Veillonella dispar* displayed a negative association ($p = 0.01$). They are summarized in Table 2. The Supplementary Table 3 lists all the associations exported by MaAsLin with the difference CBCL scores. However, for other taxonomic levels including phylum, class, order, family and genus, we could not observe significant associations and the results are listed in Supplementary Table 4.

4 Discussion

The composition of the gut microbiome showed significant relationships with DSM-based behavioral scales from the CBCL. In this sample, the alpha diversity was not significantly associated with the adjusted CBCL scores. However, specific Amplicon Sequence Variants analyses were significantly associated with the adjusted CBCL scores, some with positive and some with negative associations.

Enterococcus and *Veillonella dispar* showed significant associations with the CBCL adjusted scales of depression and anxiety. Additionally, there was a significant association of the anxiety CBCL scale with the presence of *Escherichia Coli* and *Ruminococcus*.

Aerobic microbes such as *Enterococcus* and *Escherichia* are the first to colonize the newborn under normal conditions, with the shift occurring to more anaerobic microbes (including *Veillonella*) by 4 months of age (36). However, our sample had high abundance of *Veillonella* within the first weeks in the NICU. Additionally, children born via C-section, which was true for most of the infants, have a higher pathogen abundance of *Klebsiella* and *Enterococcus*, which are associated with a later higher incidence of respiratory infections within the first year of life (37).

The gut microbiome's relevance to mood disorders is supported by the relationship of microbes with mechanisms affecting mood and behavior. *Enterococcus faecalis* converts naturally occurring levodopa into dopamine via a decarboxylation reaction. Because levodopa is able to cross the blood-brain barrier, but not dopamine, the conversion may lead to reduced central dopamine availability in the brain (38). *Veillonella* abundance has been associated with negative emotions at school age and to cognitive outcomes from birth to adolescence (39). *Veillonella* has also been linked to increased stress levels, as indicated by negative events and emotions reported by parents, emotional problems and low happiness reported by children, and a parasympathetic response to stress. These findings were independent of age, gender, parental education, BMI z-score, fiber, protein, sweet and fat food intake, physical activity, and sleep (40). The links between behavior and ASVs exists, but the direction may differ. Further research is necessary to understand better how gut bacteria and moods are

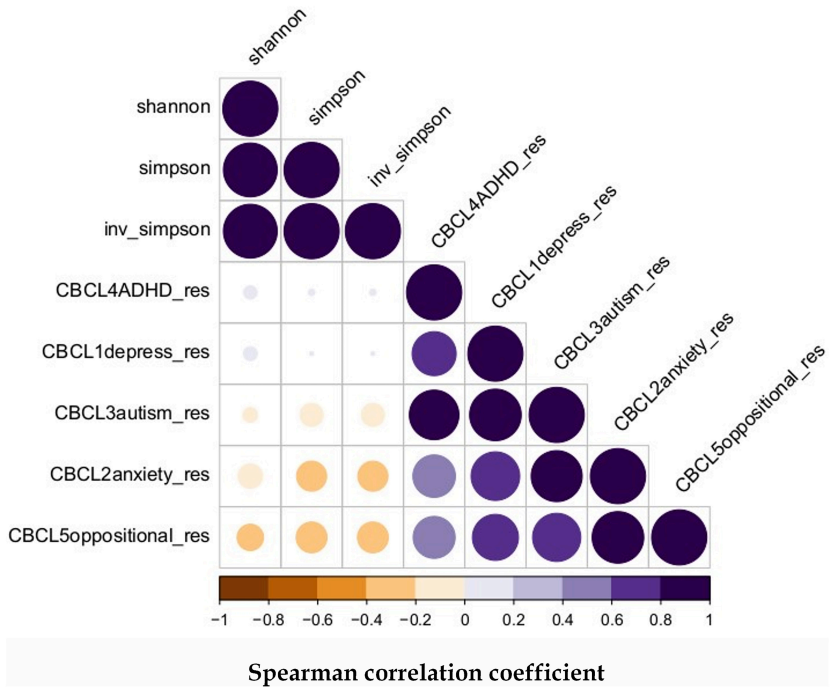

FIGURE 1
Spearman correlation between microbiome alpha diversity and later childhood behavior: The alpha diversity were derived from ASVs distribution in the samples and all the CBCL scores were adjusted for gender, delivery method, gestational age, infant birth weight, occurrence of sepsis, and days on antibiotics.

connected. Studies that follow individuals over time would be beneficial in exploring these relationships.

A lower abundance of *Ruminococcus* was related to higher scores in the depression DSM-related scale of the CBCL. Major Depressive Disorder in adults was associated with lower abundance of *Ruminococcus* (41). Contradictory results have been reported for other taxa, but not for *Ruminococcus* (41),

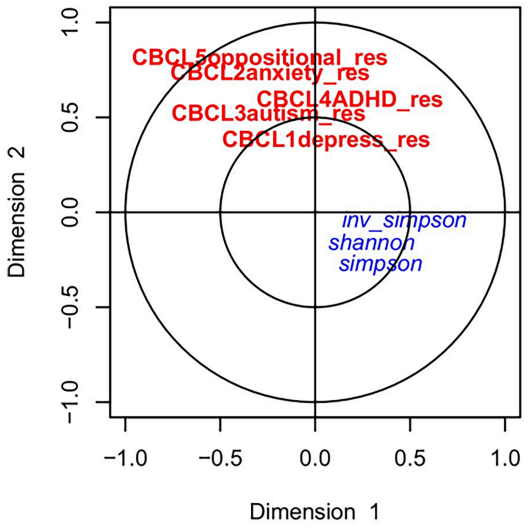

FIGURE 2
Canonical correlation analyses between alpha diversity at early life and childhood behavior at 4 years: The red and blue color represents the CBCL scores and alpha diversity, respectively. No strong associations between the CBCL scores and alpha diversity were observed.

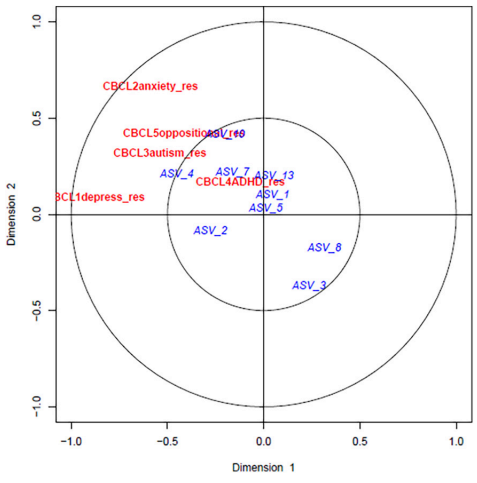

FIGURE 3
Canonical correlation analyses between predominant bacterial ASVs at early life and childhood behavior at 4 years: The red and blue color represents the CBCL scores and ASVs, respectively. ASV_1 (family *Enterobacteriaceae*), ASV_5 (*Streptococcus*), ASV_7 (*Staphylococcus*) and ASV_13 (*Enterococcus*) was all strongly associated with CBCL_ADHD adjusted values. Similarly, ASV_10 (*Citrobacter*) was related to CBCL_oppositional. ASV_4 (*Enterobacteriaceae*) was associated with both CBCL autism and CBCL ADHD adjusted scores.

TABLE 2 Association between early-life microbiome and later behavior at 4 years.

Variable	Feature	Bacterial_classification	Coefficient	*P*-value
CBCL1depression	ASV_24	*Veillonella.dispar*	0.024101364	0.000768758
CBCL1depression	ASV_2	*Escherichia coli*	0.086191646	0.024760203
CBCL1depression	ASV_179	*Enterococcus*	−0.005181727	0.038295599
CBCL1depression	ASV_169	*Escherichia coli*	0.045086345	0.038546534
CBCL1depression	ASV_1227	*Ruminococcus*	−0.010014289	0.042389942
CBCL2anxiety	ASV_24	*Veillonella.dispar*	−0.017270338	0.013292532
CBCL2anxiety	ASV_179	*Enterococcus*	0.005296564	0.042749136

Only significant associations are listed.

indicating that this microbe may be a potential biomarker for depressive disorder.

These children, as infants in the NICU, had dysbiotic gut microbiomes with an overabundance of *Gammaproteobacteria* (10). Dysbiosis could disrupt the normal gut-brain axis in developing infants. Gut microbial products reach the blood and then the brain. These chemicals are capable of modulating neuronal signaling and possibly neurodevelopment. Metabolites of bacterial origin were found in blood samples of children with autism spectrum disorder (ASD), which may cause oxidative stress, mitochondrial dysfunction, and structural changes in the amygdala, cortex, hippocampus, and cerebellum (42).

In conclusion, it is hypothesized that a connection exists between the gut microbiome and behavior. However, most previous studies (43–45) have concentrated on term infants and assessed behavior at 2 years of age or even earlier, which might not entirely align with the conditions at 4 years. Our aim was to investigate gut dysbiosis in VLBW infants and explore the relationships between the early-life gut microbiome and behavior during the preschool years at the age of 4. There were significant relationships between gut microbiome ASVs and DSM-based behavioral scales from the CBCL. Adjusted CBCL scores were significantly associated with ASVs representing *Veillonella dispar, Enterococcus, E. coli*, and *Rumincococcus*. It appears that the gut microbiome dysbiosis of VLBWs may have relationships to later childhood behavior. This study contributes to the gut microbiome literature by adding analyses related to different behavioral domains using a standardized tool linked to the DSM.

These results are preliminary due to the limited sample size. Other factors aside from gender, delivery method, gestational age, infant birth weight, occurrence of sepsis, and days on antibiotics are potentially important in these later childhood relationships. These include human milk, growth, parenting, and development characteristics after discharge.

Dysbiosis in the gut microbiome has shown associations with diverse behavioral disorders, encompassing conditions such as anxiety, depression, and even neurodevelopmental disorders like autism. A comprehensive understanding of the mechanisms through which the infant gut microbiome influences these disorders is of paramount importance for timely intervention and proactive prevention strategies.

While this pilot study offers insights into the association between infancy gut dysbiosis and preschool behavioral functions, a limitation of this manuscript is the small sample size utilized in the study. To validate these findings, further investigation with a larger sample size is necessary. Additionally, there may be other potentially significant factors in these later childhood relationships, such as human milk, growth, parenting, and developmental characteristics after discharge, which were not accounted for in this study.

Author's note

SO also worked on this research while affiliated with the University of South Florida, College of Nursing and the University of Tennessee-Knoxville, College of Nursing. AS, JY, and MG also worked on this research while affiliated with the University of South Florida, College of Nursing.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/gap/, phs001578.

Ethics statement

The studies involving humans were approved by the University of South Florida Institutional Review Board. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

SO: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing-original draft, Writing-review and editing. AS: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing-original draft, Writing-review and editing. JY: Investigation, Methodology, Resources,

Validation, Writing-review and editing. ES-H: Conceptualization, Methodology, Resources, Validation, Writing-review and editing. MG: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing-original draft, Writing-review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1294549/full#supplementary-material

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Exploring the impact of antenatal micronutrients used as a treatment for maternal depression on infant temperament in the first year of life

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Antenatal depression and maternal nutrition can influence infant temperament. Although broad-spectrum-micronutrients (BSM: vitamins and minerals) given above Recommended Dietary Allowances during pregnancy can mitigate symptoms of antenatal depression, their associated effects on infant temperament are unknown. One hundred and fourteen New Zealand mother-infant dyads (45 infants exposed to BSM during pregnancy (range of exposure during pregnancy: 12–182 days) to treat antenatal depressive symptoms (measured by Edinburgh Postnatal Depression Scale) and 69 non-exposed infants) were followed antenatally and for 12 months postpartum to determine the influence of *in utero* BSM exposure on infant temperament. The Infant Behavior Questionnaire–Revised: Very Short-Form assessed temperament at 4 (T1), 6 (T2) and 12 (T3) months postpartum via online questionnaire. Latent growth curve modeling showed BSM exposure, antenatal depression and infant sex did not statistically significantly predict initial levels or longitudinal changes in orienting/regulatory capacity (ORC), positive affectivity/surgency (PAS) or negative affectivity (NEG). Higher gestational age was positively associated with initial PAS, and smaller increases between T1 and T3. Breastfeeding occurrence was positively associated with initial NEG. Although not significant, BSM exposure exerted small, positive effects on initial NEG ($\beta = -0.116$) and longitudinal changes in ORC ($\beta = 0.266$) and NEG ($\beta = -0.235$). While BSM exposure did not significantly predict infant temperament, it may mitigate risks associated with antenatal depression. BSM-exposed infants displayed temperamental characteristics on par with typical pregnancies, supporting the safety of BSM treatment for antenatal depression.

KEYWORDS

antenatal, prenatal, nutrient, supplement, temperament, infant

1 Introduction

The antenatal environment substantially impacts fetal development, with research showing maternal behavior and emotional states during pregnancy influences fetal programming (1, 2). As a consequence, maternal psychiatric status has become a central component of antenatal care, particularly with respect to understanding its effect on long term social, emotional and

behavioral infant development (3). Initially proposed to explain the relation between maternal antenatal health and the emergence of later diseases in offspring (4), the fetal programming hypothesis has been applied to behavioral and psychological development of infants, notably infant temperament (5).

