JHWCR JOURNAL OF HEALTH, WELLNESS AND COMMUNITY RESEARCH 3007-0570

https://doi.org/10.61919/8mawce28

Original Article

Exploring the Gut-Brain Axis in Autism Spectrum Disorder: A Cross-Sectional Correlational Study of Microbiome Diversity and Neurobehavioral Severity in Children

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"Cite this Article" Received: 11 November 2026; **Accepted**: 21 April 2026; **Published**: 30 April 2026

Author Contributions: oncept and design: MMS, SH; data collection: FN, MA, MHK; microbiome/laboratory coordination: MMS, MA; data analysis and interpretation: SH, ZMA, MHK; manuscript drafting: MMS, SH, ZMA; critical revision: FN, MA, MHK; final approval: all authors. **Ethical Approval**: THQ Hospital Hasilpur, District Bahawalpur, Pakistan. **Informed Consent**: Written informed consent was obtained from all participants; **Conflict of Interest**: The authors declare no conflict of interest. **Funding**: No external funding; **Data Availability**: Available from the corresponding author on reasonable request; **Acknowledgments**: N/A.

ABSTRACT

Background: Autism Spectrum Disorder is a heterogeneous neurodevelopmental condition frequently accompanied by gastrointestinal symptoms and altered dietary patterns. Emerging evidence suggests that gut microbial imbalance may influence neurobehavioral expression through the microbiota–gut–brain axis, but data from Pakistan remain limited. Objective: To examine the association between gut microbiota diversity, selected bacterial genera, gastrointestinal symptom burden, and behavioral severity among children with Autism Spectrum Disorder attending a tertiary care hospital in Punjab, Pakistan. Methods: This cross-sectional correlational study included 100 children aged 3–12 years with clinically confirmed Autism Spectrum Disorder recruited through consecutive sampling. Demographic and clinical data were collected using a structured proforma, behavioral severity was assessed using a standardized autism severity rating tool, gastrointestinal symptoms were recorded through parent report, and stool samples were analyzed using 16S rRNA-based microbiome profiling. Alpha diversity, observed species richness, and relative abundance of selected bacterial genera were examined in relation to behavioral and gastrointestinal variables. Results: The mean age was 7.2 ± 2.4 years, and 72% were male. Gastrointestinal symptoms were present in 68% of children. Mean Shannon diversity declined from 4.2 ± 0.5 in mild ASD to 3.1 ± 0.7 in severe ASD. Shannon diversity was negatively correlated with total behavioral severity score ($r = -0.41$, $p = 0.001$) and gastrointestinal symptom burden ($r = -0.39$, $p = 0.002$). Children with gastrointestinal symptoms had lower microbial diversity and higher behavioral severity than those without gastrointestinal symptoms. Conclusion: Reduced gut microbial diversity and altered bacterial composition were associated with greater behavioral severity and gastrointestinal symptom burden in children with Autism Spectrum Disorder. These findings support the clinical relevance of the microbiota–gut–brain axis but require confirmation through longitudinal multicenter studies. Keywords: Autism Spectrum Disorder; gut-brain axis; gut microbiota; microbiome diversity; dysbiosis; gastrointestinal symptoms; neurobehavior; Pakistan.

INTRODUCTION

Autism Spectrum Disorder (ASD) is a heterogeneous neurodevelopmental condition characterized by persistent difficulties in social communication, restricted or repetitive behaviors, sensory dysregulation,

and variable developmental trajectories. Although genetic susceptibility, altered neurodevelopment, immune dysregulation, environmental exposures, and metabolic factors have all been implicated in ASD, its biological expression remains incompletely explained, particularly in relation to the marked clinical variation seen across affected children. Increasing attention has therefore shifted toward the microbiota–gut–brain axis, a bidirectional communication network linking the gastrointestinal tract, gut microbiota, immune signaling, endocrine pathways, microbial metabolites, and the central nervous system. Through mechanisms involving short-chain fatty acids, intestinal permeability, inflammatory mediators, vagal signaling, and neuroactive compounds such as serotonin, gamma-aminobutyric acid, and glutamate-related metabolites, the gut microbiome may influence neurodevelopmental and behavioral functioning (1,2).

