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Altered gut microbiota correlates with behavioral problems but not gastrointestinal symptoms in individuals with autism

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ABSTRACT

Background: Despite inconsistent results across studies, emerging evidence suggests that the microbial micro-environment may be associated with autism spectrum disorder (ASD). Geographical and cultural factors highly impact microbial profiles, and there is a shortage of data from East Asian populations. This study aimed to comprehensively characterize microbial profiles in an East Asian sample and explore whether gut microbiota contributes to clinical symptoms, emotional/behavioral problems, and GI symptoms in ASD.

Methods: We assessed 82 boys and young men with ASD and 31 typically developing controls (TDC), aged 6–25 years. We analyzed the stool sample of all participants with 16S V3-V4 rRNA sequencing and correlated its profile with GI symptoms, autistic symptoms, and emotional/behavioral problems.

Results: Autistic individuals, compared to TDC, had worse GI symptoms. There were no group differences in alpha diversity of species richness estimates (Shannon-wiener and Simpson diversity indices). Participants with ASD had an increased relative abundance of \textit{Fusobacterium}, \textit{Ruminococcus torques group} (at the genus level), and \textit{Bacteroides plebeius DSM 17135} (at the species level), while a decreased relative abundance of \textit{Ruminococcaceae UCG 013}, \textit{Ervsipelotrichaceae UCG 003}, \textit{Parasutterella}, \textit{Clostridium sensu stricto 1}, \textit{Turicibacter} (at the genus level), and \textit{Clostridium spiroforme DSM 1552} and \textit{Intestinimonas butyriciproducens} (at the species level). Altered taxonomic diversity in ASD significantly correlated with autistic symptoms, thought problems, delinquent behaviors, self-dysregulation, and somatic complaints. We did not find an association between gut symptoms and gut microbial dysbiosis.

Conclusions: Our findings suggest that altered microbiota are associated with behavioral phenotypes but not GI symptoms in ASD. The function of the identified microbial profiles mainly involves the immune pathway, supporting the hypothesis of a complex relationship between altered microbiome, immune dysregulation, and ASD that may advance the discovery of molecular biomarkers for ASD.

1. Introduction

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder affecting up to 1.5% of children and youth worldwide (Chen et al., 2019; Chiarotti and Venerosi, 2020; Rosenfeld, 2015). In addition to functional impairment in social communication, interactions, and necessary life skills (Lai et al., 2014), Autistic individuals have more co-occurring gastrointestinal (GI) symptoms such as constipation, abdominal pain, chronic diarrhea, and flatulence as compared to those typically developing populations across wide age ranges (Lanyi et al., 2022;
Lefter et al., 2019; Molloy and Manning-Courtney, 2003; Penzol et al., 2019; Wasilewska and Klukowski, 2015). Elevated anxiety (Mazurek et al., 2013) and emotional/behavioral problems (Mazeyek et al., 2014) in children with ASD are commonly associated with their GI symptoms. Such GI distress may be related to alterations in the gut microbiota (Adams et al., 2011; Coury et al., 2012), which are microbial communities inhabiting the GI tracts (Morais et al., 2021b). Reversely, stereotyped and restricted behaviors/interests of autistic individuals may also contribute to idiosyncratic dietary styles and irregular bowel movements, thereby influencing the taxonomic diversity in their gut (Yap et al., 2021).

ASD is characterized by aberrant brain development, partly underpinned by the functional interplay between immune dysregulation and abnormal neural synaptic development (Bourgeron, 2015; Ebrahimi-Fakhari and Saitoh, 2015; Matta et al., 2019). Notably, the gut microbiota may play an essential role in these pathophysiological processes by participating in the microbiota-gut-brain axis, multidirectional communications between the brain, the autonomic nervous system, the GI tract, and microbial communities, likely through mechanisms such as endocrine, vagal, immune, and microbiota-generated metabolite signaling (Fried et al., 2021; Niesler et al., 2021). Imbalanced gut microbiota (i.e., dysbiosis) may be implicated in many biological mechanisms, especially neuroplasticity, epigenetic and gene expression, and the neuroimmune system, that is associated with ASD (Davoli-Ferreira et al., 2021; Han et al., 2021; Kushak et al., 2022; Morais et al., 2021a; Zengeler and Lukens, 2021).

Over the past decade, dozens of studies have focused on characterizing microbial features associated with ASD. In humans, multiple bacterial species, such as a higher abundance of genus Lactobacillus (Tomova et al., 2015), altered (either higher (Coretti et al., 2017; Zhang et al., 2018) or lower (Strati et al., 2017)) abundances of Bacteroides, a higher abundance of phylum Firmicutes (Agarwala et al., 2018; Strati et al., 2017; Tomova et al., 2015), etc., have been linked to ASD. However, the involved species of the altered gut microbial compositions are inconsistent across the studies (Peralta-Marzial et al., 2021). For example, the diversity of gut microbiota (quantified by alpha-diversity), which is thought to promote the host’s health if highly diverse (Huttenhower et al., 2012), either is increased (Coretti et al., 2017; De Angelis et al., 2013; Finegold et al., 2010) or is decreased (Dan et al., 2020) in children with ASD when compared to typically developing control (TDC) children. Moreover, a ratio between Firmicutes and Bacteroidetes, which are two dominant bacterial phyla representing around 90% of the total bacterial species in the human gut and both are responsible for carbohydrate metabolism (Qin et al., 2010), is critical in maintaining the homeostasis of the microbiota-gut-brain axis (Huttenhower et al., 2012). Changes in this ratio in children with ASD are conflicting in directions (i.e., lower (Coretti et al., 2017; De Angelis et al., 2013; Finegold et al., 2010; Zhang et al., 2018) vs higher (Strati et al., 2017; Tomova et al., 2015) ratios). Another caveat is that although there is a clear causal link between autistic-like behaviors and gut dysbiosis in a mice model (Sharon et al., 2019), as well as emerging human evidence suggesting altered microbiota compositions in ASD, most existing human studies (Nitschke et al., 2020; Peralta-Marzial et al., 2021) have failed to establish a link between the fecal microbial profile and the severity of core symptoms or other associated clinical features in ASD (c.f. (Finegold, 2011; Tomova et al., 2015)). These inconsistencies may arise from several factors such as sampling variations (Mottron and Bzdok, 2020), sample sizes, variable phenotyping endeavors, techniques used to characterize the gut microbiota composition (Nitschke et al., 2020), and how GI symptoms are accounted in the analysis (Davies et al., 2021). For example, Dan et al. (2020) reported that the gut microbiota profile is only altered in children with ASD plus constellation. In addition, cultural/behavioral-related geographical/ethnic variations such as diet, environmental exposure, hygiene, etc., and innate differences in host genetics and immunity (Gupta et al., 2017) may contribute to heterogeneity in gut dysbiosis associated with ASD. Therefore, it is imperative to expand the literature to non-Western populations (Pulikkan et al., 2018; Zhang et al., 2018) to provide a fuller picture of the relationship between gut microbiota and ASD.

In this context, based on a deep-phenotyping autistic cohort with a relatively large sample size in Asia, first, we aimed to examine the gut microbiome dysbiosis in ASD in several domains, including taxonomic diversity, differential microbiota abundance, and the predictive function and pathway. Second, we aimed to investigate the relationship between this autism-associated gut microbiome dysbiosis and clinical symptoms, including autistic features, emotional/behavioral problems, and GI problems. We expected to find differences in the relative abundance of specific microbiota between ASD and TDC and the predictive functions and pathways. Further, we hypothesized that the identified altered microbial profiles in ASD would be associated with autistic symptoms and emotional/behavioral problems but not GI problems. Given the inconsistent results, we did not hypothesize that the specific species would be associated with ASD.

2. Methods and materials

2.1. Participants and procedure

We recruited eighty-two boys and young men (aged 6–25 years) with ASD from the National Taiwan University Hospital (NTUH), Taipei, Taiwan. The corresponding author made the clinical diagnosis of ASD according to the criteria of ASD in the DSM-5 (American Psychiatric Association, 2013). It was further confirmed by the interview with parents using the Autism Diagnostic Interview-Revised (ADI-R) and by the assessments with participants using the Autism Diagnostic Observation Scale (ADOS). The Western Psychological Services approved the Chinese version of ADI-R and ADOS in June 2007 and April 2008, respectively, for assessing ASD in clinical and research settings in Taiwan (Chien et al., 2010; Gau et al., 2010; Lo et al., 2019). We further transformed an ADOS-2 algorithm total raw score (Gotham et al., 2007) into a standardized calibrated severity score (CSS) (Gotham et al., 2009; Hus and Lord, 2014) to allow for the cross-module analysis. Ten participants with ASD did not reach the ADOS-CSS cutoff of the autism spectrum (labeled as ‘non-spectrum’ in Table 1). Still, the clinical diagnosis of ASD in all participants in their childhood was confirmed by interviews with parents using the ADI-R. No autistic participants in this study were from the same families; we did not recruit siblings to prevent the lack of independence within the same family.