Over the past three decades, several definitions and approaches on the development of temperament have been proposed (6). Despite their differences, researchers have agreed that temperament: (1) is not a trait, rather a collection of traits, (2) can be thought of as behavioral tendencies rather than specific behaviors, (3) is biologically based, (4) refers to individual differences, and (5) can be shaped through experience (7, 8). One widely accepted definition describes temperament as "constitutional differences in reactivity and regulation influenced over time by heredity, maturation, and experience" (9). This emphasizes the combination between biology and environment: an individual's temperament is genetically influenced and thus relatively stable; however, it is still shaped by the environment individuals develop in and interact with over time. Infant temperament has been positively associated with later social competence (10), identified as a risk factor in the development of future psychopathology including ADHD (11) and externalizing/internalizing behavioral problems (e.g., anxiety, depression) (12), and is often considered the building blocks of adult personality (13). Given this understanding of how temperament originates and its impact on long term development, it is not surprising the connection among temperament, fetal programming, and antenatal depression is being increasingly explored.

Thomas and Chess (14) initially proposed nine dimensions to measure and explain traits of infant temperament. Over time, these dimensions have been altered through factor analysis and investigators have determined that temperament could be broadly measured over three dimensions: (1) negative affectivity (NEG), (2) positive affectivity/surgency (PAS) and; (3) orienting/regulatory capacity or effortful control (ORC) (15). NEG includes displays of typically negative behaviors, e.g., sadness, fear, distress to limitations; PAS contains typically positive behaviors, e.g., approach, smiling and laughter; and ORC includes regulatory functioning, e.g., orienting, soot ability, cuddliness (15, 16).

Detangling the effects of antenatal depression from the effects of postpartum depression is complex, as many studies fail to separate perinatal depression into two distinct periods, antenatal or postnatal. The existing limited evidence suggests that antenatal depression is associated with, and may even predict, aspects of infant temperament, most notably negative affectivity, which is of particular importance given the dyadic nature of the mother-infant relationship and the impact affect has in transactional processes within the wider family system (17). Antenatal depression has been associated with increased risk of infant irritability and fussiness (17), as a predictor of emotional reactivity (18), and negative affectivity characterized by a lack of smiling, difficulty soothing, and increased sadness (19–21). Infant negative affectivity has been implicated as a risk factor for future psychopathology (21, 22).

Five systematic reviews have examined the association between antenatal maternal mental disorders and infant temperament (5, 19, 23–25). These reviews provide conflicting results, with four suggesting antenatal depression was associated with difficult or more negative temperament (19, 23–25), and the other concluding the evidence was equivocal (5).

Rouse and Goodman (17) identified that the timing of exposure to antenatal depression is an important variable influencing infant temperament, suggesting a window of vulnerability in mid pregnancy, while two other studies have found the impact of antenatal depression on infant negative affectivity was moderated by genetic factors (26, 27) indicating an interaction among maternal psychiatric status, genetics, and infant temperament.

Given the negative effects of maternal depression on infant temperament such as increased displays of negative affectivity (21) and emotional reactivity (28), it is expected that treating depression during pregnancy may mitigate these negative effects on the infant. Current treatment recommendations include psychological treatments for mild to moderate antenatal depression, such as cognitive behavioral therapy (CBT) and interpersonal psychotherapy (IPT) (29), with some evidence for a small but positive effect on offspring outcomes, although findings on these benefits are inconsistent (30, 31). However, women often do not access these treatments due to issues with time, cost, stigma, and childcare issues. As far as we are aware, there are no studies that have explored the effect of psychological treatments for antenatal depression specifically on infant temperament.

For more severe depression antidepressant medication (AD), such as selective serotonin reuptake inhibitors (SSRI) or selective norepinephrine reuptake inhibitors (SNRI) are recommended (29, 32). The effects of AD use in pregnancy on anthropometric outcomes have been explored, with some observational studies suggesting an increased risk of preterm birth (33), and reduced birth weight (34). While negative effects may be transient, and with preliminary findings suggesting ADs given antenatally do not appear to exert significant effects on temperament (35), the scarcity of research and no RCTs exploring the effect of medication use in the pregnant population, makes the safety of ADs in the long-term difficult to determine. Indeed, there is some hesitancy with continued use of ADs within the pregnant population (36) and psychiatric medication use can reduce by 80 percent during pregnancy (37), highlighting the importance of a careful risk–benefit analysis as well as the need for more research on alternative treatment options in pregnancy, and their subsequent effects on infant outcomes.

Growing attention is being given to the intrauterine nutritional environment, particularly improving maternal nutrient status during pregnancy (38) as the body's nutritional requirements increase to support the metabolic and hormonal changes of the mother and growth and development of the fetus. As a result of the increased nutritional demand, it is likely that many pregnant people are vulnerable to inadequate nutrient intake (39), thus supplementation with vitamins and minerals have become commonplace in obstetric care (40).

The effects of poor nutrition during pregnancy has been extensively explored, particularly given the outcome of The Dutch Famine Birth Cohort Study, where *in-utero* undernutrition was predictive of future psychopathology (41). Since then, numerous studies have documented the effects of dietary intake on infant outcomes (42–46), although only three in the past decade focused on infant temperament (47–49), with higher adherence to healthier diets being associated with higher scores on temperament dimensions of positive affectivity and orienting/regulatory capacity.

A newer line of research is investigating the effects of supplementation with vitamins and minerals (broad spectrum

micronutrients or BSM) on antenatal depression, based on extensive studies showing that BSM can positively impact on symptoms of depression in non-pregnant populations (50). Although several of the interventions were conducted within physically and psychologically well populations, participants who experienced psychological distress or severe physical illness tended to improve more with nutritional supplementation compared to participants who were well (50), thus providing support for BSM as a treatment option, which could extend into pregnant populations.

As far as we are aware, there is no literature on the relation between antenatal nutrient supplementation with BSM and infant temperament; however, there is significant evidence for the benefits of nutrient supplementation in pregnancy for overall infant development (51–53). The effects of antenatal supplementation with single nutrients such as folic acid, iron and iodine on infant outcomes although mixed, report improvements in birth outcomes (53), cognitive and motor performance in the first year (54, 55) and reduced behavioral problems later in life (56, 57). Despite these improvements, there are some reports of detrimental effects to infant outcomes related to excessive supplementation with one nutrient given over the recommended dietary allowance (58–60). Further, where no associations have been found, there are also no adverse effects reported for infant outcomes (61) suggesting that with careful monitoring of dosage, the potential benefits to infant development could outweigh the potential risks.

Supplementation with multiple micronutrients, although limited, has been found to be superior to single nutrient and iron+folic acid/iodine+folic acid supplements for improving birth outcomes (62), cognitive and motor development at 7 months (63) and increased scores of communication, motor and social skills at 36 months old (64). Multiple nutrients are known to work in combination with each other to exert their effect rather than in isolation, providing a potential explanation for this observed superiority over single-nutrient supplementation (65).

Despite the reported association between antenatal depression and infant temperament (17, 19–25), the specific mechanisms of the association remain inconclusive. Negative affectivity and poor regulatory capacity have been strongly associated with maternal antenatal mood state (26, 66, 67). Thus, targeting antenatal depression may improve maternal mental health thereby resulting in a chain of biological and environmental changes which could positively impact infant temperament and developmental outcomes.

Healthier dietary patterns in pregnancy have been associated with improvements in infant affectivity and regulatory capacity, characteristic of an "easier" infant temperament and although not directly comparable to diet studies, improving maternal nutritional status via supplementation may have similar effects on infant temperament. Several nutrients contained within the BSM formula used in the current study are known co-factors required for the synthesis of serotonin, a neurotransmitter linked to emotion regulation (68). It is possible that increasing maternal concentrations of vital nutrient co-factors in pregnancy may influence both maternal and fetal serotonin production (69), impacting emotion regulation a key component of temperament.

The current study aimed to identify whether BSM supplementation given above the Recommended Dietary Allowance but typically below the Tolerable Upper Level (the highest level of daily nutrient intake that is likely to pose no risk of adverse health effects to almost all individuals in the general population) in a sample of pregnant women with antenatal depression was associated with any adverse risk to infant temperament (such as high negative affectivity or low regulatory capacity, characteristics of a more difficult temperament) (48), or differences in initial levels or developmental changes in infant temperament dimensions (NEG, PAS and ORC) across the first year of life.

Given the existing literature finding a positive association between healthier maternal nutrition and infant temperament, and the evidence for BSM as a treatment for improving psychiatric symptoms, we hypothesized BSM exposure would pose no adverse risk to infant temperament, predict higher initial levels of positive temperament behaviors (ORC and PAS) and be associated with developmental changes on par or better than non-exposed infants on measures of temperament across the three time points; specifically with lower scores on NEG, and higher scores of PAS and ORC on the IBQ-R:VSF.

2 Methods

In this longitudinal follow up study, a sample of 123 infants were followed for 12 months in Aotearoa, New Zealand. A final sample of 114 mother-infant dyads were included in data analysis. Further information on detailed grouping and flow of participants during data collection, reasons for non-completion and exclusions from data analysis are shown in Figure 1.

A portion of the current sample included 46 infants whose mothers had previously participated in a randomized, placebo-controlled trial (RCT) conducted between 2017 and 2021 (NUTRIMUM Trial: (70, 71)). For the RCT, participants between 12 and 24 weeks' gestation identified with depressive symptomology during pregnancy (Edinburgh Postnatal Depression Scale: EPDS ≥13), and not taking any psychiatric medication, were randomized to receive daily BSM or a placebo for 12 weeks during their pregnancy The BSM formula used in the NUTRIMUM study, Daily Essential Nutrients (DEN), contains 36 essential vitamins, minerals, amino acids and antioxidants, and this combination of nutrients has been explored as a treatment for other psychiatric illnesses in non-pregnant populations (65). For the full list of ingredients contained within DEN, see Table 1. The RCT phase was followed by an open-label phase of BSM until the birth of the infant, providing an opportunity for naturalistic observation of infant temperament in a micronutrient exposed group of infants.

Infants born to participants enrolled in the NUTRIMUM trial (BSM-exposed group) were either exposed to micronutrients during *both* the RCT phase and the open label phase or exposed to the active placebo during the RCT phase and only exposed to micronutrients in the open label phase. Additionally, given that mothers could be between 12 to 24 weeks gestation when they started the RCT, this resulted in varying days of possible exposure to the micronutrients *in-utero*, from zero days up to 196 days.

For example, if a participant entered the study at 19 weeks' gestation and was randomized to the active placebo group during RCT and gave birth at 40 weeks' gestation, they would enter the open label phase at 31 weeks' gestation and micronutrient exposure would be 70 days. However, if a participant entered the study at 12 weeks' gestation and was randomized to the micronutrient group during RCT, and gave birth at 36 weeks' gestation, they would enter the open label phase at 24 weeks' gestation and micronutrient exposure would be 168 days.

The NUTRIMUM trial was prospectively registered: Australian and New Zealand Clinical Trials Registry; ACTRN12617000354381, and the overall study received ethical approval from relevant university and national ethical review boards.

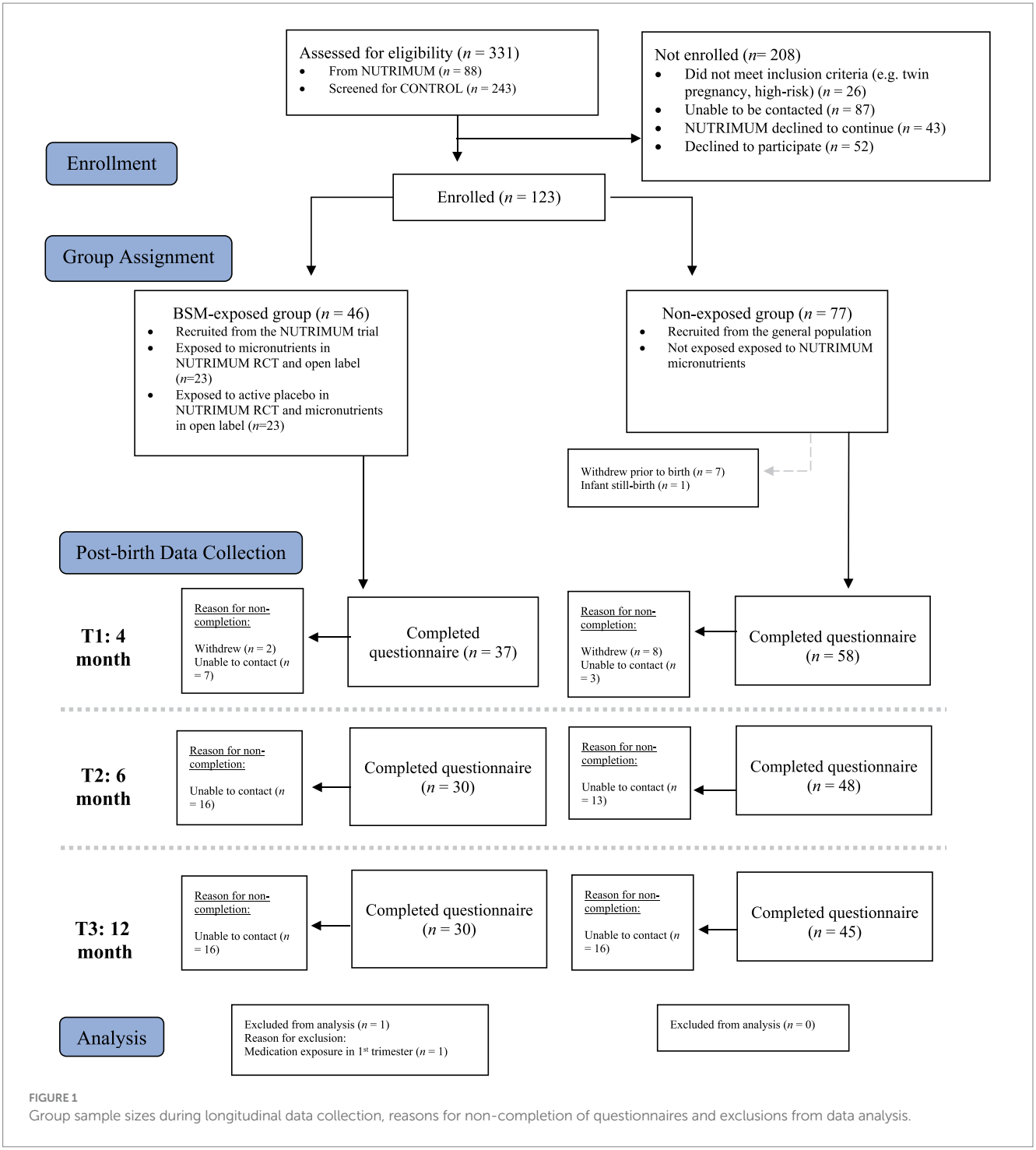

FIGURE 1
Group sample sizes during longitudinal data collection, reasons for non-completion of questionnaires and exclusions from data analysis.

