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ORIGINAL RESEARCH

Serum Metabolic and Gut Microbiome Differences in Age-Associated Fragile X Syndrome (FXS) Pediatric Patients May Benefit Clinical Therapy Development

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Background: Fragile X syndrome (FXS) is a rare, genetically based neurodevelopmental disorder characterized by intellectual disability. While previous research has largely focused on its genetic mechanisms, the role of metabolism and the gut microbiome in FXS remains underexplored. This study aimed to investigate age-related metabolic differences in the gut flora and serum metabolites of children with FXS and their associations with clinical behavioral outcomes.

Methods: A total of 32 children with FXS under 18 years were enrolled and divided into two age groups: younger (3–8 years) and older (8–18 years). Intestinal microbiota composition was analyzed using 16S rDNA gene sequencing, and serum metabolite profiles were assessed via ultra-performance liquid chromatography-mass spectrometry (UPLC-MS). Spearman correlation analysis was used to assess associations among gut flora, serum metabolites, and scores from the Social Responsiveness Scale (SRS) and Child Behavior Checklist (CBCL).

Results: Significant differences in gut bacterial genera and 1,352 serum metabolites were observed between the age groups. The older group exhibited higher levels of phospholipids, steroids, and peptides, and enrichment in the steroid hormone biosynthesis pathway. Several metabolites were significantly correlated with SRS and CBCL scores, indicating potential links between metabolic changes and behavioral symptoms.

Conclusion: Age-associated metabolic and gut microbiota alterations in FXS may contribute to variations in clinical presentation. These findings suggest a metabolic basis for FXS and provide a foundation for future research into microbiome-targeted interventions in FXS management.

Keywords: fragile X syndrome, gut microbiome, metabolites

Introduction

Fragile X syndrome (FXS) is the most common monogenetic autism spectrum disorder (ASD) as well as the second main cause of intellectual impairment after Down syndrome.[1] This neurodevelopmental condition is caused by a mutation that amplifies the CGG trinucleotide repeat sequence in the 5' untranslated region of the fragile X messenger ribonucleoprotein 1 (*FMR1*) gene on the X chromosome.[2] The CGG repeats range from 5 to 44 in unaffected individuals, expanding to 45–55 in individuals with intermediate FXS, to 55–200 in individuals with premutation, and exceeding 200 in those with the full mutation.[3,4] This expansion causes aberrant methylation of *FMR1*, which in turn compromises the expression of fragile X messenger ribonucleoprotein protein (FMRP).[5] While FXS is well-characterized genetically, its metabolic and microbiome landscapes remain understudied, with most prior work focusing on genetic mechanisms.

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As an X-linked genetic disorder, FXS has a prevalence of approximately 1 in 7,000 for males and 1 in 11,000 for women, and its clinical manifestations are usually more severe in male patients than in female patients due to dynamic mutations in *FMR1*.[6–8] FXS lacks typical clinical manifestations in its early stages, and its diagnosis primarily relies on molecular genetic testing, which is an unconventional clinical examination; therefore, research on FXS remains limited. Consequently, large-sample-size data on the incidence of FXS in the general population are unavailable.[9] While comprehension of the pathophysiology of FXS has advanced over the past years, no specific treatments are currently available. However, early diagnosis and intervention can improve the prognosis and quality of life of patients, help advise affected families in their future reproductive decision-making, and reduce the economic and medical burdens on society. Therefore, further research is required into the pathogenesis of FXS to identify simpler, specific diagnostic tools and possible therapeutic targets.

Several studies have found that gut microbes affect the nervous system by altering metabolites through the microbiota-gut-brain axis, a bidirectional communication channel between the gastrointestinal tract and the central nervous system. This axis involves interaction through the gut flora as well as their metabolites, modulating the host's brain and behavior.[10] Previous research has confirmed that both the development and function of the nervous system are influenced by the gut flora and its metabolites.[11] Among these, p-cresol sulfate, a specific metabolite derived from the microbiota, plays a role in neuroinflammation and microglial phagocytosis in neurodevelopmental disorders.[12] In recent years, the role of the gut microbiota in human neurological disorders has gained increasing interest. Dysregulation of the gut microbiota has been connected to various neurological disorders, including ASD, Parkinson's disease, schizophrenia, multiple sclerosis, Alzheimer's disease, epilepsy, and stroke. Microbial interventions, such as fecal transplants, probiotic interventions, prebiotic interventions, and postbiotic interventions have shown great potential and promise in the treatment of neurological disorders.[13–15] While the pathogenesis of FXS is genetically determined, potential therapeutic targets have been identified within the gut flora and their metabolites that affect nervous system function and alleviate the symptoms of FXS.[16] Preclinical studies in FXS mouse models have shown that gut microbiota modulation via dietary interventions improves autistic behaviors, supporting gut flora as potential therapeutic targets.[17] However, studies on the relationship between FXS and gut microorganisms and their metabolites, especially those conducted on clinical patients, are lacking. This research therefore aimed to study whether metabolic variations in the gut flora as well as serum metabolites correlate with age and to analyze whether these metabolic changes affect the clinical outcomes of patients with FXS.

Materials and Methods

Patients with FXS

This study recruited 32 children with FXS from various regions of China. All children were diagnosed based on the results of the CGG repeat number testing, conducted during previous clinical visits or at enrollment. In addition, participants were excluded if they had used antibiotics/probiotics within 4 weeks or reported diarrhea/constipation within 1 week prior to sampling. Detailed gastrointestinal symptom histories were collected, and all enrolled children had normal bowel habits. All children with FXS were categorized into two groups according to age: the younger group comprising children aged 3–8 years (15 children) and the older group comprising children aged 8–18 years (17 children). In accordance with the convention in child development research that designates 8 years of age as the boundary between early childhood and adolescence, and considering that metagenomic analyses have shown significant gut microbiota dysbiosis in children with ASD aged 3–8 years, which is correlated with symptom severity, the study subjects were divided into a younger group aged 3–8 years and an older group aged 8–18 years, to observe the differences in metabolomics and gut microbiota across different developmental stages. Due to the age range of the developmental scales, only children in the younger group were assessed for the degree of developmental delay using the Griffiths Development Scales-Chinese. Parents of all children with FXS were given two assessment tools, the Achenbach Child Behavior Checklist (CBCL) as well as SRS. Scales were provided in Chinese, validated for cultural and linguistic relevance to the study population. These scales included questions about behavioral disorders, emotional problems, and social interaction skills. Achenbach CBCL was also provided to parents or primary caregivers to estimate the degree of attention deficits, behavioral disorders, and emotional instability in children's daily lives. CBCL consists of two areas including social competence and behavioral problems. Higher social competence scores indicated better social competence, whereas scores below 30 were considered indicative of social interaction disorder. The behavioral

problems section of the CBCL was built upon a four-point scale (never, occasionally, often, and always), with scoring based on their performance in each program. The higher the score, the more severe the child's problem with the program. The CBCL results for children with FXS are shown in Figure 1. No significant remarkable variations were found between the two groups, regardless of social competence or behavioral problems. In addition, both groups revealed social disability, attention deficits, varying irritability, and behavioral or emotional problems. Taken together, these findings indicate that as children with FXS get older, their social abilities and behavioral disorders show limited improvement compared to their condition at the age of 8 years. However, evaluating the findings of these scales has subjective errors due to the differences in the educational attainment of parents or primary caregivers, who may have answered the questions inaccurately.