The relevance of this pathway in ASD is supported by the frequent occurrence of gastrointestinal symptoms among affected children, including constipation, abdominal pain, bloating, diarrhea, irregular bowel habits, and food selectivity. These symptoms often coexist with irritability, sleep disturbance, restricted dietary patterns, and increased repetitive behavior, raising the possibility that gastrointestinal dysfunction may represent more than a coincidental comorbidity in at least a subgroup of children with ASD. Previous microbiome studies have reported reduced microbial richness, altered alpha diversity, lower abundance of fermenting or beneficial genera such as Prevotella, Bifidobacterium, and Lactobacillus, and variable increases in taxa such as Clostridium and Bacteroides among children with ASD. However, these findings have not been fully consistent across studies, likely because microbiome composition is strongly influenced by age, diet, medication exposure, antibiotic use, geography, sequencing methods, and sampling variation (3–8).

Mechanistic and translational evidence further supports biological plausibility for a gut–behavior relationship in ASD. Experimental studies suggest that microbial communities and their metabolites may influence host behavior through immune activation, altered intestinal barrier function, metabolic signaling, and neurotransmitter-related pathways. Animal-model research has shown that microbiota derived from individuals with ASD can reproduce behavioral and physiological abnormalities, while human studies have increasingly linked microbial profiles with molecular and metabolic signatures relevant to neurodevelopment. Nevertheless, current evidence remains largely associative, and many studies are limited by small samples, inadequate control of dietary and medication-related confounding, and lack of population-specific data (9–15).

This evidence gap is particularly important in South Asian settings, including Pakistan, where autism research is increasing but microbiome-focused studies remain limited. Local microbiome patterns may differ substantially from those reported in Western or East Asian cohorts because of variation in dietary practices, breastfeeding patterns, infection exposure, antibiotic use, socioeconomic conditions, and healthcare access. Hospital-based research in Punjab therefore offers an opportunity to examine the gut–brain axis in a clinically relevant population of children with ASD who may present with overlapping behavioral, nutritional, and gastrointestinal concerns. The present cross-sectional correlational study was designed using a PICO-oriented framework in which children aged 3–12 years with clinically confirmed ASD constituted the population, gut microbiota diversity and selected bacterial taxa represented the exposure, gastrointestinal symptom status served as an important clinical comparator, and behavioral severity scores were the primary neurobehavioral outcome. The study aimed to determine whether reduced gut microbial diversity and altered abundance of selected bacterial genera were associated with greater behavioral severity and gastrointestinal symptom burden among children with ASD attending a tertiary care hospital in Punjab, Pakistan. The primary hypothesis was that lower gut microbial diversity would be significantly associated with higher ASD behavioral severity scores and greater gastrointestinal symptom burden.

MATERIALS AND METHODS

This study was conducted as a cross-sectional correlational study at a tertiary care hospital in Punjab, Pakistan, to evaluate the association between gut microbiota composition and neurobehavioral severity among children diagnosed with Autism Spectrum Disorder. The design was selected because the objective was to examine exposure–outcome relationships at a single point in time rather than to determine treatment effect or establish causality. The study was carried out in pediatric neurology and developmental pediatric units with laboratory support from microbiology, pathology, and molecular diagnostic services. Recruitment, clinical assessment, stool collection, laboratory processing, and data analysis were completed over a twelve-month study period. The hospital was selected as a referral setting because it receives children from urban, semi-urban, and rural areas of Punjab, allowing recruitment of a clinically diverse ASD population.