The remainder of the sample ($n = 77$) was recruited from the general population and included infants born to mothers not receiving the NUTRIMUM trial supplement although could be experiencing mood symptoms (measured on a continuum) or being treated for antenatal mood symptoms with antidepressants (SSRI: $n = 21$; SNRI: $n = 3$). Current nutrient supplementation status was collected at study entry: 71.8% of the sample not enrolled in the NUTRIMUM trial (non-exposed group) reported taking a daily nutrient supplement (e.g., folic acid, iodine, B vitamins, pregnancy multivitamin), below the Recommended Dietary Allowance (RDA), and significantly lower doses than those provided to the MN group as part of the NUTRIMUM trial.

Inclusion criteria for all participants: (1) pregnant and between 12–34 weeks' gestation, (2) aged ≥16 years, and (3) a low-risk singleton pregnancy. Exclusion criteria for all participants included: (1) women with pregnancy complications or high-risk pregnancy (e.g., placenta previa, preeclampsia), (2) known fetal abnormalities, (3) serious current or historical medical condition (e.g., hypertension, kidney disease), (4) known metabolic conditions (e.g., Wilson's disease, hemochromatosis), and (5) untreated or unstable thyroid disease and

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TABLE 1 Ingredients of micronutrient (BSM) intervention from the NUTRIMUM trial.

Daily Essential Nutrients Supplement Facts	
Total dose (4 capsules, 3 times daily)	
Vitamin A (as retinyl palmitate)	5,760 IU
Vitamin C (as ascorbic acid)	600 mg
Vitamin D (as cholecalciferol)	3,000 IU
Vitamin E (as d-alpha tocopheryl succinate)	360 IU
Vitamin K (75% as phylloquinone; 25% as menaquinone-7)	120 mcg
Thiamin (as thiamin mononitrate)	60 mg
Riboflavin	18 mg
Niacin (as niacinamide)	90 mg
Vitamin B6 (as pyridoxine hydrochloride)	69.9 mg
Folate (as L-methylfolate calcium)	801 mcg
Vitamin B12 (as methylcobalamin)	900 mcg
Biotin	1,080 mcg
Pantothenic acid (as d-calcium pantothenate)	30 mg
Calcium (as chelate)	1,320 mg
Iron (as chelate)	13.8 mg
Phosphorus (as chelate)	840 mg
Iodine (as chelate)	204 mcg
Magnesium (as chelate)	600 mg
Zinc (as chelate)	48 mg
Selenium (as chelate)	204 mcg
Copper (as chelate)	7.2 mg
Manganese (as chelate)	9.6 mg
Chromium (as chelate)	624 mcg
Molybdenum (as chelate)	144 mcg
Potassium (as chelate)	240 mg

Proprietary blend: Choline bitartrate, Alpha-lipoic acid, Mineral wax, Inositol, Acetyl-L-carnitine, Grape seed extract, Ginkgo biloba leaf extract, L-methionine, N-acetyl-L-cysteine, Boron (as chelate), Vanadium (as chelate), Lithium orotate (as chelate), Nickel (as chelate). Other ingredients: Vegetarian capsule (hypromellose), Microcrystalline cellulose, Magnesium stearate, Silicon dioxide, Titanium dioxide.

known neurological disorders (e.g., epilepsy, multiple sclerosis, narcolepsy). Initial recruitment was confined to participants residing in Canterbury, New Zealand; however, due to the COVID-19 lockdown in March 2020, the study was adapted to work remotely, and enrolments were opened to anyone residing anywhere in New Zealand who met initial inclusion criteria ($n = 7$).

3 Procedure

Eligible participants were invited to attend an initial appointment either at the study site or via telephone/video call, where the study was explained and written informed consent was obtained. Individuals who screened but not eligible were informed via email and provided links to support services and encouraged to contact their GP or lead maternity carer for any additional psychological support.

Enrolled participants were monitored throughout pregnancy via online questionnaires every 4 weeks using Qualtrics Survey software. The BSM exposed group were monitored at a higher frequency (every 2 weeks) via online questionnaires and met with a clinician either in-person or via telephone/video call every 4 weeks as part of their enrolment in the NUTRIMUM Trial to monitor mood and any potential side effects of the RCT intervention. After birth, all participants completed questionnaires at 4 and 6 months postpartum, either completed at the study site or online via email link for those who did not live locally. Participants who traveled to the study site received a NZ$10 petrol voucher for each visit to cover travels costs. At 12 months postpartum, participants were sent an email link to an online questionnaire and upon completion, received a $20 petrol voucher via mail to thank them for their time.

4 Measures

4.1 Primary measure

Maternal perceptions of infant temperament was assessed using the Infant Behavior Questionnaire–Revised: Very Short Form

(IBQ-R:VSF) (15), which is a 37-item self-report questionnaire based on the Infant Behavior Questionnaire–Revised (IBQ-R) (16). It contains three subscales: *PAS* (e.g., How often during the last week did the baby smile or laugh when given a toy?), *NEG* (e.g., When you were busy with another activity, and your baby was not able to get your attention, how often did s/he cry?) and *ORC* (e.g., When showing the baby something to look at, how often did s/he soothe immediately?).

The Infant Behavior Questionnaires (IBQ) (72) are the most widely used measure of infant temperament (15) and the revised very short version (IBQ-R:VSF) was most appropriate given it was originally developed for use in longitudinal studies and is suitable for repeated measures and time-sensitive administration (15).

Mothers completed the IBQ-R:VSF at 4-, 6- and 12-months where they were asked the frequency of specific behaviors over a seven-day period. Each question was answered on an eight-point scale with responses ranging from (1) never to (7) always. In the event certain behaviors did not arise within the past week, a "does not apply" option was available. Responses for each of the three subscales were averaged, and interpreted on a continuum, with higher scores indicating greater display of that temperament dimension. The internal consistency of the IBQ-R:VSF scales is between 0.70 to 0.92 (15).

4.2 Additional measures

Information about maternal mental health and nutritional status was collected via online questionnaires at study entry, throughout pregnancy and post-birth. Information about infant anthropometric characteristics (e.g., gestational age, weight) was obtained through hospital records. Full details and references on measures used are explained elsewhere (70).

The Edinburgh Postnatal Depression Scale (EPDS) has shown strong validity for use in measuring depressive symptoms during pregnancy and the postpartum period (73, 74) and has a Cronbach's alpha of 0.83, indicating good internal consistency (75). A cutoff of 13 was used to identify the presence of moderate depressive symptoms (75). An average antenatal depression score was calculated for each participant based on monthly EPDS scores collected during pregnancy.

A variable was created to determine the occurrence of breastfeeding postnatally. Participants were grouped (lowest to highest level of occurrence) based on whether they had never breastfed, breastfed on and off (e.g., used a combination of breast and formula feeding) or exclusively breastfed. Breastfeeding occurrence has previously been associated with temperament.

4.3 Statistical analysis

Latent growth curve modeling using MPlus 8 was used to determine changes in temperamental outcomes across time. To start, we screened for univariate outliers, with criteria set to absolute values of skew <2 and kurtosis <7 (76). We examined whether micronutrient exposure was related to children's temperamental development using latent growth curve (LGC) modeling (77) in two main stages. In the first stage, we identified normative patterns of development for each temperament outcome (i.e., NEG, ORC and PAS) from T1 to T3 (T1: 4 month; T2: 6 month; T3: 12 month) by modeling two latent factors representing the initial status (i.e., intercept) and longitudinal change (i.e., slope). We identified the best fitting unconditional models by comparing three nested models using the χ^2 difference test. The models we compared were: (a) stability only model, wherein we only estimated an intercept factor, (b) linear change model, wherein we added a slope factor and fix loadings to 0, 1, and 4 (to account for the unequal spacing between timepoints—i.e., 4, 6, and 12 months), and (c) a nonlinear change model, wherein we freely estimated the T2 factor loading. In the second step, we ran a conditional growth curve model, which included our independent variables: micronutrient exposure as our focal predictor, as well as gestational age at birth, infant sex (0 = female; 1 = male), mean antenatal depression and breastfeeding occurrence as our control variables.

Model fit was evaluated using standard indices (78, 79). We considered the following criteria as reflective of acceptable fit: a non-significant chi-square test, a comparative-fit-index (CFI) and Tucker-Lewis-Index (TLI) > 0.90, root-mean-square-error-of-approximation (RMSEA) < 0.08 with 90% confidence intervals (CI), standardized root mean square residual (SRMR) < 0.08. Analyses were run using Mplus 8 (80) using maximum likelihood estimation of the parameters (ML). We handled missing data using Full Information Maximum Likelihood (FIML) estimation because it is preferable to traditional approaches (e.g., listwise deletion, mean substitution) which have been shown to reduce power, underestimate variability, undermine the validity of sample characteristics, or a combination thereof (81, 82). However, listwise deletion was used to handle participants who provided no data at any of the timepoints of interest. We ran Little's MCAR test to evaluate whether our data were missing completely at random (MCAR), which would suggest that our missing data could be estimated reasonably using observed data.

5 Results

5.1 Sample characteristics

Mean maternal age was 31.4 years, and 77.2% European. Mean length of exposure to BSM was 104 days ($SD = 44.17$; range = 12–182 days). Mean infant gestational age was 39.4 weeks ($SD = 1.5$). Further sample information can be found in Table 2.

5.2 Orienting/regulatory capacity

For orienting/regulatory capacity, the best fitting unconditional model was the linear change model, which fit the data well $\chi^2(1) = 1.175$, $p = 0.278$, $CFI = 0.997$, $RMSEA = 0.041$ (90% [CI = 0.000, 0.269]), $SRMR = 0.019$. The variance ($s^2 = 0.289$, $p = 0.000$) of the intercept was significant, suggesting that participants started with different initial levels of orienting/regulatory capacity at T1. The mean of the slope revealed a decrease on orienting/regulatory capacity from T1 to T3 ($M = -0.069$, $p = 0.001$), while the variance on the slope was not significant ($s^2 = 0.023$, $p = 0.081$) indicating although there was an overall group decrease in orienting/regulatory capacity over time, there were not significant interindividual differences in how participants' scores varied across time.

Next, we tested a conditional model assessing whether micronutrient exposure predicted the intercept or the slope of orienting/regulatory capacity (results displayed in Table 3). The

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TABLE 2 Maternal demographic characteristics at study entry*.

	Full sample ($N = 114$)		BSM-exposed ($n = 45$)		Non-exposed ($n = 69$)		Group effect	
	n / M	*% / SD*	*n / M*	*% / SD*	*n / M*	*% / SD*	χ^2/η_p^2	*p*
Maternal ethnicity							9.78	0.082
NZ Māori	7	6.1	2	4.4	5	7.2		
Pacific Island (Tongan, Samoan, Fijian, Niuean)	3	2.6	2	4.4	1	1.4		
Asian	6	5.3	5	11.1	1	1.4		
MELAA	7	6.1	3	6.7	4	5.8		
European (New Zealand, British, Australia, Italian)	88	77.2	30	66.7	58	84.1		
Household income							2.14	0.344
Low ($0 - $39,999)	19	16.7	10	22.2	9	13		
Middle ($40,000 - $79,999)	39	34.2	16	35.5	23	33.3		
High ($80,000+)	56	49.1	19	42.2	37	53.6		
Maternal background characteristics								
Young mother (< 21 years)	3	2.6	1	2.2	2	2.9	0.049	0.825
Single parent family	8	7.0	4	8.9	4	5.8	0.399	0.528
Ethnic minority	21	18.4	11	24.4	10	14.5	1.79	0.180
Low educational qualification	16	14.0	8	17.8	8	11.6	0.863	0.353
Low SES (NZSEI-13)	9	7.9	4	8.9	5	7.2	0.101	0.751
Total social risk score (M)	0.5	0.7	0.6	0.7	0.4	0.7	0.020	0.137
Maternal clinical characteristics								
Age (M)	31.4	4.6	32.1	4.7	30.9	4.6	0.016	0.177
SES status (NZSEI-13) (M)	56.9	16.8	54.8	17.6	58.3	16.3	0.010	0.290
Pregnancy Alcohol use	21	18.4	8	17.8	13	18.8	0.021	0.886
Current Smoker	3	2.6	0	0	3	4.3	2.01	0.156
Pregnancy Drug use	5	4.4	2	4	3	4.3	0.001	0.980
Maternal antenatal wellbeing (at study entry)								
EPDS score	11.9	6.4	16.5	2.71	8.9	6.35	0.337	**<0.001**
GAD-7	7.1	5.0	8.8	4.2	6.1	5.15	0.072	0.004
PSS [a]	17.6	7.2	21.6	4.8	15.1	7.4	0.193	**<0.001**
DASS-21								
Depression	8.9	7.6	12.4	7.2	6.6	7.0	0.142	**<0.001**
Anxiety	6.5	5.8	6.9	5.9	6.2	5.8	0.004	0.530
Stress	14.2	8.0	17.1	6.1	12.3	8.5	0.087	**0.001**
Nutrition score (DST) (M)[b]	66.5	9.4	66.2	7.5	66.8	10.5	0.001	0.733
Not at risk (n, %)[b]	12	10.5	2	4.55	10	14.3		
Possible risk (n, %)[b]	77	67.5	34	75.5	43	62.3		
At risk (n, %)[b]	23	20.2	9	20.45	14	20.0		
Mean EPDS score through pregnancy	8.6	4.2	9.5	2.5	8.0	4.9	0.032	0.058
Infant clinical characteristic (at birth)								
Female sex	54	47.4	18	40	36	52.17	1.62	0.203
Gestational age (M)	39.4	1.5	39.6	1.3	39.3	1.7	0.010	0.298

