scientific reports

Check for updates

OPEN

Characterization of gut microbiota on gender and age groups bias in Thai patients with autism spectrum disorder

Benjaporn Bhusri[1,11], Sawannee Sutheeworapong[2,11], Weerayuth Kittichotirat[1,2], Kanthida Kusonmano[8], Chinae Thammarongtham[5], Supatcha Lertampaiporn[5], Peerada Prommeenate[5], Verayuth Praphanphoj[6], Wiranpat Kittitharaphan[7], Sudarat Dulsawat[3], Prasobsook Paenkaew[9] & Supapon Cheevadhanarak[2,3,4]✉

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by social communication and interaction problems. The prevalence of ASD is increasing globally, with a higher ratio of males to females. Gastrointestinal symptoms are common in individuals with ASD, and gut microbiota has been implicated in the disorder's development. This study aimed to investigate the gut microbiota alteration in Thai individuals with ASD compared to healthy controls using 16S rRNA gene sequencing. The influence of gender and age on gut microbiota composition and function was also examined. A total of 65 ASD individuals and 30 neurotypical (NT) individuals were included in the analysis. The results revealed notable differences in gut microbiota composition between the ASD and NT groups, with variations observed in microbial richness and the presence of enriched microbial taxa. These differences were influenced by both gender and age. Fusobacteriota, Fusobacteriaceae, and *Fusobacterium* were found to be enriched in individuals with ASD. Furthermore, the study identified gender-related taxa, such as *Bacteroides plebeius*, enriched in ASD females. Age-related taxa, including *Veillonella*, known to be associated with poor oral hygiene, were also observed in ASD children. The analysis of differentially abundant pathways highlighted the enrichment of various metabolic pathways in individuals with ASD, including those related to endocrine-disrupting chemicals. These findings underscore the importance of considering gender and age when studying gut microbiota in ASD. They provide valuable insights into the potential role of gut microbiota dysbiosis in ASD pathogenesis and highlight the influence of environmental factors.

Autism spectrum disorder (ASD) is a neurodevelopmental disorder resulting from abnormal brain organization in early development. Although ASD symptoms and severity can vary from person to person, the main features are social communication and interaction problems as well as restricted and repetitive behaviors or interests[1]. According to a recent study, the prevalence of ASD is on the rise and affects 1 in 100 children around the world[2]. From 2000 to 2016, the ASD prevalence has increased by approximately 2.8 times. In the United States, 1 in 54 children aged 8 years was diagnosed with ASD[3]. It is difficult to determine the cause of the disease, as its

[1]Bioinformatics and Systems Biology Program, School of Bioresources and Technology, and School of Information Technology, King Mongkut's University of Technology Thonburi, Bangkok 10150, Thailand. [2]Systems Biology and Bioinformatics Unit, Pilot Plant Development and Training Institute, King Mongkut's University of Technology Thonburi, Bangkok 10150, Thailand. [3]Fungal Biotechnology Unit, Pilot Plant Development and Training Institute, King Mongkut's University of Technology Thonburi, Bangkok 10150, Thailand. [4]School of Bioresources and Technology, King Mongkut's University of Technology Thonburi, Bangkok 10150, Thailand. [5]Biochemical Engineering and Systems Biology Research Group, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency at King Mongkut's University of Technology Thonburi, Bangkok 10150, Thailand. [6]Medical Genetics Center, Sai Mai District, Bangkok 10220, Thailand. [7]Yuwaprasart Waithayopathum Child and Adolescent Psychiatric Hospital, Ministry of Public Health, Samut Prakan 10270, Thailand. [8]Bioinformatics and Systems Biology Program, School of Bioresources and Technology, King Mongkut's University of Technology Thonburi, Bangkok10150, Thailand. [9]Department of Microbiology, Faculty of Medicine, Chiang Mai University, Chiang Mai50200, Thailand. [11]Benjaporn Bhusri and Sawannee Sutheeworapong have equally contributed to this work. ✉email: supapon.che@kmutt.ac.th

complexity includes a wide range of symptoms and severity. It is likely that multiple factors, including genetic, epigenetic, and environmental risk factors, are involved[4,5]. Approximately 46.8% of people with ASD experience gastrointestinal (GI) symptoms, including constipation, diarrhea, abdominal pain, gaseousness/bloating, and vomiting[6]. ASD children with frequent GI symptoms exhibited more irritability, social disengagement, hyperactivity, and stereotypic behaviors compared with those without frequent GI symptoms[7]. Because of the link between ASD and GI issues, scientists have become interested in gut microbiota as an etiology for ASD. Several recent studies have discovered an altered gut microbiota composition in ASD individuals, which is generally known as "dysbiosis"[8]. Dysbiosis of gut microbiota can induce alterations in bacterial metabolites and a leaky gut, which could be a potential factor for generating ASD[9]. Kang et al.'s 2017 study found that Microbiota Transfer Therapy (MTT) improved GI issues and autism-related symptoms in children with ASD[10]. In 2019, a follow-up with the same 18 children revealed that these improvements in both GI and core ASD symptoms were sustained even two years after the treatment had ended[11]. In another study by Wong et al. (2022), researchers examined 92 boys with ASD and 112 typically developing controls, finding significant differences in gut microbiota composition between the two groups, regardless of the presence of functional gastrointestinal disorders (FGID). This suggests that gut dysbiosis in ASD could influence neuropsychiatric symptoms independent of GI conditions[12]. The gut microbiota plays a crucial role in human health and can impact brain development and behavior through the gut-brain axis[13].

ASD is approximately four times more prevalent in males than in females. While the reasons for this gender difference are not fully understood, sex-specific genetic and hormonal factors have been suggested to be involved[14]. Moreover, gender has been found to influence the composition of gut microbiota[15,16]. However, a comparative analysis of the gut microbiota in males and females with ASD is often overlooked. Another critical consideration in gut microbiota dysbiosis in ASD is age, which exerts a notable influence on the composition and functions of gut microbiota, particularly in early life[17]. Despite ASD being a lifelong condition, there is comparatively less research on changes in gut microbiota profiles in adolescents and adults. The findings from studies examining gut microbiota in individuals with ASD have been inconsistent[18]. Factors such as geographic location have been identified as potential influences on gut microbiota composition[19]. A recent study by Fouquier et al. underscored the significant association between gut microbiota composition and ASD, especially when accounting for the geographical area under study[20].

In Thailand, research on altered gut microbiota in individuals with ASD has been infrequently mentioned. Hence, this study employs 16S rRNA gene sequencing to investigate the modified gut microbiota in Thai individuals with ASD compared to healthy controls. The putative microbial biomarkers and changes in gut microbiota functions were investigated in additionally considering gender and age effects. These findings offer insight into the potential correlation between compositional and functional changes in the gut microbiota and ASD, as well as the significance of gender and age differences. Moreover, this research may advance our understanding of potential biomarkers or treatment targets for ASD.

