Research Square

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Excess Escherichia elicits mild autism spectrum disorder in young subjects via disturbing the balance of gut microbial GABA metabolism

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Abstract

Background: Accumulating clinical evidence demonstrated that gut microbiota (GM) transfer substantially ameliorated autistic symptoms, implying a role of GM in autism spectrum disorder (ASD). Continuous investigation reported GM alteration in ASD individuals, while inconsistency also persists in the abundance of bacterial taxon, as well as their metabolites. Due to deficiencies of stringent study designs, multi-omics assessment, and even animal tests, such data heterogeneity is yet to be addressed, which impedes establishing a causal effect of a specific microbiome-metabolome axis on pathogenesis of ASD.

Results: We developed a participant recruitment and sampling procedure that fully considered possible intergroup and intragroup confounders, including age, diet, severity of ASD, and stool consistency. To tease out biological features of ASD from an overarching investigation on metabolome, composition and function of GM, we compared faecal samples collected from 123 children with either mild ASD (n=56) or typical development (TD) (n=67). Metabolite profiling of targeted metabolomics screened and validated a significantly higher ratio of gamma-aminobutyric acid (GABA) to glutamate (Glu) as a metabolic signature of ASD. Linked to autistic symptomatology, the microbial GABA/Glu ratio independently distinguished ASD from TD subjects, particularly at 3-5 years. The 16S rRNA gene sequencing displayed substantially declined GM richness but over-presented genus *Escherichia* in ASD, which was profoundly associated with the increased GABA/Glu ratio. Metagenomics confirmed both structural and metabolic dysbiosis of GM via revealing consistent changes in microbial components and *Escherichia* abundance, and highlighting overall hypofunction of the GM and imbalanced GABA/Glu synthesis in ASD. We further identified a series of *E. coli*-specific adhesins that linked *Escherichia* colonization with GABA production. Quantitative PCR validation supported increment of *Escherichia*-specific glutamate decarboxylase, promoting GABA synthesis. Further, challenge of healthy weaning mice with gut commensal *E. coli* induced significant social deficiency, but not abnormalities in repetitive, locomotor, recognition memory- and anxiety-related behaviors.

Conclusions: Our multi-omics study elucidated a microbe-metabolite axis out of GM dysbiosis in mild ASD, characterized by over-presented *Escherichia* and concomitant GABA/Glu imbalance, which were well-marked features of ASD-linked GM at an early age, and potential causal factors of autistic symptomology. Our study provided insight into importance of microbial excitatory/inhibitory neurotransmitters imbalance in diagnosis and therapeutic treatment of ASD.

Background

Autism spectrum disorder (ASD) is a multifactorial neurodevelopmental disorder characterized by impaired social interaction, repetitive behavior, and stereotyped interests [1]. Recent epidemiological data show that 1-2% of children worldwide are currently diagnosed with ASD [2]. On one hand, behavioral intervention at an early age may achieve positive outcomes [3]; On the other, early diagnosis is strenuous as the very beginning onset of autistic symptoms is usually mild and barely detectable until 3 years of age or older. Current diagnosis primarily relies on behavioral observations, which inevitably generate inter- or intra-observer variation and thus under-report ASD in very young children or mild cases [4]. Neither molecular diagnosis nor targeted therapy has yet become available as the etiology of ASD remains largely unclear. Recently, emerging

evidence showed that transferring healthy gut microbiota (GM) to ASD children could substantially improve their autistic symptoms [5, 6]. These breakthroughs have inspired intensive investigation on GM in ASD.

Advances in dissecting the distinct GM between autistic and neurotypical individuals revealed polymicrobial features of ASD. However, results from different studies were usually inconsistent and sometimes contradictory. For example, a systematic review in 2019 summarized a concurrent decrease in the abundance of *Bifidobacterium*, *Blautia*, *Dialister*, *Prevotella*, *Veillonella*, and *Turicibacter* in ASD patients [7], whilst according to a recent large-scale study in 2020, most of these genera remained unchanged in abundance and the *Bifidobacterium* was even increased in ASD [8]. The data heterogeneity has thus raised worldwide debate on whether aberrant GM is truly linked to the pathogenesis of ASD. As is known, human GM is dynamic and sensitive to variations of hosts (e.g. developmental stages, physiological or pathophysiological status) and of environment (e.g. geography, diet, drug use) [9]. We noticed that most previous reports didn't clearly show or consider an exhaustive procedure to control pivotal inter- and intra-group confounders during participant recruitment, such as age [10, 11], sex [12], dietary preference [8, 13], severity of ASD [14], and even antibiotic or prebiotic administration [15, 16]. Equally, stool consistency (i.e. quality and shape) is also important in determining data quality [17], but faecal sampling is laborious, which largely relies on either patients or guardians. However, stool specimen collection from children on the autistic spectrum is far more complicated than that from neurotypical participants. To our knowledge, only a few studies mentioned strategies to control stool consistency [18]. These limitations on study design may generate analytic bias and data heterogeneity among studies. Therefore, a standardized recruitment and sampling procedure appears to be particularly indispensable for controlling confounders and teasing out the key distinct features of GM in ASD.

Microbial metabolites constitute a dynamic network that maintains the homeostasis of both GM community and host [19, 20]. Thus, changes in metabolite level may reflect the well-timed function exertion of GM under different conditions. Thus far, implications for the importance of microbial metabolites in ASD were mostly obtained from animal studies. Functional compounds, such as short-chain fatty acids (SCFAs), neurotransmitters and bile acids (BAs), were found involved in regulating social brain via affecting energy metabolism, immune response and neuroactivity in ASD models [20, 21]. Nevertheless, those that potentially triggered autistic-like behaviors in experimental animals, such as propionic acid [22], dopamine [23] and lithocholic acid [24], were yet to be supported by data from clinical GM studies. Metabolic signature of faecal microbiome in autistic children is still inadequate. Several good attempts using metagenomics identified metabolic abnormalities of autistic GM from the perspective of associated genetic pathways, including neurotransmitter synthesis [8, 12, 14, 25] and detoxification [26], while the abundance levels of the relevant metabolite were nonetheless unmentioned or unvalidated biochemically in these studies. Therefore, cross-examination using multi-omics has become essential to obtain and assemble a complete chain of evidence for identifying functional changes of autistic GM. Meanwhile, concomitant verification in animal experiments would further reveal the role of GM dysbiosis in autistic symptoms.

Here, we established a standardized procedure that fully considered possible confounders during participant recruitment and stool sampling. We collected faecal samples from 123 children with mild ASD or with typical development (TD), and thoroughly compared the microbiome and metabolome between the two groups by incorporating targeted metabolomics, 16S rRNA gene sequencing, metagenomics, and quantitative polymerase chain reaction (PCR) analysis. Altogether, from evidence of GM dysbiosis in mild ASD, we

established a well-marked microbiome-metabolome axis, characterized by increased abundance of *Escherichia* and aberrant GABA/Glu metabolism, which may play a causal role in the early onset of ASD.

Methods

Human study design

Procedural standards for participant recruitment

Subjects with diagnosed ASD according to the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5), as well as TD peers, were continuously enrolled through ARK Autism & Rehabilitation Institute, Shanxi, China. The severity of ASD was evaluated as per the Childhood Autism Rating Scale (CARS) by two experienced pediatricians. Information on all participants, including age, gender, dietary habit, manner of birth, administration of drug/healthcare products and common pathological conditions (neurologic, psychiatric and gastrointestinal diseases), were gathered via questionnaire. Next, in order to control the associated biomedical and environmental confounders that may affect the subsequent analyses, exclusion criteria were set up as follows: 1) on unbalanced diets (completely refusing or extremely favoring of specific staple foods); 2) diagnosed with severe ASD; 3) suffering from other neurologic or psychiatric disorders including epilepsy, schizophrenia, depression and attention-deficit/hyperactivity disorder (ADHD); 4) suffering from intestinal infectious diseases; 5) antibiotic or probiotic administration within a month before sampling. Finally, two cohorts, namely a main cohort (ASD vs. TD = 50 vs. 55) and an independent cohort (6 vs. 12), were successfully recruited for autistic GM profiling and validation.

Faecal sampling principles and specimen consistency

To ensure the sample quality, faecal sampling work was conducted by the guardians of the research participants. They were trained on the very basics of asepsis, recognition of stool consistency, and sampling operation. First, assisted by their guardians, participants were all required to defecate on a prepared cellulose core diaper for faecal separation from urine. Then, the stool consistency of each excreta was evaluated as per the Bristol Stool Chart and subsequently controlled by sampling only corn-on-cob-like (type 3) or sausage-like (type 4) faeces as normal stool. Next, to avoid any possible air-borne contamination, the surface part of the faeces was not harnessed. A sterile sampling scoop was used to withdraw the inner core of the stool. Finally, about 2 grams of quality specimen were collected in a sterile collecting tube. Samples were immediately refrigerated for 30 min, and then handled over to one of our staff for snap freezing and shipping by dry ice. For biological replicates, faecal sampling from each participant was performed two to three times with a minimum of 48-hour intervals. Each faecal sample was aliquoted and stored at -80 ℃ before being subjected to Ultra Performance Liquid Chromatography with Tandem Mass Spectrometry (UPLC-MS/MS), Gas Chromatography-Mass Spectrometry (GC-MS), 16S rRNA gene sequencing, metagenomics or quantitative PCR.

Sample grouping for multi-omics investigation

Each sampling of selected participants brought about a collection of specimens that was named as a sample set. To avoid analytic bias resulted from random events during sampling, such as emotional fluctuation,

subtle changes of daily diets, and weather variations, different sample sets from the main cohort was exploited for multi-omics studies: the first sample set was gathered for analyzing metabolic and structural features of GM and screening distinct metabolites or taxon; the second and third sample sets were used for functional analysis of changes in autistic GM. The samples from the independent cohort were used for validation of key findings.