MN, exposed to BSM in-utero; CONTROL, not exposed to BSM in-utero; SES, socioeconomic status; NZSEI-13, New Zealand socioeconomic status index 2013; EPDS, Edinburgh Postnatal Depression Scale (≥13 is presence of moderate depressive symptoms; GAD-7, Generalized Anxiety Scale; PSS, Perceived Stress Scale; DASS, Depression, Anxiety, Stress Scale). *This table indicates demographics for mother-infant dyads that were still currently active at the time of infant birth, which is considered the beginning of the postpartum period; a, missing data; MN ($n = 43$); b, missing data; Control ($n = 67$). The Generalized Anxiety Scale (GAD-7) is a self-report measure used to identify generalized anxiety symptoms over the past 2 weeks; scores range from 0 to 21, with higher scores identifying more severe symptoms. The Perceived Stress Scale (PSS) is a self-report measure used to identify an individual's perceived level of stress over the past 4 weeks. Scores range from 0 to 40 (with higher scores indicating higher stress). The Depression, Anxiety, Stress scale (DASS-21) is a 21-item self-report measure with three subscales, each measuring the severity of depression, anxiety and stress ranging from normal to extremely severe. Each scale has a maximum score of 21, with elevated scores indicating more severe symptoms on that domain. The Dietary Screening Tool (DST) was adapted for a New Zealand population and used to collect information on nutritional status and dietary intake at study entry. It has a total score of 100, with higher scores indicating a healthier dietary pattern and scores below 60 indicating at nutritional risk.

TABLE 3 Conditional latent growth curve model—orienting/regulatory capacity.

	Intercept			Slope		
	b	β	*p*	*b*	β	*p*
BSM Exposure	−0.001	0.057	0.626	0.001	0.266	0.060
Gestational age	−0.025	−0.058	0.611	−0.016	−0.169	0.158
Infant sex	0.227	0.179	0.065	−0.064	−0.227	0.085
Mean antenatal depression	−0.018	−0.115	0.216	−0.003	−0.097	0.433
Breastfeeding occurrence	0.142	0.143	0.197	−0.036	−0.163	0.231

TABLE 4 Conditional latent growth curve model—positive affectivity/surgency.

Positive affectivity/ surgency	Intercept			Slope		
	b	β	*p*	*b*	β	*p*
BSM Exposure	−0.001	−0.091	0.367	0.000	0.008	0.943
Gestational age	**0.102**	**0.171**	**0.048**	**−0.041**	**−0.311**	**0.000**
Infant sex	0.207	0.116	0.259	−0.038	−0.095	0.366
Mean antenatal depression	−0.002	−0.010	0.925	0.002	0.035	0.735
Breastfeeding occurrence	0.078	0.056	0.566	−0.013	−0.042	0.717

For bolded parameters, $p < 0.05$.

conditional model fit the data well $\chi^2(6) = 4.519$, $p = 0.606$, $CFI = 1.000$, $RMSEA = 0.000$ (90% CI [0.000, 0.109]), SRMR = 0.060). BSM exposure did not predict initial levels of orienting/regulatory capacity ($b = -0.001$, $\beta = -0.057$, $p = 0.626$). Infant sex ($b = 0.227$, $\beta = 0.179$, $p = 0.065$), gestational age at birth ($b = -0.025$, $\beta = -0.058$, $p = 0.611$), breastfeeding occurrence ($b = 0.142$, $\beta = 0.143$, $p = 0.197$) and mean antenatal depression ($b = -0.018$, $\beta = -0.115$, $p = 0.216$) also did not predict the intercept. Similarly, BSM exposure did not significantly predict longitudinal changes in orienting/regulatory capacity, though the effect was small and in the expected direction ($b = 0.001$, $\beta = 0.266$, $p = 0.060$). Infant sex ($b = -0.064$, $\beta = -0.227$, $p = 0.085$), gestational age at birth ($b = -0.016$, $\beta = -0.169$, $p = 0.158$), breastfeeding occurrence ($b = -0.036$, $\beta = -0.163$, $p = 0.231$), and mean antenatal depression ($b = -0.003$, $\beta = -0.097$, $p = 0.433$) also did not predict the slope of orienting/regulatory capacity. The model accounted for a moderate part of the variance of the intercept ($R^2 = 0.063$) and the slope ($R^2 = 0.164$) of orienting/regulatory capacity behavior.

5.3 Positive affectivity/surgency

For positive affectivity/surgency, the best fitting unconditional model was the nonlinear change model, which fit the data well, $\chi^2(1) = 0.216$, $p = 0.642$, $CFI = 1.000$, $RMSEA = 0.000$ (90% CI [0.000, 0.203]), $SRMR = 0.045$. In this model, the variance ($s^2 = 0.787$, $p = 0.000$) of the intercept was significant, suggesting that participants started with different initial levels of positive affectivity/surgency at T1. The mean and variance of the slope were significant, with the slope revealing an increase in positive affectivity/surgency from T1 to T3 ($M = 0.283$, $p = 0.000$) and the variance ($s^2 = 0.036$, $p = 0.000$) indicating significant interindividual differences in how participants' scores varied on positive affectivity/surgency across time.

We then tested a conditional model with the same predictor and control variables as above, to see whether they were predictive of the intercept or slope (results displayed in Table 4). The conditional model fit the data well $\chi^2(6) = 7.436$, $p = 0.282$, $CFI = 0.978$, $RMSEA = 0.048$ (90% CI [0.000, 0.144]), $SRMR = 0.045$). Gestational age at birth ($b = 0.102$, $\beta = 0.171$, $p = 0.048$) significantly predicted intercept, indicating higher gestational age was associated with a higher initial level of positive affectivity/surgency behavior. BSM exposure ($b = -0.001$, $\beta = -0.091$, $p = 0.367$) did not predict initial levels of positive affectivity/surgency. Infant sex ($b = 0.207$, $\beta = 0.116$, $p = 0.259$), breastfeeding occurrence ($b = 0.078$, $\beta = 0.056$, $p = 0.566$), and mean antenatal depression ($b = -0.002$, $\beta = -0.010$, $p = 0.925$) also did not predict the intercept. Similarly, BSM exposure did not predict longitudinal changes in positive affectivity/surgency ($b = 0.000$, $\beta = 0.008$, $p = 0.943$). Infant sex ($b = -0.038$, $\beta = -0.095$, $p = 0.366$), breastfeeding occurrence ($b = -0.013$, $\beta = -0.042$, $p = 0.717$), and mean antenatal depression ($b = 0.002$, $\beta = 0.035$, $p = 0.735$) also did not predict the slope of positive affectivity/surgency. Gestational age at birth ($b = -0.041$, $\beta = -0.311$, $p = 0.000$) significantly predicted the slope positively, indicating that infants with a higher gestational age at birth showed lower intraindividual increases in positive affectivity/surgency over time. The model accounted for a moderate part of the variance of the intercept ($R^2 = 0.055$) and the slope ($R^2 = 0.121$) of positive affectivity/surgency behavior.

5.4 Negative affectivity

Finally, we tested the unconditional models for negative affectivity. The linear change model fit the data well, $\chi^2(1) = 0.028$, $p = 0.868$, $CFI = 1.000$, $RMSEA = 0.000$ (90% CI [0.000, 0.138]), $SRMR = 0.004$. In this model, the variance ($s^2 = 0.511$, $p = 0.000$) of the intercept was significant, suggesting that participants started with different initial

TABLE 5 Conditional latent growth curve model—negative affectivity.

	Intercept			Slope		
	b	β	*p*	b	β	*p*
BSM Exposure	−0.001	−0.116	0.368	0.000	−0.235	0.490
Gestational age	−0.013	−0.026	0.807	0.008	0.118	0.653
Infant sex	0.007	0.005	0.971	0.050	0.250	0.597
Mean antenatal depression	0.028	0.163	0.197	0.006	0.243	0.508
Breastfeeding occurrence	**0.248**	**0.220**	**0.045**	0.039	0.245	0.553

For bolded parameters, $p < 0.05$.

levels of negative affectivity at T1. The mean of the slope revealed an increase on average negative affectivity from T1 to T3 (M = 0.123, $p = 0.000$); however, the variance on the slope was not significant indicating there were not significant differences between participants in the overall increase ($s^2 = 0.015$, $p = 0.608$).

In the conditional model for negative affectivity, we included the same control variables as in previous models to determine if they predicted the slope or intercept of negative affectivity (results displayed in Table 5). The conditional model fit the data well $\chi^2(6) = 2.347$, $p = 0.885$), *CFI* = 1.000, *RMSEA* = 0.000 (90% CI [0.000, 0.061], *SRMR* = 0.022 (results displayed in Table 3). BSM exposure did not predict initial levels of children's negative affectivity ($b = -0.001$, $\beta = -0.116$, $p = 0.368$). Infant sex ($b = 0.007$, $\beta = 0.005$, $p = 0.971$), gestational age at birth ($b = -0.013$, $\beta = -0.026$, $p = 0.807$), and mean antenatal depression ($b = 0.028$, $\beta = 0.163$, $p = 0.197$), also did not predict the intercept. Breastfeeding occurrence ($b = 0.248$, $\beta = 0.220$, $p = 0.045$) significantly predicted the intercept, indicating higher occurrences of breastfeeding were associated with higher initial levels of negative affectivity. BSM exposure ($b = 0.000$, $\beta = -0.235$, $p = 0.490$) did not significantly predict longitudinal changes in negative affectivity. Infant sex ($b = 0.050$, $\beta = 0.250$, $p = 0.597$), gestational age at birth ($b = 0.008$, $\beta = 0.118$, $p = 0.653$), breastfeeding occurrence ($b = 0.039$, $\beta = 0.245$, $p = 0.553$), and mean antenatal depression ($b = 0.006$, $\beta = 0.243$, $p = 0.508$), also did not predict the slope of negative affectivity. The model accounted for small part of the variance of the intercept ($R^2 = 0.096$) and the slope ($R^2 = 0.222$) of negative affectivity.

6 Discussion

Antenatal depression is a significant public health issue, and the limited treatment options available have significant limitations with respect to infant outcomes. Untreated, antenatal depression is associated with a more difficult temperament in the infants which is a risk factor for future psychopathology. For these reasons, we explored the use of BSM, given as a treatment for symptoms of antenatal depression, and its effect on infant temperament in the first year of life. This is the first study of its kind, and although there has been some investigation into the effect of *in-utero* micronutrient supplementation on infant development (83–86), influence on infant temperament has not been the main focus.

Across the three temperament dimensions assessed using the IBQ-R:VSF, the general trend over time within our sample was consistent between exposed and unexposed infants, with no significant differences, suggesting no adverse effects of *in-utero* BSM on infant temperamental outcomes in the first year of life. Given exposure to antenatal depression is associated with more negative displays of temperament, BSM-exposed infants may have been at greater risk of poorer outcomes; however, it appears *in-utero* exposure to BSM may mitigate the known risks associated with antenatal depression, as BSM-exposed infants displayed temperamental characteristics on par with typical pregnancies where symptoms of depression were not present.

The ORC unconditional model revealed a significant overall group decrease in orienting/regulatory capacity over time, and while this decrease may seem unexpected, given the general understanding that regulatory capacity increases over time as an infant develops, it is consistent with the effect of increased mobility. This increase in mobility as the infant ages likely leads to greater dissatisfaction with remaining stationary, and a growing desire for independence from a caregiver. This results in fewer behaviors associated with high loading on the ORC scale, typically seen more in younger infants, which contribute to the overall decreasing ORC score, e.g., measures of perceptual sensitivity, duration of orienting, cuddliness and low intensity pleasure.

The decrease in ORC observed in our results are consistent with existing literature suggesting that older infants are less likely to enjoy being held closely by a caregiver or be involved in quiet activities, have a preference for high intensity stimulation (16), possess increased ability to habituate to objects more rapidly and more control over attentional processes which allow infants to disengage from stimuli more efficiently as they develop (87).

Within our sample, higher gestational age at birth predicted smaller individual increases in positive affectivity over time. From a developmental perspective, it is purported that infants with an increased gestational age may have been marginally more developmentally advanced initially. Given this initial advantage, it appears these infants displayed a slower rate of growth compared to those of a lower gestational age, which results in the observed lack of longitudinal change.

The significant association between higher breastfeeding occurrences and negative affectivity is also consistent with previous literature (87, 88). Infants of breast-fed mothers have been identified as more irritable, displaying more negative affect and fussiness compared to mixed-fed and formula-fed infants (89–92). The increased displays of negative affect (e.g., crying and irritability) may stem from the perceived stress associated with mastering a successful latch for both mother and baby.