Blood and Stool Sample Collection

Nurses collected blood samples from all patients after fasting from drinks and solids for at least 4 hs for both drinks and solids. Samples were centrifuged at 1,000 rpm for 10 min at 4°C and then at 3,000 rpm for 10 min at 4°C to extract serum. Subsequently, the serum was collected and stored at −80°C until profiling. Within 3 days of enrollment, stool samples were collected without any water or urine contamination. The feces samples were then stored at −80°C after collection.

Untargeted Metabolomic Profiling of Serum Samples

Ultra-performance liquid chromatography-mass spectrometry (MS) analysis-based untargeted metabolomic profiling was used to analyze serum samples. An equal volume of serum (10 μL) from each of the 32 serum samples was pooled and aliquoted to serve as the quality control (QC) sample for untargeted metabolomic detection. One QC sample was inserted after every eight individual samples to ensure data consistency. For metabolite extraction in untargeted metabolomic detection, both QC and individual samples were prepared using a salting-out approach. Metabolic extraction data were collected and analyzed using reversed-phase liquid chromatography-MS in both positive as well as negative ionization modes. Metabolomic feature extraction, alignment, and quantification were performed with the XC-MS software (Nonlinear Dynamics, Durham, NC, USA). Metabolites with an abundance of less than 50,000 in all individual samples or equal to zero in more than 85% of the individual samples were filtered out to eliminate background signals. To remove inter-batch variation in subsequent analyses, the peak areas of metabolites from all individual samples in the untargeted metabolomic profiling were normalized to a common QC sample. The R pre-process Core software package (v1.47.1) was employed for Robust Multi-array Average (RMA) normalization to calculate the relative abundance ratio of metabolites in each individual sample. After calculating the coefficient of variance (CV%) for every metabolic feature based on the abundance in the QC samples, the metabolites with CV% below 30% were retained for downstream analysis.

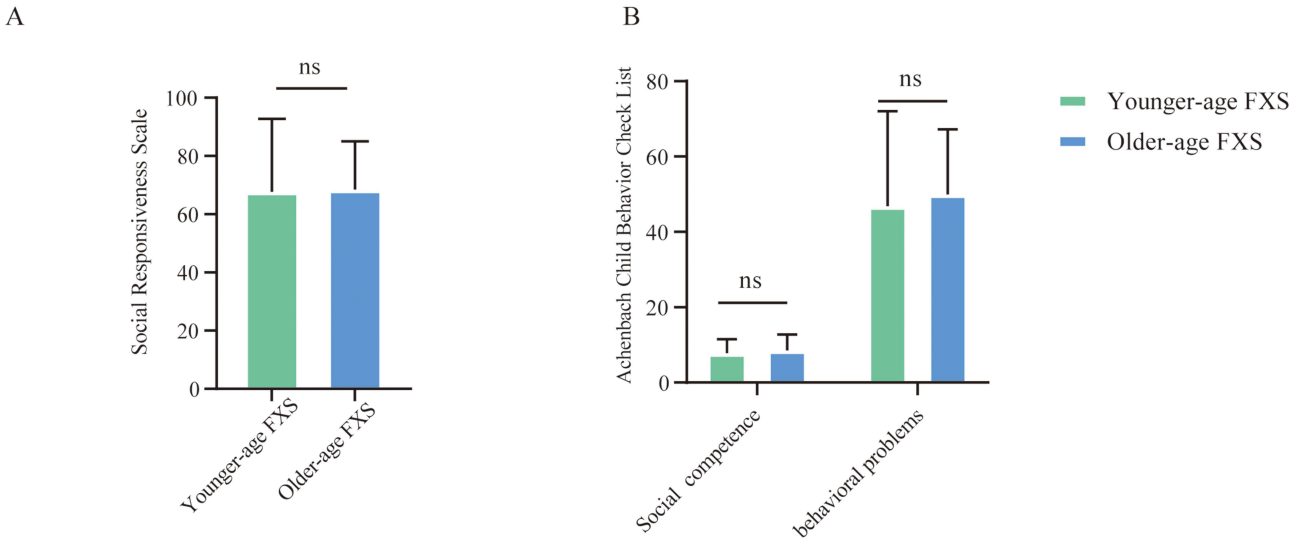

Figure 1 SRS and CBCL scores of patients with FXS. (**A**) SRS scores of children with FXS. (**B**) CBCL scores, including social competence and behavioral problem scores, of children with FXS. Ns indicates no statistically significant difference.
Abbreviations: CBCL, Child Behavior Checklist; FXS, fragile X syndrome; SRS, Social Responsiveness Scale.

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Serum metabolites were analyzed using an UltiMate 3000 UPLC system (Thermo Fisher Scientific, Waltham, MA, USA) coupled with a Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Chromatographic separation employed a BEH C8 column (2.1 × 100 mm, 1.7 μm, Waters, Milford, MA, USA) for positive ion mode and an HSS T3 column (2.1 × 100 mm, 1.8 μm, Waters, Milford, MA, USA) for negative ion mode. The mobile phase consisted of water with 0.1% formic acid (FA, Phase A) and acetonitrile with 0.1% FA (Phase B), following a gradient program: 0.0–1.0 min (95% A), 1.1–11.0 min (linear gradient to 5% A), 11.1–13.0 min (isocratic at 95% B), and 15.0 min (re-equilibration to 95% A). Flow rate was set at 0.35 mL/min, with a 5 μL injection volume.

For mass spectrometry, electrospray ionization (ESI) was used in both positive (ESI+, 3.8 kV spray voltage) and negative (ESI−, −3.0 kV spray voltage) modes, with a capillary temperature of 320°C, sheath gas flow of 35 arb units, auxiliary gas heater temperature of 350°C, and S-lens RF level of 50. The mass range was 100–1200 m/z (ESI+) and 70–1060 m/z (ESI−), with full MS resolution at 70,000 (FWHM at m/z 200) and MS/MS resolution at 17,500 (FWHM at m/z 200). Stepped collision energies (20 and 40 NCE) were applied for fragmentation. Daily mass calibration utilized Pierce™ LTQ Velos™ ESI Positive/Negative Ion Calibration Solutions (Thermo Fisher Scientific), ensuring mass accuracy ≤2 ppm across the mass range.