Thirty-one TDC boys and young men were recruited from the schools or communities geographically and socioeconomically similar to the ASD group. TDC had an equal age range (aged 6–25 years) with that of ASD. All participants and their parents were interviewed to comprehensively survey the psychiatric diagnoses of participants using the Chinese version of the Schedule for Affective Disorders and Schizophrenia for School-Age Children – Epidemiological Version (K-SADS-E) interview for DSM-5 (Chen et al., 2017b). Exclusion criteria for both groups included having current neurological or systemic medical illness or major mental disorders, including any substance use disorders (including alcohol, tobacco, marijuana, or any hard drugs), schizophrenia, mood disorders, anxiety disorders; current use of psychotropic medication except methylphenidate; receiving any supplemental probiotics or prebiotics or being treated with any antibiotics within one month before the study assessment.

Methylphenidate is commonly used in individuals with ASD (18 participants were using it herein), and there is no evidence suggesting methylphenidate could affect the gut microbiota (Vich Vila et al., 2020). We thus decided not to exclude the participants who were taking methylphenidate. All TDC participants were clinically confirmed to ensure they had no mental disorders. Participants with ASD, who had co-occurring attention-deficit hyperactivity disorder (ADHD), obsessive-compulsive disorder (OCD), tic disorders, or learning disorders, were still included in the studies considering the common co-occurrence of
Table 1
Demographics, intelligence, and clinical symptoms for the autism spectrum disorder (ASD) and typically developing control (TDC) groups.

|                          | ASD (N = 82) | TDC (N = 31) | Statistics |
|--------------------------|--------------|--------------|------------|
| Male, n (%)              | 82 (100%)    | 31 (100%)    |            |
| Age (year), mean ± SD    | 17.16 ± 4.91 | 13.02 ± 3.86 | F = 17.86, p < 0.001 |
| Body Mass Index (BMI)    | 20.86 ± (6-25) | 17.65 ± (6-25) | F = 13.29, p < 0.001 |
| Full-scale IQ            | 100.48 ± 4.45 | 112.16 ± 3.33 | F = 10.79, p < 0.001 |
| Social awareness         | 6.67 ± 2.64  | n.a. ± 3.9   |            |
| Stereotyped behaviors/ interest | 3.86 ± 2.15 | n.a. ± 3.06  |            |
| Social awareness         | 20.81 ± 4.00 | 11.45 ± 6.18 | F = 95.15, p < 0.001 |
| Social emotion           | 11.99 ± 4.63 | 5.19 ± 4.45  | F = 55.62, p < 0.001 |
| Total score (0–195)      | 86.24 ± 28.98 | 33.21 ± 25.97 | F = 87.81, p < 0.001 |

Table 1 (continued)

|                          | ASD (N = 82) | TDC (N = 31) | Statistics |
|--------------------------|--------------|--------------|------------|
| Social Responsiveness Scale (SRS) |              |              |            |
| Social communication     | 35.45 ± 16.11 | 11.35 ± 11.59 | F = 65.12, p < 0.001 |
| Stereotyped behaviors/interest | 35.45 ± 16.11 | 5.26 ± 6.03    | F = 79.15, p < 0.001 |
| Social awareness         | 20.81 ± 4.00 | 11.45 ± 6.18 | F = 95.15, p < 0.001 |
| Social emotion           | 11.99 ± 4.63 | 5.19 ± 4.45  | F = 55.62, p < 0.001 |
| Total score (0–195)      | 86.24 ± 28.98 | 33.21 ± 25.97 | F = 87.81, p < 0.001 |

Table 1 (continued)

|                          | ASD (N = 82) | TDC (N = 31) | Statistics |
|--------------------------|--------------|--------------|------------|
| Food preferences (0–30)  | 5.09 ± 4.88  | 3.29 ± 4.37  | F = 4.03, p = 0.047 |
| (0–17)                   | (0–12)       |              |            |

The SRS and CBCL statistics were controlled for age, ADHD comorbidity, and methylphenidate use. The range of each GI index is presented in parentheses. The statistical values of group comparison of GI symptoms were controlled with self-reported types and age. We used ANOVA to compare these variables between groups. Abbreviation: IQ = intelligence quotient; SD = standard deviation; RRSBI = Restricted, repetitive/stereotyped behaviours and interests; CSS = calibrated severity score; ASD = autism spectrum disorder, TDC = typically developing control.

these neurodevelopmental disorders in ASD. Because these co-occurring neurodevelopmental disorders may affect the findings associated with ASD differently, we implemented several strategies to account for confounding comorbidities as detailed in the following sections (that is, ADHD and methylphenidate usage as categorical nuisance covariates; additional subgroup analysis for tic and OCD that may be associated with pediatric acute-onset neuropsychiatric syndrome (PANS) or pediatric autoimmune neuropsychiatric disorder associated with streptococcal infections (PANDAS) (Quaglialiero et al., 2018). As GI symptoms and dietary styles may impact the diversity of the gut microbiome (Yap et al., 2021), GI problems were assessed by the gastrointestinal symptoms questionnaire (Bovenschen et al., 2006), detailed as follows, to ensure that all of the participants did not have any GI diseases diagnosed in the past three months and did not use any supplement probiotics. All participants (n = 113) received screening assessments of the history of physical health conditions including: asthma (n = 0), thyroid disease (n = 0), allergies (n = 42), hematological diseases (n = 1), hepatitis B or C carriers (n = 0), diabetes (n = 0), epilepsy (n = 5), heart diseases (n = 2), hypertension (n = 0), autoimmune diseases (n = 0) and glucose-6-phosphate dehydrogenase deficiency (G6PD; n = 0). In our samples, we also found nine autistic participants had a history of GI disease, which was resolved at least one year before the study. The presence of any history of physical conditions was also included as a nuisance covariate in all analyses, as detailed below.

The Wechsler Intelligence Scale assessed participants’ intellectual function for Children’s-3rd edition (WISC-III) (Wechsler, 1991) for those aged younger than 16 years, and the Wechsler Adult Intelligence Scale-3rd edition (WAIS-III) (Wechsler, 1997) for those above 16 years. Handedness was assessed by the Edinburgh Handedness Inventory (Oldfield, 1971).

The Research Ethics Committee of the NTUH (approval number, 201707041RINA; ClinicalTrials.gov number, NCT02719067, and NCT04873674) approved this study before implementation. Considering difficulties in implementing both projects and statistical power, we decided to pool the participants from both projects listed on ClinicalTrials.gov. Sixty participants were recruited from NCT02719067, and 53 participants enrolled in NCT04873674. The inclusion/exclusion criteria and baseline cross-sectional assessments were identical for every participant from these two projects. The procedures and purpose of the study were explained face-to-face to the participants and their parents, who then provided written informed consent.

2.2. Measures

2.2.1. Social Responsiveness Scale assessments (SRS)

The SRS quantifies autistic traits and associated social/communication/emotion symptoms and demonstrates a continuous distribution of autistic traits in the general population (Constantino and Todd, 2000; Fombonne et al., 2012), which is not affected by intelligence, age, race, or the education level of respondents (Constantino et al., 2003). The psychometric properties of its Chinese version have been established,
with a validated four-factor structure (Gau et al., 2013), including social awareness, social communication, social emotion, stereotyped behaviors, and a total score, and has been widely used in autism research in Taiwan (Tung et al., 2021).

3. Children behavior checklist (CBCL)

The CBCL is a parent/caregiver-rated questionnaire used to measure the emotional/behavioral problems in youth aged 4–18. Each item is scored 0 if not true, 1 if somewhat or sometimes true, and 2 if very true or often true. Eight emotional/behavioral domains were quantified on the CBCL, including Anxious/Depressed symptoms, Attention Problems, Aggressive Behaviors, Delinquent Behaviors, Social Problems, Thought Problems, Somatic Complaints, and Withdrawn, which can be further summarized as two subscales of internalizing (emotional) problems and externalizing (behavioral) problems (Pandolfi et al., 2009). The Child Behavior Checklist (CBCL) is a widely-used parent-reported scale used for evaluating behavioral problems of children aged 4–18. Eight subscales were derived from the 118 items: attention, anxiety/depression, aggression, delinquency, social problems, somatic symptoms, thought problems, and withdrawn (Achenbach, 1991). Raw scores on each subscale were transformed into T-scores with a mean of 50 and a standard deviation (SD) of 10. The Chinese version of CBCL was established according to the norm established in Taiwan and has been proved reliable and valid (Yang et al., 2000). The summed T-scores of the Anxious/Depressed, Aggression, and Attention subscales (dysregulation profile) of the CBCL are defined as CBCL-DP (Ayer et al., 2009). The CBCL-DP includes manifestations of the three components of self-regulation: affect, behavior, and cognition; emotional dysregulation (ED) could be conceptualized as a combination of these problems. The CBCL-DP has been extensively used in clinical and non-clinical subjects (Kim et al., 2012) to characterize emotional and behavioral dysregulation. This proxy measure of the CBCL-DP is also a well-validated and replicated estimate of ED in ADHD (Biederman et al., 2012; Donfrancesco et al., 2015; Uchida et al., 2014) and ASD (Keefer et al., 2020; Vasa et al., 2012). Prior studies have shown that children with ADHD who have CBCL-DP sum T-scores above 180 have greater clinical severity and psychosocial impairments later in life (Biederman et al., 2009; Biederman et al., 2012; Peyre et al., 2015; Spencer et al., 2011).