Targeted metabolomics

Measurements of neurotransmitters, BAs, and GABA metabolism-specific metabolites were subjected to the UPLC-MS/MS procedure, whilst SCFAs assessment subjected to GC-MS.

Sample preparation & extraction

To investigate the absolute abundance of different types of metabolites, faecal samples were prepared differently. For neurotransmitters, samples were prepared in pre-cooled acetonitrile with 1% (v/v) formic acid (FA); for BAs, samples were added to pre-cooled methanol; for specific metabolites in GABA metabolism, samples were added to a pre-cooled solution of acetonitrile and methanol in water (2:2:1, v/v/v). After vortexing, the obtained sample homogenate was incubated for 20 min at -20 °C to induce protein deposits, and then centrifuged at 14,000 *g* for 15 min at 4 °C. The supernatants were collected and dried under vacuum. Next, the above sample extracts were in turn redissolved in solutions of acetonitrile in water (1:1, v/v), methanol in water (1:1, v/v), and acetonitrile and methanol in water (2:2:1, v/v/v), and centrifugated as described above. The supernatants were collected and subjected to UPLC-MS/MS. Faecal samples for assessing SCFAs were prepared in 15% (v/v) phosphoric acid, and subjected to GC-MS.

UPLC-MS/MS procedure

For neurotransmitters measurement, an Agilent 1290 Infinity UPLC system (Agilent, USA) equipped with an Acquity UPLC BEH C18 column (1.7 μm × 2.1 mm × 100 mm, Waters, Canada) was used to analyze the samples. The samples were put in an auto-sampler (chromatographic column temperature, 45 °C; flow velocity, 300 μL/min). The mobile phase consists of 0.1% (v/v) ammonium formate (liquid A) and acetonitrile with 0.1% (v/v) FA (liquid B). A gradient-elution program was set as follows: starting from 90% B at 0 min, linear gradient was decreased to 40% B over 18 min; then eluent B was returned to 90% within 1 s and maintained for 5 min. GABA and glutamate (Glu) were used as the standards for chromatographic retention time correction. Subsequently, an electrospray ionization (ESI)-triple 5500 quadrupole-linear ion trap (QTRAP)-mass spectrometer (AB SCIEX, USA) was applied to conduct mass spectrometry (MS) analysis in positive ion mode (ESI^+). The ESI^+ source conditions were as follows: ion spray voltage floating (ISVF), 5000 V; ion source gas1 (Gas1), 60; ion source gas2 (Gas2), 60; curtain gas (CUR), 30; source temperature, 450 °C.

For bile acids measurement, a Waters Acquity UPLC I-Class system (Waters, USA) equipped with the Acquity UPLC BEH C18 column was used to analyze the samples. Samples were added with deuterated bile acids as internal standards (100 ppm; Thermo Fisher Scientific, USA) for chromatographic retention time correction, and put in an auto-sampler (chromatographic column temperature, 45 °C; flow velocity, 300 μL/min). The mobile phases were pure water with 0.1% (v/v) FA (liquid A) and methanol (liquid B). A gradient-elution program was set as follows: starting from 60% B at 0 min, linear gradient was increased to 65% B over 6 min

and further to 80% B within 5 s; then, eluent B was returned to 90% within 1 s and maintained for 9 min. Subsequently, the 5500 QTRAP-mass spectrometer was applied to conduct MS analysis in negative ion mode (ESI^-). The ESI^- source conditions were as follows: ISVF, -4500 V; Gas1, 55; Gas2, 55; CUR, 40; source temperature, 550 °C.

To measure GABA metabolism-specific metabolites, the Waters UPLC system was equipped with the same sampler using the same column conditions as described above for bile acids measurement. Samples were added with a mixture of relevant compounds as internal standards (100 ppm; Shanghai Applied Protein Technology, China) for chromatographic retention time correction. The mobile phases were pure water with 1.2% (v/v) ammonium (liquid A) and methanol with 0.2% (v/v) FA (liquid B). A gradient-elution program was set as follows: starting from 75% B at 0 min, linear gradient was first decreased to 62% B over 10 min and further to 40% B over next 5 min; then, eluent B was returned to 75% within 30 s and held for 17 min. Subsequently, the 5500 QTRAP-mass spectrometer was applied to conduct MS analysis in both ESI^+ and ESI^- as described above.

The multiple reaction monitoring (MRM) was used for acquisition, detection and quantification of the metabolites in this study. Multi-Quant software (version 3.0.2) was used to extract and correct the extract the peak area and retention time of the chromatogram, and the relative content of the corresponding metabolite was represented as the area of each peak.

GC-MS procedure

For SCFAs, Agilent 6890N/5975B GC-MS spectrometer (Agilent, USA) was applied. Samples were added with 4-methylvaleric acid as internal strandards (83 ppm; Thermo Fisher Scientific), and put in an automatic sampler (carrier gas, helium; flow velocity, 1.0 mL/min; injection port temperature, 250 °C; split injection, split ratio 10:1; solvent delay, 2.2 min.) A HP-INNOWAX capillary GC column (30 m × 0.25 mm × 0.25 μm, Agilent) was used to separate the samples. Temperature programming was as follows: the initial temperature of the column oven was set at 90 °C, and then increased to 120 °C at a speed of 10 °C/min, to 150 °C at 5 °C/min and finally to 250 °C at 25 °C/min, where it was held for 2 min. Subsequently, MS conditions were set as follow: electron bombardment ionization source; iron source temperature, 230 °C; quadrupole temperature, 150 °C; electron energy 70 eV. The selected ion monitor (SIM) was to detect SCFAs. The MSD ChemStation software (version 2.0) was used to extract and correct the peak area and retention time of the chromatogram, representing the relative content and identification of SCFAs, respectively.

Genomic analysis

Extraction of Genomic DNA

Bacterial DNA was isolated from faecal stools using a QIAamp DNA Stool Mini Kit (QIAGEN, Germany) following the manufacturer's instructions. Genomic DNA was detected by 1% (w/v) agarose gel electrophoresis.

Bacterial 16S rRNA gene sequencing and annotation

The barcoded primers used to amplify 16S rRNA gene (V3-V4) were 319F/806R [27]. Sequencing library was generated using NEB Next®Ultra™DNA Library Prep Kit for Illumina (NEB, USA) following manufacturer. The library was sequenced on an Illumina HiSeq2500 platform (Illumina, USA) and paired-end reads were generated. Paired-end reads were merged into consensus fragments using FLASH (v1.2.11) [28], and then were assigned to each sample according to the unique barcodes. Clustering of operational taxonomic units (OTUs) was performed in Usearch (version 10.0) using the UPARSE-OTU and UPARSE-OTU ref algorithms [29]. Sequences with ≥97% similarity were assigned to the same OTUs. The Ribosomal Database Project (RDP) classifier was used for taxonomic annotation of OTUs [30].

Metagenome sequencing and annotation

Sequencing library was generated using TruSeq™ DNA Sample Prep Kit (Illumina) following manufacturer. Paired-end sequencing was performed on the Illumina HiSeq2500 platform. Host sequences were filtered out using BWA (http://bio-bwa.sourceforge.net) and remained reads were assembled using Megahit (https://github.com/voutcn/megahit). Annotations of taxonomy, functional pathway and bacterial virulence factors were performed with BLASTP (Version 2.2.31+) according to Non-Redundant Protein Sequence Database (https://ftp.ncbi.nlm.nih.gov/blast/db/FASTA/), Kyoto encyclopedia of genes and genomes (KEGG) (http://www.genome.jp/kegg/) and virulence factor database (VFDB) (http://www.mgc.ac.cn/) respectively.

Bacterial profiling

QIIME (v1.9.1) was used for analyzing microbial a-density and b-density. Based on a-diversity, Chao index and Shannon index were used for presenting microbial diversity and richness respectively in each sample. Based on b-diversity, principal coordinates analysis (PCoA) was used to visualize dissimilarities of GM component among samples. Linear discriminant analysis effect size (LEfSe) was used to identify differential taxon between groups [31].

Quantification of glutamate decarboxylase in genera Escherichia and Bacteroides

Primers were designed against *Escherichia*- or *Bacteroides*- specific glutamate decarboxylase (GAD) DNA sequences from the National Center of Biotechnology Information (NCBI). Two orthologs of *gad* in *Escherichia* were detected by *Escherichia-gadA*-qF/qR and *Escherichia-gadB*-qF/qR; *Bacteriodes*-specific *gad* was detected by primer pair *Bacteroides-gad*-qF/qR (Table S6). The reaction mixture for amplification consisted of 2 μl of Roche Fast Start LightCycler Mastermix, forward and reverse primers (0.5 mM each), 3.2 mM $MgCl_2$ and nuclease-free water to a final volume of 15 μl. The amplification cycles consisted of incubation at 95°C for 30s, at 57°C for 30s, and 72°C for 30s. Cycle threshold was measured and target gene concentration was analysed. Generation of a standard curve, determination of the qPCR efficiency, and calculation of the copy number were carried out with 7500 Fast System SDS v1.4 software (Thermo Fisher Scientific).

Animal study design

MaleC57BL/6J mice were housed in a pathogen-free facility under a 12-h light/12-h dark cycle. Mice were randomly divided into an experimental group and a control group and then housed separately. At postnatal day (P) 21, the experimental group was challenged with gut commensal *E. coli* (CICC20658, China Center of Industrial Culture Collection, China) by daily gavage at a dose of 10^8 per mice for 5 consecutive days. Meanwhile, phosphate buffered saline (PBS) was used as placebo in the control group. Subsequently, mice were housed for another 7 days for *E. coli* colonization. At P32, faecal samples from mice were collected and all mice were subjected to behavioral tests.