MS/MS spectra of the QC samples were obtained at various energy fragmentation (25 as well as 50 NCE) of the top 10 parent ions. The QC MS1/MS2 spectra were imported into the MS-DIAL 4.24 program and public databases, including the Human Metabolome Database (HMDB) and PubChem. Chemical structures of metabolites of interest were putatively identified by matching their experimental retention times (when available) and MS/MS spectra to reference spectra in the METLIN and HMDB databases. The combination of retention time and MS/MS spectra provides a unique fragmentation signature for each metabolite, determined by its molecular structure, functional groups, and collision energy. Using in silico matching algorithms, including GNPS, chemical structures were inferred based on spectral similarity. These structures were then classified into chemical classes based on their structural motifs and functional groups. Consequently, the metabolites were grouped according to the chemical classes of those corresponding chemical structure annotations.

Data preprocessing, statistical analysis, as well as forecast modeling were conducted using the R software (4.2.1). The relative abundance of each metabolite was determined. After normalizing the raw abundances of metabolites for all individual samples using RMA, their relative abundances were calculated and used for subsequent analysis. Using analysis of variance, we chose metabolites with a trimmed p-value of <0.05 as markedly altered. Built upon our results, metabolites with fold changes of <1.5 and >0.7 between the younger and older groups were screened out. Multiple comparisons were corrected using the Benjamini-Hochberg false discovery rate (FDR, $q < 0.05$) to control false positives, in addition to fold change (>1.5 or <0.7) criteria. KEGG pathway enrichment analysis was conducted using MetaboAnalyst 5.0, mapping annotated metabolites to KEGG pathways. The hypergeometric test was applied to identify enriched pathways, with significance set at a false discovery rate (FDR) < 0.05 to correct for multiple testing.

Metagenomic Analysis of Stool Samples

Using the OMEGA Mag-Bind Soil DNA Kit (M5635-02; Omega Bio-Tek, Norcross, GA, USA), microbial genomic DNA was recovered from each sample. To create metagenome shotgun sequencing libraries with insert sizes of around 400 bp, the collected microbial DNA was treated. Primers were cut using the cutadapt plugin, and then raw sequence data were demultiplexed using the demux plugin. The sequences were then quality-filtered, denoised, and merged as well as the chimera was removed via the DADA2 plugin and other data processing. The Illumina NovaSeq platform (Illumina, USA) was used to sequence each library using the PE150 technique. Mass-filtered readings were obtained by processing raw sequencing data. Mass-filtered metagenomic sequencing reads were categorized based on the GTDB-derived database. Reads from each sample were assembled into contigs (longer than 300 bp) and clustered based on a sequence identity threshold of 0.95, with 90% coverage of the shorter contigs, using mseqs2. Non-redundant contigs were compared against the National Center for Biotechnology Information (NCBI)-nt database using mmseqs2 to obtain the lowest common ancestor taxonomy and gene abundance. Linear discriminant analysis (LDA) effect size (LEfSe) was leveraged to test for differentially abundant taxa as well as functions across groups. Beta diversity analysis was employed

to test for variations in the composition as well as function profiles of microbial communities between the groups using the Bray–Curtis distance metrics.

Spearman Correlation Analysis

Spearman correlation analysis was performed to evaluate the correlations among the SRS scores, CBCL social competence scores, CBCL behavioral problem scores, gut microflora, and the 1,995 annotated metabolites. Statistical significance was set at a p-value of <0.05. Correlation coefficients (r) < 0 suggested a negative connection, whereas r > 0 showed a positive one.

Results

SRS and CBCL Scores of the Older and Younger Groups

This study recruited 15 children with FXS aged 3–8 years as the younger group and 17 children aged 8–18 years as the older group. The baseline demographic and clinical characteristics of the enrolled children with FXS are displayed in Table 1.

SRS was completed by the parents or primary caregivers of children with FXS. The SRS results were used to estimate social acceptability and the degree of social interaction disorder. Total SRS scores ranged from 0 to 195, with higher scores suggesting a higher degree of social interaction disorder. The SRS results were divided into four categories: a score of ≤60 was considered normal, 60–75 indicated mild difficulty, 75–90 indicated moderate difficulty; and ≥90 indicated severe difficulty. The SRS results for children with FXS are presented in Figure 1A. No significant remarkable variations were found between the two groups about social acceptability or mild difficulty in social interaction. The Achenbach CBCL was also provided to parents or primary caregivers to estimate the degree of attention deficits, behavioral disorders, and emotional instability in children's daily lives. CBCL consists of two areas including social competence and behavioral problems. Higher social competence scores indicated better social competence, whereas scores below 30 were considered indicative of social interaction disorder. The behavioral problems section of the CBCL was built upon a four-point scale (never, occasionally, often, and always), with scoring based on their performance in each program. The higher the score, the more severe the child's problem with the program. The CBCL results for children with FXS are shown in Figure 1B. No significant remarkable variations were found between the two groups, regardless of social competence or behavioral problems. In addition, both groups revealed social disability, attention deficits, varying irritability, and behavioral or emotional problems. Taken together, these findings indicate that as children with FXS get older, their social abilities and behavioral disorders show limited

Table 1 Baseline Demographic and Clinical Characteristics of Patients with FXS

Characteristics	Younger (n=15)	Older (n=17)	p-value
Male, No. (%)	15 (100%)	16 (94.12%)	1
CBCL score, mean (SD)[a]			
Social competence	7.80 (3.68)	8.59 (4.17)	0.579
Behavioral problems	46.8 (25.26)	49.94 (17.26)	0.681
SRS score, mean (SD)[b]	67.73 (25.09)	68.47 (16.55)	0.922
FMR1 gene mutation types, mean (SD)[c]			
Full mutation	11 (73.3%)	15 (88.2%)	0.533
Premutation	1 (6.7%)	1 (5.9%)	1
Mosaicism	3 (20.0%)	1 (5.9%)	0.503

Notes: [a]The CBCL scale is extensively employed for assessing behavioral and emotional problems in children as well as adolescents, and it is mainly divided into general items, social competence, and behavioral problems. The higher the score, the more serious the child's behavioral problems are. [b]The SRS is leveraged to assess the social interaction abilities of children and adolescents, and it is split into 5 dimensions: social awareness, social cognition, social communication, social motivation, as well as autistic behavior patterns. The higher the total score of the scale, the more serious the individual's social impairments are. [c]The gene mutations in FXS mainly include the following three types: premutation: the number of CGG trinucleotide repeats is between 55 and 200; full mutation, the number of CGG repeats exceeds 200; and mosaicism: different numbers of CGG repeats exist in an individual's cells, which may include both premutation and full mutation.