Given the role that temperament plays in the dyadic nature of the mother–infant relationship, every effort should be made to protect vulnerable infants from challenges that could arise related to social and emotional development. BSM appears to be a promising option

given its success in treating antenatal depression (71), and the current study provides reassurance in its safety with relation to infant temperament, suggesting that by mitigating the risks associated with antenatal depression, we can set these potentially at-risk infants on a more positive developmental trajectory.

7 Strengths, limitations and future research

The present study involved a longitudinal multi-trait assessment of infant temperament across the first year of life. Using latent growth curve modeling, we could disentangle within- and between-child effects to closely examine whether BSM exposure predicted either higher initial levels or developmental changes in temperament. To our knowledge, this is the first study to examine how BSM supplementation during pregnancy relates to infant temperament. Antenatal depression is a known risk factor for a more difficult infant temperament, thus assessing the impact of a nutritional intervention used as a treatment for antenatal depression provides vital information on whether some of those risks can be mitigated.

Limitations included a modest sample size with likely underpowered statistical analyses which may explain the lack of significant findings. Future studies should sample a larger group of mothers to provide adequate statistical power to evaluate these research questions and include a more diverse population (e.g., ethnic minority, low socioeconomic status). Still, the findings from this study could be preliminarily informative, for instance, by examining the findings from a perspective focused on effect sizes rather than null hypothesis significance tests. Through this lens, the role of BSM exposure appears more positive: The three effects that met Cohen's 1988 criteria for a "small" standardized beta (i.e., ≥ 0.10) (93) were all favorable: BSM exposure on longitudinal changes in orienting/regulatory capacity ($\beta = 0.266$) as well as on initial levels and longitudinal changes in negative affectivity ($\beta s = -0.116$ and -0.235). The direction of these correlations indicate that BSM exposure does not negatively impact infant temperament in the first year of life and may exert a small positive influence.

Another potential limitation involved using mothers who had experienced antenatal depression as informants of their children's temperament as maternal depression has been associated with informant discrepancies of children's functioning (94). It is possible that mothers whose antenatal depression improved via BSM exposure tended to perceive and rate their children's temperament more favorably than those whose depression did not improve at all or did improve but not to the same degree. Nevertheless, we expect that any effects of maternal depression would remain relatively stable across assessments and therefore be partial out by the intercept. This means that inter-individual differences in maternal depression would not account for the links between BSM exposure and within-infant changes in temperament over time [i.e., latent slopes; for a related discussion, see (95)].

Finally, there is existing evidence that maternal diet can impact temperament, thus it is plausible that diet may impact infant temperament differently to nutrient supplementation. Although information was collected on maternal nutritional risk based on dietary intake at study entry, the questionnaire only assessed nutritional risk based on food consumed within the past 7 days, thus is not appropriate to infer a dietary pattern across gestation.

8 Conclusion

Managing maternal mental health has become a central component in antenatal care, and a recent RCT showed that introducing a BSM regimen for pregnant women with antenatal depression could result in meaningful improvements in their mental health (71) in addition to positively influencing infant birth and neurobehavioral outcomes in the first weeks of life (71, 86). The current study investigated whether this BSM exposure had any impact on the temperament of these women's infants across their first year of life. At the very least, our results indicate that BSM is effective in mitigating the risks associated with untreated antenatal depression, do not appear to increase any adverse risk to the infant temperament longitudinally, and may even indicate a small but positive effect.

Data availability statement

The raw data supporting the conclusions of this article are not readily available as the participant's consent has not been provided. The original MPlus data files are available. Requests to access these files should be directed to the corresponding author, julia.rucklidge@canterbury.ac.nz.

Ethics statement

The studies involving humans were approved by Southern Human and Disabilities Ethics Committee (ref: 16/STH/187) and the Standing Committee on Therapeutic Trials (ref: 16/SCOTT/131). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

SC: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing. SD: Formal analysis, Supervision, Writing – review & editing. JH: Conceptualization, Data curation, Methodology, Supervision, Writing – review & editing. HB: Conceptualization, Data curation, Methodology, Writing – review & editing. JR: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Microbiota gut-brain axis: implications for pediatric-onset leukodystrophies

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Neurodegenerative disorders are a group of diseases characterized by progressive degeneration of the nervous system, leading to a gradual loss of previously acquired motor, sensory and/or cognitive functions. Leukodystrophies are amongst the most frequent childhood-onset neurodegenerative diseases and primarily affect the white matter of the brain, often resulting in neuro-motor disability. Notably, gastrointestinal (GI) symptoms and complications, such as gastroesophageal reflux disease (GERD) and dysphagia, significantly impact patients' quality of life, highlighting the need for comprehensive management strategies. Gut dysbiosis, characterized by microbial imbalance, has been implicated in various GI disorders and neurodegenerative diseases. This narrative review explores the intricate relationship between GI symptoms, Gut Microbiota (GM), and neurodegeneration. Emerging evidence underscores the profound influence of GM on neurological functions via the microbiota gut-brain axis. Animal models have demonstrated alterations in GM composition associated with neuroinflammation and neurodegeneration. Our single-centre experience reveals a high prevalence of GI symptoms in leukodystrophy population, emphasizing the importance of gastroenterological assessment and nutritional intervention in affected children. The bidirectional relationship between GI disorders and neurodegeneration suggests a potential role of gut dysbiosis in disease progression. Prospective studies investigating the GM in leukodystrophies are essential to understand the role of gut-brain axis dysfunction in disease progression and identify novel therapeutic targets. In conclusion, elucidating the interplay between GI disorders, GM, and neurodegeneration holds promise for precision treatments aimed at improving patient outcomes and quality of life.

KEYWORDS

neurodegenerative disorders, leukodystrophies, gut-brain axis, dysbiosis, gut microbiota

1 Introduction

Neurodegenerative disorders are a group of diseases characterized by progressive degeneration of the structures composing the central and/or peripheral nervous system, leading to a gradual loss of previously acquired motor, sensory and/or cognitive functions. Most common neurodegenerative disorders are typical of adulthood, such as Parkinson disease, and

some forms of dementia (i.e., Alzheimer disease, Lewy body dementia, etc), while they are rarer entities in children. Childhood-onset neurodegenerative diseases pose unique challenges for paediatric neurologists as they may show overlapping symptoms with other neurological conditions; loss of motor skills, cognitive deterioration, feeding difficulties, and vision and/or hearing impairment are common features of different neurological diseases, and often the same disease may display different clinical presentations. An algorithm for the management of children with suspected neurodegenerative disorders and a classification system for these conditions, based on the prominently involved structures (i.e., disorders with prominent involvement of cerebral grey matter, leukoencephalopathies, etc.) has been developed (1). Leukodystrophies make up a significant proportion of pediatric-onset neurodegenerative conditions (2).

2 Leukodystrophies

Leukodystrophies are a heterogeneous group of rare genetic neurodegenerative disorders that affect children, primarily involving the white matter of the brain (3). Leukodystrophies can be classified upon the white matter component primarily involved and can be distinguished in myelin disorders, astrocytopathies, leukoaxonopathies, microgliopathies and leukovasculopathies (4). According to the neuroradiological patterns we can define hypomyelinating forms, characterized by an arrest of the formation and maturation of myelin, and other disorders, mainly represented by demyelinating forms, characterized by progressive degeneration of the white matter (4). A consensus has been built among a panel of leukodystrophy specialists regarding the definition of the term leukodystrophy. The panel comprehensively identified disorders that align with the established definition, creating a list of known leukodystrophies. Additionally, the group introduced the term 'genetic leukoencephalopathy (gLE)' to describe hereditary disorders causing white matter abnormalities that do not strictly meet the criteria for leukodystrophies (3). Although aetiology varies across conditions, an alteration in metabolic/cytohistological processes commonly represents the disease cause, and neuroinflammation might boost disease progression (5).

From a clinical point of view, the involvement of white matter tracts almost always impacts motor abilities, leading to various degrees of motor impairment usually related to pyramidal signs and/or ataxia. Other variable symptoms may include extrapyramidal movement disorders (mainly dystonia), seizures, delays or changes in cognitive development over time, visual and auditory impairment, extra neurological signs and symptoms depending on the specific disorder (6).

The hereditary nature of leukodystrophies, combined with their monogenic origin, has facilitated the development of some animal models. These are extensively employed in biomedical research because of their potential to replicate some aspects of human diseases, thus enabling an in-depth investigation of pathophysiological processes. Rutherford and Hamilton (7) provided a review of animal models of some of the most common leukodystrophies, such as X-linked adrenoleukodystrophy (X-ALD), metachromatic leukodystrophy (MLD), Krabbe's disease (KD), Alexander disease (AxD), and Aicardi-Goutières syndrome (AGS) and highlighted their usefulness in identifying new cellular drivers and their potential target for new therapeutic strategies (7). Though, despite their significant contribution in understanding leukodystrophies pathogenesis, reliability on disease progression and response to experimental treatments remain scarce, largely due to the lack of animal models that fully and adequately mimic human disease, particularly white matter pathology. The translational gap necessitates the use of complementary methodologies, such as computational models, human cell-based systems, and clinical studies, to enhance the relevance and applicability of preclinical findings to human health and disease.

3 GI disorders in leukodystrophies and nutritional interventions: insights from literature

Leukodystrophies often entail life-challenging gastrointestinal (GI) complications, with gastroesophageal reflux disease (GERD), recurrent vomiting, and bowel dysfunction being the most frequent concerns, often affecting appetite and growth patterns (8). In addition, dysphagia is a very frequent, disabling and sometimes fatal symptom. It is linked with the risk of malnutrition and exposes patients to the dangers of aspiration pneumonia or airway obstruction (9). It recognizes a multifactorial origin (neurogenic, postural, iatrogenic, upper gastrointestinal tract dysfunction) and can cause dehydration, chronic malnutrition, failure to thrive, and depletion of essential nutrients (10). Anorexia has also been described in leukodystrophies (11).

GI disorders represent a challenging problem and significantly increase the burden of disease in these patients. They can primarily be related to disease pathogenesis, such as in AxD (12), or can be a consequence of severe neurological disability, like what is usually observed in cerebral palsy (13). Sometimes, an earlier onset of GI complications has been related to an earlier disease onset, as described in MLD (14). A proper nutritional assessment and intervention can ameliorate the nutritional status of children with leukodystrophies (10). Given the extreme phenotypic variability, nutritional intervention must be directed to meet the individual patient's needs, usually targeting the specific symptoms and complications to improve patients' quality of life. Specific dietetic approaches have been explored as therapeutic intervention for some leukodystrophies. Ketogenic diet has shown to promote myelination in mouse models of Pelizaeus Merzbacher Disease (15), and has been administered in isolated cases of leukodystrophy (16, 17). Additionally, it is well known that dietary intervention plays a significant role in X-ALD, with a diet that is primarily characterized by the restriction of Very Long Chain Fatty Acids (VLCFA) and the augmentation of peroxisomal beta-oxidation through the administration of a combination of antioxidant compounds, conjugated linoleic acid (CLA), and Lorenzo's oil (LO) [a 4:1 mixture of glyceryl trioleate (GTO) (C18:1 n-9) and glyceryl trierucate (GTE) (C22:1 n-9)], conjugated linoleic acid (CLA), and antioxidants (18).

4 GI disorders in leukodystrophies: an Italian single center experience

Out of 175 patients referred to our Centre for Diagnosis and Care of Leukodystrophies and Associated Conditions (C.O.A.L.A.) at *V. Buzzi* Children's Hospital in Milan, Italy, who were diagnosed with either a leukodystrophy or a genetic leukoencephalopathy (Supplemenatry Table S1), data on gastrointestinal symptoms were available for 133 (76%). More than half of our cohort (75 patients, 56.4%) had GI manifestations. 35 individuals (26.3% of the cohort)

TABLE 1 Distribution of GI symptoms in the population affected by leukodystrophies or genetic leukoencephalopathies referred to the centre for diagnosis and care of leukodystrophies and associated conditions (C.O.A.L.A.) at V. Buzzi Children's Hospital in Milan, Italy.

	N (%)	Mean age (y) at onset (range)	Age at onset (median, y)
Dysphagia/Feeding intolerance	49 (37.12)	5.77 (0–24)	3.67
Failure to thrive	36 (27.48)	1.66 (0–10)	0.75
GERD	29 (22.14)	1.47 (0–13)	0.40
Feeding tube placement	20 (15.27)	6.34 (0.13–21)	3.25
Complete reliance on feeding tube	12 (9.16)		
Recurrent vomiting	8 (6.11)	1.46 (0–6)	1
Liver dysfunction	4 (3.10)		
IBD	2 (1.54)		
Other GI symptoms	8 (6.2)		

reported one GI symptom, 15 (11.3%) were diagnosed with 2 gastrointestinal symptoms, while 3 or more manifestations were observed in 25 individuals (18.8%) (Table 1).

Dysphagia or feeding intolerance was the most reported manifestation, accounting up to 37.1% of our cohort, with a mean age at onset of 5.7 years (median 3.7). Failure to thrive (according to WHO or CDC growth charts)[1] was observed in more than a quarter of our patients (27.5%) and was reported at a mean age of 1.7 years, even if half of these patients had growth failure noted within the first year of life (median 0.75 year). GERD was also diagnosed early in life (mean age at onset 1.5 years, median 0.4 years) in 22.1% of our patients. 20 patients (15.3%) required feeding tube placement at a mean age of 6.3 years (median 3.25 years) and 12 (60%) had a complete reliance on gastric feeds (9.2% of the whole cohort). Recurrent vomiting (6.1%), liver dysfunction (3.1%), and inflammatory bowel disease (1.5%) were also reported. Other gastrointestinal abnormalities (e.g., stypsis, recurrent diarrhoea, abdominal pain, vomiting, nausea) were noted in 6.2% of patients (Table 1).