Methods

Study participants

All experiments and methods were performed following the relevant guidelines and regulations, as approved by the ethics committee of Yuwaprasart Waithayopathum Child and Adolescent Psychiatric Hospital, and all participants provided written informed consent before the study commenced. Informed consent was also obtained from a parent and/or legal guardian. In 2019, we recruited a total of 95 Thai participants aged 3 to 35 years. This cohort included 33 neurotypical (NT) individuals, who were siblings of ASD participants, and 62 individuals with ASD, all from Yuwaprasart Waithayopathum Child and Adolescent Psychiatric Hospital. The research was conducted under the project titled "Discovery of biomarkers for autism spectrum disorders from exome sequencing and gut microbiome using big data technology for life science approach" at King Mongkut's University of Technology Thonburi. Among the 62 ASD participants, there were 35 males and 27 females, while the 33 NT participants included 30 males and 3 females. Participants were categorized into three age groups based on the Medical Subject Headings (MeSH) definition: children aged 6 to 12 years (ASD = 19, NT = 12), adolescents aged 13 to 18 years (ASD = 15, NT = 14), and adults aged 19 to 35 years (ASD = 26, NT = 6). ASD diagnoses were made by an experienced child psychologist using the Autism Diagnostic Observation Schedule (ADOS). Participants had not received antibiotics and probiotics for at least three months before sampling.

Fecal sample collection and DNA extraction

Fecal samples of approximately 1 gram were collected in DNA/RNA Shield™ Fecal Collection Tubes (Zymo Research) by participants or their guardians, as appropriate. The samples were transported to the laboratory within 1 month of collection. DNA/RNA Shield™ reagent ensured sample stability during transport and storage at ambient temperatures. Upon arrival, they were stored at −20 °C until DNA extraction. DNA was extracted from the fecal samples using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions, which incorporated both enzymatic and mechanical cell lysis. Extracted DNA was determined for DNA quality and quantity by the NanoPhotometer® N60/N50 spectrophotometer. DNA quantity and quality criteria were 1.8–2.0 for A_{260}/A_{280} and 2.0–2.2 for A_{260}/A_{230}. Only samples with DNA concentrations greater than 10 ng/µl were further analyzed for 16S rRNA sequencing.

16S rRNA gene sequencing analysis

DNA library for sequencing was prepared using MiSeq Reagent Kit v2 following the manufacturer's instructions. The DNA was amplified by polymerase chain reaction (PCR) to target the V4 hypervariable region of the 16 S rRNA gene. The forward primer was 515 F (5′-GTGCCAGCMGCCGCGGTAA-3′) and the reverse primer was 806R (5′-GGACTACHVGGGTWTCTAAT-3′). The 16 S rRNA amplicons were sequenced by the Illumina

NovaSeq platform (Genome Quebec, Canada), generating demultiplexed paired-end (250 × 2 bp) reads. The sequence reads were supplied as FASTQ-format files and were processed through QIIME2 (Quantitative Insights into Microbial Ecology 2) pipeline version 2021.4[21]. Under QIIME2 plugins, adapters and primers were removed by *q2-cutadapt*. The sequence reads were processed to remove adapters and primers. The data were filtered by truncating the forward and reverse reads at position 200, based on quality profiles and amplicon length, to eliminate low-quality regions. Chimeras were checked, paired-end reads were merged, and amplicon sequence variants (ASVs) were identified using the DADA2 method. Singleton ASVs, defined as sequences appearing only once across all samples, were removed to reduce the impact of potential sequencing errors. Taxonomic assignment was carried out by *feature-classifier classify-sklearn*, which is based on machine learning classification and requires trained classifiers of reference databases. The naïve Bayes classifier was specifically trained using the SILVA 138 reference database on the V4 region of the 16 S rRNA. The assignment of eukaryotes, unassigned kingdoms, mitochondria, and chloroplast from ASVs was eliminated. The Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to predict metabolic functions based on their 16 S rRNA genes using Phylogenetic investigation of communities by reconstruction of unobserved states 2 (PICRUSt2) version 2.4.1[22–25].

Microbiome diversity analysis

This study examined the differences in gut microbiota composition between ASD and NT. Additionally, we examined the impact of this alteration on different gender and age groups. The data were analyzed using MicrobiomeAnalyst for comprehensive statistical, graphical, and meta-analysis of microbiome[26]. All data were normalized by rarefaction to provide the same number of reads in each sample.

The diversity of the gut microbiota community was assessed through alpha diversity (within-sample) and beta diversity (between-sample). The alpha diversity indices, including Observed ASVs, Shannon's diversity index, and Simpson's diversity index, were statistically tested by the Wilcoxon rank-sum test to compare between ASD and NT. Additionally, these statistical tests were done to compare ASD and NT in each gender and age group, which could impact the outcome. The beta diversity was computed using the Bray-Curtis distance matrix and visualized through principal coordinates analysis (PCoA). Differences in the bacterial communities between ASD and NT (with and without separating by gender and age group) were investigated using Permutational multivariate analysis of variance (PERMANOVA) with a P-value of 0.05. The alpha and beta diversities were also explored in addition to age range consideration.

Differential abundance bacteria and functional enrichment analysis

Bacteria with a prevalence of less than 10% across all samples were excluded from the discovery of biomarkers. The linear discriminant analysis (LDA) effect size (LEfSe) tool was utilized to characterize differentially abundant bacteria between ASD and NT with and without separating by gender and age group. Differentially abundance bacteria with significance were discovered based on a Kruskal–Wallis statistical analysis and a Linear Discriminant Analysis (LDA) model implemented in LEfSe[27]. Taxa with LDA > 2.0 and a P-value of 0.01 were examined as putative microbiome biomarkers for ASD. We also employed the LEfSe to identify differentially abundant bacterial functions, using a cutoff of LDA > 2.0 and a P-value of 0.05.

Ethics approval and consent to participate

This study protocol was approved by the Ethics Committee of the Yuwaprasart Waithayopathum Child and Adolescent Psychiatric Hospital and the King Mongkut's University of Technology Thonburi. All participants provided written informed consent to participate in the study.

Results

Characteristics of study participants

In this study, a total of 95 subjects were recruited between March to July 2019, comprising 62 individuals diagnosed with ASD and 33 neurotypical (NT) individuals. Of these, 28 pairs of siblings were included. The age of the participants ranged from 3 to 35 years old, and there were 65 males and 30 females. The male-to-female ratio was 35:27 for those diagnosed with ASD and 30:3 for those without ASD (NT). The study included 31 children, 29 adolescents, 32 adults, as well as 2 individuals whose age was not specified, and one preschool-aged child. The information on the study participants is presented in Table 1.

Characteristics	ASD	NT
Subjects (n)	62	33
Male/Female	35/27	30/3
Age range		
Child (6 to 12 years)	19	12
Adolescent (13 to 18 years)	15	14
Adult (19 years and older)	26	6

Table 1. Characteristics of study participants.