Abbreviations: CBCL, Child Behavior Checklist; FMR1, fragile X messenger ribonucleoprotein 1; FXS, fragile X syndrome; SD, standard deviation; SRS, Social Responsiveness Scale.

improvement compared to their condition at the age of 8 years. However, evaluating the findings of these scales has subjective errors due to the differences in the educational attainment of parents or primary caregivers, who may have answered the questions inaccurately.

Serum Untargeted Metabolomic Profiling

To study the connection between the serum metabolome and FXS in children of different ages, we performed untargeted metabolomic detection in the serum samples of all children enrolled in this cohort. Among the metabolites that could be reliably detected (abundance > 50,000 in QC samples, CV < 30% in QC samples), 1,352 were found to be significantly altered between the younger and older groups (Figure 2A). The distribution of all samples in the principal component analysis plot based on these altered metabolites revealed that the younger group could be clearly distinguished from the older one (Figure 2B). Of the 1,352 metabolite features, 99 were annotated, and the relative abundances of these metabolites in the cohort are presented in Figure 2C. Analysis of these 99 significantly altered metabolites (FDR < 0.05) revealed that the differences in the older group compared with the younger group primarily involved phospholipids, steroids, and peptides (Figure 2D). To further characterize the biological implications of these altered serum metabolomes, we performed the Kyoto Encyclopedia of Genes as well as Genomes pathway enrichment analysis via metabolites with specific compound annotations. We observed an enrichment of the steroid hormone biosynthesis pathway in upregulated metabolites in the older group. The metabolites involved in this pathway, including dehydroepiandrosterone sulfate, cortisol, 21-deoxycortisol, 3beta-hydroxypregn-5-en-20-one sulfate, and etiocholanolone glucuronide, were also consistently upregulated in the older group (Figure 2E).

Intestinal Metagenomic Analysis

Using colonic fecal samples from children in both groups, we performed 16S rDNA amplicon sequencing to assess the variations in intestinal flora structure between the two groups. Operational taxonomic units (OTUs) as well as abundance data tables were created by merging the acquired sequences at 100% sequence similarity. In total, 16,112 OTUs were identified, with 1,328 OTUs overlapping between the younger and older groups (Figure 3A). The number of OTUs specific to the older group was slightly higher than that of the younger group, suggesting a richer microbiota in the older group. Rarefaction curves were generated for the samples based on species-associated OTUs and sequence numbers. As shown in Figure 3B, a flat trend indicated that the sampling amount was appropriate. This flattening of the curve illustrates how sequencing depth affects the observed samples' diversity and species richness. The diversity in the present sample is better reflected by the sequencing findings when the rarefaction curve is flatter. To further evaluate the richness and diversity of the microbial community between these two groups, α-diversity indices including Chao1, observed species, Shannon, Simpson, Faith's PD, Pielou's evenness, as well as Good's coverage were employed. No significant variations were observed between the groups (Figure 3C). β-diversity was analyzed using the Bray–Curtis metrics to assess changes in microbial community structure between samples. The unweighted pair-group method with arithmetic means was applied to assess the similarity between samples in the form of hierarchical trees (Figure 3D). This research observed higher β-diversity in the gut flora of the older group (Bray-Curtis distance: 0.68 ± 0.05 vs younger: 0.59 ± 0.04, $p = 0.017$, Figure 3E), indicating that the community structure was more heterogeneous in the older group than in the younger one. Additionally, the gut microbial composition in fecal samples was analyzed by selecting the top 10 species in terms of abundance at the phylum and genus levels (Figures 3F and G). Firmicutes and Bacteroidetes were the predominant phyla. No significant difference was found in the abundance of microbes at the phylum level between the two age groups. The microbiota were predominantly composed of Firmicutes (overall mean, 56.01%), Bacteroidetes (32.72%), Actinobacteria (6.45%), and Proteobacteria (4.30%). At the genus level, the relative abundance of *Roseburia* as well as *Prevotella* increased in the older group, whereas in the younger one, the abundance of *Bifidobacterium* increased. The LEfSe method was used to identify gut microorganisms remarkably different between the two groups, and the results revealed that c_Verrucomicrobiae, *g_Akkermansia*, o_Verrucomicrobiales, p_Verrucomicrobia, *f_Verrucomicrobiaceae*, and *g_Veillonella* were the most abundant in the younger group (LDA score (log10) > 2.5). However, the older group was mainly characterized by a higher abundance of *f_S24_7, g_Megamonas, g_Collinsella*, and *f_Micrococcaceae* (LDA score (log10) > 2.5) (Figure 3H and I). The findings revealed remarkable variations in the microbiota between the two groups.