4. Evaluation of GI severity index

All participants reported their GI symptoms and the disturbance frequency over the past four weeks before assessments, and then the investigators rated the severity score. Expressly, the self-report was provided either by the participant only (for younger participants who were younger than 15 years old, who may need assistance to understand the questions) or by the participant with his/her parents. The sample distributions of the two self-report types did not differ between the diagnostic groups ($X^2 = 2.19; p = 0.139$; Table 1). The gastrointestinal symptoms questionnaire, developed by Bovenschen et al. (2006), includes questions about the severity of GI symptoms during the last four weeks. It contains 23 questions and covers the details of frequency and duration of symptoms (e.g., day or night), including abdominal pain (4 items), epigastric pain (3 items), heartburn, regurgitation, abdominal rumbling, bloating, empty feeling, nausea, vomiting, loss of appetite, postprandial fullness, belching, flatulence, haematemesis, and dysphagia summarized as upper GI symptoms (13 items). The bowel habits (11 items) include melaena, bloody stool, mucus in stool, frequent hard stool, diarrhoea, mixed stool consistency, constipation, frequent pain experience, urgent stools, incomplete evacuation, and steatorrhoea. Food preferences (5 items) include meat, vegetable, fruit, grain, and dessert. Each item is rated 0–6, whereby 0 means “no complaints,” and 6 represents the worst unbearable severity of a specific symptom. The investigators scored each symptom based on the interviews with the participants and their parents.

4.1. Fecal sample collection and DNA extraction

Fresh stool samples were collected from all of the participants. The samples were placed into sterile stool tubes, frozen at $-20\,\degree\text{C}$ for temporary preservation for less than seven days, and then transported in storage with a large quantity in a dry ice box within two hours to the lab, where the samples were frozen at $-80\,\degree\text{C}$ for future use. The details are described in supplementary method (b).

4.2. 16S rRNA amplicon library construction and illumina V3V4 sequencing analysis

Amplification sequencing was performed using 300 bp paired-end raw reads and demultiplexed each sample based on barcode identification. After demultiplexing, the paired-end reads removed primer and adapter sequences using the QIME2 cutadapt plugin (Martin, 2011). For the ASVs (Amplificon Sequence Variants) construction, the denoising pipeline was performed with the QIME2 DADA2 plugin ($v2021.4$; https://qiime2.org/) (Callahan et al., 2016; Quin et al., 2018), including quality filtering, dereplication, dataset-specific error model learning, denoising, paired-end reads joining and chimeras removing. There were no group differences in overall quality control and sequencing effectiveness (Table 2). The trimming and filtering were performed with a maximum of two expected errors per reading (maxEE = 2). DADA2 algorithm resolves exact merged paired-end reads with overlapping 12 base pairs with near-zero error rate. For each representative sequence, the feature-classifier (Bokulich et al., 2018) and algorithm in QIME2 (Bolyen et al., 2019) were employed to annotate taxonomy classification based on the information retrieved from the Silva database. To analyze the sequence similarities among different ASVs, we performed multiple sequence alignment using the QIME2 alignment MAFFT (Katoh and Standley, 2013) against the Silva database (Balvoociute and Huson, 2017; Gyarmati et al., 2016; Hong et al., 2016). A phylogenetic tree was constructed with a set of sequences representative of the ASVs using the QIME2 phylogeny fasttree (Price et al., 2009, 2010). To normalize the variations in sequence depth across samples, ASVs abundance information was rarefied to the minimum sequence depth using the QIME2 script (single_rarefaction.py).

4.3. Statistical analysis

4.3.1. Covariate variables

To provide a more precise overview, we outlined the procedures and rationales for choosing nuisance covariates before explaining the statistical models. Namely, the choice of the nuisance covariates for analyses on study objectives was decided considering demographic differences and the potential biologically relevant confounding factors. First, the group comparisons of the gut microbiome were controlled for the factors which may influence the gut microbiome: age, body mass index (BMI), health conditions of allergies, hematological diseases, epilepsy, heart diseases, GI symptoms (upper GI symptoms and bowel habits, which were significantly different between groups), food preferences, historical GI diseases, ADHD comorbidity, and methylphenidate use. Second, the correlations of behavioral symptoms of SRS and CBCL with microbial profiles were controlled for age, ADHD comorbidity, and methylphenidate use. In contrast, the correlational analysis between microbial profiles and GI symptoms was additionally controlled for self-reported type and age. We performed the covariates analysis using a generalized linear model (GLM) to control the effects and extract the residuals from the ASV table generated from QIIME 2, as well as clinical symptoms, yielding the adjusted ASV table, alongside the adjusted clinical symptoms, for the subsequent analyses.

4.3.2. Taxonomic diversity analysis

Alpha diversity was indicative of the species complexity (i.e., the number of different species represented in the microbial community)
within individual samples based on five other criteria output from the QIIME2 pipeline, including Shannon, Simpson, PD whole tree, and Good-coveragen (Whittaker, 1972). Community richness was assessed by the species richness and the relative abundance and evenness accounting for diversity, which was evaluated by Pielou’s evenness, Shannon, and Simpson indices. A rarefaction curve was constructed by randomly selecting a certain amount of sequencing data from each sample to represent the number of the observed species (Schloss et al., 2009). The details of these methods are provided in Supplementary Methods.

Beta diversity analysis was used to evaluate the differences among samples in terms of species complexity. Two beta diversity parameters, the weighted and unweighted UniFrac (Lozupone and Knight, 2005; Lozupone et al., 2011), were calculated using the QIIME2 pipeline. The principal component analysis (PCA) of beta diversity via unadjusted and non-transformed covariance matrix assessed which variables with dominant ASVs contributed most of the data variances. PCA was applied to reduce the dimensions of the multiple variables using the FactoMineR package and ggplot2 package in R software. Principal Coordinate Analysis (PCoA) was performed using the distance matrix to acquire principal coordinates to visualize sophisticated and multidimensional data in both groups, aiming to discrete the samples in different dimensions (Jiang et al., 2013). A distance matrix of weighted, unweighted UniFrac and Bray-Curtis dissimilarity among samples obtained previously was transformed into a new set of orthogonal axes, by which the most influential variable was represented by the first principal coordinate and the second most influential one by the second principal coordinate, and so on. We conducted PCoA analysis using the stat and ggplot2 packages in R. To further increase the group distinction, the supervised partial-least-squares discriminant analysis (PLS-DA) was used to evaluate and visualize variance based on the level of gut microbiota composition among the groups. PLS-DA was performed using the R package mixOmics (https://mixomics.org/). The post hoc analysis of group differences was tested by Tukey’s test and Fisher’s Least Significant Difference (LSD).

4.3.3. Microbiota differential abundance and pathway analysis
We compared all species at various taxonomic levels between the two groups using Wilcoxon rank in the STAMP software (v2.1.3) (Parks et al., 2014). Statistically significant biomarkers were identified using the LEfSe analysis Field (Segata et al., 2011), which assisted in identifying genomic biomarkers that characterize statistical differences among biological groups (Chang et al., 2022). In brief, LEfSe is an approach based on an algorithm that performs the non-parametric Wilcoxon rank-sum test to identify bacterial taxa whose relative abundance is significantly different between the case and control groups. LEfSe applies linear discriminant analysis (LDA) to those bacterial taxa identified as significant differences and further assesses the effect size of each differentially abundant taxon. This study considered taxa with an LDA score (log 10) > 2 as statistically significant. To determine whether the community structures significantly differ between and within groups of categorical metadata, we used Anosim, which is a non-parametric multivariate approach (Bruno et al., 2008; Clarke, 1993), multiple response permutation procedure (MRPP) analysis (Xu et al., 2017), and Adonis analysis (Anderson, 2001; Chan et al., 2016), which corresponds to the permutational multiple ANOVA. Additionally, ANCOMv2.1 (Mandal et al., 2015) was used to compare microbial communities based on differential abundance analysis for absolute microbial abundances. It accounts for the compositional nature of microbiome data by performing the compositional log-ratio (CLR) transformation. ANCOM employs a heuristic strategy to declare taxa that are significantly differentially abundant. For a given taxon, the output W statistic represents the number of CLR transformed models where the taxon is differentially abundant about the variable of interest. The larger the value of W, the more likely the taxon is differentially abundant (Everard et al., 2013; Morton et al., 2019; Zhu et al., 2020). This study’s cutoff value corrected by false discovery rate (FDR) is Wdetected 0.6, indicating significance. ALDEx2 (ANOVA-Like Differential Expression analysis version 2) was used to detect species with significant differences in microbial communities between groups (Fernandes et al., 2014). ALDEx2 used CLR as data transformation and estimated using Dirichlet distribution and the Monte-Carlo method for the comparison (Fernandes et al., 2013). Data were transformed into log-ratio distributions to account for the compositional nature of the data. In this study, the significant effect size had to be over 1.