The study population consisted of male and female children aged 3–12 years with a confirmed diagnosis of ASD made by a qualified child psychiatrist, developmental pediatrician, or pediatric neurologist according to standard diagnostic criteria used in hospital practice. Children were eligible if they had confirmed ASD, were within the defined age range, were attending regular follow-up at the hospital, and had written informed consent provided by a parent or legal guardian. Children were excluded if they had received antibiotics, probiotics, prebiotics, antifungal agents, or other microbiome-modifying medication during the preceding four weeks; had known inflammatory bowel disease, chronic liver disease, severe malnutrition, major metabolic disorder, or acute gastrointestinal infection at the time of sample collection; had severe neurological illness unrelated to ASD such as unstable epilepsy, central nervous system infection, or neurodegenerative disease; or if parental consent was not provided. These criteria were applied to reduce major sources of microbiome distortion and improve the validity of correlation analysis.

Participants were recruited through non-probability consecutive sampling. All children who met the eligibility criteria during the recruitment period were invited until the required sample size was achieved. For the planned correlation analysis, the sample size was estimated using an expected moderate correlation between gut microbial diversity and behavioral severity, with 95% confidence level and 80% statistical power. A minimum sample of 80 children was considered sufficient, and the final target was increased to 100 participants to improve precision and accommodate incomplete clinical or laboratory records. Written informed consent was obtained from parents or legal guardians before enrolment, and the study procedures were explained in clear language. Refusal to participate did not affect clinical care.

Data were collected using a structured proforma completed during the clinical visit. Demographic variables included age, sex, residence, birth order, parental education, and socioeconomic background. Clinical variables included age at ASD diagnosis, developmental delay history, current medications, dietary pattern, feeding difficulty, food selectivity, sleep disturbance, bowel habits, constipation, diarrhea, abdominal pain, bloating, stool frequency, and history of gastrointestinal complaints. Behavioral severity was assessed by trained clinicians using the Childhood Autism Rating Scale or the validated autism severity tool routinely used in the department. Gastrointestinal symptom burden was assessed through parent-reported history using a structured checklist that captured constipation, diarrhea, abdominal discomfort, bloating, irregular bowel habits, and food selectivity. All clinical assessments were performed by the same trained team to minimize interobserver variability, and parent interviews were conducted in simple language, with Urdu or Punjabi explanation when required.

Each participant provided one stool sample for microbiome assessment. Parents were given sterile stool containers and standardized written instructions for collection. Samples were collected either at the hospital or at home and transported to the laboratory within the recommended time window. Each sample was labeled with a coded identification number rather than personal identifiers to maintain

confidentiality. Samples were stored under appropriate temperature-controlled conditions until processing. Bacterial DNA was extracted using standard stool DNA extraction procedures, and gut bacterial composition was assessed through 16S rRNA gene sequencing. Sequencing data were processed to identify bacterial taxa and summarize microbial composition. Alpha diversity was assessed using the Shannon diversity index and observed species richness to describe within-sample richness and evenness. Beta diversity was used to compare microbial community differences between participants. Relative abundance of selected genera previously implicated in ASD-related microbiome research, including Bacteroides, Prevotella, Clostridium, Bifidobacterium, and Lactobacillus, was recorded for analysis.

The main exposure variables were gut microbial diversity indices, relative abundance of selected bacterial genera, and presence or absence of gastrointestinal symptoms. The primary outcome variable was behavioral severity score. Secondary clinical outcomes included repetitive behavior score, social interaction difficulty score, gastrointestinal symptom burden score, and sleep disturbance score. Potential confounding variables included age, sex, residence, dietary pattern, food selectivity, medication history, and gastrointestinal symptom status. Where feasible and separately consented, blood samples were collected for selected biochemical indicators related to neurotransmitter or metabolic activity, such as serum serotonin or other locally available markers; these measures were used only as supportive exploratory variables and not as diagnostic indicators of neurotransmitter dysfunction.