Principles for mouse faecal sampling and storage are similar to that for human studies. Each mouse was placed in individual sterilized box for defecating. During sampling, urine was wiped off immediately with an antiseptic swab where urination was found. Stool consistency was evaluated as previously described [32]. Accordingly, hard-formed faecal stool from each mouse was considered as normal stool and was subsequently clipped into a tube with sterilized tweezers. Soft-formed, unformed or urine-mixed samples were discarded.

Quantification of *E. coli* and *Escherichia*- -specific glutamate decarboxylase

Primers against the 16S rDNA sequence of gut commensal *E. coli* were designed as previously described [33]. The primers for *Escherichia*-specific *gadA* and *gadB* in human study were reused in current animal study. A same qPCR procedure was performed as described above.

Behavioral tests on mice

Three-chamber test Social preference and social recognition of mice were assessed in a three-chamber apparatus (60 x 40 x 20 cm, L x W x H) as previously described [34, 35]. Briefly, the apparatus was divided into three interconnected chambers with the left and right chambers contained one small cage each, while the middle chamber was empty. For habituation, the test mouse was first placed in the apparatus for a 10-min period. For evaluating social preference, the test mice could possibly interact with an age- and sex-matched stranger mouse (Stranger 1) that was placed in the cage of the left chamber, or stay with the empty cage of the right chamber during the second 10 min. For evaluating social recognition, a second stranger mouse (Stranger 2) was introduced into the previous empty cage of the right chamber in the third 10 min. The accumulative time that the test mouse spent in interacting with the empty cage, stranger 1 or stranger 2 was respectively recorded by EthoVision XT software (Noldus Information Technology, Leesburg, USA).

Marble-burying test Repetitive behavior of mice was assessed in a mouse cage (42 × 24 × 12 cm, L x W x H) laid with 5 cm-thick corncob padding as previously described [36]. Briefly, 20 glass beads (15 mm, diameter) were put into the cage and regularly divided into five rows (4 beads in each row). Then, the test mouse was placed in the cage for 30 min. The number of buried glass beads (being buried more than 50% of volume) was counted.

Open field test Grooming, voluntary movement and anxiety behavior of mice were assessed in an open box (40 x 40 x 40 cm, L x W x H) as previously described [35, 37]. Briefly, the open box was divided equally into 16 smaller grids and the central 4 grids were set as the central area (20 x 20 cm). Then, the test mouse was placed in the cage for 10 min. The grooming time of each mouse was recorded artificially. The speed,

travelling distance and the time of each mouse that spent in the central area were calculated by EthoVision XT software.

Novel object recognition Recognition memoryof mice was assessed in a box (40 x 40 x 40 cm, L x W x H) as previously described [35, 38]. After 10-min habituation in the box, the test mouse was exposed to two identical objects for another 10 min. Then, one object was replaced with a novel object and the mouse was subsequent allowed to explore the objects for 10 min. The sniffing time at the proximity of each object within 2 cm or directly in touching the objects was recorded by EthoVision XT software.

Elevated plus maze The anxiety behavior of mice was assessed in a 1 m height platform consisting of 4 arms (two open arms and two closed arms crossed together) as previously described [35]. The test mouse was initially placed in the central area and their trails were recorded for 5 min. The accumulative time of the mouse in open arms and closed arms was calculated by Noldus EthoVision XT10 software.

Reciprocal Social Interaction As previously reported [39], the test mouse was placed in a new cage and exposed to an age- and sex-matched stranger mouse for 10 min. The time of social interactions between the two mice (e.g. close following, touching, nose-to-nose sniffing, nose-to-anus sniffing, and crawling over/under each other) was calculated.

Statistical analyses

Statistical analyses were performed by using R statistical software (v3.6.3) (www.R-project.org/) and SPSS (version 22). Variables of metabolite level, including the ratio of metabolites, gene abundance, and pathway abundance were normalized by "value/SD" before further statistical analyses. Wilcoxon rank-sum test and Chi-square test were used to compare the average of continuous variable and frequency of nominal variable, respectively, between ASD and TD, with $p< 0.05$ as significant. An obtained p-value of pathways and genes were adjusted by the Benjamini-Hochberg procedure, with false discovery rate (FDR) < 0.1 as significant. Partial least-squares discrimination analysis (PLS-DA) was exploited to compute the predicted potential biomarkers for ASD among metabolites, with the value of variable importance for the projection (VIP) > 1 as significant. Permutational multivariate analysis of variance (PERMANOVA) was used to address the difference of PLS-DA and PCoA plots, with $p < 0.05$ as significant. Receiver operating characteristic (ROC) analysis was used to test the performance of potential markers for ASD. Pearson correlation analysis and linear regression were performed to evaluate the correlation among GM abundance, metabolites levels, pathway abundance, and ASD rating score, with coefficient r as the indicator of relationship and $p < 0.05$ as significant.

Results

Control of confounders during participant recruitment and sampling

In an attempt to identify GM features aiding early diagnosis of ASD, we set out our investigation on mild cases that represent the main population in ASD patients worldwide according to the recent estimation [40-42]. To control the confounding factors, we established a procedure and followed our principles during participant recruitment and sampling as described above. In keeping with such standards, we managed to

enroll a total of 123 children (ASD=56; TD=67) aged 3-8 years, which was grouped as the main cohort (ASD=50; TD=55) and the independent cohort (ASD=6; TD=12) (**Table 1**) in this study. CARS evaluation confirmed that all the enrolled ASD subjects were mild cases (34.44 ± 1.74 in the main cohort and 33 ± 1.67 in the independent cohort). All research participants kept healthy, balanced diets with similar staple foods and cooking style, but avoiding stimulating foods (**Table S1**), which would minimize the influence of variation of diet habits on the subsequent analyses. Further, the age of ASD group was matched against that of TD, showing comparable mean values ($p > 0.05$) and consistent distributions (**Figure S1A and B**) in both the main cohort (5.16 ± 0.21 vs. 4.69 ± 0.15) and the independent cohort (4.17 ± 0.31 vs. 4.08 vs. 0.19). The main cohort presented a higher male proportion (88% vs. 47.27%, $p < 0.0001$) and a higher constipation occurrence (40% vs. 16.16%, $p = 0.007$), along with comparable caesarean-born condition (38% vs. 32.73%, $p > 0.05$) in ASD vs. TD subjects. Considering high prevalence of ASD in males worldwide, the independent cohort was all male with consistent constipation occurrence and delivery methods for a prudential, independent investigation on GM. During sampling, stool consistency was strictly controlled via collecting corn-on-cob-like and sausage-like faecal samples (**Table S2**), which are classified as normal stool as per the Bristol Stool Chart. Our subsequent analysis revealed that age, but not other aforementioned host factors, was associated with individual variants of GM components, including diversity and richness (**Figure S1C**), suggesting that the possible ingroup and intergroup confounders that may influence extracting GM features were well controlled.

Gut microbial GABA/Glu imbalance was identified as a key metabolic signature of children with ASD

To investigate the metabolic features of GM in ASD, we first determined a total of 32 well-described metabolites from neurotransmitters, SCFAs and BAs that are broadly applicable to ASD symptomatology as per literature or clinical inference [43]. We then collected 105 (ASD = 50; TD = 55) faecal samples via a first sampling from the main cohort as a screening set, and exploited targeted MS analysis (**Figure 1A**) to quantify the faecal metabolites. Plots based on PLS-DA showed a clear separation of the metabolites between ASD and TD ($p = 0.0004$) (**Figure 1B**). Upon the screening criteria for significance set at $p < 0.05$ and VIP > 1, we identified 7 ASD candidate biomarkers including 5 neurotransmitters (GABA, norepinephrine, dihydroxyphenylalanine, histamine and tyramine), 1 SCFA (acetic acid) and 1 BA (chenodeoxycholic acid) (**Figure 1C**). Further, we asked if there was an intrinsic association among the 7 putative metabolite markers. We thus performed linear regression analysis, setting 1 metabolite as a dependent variable and the other independent variables alternately. Multiple correlation coefficients suggested that GABA was most associated with other candidate markers in the ASD-specific metabolic profiling (**Figure 1D**).

Considering ad-hoc or one-off changes that may lead to possible sample/data variation from the screening set, such as daily dietary changes, weather, cultivation conditions and unforeseen events, to name a few, we set out a second sampling via a collection of 102 faecal samples (ASD = 49, TD = 53) from the main cohort as our validating set. With these samples, we performed targeted metabolomics focusing on metabolites associated with GABA metabolism (**Figure 1E**). In keeping with the results from the screening set, ASD subjects showed a significant augment of GABA, alongside a significant decline of Glu, glutamine, α-ketoglutarate, fumarate, malate, and oxaloacetate, compared to TD (**Figure 1F**). The substantial change of individual metabolites revealed an overall imbalance of GABA metabolism in ASD.

We postulated that the metabolic features of GM could be linked to early diagnostics of ASD as it reliably reflects subtle pathophysiological changes and even symptomatic characteristics of autism. Subsequently, we assessed the diagnostic performance of the aforementioned metabolites; however, GABA and other differential metabolites showed suboptimal diagnostic efficiency (areas under the curves [AUC] < 0.75, **Figure S2**) using ROC analysis. It raised the possibility that the level change of a single metabolite may not be sufficient to capture the dynamic traits of the gut microbial metabolism in ASD. It was reported that specific stages of multiple sclerosis development could be assessed by the imbalance of glutathione metabolism in GM, presented as a ratio of cysteine persulfide to cysteine [44]. Hence, by evaluating metabolites and their known precursors, we developed ratios of GABA to Glu and of Glu to glutamine, which are thought to recapitulate the dynamics of GABA metabolism. To acquire the key feature of microbial metabolism in ASD from a wider range, we also calculated ratios of dopamine to norepinephrine or to dihydroxyphenylalanine in tyrosine metabolism, serotonin to 5-hydroxyindole-3-acetic acid in tryptophan metabolism, and secondary to primary BAs in BAs metabolism (**Figure S3**). By comparing all the above-proposed ratios between ASD and TD, we identified that the GABA/Glu ratio ($p < 0.001$, VIP = 1.37) amongst the others was the only potential biomarker for ASD (**Figure 1G**, **Table S3**). It is worth noting that GABA and Glu are the primary pair of inhibitory and excitatory neurotransmitters in the nervous system, the imbalance of which triggers neuropsychiatric disorders [45]. Here, imbalance of the gut microbial GABA metabolism, represented by an increased GABA/Glu ratio, revealed a metabolic signature of ASD.