For the predictive functional and pathway analyses, functional abundances from 16S rRNA sequencing data were analyzed for the prediction of functional genes with the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2; version 2.3.0) (Douglas et al., 2020), Tax4Fun2 (Ajhauer et al., 2015; Wemheuer et al., 2020), and FAPROTAX (v1.1) (Loca et al., 2016). In addition, inferred microbial community phenotypes were predicted using BugBase on six phenotype categories, including Gram staining, oxygen tolerance, biofilm formation, mobile element content, pathogenicity, and oxidative stress tolerance (Ward et al., 2017). FDR-adjusted p values ($p_{\text{adj}} \leq 0.05$) were considered statistically significant (Shaffer, 2007).

All preceding microbiome indices and their implications are summarized in Table 3.

4.3.4. Association between symptoms and microbiota abundance analysis
First, correspondence and multiple regression analyses were combined to yield the microbial community structure representing the overall taxonomic diversity, constrained Ordination, or Multivariate Direct Gradient Analysis. This approach was used to reflect the relationships between species and behavioral symptoms and detect essential driving variables affecting individual taxonomic diversity distribution. Each step of the calculation is controlled for the covariate variables. The redundancy analysis (RDA) process was applied, mainly including: (1) calculating the Euclidean distance matrix according to the selected algorithm; (2) performing the PCoA on the distance matrix; and (3) RDA analysis using the eigenvalues obtained by PCoA and variables of symptoms. In brief, we used the Bray-Curtis distance matrix as a microbial community. As to another distance matrix, we calculated the Euclidean distance of the behavioral symptoms, including metrics from SRS, GI symptoms, and CBCL. The Mantel test Field analyzed the association between symptoms and microbial community (Mantel, 1967). The distance matrix-based mantel test can test a single or a group of factors relevant to the entire microbial community. The value of the distant attribution was calculated and converted into a distance matrix for analysis (Rui et al., 2015; Smith et al., 2015). We used the vegan package in R (https://github.com/vegandevs/vegan) to implement the Mantel test. The results were generated with 10,000 permutations and corrected by FDR. Further, we also used a generalized linear model to test the correlation between the significant microbiota, which showed group differences identified from diversity and differential abundance analyses, and behavioral symptoms.

5. Results
5.1. The sample characteristics
Table 1 shows demographics, autistic symptoms, and GI symptoms for the ASD and TDC groups. The Shapiro Wilk test revealed that most distributions of emotional/behavioral problems did not deviate from the normality assumption (Table 4). Despite within the same age range, the ASD group (mean age 17.16 ± 4.91 years) was older than the TDC group (13.02 ± 3.86 years) ($p < 0.001$). In addition, we found that SD had a higher BMI than TDC. Therefore we included age and BMI as covariates while examining the microbiome diversity. The ASD group had a lower IQ than TDC. We further compared SRS and CBCL after controlling for ADHD comorbidity, methylphenidate use, and age and showed that all the CBCL subscales scored higher in ASD than TDC,
except for somatic complaints of CBCL ($F = 1.47, p = 0.228$; Table 1). All autistic symptom domains (SRS) scored significantly higher in ASD than TDC. The GI symptoms were compared after controlling for the self-reported types and age effect. We found that the overall upper GI symptoms ($F = 5.63, p = 0.019$) in the ASD group were more severe than in the TDC group, especially in bloating, empty feeling, nausea, and postprandial fullness (Table 5). As for bowel habits, the ASD group was more severe than the TDC group ($F = 5.70, p = 0.019$), particularly in constipation and incomplete evacuation but not diarrhea (Table 5). ASD group reported more food preferences in meat ($F = 6.82, p = 0.010$; Table 5).

A. Venn diagram of observed ASVs

![Venn diagram](image1)

B. *Firmicutes* to *Bacteroidetes* ratio

![Ratio graph](image2)

C. Top 10 taxa of relative abundance (%)

![Relative abundance graph](image3)

D. Beta diversity

![Beta diversity graph](image4)
5.2. The taxonomic diversity between ASD and TDC

We assessed 4198 ASVs and found 2285 and 683 uniquely observed ASVs in ASD and TDC, respectively (Fig. 1A). They included 342 genus-level microbiota and 146 species-level microbiota in ASD and TDC. There was no significant group difference in the quality measures of observed sequence length and effective tag (Table 2). No sample sequencing bias in further analyses can be confirmed, while the rank relative abundance curve showed no differences between groups (Fig. 1B). Further, we compared the Firmicutes to Bacteroidetes ratio (F/B ratio), top 10 relative abundance of microbiota in the genus, and alpha and beta diversity between ASD and TDC after controlling for the covariates (age, ADHD comorbidity, methylphenidate use, upper GI symptoms, bowel habits, food preferences, BMI, and history of GI diseases and physical health conditions and BMI). The F/B ratio (Fig. 1B) was not significantly different between the two groups ($p_{\text{FDR}} = 0.858$). The relative abundance of the top 10 genera of microbiota (Bacteroides, Escherichia/Shigella, Bifidobacterium, Faecalibacterium, Blautia, Subdoligranulum, Collinsella, Eubacterium hallii group, Ruminococcus 2, Agathobacter and others) were not significantly different between ASD and TDC ($p_{\text{FDR}} > 0.05$; Fig. 1C). In contrast, we observed that the taxa relative abundance and the dominant species were different between ASD and TDC by visualization of a taxonomy tree using GraPhiAn (Asnicar et al., 2015) (Fig. 2F), in which the critical nodes of microbiota were also distinct at the cluster level evolution heat tree (Fig. 3). The Unweighted UniFrac distance of beta diversity was not different between ASD and TDC ($p_{\text{FDR}} = 0.054$; Fig. 1D), while the weighted UniFrac distance of beta diversity in ASD was significantly higher than TDC ($p_{\text{FDR}} < 0.001$; Fig. 1D).

We also found no significant differences in bacterial richness, evenness, and diversity of the microbial communities between the ASD and TDC groups based on alpha-diversity indices ($p_{\text{FDR}} > 0.05$, Table 2).

Next, we compared the relative abundance of microbiota community between ASD and TDC from phylum to genus level (Fig. 4). After controlling for nuisance covariates (age, ADHD comorbidity, methylphenidate use, upper GI symptoms, bowel habits, food preferences, BMI, and history of GI diseases and physical health conditions), the Wilcoxon test identified four microbiota, Ruminococcaceae UCG 013 ($p_{\text{FDR}} = 0.006$), Ruminococcus torques group ($p_{\text{FDR}} = 0.003$), Erysipelotrichaceae UCG 003 ($p_{\text{FDR}} = 0.025$) and Parasutterella ($p_{\text{FDR}} < 0.001$) were significantly different in relative abundance between ASD and TDC (Fig. 2A1). Further, we carried out a Spearman correlation between these four microbiota and other 339 microbiota at the genus level separately in ASD and TDC. The resulting correlation matrices further underwent Fisher Z transformation to facilitate the group comparisons, corrected by FDR. We found six of these 339 microbiota (aswinbacter, Allisonella, Lachnospiraceae UCG 001, Intestinimonas, Turicibacter and Ruminoclostridium) showed significant group differences ($Z$ score either $<-3$ or $>3$) in their correlations with this four microbiota (Fig. 2A2). Next, at the species level, we found the top five microbiota loading based on the PCA analysis of Principal Component 1 (34.2%) and 2 (19.8%) (Fig. 2B), including Bacteroides fragilis, plebeius DSM 17135, coprocola DSM 17136 and stercoris ATCC 43,183 and Ruminococcus sp N15MGS 57. Though the samples of ASD and TDC were not well separated, we used the Wilcoxon test and found one of these five microbiota in relative abundance, Bacteroides plebeius DSM 17135, was significantly higher in ASD than TDC ($p_{\text{FDR}} = 0.015$).

Furthermore, we investigated the overall microbiota community differences from phylum to species levels between ASD and TDC to discover potential evolutionary biomarkers associated with ASD based on the gut microbiome. As shown in Fig. 2C, LefSe analysis identified predominant bacterial biomarkers contributing to the group differences. Specifically, the ASD group had significant increases (LDA $> 2$ with a total of 20 potential biomarkers; Table 8) in the relative abundance of Intestinibacter, Allisonella, Fusobacterium (at the genus level), as well as Parabacteroides merdae and Megaphaecalis elsdeni (at the species level). On the other hand, significant decreases in Clostridium sporofera DSM 17135.