Summary of sequencing data characteristics

A total of 47,004,242 paired-end reads were obtained from the 95 subjects, with an average of 494,781 reads per sample. After processing the sequence data, 46,951,861 high-quality amplicon sequences were classified into 6,122 ASVs. The average sequence read per sample was 494,230, with a range of 277,860 to 747,549 reads. To account for differences in sequencing depth, the ASV abundance was normalized by rarefying at a depth of 277,860, resulting in the retention of all 95 samples for downstream analysis. The taxonomic classification revealed 34 phyla, 76 classes, 174 orders, 301 families, 657 genera, and 1,137 species.

Gut microbiota diversity in ASD and NT

The alpha diversity measures, including observed ASVs, Shannon's, and Simpson's diversity indices, did not show any significant difference between ASD and NT groups, as illustrated in Fig. 1a. Beta diversity was evaluated using the Bray-Curtis dissimilarity metric and visualized through a PCoA plot, which revealed that ASD and NT individuals shared a similar gut microbiota community (Fig. 2a). The statistical significance of these results was further confirmed by the PERMANOVA analysis, which showed no significant difference in the overall microbial community structure between ASD and NT groups (PERMANOVA, P-value = 0.313).

Identification of gut microbiota biomarkers of ASD

In order to investigate potential gut microbiota biomarkers associated with ASD, we conducted LEfSe analysis to investigate the potential gut microbiota biomarkers associated with ASD by comparing the differential abundance of bacteria between individuals with ASD and NT at various taxonomic levels, including phylum, family, genus, and species. The results of the LEfSe analysis indicated that Fusobacteriota, Fusobacteriaceae, and *Fusobacterium* were taxa differentially abundant and enriched in the ASD group compared to the NT group. In contrast, no taxa were found to be significantly differentially abundant (p-value = 0.01, LDA > 2) in the NT group (Fig. 3, Supplementary Fig. S1).

Gender-related alterations in gut microbiota composition in ASD

To investigate whether there were any gender-related differences in gut microbiota composition between individuals with ASD and NT, we analyzed the alpha and beta diversities separately in the male and female groups. The observed ASVs indicated significantly higher richness in NT males than in ASD males (P-value = 0.0149). In females, NT also had higher observed ASVs, but the difference was not significant (Fig. 1b). We observed no significant differences in Shannon's and Simpson's diversity indices between the ASD and NT groups in males or females (data not shown). Beta diversity analysis, taking gender into account, showed no significant difference in the microbial community between the two groups. The statistical significance of this similarity was confirmed by the PERMANOVA, with a non-significant P-value of 0.186 (Fig. 2b).

To identify gender-related putative biomarkers of ASD, we employed LEfSe analysis separately in male and female groups. The results showed that the differentially abundant taxa between ASD and NT were different depending on gender, as shown in Fig. 3 and Supplementary Figs. S2-S3. *Ruminococcus gnavus* group was a differentially abundant genus enriched in ASD males. Conversely, in NT males, the Synergistota phylum, along with the Synergistaceae and Clostridiales vadinBB60 group families, exhibited significant enrichment. Several genera, including *Cloacibacillus*, Oscillospiraceae UCG-002, Christensenellaceae R7 group, *Eubacterium ruminantium* group, *Ruminococcus gauvreauii* group, and *Clostridia* vadinBB60 group, were also found to be significantly enriched in NT males. In the female group, Fusobacteriota, Fusobacteriaceae, *Fusobacterium*, and *Bacteroides plebeius* were differentially abundant taxa enriched in ASD. On the other hand, there were not significantly differentially abundant taxa identified in the NT females compared with ASD females.

Age-related alterations in gut microbiota composition in ASD

To investigate whether there were any age-related alterations in gut microbiota composition between ASD and NT of different age groups. We analyzed the alpha and beta diversities separately in child, adolescent, and adult groups to compare the observed ASVs, Shannon's and Simpson's diversity indices, and microbial community between ASD and NT. The results revealed no significant differences in observed ASVs, Shannon's and Simpson's diversity indices between ASD and NT in any of the three age groups. (Fig. 1c). Furthermore, beta diversity analysis, taking age into account, showed no significant difference in the microbial community between ASD and NT. The similarity between ASD and NT in each age group was found to be non-significant through PERMANOVA analysis with a P-value of 0.378 (Fig. 2c).

To identify age-related putative biomarkers of ASD, LEfSe analysis was used separately in the three age groups. The results showed that the differentially abundant taxa between ASD and NT were different depending on age (Fig. 3, Supplementary Figs. S4-S5). *Veillonella* demonstrated significant enrichment in ASD children, while Acidaminococcaceae, *Phascolarctobacterium*, *Tyzzerella*, and *Bacteroides clarus* were significantly enriched in ASD adolescents. However, no significant bacterial enrichment was observed in ASD adults. Additionally, there were no identified bacterial taxa that significantly enriched in NT individuals across each age group.

Enriched functions of gut microbiota in ASD

The functional contribution of gut microbiota to ASD and NT individuals was analyzed using PICRUSt2 based on ASVs. A total of 179 KEGG pathways were identified across all samples. Following the prediction of functional pathways, we utilized LEfSe analysis to identify differentially abundant pathways between ASD and NT individuals as shown in Fig. 4 and Supplementary Figs. S6-S10. The results showed that when age and gender were not considered, fluorobenzoate degradation and carotenoid biosynthesis pathways were enriched in ASD. However, when gender was considered, styrene degradation was enriched in male ASD, while linoleic acid metabolism was enriched in female ASD. Additionally, when age was considered, nitrotoluene degradation and

