ORIGINAL ARTICLE

Psychiatry and Clinical Psychopharmacology 2025;35(1):6-13
DOI:10.5152/pcp.2025.24966

The Impact of Valproic Acid on Microbiota in a Mouse Model of Autism Spectrum Disorder

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ABSTRACT

Background: Autism spectrum disorder (ASD) is a complex neuropsychiatric condition with a multifactorial etiology, involving both genetic predisposition and environmental factors. Valproic acid (VPA), a commonly used antiepileptic drug, has been shown to induce ASD-like behaviors in rodent models, making it a valuable tool for studying the pathophysiology of ASD. This study aims to explore the effects of VPA on behavior and the microbiota in a mouse model of ASD.

Methods: C57BL/6 mice were used in this study, with pregnant females receiving a single intraperitoneal injection of VPA (450 mg/kg) or a saline solution on gestational day E12.5. Behavioral assessments, including the Three-Chamber Social Test, Elevated Plus Maze, Marble Burying Test, Open Field Test, and Light-Dark Box Test, were conducted on 8-week-old mice. Oral and fecal samples were collected for microbiota analysis, and gene expression profiling was performed on brain samples.

Results: VPA-treated mice exhibited significant deficits in social interaction, anxiety-like behaviors, and repetitive actions. Microbiota analysis revealed significant shifts in the composition of both oral and fecal microbial communities in VPA-treated mice, with reductions in alpha diversity and changes in the relative abundance of specific taxa. Gene set variation analysis of mice harboring VPA-induced microbiota identified notable discrepancies in metabolic pathways, suggesting that the dysbiosis may modulate the expression of genes involved in critical metabolic processes.

Conclusion: The present study provides evidence that VPA exposure during early development can induce ASD-like behaviors in mice, along with significant changes in the composition of the microbiota. These findings underscore the complex interplay between environmental factors, such as VPA, and the microbiota in the pathophysiology of ASD. The study lays the groundwork for future research aimed at developing targeted interventions to mitigate the symptoms of ASD and other neuropsychiatric disorders, potentially through modulating the microbiota-gut-brain axis.

ARTICLE HISTORY

Received: August 29, 2024
Revision Requested: October 10, 2024
Last Revision Received: November 11, 2024
Accepted: November 12, 2024
Publication Date: March 17, 2025

INTRODUCTION

The intricate relationship between environmental factors, microbial communities, and behavioral outcomes has emerged as a pivotal area of interest in the field of neuropsychiatric research.[1,2] Autism spectrum disorder (ASD), characterized by deficits in social interaction, heightened anxiety, and repetitive behaviors, presents a complex and multifaceted challenge for which the underlying mechanisms remain partially elusive.[3] Valproic Acid (VPA), a widely used antiepileptic drug, has been recognized for its potential to induce behavioral alterations reminiscent of ASD when administered during early development.[4] This has positioned VPA as a valuable tool for exploring the pathophysiology of ASD and the role of environmental exposures in its onset and progression.

The microbial ecosystem, both within the oral cavity and the gastrointestinal tract, represents a dynamic and complex environment that has been increasingly recognized for its role in modulating behavior and mental health.[5] The shifts in microbial composition observed in VPA-treated mice, including reductions in alpha diversity and changes in the relative abundance of specific taxa, suggest that environmental factors such as VPA may exert a profound influence on the microbiota, potentially contributing to the behavioral manifestations of ASD.[6,7] Moreover, the transcriptional profiling of mice harboring VPA-induced microbiota reveals disrupted metabolic pathways and altered gene expression patterns, highlighting the intricate connections between the microbiome and host physiology.[8,9] These findings underscore the potential for microbial dysbiosis to modulate not only behavior but also the underlying neural and molecular mechanisms that drive these behaviors. This study is a comprehensive

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Cite this article as: Li B, Xiong Y, Li Y. The impact of valproic acid on microbiota in a mouse model of autism spectrum disorder. *Psychiatry Clin Psychopharmacol.* 2025;35(1):6-13.

investigation into the impact of VPA on the behavioral characteristics and microbial composition of mice. By adopting a multifaceted approach that integrates behavioral assessments, microbial analysis, and gene expression profiling, the aim was to uncover the intricate interplay between these variables and their collective influence on the manifestation of ASD-like behaviors.