### Table 2

| Alpha diversity indexes | ASD (N = 82) | TDC (N = 31) | $p$-value ($p_{\text{FDR}}$) |
|-------------------------|--------------|--------------|-----------------------------|
| Species richness        | 189.59 ± 56.38 | 194.90 ± 57.10 | 0.563                       |
| Shannon-Wiener diversity index | 5.03 ± 0.91 | 5.29 ± 0.68 | 0.210                       |
| Simpson diversity index | 0.91 ± 0.10 | 0.94 ± 0.05 | 1.50                         |
| Pioulo’s evenness index  | 0.67 ± 0.10 | 0.70 ± 0.07 | 0.182                       |
| Phylogenetic diversity (PD) | 13.71 ± 4.84 | 14.09 ± 4.58 | 0.548                       |
| whole tree               | 3.48 | 3.71 | 0.639                       |
| Goods coverage index     | 0.99 ± 0.0006 | 0.99 ± 0.0009 | 0.639                       |

Total six indexes of alpha-diversity showed no group differences between ASD and TDC after controlling for age, BMI, health conditions (allergies, hematological diseases, epilepsy, heart diseases, and autoimmune disease), GI symptoms (upper GI symptoms, and bowel habits, which significantly differed between groups), food preferences, historical GI diseases, ADHD comorbidity, and methylphenidate use. Note: Species richness estimators were used to estimate the indexes of the total number of species in the community. Shannon-Wiener diversity index & Simpson diversity index & Pioulo’s evenness were used to measure the microbial diversity in the sample. PD whole tree was the diversity index calculated the distance of the evolutionary tree. ASD = autism spectrum disorder, TDC = typically developing control.
A. The correlation heatmap of top 35 genera

B. PCA analysis of microbiota in species level

C. Linear discriminant analysis (LDA) effect size test

D. Differential microbiota abundance between ASD and TDC

1. Metagenome sequence analysis
2. Effect plot for differential abundance analysis (ALDEX2)
3. Volcano plot for the analysis of composition of microbiomes (ANCOM) test

Fig. 2. The differential relative abundance and correlations of gut microbiota compositions in the ASD and TDC groups. (A) The correlation heatmap of the top 35 genera. The heatmap indicates the correlation coefficient of relative abundance of the microbiota of ASD and TDC. Ruminococcaceae UCG 013, Erysipelotrichaceae UCG 003, Ruminococcus torques group, and Parasutterella significantly altered in the ASD group using the Wilcoxon test (corrected by FDR). To compare correlation matrices of two groups, we implemented r to z transformation and tested the difference (significance: absolute Z score > 3; p_{FDR} < 0.05). (B) Principal component analysis of microbiota at the species level. We used the value of weighted UniFrac distance of beta diversity as input for PCA. The X-axis represents principal component (PC) 1 (34.2 % of variance), and the Y-axis represents PC2 (19.8 % of variance), with the top 5 loadings, including Bacteroides fragilis, plebeius DSM 17135, coprocola DSM 17136 and stercoris ATCC 43183, and Ruminococcus sp N15MGS 57. Among this five microbiota, the Wilcoxon test showed only the relative abundance of Bacteroides plebeius DSM 17135 was significantly greater in ASD. (C) Linear discriminant analysis (LDA) effect size test. The LEfSe analysis showed that the LDA score > 3 reaches significance. The left panel denotes the evolution composition between groups, and the right represents the significant level. The alphabets next to the name of microbiota correspond to the evolution cladogram. (D) Differential microbiota abundance between ASD and TDC: (1) Metagenome sequence analysis directly compared the species level of microbiota after controlling for covariates and corrected p value via FDR. Clostridium sordoiforme DSM 155 and Intestinimonas butyriciproducens were significantly higher in TDC. (2) The ALDEX2 analysis identified higher Erysipelotrichaceae UCG 003 at the genus level in TDC than ASD with an effect size > 1. The details of ALDEX2 analysis are described in Supplementary Method. The expected value of the distributional difference between groups (Y axis: median log2 btw-condition diff = median log2 between-group difference) and the expected value of the overall variance (X axis: median log2 win-condition diff = median log2 within-group difference) was calculated. The effect size was used to assess the reproducibility of differences between groups. (3) The ANCOM analysis at the family level found the Prevotellaceae passed the cutoff (W_{detected} = 0.6, which corresponded to p_{FDR} < 0.05. The ANCOM analysis of the details described in Supplementary Method. The X axis, compositional log-ratio (CLR) mean difference, is transformed from species abundance of samples and plotted F-statistic score. Y axis represents the W value.
1552 (at the species level) and Erysipelotrichaceae UCG 003, Clostridium sensu stricto 1, Turicibacter, Ruminococcus 1, Lachnospiraceae UCG 001, and Coprococcus 2 were observed in ASD relative to TDC. As indicated by LDA > 3, a total of 11 potential microbiota (as presented by a cladogram of gut microbial structure and their predominant bacteria in Fig. 2C) displayed the most significant difference in taxa between ASD and TDC ($p_{FDR} < 0.05$). We found a related microbiota from Fusobacteria (at the phylum level), Fusobacteria (at the class level), Fusobacteriaceae (at the order level), Fusobacteriaceae (at the family level) to Fusobacterium (at the genus level), as well as Prevotellaceae (at the family level) was increased in ASD, compared to TDC. In contrast, Clostridiales 1, Eggerthellaceae (at the family level), Turicibacter, Clostridium sensu stricto 1, and Erysipelotrichaceae UCG 003 (at the genus level) were increased in TDC compared to ASD. No potential biomarker at LDA > 4 was observed.

To test whether group-level proportional differences also existed across the different levels, especially those microbiota observed in the principal component and LefSe analysis, we directly compared the 146 species-level microbiota (adjusted with covariates of ASV table). ANCOM through CLR and W statistics were used to decide group differences, and ALDE2 estimated the effect size of between-group differences for cross-validation (Fig. 2D). We found that at the species level, Clostridium spiroforme DSM 1552 ($p_{FDR} = 0.002$) and Intestimonas butyriciproducens ($p_{FDR} = 0.034$) displayed lower relative abundances in ASD compared to TDC (Fig. 2D1). Family Prevotellaceae was identified in over $\omega_{\text{L1}}$ of our samples (Fig. 2D2), in which the ASD group had a higher abundance than the TDC group ($p_{FDR} = 0.008$). To determine differential abundances of features and the effect size > 1 via ALDE2, we found Erysipelotrichaceae UCG 003 (at the genus level) was significantly different between the diagnostic groups (effect size = 1.532, $p_{FDR} = 0.039$; Fig. 2D3). Overall, the findings of LefSe were similar to ANCOM and ALDE2 after validation.

5.4. The predicted function and pathway between ASD and TDC

The bacterial diversity of the functional microbiota, such as aerobic ($p_{FDR} = 0.051$), anaerobic ($p_{FDR} = 0.349$), Gram-negative ($p_{FDR} = 0.622$), and Gram-positive ($p_{FDR} = 0.082$) did not demonstrate group differences (Fig. 3A). Further, we explored the predicted function of microbiota via FAPROTAX, PICRUSt2, and Tax4fun2. Firstly, we aimed to explore the differences for cross-validation (Fig. 2D). According to the length value, the length of the first axis (DCA1) was 1.28 (smaller than 3) (Hill and Gauch, 1980), suggesting a fitting of the linear model to our data. The redundancy analysis (RDA) thus was applied to a linear model. We found that component 1 yielded from the RDA only explained 2 % variance, and component 2 only explained 1 %, which did not show group differences based on the ANOVA ($F = 0.87, p = 0.913$) (Fig. 4A). Further, the Mantel test showed neither ASD nor TDC had any significant correlation between the microbial community structure and GI symptoms (Table 4). Because we found no significant correlations between microbiota and GI symptoms, we did not conduct further analysis to explore the association between GI symptoms and predicted microbiota functions.