Faecal GABA/Glu ratio was validated as an early hallmark of ASD and associated with core ASD symptomatology

To delineate the clinical significance of the microbial GABA/Glu ratio in ASD, we plotted ROC curves for the two faecal sampling sets from the main cohort and calculated the corresponding AUC thereafter. Subsequently, by analyzing the screening set (AUC = 0.82) and the validating set (AUC = 0.85) (**Figure 2A**), we found the GABA/Glu ratio an overall satisfactory biomarker for ASD, far outweighing the aforementioned individual metabolites in terms of independently serving as an essential feature of ASD.

Considering the stages of human development and growth, followed with pertinent adaptation of dietary structure, that may influence the clinical applicability of the faecal GABA/Glu ratio in the early onset of ASD, we stratified the main cohort into preschoolers (3-5 year olds) and schoolers (6-8 year olds). Intriguingly, the diagnostic efficiencies of the GABA/Glu ratio in the preschooler subgroup (**Figure 2B**, red and blue lines) were much higher than that in the schooler subgroup (**Figure 2C**, red and blue lines). To corroborate this finding, we conducted targeted metabolomics with samples from the independent cohort that consisted of preschoolers, and assessed the ROC performance of the GABA/Glu ratio thereafter. As expected, a consistent effective ROC performance (AUC=0.92) was achieved (**Figure 2B**, cyan line).

Next, we asked if the GABA/Glu ratio was informative for recognition of the core ASD symptomatology. We performed a Pearson correlation analysis between GABA/Glu ratio and clinical manifestations of ASD evaluated by CARS score. The GABA/Glu ratio was positively correlated ($p < 0.05$) with the severity of the distinguishing symptoms at the two stages of child development. In preschoolers, the microbial GABA/Glu ratio was correlated with emotional and intellective abnormalities (**Figure 2D and E**) that were known

obstacles for early development of social skills [46]. In schoolers, the faecal GABA/Glu ratio was linked to aberrance in auditory signal transduction (**Figure 2F**), indicative of a cerebral GABA/Glu imbalance in auditory cortex as previously reported [47]. The association of faecal GABA/Glu ratio with pertinent neuropsychiatric symptoms at distinct developmental stages suggested a role of microbial GABA/Glu imbalance in abnormal development of the social brain. Taken together, our results showed that the faecal GABA/Glu ratio was a potent independent biomarker for the early diagnosis of ASD.

ASD-linked GM dysbiosis was associated with the elevated microbial GABA/Glu ratio

To ferret out the underlying difference in GM composition between ASD and TD with concomitant alteration in microbial GABA metabolism, we applied the same faecal samples that were used for the metabolomic screening to 16S rRNA gene sequencing (**Figure 3A**). Plots of PCoA based on β diversity analysis showed no obvious distinction of ASD-specific GM from the TD controls ($p = 0.9$) (**Figure 3B**). However, via α-diversity-based analysis, we found that the ASD-linked GM community exhibited a significantly lower diversity (Chao index, $p < 0.05$) and comparable richness (Shannon index, $p > 0.05$) relative to that in TD **(Figure 3C**). Further, through LEfSe analysis, we identified 35 differential genera, including ASD-enriched *Escherichia/Shigella*, *Lachnoclostridium*, *Megamonas*, *Megasphaera*, and *Veillonella*, as well as TD-enriched *Bacteroides*, *Akkermansia*, *Parabacteroides*, and *Rothia* (**Figure 3D**, **Table S4**), indicative of GM dysbiosis in ASD as previously reported [8, 16]. Next, we asked if there was possible correlation between GM dysbiosis and the imbalanced GABA metabolism. We began with assessing the 35 differential genera between ASD and TD groups, and subsequently sought out 3 genera that were significantly correlated with the GABA/Glu ratio, including *Escherichia/Shigella*, *Megasphaera*and *Veillonella* (**Figure 3E**). Intriguingly, among the above 3 candidate genera, *Escherichia/Shigella* was the only known genus with the capacity of producing GABA in human gut [9], implying a biological relation between over-presented *Escherichia/Shigella* and microbial GABA/Glu imbalance.

Considering the intrinsic individual heterogeneity in faecal samples that may disguise the bona fide traits of GM dysbiosis in ASD, we argued that deteriorated GABA/Glu metabolic imbalance might render such features more recognizable. To test this, we divided ASD subjects into four subgroups as per quartile of the GABA/Glu ratio (25% = 1.1; 50% = 1.81; 75% = 2.27) compared to the basal level (i.e. baseline) in TD, and sought to draw out the concomitant changes in GM assembly and structure, including richness and diversity of the ASD-linked GM, as well as the key differential bacterial genera at play. Of note, the top quartile of the autistic subjects showed most obvious GM dysbiosis and augmented *Escherichia/Shigella* abundance(**Figure 3F**). Collectively, the data suggest that the microbial GABA/Glu imbalance is reliably linked to the GM dysbiosis in ASD, in particular to the over-presented *Escherichia/Shigella*.

Imbalanced GABA metabolism was associated with overall hypofunction of the GM community in ASD

To elucidate the molecular mechanism that underlies the GM dysbiosis in association with the imbalanced GABA metabolism, we set out metagenomic analysis followed by qPCR validation on faecal samples (**Figure 4A**). Hence, from the main cohort we selected 7 ASD subjects with relatively high GABA/Glu ratios (hASD, GABA/Glu = 0.64 ± 0.19) along with 8 age- and gender-matched TD subjects with normal GABA/Glu

ratios around the basal level (nTD, GABA/Glu = 0.25 ± 0.14). For data quality and confidence, a third faecal sampling was conducted from these participants who were all male and aged from 3 to 5 years (**Figure 4A**).

Subsequently, we compared the structural and functional traits of the GM between the above two groups. Bacterial taxonomy analysis confirmed a distinctive structural alteration (**Figure 4B**), alongside a significant decline of both richness and diversity of GM in the hASD children compared to their neurotypical peers (**Figure 4C**), which was consistent with our previous results obtained from the first faecal sampling **(**Figure 3B and F**)**. Strikingly, KEGG pathway analysis revealed that multiple molecular pathways relating to cellular metabolism and homeostasis exhibited significantly decreased abundance, suggesting a deteriorated viability of the GM in hASD. These signaling categories included protein processing, longevity regulation, tricarboxylic acid cycle, and Glu, aspartate, alanine, and histidine metabolism ($p < 0.05$, FDR < 0.1) (**Figure 4D**), implying disruption of energy and amino acids metabolism, proteostasis, survival and growth in hASD. In addition, we identified a plethora of profoundly altered stress response genes which as a whole was significantly decreased in the hASD subjects **(Figure 4D)**. Of note, some of these stress response genes, such as *gor*, *gpx*, *sod1* and *katG* (**Figure S4, Table S5**), encode enzymes for detoxifying superoxidase and peroxide, which are primary intracellular reactions of microbes for overcoming extracellular stresses [48-50]. These data suggested a suboptimal living environment wherein the overall hypofunction of the hASD-linked GM community occurred.

Among the identified of ASD-associated pathways, the category of Glu, aspartate and alanine metabolism comprised a series of enzyme-encoding genes involved in regulation of GABA metabolism, such as *gabT* (GABA aminotransferase) and *gabD* (succinate semialdehyde dehydrogenase) for GABA degradation, and *gltD* (Glu synthase) and *purF* (amidophosphoribosyltransferase) for synthesis of Glu and glutamine, respectively. To further reveal the molecular basis of the imbalanced GABA metabolism in ASD-linked GM, we analyzed abundance of these genes (**Figure 4E, i, abundance**). We found significant increment of *gabT* and *gabD*, concomitant with significant decrement of *gltD* and *purF* in hASD relative to nTD. Of note, transcription of *gabT* and *gabD* is GABA-dependent in microbes, such as species of *Escherichia* and *Bacillus* [51, 52], suggesting that adequate supplies of GABA ensured GABA consumption in the GM community of ASD. Besides, other genes, including *aspB*, *argG*, *asdA* and *ala*, which are involved in metabolic interactions among Glu, aspartate and alanine, were consistently declined in hASD. Further, most of these aforementioned genes were significantly correlated with the GABA/Glu ratio (**Figure 4E, i, Coefficient**). In keeping with our finding of the augmented GABA/Glu ratio in the metabolomic study, these results at the molecular level verified the imbalanced microbial GABA metabolism in ASD.

To evaluate host-bacterial interactions, we mapped the metagenomics results against the virulence factor database including all known bacterial factors involving colonization on host intestinal epithelium, microbe-host communication, and reciprocal responses. We identified a panel of 21 available *E. coli*-specific adhesins, whose role was reported in facilitating bacterial colonization of intestinal epithelium [53]. By comparing the abundance of these adhesins between hASD and nTD, we determined that 7 molecules, such as P fimbriae, S fimbriae, and F1C fimbriae, were significantly increased in hASD (**Figure 4E, ii, Abundance**). The rest of them showed an ascending trend in abundance although not significant (**Table S6**). Further, we found that the abundance of each of all the 7 molecules was significantly correlated associated with the the GABA/Glu ratio

(**Figure 4E, ii, Coefficient**), implying that microbial GABA/Glu ratio was influenced by colonization status of *Escherichia* species.