Autism spectrum disorder is a heterogeneous disorder, and its etiology is likely multifactorial, involving a combination of genetic predisposition and environmental factors.[10] The VPA mouse model offers a unique opportunity to study the impact of an environmental exposure on the development of ASD-like behaviors and to dissect the underlying biological mechanisms.[11,12] The findings from this study may not only enhance the understanding of the role of the microbiome in ASD but also provide critical insights into the plasticity of the brain and the potential for interventions that target the microbiota-gut-brain axis.

This study represents a significant step forward in the understanding of the complex relationships between environmental factors, the microbiome, and neuropsychiatric disorders. By unraveling the effects of VPA on behavior and the microbiome, the groundwork for future research that may lead to the development of targeted interventions to mitigate the symptoms of ASD and other related conditions is laid. The potential for microbial-based therapies to modulate behavior and improve mental health outcomes is an exciting area of exploration that could revolutionize the way neuropsychiatric disorders are approached.

MATERIAL AND METHODS

Animals

C57BL/6 mice were acquired from Shanghai SLAC Laboratory Animal Co., Ltd. The animals were housed under specific pathogen-free conditions (12-hour light/darkness cycle) and had access to standard chow and water and allowed to feed ad libitum. As previously described, pregnant females received a single intraperitoneal injection of VPA (450 mg/kg) or a saline solution (0.9% NaCl) on gestational day E12.5.[13,14] saliva and fecal samples were collected from the animals at 6 weeks of age, and Three-Chamber Social Test (TCST), Elevated Plus Maze (EPM), Marble Burying Test (MBT), Open Field Test (OFT), and Light-Dark Box Test (LDBT) were conducted at 8 weeks of age. The collection of experimental data is illustrated in Figure 1A. All experimental procedures were carried out in accordance with the ARRIVE 2.0 guidelines for preclinical animal research. The study was approved by the Ethics Committee of Tongji University Affiliated Dental Hospital (Approval Number:[2024]-SR-43).

MAIN POINTS

- Valproic acid (VPA) induced ASD-like behaviors in a mouse model, offering insights into the pathophysiology of ASD.
- Valproic acid exposure led to significant alterations in the oral and fecal microbiota of mice, indicating a potential role of the microbiome in ASD.
- Mice with VPA-induced microbiota exhibited transcriptional differences, particularly in metabolic pathways, suggesting dysbiosis may impact gene expression.
- This study highlights the intricate interplay between environmental factors like VPA, the microbiota, and behavior, providing new insights into ASD pathophysiology.
- The findings suggest potential targets for microbiota-based interventions to mitigate ASD symptoms and other neuropsychiatric disorders.

Behavioral Assessment

Three-Chamber Social Test: Social interactions were carried out as outlined in previous studies.[15] Briefly, the mice underwent an acclimation phase lasting 10 minutes within an unoccupied 60 × 40 × 23 cm Plexiglas enclosure, which was segmented into 3 contiguous compartments (left, central, right). The animals' sociability was assessed during a subsequent 10-minute interval, during which they had the option to engage with an unoccupied wire basket or one that housed an unfamiliar conspecific that was genetically, chronologically, and sexually identical to the subject (referred to as Mouse 1). The duration of interaction was quantified by observing the amount of time the test mouse devoted to sniffing or climbing on either the vacant basket or the one with the unfamiliar mouse. The placement of the unoccupied basket or the unfamiliar mouse in either the left or right compartment during the sociability phase was alternated between trials to prevent any positional prejudice. The preference for social novelty was tested in an additional 10-minute session by introducing a second unfamiliar mouse (denoted as Mouse 2) into the space previously occupied by the empty wire basket. The time spent interacting with the empty basket, Mouse 1, or Mouse 2 was documented and analyzed utilizing the automated AnyMaze system by trained, unbiased observers. The human observers remained unaware of the treatment assignments.

Elevated Plus Maze: The Elevated Plus Maze (EPM) assessment was carried out following a previously established protocol with minor adjustments.[16] In brief, the apparatus consists of 2 open arms (each measuring 30 cm in length and 6 cm in width), 2 enclosed arms (30 cm in length, 6 cm in width, and 15 cm in height), and a central square (6 cm × 6 cm) made of plexiglass. Each subject was positioned at the center of the maze, oriented towards one of the open arms. The number of arm entries and the duration of time spent in each arm were recorded over a 5-minute period. To remove the scent of the previously tested mouse, the maze was meticulously sanitized with a tissue moistened with 70% alcohol.