5.5. Stratified subgroup analysis excluding people with comorbid OCD or tics

Using constrained ordination analysis to investigate the correlation between the microbial community structure and CBCL in the ASD and TDC groups, we found that at the species level, Component 1 yielded from the RDA explained 90.12 % of the variance (2.05 % with type II scaling) (Fig. 4B; $F = 24.51, p_{FDR} < 0.001$), while the DCA was 1.84. We implemented a post-hoc LSD analysis following the RDA results to explore the correlations between CBCL and a set of microbial multivariables including Bidifodobacterium longum subsp longum, swine fecal bacterium SD Pec10, Bacteroides plebeius DSM 17135, serratotica ATCC 43183, fragilis and coprocopora DSM 17136, Clostridiales bacterium 42.27, Ruminococcus sp N15MGS 57 and Parabacteroides merdae (Fig. 4B). As for the microbial community and subscores of SRS, we found that Component 1 yielded from the RDA explained 82.35 % of the variance (9.17 % with type II scaling) (Fig. 5C; $F = 16.37; p_{FDR} < 0.001$). The post-hoc LSD analysis found significant correlations between the subscores of SRS and a set of Bacteroides plebeius DSM 17135, fragilis and serratotica ATCC 43183, Bidifodobacterium longum subsp longum, swine fecal bacterium SD Pec10, Ruminococcus sp N15MGS 57, Parabacteroides merdae, and Megasphaera micronucleiformis. Further, the Mantel test showed that the ASD group had overall positive correlations between microbial communities and all SRS subscores (Table 5), while there was no such relationship in the TDC group. Similarly, the Mantel test also displayed that microbial communities were positively correlated with aggressive behavior ($\rho = 0.56, p_{FDR} = 0.003$), anxious/depressed symptoms ($\rho = 0.63, p_{FDR} = 0.002$) and withdrawn ($\rho = 0.72, p_{FDR} < 0.001$), and were negatively correlated with delinquent behavior ($\rho = -0.67, p_{FDR} = 0.002$), somatic complaints ($\rho = -0.45, p_{FDR} = 0.038$) and dysregulation profile ($\rho = -0.62, p_{FDR} = 0.002$) in ASD. No such correlation was observed in TDC.

Furthermore, a Spearman correlation was conducted between the significant predictive functions of seven KEGG L3 pathways and the SRS and CBCL scores significantly related to the microbial community in ASD (Table 5 and Fig. 4B&C). We found social communication was carried out supplemental stratified analyses on all the preceding group comparisons by excluding the participants with comorbid OCD or tics (n = 5). These supplementary findings were similar to the results of the entire samples (SFigure 5–8, and STable 9). The only remarkable exception was that the top 10 relative abundance was slightly different: Streptococcus replaced Agathobacter as the top 10 genera of microbiota (SFigure 5C).

5.6. The relationship between taxonomy relative abundance, predicted functional pathway, and GI symptoms

We aimed to observe the correlation of specific microbiota and the symptoms of GI symptoms differently between ASD and TDC. Therefore, in this section, we controlled for the covariates of age, BMI, history of GI diseases and physical health conditions, ADHD comorbidity, and methylphenidate use. According to the constrained ordination analysis, to identify the optimal distribution of our data model (either a linear, unimodal, or bimodal distribution), we implemented a detrended correspondence analysis (DCA). According to the length value, the length of the first axis (DCA1) was 1.28 (smaller than 3) (Hill and Gauch, 1980), suggesting a fitting of the linear model to our data. The redundancy analysis (RDA) was thus applied to a linear model. We found that component 1 yielded from the RDA only explained 2 % variance, and component 2 only explained 1 %, which did not show group differences based on the ANOVA ($F = 0.87, p = 0.913$) (Fig. 4A). Further, the Mantel test showed neither ASD nor TDC had any significant correlation between the microbial community structure and GI symptoms (Table 4). Because we found no significant correlations between microbiota and GI symptoms, we did not conduct further analysis to explore the association between GI symptoms and predicted microbiota functions.

5.7. The taxonomy relative abundance and predicted functional pathway correlated with autistic symptoms and emotional/behavioral problems

As PANDAS and related conditions may affect microbial profiles in their distinct ways (Quaglariello et al., 2018; Wang et al., 2022), we carried out supplemental stratified analyses on all the preceding group comparisons by excluding the participants with comorbid OCD or tics (n = 5). These supplementary findings were similar to the results of the entire samples (SFigure 5–8, and STable 9). The only remarkable exception was that the top 10 relative abundance was slightly different: Streptococcus replaced Agathobacter as the top 10 genera of microbiota (SFigure 5C).
A. Predicted phenotypes of microbiota

Fig. 3. The predicted phenotypes and their predicted functions of microbiota between groups. (A) No group differences were found in predicted phenotypes, including aerobic, anaerobic, Gram-positive and -negative microbiota. (B) Functional pathways predicted by PICRUSt2. KEGG biomarkers are listed from L1 to L3. The Wilcoxon test was performed and corrected with FDR ($p_{FDR} < 0.05$). (C) KEGG orthology was predicted using Taxa4fun2. The p value of group differences from the Wilcoxon test was not corrected by FDR. No significant differences were noted after being corrected for multiple tests.

B. Functional pathways predicted by PICRUSt2

| KEGG level 1 | KEGG level 2 | KEGG level 3 | Statistic pFDR value |
|--------------|--------------|--------------|----------------------|
| Metabolism   | Lipid Metabolism | Arachidonic acid metabolism | $p=0.009$ |
|              | Amino Acid Metabolism | Valine, leucine and isoleucine degradation | $p < 0.001$ |
|              | Metabolism of Other Amino Acids | D-Arginine and D-ornithine metabolism | $p < 0.001$ |
|              | Biosynthesis of Other Secondary Metabolites | Isoquinoline alkaloid biosynthesis | $p=0.002$ |
|              | Energy Metabolism | Nitrogen metabolism | $p < 0.001$ |
| Organismal Systems | Digestive System | Mineral absorption | $p=0.001$ |
|              |              | Protein digestion and absorption | $p=0.013$ |

C. KEGG orthology

Fig. 3. The predicted phenotypes and their predicted functions of microbiota between groups. (A) No group differences were found in predicted phenotypes, including aerobic, anaerobic, Gram-positive and -negative microbiota. (B) Functional pathways predicted by PICRUSt2. KEGG biomarkers are listed from L1 to L3. The Wilcoxon test was performed and corrected with FDR ($p_{FDR} < 0.05$). (C) KEGG orthology was predicted using Taxa4fun2. The p value of group differences from the Wilcoxon test was not corrected by FDR. No significant differences were noted after being corrected for multiple tests.
positively correlated to the pathways of protein digestion and absorption and mineral absorption in ASD. Social emotion was positively correlated with mineral absorption in ASD. The predictive functions of valine, leucine, and isoleucine degradation, mineral absorption, and nitrogen metabolism were negatively correlated with dysregulation profile, anxious/depressed symptoms, and aggressive behavior, respectively, in ASD (Fig. 4D).

Table 4
The Mantel test of correlations between microbial community and GI symptoms.

| Environmental variables | Abdominal pain | Epigastric pain | Upper GI symptoms | Bowel habits | Food preferences |
|-------------------------|---------------|----------------|-------------------|-------------|-----------------|
| ASD: Correlation (rho)  | -0.02         | -0.03          | 0.02              | 0.04        | -0.02           |
| p-value (FDR corrected) | 0.676         | 0.725          | 0.252             | 0.171       | 0.684           |
| TDC: Correlation (rho)  | -0.06         | -0.03          | -0.04             | 0.02        | -0.06           |
| p-value (FDR corrected) | 0.964         | 0.757          | 0.815             | 0.327       | 0.983           |

There were no significant correlations between microbial species abundance and GI symptoms in the ASD and TDC groups controlling for age, BMI, health conditions of allergies, hematological diseases, epilepsy, heart diseases, historical GI diseases, ADHD comorbidity, and methylphenidate use and GI symptoms were controlled age and self-reported type. To calculate the Spearman correlations via Mantel test between the microbial community structure and GI symptoms, we calculated the Bray-Curtis dissimilarity matrix for species abundance and Euclidean distance matrix for GI symptoms as the x matrix and y matrix of the equation, respectively, to enter in Mantel test. GI = Gastrointestinal.

Fig. 4. The microbiotas and their predicted functions in correlation with clinical symptoms between groups. (A) (B) (C) The direction of the element vectors and the coordinates of the eleven genera in the biplot reveals their relationships between group differences via RDA analysis and ANOVA. (A) The microbial community of GI symptoms. No group difference was identified. (B) The microbial community of behavioral problems (CBCL). The seven genera have a relatively negative correlation to somatic complaints, aggressive behavior, anxiety behavior, dysregulation profile, and delinquent behavior. (C) The microbial community of social symptoms (SRS). The eight genera positively correlated with social communication, stereotyped/interest and social emotion. (D) Differential correlation patterns of the functional categories of microbiota with symptoms in ASD. The circle size represents the value of correlation coefficient (R). The blue color indicates the negative correlation, and red color denotes the positive correlation. The * symbol represents the significant correlation with correction by FDR, which the R score has >0.57. Abbreviation: Abdomen: abdominal pain; Bowel: bowel habits; Upper.GI: upper GI symptoms; Epigastr: Epigastric pain; Food.pre: food preferences; CBCL.SOM: somatic complaints of CBCL; CBCL.WIT: withdrawn of CBCL; CBCL.AGS: aggressive behavior of CBCL; CBCL.ANX: anxious/depressed symptoms; CBCL.DP: dysregulation profile of CBCL; CBCL.DEL: delinquent behaviors of CBCL; SRS.SC: social communication of SRS; SRS.E: social emotion of SRS; SRS.STER: Stereotyped behaviors/interests of SRS; SRS.SA: social awareness of SRS. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
The microbial species abundance and behavioral symptoms showed a significantly correlated, which was controlled by age, BMI, health conditions of allergies, hematological diseases, epilepsy, heart diseases, historical GI diseases, ADHD comorbidity, and methylphenidate use, and behavioral symptoms were controlled by age, ADHD comorbidity, and methylphenidate use. To get rho we examined Spearman correlation via Mantel test between the microbial community structure and behavioral symptoms. We calculated the Bray-Curtis dissimilarity matrix for species abundance and Euclidean distance matrix for behavioral symptoms as x matrix and y matrix of the equation to enter in Mantel test. The bold text indicates the significance, which is pFDR < 0.05. The bold text indicates the significant index.