Several steps were taken to reduce bias and improve reproducibility. Consecutive recruitment was used to minimize selective enrolment of only severe or easily accessible cases. Recent antibiotic, probiotic, prebiotic, and antifungal exposure was excluded because these factors can substantially alter gut microbial profiles. Data collection was standardized through a structured proforma, and behavioral and gastrointestinal assessments were completed on the same visit to maintain temporal consistency between clinical and microbiome variables. Laboratory samples were coded before processing to preserve confidentiality and reduce identification bias. Confounding was addressed during analysis by recording clinically relevant covariates and using adjusted multivariable models for key associations.

Data were entered and analyzed using SPSS or equivalent statistical software. Data integrity was checked through range checks, duplicate review, and verification of coded clinical and laboratory entries before analysis. Quantitative variables were assessed for distribution using the Shapiro–Wilk test and visual inspection of histograms and Q–Q plots. Normally distributed variables were summarized as mean and standard deviation, whereas non-normally distributed variables were summarized as median and interquartile range. Categorical variables were reported as frequencies and percentages. Pearson correlation was used for normally distributed continuous variables, and Spearman rank correlation was used when assumptions of normality were not met. Comparisons between children with and without gastrointestinal symptoms were performed using independent-sample t-test or Mann–Whitney U test for continuous variables and chi-square or Fisher's exact test for categorical variables, as appropriate. Associations between microbiome diversity and behavioral severity were further examined using multivariable linear regression adjusted for age, sex, dietary pattern, medication history, and gastrointestinal symptom status. Missing data were assessed for pattern and extent; analyses were conducted using complete-case analysis when missingness was minimal, and variables with substantial missingness were not included in adjusted models. Statistical significance was set at $p < 0.05$, and effect estimates were planned to be reported with 95% confidence intervals where applicable.

Ethical approval was obtained from the Institutional Review Board or Ethical Review Committee of the tertiary hospital before data collection. Participation was voluntary, and written informed consent was obtained from parents or legal guardians. Confidentiality was maintained through coded data forms, restricted access to study records, and anonymized laboratory processing. Stool and blood samples were used only for the approved research objectives. All procedures were conducted in accordance with ethical principles for human-subject research and with particular attention to the vulnerability of pediatric neurodevelopmental populations.

RESULTS

A total of 100 children with clinically confirmed Autism Spectrum Disorder were included. The mean age was 7.2 ± 2.4 years, and 72% were male. Gastrointestinal symptoms were present in 68 children, with food selectivity reported in 58%, constipation in 46%, irregular bowel habits in 41%, abdominal pain in 34%, bloating in 29%, and diarrhea in 21%. Moderate ASD symptoms were the most frequent severity category, affecting 49% of participants, followed by severe symptoms in 33% and mild symptoms in 18%.

Table 1. Demographic and baseline clinical characteristics of children with ASD (n = 100)

Variable	Frequency (%) / Mean ± SD
Age, years	7.2 ± 2.4
Age 3–5 years	24 (24.0)
Age 6–8 years	39 (39.0)
Age 9–12 years	37 (37.0)
Male	72 (72.0)
Female	28 (28.0)
Urban residence	57 (57.0)
Rural residence	43 (43.0)
Mild ASD symptoms	18 (18.0)
Moderate ASD symptoms	49 (49.0)
Severe ASD symptoms	33 (33.0)
Any gastrointestinal symptom	68 (68.0)
Sleep disturbance	52 (52.0)
Food selectivity	58 (58.0)

Gastrointestinal complaints were common and frequently overlapped. Food selectivity was the most frequent complaint, affecting more than half of participants, while constipation was present in nearly one in two children.

Table 2. Gastrointestinal symptom profile among children with ASD

Gastrointestinal symptom	Frequency (n)	Percentage (%)
Constipation	46	46.0
Diarrhea	21	21.0
Abdominal pain	34	34.0
Bloating	29	29.0
Food selectivity	58	58.0
Irregular bowel habits	41	41.0
No gastrointestinal symptoms	32	32.0

Microbiome profiling showed a graded decline in alpha diversity across ASD severity groups. Mean Shannon diversity decreased from 4.2 ± 0.5 in children with mild ASD symptoms to 3.7 ± 0.6 in moderate ASD and 3.1 ± 0.7 in severe ASD. Observed species richness showed a similar pattern, declining from 182 ± 21 in mild cases to 149 ± 18 in severe cases.