Marble Burying Test: The animal was introduced into a cage that had been lined with 4-cm-thick fresh bedding and was given 10 minutes to acclimate. Subsequently, 20

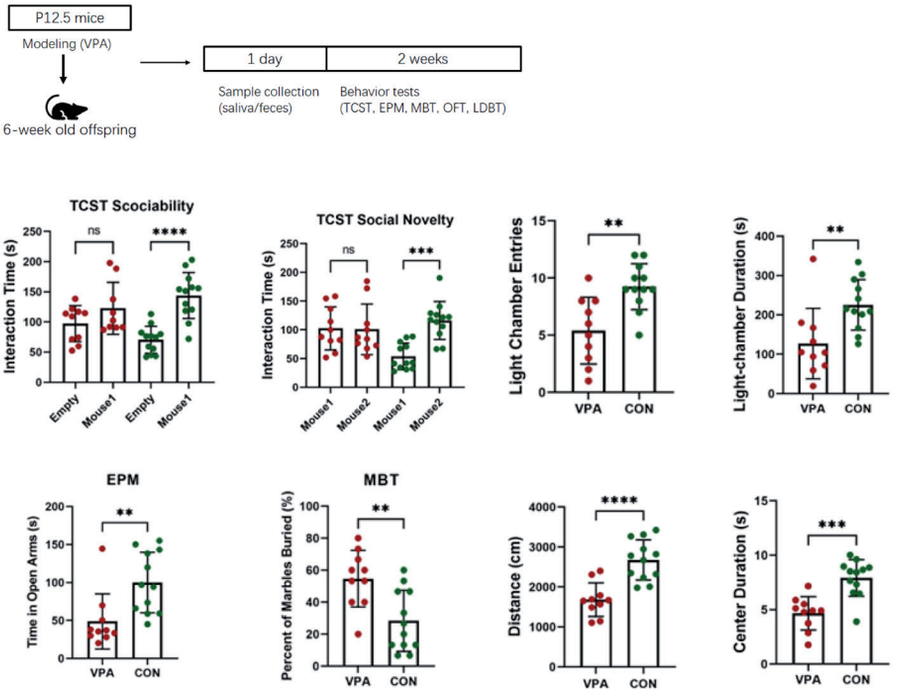

Figure 1. The valproic acid (VPA) treatment on mouse behavior. (A) Overview of the Experimental Layout. TCST represents the Three-Chamber Social Test, EPM refers to the Elevated Plus Maze; MBT stands for the Marble Burying Test, OFT is the Open Field Test, and LDBT denotes the Light-Dark Box Test. The results of the behavioral assessments are depicted: (B and C) TCST, (D) EPM, (E) MBT, (F, G) LDBT, and (H-J) OFT. *Indicates $P < .05$. **Represents $P < .01$. ***Denotes $P < .001$. ****Indicates $P < .0001$.

black marbles were positioned within the cage in a 4 ri5 grid, spaced evenly apart, with the mouse temporarily excluded from the area. Following this, the mouse was reintroduced to the cage for a 10-minute trial. The quantity of marbles that were buried was tallied by a pair of observers who were unaware of the experimental details. A marble was deemed buried if over half of it was obscured by the bedding material. Post-test, the marbles were sanitized using a 70% ethanol solution.

Open Field Test: The rodents were introduced into an open field (dimensions 40 × 40 × 20 cm) and given the opportunity to explore unhindered for a duration of 10 minutes, with their movements tracked in real-time via the AnyMaze tracking application. Parameters such as the distance covered, velocity, and duration of stay within the central region of the arena were captured and calculated automatically throughout the experiment. The central zone was designated as the inner area measuring 20 × 20 cm.

Light-Dark Box Test: Gently place the mouse within the dark sector of the light-dark apparatus, enabling it to roam unhindered, and activate the software's video stream for monitoring the mouse's behavior. Document and evaluate the number of times the mouse enters the light chamber and the length of time it remains there over a 10-minute period.

Sample Collection

Each mouse was sequestered in a sterile, autoclaved cage and permitted to defecate without restraint. Oral specimens were acquired by swabbing the inside of the mouth for a duration of 30 seconds using sterile cotton swabs. Fecal matter was gathered as it was produced. All collected samples were promptly preserved at a temperature of −80°C.