Given the association between the GABA/Glu ratio and *Escherichia/Shigella* found in our 16S rRNA gene sequencing analysis (**Figure 3F**), here we further investigated if there was such a microbe-metabolite axis. We first analyzed the relative abundance of genera *Escherichia* and *Shigella*, which could not be individually distinguished by 16S rRNA gene sequencing. The abundance of *Escherichia* in hASD subjects was consistently higher than that in nTD controls (hASD vs. nTD = 3.27% ± 1.18% vs. 0.13% ± 0.04%), while the relative abundance of *Shigella* was very low in both groups (0.17% ± 0.07% vs. 0.006% ± 0.002%) (**Figure 4F**). To consolidate that *Escherichia* is the bona fide contributor of GABA production, we investigated the key enzyme of the microbial GABA-producing pathway through qPCR testing the gene abundance of the genus-specific GAD of *Escherichia*. The abundance of *gadA* and *gadB* were specifically increased in *Escherichia* (**Figure 4G**). Taken together, the data suggested that over-presented *Escherichia* gave rise to excess GABA and thus led to a GABA/Glu ratio in children with ASD.

Bacterial challenge with *E. coli* elicited social deficiency in mice associated with altered GABA metabolism

To further verify the relationship among over-presented *Escherichia*, GABA metabolic imbalance and autistic symptomology, we challenged male C57BL6/J mice with a non-pathogenic *E. coli* strain. Intragastric administration of gut *E. coli* was performed from P21 to P25 to mimic *Escherichia* over-presentation in gut of preschoolers (**Figure 5A**). After 7 days colonization, qPCR of faecal microbiome DNA demonstrated substantial increment of abundance of *E. coli* (FC = 33.16, $p < 0.05$) (**Figure 5B**) and *Escherichia*-specific *gad* (FC of *gadA* = 7.13, $p < 0.0001$; FC of *gadB* = 7.26, $p < 0.0001$) (**Figure 5C**) resulted from *E. coli* challenge in mice. These results suggested that we had successfully induced over-presented *E. coli* and imbalanced GABA metabolism in mice, as our findings in children with ASD (**Figure 4F and G**).

Furthermore, the three-chamber test (**Figure 5D**) show normal social preference (**Figure 5E**) but obviously deficit social recognition (**Figure 5F**) in *E. coli* challenged mice, suggesting substantial deficiency in social activity of mice caused by *E. coli*. In support of this, subsequent test of reciprocal social interaction (**Figure 5G**) depicted that the time of interaction with stranger mouse was significantly decreased in *E. coli* group than that in PBS group (**Figure 5H**). In the open field test (**Figure 5I**), comparable grooming time (**Figure 5J**) in the two groups suggested that there was no repetitive behavior in *E. coli* challenged mice, as we observed in subsequent marble-burying test (**Figure S6A**). On the other side, comparisons of the time spent in center (**Figure 5K**), travelling distance (**Figure 5L**) and mean velocity (**Figure 5M**) between PBS group and *E. coli* group were all not significant. Other behaviors, including novel object recognition (**Figure S6B**) and elevated plus maze (**Figure S6C**), were also not changed in *E. coli* group. These data indicated that the social deficiency elicited by gut *E. coli* challenge was independent of either anxiety or abnormalities in recognition memory and locomotor activity.

Discussion

Via establishing a set of research principles, we strictly controlled confounders from participant recruitment and faecal sampling that may affect data analysis. Through targeted metabolomics, we identified microbial

GABA/Glu imbalance as a metabolic signature (**Figure 1**) in the mild ASD subjects relative to the neurotypical peers. This outcome, formulated as an increased GABA/Glu ratio, was confirmed using different samples from the main cohort, as well as exploiting samples from an independent cohort. Importantly, the GABA/Glu ratio was validated as an independent biomarker for ASD with a very high degree of accuracy, and was correlated to core symptomatology in autistic children, particularly in those preschoolers aged 3-5 years (**Figure 2**). Further, by 16S rRNA gene sequencing, we showed ASD-featured GM dysbiosis, highlighted by excessively abundant genus *Escherichia/Shigella*, which was closely related to the GABA/Glu imbalance (**Figure 3**). Of note, at a deteriorated GABA/Glu ratio, the structural and functional changes in GM between ASD and TD individuals became more recognizable (**Figure 4B and C**), which was characterized by overall hypofunction of the ASD-linked GM community via our metagenomic analysis (**Figure 4D**). The subsequent qPCR validation elucidated that over-presented *Escherichia*, a known intestinal GABA producer, was the major contributor of the augmented faecal GABA/Glu ratio in ASD (**Figure 4E-G**). At the end of our study, we verified that bacterial challenge of weaning mice with commensal *E. coli* induced social deficiency, concomitant with faecal GABA augmentation (**Figure 5**). As was shown via multi-omics analyses and multi-level validation, our findings provided an integrated chain of evidence, supporting that gut *Escherichia* elicited abnormal GABA metabolism as the key feature of mild ASD.

Our findings of the increased microbial GABA/Glu ratio in mild ASD were partially supported by evidence from previous metabolomic observations in faecal samples of ASD patients, including elevated carboxyethyl GABA [8] and declined a-ketoglutarate [25], which are intermediate metabolites during synthesis of GABA and Glu, respectively. We also noted a few studies that claimed a decreased tendency of faecal GABA level in ASD, albeit without statistical significance [54, 55]. This could be due to confounding effects, smaller sample size, or unclear stratification. Strikingly, when split into two age groups of participants, we found a better ROC performance of the GABA/Glu ratio in preschoolers (aged 3-5 years) than that in schoolers (aged 6-8), suggesting a strong diagnostic value especially for the younger children on the autistic spectrum. Therefore, observations in untargeted age range in different studies may have resulted in data inconformity as such. Besides, multiple reports using metagenomic studies, including ours, documented decrement of Glu metabolism-associated pathways as critical indicators of microbial functional changes in ASD subjects around 4 years old [14, 25, 26]. Further, via integrating our genetic pathway analysis with metabolomic verification, we demonstrated an enhanced supply of GABA (increment of *gabT* and *gabD*) and a declined Glu synthesis (decrement of *gltD* and *purF*) in the ASD-linked GM community, confirming the imbalanced GABA metabolism as a consistent GM signature in young children with ASD.

In recent years, potential GM-associated biomarkers for ASD were continuously reported and often presented as a collection of bacteria [8, 14], a group of microbial metabolites [56], or a set of GM-associated genes [26], suggesting that indexes of the same category formed a composite marker and collectively predicted ASD symptoms. Here, we identified the faecal GABA/Glu ratio as an independent biomarker with significant correlation to specific autistic symptoms, especially at an early age. As is known, ASD is a neurodevelopmental disorder with cerebral imbalance between excitation and inhibition. Altered levels of GABA and Glu in cerebra ultimately underlie the excitatory/inhibitory imbalance of neurotransmission in ASD pathogenesis [57]. However, is the faecal microbial GABA/Glu imbalance indispensable for neuronal activities in the brain? It is thought that GM-derived metabolites are an important source of GABA supply to the central

nervous system, as studies in animals revealed that GM alterations could affect both circulatory and cerebral levels of GABA [58, 59]. Accordingly, as a non-invasive biomarker for early diagnosis of ASD, the microbial GABA/Glu ratio is advantageous over the cerebral counterpart, for it can be accurately measured via *in vitro* tests on accessible faecal samples. By contrast, due to distinct functional cerebral regions and low resolution of the current measuring techniques for *in vivo* detection, it is not straightforward to obtain such cerebral data from human. For example, some previous clinical studies observed an increased GABA/Glu ratio while others a decreased ratio in either different cerebral regions or even the same region [60-65].

We found an overall hypofunction of GM in autistic subjects with an imbalanced GABA/Glu metabolism, which was in keeping with accumulating evidence showing a suboptimal, inefficient state of ASD-linked GM, such as impaired detoxifying ability, decreased neurotransmitter synthesis, and underdeveloped maturity [8, 14, 25, 26, 56]. Indeed, the increased faecal GABA/Glu ratio was correlated to a series of lower-abundant viability-related pathways (**Table S4**), of which tricarboxylic acid cycle and GABA metabolism-associated pathways are known essential to fueling GM growth [9]. In addition, our finding of the enriched stress response genes in the ADS-linked GM confirmed the role of GABA metabolism in bacterial defense of homeostasis and response to stress stimuli [66]. Collectively, our metagenomic and metabolomic data suggested a sub-health intestinal ecosystem in ASD subjects, wherein a high level of the faecal GABA/Glu ratio was likely to be a metabolic messenger for the GM-host interaction. Albeit mechanistically unelucidated yet, our findings have important implications for a better understanding of the role of GABA metabolism in the entire GM metabolism in autistic children.

As was discussed above, changes in the gut bacterial taxonomic abundances between ASD and TD were frequently reported, such as increased abundance of *Escherichia* [8, 12, 16]; however, the direct link of the microbiome with its metabolites remains to be elucidated. In this study, we established an axis of excess *Escherichia* and elevated GABA production. Converting Glu to GABA in *Escherichia* species was known as an important means of adaptation to an acidic environment [67], like the intestinal tract. Interestingly, we found that both *Escherichia* abundance and *E. coli*-specific adhesins were positively correlated with the GABA/Glu ratio, indicative of metabolic adaptation of *Escherichia* for its survival and replication during intestinal colonization. We also asked if other GABA producers in the gut, including *Bacteroides*, *Parabacteroides*, *Bifidobacterium* and *Lactobacillus* [9, 68], could influence the GABA metabolism in ASD. However, we found that these genera were decreased or remained unchanged in ASD subjects, and were not associated with the faecal GABA/Glu ratio **(Figure S6A)**. Thus, we postulated that individual taxa, like *Escherichia*, rather than the whole GM community, might account for GABA production. Indeed, we observed a significantly increased *Escherichia*-specific *gad*, the enzyme catalyzing Glu to GABA (i.e. GABA synthesis) in children with ASD, which strongly supported our hypothesis. On the contrary, GM-genomic *gad* was unchanged, and thus became less likely to contribute to the augment of the GABA/Glu ratio. In addition, the *Bacteroides*-specific *gad* as a control exhibited a decreasing tendency **(Figure S6B)**. Taken together, our integrated analyses elucidated a previously unreported axis of *Escherichia*-GABA metabolism in the GM of children with ASD.