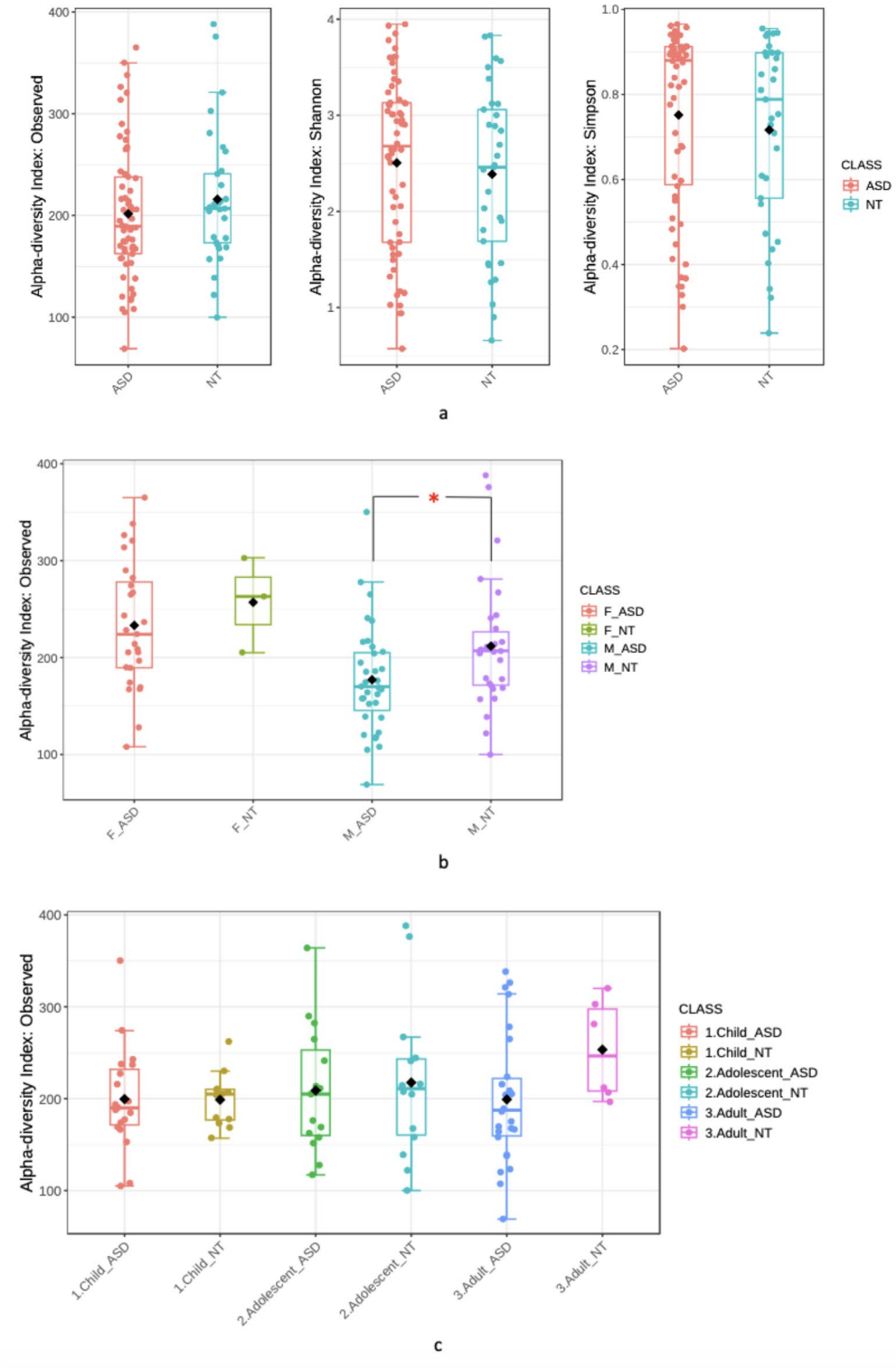

Fig. 1. Alpha diversity indices in individuals with ASD and NT. The box plots compare the observed ASVs, Shannon index, and Simpson index as measures of alpha diversity between individuals with ASD and NT individuals (**a**). Additionally, the observed ASVs are compared between individuals with ASD and NT, considering gender (**b**) and age (**c**).

fluorobenzoate degradation were enriched in ASD children, whereas apoptosis was enriched in NT children. Geraniol degradation and atrazine degradation were enriched in ASD adolescents. However, no significant functional pathways were observed in ASD adults. After False Discovery Rate (FDR) correction, no significant functional differences were found between the ASD and NT.

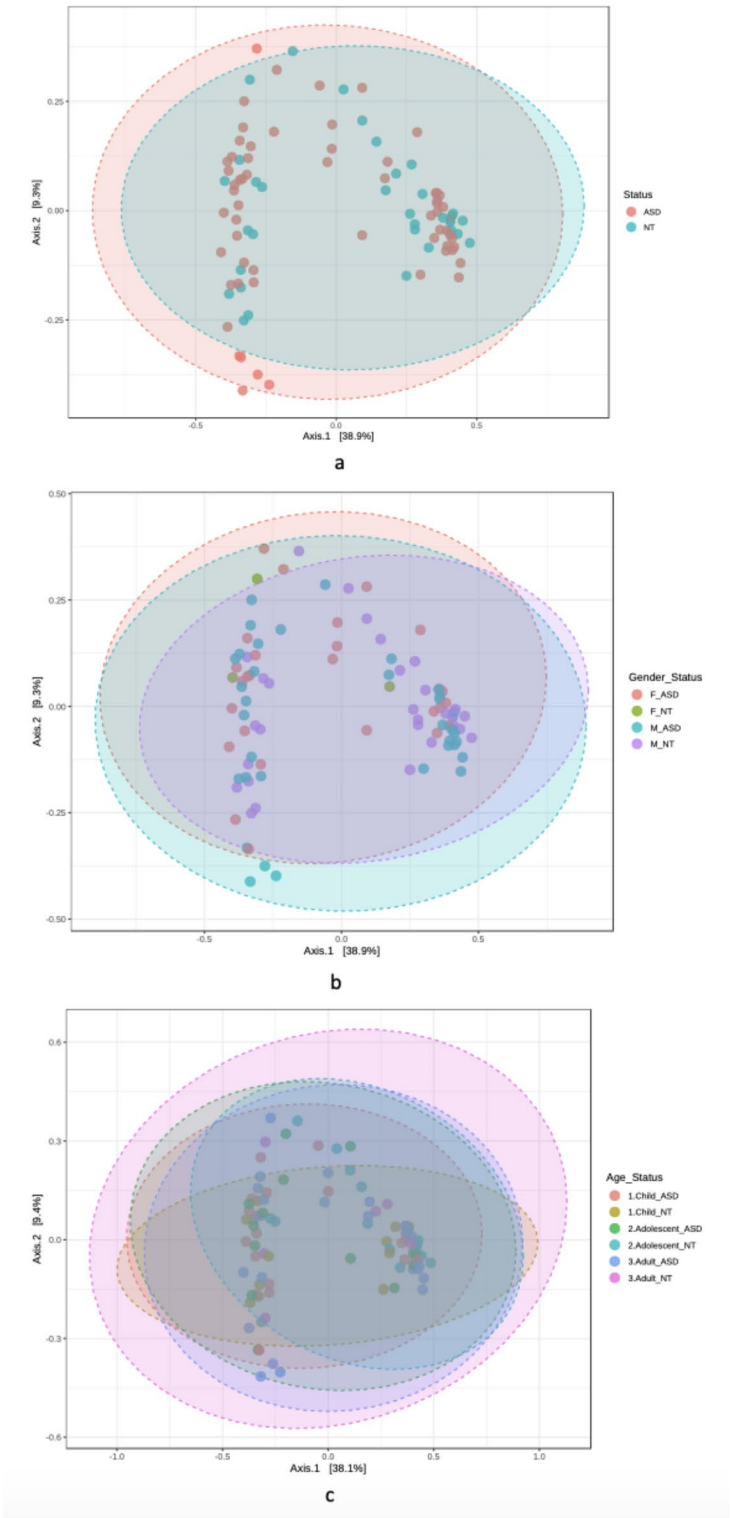

Fig. 2. Beta diversity of gut microbiota in individuals with ASD and NT. The PCoA plot illustrates the beta diversity of gut microbiota based on Bray-Curtis dissimilarity, comparing individuals with ASD and NT individuals (**a**). Furthermore, the beta diversity was examined by considering gender (**b**) and age (**c**) in individuals with ASD and NT.