Our series highlights the relevance of GI disorders in patients affected by leukodystrophies. Emerging evidence underscores the intricate interplay between GI disorders and Gut Microbiota (GM), highlighting the bidirectional nature of this relationship, wherein GI disorders can perturb the delicate balance of GM composition (19). Alterations in GM, in turn, have been implicated in influencing the pathophysiology of neurodegenerative diseases (20). These findings underscore the critical importance of understanding and potentially modulating GM in the context of both GI and neurological health, thereby modulating the clinical outcomes (21). However, no studies have been conducted so far on GM and disease outcomes in patients with leukodystrophies.

5 Gastrointestinal disorders and gut microbiota

The human GI tract is one of the biggest interfaces between the host and the environment, with symbiotic microorganisms that offer many benefits to the host. The GM composition varies between individuals and evolves through the host's lifespan, and it is influenced by intrinsic and extrinsic factors (22). Among the major factors able to influence GM composition are the composition of maternal microbiota, maternal health and nutrition status before and during pregnancy, lactation, type of childbirth and diet. Geographic area of residence, antibiotic use, smoking exposure, as well as the health of immune system are also proven to impact GM (23, 24). Diet represents one of the main variables that affect the composition of GM, possibly leading to diversification of the microbial populations. The microbial composition of the small intestine plays an important role in modulating gastrointestinal processes such as secretion and motility and digestive functions, in addition to maintaining a tight communication with the CNS via the microbiota-gut-brain axis (MGBA) (25, 26).

The association between gastrointestinal disorders and microbiota alterations has been analysed in animal models. Kashyap et al. (27) utilized controlled mouse models to investigate the relationship between diet, transit time and GM. They demonstrated changes in gut microbial communities associated with variations in gut transit time by either speeding up or slowing down host gastrointestinal transit, administering polyethylene glycol or loperamide, respectively. These alterations in microbiota returned to normal levels after discontinuing the treatments. In contrast, introducing a diverse fecal microbiota from healthy humans into germ-free mice significantly reduced gastrointestinal transit time and enhanced colonic contractility. The different response depended on the quality and quantity of carbohydrates consumed with diet, as fermentable polysaccharides alter the composition of gut microbiota and the production of metabolites, i.e., short chain fatty acids (SCFAs) (27).

The intricate relationship between GI disorders and the GM is also the focus of several recent clinical studies, that explore the complex interplay between different microbial communities and various GI conditions. Irritable Bowel Syndrome (IBS) is a prevalent functional GI disorder characterized by recurrent abdominal pain and altered bowel function. It represents a good example of GI disorder, given the complex pathogenesis, that potentially involves genetic predisposition, environmental factors, and gut dysbiosis (28, 29). Through metagenomic analyses and 16S rRNA gene sequencing, a dysregulated GM composition has been unveiled in these patients, characterized by alterations in microbial diversity, abundance, and metabolic function (29). Dietary interventions have emerged as promising avenues for modulating gut microbial composition and alleviating IBS symptoms,

1 https://www.cdc.gov/growthcharts/clinical_charts.htm

underscoring the bidirectional relationship between diet, GM, and clinical outcomes (28, 29).

GM has been extensively studied in GERD as well. Indeed, intestinal dysbiosis has been described in cohorts of patients with GERD and seems to be associated not only to the pathogenesis of this condition itself (30), but also to the specific pharmacological treatment to which these patients are subjected (31). A recent review by Kiecka et al. provides an overview of the most important effects of long-term proton pump inhibitors (PPIs) use (32). Among them, gut dysbiosis, probably due to their mechanism of function, is reported. In fact, PPIs exert profound effects on gastric acid secretion, thereby altering the luminal pH and perturbing microbial equilibrium within the GI tract. To support this evidence, probiotic supplementation has emerged as a promising strategy for restoring gut microbial homeostasis and ameliorating adverse sequelae associated with PPI-induced dysbiosis by replenishing beneficial microbial strains and enhancing mucosal barrier function (32). Indeed probiotic strains, such as *Lactobacillus reuteri* (DSM 17938), have appeared promising, showing mitigating efects in children on PPIs therapy. Other interesting strains with potential protective function include *L. rhamnosus* LR06 (DSM 21021) or *L. pentosus* LPS01 (DSM 21980) (32).

Finally, numerous microbial products have been recognized as regulators of GI motility and are implicated in the pathogenesis of colonic motility disorders. These include short-chain fatty acids (SCFAs), bile acids, tryptamine, as well as various gaseous byproducts such as methane, hydrogen sulfide, and hydrogen gas (33, 34).

6 Gastrointestinal function and microbiota-gut-brain axis (MGBA): the bidirectional communication

The well-known close and bidirectional communication between brain and intestine happens via the microbiota-gut-brain axis (MGBA). GM can influence the systemic health by contributing to the signaling along the GBA, whereas the Central Nervous System (CNS), Enteric Nervous System (ENS), neuroendocrine and neuroimmune pathways are all involved in the bidirectional communication between the CNS and the GI tract (35, 36). Top-down communication refers to the transmission of information from brain-to-gut whereas the bottom-up to the one from gut-to-brain (37) (Figure 1).

6.1 The top-down communication

Recently, several studies have highlighted the influence of modulations in the GM on behavior and disease severity in animal models of neurodevelopmental, neurodegenerative, and psychiatric disorders (38, 39). It is fully understood that a communication between GM and CNS does exist, and it is referred to as MGBA, which plays a pivotal role in maintaining homeostasis in the gastrointestinal tract, CNS, and microbial systems. This regulation is achieved through a complex network of chemical transmitters, including endocrine hormones, microbial molecules, and metabolites (40). GM plays an important role in the regulation of neurodevelopmental processes, including blood–brain barrier (BBB) formation and integrity, microglial maturation and function, and myelination, whose disruption could have a role in neurodegenerative diseases (41). According to recent studies, the MGBA is essential for controlling several physiological functions as well as pathophysiologic processes (21). It is now evident that the gut has direct control over the brain, and the brain exerts an effect over the gut functions. Evidence in animal research derives from investigations on infections, antibiotics, and fecal transplants, as well as from germ-free animal models (21). Via the ENS, the Vagus nerve directly regulates different gut processes, many of which have an impact on the GM, gut motility, intestinal permeability, bile and enzyme secretion, mucus production, nutrient absorption, and satiation. In addition, the Vagus nerve regulates inflammation. To maintain equilibrium in the human organism, a balanced and healthy microbiota is crucial. The disruption of eubiosis (i.e the dysbiosis status) causes the loss of homeostasis, richness, and evenness of microbial species, favoring disease onset.

6.2 The bottom-up communication

Gut dysbiosis may result in chronic inflammation, which has critical effects on the brain. In fact, it promotes the aggregation of misfolded proteins around neurons at the CNS level, disrupting neuronal function, survival, and hence synaptic integrity. The death of neuronal cells leads to the release of misfolded neurotoxic aggregates, further exacerbating neuroinflammation (42).

Chronic inflammation and oxidative stress determined by gut dysbiosis have been explored in several neurodegenerative disorders, such as Parkinson disease (PD), Alzheimer's Disease (AD), Multiple sclerosis (MS) and Amyotrophic Lateral Sclerosis (ALS) (43). In PD, gut dysbiosis has been shown to trigger and promote α-synuclein fibril formation and dissemination, and the transplantation of fecal microbiota from PD patients to α-synuclein-overexpressing mice worsened inclusion bodies and parkinsonian symptoms compared to mice receiving healthy donor microbiota (40).

Research by Raval et al. (43) suggests a connection between GM dysbiosis and heightened inflammation and intestinal permeability in AD progression. Inflammatory reactions resulting from GM dysbiosis contribute to the breakdown of gut epithelial barriers, facilitating the entry of gut bacteria, fungi, and their products into the brain. Individuals with AD exhibit elevated bacterial levels within the brain compared to those without the condition. This invasion of GM components into the brain may contribute to both peripheral and central innate immune system dysfunction, characteristic of AD pathology (44). Furthermore, products derived from GM, such as lipopolysaccharides (LPS), microbial amyloid, and neurotoxins, have been implicated in neurodegeneration, amyloid-beta aggregation, neurofibrillary tangle formation, and neuroinflammation within the brain (45, 46).

Perturbations in the GM of children affected by MS compared to children without MS (47) and associations between GM and MS activity in children have been demonstrated (48). Studies about transplantation of MS patients' microbiota into two different animal models of MS have highlighted the importance of interleukin IL10-producing CD4 T cells in the immunomodulatory effects of the GM (49, 50). Furthermore, the presence of specific Gram-positive bacteria in the gastrointestinal tract, which activate Th17 cells, significantly affected the severity of the disease in mice (49). In addition, converging data from germ-free mice and antibiotic preclinical studies have

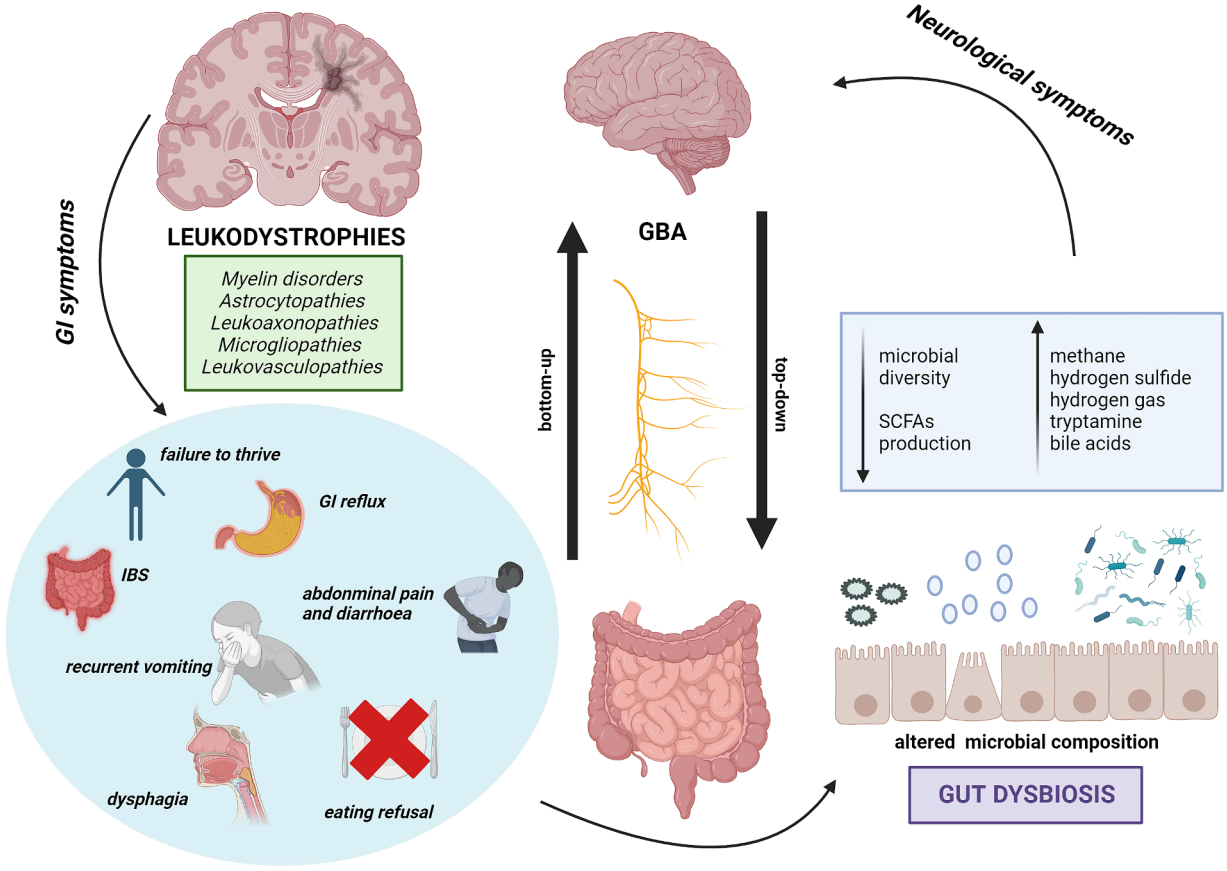

FIGURE 1
The bidirectional communication between GI disorders in Leukodystrophies and MGBA. Representation of the relationship between gastrointestinal symptoms in leukodystrophies and their potential impact on neurological severity. Key GI symptoms may determine alterations in GM, suggesting a link between dysbiosis and worsening of neurological symptoms. This implies a possible connection between GI issues in leukodystrophies and neurological severity.

implicated the microbiota in regulating myelin production in mouse prefrontal cortex (50, 51).

The relevance of GM has been also demonstrated in animal models of neurodegenerative disorders which usually have their onset in childhood. For example, autophagic dysfunction and GM dysbiosis have been demonstrated to cause chronic immune activation in the Drosophila model of Gaucher disease, through chronic activation of NF-kB signaling in the Gba1 loss-of-function model. Atilano et al. (52) observed that restoring microbiota or stimulating autophagy to remove immune mediators, rather than administering prolonged immunosuppression, may represent effective therapeutic avenues for GBA1-associated disorders. Kovàcs et al. (53) reported that the GM of mouse models of ceroid lipofuscinosis is altered as compared to wild-type mice. They demonstrated that acidified drinking water markedly changed the GM composition of Cln1 mice, reduced the abundance of the pro-inflammatory microorganisms, determined a decrease in the amount of lysosomal storage material in every brain region examined, reduced astrocytosis in the striatum and somatosensory cortex, attenuated microglial activation in the thalamus, and preserved the ability of Cln1 mice to climb down a vertical pole as quickly and proficiently as wild-type mice (53).