### 6. Discussion

As the gut microbiome study in Asia with a deep-phenotyping and geographically and ethnically novel cohort, we compared the gut microbiota taxa between the ASD and TDC groups. Specifically, both groups showed similar alpha diversity in the gut microbiota, while ASD had a higher weighted UniFrac distance of beta diversity. We identified some of the relative abundance of microbiota, including *Fusobacteria, Fusobacteriaceae, Fusobacterium, and Prevotellaceae* increased in ASD as compared to TDC. Conversely, *Turicibacter, Erysipelotrichaceae, Erysipelotrichaceae*, and *Prevotellaceae* decreased in ASD as compared to TDC. The differential microbial abundance analysis identified several microbial taxa between individual groups (Ruminococcaceae UCG 003, *Clostridium sensu stricto 1*, and *Fusobacteria* at phylum level, *Intestinibacter, Allisonella, Lachnospiraceae UCG 001*, and *Ruminococcus torques* group at genus level were increased in the relative abundance of ASD. In contrast, the relative abundance of *Eggerthellaceae* and *Clostridiaceae 1* at the family level, *Parasutterella, Turicibacter, Ruminococcaceae UCG 013, Clostridium sensu stricto 1*, and *Erysipelotrichaceae* *UCCG 003* genus at the genus level, *Clostridium* *spiroforme DSM 1552* and *Intestinimonas butyriciproducens* at the species level are decreased in ASD.

The differential microbial abundance analysis identified several microbiota, from *Fusobacteria* at the phylum level, *Fusobacterium* at class level, *Fusobacteriaceae and Prevotellaceae* at family level, *Fusobacterium, Intestinibacter, Allisonella, Lachnospiraceae UCG 001*, and *Ruminococcus torques* group at genus level were increased in the relative abundance of ASD. In contrast, the relative abundance of *Eggerthellaceae* and *Clostridiaceae 1* at the family level, *Parasutterella, Turicibacter, Ruminococcaceae UCG 013, Clostridium sensu stricto 1*, and *Erysipelotrichaceae* *UCCG 003* genus at the genus level, *Clostridium spiroforme DSM 1552* and *Intestinimonas butyriciproducens* at the species level are decreased in ASD. The altered microbiota was largely consistent with previous reports of altered microbiome associated with ASD (Bezawada et al., 2020; Ho et al., 2020; Iglesias-Vazquez et al., 2020; Liu et al., 2019a; Liu et al., 2019b). Specifically, we identified that ASD had a higher relative abundance in the microbiota from *Fusobacteria to Fusobacterium-associated* phylum evolution trees. *Fusobacterium* has been reported as a novel higher abundance in Chinese populations (Yeoh et al., 2020). The enrichment of *Fusobacterium* is related to reduced immune recovery and persistent immune dysfunction in particular patients (Lee et al., 2018). Moreover, one of *Fusobacterium species, Fusobacterium nucleatum*, has been identified as a proinflammatory autochthonous bacterium implicated in human colorectal cancer and abundantly found in patients suffering from chronic gut inflammation (Hashemi Goradel et al., 2019). Interestingly, an increase of *Ruminoclostridium 6* may be associated with accelerating immune response with probiotic treatments (Santos et al., 2020). *Ruminoclostridium 6* has also been suggested as one of the bacteria to cause autistic behaviors in mice (Kong et al., 2021) and altered in Chinese children with ASD (Ma et al., 2019). *Ruminococcaceae UCG 13*, which decreased in ASD, is also related to immune response (Hakozaki et al., 2020; Malini et al., 2019). Many other identified genera also are involved in the inflammatory responses. For example, the *Ruminococcus torques group* has been linked to altered immune responses and intestinal inflammatory diseases triggered by disrupted barrier function (Malini et al., 2019). Similar findings of increasing *Ruminococcus torques group* in the ASD group have been reported by Wang et al. (2013). *Turicibacter*, which was decreased in ASD, is linked to a serotonin-related inflammatory response (Hoffman and Margolis, 2020). Conversely, genera such as *Intestinibacter* and

### Table 5

The Mantel test of correlations between microbial community and behavioral symptoms (CBCL and SRS).

| Environmental variables | ASD: Correlation rho | p value | TDC: Correlation rho | p value |
|-------------------------|----------------------|---------|----------------------|---------|
| Social communication    | 0.72                 | <0.001  | 0.02                 | 0.821   |
| Social behavior         | 0.65                 | 0.002   | 0.08                 | 0.637   |
| Social awareness        | 0.56                 | 0.003   | 0.03                 | 0.792   |
| Social emotion          | 0.53                 | 0.003   | 0.03                 | 0.784   |
| Aggressive behavior     | 0.56                 | 0.003   | 0.06                 | 0.594   |
| Anxious/ Depressed behavior | 0.63              | 0.002   | 0.06                 | 0.602   |
| Attention problems      | -0.01                | 0.894   | -0.01                | 0.894   |
| Delinquent behavior     | -0.67                | 0.002   | -0.08                | 0.682   |
| Social problems         | -0.23                | 0.083   | -0.02                | 0.803   |
| Somatic complaints      | -0.45                | 0.038   | -0.04                | 0.732   |
| Thought problems        | 0.03                 | 0.393   | 0.01                 | 0.891   |
| Withdrawn               | 0.72                 | <0.001  | 0.07                 | 0.598   |
| Dysregulation profile   | -0.62                | 0.002   | -0.06                | 0.605   |
| Internalizing           | -0.15                | 0.103   | -0.05                | 0.681   |
| Externalizing           | 0.19                 | 0.125   | 0.04                 | 0.759   |
Lachniporaceae UCG 001 are reported to associate with anti-inflammatory response. Namely, *Intestinibacter* is a potential protective factor for autoimmune diseases (Russell et al., 2019) and *Lachniporaceae UCG 001* has been reported to hydrolyze starch and other sugars to produce butyrate and other short-chain fatty acids (SCFAs) (Biddle et al., 2013; Devillard et al., 2007) and as anti-inflammatory bacteria (Wei et al., 2018). In addition to involvement in immune modulation, some of the ASD-associated microbiota observed herein also are implicated in obesity and related metabolism. For example, *Prevotellaceae* is abundant in carbohydrates and fibers, which may serve as a biomarker for homeostasis or disease state through its metabolite signature (Preocup and Vodnar, 2019). Both *Allisonella* (Haro et al., 2016) and *Prevotellaceae* (Jang et al., 2017) are associated with risk for obesity. Interestingly, Kim et al. (2020) reported that after probiotic supplementation, the elder improves cognitive function and mood with a decrease in *Allisonella* and *Prevotellaceae*. These mechanisms may, in part, explain why they both were increased in our samples of ASD, who also had higher BMI than TDC. *Parasutterella*, which showed lower relative abundance in ASD, has been reported to reduce low-density lipoprotein when its abundance increases (Bush and Alfa, 2020). In addition, *Fusobacteriaceae* (Rau et al., 2018), *Prevotellaceae* (Rau et al., 2018), *Intestinibacter* (Tsukuda et al., 2021), *Lachniporaceae UCG 001* (Vacca et al., 2020), *Ruminococcus torqueus group* (Rau et al., 2018), *Clostridiales 1* (Poll et al., 2020), *Parasutterella* (Ju et al., 2019), *Turicibacter* (Granado-Serrano et al., 2019), *Lachniporaceae UCG 001* (Biddle et al., 2013; Devillard et al., 2007), and *Intestiminonas butyriciproducens* (Bui et al., 2016) are all involved with SCFAs metabolism, which is speculated to be linked with ASD (Silva et al., 2020). This microbiota with diagnostic differences in relative abundance is mainly consistent with prior literature. Functionally, they are generally implicated in the inflammatory or anti-inflammatory response as well as obesity and associated metabolism (Dhaliwal et al., 2019), which both are linked to the expression of ASD phenotypes (Robinson-Agramonte et al., 2022).