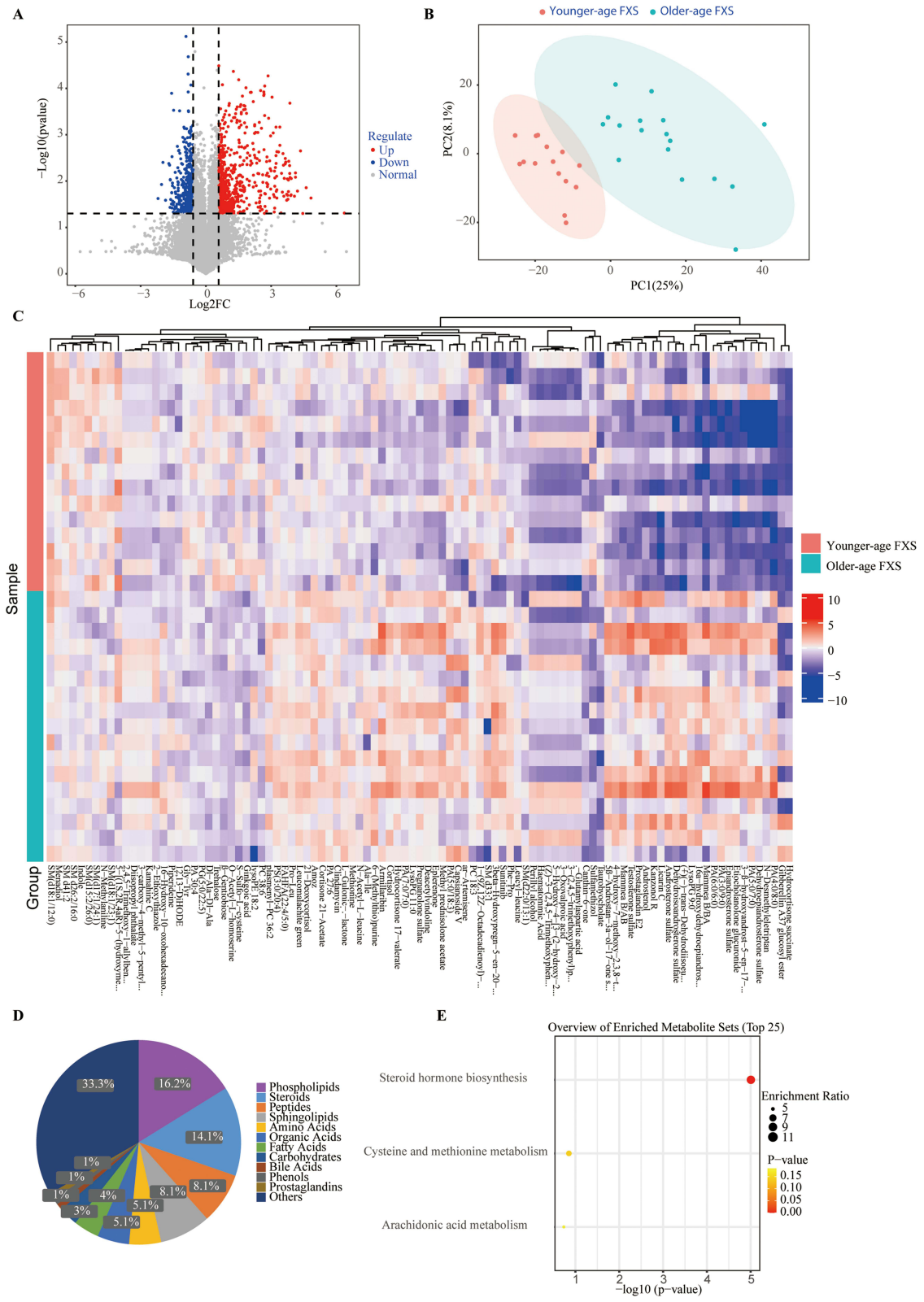

Figure 2 Serum metabolomics analysis. (**A**) RPLC-MS analysis of metabolites that showed significantly altered abundances between the different pairs of the population (A vs B, $p<0.05$, fold change >1.5 or <0.7). (**B**) PCA plot based on all altered metabolites. (**C**) Heatmap of annotated metabolites in differential metabolites. (**D**) Pie graph of the class composition according to the number of altered metabolites. (**E**) Bubble chart of the KEGG pathway enrichment analysis of metabolites with specific compound annotation.

Abbreviations: RPLC-MS, reversed-phase liquid chromatography-mass spectrometry; PCA, principal component analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes.

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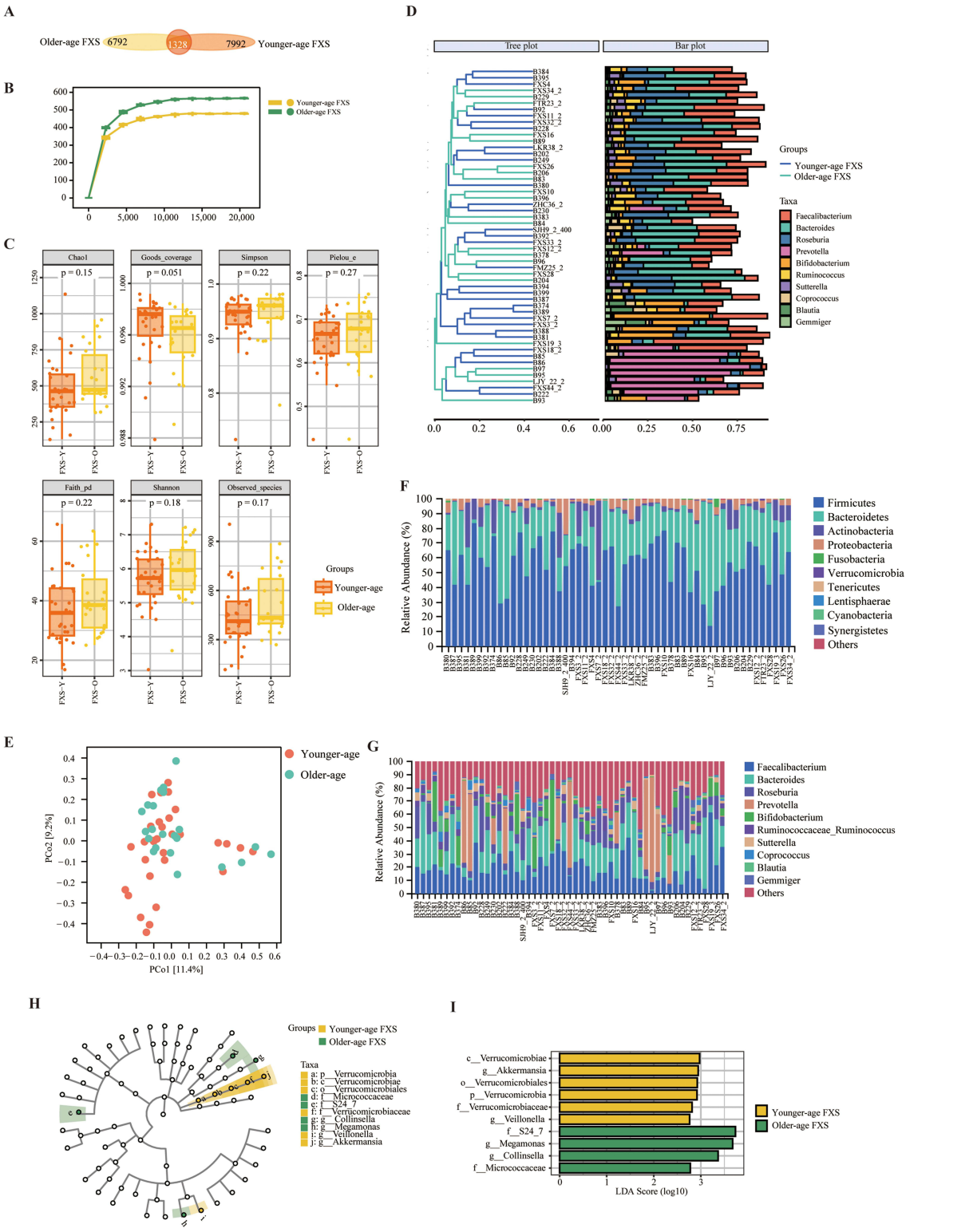

Figure 3 Microbiome analysis. (**A**) OTU Wayne diagrams based on its presence or absence across samples (groups) to count the membership of each set separately (**B**) The rarefaction curve of random sequences per sample and their corresponding number of observed species. (**C**) Characterized by Chao1, Observed species index, Shannon, Simpson index, Faith's PD index, Pielou's evenness index, Good's coverage index Coverage-estimated α-Diversity indices. (**D**) UPGMA cluster analysis of 53 samples from 32 FXS children at the genus level. (**E**) Principal coordinates analysis (PCoA) constructed on the basis of Bray-Curtis metrics between the two groups. (**F**) Stacked histograms of species composition at the Phyla Level. (**G**) Stacked histograms of species composition at the genus level. (**H**) Taxonomic branching map of species based on LEfSe analysis. (**I**) Histogram of the distribution of LDA values for significantly different species.