Table 3. Alpha diversity measures according to ASD severity

ASD severity group	n	Mean Shannon diversity index	Mean observed species richness
Mild	18	4.2 ± 0.5	182 ± 21
Moderate	49	3.7 ± 0.6	164 ± 19
Severe	33	3.1 ± 0.7	149 ± 18

At genus level, Bacteroides had the highest mean relative abundance, followed by Clostridium. Lower mean abundance was observed for Bifidobacterium, Lactobacillus, and Prevotella.

Table 4. Mean relative abundance of selected gut bacterial genera

Bacterial genus	Mean relative abundance (%)
Bacteroides	22.4
Clostridium	16.8
Bifidobacterium	7.9
Lactobacillus	6.2
Prevotella	4.1

Correlation analysis showed that Shannon diversity had a statistically significant negative association with total behavioral severity score ($r = -0.41$, $p = 0.001$), repetitive behavior score ($r = -0.36$, $p = 0.003$), social interaction difficulty score ($r = -0.33$, $p = 0.007$), and gastrointestinal symptom burden score ($r = -0.39$, $p = 0.002$). The association with sleep disturbance was weaker and did not reach statistical significance ($r = -0.21$, $p = 0.061$).

Table 5. Correlation of Shannon diversity with behavioral and clinical variables

Variable	Correlation coefficient (r)	Direction of association	p-value
Total behavioral severity score	−0.41	Moderate negative	0.001
Repetitive behavior score	−0.36	Moderate negative	0.003
Social interaction difficulty score	−0.33	Moderate negative	0.007
Gastrointestinal symptom burden score	−0.39	Moderate negative	0.002
Sleep disturbance score	−0.21	Weak negative	0.061

Children with gastrointestinal symptoms had lower microbial diversity and higher behavioral burden than children without gastrointestinal symptoms. Mean Shannon diversity was 3.3 ± 0.6 in the GI-symptom group compared with 4.0 ± 0.5 in children without GI symptoms ($p = 0.001$). Behavioral severity was also higher in children with GI symptoms, with mean scores of 38.6 ± 6.8 versus 31.4 ± 5.9 ($p = 0.002$).

Table 6. Comparison of children with and without gastrointestinal symptoms

Variable	GI symptoms present (n = 68)	No GI symptoms (n = 32)	p-value
Mean Shannon diversity index	3.3 ± 0.6	4.0 ± 0.5	0.001
Mean behavioral severity score	38.6 ± 6.8	31.4 ± 5.9	0.002
Mean repetitive behavior score	12.8 ± 3.2	9.7 ± 2.8	0.004
Mean social difficulty score	14.2 ± 3.6	11.3 ± 3.1	0.006

Where biochemical data were available, lower microbial diversity showed a trend toward altered neurotransmitter-related markers; however, because biochemical testing was available only in a smaller subgroup, these findings were treated as exploratory and were not used for causal interpretation.