Regarding the predicted functions of microbiota via KEGG pathway, we identified relatively higher enrichment of interdependent metabolic pathways involving amino acid metabolism (valine, leucine, isoleucine degradation and β-Arginine and γ-ornithine metabolism), lipid metabolism (arachidonic acid metabolism), energy metabolism (nitrogen metabolism) and biosynthesis of other secondary metabolites (isoquinoline alkaloid biosynthesis), and digestive system (mineral absorption and protein digestion and absorption), in ASD (Fig. 3B and C). These identified metabolic functions were consistent with previous reports (El-Ansary et al., 2014; Sanctuary et al., 2018; Smith et al., 2020) and are related to ASD. For example, valine, leucine, and isoleucine belong to the branched-chain amino acids (BCAAs), which are associated with the tricarboxylic acid cycle of the energy reproduction (Gojda and Cahova, 2021). Dysregulation of BCAAs may contribute to behavioral characteristics of ASD (Smith et al., 2019) and autistic brain (Maynard and Manzini, 2017). Orozco et al. (2019) reported lower levels of arginine and ornithine in ASD. Arachidonic acid plays an important role in neural development (Rapoport, 2008; Tallima and El Ridi, 2018). Deficits associated with the release of arachidonic acid from the membrane phospholipids and its subsequent metabolism are linked to the expression of autistic traits (Tamiji and Crawford, 2016). Earlier clinical trials also suggest that arachidonic acid intake may improve the social impairments of ASD (Yui et al., 2016; Yui et al., 2012). Further, nitrogen-related metabolism has been reported to be related to the psychiatric dysfunction in ASD (Abuaish et al., 2021). Nitrogen compounds are involved with metabolic dysfunction in ASD (Gao et al., 2021). Our findings indicate that disemaggregating interactions among microbiota, energy and amino acid metabolisms, and expression of autistic psychopathology could be a future research direction.

Emerging literature suggests the link between behavior/symptoms and gut microbiota in ASD (Ding et al., 2017; Xu et al., 2019). The present RDA observed part of the variation in microbiota composition, including the *Bacteroides* with multiple species (i.e., *fragilis*, *stercoris* ATCC 43183, caprocola DSM 17136, and plebeius DSM 17,135 group), *Bifidobacterium longum subsp longum*, *swine fecal bacterium SD Pec10*, *Ruminococcus sp* N15MGS 57, and *Parabacteroides merdae* could be explained by the diagnostic groups and social and emotion/behavior symptoms. These various species contribute to varied functions in the metabolism and immune system (Wexler, 2007). For example, *Bacteroides fragilis* is associated with carbohydrate metabolism in a wide range of dietary polysaccharides (Wexler et al., 2002). *Bacteroides fragilis* has been used to improve autistic behaviors in mice (Hsiao et al., 2013). Furthermore, *Bacteroides caprocola DSM 17,136* is related to glycosyl hydrolases (Chen et al., 2017a), which is linked to carbohydrate digestion disturbance of ASD (Williams et al., 2011). In combination with four other *Bacteroides* species, *Bacteroides caprocola DSM 17,136* also interacts with polysaccharides to mediate antigen-presenting immune reactions. Along with the findings above at the species level, the *Bacteroides* predominant findings in ASD may support the complex relationship between altered microbiome, immune dysregulation, and ASD (Ancona et al., 2021; Carpita et al., 2020; Lungba et al., 2020b; Suganya and Koo, 2020). Like *Bacteroides*, *Bifidobacterium longum subsp longum* was also used to treat autistic mice (Abuaish et al., 2022; Abuaish et al., 2021). In brief, this microbiota, whose variance could be driven by an autism diagnosis and poor social and emotional behaviors, is involved in metabolic pathways associated with the pathophysiology of ASD and has been used as a treatment for autistic symptoms in preclinical studies.

The correlations between SRS scores and microbiota composition of ASD was consistent with previous studies (Hughes et al., 2018; Iovene et al., 2017; Zhou et al., 2020). Nonetheless, an earlier study reported no correlation of CBCL with gut microbiota (Strati et al., 2017), which is incompatible with our findings. Sample sizes, cultures, and sampling heterogeneity might partially explain the inconsistency. Notably, the follow-up Mantel test (Table 5) showed that subscales of SRS and aggressive behavior, anxious behavior, and withdrawal on the CBCL positively correlated to the microbial community in ASD but not in TDC. In contrast, the delinquent behavior, somatic complaints, and impaired self-dysregulation (CBCL-dysregulation profile) on the CBCL exhibited a negative link with microbial abundance. This shared pattern between impaired self-dysregulation and SRS may indirectly support the hypothesis that emotion dysregulation is intrinsic to autism (Lin et al., 2020; Mazefsky et al., 2013; Ni et al., 2018; Ni et al., 2020). A previous clinical trial (Liu et al., 2019c) showed that participants with ASD had reduced SRS scores but did not show changes in CBCL scores after probiotic treatment. Similarly, Santocchi et al. (2020) demonstrated probiotic intakes could improve ASD core symptoms and social functions rather than other behavioral problems. Taken together, different symptoms, behaviors, or cognitive functions may relate to various microbial profiles distinctly. Our findings suggest that the future development of treatment targeted at microbiota should be tailored to unique behavior-microbiota pairing relationships.

Zooming in on the relationship between microbial function and symptom/behavior, we found social communication and social emotion were positively correlated to the digestive system and dysregulation profile. In contrast, aggressive and anxiety behaviors were negatively related to BCAA metabolism, nitrogen metabolism, and mineral absorption sequentially. Despite the nature of cross-sectional correlation, our findings provide empirical evidence that microbiota composition plays a role in the presentation of phenotypes of autism and co-occurring emotion/behavior problems (Abuaish et al., 2012). Our study’s strengths are a Taiwanese sample with a relatively large size and deep phenotyping. At the same time, we excluded participants with other physical problems, inheritable diseases, or severe GI symptoms (i.e., a confirmed GI diagnosis) (Gupta et al., 2017) as well as those with active use of probiotics, prebiotics, and symbiotics (Andreo-Martinez et al., 2020). This approach may preclude some patients with more aberrant microbiota compositions, thus precluding the detection of correlations between GI symptoms/bowel movement/food style and
microbial profiles.

The study has several limitations. First, gut microbial composition by itself does not provide a predictive biomarker for ASD, and the single technology of high-throughput sequencing will need to be integrated with multiple sources of omics data (e.g., proteomics, transcriptomics, metabolomics, microRNAs, and exomes) to identify potential signatures for the spectrum of symptoms in individuals with ASD. Second, our sample only comprised male participants, which may contribute to inconsistencies in our findings with earlier results. Third, the study was a cross-sectional design, preventing us from confirming the causal directions between the correlational link between the ASD diagnosis and microbial profiles. Fourth, the current findings from male-only samples may not be able to be generalized to other subgroups on the spectrum. Fifth, the age range (6–25) of the current sample was wide, and the study was not sufficiently powered to implement the age-stratified analysis. This developmental effect on life and diet styles may not be fully accounted for by including age as a nuisance covariate. Nonetheless, Taiwan’s national nutrition intake survey from 2013 to 2016 (Wen-Harn, 2013) indicates that the dietary style and nutrition intake are not significantly different among age groups (Wen-Harn, 2017). This fact may justify our approach of lumping children and emerging adults in the current analysis. Sixth, the GI assessments were reported by either emerging adults or children and their parents, although the ratio of these two self-report types did not differ between groups. Moreover, the current measure of GI assessments is not the most comprehensive questionnaire compared to other reports (Gorrindo et al., 2012). Seventh, although we statistically controlled for a history of GI diseases, these earlier GI experiences and associated dietary changes may still lingeringly affect the microbial community. Last, we did not collect data on prenatal or perinatal events, which could affect the gut microbiome (Yao et al., 2020).

In conclusion, we found that the gut microbiome in specific microbiobta significantly differed from ASD and TDC. Further, the present study showed microbial profiles strongly associated with phenotypes such as social problems, thought problems, delinquent behavior, dysregulation profile, and somatic complaints. Interestingly, we did not find evidence that GI symptoms were associated with microbiota alteration in ASD. If replicated, our results suggest that there may be an upstream role of the gut microbiome in ASD-related psychopathology and emotional/behavioral problems, regardless of the status of comorbid GI symptoms. The function of the identified microbial alterations mainly involves the immune pathway, suggesting the complex relationship between altered microbiome, immune dysregulation, and ASD. Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

Acknowledgments

The study was approved by the Research Ethics Committee of National Taiwan University Hospital, Taipei, Taiwan, and written informed consent and/or child assent were obtained from the participants or their parents. This work was supported by grants from the Ministry of Science and Technology, Taiwan (MOST108-2321-B-002-034, MOST109-2327-B-002-004, MOST110-2327-B-002-004, MOST111-2327-B-004-013). The National Health Research Institute, Taiwan (NHRI-EX110-11002P1), and National Taiwan University Hospital (NTUH108-S4528) to SSY. HYL is supported by the Azrieli Adult Neurodevelopmental Centre, Centre for Addiction and Mental Health, and an Academic Scholar Award from the Department of Psychiatry, University of Toronto. The authors are grateful to all the participants and their parents and their research and participation for data collection.

Appendix A. Supplementary data

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