Abbreviations: OTU, operational taxonomic unit; UPGMA, unweighted pair-group method with arithmetic means; LEfSe, linear discriminant analysis effect size; LDA, linear discriminant analysis.

Correlations Between Serum Metabolites and Scales

Spearman correlation analysis was conducted to assess the connection of the scale scores with the 99 signature annotated metabolites (Figure 4A). Among the serum metabolites, β-gentiobiose and SM (d15:2/26:0), were negatively correlated with the SRS scores, suggesting that variations in the levels of these metabolites may be associated with the decline in social interaction abilities (Figure 4B and C). Piperideine, 3-carboxy-4-methyl-5-pentyl-2-furanpropanoic acid, and 12,13-DiHODE, were positively correlated with the social competence scores in the CBCL, indicating their possible roles in the development and manifestation of children's social competence. SM d41:2 was negatively correlated with the social competence scores in the CBCL, indicating that it plays a certain inhibitory or ameliorative role in the development and manifestation of children's social competence (Figure 4D–G). Pro-Leu, androstenol, dehydroisoandrosterone sulfate, PA (6:0/6:0), dehydroepiandrosterone sulfate, and leucyl-leucine, were positively correlated with the behavioral problem scores in the CBCL, indicating their potential roles in promoting social disability, attention deficit, and varying irritability (Figure 4H–M).

Correlations Between Gut Microbiota and Serum Metabolites

To study the potential correlations of microbiome composition with serum metabolome, Spearman correlation analysis was employed to study the connection between the two datasets. Forty-seven annotated metabolites correlated with four genera in the gut flora of children with FXS (Figure 5A). Enrichment of the members of the *Megamonas* genus in fecal samples from children in the older age was positively correlated with six serum metabolites that increased with age, including PA (4:0/8:0), epiandrosterone sulfate, cortisol, etiocholanolone glucuronide, PA (5:0/18:3), and SM d33:1. Enrichment of the members of the *Bifidobacterium* genus in fecal samples from children in the younger group was negatively correlated with four serum metabolites whose levels decreased with age, including PG (5:0/22:5), SM (d15:2/26:0), PC 38:6, and indole. Additionally, enrichment of the members of the *Veillonella* genus was positively correlated with five serum metabolites, with the most significant correlations found between the *Veillonella* genus and 16α-hydroxydehydroepiandrosterone 3-sulfate. Only indole negatively correlated with the *Prevotella* genus. Analysis of these metabolites in relation to the intestinal flora revealed that the shift in the older one compared with in the younger group predominantly involved phospholipids, amino acids, and sphingolipids (Figure 5B).

Discussion

FMR1 protein expression in children with FXS has been associated with intelligence level and the degree of social impairment; however, the metabolic mechanisms underlying this phenomenon are still poorly understood. In this study, we comprehensively analyzed the gut microbiome as well as host metabolites in older and younger groups of children with FXS. At the phylum level, we found no marked differences in the abundance of gut microbial composition between children with FXS in different age groups. The gut microbiota mainly consisted of the phyla Firmicutes, Bacteroidetes, Actinobacteria, as well as Ascomycetes. At the genus level, the relative abundance of *Collinsella* and *Megamonas* was higher in the older group, whereas the relative abundance of *Veillonella* and *Akkermansia* was higher in the younger group. Furthermore, we analyzed the metabolites in conjunction with the SRS and CBCL scale scores and found that two metabolites were negatively correlated with the SRS scores; three and one metabolites were positively and negatively correlated with the social competence scores in the CBCL, respectively; and six metabolites were positively correlated with the behavioral problem scores in the CBCL. In addition, we identified metabolic pathways that were causally related to the SRS and CBCL scale scores, providing an important and reliable basis for subsequent studies.

The gut microecosystem is the most important and complicated microecosystem in the human body. The development of different neurodevelopmental disorders, such ASD, attention deficit hyperactivity disorder, and Rett's syndrome, is closely associated with disturbances in the gut microbiota.[11] As FXS is categorized as a rare disease, its incidence is relatively low. From a traditional perspective, the disease is mainly caused by genetic factors, leading to the relative rarity of studies on FXS, both domestically and internationally. Moreover, existing studies have primarily focused on genetic aspects, whereas the effects of intestinal flora on the central nervous system have not yet been thoroughly investigated. Currently, some progress has been made in studying the gut microbiota in an animal model of FXS (Fmr1 KO mice). In a previous research, 16s rRNA