Microbial Analysis

Deoxyribonucleic acid was isolated from both oral and fecal specimens using the OMEGA Soil DNA Kit (manufactured by Omega Bio-Tek), adhering to the provided protocol. The V3-V4 segment of the bacterial 16S rRNA gene sequence was amplified via polymerase chain reaction (PCR), utilizing the primer set 338F (ACTCCTACGGGAGGCAGCA′) and 806R (CGGACTACHVGGGTWTCTAAT). Following PCR amplification, the resulting amplicons were separated, purified, and measured before undergoing paired-end sequencing (2 × 250 bp) on the Illumina NovaSeq 6000 sequencer. The raw sequence data were deconvoluted using the demux plugin, and primers were excised with the cutadapt plugin. The sequences were then subjected to quality filtering, noise reduction, merging, and chimera elimination through the DADA2 plugin. Non-singleton amplicon sequence variants were aligned using mafft and utilized to generate a phylogenetic tree with fasttree2. Taxonomic classification of the amplicon sequence variants was performed using the classify-sklearn naive Bayes classifier within the feature-classifier plugin.

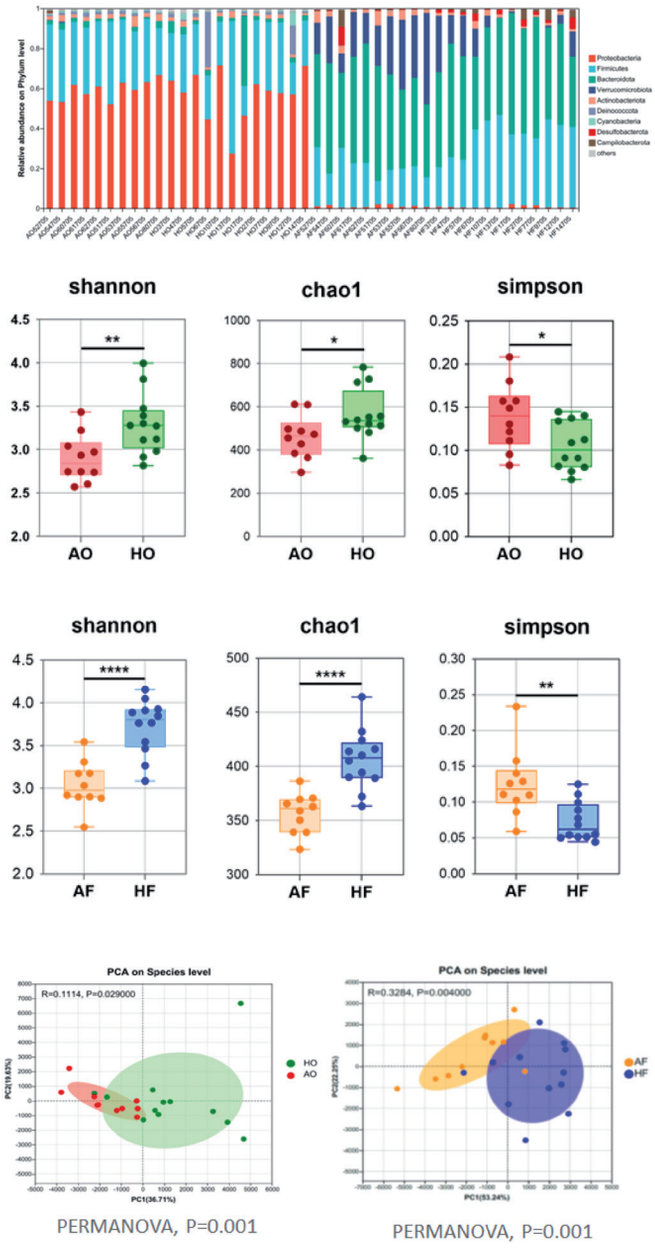

Figure 2. Analysis of Microbial Communities in Donor and Recipient Mice. (A) Graphical Representation of Taxonomic Distribution. The community structure of each sample from the donors and recipient mice is illustrated at the phylum level, showcasing the top 10 phyla. Each taxonomic group is represented by a unique color. (B) Assessment of Bacterial Richness Using the Shannon, Chao1 Index, and Simpson. Significant variations between groups were evaluated using the Welch t-test. *Indicates $P < .05$. ***Indicates $P < .001$. ****Indicates $P < .0001$. (C) Principal Component Analysis (PCA) Scatter Plots.