In this study, we found that the age was a profound factor associated with individual variation of the GM components, lending it appropriately to the notion that the development of host and GM substantially influenced each other in both ASD and TD subjects [14]. This enlightened our analysis based on age

stratification that revealed peak levels of *Escherichia* and the GABA/Glu ratio in children at 4 years **(Figure S7)**, right within the critical time window of neurodevelopment and GM assembly [69]. It was likely that particular features of GM at an early age may play a causal role in ASD. Indeed, our study demonstrated that the bacterial challenge of commensal *E. coli* in the healthy weaning mice generated an abnormal GABA metabolism in the GM, in support of our clinical findings. Furthermore, social deficiency in the *E. coli* challenged mice was specifically induced, independent of repetitive behavior, anxiety, recognition memory and locomotor abnormity, which was in keeping with the GABA/Glu ratio-associated symptomology identified in autistic preschoolers. Besides, the behavioral phenotype of our mouse model is similar to that observed in mice with a global deficit of the *chd8* gene[70], known as one of the top ASD-associated genes.

Overgrowth of *Escherichia* at early age may disrupt the homeostasis of GABA metabolism in GM community, which had the potential to induce serous and neural GABA/Glu imbalance and thus impeded the development of social brain. Interestingly, a recent clinical trial showed that lowering the GABA/Glu ratio in the insular cortex and visual cortex by oral bumetanide attained to symptomatic improvement in the preschooler ASD subjects [71]. Although no GM investigation was involved in this trial, the oral drug could theoretically affect the faecal, serous and neural GABA/Glu ratio, suggesting that targeted therapeutic strategies for ASD through restoration of the gut microbial GABA/Glu imbalance could be promising. Therefore, we consider that the microbial GABA/Glu dynamic might serve not only as an early diagnostic marker but also as a therapeutic target for ASD.

Conclusions

In conclusion, our study elucidated a conspicuous axis of *Esherichia*-GABA metabolism in both mild ASD patients and the mouse model, which featured ASD-linked structural and functional changes of GM at an early age, and potentially played a causal role in social deficiency of autistic symptomology. These findings highlighted the role of GM in regulating excitatory/inhibitory neurotransmitter balance in the host, and facilitated the diagnostic and therapeutic strategies for ASD from the perspective of the gut-microbiota-brain axis.

Declarations

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Availability of data and materials

The datasets supporting the conclusions of this article will be included as additional file before peer-review.

Author contributions

D.W., N.L., H.L., Y.P., and Y.H. conceived and designed this research.

D.W., L.L., S.F., H.Y., and Y.H. conducted participant recruitment.

D.W., N.L., T.W., S.F., and H.Y. conducted the sample collection.

D.W., W.Z. and X.Z. conducted the experiments of metabolomics and genomics.

D.W. and Y.P. performed the bioinformatics analysis of metabolomics and genomics.

D.W., W.Z. and Y.J. performed animal experments.

D.W., N.L., H.L. and Y.P. collected and analyzed the experimental data.

D.W. and N.L. wrote draft preparation.

D.W., N.L., H.L. and X.Z. wrote and edited the manuscript.

N.L., H.L., and Y.H. reviewed and supervised the manuscript.

N.L. oversaw the project.

All authors have read and approved the published version of the manuscript.

Competing interests

The authors have declared no competing of interest.

Consent for publication

All authors have read and approved the paper for submission.

Ethics approval and consent to participate

The human gut microbiota study was reviewed and approved by the Ethical Committee of The Seventh Affiliated Hospital of Sun Yat-Sen University. Written informed consent was obtained from the guardians of the participants. The animal study was reviewed and approved by Southern University of Science and Technology Animal Care and Use Committee.

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Tables

Table 1. Clinical characteristics of ASD and TD subjects.

		Main cohort			Independent cohort		
		ASD	**TD**	**p-value**	**ASD**	**TD**	**p-value**
Subjects (n)		50	55	-	6	12	-
Male/Female (n/n)		44/6	26/29	0.00	6/0	12/0	-
Average Age (years)		5.16(0.21)	4.69(0.15)	0.13[b]	4.17(0.31)	4.08(0.19)	0.93[b]
Severity	CARS[a]	34.44(1.74)	-	-	33.00(1.67)		-
Constipation (n)		20	9	0.007	2	2	1.00[c]
Delivery (n)	Caesarean	19	18	0.45	3	4	0.63[c]

Data are presented as “mean (SD)”, unless otherwise stated. **a** CARS = childhood autism rating scale: <30 normal, 30–36 mild ASD, ⩾36 severe ASD. **b** p-value = Wilcoxon rank sum test between groups. **c** p-value = Chi-square test between groups. ASD = autism spectrum disorder. TD = typical development.

Figures

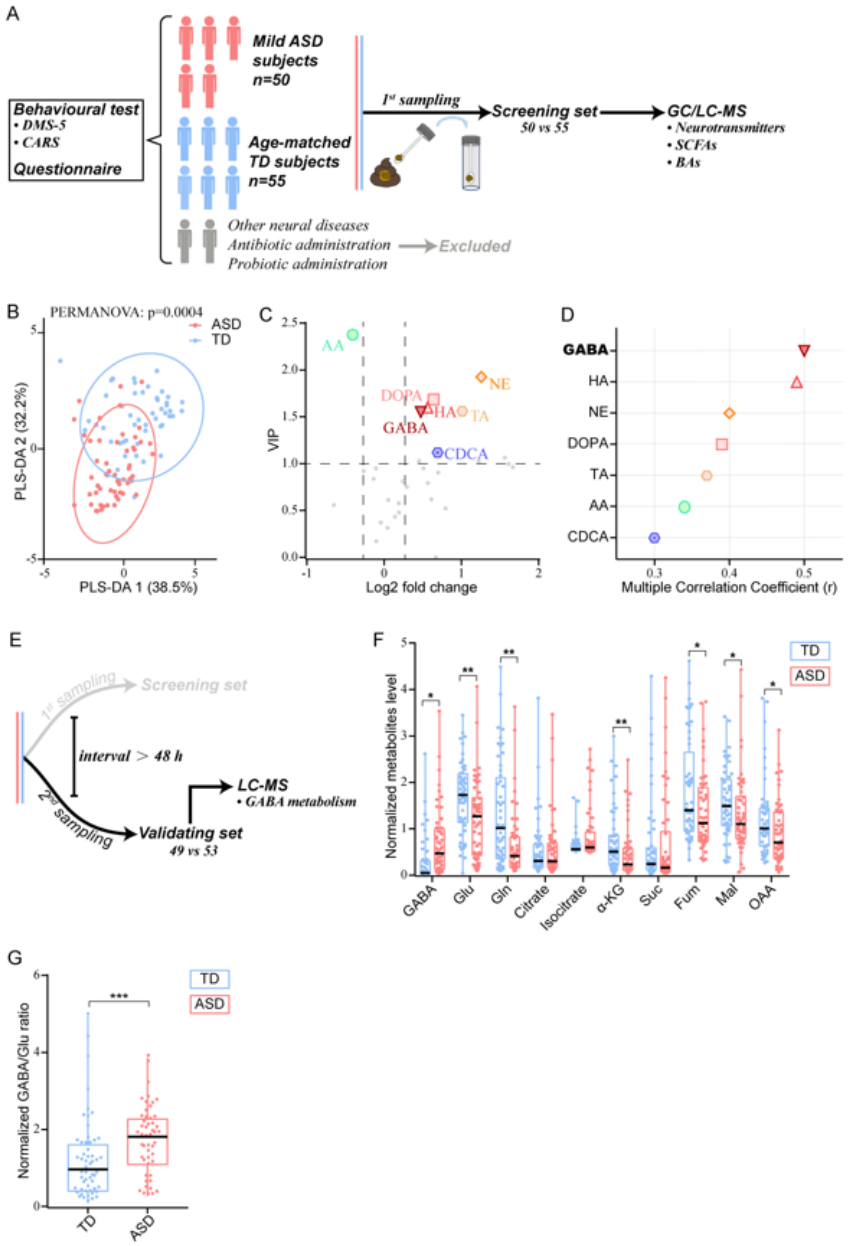

Figure 1

GABA/Glu imbalance was identified as a key metabolic signature of gut microbial metabolism in ASD. (A) Work flow for participant recruitment, screening set sampling and targeted metabolomics. (B) Plots of microbial metabolic profiling based on PLS-DA significantly distinguished TD and ASD. (C) According to VIP, 7 metabolites were identified as potential biomarkers ($p < 0.05$, VIP > 1) for ASD, among which GABA was most

associated with the metabolic disturbance of GM (D). (E) Work flow for validating set sampling and GABA metabolism-targeted metabolomics. Compared to TD, validating for metabolites involving GABA metabolism (F) depicted a disturbed GABA metabolism which was characterized by a significant increment of GABA-to-Glu ratio (G) in ASD. Data of plots box were normalized by value/SD. Significance was tested by Wilcoxon rank sum test with * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. DSM-5 = diagnostic and statistical manual of mental disorders, fifth edition. CARS = childhood autism rating scale. ASD = autism spectrum disorder. TD = typical development. GC = gas chromatography. LC = liquid chromatography. MS = mass spectrometry. SCFAs = short chain fatty acids. BAs = bile acids. GABA = g-aminobutyric acid. DOPA = dihydroxyphenylalanine. HA = histidine. TA = tyramine. NE = noradrenaline. CDCA = chenodeoxycholic acid. AA = acetic acid. Glu = glutamate. PLS-DA = partial least-squares discrimination analysis. VIP = variable importance for the projection.