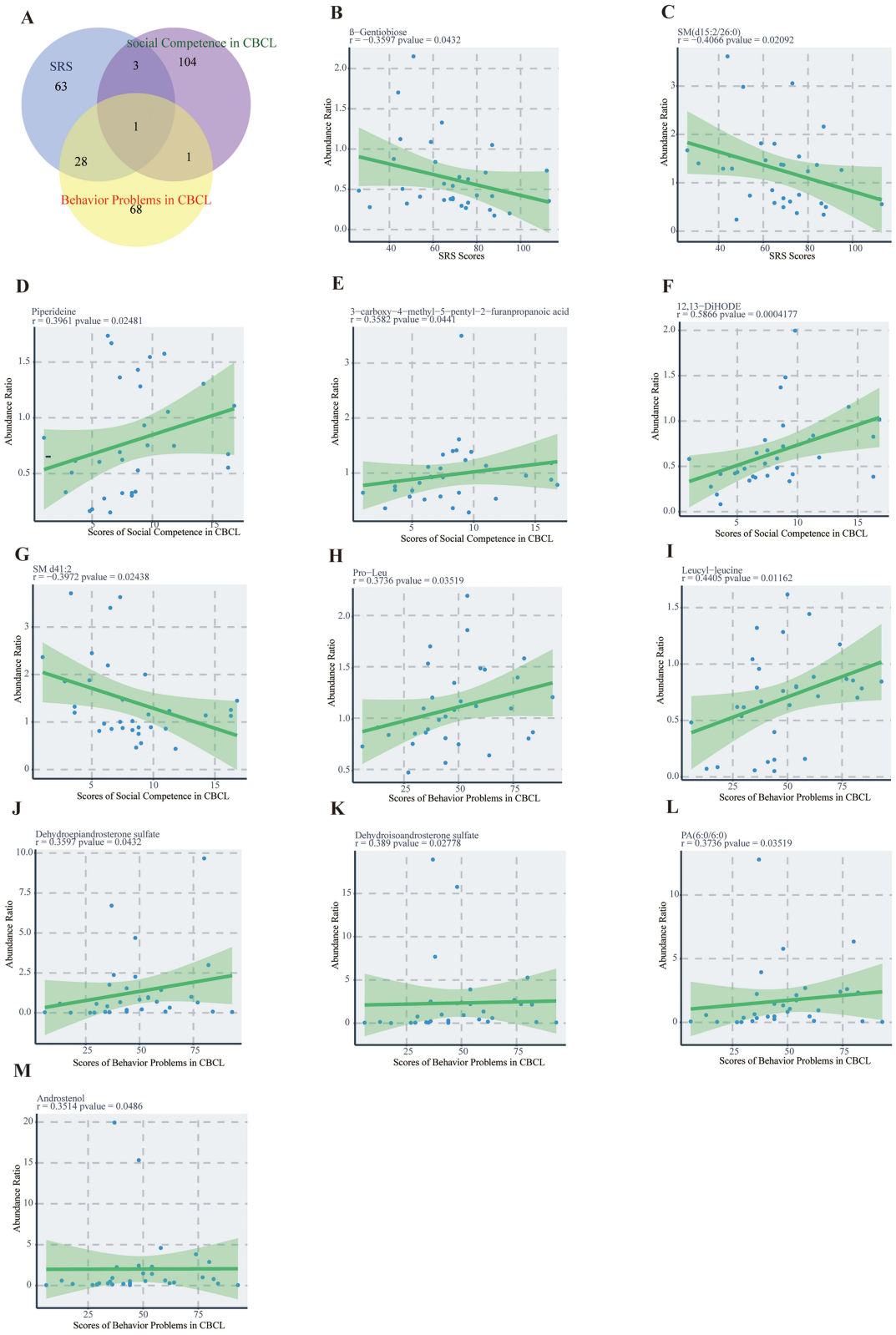

Figure 4 Correlations between serum metabolites and scales. (**A**) The core-pan graph of the number of metabolites correlated with the SRS and CBCL scores. (**B** and **C**) Metabolites among the 99 annotated metabolites that negatively correlated with the SRS scores. (**D-G**) Metabolites among the 99 annotated metabolites that correlated with the social competence scores in the CBCL. (**H-M**) Metabolites among the 99 annotated metabolites that positively correlated with the behavioral problem scores in the CBCL.
Abbreviations: CBCL, Child Behavior Checklist; SRS, Social Responsiveness Scale.

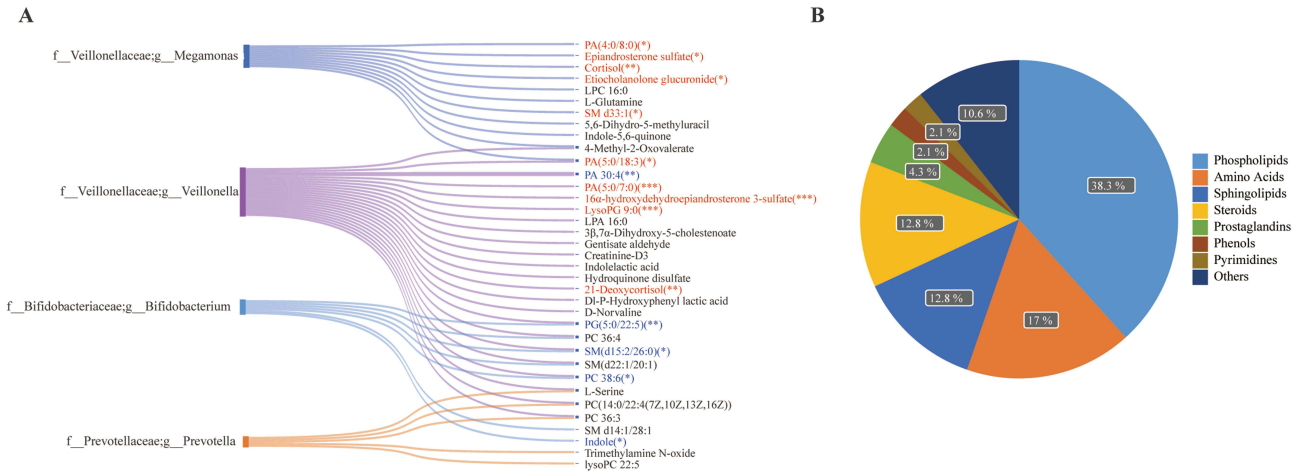

Figure 5 Correlations between gut microbiota and serum metabolites. (**A**) Spearman correlation coefficients between genera (*Megamonas, Veillonella, Bifidobacterium, Prevotella*) and serum metabolites. Red nodes represent upregulated metabolites, and blue nodes represent downregulated metabolites (fold change > 1.5 or < 0.7, FDR < 0.05). Line width reflects correlation strength, and correlation significance is denoted by asterisks: * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$ (all FDR-corrected for multiple testing). (**B**) Pie graph of the class composition according to the number of altered metabolites.

sequencing was performed on the fecal samples of Fmr1 KO mice, revealing the following average relative abundances of the most represented phyla are Bacteroidetes (54.23%) and Firmicutes (36.49%).[16] The gut flora of mice as well as patients with FXS revealed a high degree of similarity, suggesting the feasibility of using the Fmr1 KO mouse model to study the gut flora in FXS. Notably, while age-associated gut microbiota β-diversity in the general population typically exhibits stability, the older FXS group showed a significant increase ($p = 0.017$), indicating abnormal community heterogeneity associated with FXS. Additionally, enrichment of the steroid hormone biosynthesis pathway in older FXS patients, unreported in normal development, highlights disease-specific metabolic remodeling.

Other research has shown that dietary fish oil, an important supplemental source of omega-3 polyunsaturated fatty acids, can have a remarkable role in modulating the gut microbiota in a mouse model of FXS. This modulation effectively improves autistic behaviors and promotes the return of gut homeostasis to normal in mice. One study confirmed that the intestinal flora of Fmr1 KO mice differed significantly from that of normal mice in composition, both at the phylum and genus levels, with a significantly lower percentage of Verrucomicrobiota in Fmr1 KO mice than in normal mice.[17] Different characteristics of the bacterial flora that are closely related to inflammation and synthesis of short-chain fatty acids were found between Fmr1 KO and normal mice. However, no differences were found in microbial abundance and diversity indices. The absence of FMRP expression was shown to interfere with the normal homeostasis of the intestine. In contrast, following fecal transplantation from dietary fish oil-fed Fmr1 KO mice into recipient Fmr1 KO mice, the recipient mice showed improved cognitive, social, and anxiety behaviors; decreased TNF-α mRNA expression in their colon; increased mRNA as well as protein levels of TJP3; decreased serum lipopolysaccharide levels; as well as increased abundance of *Akkermansia* and *Gordonibacter*. These findings indicate that dietary fish oil can elevate gut homeostasis as well as autistic behaviors by modulating the gut microbiota.[17] This study inspires enthusiasm and confidence in the search for colony-based therapies to alleviate FXS and provides direction for future research and practice.