Discussion

Based on an analysis of the 16S rRNA sequence data obtained from 65 individuals diagnosed with ASD and 30 neurotypical individuals across gender and age groups, including children, adolescents, and adults, we observed notable differences in the composition of gut microbiota between ASD and NT. These differences encompassed

	Differentially abundant taxa	Overall	Male	Female	Child	Adolescent	Adult
Phylum	Fusobacteriota	3.22		3.06			
	Synergistota		-3.01				
Family	Fusobacteriaceae	3.22		3.06			
	Clostridiales vadinBB60 group		-2.08				
	Synergistaceae		-3.01				
	Acidaminococcaceae					3.51	
Genus	*Fusobacterium*	3.22		3.06			
	Ruminococcus gnavus group		2.1				
	Clostridia vadinBB60 group		-2.08				
	Ruminococcus gauvreauii group		-2.09				
	Eubacterium ruminantium group		-2.22				
	Christensenellaceae R7 group		-2.91				
	Oscillospiraceae UCG-002		-3				
	Cloacibacillus		-3.01				
	Veillonella				3.07		
	Phascolarctobacterium					3.48	
	Tyzzerella					2.39	
Species	*Bacteroides plebeius*			2.84			
	Bacteroides clarus					2.62	

Fig. 3. Summary of differentially abundant bacteria by LEfSe analysis in each subject group enriched in ASD and NT. The LEfSe analysis at the phylum, family, genus, and species level identified taxa with an LDA score (log10) > 2.0 and a P-value of 0.01, revealing significant differences between individuals with ASD and NT individuals. The LEfSe analysis was performed considering gender (male and female) and age groups (child, adolescent, and adult) in individuals with ASD and NT. Red boxes represent enrichment in ASD, while blue boxes represent enrichment in NT. The numbers indicate the LDA score, with negative values indicating enrichment in NT individuals.

Differentially abundant functions	Overall	Male	Female	Child	Adolescent	Adult
Fluorobenzoate degradation	2.13			2.59		
Carotenoid biosynthesis	2.07					
Styrene degradation		2.85				
Linoleic acid metabolism			3.87			
Nitrotoluene degradation				3.56		
Apoptosis				-2.94		
Geraniol degradation					3.41	
Atrazine degradation					2.86	

Fig. 4. Summary of KEGG pathway differential abundance between ASD and NT based on LEfSe analysis. The significant pathway enrichments were identified based on an LDA score (log10) > 2.0 and a P-value of 0.05. Additionally, the LEfSe analysis was further performed, considering gender (male and female) and age groups (child, adolescent, and adult) in individuals with ASD and NT. Red boxes represent enrichment in ASD, while blue boxes represent enrichment in NT. The numbers indicate the LDA score, with negative values indicating enrichment in NT individuals.

variations in microbial community structure, as well as the presence of differentially enriched microbes. These differences were influenced by both gender and age. Importantly, our results suggest that gender and age play a crucial role in shaping the differences observed in the gut microbiota between individuals with ASD and NT.

In this study, the alpha diversity analysis revealed a significant difference in microbiota richness between males with ASD and NT males. Specifically, males with ASD had a lower species richness compared to NT males, which is consistent with previous studies that have also reported lower diversity and richness in individuals with ASD[28,29]. However, this significant difference in richness was observed only within the male group, and no significant difference was found between ASD and NT individuals when considering different age groups. The limited sample size of only three neurotypical females in our study might have contributed to the lack of a significant difference in microbial richness between female individuals with ASD and NT females. Therefore, larger sample sizes are needed to draw more reliable conclusions about microbial richness in female individuals

with ASD compared to NT. Nevertheless, when gender was not taken into account, there was still no significant difference in microbial richness. This suggests that gender bias may impact the measurement of microbial diversity between individuals with ASD and NT individuals.

Regarding beta diversity, a previous study by Strati found that the gut microbiota community of individuals with ASD was different from that of NT individuals[30]. However, our study's findings did not align with this. Instead, we observed a shared community between individuals with ASD and NT individuals in terms of beta diversity. This discrepancy could arise from various factors. A potential explanation could be that the previous study matched participants based on age and sex between ASD and NT groups, while in our study, approximately half of the total subjects were paired siblings. These siblings may share genetic and lifestyle patterns that could have influenced the observed results.

Our study, which investigated changes in gut microbiota at taxonomic levels, revealed an enrichment of Fusobacteriota, Fusobacteriaceae, and *Fusobacterium* in individuals with ASD. These enrichments were also observed when considering gender, specifically in females with ASD. However, they did not reach significance in males with ASD. The studies on sex differences in the oral microbiome and gut microbiome found that *Fusobacterium*was present in higher numbers in males compared to females[31,32]. This higher baseline in healthy males may contribute to the inability to observe significant differences between ASD and NT in the male group.

In males, *Ruminococcus gnavus* group was found to be enriched in ASD. Conversely, in males, we observed numerous bacteria enriched in NT individuals, including Synergistota phylum, as well as the families Synergistaceae and Clostridiales vadinBB60 group. Several genera, such as *Cloacibacillus*, Oscillospiraceae UCG-002, Christensenellaceae R7 group, *Eubacterium ruminantium* group, *Ruminococcus gauvreauii* group, and *Clostridia* vadinBB60 group, were also enriched in the NT group. Previous studies have also linked *Fusobacterium* and *Ruminococcus* with ASD[33,34]. Certain species of *Fusobacterium* and *Ruminococcus gnavus* have been linked to inflammation. However, the processes involved are complex and probably involve a combination of factors, including potential interactions with the immune system through surface molecules and toxins production[35,36].

Among gut microbiota enriched in males with NT, *Ruminococcus gauvreauii* group and *Clostridia* vadinBB60 group belong to the *Ruminococcus* and Clostridium, respectively. Both groups are known to include butyrate-producing bacteria[37]. However, no significant change was observed in the butyrate metabolism pathway. Even with the enrichment of specific bacteria, other bacteria may assume a more dominant role in butyrate production, potentially resulting in similar overall effects on butyrate metabolism[38]. A previous study conducted by Zheng et al. discovered that individuals with ASD and constipation have lower levels of beneficial bacteria that produce butyrate, a short-chain fatty acid (SCFA) that supports gut health. They also tend to show elevated levels of *Fusobacterium*, a bacterium that may induce gut inflammation[39]. Furthermore, the earlier investigation revealed a connection between a lower risk of depression and a higher abundance of Oscillospiraceae UCG-002, which was enriched in individuals with NT males[40]. Therefore, our study on ASD implies that the gut microbiota may play a crucial role in its development by impacting inflammation and mental health, which underscores the complexity of these relationships in ASD.