RNA Sequencing

Total RNA was isolated from brain samples individually using the RNeasy Mini Kit, following the manufacturer's guidelines. Complementary DNA libraries were prepared with the TruSeq RNA Sample Preparation Kit (Illumina). These libraries were then subjected to sequencing on the Illumina NovaSeq 6000 platform. The raw sequenced reads were processed to remove adapter sequences, unknown bases (N), and low-quality reads. The high-quality clean reads were subsequently assembled into contigs, transcripts, and unigenes using the Trinity program. The assembled unigenes were annotated using BLAST, with an E-value threshold of < 0.00001. Ultimately, both the 16S rRNA gene sequencing and transcriptome sequencing were carried out at Shanghai Personal Biotechnology Co., Ltd.

Statistical Analyses

The analysis of microbial sequence data was primarily carried out using QIIME2 and R software packages (version 3.2.0). Alpha-diversity metrics, including Chao1 and Shannon indices, were determined based on the amplicon sequence variant profiles. A principal component analysis (PCA) was performed based on the compositional profiles at the species level. The permutation-based multivariate analysis of variance (PERMANOVA) was employed to evaluate the significance of the differences in microbiota structure across different groups. The ANCOM statistical framework,[17] designed for the analysis of the composition of microbiomes, was utilized to pinpoint differentially abundant taxa among groups. The ANCOM generated the W value, which represents the count of subhypotheses tested.[18]

Regarding transcriptome sequencing, the expression levels of unigenes were compared across groups using FPKM (fragments per kilobase per million mapped reads).[19] DESeq software was applied to identify differentially expressed genes (DEGs). Gene set variation analysis (GSVA) was conducted to detect differential pathways among groups. The GSVA scores were computed using a nonparametric method, involving a Kolmogorov–Smirnov-like random walk statistic. All presented data are reported as the mean values ± SEM.

RESULTS

VPA-Induced Deficits in Social Interaction and Anxiety-Like Behaviors, Along with Repetitive Actions, Were Observed in Mice

In TCST testing, the VPA-exposed cohort failed to exhibit a preference for the unfamiliar mouse (mouse 1) over an empty container, suggesting a disruption in social behavior following VPA administration (Figure 1B). Moreover, the control subjects typically demonstrated a robust inclination towards novel social interactions, investing a significantly greater duration in engagement with a new mouse (mouse 2) compared to a familiar one (mouse 1), as shown in Figure 3C. Notably, the VPA-treated rodents allocated a reduced amount of time in the EPM relative to the controls, with the control group's data replicated for comparison (Figure 1D). Consequently, the