To the best of our knowledge, this study is among the first to conduct an in-depth analysis of the intestinal flora and metabolic profiles of children with FXS using fecal and serum samples from clinical patients. Herein, we collected samples from 32 children with FXS, a rare disease, to investigate the characteristics of the gut flora in the older and younger groups and explored the metabolites and metabolic pathways that are causally related to children's social skills, behavioral problems, and emotional management. These findings provide a basis for the accurate establishment of disease prognostic models and the search for therapeutic targets, providing a direction for subsequent in-depth studies. Overall, the elevated abundance of *Akkermansia* in younger FXS children corroborates previous findings of significantly increased *Akkermansia* levels in FXS mouse models and aligns with its established roles in gut-barrier maintenance and immune modulation.[15,16] In our study, this genus was negatively correlated with serum metabolites like indole and SM (d15:2/26:0), suggesting a potential early-life

interaction between *Akkermansia* and metabolic pathways that may influence neurodevelopment via the microbiota-gut-brain axis, warranting further investigation into its therapeutic potential. We found that upregulated metabolites in the serum of patients in the older group revealed significant enrichment in the steroid hormone biosynthetic pathway. Further investigation revealed that metabolites involved in this pathway, such as dehydroepiandrosterone sulfate, cortisol, 21-deoxycortisol, 3beta-hydroxypregn-5-en-20-one sulfate, and etiocholanolone glucuronide, were upregulated in the older group. As shown in Figure 2E, the steroid hormone biosynthesis pathway was significantly enriched in metabolites positively correlated with age. Correlation analysis (Figure 4) further demonstrated associations between these pathway metabolites and SRS/CBCL scores, providing a basis for their role in FXS clinical outcomes. This indicates that steroid hormone biosynthesis abnormalities may occur in patients with FXS during adolescence. Steroid hormones participate in the normal development as well as function of the nervous system through mechanisms such as modulation of neurotransmitter systems, rapid modulation of neuronal excitability as well as function, and brain plasticity as well as behavior.[18,19] In this study, higher levels of dehydroisoandrosterone sulfate were positively associated with improved behavioral problems in children with FXS. Previously, both dehydroisoandrosterone sulfate and its unsulfated form—classified as neurosteroids—have been recognized to be synthesized in the brain and to exert a range of neuroprotective and neuromodulatory effects. These neurosteroids can modulate neuronal excitability, plasticity, and survival, although the precise mechanisms remain to be elucidated.[20] Four previous large cohort studies on vitamin D, a neurosteroid hormone involved in neurodevelopment, each with more than 300 individuals diagnosed with ASD, revealed that the median serum 25-hydroxyvitamin D3 levels in neonatal dried blood samples were remarkably lower in newborns later diagnosed with ASD than in those without ASD.[21,22] Recent serum metabolomics studies have identified 3beta-hydroxypregn-5-en-20-one sulfate as a contributor to cognitive impairment induced by chronic sleep deprivation, highlighting its potential impact on neurological function. This finding aligns with our results, suggesting that further investigation into the role of 3beta-hydroxypregn-5-en-20-one sulfate in the cognitive deficits of FXS may uncover a promising target for elucidating the pathogenesis and developing effective therapies for FXS.[23] Additionally, elevated serum steroid-related biomarkers in pregnant women, such as estradiol and sex hormone-binding globulin, have been associated with a higher risk of autism in the offspring.[24] Future studies should explore whether these hormones play a role in the pathogenesis or clinical severity of FXS and examine their specific mechanisms of action.

This study has some limitations. First, the CBCL and SRS scores are highly subjective and may be influenced by the cultural background of the participants who completed the assessments, which affects the objectivity of the results in reflecting the severity of the disease in children. And a notable limitation of this study is the absence of a healthy control group, which means we cannot definitively distinguish FXS-specific changes from age-related physiological variations in the general population, such as typical hormonal shifts during adolescence. Future studies incorporating age-matched healthy controls will be critical to validate whether the observed metabolic and microbiota differences are disease-specific or reflective of normal developmental trajectories. However, the enrichment of the steroid hormone biosynthesis pathway in older FXS patients—unreported in normative pediatric cohorts—suggests a potential FXS-specific dysregulation, warranting further investigation. Additionally, there are no in-depth studies on the effectiveness of colony-based interventional therapy in improving intellectual disability and social impairment in patients with FXS. Further, this study did not incorporate CGG repeat number or mutation type (premutation/mosaicism) as variables due to limited clinical data availability. It is necessary for future genotype-stratified analyses to explore how repeat expansion influences metabolic and microbiota profiles. Finally, the potential metabolic pathways identified in this study need further validation through controlled experiments using animal models. The absence of a healthy control group limits conclusions about disease specificity. Future studies with controls are warranted. Due to the rare nature of FXS and small sample size, we could not analyze differences by FMR1 mutation type (full mutation, premutation, mosaicism). Future studies with larger cohorts will explore genotype-specific metabolic/microbiota profiles.

The gut microbiota composition stabilizes with age. In children and adolescents older than 8 years, the microbial profile increasingly resembles that of adults, with Bifidobacterium remaining a dominant genus.[25] In children younger than 8 years, our previous work has shown that, compared with ASD, levels of Bacteroidetes and Bifidobacterium are elevated in typically developing children.[26] A serum metabolomics study of healthy children under four years of age found that the differential metabolites in these children were predominantly involved in the eukaryotic ribosome

pathway.[27] These findings differ from ours, indicating that our observed alterations are more likely FXS-specific rather than reflections of normal childhood development.

In conclusion, this study demonstrated that the serum levels of metabolites, particularly gut microbe-associated metabolites, showed significant changes in children with FXS of advanced age. These findings suggest a potential metabolic basis for FXS and serve as a foundation for the further identification of biomarkers that could provide a foundation for identifying age-associated biomarkers and therapeutic targets in FXS children. These age-associated metabolic and microbiota differences also lay the groundwork for developing precision interventions targeting steroid hormone biosynthesis and gut-brain axis signaling in FXS.

Data Sharing Statement

The raw XC-MS data are available via MetaboLights with identifier MTBLS12206, while the raw 16S rDNA sequence data were deposited in the NCBI Sequence Read Archive under the accession number PRJNA1208259.

Ethics Statement

The studies involving human participants were reviewed and approved by Ethics Committee for Biomedical Research, First Hospital of Peking University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. This study complies with the Declaration of Helsinki.

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Disclosure

The authors declare that they have no conflicts of interest in this work.

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