Additionally, our results revealed notable differences in gut microbiota between genders. A previous study involving germ-free mice indicated that the manipulation of the gut microbiota had sex-specific effects on social behaviors, including aggression[41]. Therefore, our findings indicated that the bacterial taxa associated with gender further emphasize the importance of gender in influencing both the composition and outcomes of the gut microbiota.

Regarding age-related taxa, our results showed variations in the differential abundance of gut microbiota between individuals with ASD and NT across different age groups. An interesting finding was the enrichment of *Veillonella*in ASD children, a bacterium previously associated with poor oral hygiene status in Thai children, which often leads to periodontitis and dental caries[42]. Some studies have suggested a potential association between *Veillonella*and certain gastrointestinal diseases, such as inflammatory bowel disease (IBD)[43,44]. In a 20-year large-scale birth cohort study, Ahrens et al. (2024) found an association between gut microbiota and neurodevelopmental disorder (including ASD), as well as early-onset mood and gastrointestinal problems[45]. Additionally, *Tyzzerella*which exhibited enrichment in ASD adolescents in this study, has been proposed as potentially associated with IBD[46]. It corresponds with studies indicating that individuals with ASD have a higher likelihood of developing IBD compared to the general population[47,48].

Another study by Soltysova et al. found an age-related bacterial signature in people with psychiatric disorders including Attention-Deficit/Hyperactivity Disorder (ADHD), depressive disorder, Rett syndrome, and anorexia nervosa[49]. This implied that the composition of gut microbiota may exhibit age-related variations in autistic individuals, similar to observations in other psychiatric disorders."

In our analysis of differentially abundant pathways, we observed that fluorobenzoate degradation, carotenoid biosynthesis, styrene degradation, linoleic acid metabolism, nitrotoluene degradation, geraniol degradation, and atrazine degradation were enriched in individuals with ASD, considering gender and age as separate groups. Specifically, we found that atrazine degradation was enriched in adolescents with ASD. Atrazine is an endocrine-disrupting triazine herbicide that has been studied previously for its immunosuppressive effects. In mouse models, it has been observed to cause decreased delayed-type hypersensitivity (DTH) response and reduced socialization behaviors[50]. Regarding nitrotoluene degradation, while there is currently no direct link established between nitrotoluene degradation and ASD, it is worth mentioning that p-nitrotoluene, a specific compound in the nitrotoluene family, has been used in the synthesis of various chemicals, dyes, and materials such as agricultural and rubber chemicals[51]. The study by Ishido et al. found that p-nitrotoluene, an endocrine disruptor, caused hyperactivity in rats[52]. It is important to highlight those certain environmental chemicals, including atrazine and p-nitrotoluene, may have a potential association with behavioral effects in individuals with ASD.

A study conducted by Gomez in 2021 revealed that early-life exposure to environmental toxicants can lead to persistent changes in the gut microbiota of adult male mice. Throughout the research, maternal exposure

to bisphenol S (BPS) took place from pregnancy day 8 to the end of lactation (postnatal day 21)[53]. BPS, a Bisphenol compound extensively used for food storage in the form of plastic, can disrupt the neuroendocrine system within the hypothalamus. This compound is prevalent in various food packaging materials, including baby bottles, canned food/drinks, as well as sales receipts[54]. The study involved examining the offspring mice in adulthood, revealing a noteworthy alteration in fluorobenzoate degradation within their gut microbiota. Fluorobenzoate, a fluorinated aromatic compound commonly found in pesticides, demonstrated a distinct change due to the exposure[53]. As a result, maternal exposure and early-life exposure to certain environmental toxicants, particularly endocrine disruptors, may have an impact on gut microbiota changes in individuals with ASD and could be implicated in the development of the disease.

In conclusion, our study provides insights into the differences in gut microbiota composition, microbial taxa, and functional pathways between individuals with ASD and neurotypical individuals. Gender and age were found to play significant roles in shaping these differences. The gut microbiota potentially associated with ASD, as indicated in this study, has primarily been linked with other diseases, such as gastrointestinal inflammation (e.g., IBD) and mental health issues. Furthermore, certain environmental chemicals, particularly those classified as endocrine-disrupting chemicals, may have potential associations with behavioral effects in individuals with ASD. However, further research is needed to elucidate the precise mechanisms underlying these associations and their implications for individuals with ASD.

Limitations

This paper would be incomplete without addressing some of its potential limitations. First, while 16S rRNA gene sequencing is a commonly accepted and practical method for microbiota research, it has limitations compared to full-genome sequencing. This approach does not allow for precise species-level identification, and the uneven distribution of 16S rRNA gene copies across different bacterial taxa can introduce biases in relative abundance estimates[55]. Second, the study's sample sizes are quite limited, with only three neurotypical female participants and six neurotypical adults, which may impact the generalizability of the findings for these groups. A larger sample size would strengthen the conclusions regarding differences in microbiota. Third, we did not collect detailed dietary information from participants, which is a limitation given that diet can significantly influence microbiota composition. Lastly, the differential abundance analysis employed the LEfSe method, which is susceptible to null inflation and a high type I error rate when zeros are present in the data. Despite these drawbacks, we chose LEfSe for its ability to provide both statistical significance and biological relevance, effectively identifying key microbial taxa that explain differences between groups in line with our research objectives. Future studies should consider incorporating alternative methods such as ANCOM-BC or MaAsLin2[56].

Data availability

Raw sequences of the V4 hypervariable region of the 16 S rRNA gene for all samples, in FASTQ file format, are available under BioProject accession number PRJNA928241, with the BioSample accession number: SAMN33247443 - SAMN33247537.

Received: 3 August 2024; Accepted: 13 January 2025
Published online: 20 January 2025