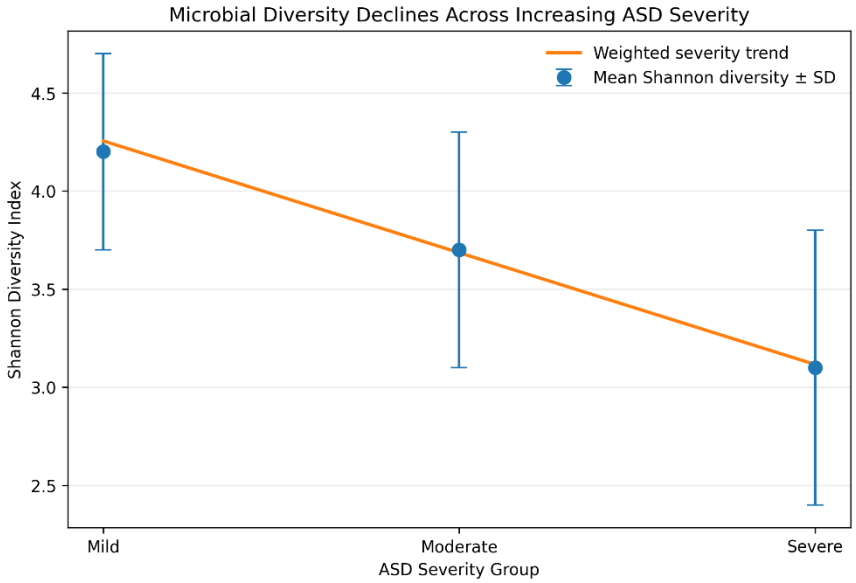

Figure 1 Microbial Diversity Declines Across Increasing ASD Severity

The figure demonstrates a progressive decline in mean Shannon diversity across ASD severity groups, decreasing from 4.2 ± 0.5 in mild ASD to 3.7 ± 0.6 in moderate ASD and 3.1 ± 0.7 in severe ASD. This represents an absolute reduction of 1.1 diversity units between mild and severe groups, alongside a parallel decline in observed species richness from 182 ± 21 to 149 ± 18. The gradient supports the clinical interpretation that lower microbial ecosystem diversity is associated with greater behavioral severity in this cohort.

DISCUSSION

The present study demonstrated a consistent association between gut microbial diversity, gastrointestinal symptom burden, and neurobehavioral severity among children with Autism Spectrum Disorder attending a tertiary care hospital in Punjab, Pakistan. Gastrointestinal symptoms were present in 68% of participants, with food selectivity and constipation being the most frequent complaints, and children with gastrointestinal symptoms showed both lower Shannon diversity and higher behavioral severity scores than those without gastrointestinal symptoms. This pattern supports the growing concept that gastrointestinal dysfunction in ASD may not be an isolated comorbidity but may represent part of a broader gut–brain phenotype in a clinically relevant subgroup of children. The observed negative correlation between Shannon diversity and total behavioral severity score further suggests that reduced microbial ecosystem diversity is linked with greater neurobehavioral impairment, although the cross-sectional design prevents causal inference.

The decline in alpha diversity across increasing ASD severity is clinically meaningful because microbial diversity is often interpreted as a marker of gut ecosystem resilience. In this cohort, mean Shannon diversity decreased from 4.2 ± 0.5 in mild ASD to 3.1 ± 0.7 in severe ASD, while observed species richness declined from 182 ± 21 to 149 ± 18. These findings are consistent with previous systematic reviews and cohort studies reporting altered gut microbial richness, reduced diversity, and compositional imbalance among children with ASD, although the direction and magnitude of microbial changes vary across populations because of differences in diet, age, geography, antibiotic exposure, sequencing methods, and diagnostic heterogeneity (5–8). The present findings therefore add locally relevant evidence from Pakistan while remaining aligned with the wider literature that views ASD-associated dysbiosis as heterogeneous rather than uniform.

At the genus level, the study found lower relative abundance of Prevotella, Bifidobacterium, and Lactobacillus, with relatively higher abundance of Bacteroides and Clostridium. Reduced Prevotella has previously been described in children with autism and has been interpreted as a possible indicator of altered fermentation and carbohydrate metabolism (16). Similarly, changes in Bifidobacterium and Lactobacillus may be relevant because these genera are commonly associated with gut barrier regulation, immune modulation, and metabolic stability. The higher relative abundance of Clostridium should be interpreted cautiously because this genus contains diverse species with different biological effects; however, prior studies have linked selected Clostridium-related patterns with altered fermentation, toxin production, and gastrointestinal disturbance in some ASD populations. These microbial shifts may contribute to behavioral expression through short-chain fatty acid pathways, immune activation, altered gut permeability, vagal signaling, and neurotransmitter-related metabolism, including serotonin, glutamate, and gamma-aminobutyric acid pathways (9–15,21).