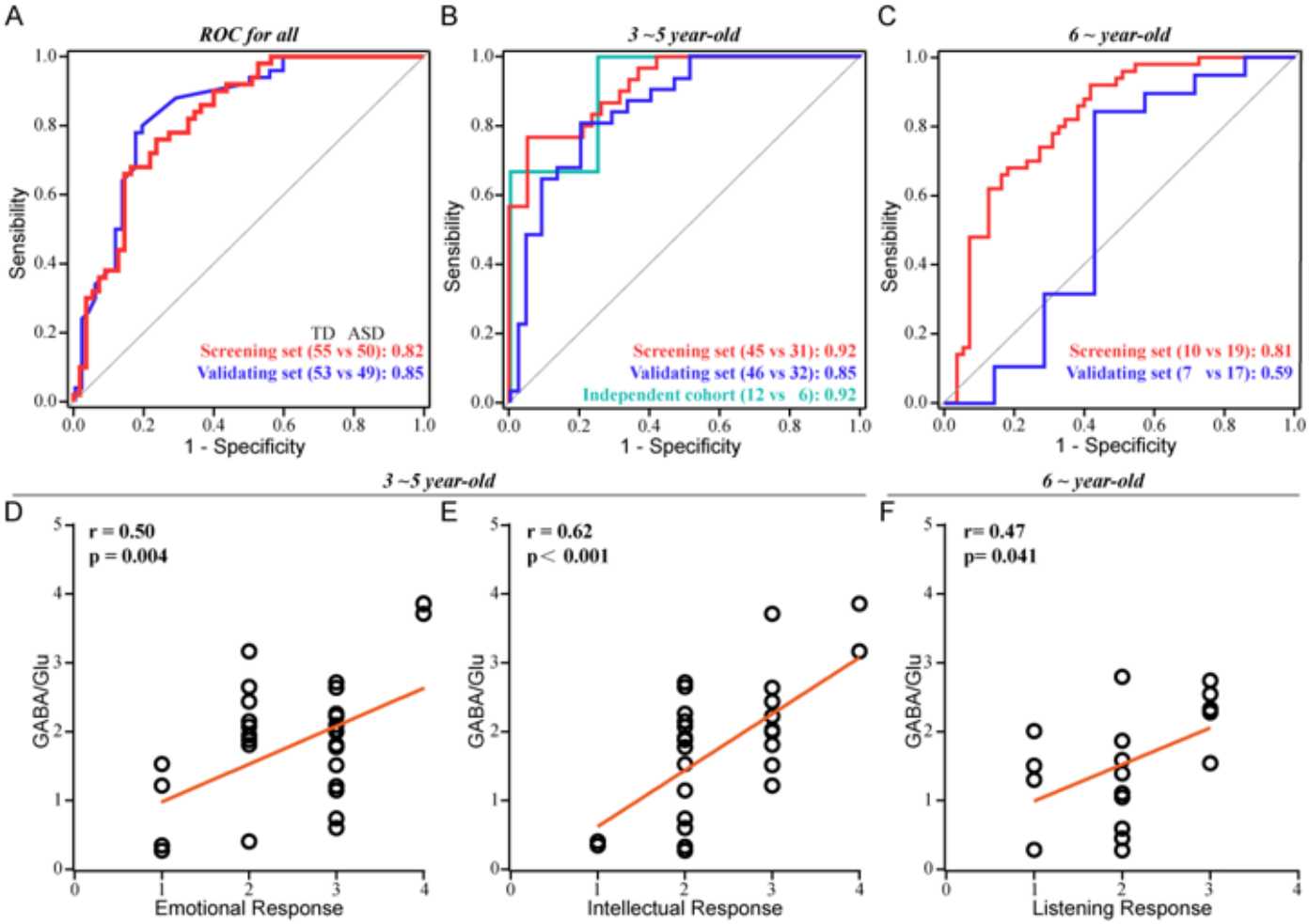

Figure 2

Clinical significance of gut microbial GABA/Glu ratio in early diagnosis of ASD. The GABA/Glu ratio was identified as an independent marker by ROC analysis for classifying ASD vs. TD for all participants (A), 3–5-year-old subgroups (B) and over 6-year-old-and-above subgroups (C). AUC values of the screening set,

validating set and independent cohort were denoted in red, blue and cyan lines respectively. Linear plots showed the GABA/Glu ratio was associated with autistic symptoms in 3–5-year-old subgroups (D-E) and 6-year-old-and-above subgroups (F). ROC = receiver operating characteristic. AUC = area under the curve

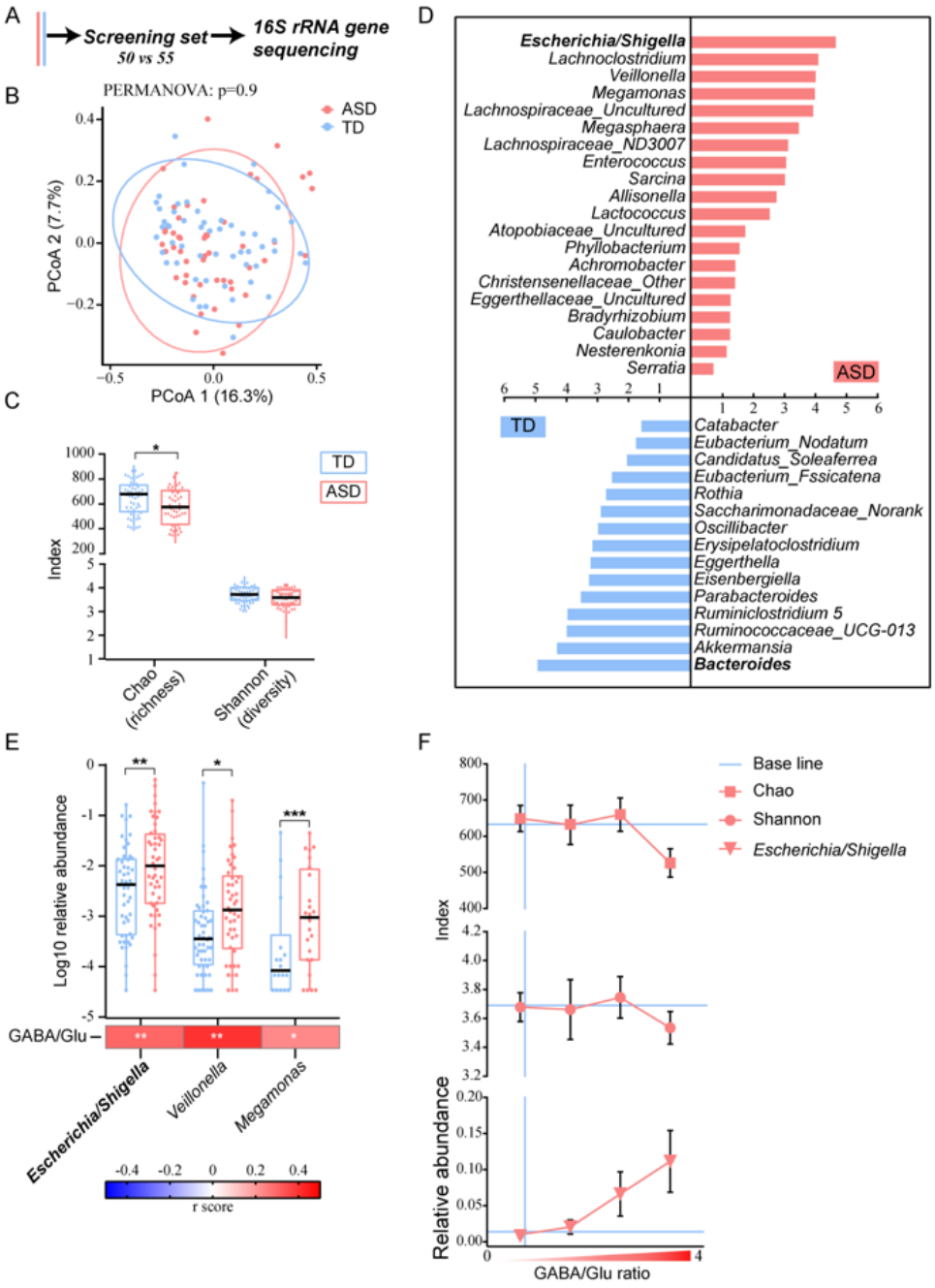

Figure 3

Structural dysbiosis of GM was related to the elevated faecal GABA/Glu ratio in ASD. (A) Samples from the screening set were used for 16S rRNA gene sequencing. (B) PCoA plots showed that the gut microbial composition could not be distinguished between the two groups. (C) Microbial α density analysis comparing ASD with TD. (D) Lefse at the genera level identified 35 differential GM enriched in ASD and TD. (E) The correlation of differential genera with the GABA/Glu ratio. Boxplots showed relative abundance of the GABA/Glu ratio associated genera (top). Significance was tested by Wilcoxon rank sum test with * $p < 0.05$. ** $p < 0.01$. Heatmap showed correlation (r score) between GM and the GABA/Glu ratio (bottom). (F) Indexes of Chao and Shannon and relative abundance of *Escherichia/Shigella* in each quartile of autistic subjects divided according to their GABA/Glu ratio. PCoA = principal coordinates analysis. LefSe = linear discriminant analysis effect size.