References

1. Lord, C., Elsabbagh, M., Baird, G. & Veenstra-Vanderweele, J. Autism spectrum disorder. *Lancet* **392**, 508–520. https://doi.org/10.1016/S0140-6736(18)31129-2 (2018).
2. Zeidan, J. et al. Global prevalence of autism: A systematic review update. *Autism Res.* **15**, 778–790. https://doi.org/10.1002/aur.2696 (2022).
3. Maenner, M. J., Shaw, K. A. & Baio, J. Prevalence of autism spectrum disorder among children aged 8 years—autism and developmental disabilities monitoring network, 11 sites, United States, 2016. *MMWR Surveill. Summ.* **69**, 1 (2020).
4. Chaste, P. & Leboyer, M. Autism risk factors: Genes, environment, and gene-environment interactions. *Dialogues Clin. Neurosci.* **14**, 281–292. https://doi.org/10.31887/DCNS.2012.14.3/pchaste (2012).
5. Yoon, S. H., Choi, J., Lee, W. J. & Do, J. T. Genetic and epigenetic etiology underlying autism spectrum disorder. *J. Clin. Med.* **9**. https://doi.org/10.3390/jcm9040966 (2020).
6. Holingue, C., Newill, C., Lee, L. C. & Pasricha, P. J. Daniele Fallin, M. Gastrointestinal symptoms in autism spectrum disorder: A review of the literature on ascertainment and prevalence. *Autism Res.* **11**, 24–36. https://doi.org/10.1002/aur.1854 (2018).
7. Chaidez, V., Hansen, R. L. & Hertz-Picciotto, I. Gastrointestinal problems in children with autism, developmental delays or typical development. *J. Autism Dev. Disord.* **44**, 1117–1127. https://doi.org/10.1007/s10803-013-1973-x (2014).
8. Lasheras, I. et al. Microbiota and gut-brain axis dysfunction in autism spectrum disorder: Evidence for functional gastrointestinal disorders. *Asian J. Psychiatr.* **47**, 101874. https://doi.org/10.1016/j.ajp.2019.101874 (2020).
9. Srikantha, P. & Mohajeri, M. H. The possible role of the microbiota-gut-brain-axis in autism spectrum disorder. *Int. J. Mol. Sci.* **20**. https://doi.org/10.3390/ijms20092115 (2019).
10. Kang, D. W. et al. Microbiota transfer therapy alters gut ecosystem and improves gastrointestinal and autism symptoms: An open-label study. *Microbiome* **5**, 10. https://doi.org/10.1186/s40168-016-0225-7 (2017).
11. Kang, D. W. et al. Long-term benefit of microbiota transfer therapy on autism symptoms and gut microbiota. *Sci. Rep.* **9**, 5821. https://doi.org/10.1038/s41598-019-42183-0 (2019).
12. Wong, O. W. H. et al. Disentangling the relationship of gut microbiota, functional gastrointestinal disorders and autism: A case-control study on prepubertal Chinese boys. *Sci. Rep.* **12**, 10659. https://doi.org/10.1038/s41598-022-14785-8 (2022).
13. Mehra, A. et al. Gut microbiota and autism spectrum disorder: From pathogenesis to potential therapeutic perspectives. *J. Tradit. Complement. Med.* **13**(2), 135–149 (2022).
14. Werling, D. M. & Geschwind, D. H. Sex differences in autism spectrum disorders. *Curr. Opin. Neurol.* **26**, 146–153. https://doi.org/10.1097/WCO.0b013e32835ee548 (2013).
15. Valeri, F. & Endres, K. How biological sex of the host shapes its gut microbiota. *Front. Neuroendocrinol.* **61**, 100912. https://doi.org/10.1016/j.yfrne.2021.100912 (2021).