The composition of GM in neurodevelopmental disorders (ND) and its potential impact on brain functions and behaviors is the topic of a recent narrative review (54), which highlighted the role of gut microbes and their metabolites in directly or indirectly influencing brain function. In particular, it was noted that an increase in Clostridium spp. can lead to elevated production of indole, which suppresses the growth of beneficial bacteria like Bifidobacteria and Lactobacilli, ultimately affecting gamma-aminobutyric acid (GABA) levels (55). This mechanism has been associated with occurrences of stereotypies, hypersensitivity, and epilepsy. Furthermore, toxins produced by Clostridia exacerbate inflammatory responses. In other NDs, certain microbial species such as Enterobacteriaceae, Sutturella spp., and Erysipelotrichaceae also contribute to inflammation, leading to alterations in gut permeability and gastrointestinal symptoms (56). Additionally, a high protein diet in ND patients promotes the production of branched chain fatty acids (BCFAs) and propionate (57), with the latter showing behavioral impairment in animal models, suggesting the potential for microbiome-based treatments.

6.3 MGBA in leukodystrophies

Composition in GM has been explored in one adult-onset leukoencephalopathy, namely cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy

(CADASIL) (58). In the GM from 15 Japanese CADASIL patients, a notable rise in the presence of certain bacteria was observed, including Lachnospira, Odoribacter, Parvimonas, unidentified genera within Barnesiellaceae and Lachnospiraceae families, compared to paired controls. Conversely, there was a significant decrease in the presence of Megasphaera and Acidaminococcus. When comparing CADASIL subgroups, those who had experienced a stroke displayed a significant decrease in Phascolarctobacterium and Paraprevotella. Potential impact of certain genera on C-reactive protein levels was highlighted, as well as their role in stimulating the production of interleukin-10 (IL-10) and transforming growth factor-beta (TGF-β) (58), suggesting that GM composition may not only affect the onset but also the progression of CADASIL.

To the authors' knowledge, no studies have been conducted to date on MGBA and disease outcomes in leukodystrophies. Expanding upon the work that has been done with CADASIL, it could be worthwhile to investigate the potential effects of MGBA on the phenotype of other leukodystrophies. Indeed, there is often no clear genotype–phenotype association in these diseases, and current research has focused on potential phenotypic modifiers. Given the significant role that GI disorders play in leukodystrophies and the intricate relationships that drive MGBA, unraveling the eventual influence of GM on disease phenotype could mark a significant advancement in comprehending the remarkable phenotypic heterogeneity that has been noted in leukodystrophies.

7 Discussion

Several studies highlight the bidirectional link between gastrointestinal disorders and altered GM, and the existence of a gut-brain axis is nowadays widely accepted. Thus, a deeper understanding of how the gastrointestinal and nervous systems interact together with the GM mediation is needed. Studies on the impact of dysbiosis and MGBA dysfunction in neurological diseases are increasing, especially in the field of neurogenerative disorders. Though, studies on the role of gut-brain axis and microbiota alterations in paediatric-onset neurodegenerative conditions are scarce.

Basing on these assumptions and focusing on leukodystrophies and genetic leukoencephalopathies, which are among the most frequent neurodegenerative disease in children, we may speculate that GI disorders in patients with leukodystrophies may contribute to dysbiosis, leading to altered processes in both the gut and brain, and contributing to neurodegeneration. The loss of blood–brain barrier integrity, which may also be influenced by the GM, promotes the translocation of gut microbes and their metabolites, potentially contributing to inflammation, oxidative stress, pathological protein aggregation, abnormal proteolysis, and neuronal death. These processes are known to play crucial roles in the pathogenesis of various neurodegenerative disorders, including some leukodystrophies (59, 60). Furthermore, considering the essential role of the GM in immune system development and maturation, it is reasonable to suspect its involvement in the pathogenesis of neurodegenerative disorders with a significant inflammatory component (61–64).

To our knowledge, the literature lacks systematic studies investigating the prevalence of GI disorders in patients affected by leukodystrophies. A study conducted by Kay-Rivest et al. (9) reported dysphagia in 7 out of 12 (58%) leukodystrophy patients recruited, with 3 (43%) being completely reliant on a gastric tube. While these results may be slightly biased due to a small sample size, they are consistent with the findings in our cohort. Our results underscore the importance of conducting a comprehensive gastroenterological and nutritional assessment in children affected by white matter disorders. All children with leukodystrophies should have their growth patterns monitored using growth charts, and accurate dietary data are essential for adjusting food intake to promote growth and maintain gut eubiosis. In children affected by neurological impairment (NI) with long-term enteral nutrition, a significant impact on gut microbiota composition was found, which was in turn linked to an aggravation of their nutritional status (65). The significant prevalence of GI symptoms, such as dysphagia and GERD, underscores the need to deepen our understanding of the influence of the gut-brain axis on the clinical phenotype of these individuals. Therefore, prospective studies aimed at analysing the GM in these disorders are crucial, as our understanding of how gut environment affects neurodegenerative disorders may reshape treatment approaches. To this aim, it becomes relevant to identify adequate biomarkers that confirm and measure the impact of dysbiosis and gut-brain axis dysfunction on disease progression and examine the efficacy of innovative treatments targeting the GM, eventually evaluating the potential role of animal models in this process.

Therapies like biotics and faecal transplants offer potential for customized treatments to improve gut health and function, potentially reducing brain inflammation, limiting protein aggregate formation, and slowing disease progression. This shift toward considering the gut-brain connection as a potential treatment may represent a significant departure from conventional methods and holds promise for improving outcomes and quality of life in patients that deal with neurodegenerative diseases.

Author contributions

YV: Data curation, Investigation, Methodology, Resources, Writing – original draft, Writing – review & editing. FB: Data curation, Investigation, Methodology, Resources, Writing – original draft, Writing – review & editing. VT: Investigation, Methodology, Writing – original draft, Writing – review & editing. MT: Investigation, Methodology, Writing – original draft, Writing – review & editing. CM: Writing – review & editing, Conceptualization. GZ: Writing – review & editing. DT: Conceptualization, Writing – review & editing. EV: Conceptualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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Pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections and gut microbiota composition: what do we know?

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Post-streptococcal autoimmune neuropsychiatric disorders (PANDAS) are a group of pathological condition characterized by sudden-onset obsessive-compulsive and tic disorders following beta-hemolytic Streptococcus group A (GAS) infection, hypothesized to be caused by autoimmune mechanisms targeting the basal ganglia. Scant literature is available regarding the microbiota composition in children with PANDAS, however few studies support the hypothesis that streptococcal infections may alter gut microbiota composition in these patients, leading to chronic inflammation that may impact the brain function and behavior. Notable changes include reduced microbial diversity and shifts in bacterial populations, which affect metabolic functions crucial for neuroinflammation. Elevated serum levels of sNOX2-dp and isoprostanes indicate oxidative stress, while the presence of lipopolysaccharides (LPS) may contribute to neuroinflammation. The aim of this narrative review is to explore the link between PANDAS and gut microbiota composition. The potential connection between gut microbiota and neuropsychiatric symptoms in PANDAS might suggest the importance of dietary interventions, such as promoting the Mediterranean diet and fiber intake, to reduce the inflammatory state of this patients and therefore improve their outcome.

KEYWORDS

PANDAS, gut microbiota, nutrition, diet, gut-brain axis, oxidative stress

Introduction

Pediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcal Infections (PANDAS) represent a group of neurological tardive complications of *Streptococcus pyogenes* (beta-hemolytic Streptococcus group A, GAS) infection in childhood (1). PANDAS were first described as conditions of brain's neurologic function impairment resulting in the sudden manifestation of obsessive-compulsive disorders (OCD), tic disorders or other behavioral symptoms due to the complications of GAS infection (1, 2). The close relation with streptococcus infection led to the hypothesis of autoimmune pathogenesis of PANDAS. An autoantibody mimicry mechanism may cause progressive damage of basal ganglia, leading to neuropsychiatric behaviors (3). The diagnosis of PANDAS should be made in presence of OCD and/or tics, complex or not observable in other disorders, with an acute onset and severe episodic changes in behavior between the age of 3 years old and puberty, associated with GAS

infection confirmed by positive pharyngeal swab and/or increased titers of anti-streptolysin-O (ASLO) or anti-DNase B. PANDAS most frequently occur in male children or adolescents (4, 5). Characteristic symptoms are tics, hyperactivity, urinary urgency, anxiety, depression, impulsiveness, oppositional defiant disorder eating disorders, and a decline in school performance (1). Studies have shown that, after initial infection, disease exacerbations could be associated with other factors than GAS, such as different bacterial or viral infections, or internal stimuli like stress. Pediatric Acute-Onset Neuropsychiatric Syndrome (PANS) refers to the onset of similar symptoms secondary to other bacteria or viral infections (6, 7).

PANDAS treatment

Treatment of PANDAS mainly includes psychoactive drugs, immunotherapeutic with steroids, antibiotics, plasmapheresis, and intravenous immunoglobins (1). The administration of antibiotics, in particular penicillin, is useful only in case of active streptococcal infection to eradicate the bacteria (1, 6). Regarding tonsillectomy as a treatment, its effectiveness in limiting OCD symptoms is still debated. Further studies are needed to demonstrate clear evidence of benefit (8).

Most frequently used antipsychotic drugs include risperidone, for severe behavioral symptoms, selective serotonin re-uptake inhibitors (SSRIs), for the improvement of OCD symptoms, atomoxetine, used in presence of attention deficit hyperactivity disorder (ADHD), and lorazepam, for the improvement of motor activity and expressive language. Unfortunately, only few studies have inquired into the effects of psychiatric therapies in PANDAS. Additionally, this treatment includes also psychoactive medications and behavioral-cognitive therapy for children who present severe stress and anxiety. Finally, there is scarce evidence concerning PANDAS treatment with immunotherapy (1, 9): this includes therapies with corticosteroids or cyclooxygenase (COX) inhibitors, probiotic treatment, IVIG, and plasma exchange. Corticosteroid or non-steroidal anti-inflammatory drugs have been observed to reduce the duration of symptom flares, while IntraVenous ImmunoGlobulin (IVIG) and plasma exchange have been demonstrated to significantly reduce Yale-Brown Obsessive Compulsive Scale scores (6). Few studies have indicated that therapeutic manipulations of the composition of the gut microbiota might be an additional treatment for some neuropsychiatric symptoms.

The role of gut microbiota

The term microbiota refers to the composition of commensal microbes (bacteria, viruses, fungi) in the body of a healthy individual (1, 6). This complex system develops during intrauterine periods and is influenced by various factors as maternal antimicrobial treatments, vaccinations, exposure to chemicals, diet, type of delivery, and infant feeding habits. Research has explored the relationship between the gut microbiota and the development of psychiatric disorders (10, 11): in fact, the hypothesis of the microbiota-gut-brain axis could explain the correlation between the development of the central nervous and gastrointestinal homeostasis (12). Their communication occurs through a variety of complex mechanisms involving microbial metabolites, immune cells, tryptophan metabolism, neural and endocrine pathway: these mechanisms are important regulators of neurotransmitters such as γ-aminobutyric acid (GABA) (2). Neuropsychiatric disorders with a possible gastrointestinal etiology include autism, anorexia nervosa, anxiety, Parkinson's disease, Alzheimer's disease, attention deficit hyperactivity disorder (ADHD), schizophrenia, bipolar disorders, alcohol dependence, or migraine pain (1). Several preclinical and clinical studies suggested that alterations of microbiota are associated with neuroinflammation (3, 13). Furthermore, this relationship is well supported by studies that have investigated the effects of probiotics, antibiotics, or even germ-free animals on brain activity and function (3, 14, 15).

This narrative review aims at exploring the current knowledge about the connection between PANDAS and the composition of gut microbiota in children, underling the role of the communication along the microbiota gut-brain axis. For this narrative review, the authors have independently searched via PubMed/MedLine database articles published in the last 15 years (2009–2024), based on the following keywords: nutrition; diet; gut microbiota; Pediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcal Infection. A narrative synthesis approach was used to summarize the results of the included studies. The main relevant case–control studies available regarding the potential association between PANDAS and gut microbiota are two: the first one analyzed the microbiota composition in 30 children with PANDAS or PANS (2), while the most recent one focused on the determination of oxidative stress markers in 30 children with the same diseases (3).

PANDAS and gut-microbiota composition

According to the articles collected, evidence suggests that streptococcal infections may alter the composition of gut microbiota in pediatric patients with PANDAS, contributing to a persistent inflammatory state that might indirectly influence brain function and individual behavior. In a case–control study by Quagliariello et al. (2), gut microbiota of 30 patients affected by PANS/PANDAS was analyzed and compared with healthy individuals. In younger PANDAS patients (y-PAN, 4–8 years old), reduced microbial diversity (α-diversity) has been observed, with a significant increase in Bacteroidetes such as Bacteroides, Odoribacter, and Oscillospira, and reduction of Firmicutes and TM7 (Saccharibacteria) in comparison to healthy controls. The group of patients who discontinued antibiotic therapy and/or probiotic intake 2 to 4 months prior to the study showed higher levels of Bacteroidaceae, Rikenellaceae, and Odoribacteriaceae (2). Conversely, some Firmicutes families including Turicibacteraceae, Tissierellaceae, Gemellaceae, and Carnobacteriaceae (Bacilli class), Corynebacteriaceae, and Lachnospiraceae were absent.