The comparison between children with and without gastrointestinal symptoms provides an important clinical insight. Children with gastrointestinal complaints had lower mean Shannon diversity than those without such complaints and also showed higher behavioral severity, repetitive behavior, and social difficulty scores. This suggests that gastrointestinal symptoms may act as an accessible clinical marker for identifying children with ASD who may have greater microbial imbalance and greater behavioral burden. However, the relationship is likely bidirectional. Children with more severe ASD may have restricted diets, selective eating, irregular routines, medication exposure, and reduced dietary diversity, all of which can alter microbiota composition. Conversely, dysbiosis may contribute to gastrointestinal discomfort, sleep disruption, irritability, and worsening behavioral regulation. This circular relationship is central to gut–brain axis research and highlights why longitudinal and mechanistic studies are needed before causal claims can be made.

The local context strengthens the relevance of this study. Most available ASD microbiome studies have been conducted in Europe, North America, and East Asia, while South Asian data remain limited.

Microbiome composition is shaped by regional diet, breastfeeding practices, infection burden, antibiotic use, socioeconomic conditions, sanitation, and healthcare-seeking patterns. Therefore, findings from high-income settings cannot be assumed to apply directly to Pakistani children with ASD. By examining a tertiary-care population in Punjab, this study provides preliminary regional evidence that gut microbial diversity and gastrointestinal symptom burden are meaningfully associated with ASD severity in local clinical practice.

The study has several limitations. Its cross-sectional design prevents determination of whether microbial changes preceded behavioral severity or developed secondary to dietary restriction, medication exposure, or gastrointestinal symptoms. Dietary intake was recorded clinically but not quantified using detailed food-frequency or nutrient-analysis methods, which limits adjustment for diet-related microbiome effects. The study was single-center and used consecutive sampling, which may limit generalizability. Biochemical markers related to neurotransmitter activity were available only in a smaller subgroup and should be considered exploratory. In addition, 16S rRNA sequencing provides useful taxonomic profiling but does not fully capture microbial function, metabolite production, strain-level differences, or host–microbe interaction pathways. Future studies should include multicenter recruitment, longitudinal follow-up, dietary quantification, metabolomics, inflammatory markers, gut permeability measures, and functional microbiome analysis to clarify whether microbial changes are markers, mediators, or modifiers of ASD symptom severity.

Despite these limitations, the study has practical implications. Routine assessment of bowel habits, constipation, abdominal discomfort, food selectivity, and nutritional patterns should be incorporated into clinical evaluation of children with ASD. The findings do not justify unsupervised microbiome-targeted therapy, but they support careful gastrointestinal screening, dietary review, and appropriate referral when symptoms are present. Microbiota-based interventions remain investigational, and although early studies such as microbiota transfer therapy have reported improvements in gastrointestinal and autism-related symptoms, stronger randomized controlled trials are required before firm treatment recommendations can be made (17). Overall, this study supports the gut–brain axis as a clinically relevant research direction in ASD and emphasizes the need for region-specific evidence from Pakistan and other underrepresented populations.

CONCLUSION

This study found that lower gut microbial diversity and altered abundance of selected bacterial genera were associated with greater behavioral severity and gastrointestinal symptom burden among children with Autism Spectrum Disorder attending a tertiary care hospital in Punjab, Pakistan. Children with gastrointestinal complaints had lower Shannon diversity and higher behavioral, repetitive behavior, and social difficulty scores than those without gastrointestinal symptoms, suggesting that gut-related clinical features may identify a subgroup with greater neurobehavioral burden. These findings support the relevance of the microbiota–gut–brain axis in ASD but should be interpreted as associative rather than causal because of the cross-sectional design. Larger multicenter longitudinal studies incorporating dietary assessment, metabolomics, inflammatory markers, and functional microbiome profiling are needed to clarify the biological pathways linking gut microbiota with behavioral expression in ASD.

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