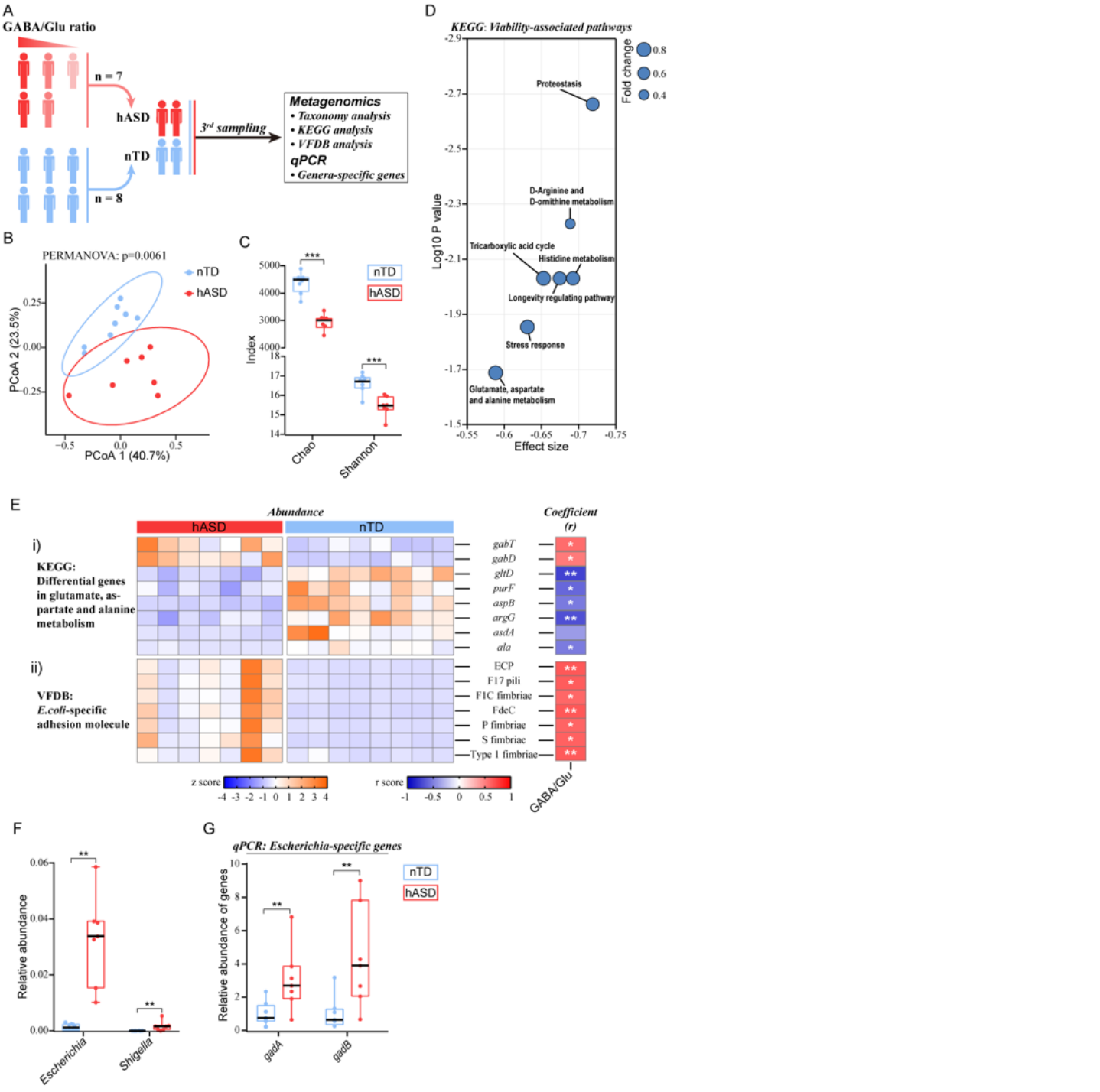

Figure 4

Over-presented *Escherichia* in the ASD-specific GM community contributed to excess GABA. (A) Autistic subjects with relatively high GABA/Glu ratio (hASD) and age-, sex- matched TD subjects with normal GABA/Glu ratio (nTD) were selected for metagenomics and qPCR analysis. Taxonomy analysis showed (B) distinguished GM structure with (C) significantly decreased richness and diversity in hASD vs. nTD. (D) Based on KEGG analysis, significant decline in multiple pathways elaborated hypofunction of GM in hASD. (E) i) GABA-metabolism-associated genes idenfied by KEGG analysis and their association with the GABA/Glu ratio;

ii) *E. coli*-specific adhesins revealed by VFDB analysis and their association with the GABA/Glu ratio. (F) Over-presented genera *Escherichia* was verified in hASD. (F) qPCR was conducted for validating genera-specific enzyme of GABA synthesis. Gene abundance of *Escherichia*-specific *gadA* and *gadB* were significantly increased in hASD. KEGG = Kyoto encyclopedia of genes and genomes. VFDB = virulence factor database. Significance was tested via Wilcoxon rank sum test with black ** p < 0.01, *** p < 0.001 and Pearson correlation analysis with white * p < 0.05, ** p < 0.01.

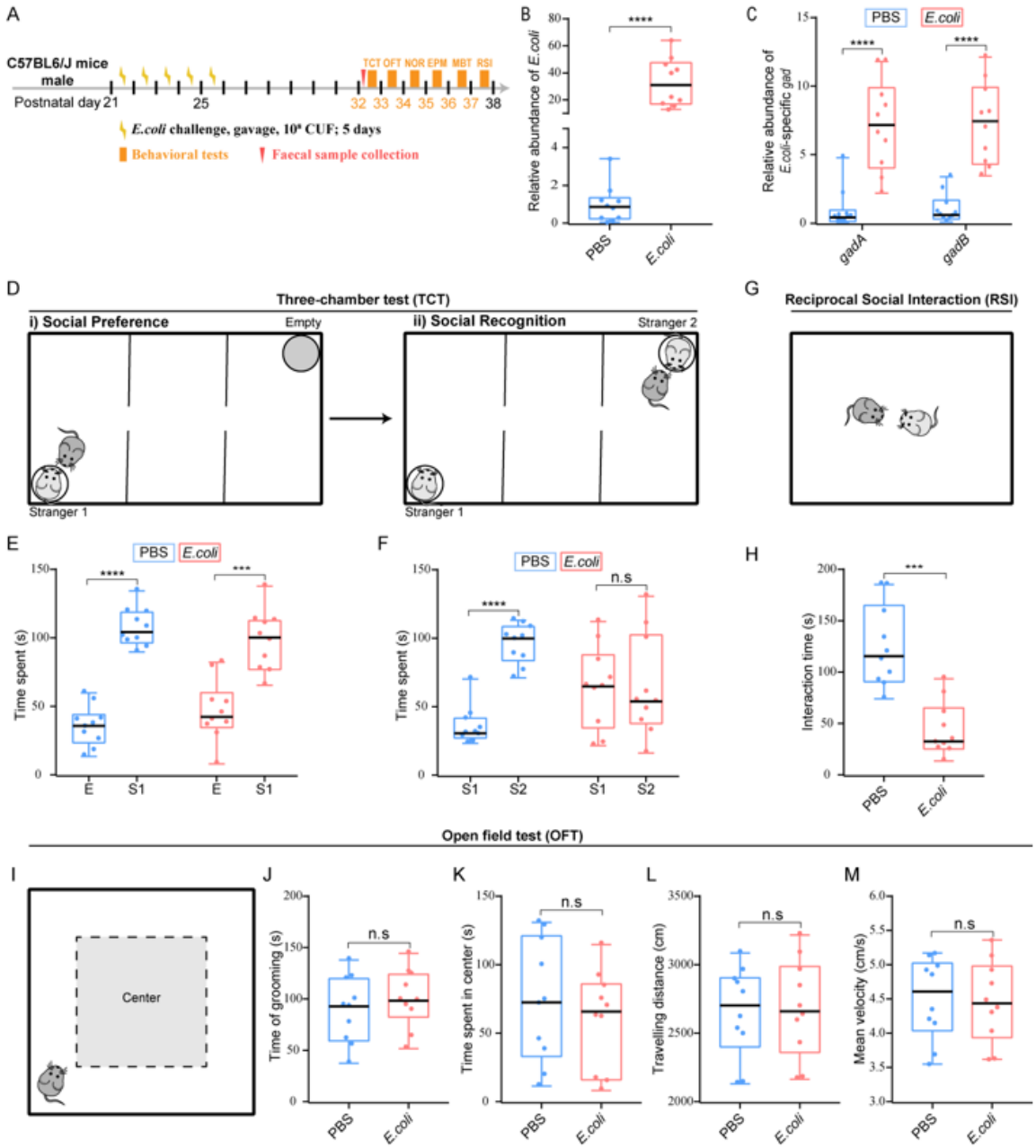

Figure 5

Challenge of gut *E. coli* led to social deficiency in mice. (A) Work flow of *E. coli* challenge and behavioural tests in animal experiments. The *E. coli* challenged group showed significantly increased relative abundance of (B) gut *E. coli* and (C) *Escherichia*-specific *gadA* and *gadB* than that in PBS group. (D) Diagram of three-chamber test (TCT). The *E. coli* challenged group showed (E) normal social preference and (F) deficit social recognition. (G) Diagram of reciprocal social interaction (RSI). (H) The *E. coli* challenged group showed significantly decreased integration time than that in PBS group. (I) Diagram of open field test (OFT). The *E. coli* challenged group showed comparable (J) time of grooming, (K) time spent in center, (L) travelling distance and (M) mean velocity with PBS group. PBS = phosphate buffer saline. NOR = novel object recognition. EPM = elevated plus maze. MBT = marble-burying test. CFU = colony forming unit. *** $p < 0.001$. **** $p < 0.0001$.

Supplementary Files

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- Supplementarytableandfigure.docx