The alteration of the microbiome is linked to crucial metabolic capacities such as glycan degradation and the production of short-chain fatty acids (SCFAs), known for their beneficial effects including anti-inflammatory properties and support for intestinal barrier integrity. When SCFA production is compromised due to altered microbiota, chronic inflammatory state in the gut may worsen, leading to neurological and behavioral alterations (2, 6). Association between high levels of Anti-Streptolysin O (ASLO) titers and specific genera of bacteria such as Dehalobacterium and Lactobacillus have also been observed in the same study, suggesting a complex interaction between immune response, intestinal microbiota, and neural functions (2).

Antibodies produced in response to streptococcal infections may influence the composition of the intestinal microbiota, potentially affecting dopamine receptors and other neural processes crucial for brain function, including those involved in tyrosine metabolism associated with neuronal dysfunctions observed in conditions like Parkinson's disease (2). Regarding treatment, while antibiotics are essential in treating acute Group A beta-hemolytic Streptococcus infections, their prolonged use raises concerns regarding their promotion of intestinal dysbiosis. Antibiotic therapy in PANDAS patients should therefore balance the need to treat the primary infection with preserving the integrity of the gut microbiota. Complementary strategies, such as the use of biotics may be considered to minimize the negative effects of antibiotics on the intestinal microbiota and potentially improve clinical outcomes (16, 17). Other studies confirm the potential link between gut microbiota and psychiatric symptoms, as highlighted by clinical studies utilizing fecal microbiota transplantation (FMT). Two studies conducted on children with Autism Spectrum Disorder (ASD) and gastrointestinal issues demonstrated significant results (18, 19). In the first study (18), 18 children received multiple sessions of FMT from healthy donors after clearing their own gut microbiota. Eight weeks after the last FMT session, gastrointestinal symptoms decreased by 77%, and ASD symptoms improved by 24%. This improvement was associated with an overall increase in microbiota diversity, including higher levels of Bifidobacterium, Prevotella, and Desulfovibrio genera. Two years after treatment, ASD and gastrointestinal symptoms had decreased by 47 and 58%, respectively, compared to pre-treatment levels. The second study (19), conducted on 40 children with ASD and gastrointestinal symptoms, reported similar findings. After 4 weeks of treatment with donor microbiota, gastrointestinal symptoms decreased by 35%, and ASD symptoms improved by 6%. It was observed that patients who responded positively to treatment showed a significant decrease in the prevalence of *Eubacterium coprostanoligenes* compared to non-responders. Additionally, a study involving adults with irritable bowel syndrome (IBS) without psychiatric diagnosis showed that FMT reduced non-clinical symptoms of depression and obsessive-compulsive symptoms. Although these studies are promising and suggest that FMT may positively influence psychiatric symptoms through changes in the composition of the intestinal microbiota, it is important to note that controlled placebo and double-blind studies are still lacking to confirm these effects and to fully understand the underlying mechanisms (6).

PANDAS and inflammation

Another interesting aspect of available evidence about children with PANDAS is the detection of elevated serum levels of soluble NOX2 derived peptide (sNOX2-dp) and isoprostanes, indicators of elevated systemic oxidative stress and relevant to the manifestation of neuropsychiatric symptoms of the disease. NOX2 activation has also been associated with other inflammatory neurological conditions, suggesting a potential mechanism through which streptococcal infection could influence the pathogenesis of PANDAS (3). Available data (3) also suggest that the immune response triggered by streptococcal infections may lead to the release of lipopolysaccharides (LPS) by gram-negative bacteria, passing from the gut into the bloodstream and potentially contributing to observed neuroinflammation. The evidence of a correlation between elevated levels of serum LPS, sNOX2-dp, and isoprostanes suggests the presence of a potential mechanism through which LPS could promote oxidative stress and neuroinflammation in the context of PANDAS.

The interaction between gut microbiota, antibiotics, and treatments such as prebiotics plays a significant role in gastrointestinal health and potentially also in psychological disorders. However, further research is needed to fully understand the mechanisms involved and optimize the clinical use of these therapies (20).

Discussion

The few studies available to date, regarding the gut microbiota composition in children with PANDAS, suggest that this group of patients may have an altered gut microbiota composition compared to healthy controls, as well as a different expression of specific metabolites involved in the inflammatory response, antibody production, and associated with brain function (Figure 1). The study by Loffredo et al. (3) has observed a correlation between increased levels of serum LPS, sNOX2-dp, and isoprostanes, suggesting that LPS might cause oxidative stress and neuroinflammation in PANDAS. Further studies are needed to analyze the association between NOX2 levels and the severity of the neurological manifestations in children with PANDAS, as well as the effects of a healthy dietary pattern useful to promote a healthy gut microbiota, or antioxidant substances on the activity of NOX2 and LPS in this population. The aim of such research would be to obtain new evidence to provide physicians with specific guidelines on managing children with PANDAS in terms of diet and appropriate pharmacological treatment, in order to prevent the worsening of leaky gut and the exacerbation of neurological symptoms.

Increasing data highlight how the severity of symptoms in many neurodegenerative diseases can be linked to gut dysbiosis, thanks to the bidirectional gut-brain communication pathway (21–23). In light of the evidence of gut dysbiosis and the inflammatory state in children with PANDAS, diet could play a role in improving the microbiota composition and thereby reducing the severity of neurological symptoms. Indeed, diet is one of the main factors influencing the composition of gut microbiota and may be responsible for the diversification of the microbial population (24, 25). However, it is important to remember that pediatric patients require an adequate intake of nutrients for proper growth and development, and it is necessary to avoid a restrictive diet that could worsen obsessive-compulsive symptoms and promote the development of eating behavior disorders. As for patients with ASD, the restrictive diet that many patients follow presents challenges in assessing the composition of the gut microbiota, adding complexity in the definition of a nutritional intervention as well. Moreover, considering the impact of antibiotics and of the restrictive diets followed by children, the intervention should be aimed at restoring the correct composition of the gut microbiota. The use of antibiotics in patients with GAS infection may also represent a significant bias in the assessment of gut microbiota composition. Indeed, it is well known that antibiotic treatment may lead to significant alterations, including reduced species diversity, altered metabolic activity and selection of antibiotic-resistant organisms, which can cause antibiotic-associated diarrhea and recurrent *Clostridioides difficile* infections (26). It could be useful to provide guidance on following an anti-inflammatory dietary pattern, such as the Mediterranean diet, while avoiding a Western diet

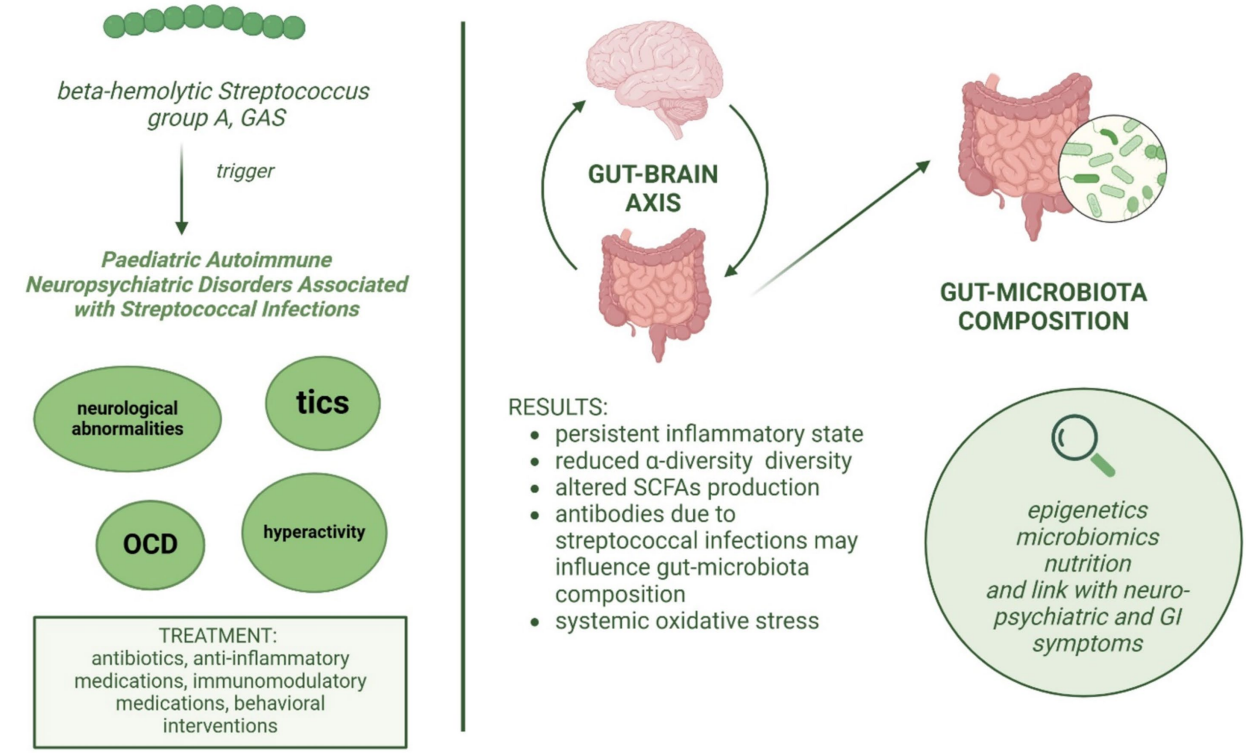

FIGURE 1
Etiopathogenesis of PANDAS and potential association between dysbiosis and neurological symptoms severity.

that is high in trans fatty acids, food additives, and ultra-processed foods (27), while ensuring an adequate intake of fibers. A targeted nutritional assessment is essential to identify individual dietary needs and habits, allowing for a personalized intervention plan that addresses each child's unique health requirements. Despite these considerations, there is currently no evidence supporting a specific diet for managing PANDAS. Therefore, following a Mediterranean diet—known for its anti-inflammatory benefits and balanced nutrient profile (28)—may be advisable, always considering the individual preferences of the children and implementing progressive and personalized interventions based on the Mediterranean diet. To improve the gut microbiota composition through food consumption, it would be advisable to recommend foods rich in fiber (29) but given the selectivity of these patients, promoting a change in dietary habits may be challenging. In addition to foods, the use of prebiotics would have an impact on the microbiota. However, postbiotics could also be considered for their immunomodulatory effects on the immune system (30).

In the pediatric population with PANDAS, a first retrospectively nutrients or food-based dietary pattern analysis could be carried out in order to understand the relationship between diet and health. Further clinical studies could be conducted to evaluate the association between gut microbiota composition and diet.

One of the limitations of the available studies is that both PANDAS and PANS were included in the analysis, providing a heterogeneous case group in terms of etiopathogenesis. Available data in literature are not sufficient to demonstrate whether a different infection than GAS may affect microbiota composition differently, however, a study conducted on murine model revealed that the gut flora presented specific changes according to different antibiotics use and treatment timing (31). Therefore, further studies including a more conspicuous group of selected PANDAS patients are needed to confirm reported data.

A close collaboration is essential among the numerous professionals involved in the pathogenesis and treatment of this complex pathological condition, including pediatricians, rheumatologists, immunologists, neuropsychiatrists, infectiologists, and nutrition experts. A multidisciplinary approach would allow a faster integration of new discoveries, aiming to improve treatment and care strategies. Additionally, it is important to widely disseminate knowledge of these results to general pediatricians, who are the first to manage these patients and can provide an initial effective intervention to modulate the inflammatory response through proper treatment and the implementation of an anti-inflammatory diet.

According to the limited data available, it may be postulated that a relationship between PANDAS and altered gut microbiota composition and that this may contribute to the severity of neuropsychiatric symptoms in these patients. Current treatments, including antibiotics and immunotherapies, are essential but may disrupt gut microbiota, necessitating careful management. Incorporating dietary strategies, aiming to increase the fiber intake could offer beneficial outcomes. A multidisciplinary approach is crucial for integrating new findings into clinical practice and optimizing treatment strategies to improve patient care and outcomes.

Author contributions

VT: Investigation, Methodology, Writing – original draft, Writing – review & editing. MT: Investigation, Methodology, Writing – original draft, Writing – review & editing. IG: Investigation, Writing – original draft. ES: Investigation, Writing – original draft. EV: Conceptualization, Methodology, Supervision, Writing – review & editing. GZ: Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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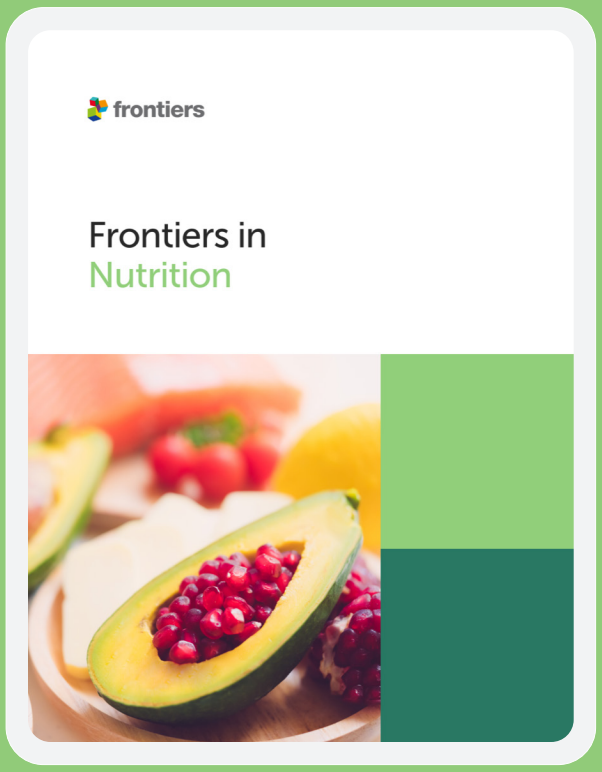

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