16. Hao, X., Pan, J., Gao, X., Zhang, S. & Li, Y. Gut microbiota on gender bias in autism spectrum disorder. *Rev. Neurosci.* https://doi.org/10.1515/revneuro-2020-0042 (2020).
17. Wan, Y. et al. Underdevelopment of the gut microbiota and bacteria species as non-invasive markers of prediction in children with autism spectrum disorder. *Gut* https://doi.org/10.1136/gutjnl-2020-324015 (2021).
18. Bezawada, N., Phang, T. H., Hold, G. L. & Hansen, R. Autism spectrum disorder and the gut microbiota in children: A systematic review. *Ann. Nutr. Metab.* **76**, 16–29. https://doi.org/10.1159/000505363 (2020).
19. Senghor, B., Sokhna, C., Ruimy, R. & Lagier, J. C. Gut microbiota diversity according to dietary habits and geographical provenance. *Hum. Microbiome J.* **7**, 1–9 (2018).
20. Fouquier, J. et al. The gut microbiome in autism: Study-site effects and longitudinal analysis of behavior change. *mSystems* **6**. https://doi.org/10.1128/mSystems.00848-20 (2021).
21. Caporaso, J. G. et al. QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods*. **7**, 335–336. https://doi.org/10.1038/nmeth.f.303 (2010).
22. Douglas, G. M. et al. PICRUSt2 for prediction of metagenome functions. *Nat. Biotechnol.* **38**, 685–688. https://doi.org/10.1038/s41587-020-0548-6 (2020).
23. Kanehisa, M. & Goto, S. K. E. G. G. Kyoto encyclopedia of genes and genomes. *Nucleic Acids Res.* **28**, 27–30. https://doi.org/10.1093/nar/28.1.27 (2000).
24. Kanehisa, M. Toward understanding the origin and evolution of cellular organisms. *Protein Sci.* **28**, 1947–1951. https://doi.org/10.1002/pro.3715 (2019).
25. Kanehisa, M., Furumichi, M., Sato, Y., Kawashima, M. & Ishiguro-Watanabe, M. KEGG for taxonomy-based analysis of pathways and genomes. *Nucleic Acids Res.* **51**, D587–D592. https://doi.org/10.1093/nar/gkac963 (2022).
26. Chong, J., Liu, P., Zhou, G. & Xia, J. Using MicrobiomeAnalyst for comprehensive statistical, functional, and meta-analysis of microbiome data. *Nat. Protoc.* **15**, 799–821. https://doi.org/10.1038/s41596-019-0264-1 (2020).
27. Segata, N. et al. Metagenomic biomarker discovery and explanation. *Genome Biol.* **12**, R60. https://doi.org/10.1186/gb-2011-12-6-r60 (2011).
28. Kang, D. W. et al. Reduced incidence of Prevotella and other fermenters in intestinal microflora of autistic children. *PLoS ONE* **8**, e68322. https://doi.org/10.1371/journal.pone.0068322 (2013).
29. Chen, Z. et al. Gut microbial profile is associated with the severity of social impairment and IQ performance in children with autism spectrum disorder. *Front. Psychiatry* **12**, 789864. https://doi.org/10.3389/fpsyt.2021.789864 (2021).
30. Strati, F. et al. New evidences on the altered gut microbiota in autism spectrum disorders. *Microbiome* **5**. https://doi.org/10.1186/s40168-017-0242-1 (2017).
31. Liu, X. et al. Sex differences in the oral microbiome, host traits, and their causal relationships. *iScience* **26**, 105839. https://doi.org/10.1016/j.isci.2022.105839 (2023).
32. Takagi, T. et al. Differences in gut microbiota associated with age, sex, and stool consistency in healthy Japanese subjects. *J. Gastroenterol.* **54**, 53–63. https://doi.org/10.1007/s00535-018-1488-5 (2019).
33. Ho, L. K. H. et al. Gut microbiota changes in children with autism spectrum disorder: A systematic review. *Gut Pathog.* **12**, 6. https://doi.org/10.1186/s13099-020-0346-1 (2020).
34. Zhang, Q. et al. Comparison of gut microbiota between adults with autism spectrum disorder and obese adults. *PeerJ* **9**, e10946. https://doi.org/10.7717/peerj.10946 (2021).
35. Henke, M. T. et al. Ruminococcus gnavus, a member of the human gut microbiome associated with Crohn's disease, produces an inflammatory polysaccharide. *Proc. Natl. Acad. Sci. U S A* **116**, 12672–12677. https://doi.org/10.1073/pnas.1904099116 (2019).
36. Liu, L., Liang, L., Yang, C., Zhou, Y. & Chen, Y. Extracellular vesicles of Fusobacterium nucleatum compromise intestinal barrier through targeting RIPK1-mediated cell death pathway. *Gut Microbes* **13**, 1–20. https://doi.org/10.1080/19490976.2021.1902718 (2021).
37. Singh, V. et al. Butyrate producers, the sentinel of gut: Their intestinal significance with and beyond butyrate, and prospective use as microbial therapeutics. *Front. Microbiol.* **13**, 1103836. https://doi.org/10.3389/fmicb.2022.1103836 (2022).
38. Louis, P. & Flint, H. J. Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. *FEMS Microbiol. Lett.* **294**, 1–8. https://doi.org/10.1111/j.1574-6968.2009.01514.x (2009).
39. Liu, S. et al. Altered gut microbiota and short chain fatty acids in Chinese children with autism spectrum disorder. *Sci. Rep.* **9**, 287. https://doi.org/10.1038/s41598-018-36430-z (2019).
40. Liang, S. et al. Multi-cohort analysis of depression-associated gut bacteria sheds insight on bacterial biomarkers across populations. *Cell. Mol. Life Sci.* **80**. https://doi.org/10.1007/s00018-022-04650-2 (2022).
41. Sylvia, K. E., Jewell, C. P., Rendon, N. M., John, S., Demas, G. E. & E. A. & Sex-specific modulation of the gut microbiome and behavior in siberian hamsters. *Brain Behav. Immun.* **60**, 51–62. https://doi.org/10.1016/j.bbi.2016.10.023 (2017).
42. Mashima, I. et al. Exploring the salivary microbiome of children stratified by the oral hygiene index. *PLoS ONE* **12**, e0185274. https://doi.org/10.1371/journal.pone.0185274 (2017).
43. Shaw, K. A. et al. Dysbiosis, inflammation, and response to treatment: A longitudinal study of pediatric subjects with newly diagnosed inflammatory bowel disease. *Genome Med.* **8**, 75. https://doi.org/10.1186/s13073-016-0331-y (2016).
44. Santoru, M. L. et al. Cross sectional evaluation of the gut-microbiome metabolome axis in an Italian cohort of IBD patients. *Sci. Rep.* **7**, 9523. https://doi.org/10.1038/s41598-017-10034-5 (2017).
45. Ahrens, A. P. et al. Infant microbes and metabolites point to childhood neurodevelopmental disorders. *Cell* **187**, 1853-1873 e1815. https://doi.org/10.1016/j.cell.2024.02.035 (2024).
46. Mah, C. et al. Assessing the relationship between the gut microbiota and inflammatory bowel disease therapeutics: A systematic review. *Pathogens* **12**. https://doi.org/10.3390/pathogens12020262 (2023).
47. Fousekis, F. S. et al. Inflammatory bowel disease and patients with mental disorders: What do we know? *J. Clin. Med. Res.* **13**, 466–473. https://doi.org/10.14740/jocmr4593 (2021).
48. Kim, J. Y. et al. Association between autism spectrum disorder and inflammatory bowel disease: A systematic review and meta-analysis. *Autism Res.* **15**, 340–352. https://doi.org/10.1002/aur.2656 (2022).
49. Soltysova, M., Tomova, A. & Ostatnikova, D. Gut microbiota profiles in children and adolescents with Psychiatric disorders. *Microorganisms* **10**. https://doi.org/10.3390/microorganisms10102009 (2022).
50. Shelton, J. F., Hertz-Picciotto, I. & Pessah, I. N. Tipping the balance of autism risk: Potential mechanisms linking pesticides and autism. *Environ. Health Perspect.* **120**, 944–951. https://doi.org/10.1289/ehp.1104553 (2012).
51. National Toxicology, P. Toxicology and carcinogenesis studies of p-nitrotoluene (CAS 99-99-0) in F344/N rats and B6C3F(1) mice (feed studies). *Natl. Toxicol. Program. Tech. Rep. Ser.* **498**, 1–277 (2002).
52. Ishido, M., Masuo, Y., Oka, S., Niki, E. & Morita, M. p-Nitrotoluene causes hyperactivity in the rat. *Neurosci. Lett.* **366**, 1–5. https://doi.org/10.1016/j.neulet.2004.04.091 (2004).
53. Gomez, M. V. et al. Early life exposure to environmental contaminants (BDE-47, TBBPA, and BPS) produced persistent alterations in fecal microbiome in adult male mice. *Toxicol. Sci.* **179**, 14–30. https://doi.org/10.1093/toxsci/kfaa161 (2021).
54. Thoene, M., Dzika, E., Gonkowski, S. & Wojtkiewicz, J. Bisphenol S in food causes hormonal and obesogenic effects comparable to or worse than bisphenol A: A literature review. *Nutrients* **12**. https://doi.org/10.3390/nu12020532 (2020).
55. Hugenholtz, P. & Tyson, G. W. Microbiology: Metagenomics. *Nature* **455**, 481–483. https://doi.org/10.1038/455481a (2008).
56. Nearing, J. T. et al. Author correction: Microbiome differential abundance methods produce different results across 38 datasets. *Nat. Commun.* **13**, 777. https://doi.org/10.1038/s41467-022-28401-w (2022).

Acknowledgements
We extend our heartfelt gratitude to Associate Professor Dr. Jeerayut Chaijaruwanich, Dr. Jittisak Senachak, Tayvich Vorapreeda, Tanawut Srisuk, and Pich Tantichukiatkul for their invaluable contributions to this project. This research was supported by King Mongkut's University of Technology Thonburi (KMUTT) for the fiscal year 2018, under project number 61000093. We also express our thanks to the Petchra Pra Jom Klao Ph.D. Research Scholarship from KMUTT for financially supporting Benjaporn Bhusri. Finally, we sincerely appreciate the participation of all the recruited participants.

Author contributions
S.C., S.S., and V.P. contributed to the conceptualization and provided overall supervision of the study. We.K., K.K., C.T., S.L., and Pe.P. provided critical insights during the conceptualization phase. Funding acquisition was managed by S.C., V.P., We.K., K.K., S.S., and Pe.P. Participant recruitment and data acquisition were overseen by V.P. and Wi.K. Pr.P. assisted in data collection, while S.D. provided expertise in DNA extraction. Data analysis and result interpretation were conducted by B.B., who also drafted the initial manuscript. All authors thoroughly reviewed and approved the final version of the manuscript for publication.

Declarations

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-86740-2.

Correspondence and requests for materials should be addressed to S.C.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.