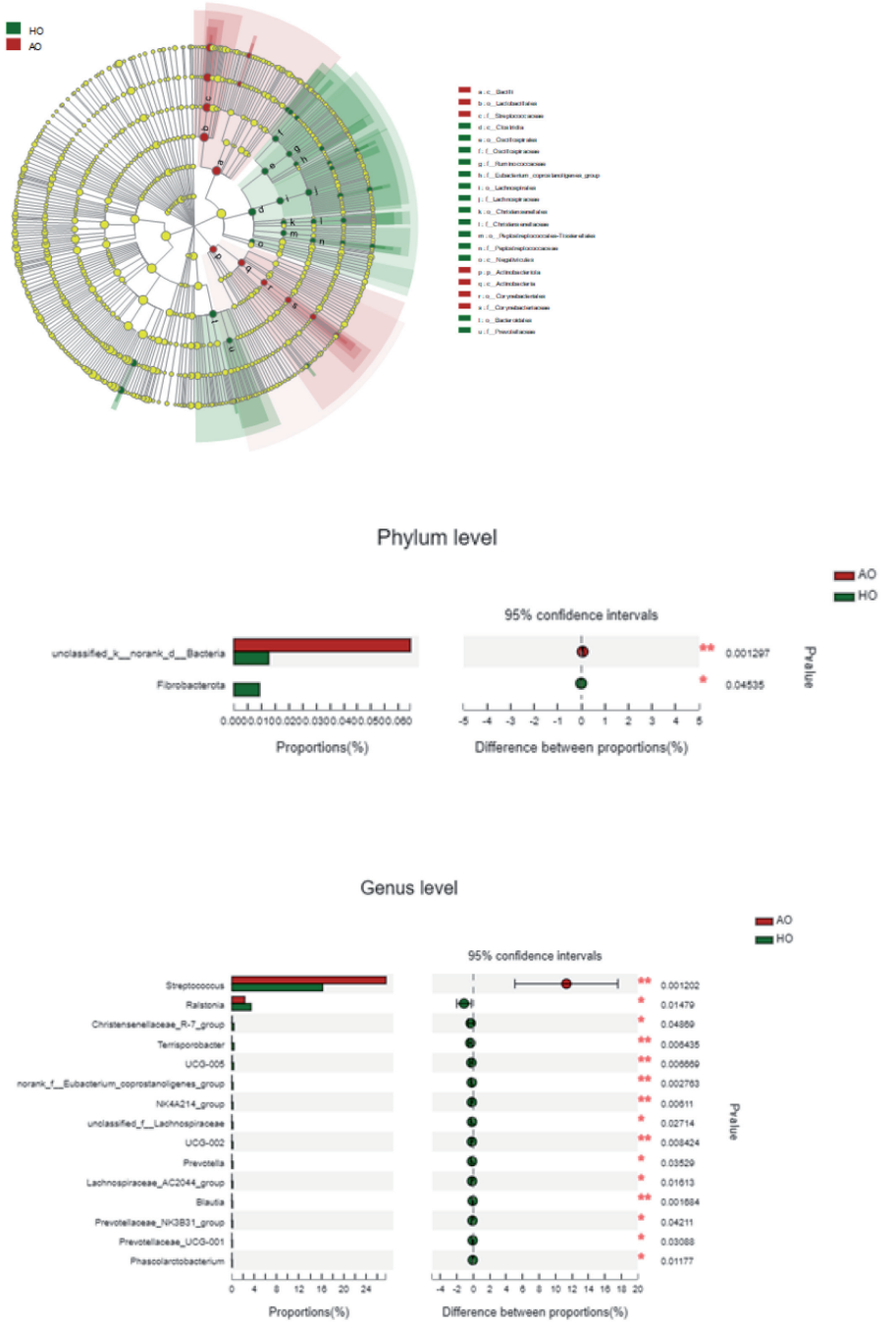

Figure 3. Examination of Microbial Disparities Across Mouse Cohorts of Oral Samples. Distinctive (A) cladogram, (B) phyla and (C) genera were identified in the oral samples from control and VPA group mice.

VPA-treated animals demonstrated heightened anxious-like responses. Regarding the MBT, the VPA group buried a disproportionately high number of marbles—a metric indicative of repetitive behavior—compared to the control subjects (Figure 1E). In the LGBT task, the VPA mice made fewer entries and spent less time in the light compartment than their control counterparts (Figure 1F and G). Analysis of the OFT revealed discrepancies in the total path lengths covered, with the VPA group showing fewer entries into and less time spent in the center area compared to controls (Figure 1H and I). This pattern suggests a dampened exploratory ethos among VPA-treated animals.

Investigation of VPA-Treated Mice Revealed Significant Shifts in the Oral and Fecal Microbial Ecosystems

To assess whether VPA administration leads to modifications in the microbial constellations within the oral cavity and gastrointestinal tract of mice, compared to their controls, 16S rRNA gene sequencing on oral and fecal samples was conducted (Figures 2A and B). Analysis of alpha diversity,

as indicated by the Shannon, Chao1, and Simpson indices, revealed a reduction in the HO group in both oral and fecal contexts when contrasted with the VPA and control cohorts. Principal Component Analysis further highlighted distinct separation between VPA and control groups, implying varying profiles of microbial populations (Figure 2C).

To pinpoint the specific taxa driving these discrepancies, ANCOM analysis was employed on taxa with a mean relative abundance exceeding 1% and an FDR threshold of < 0.05. In oral samples, a notable increase in the abundance of unclassified_k__norank_d__Bacteria within the ASD ORAL SAMPLE group at the phylum level was detected (Figures 3A and B). Moreover, at the genus level, Streptococcus demonstrated higher abundance in the ASD ORAL SAMPLE group (Figure 3C). Examining fecal samples, the principal discriminative phyla were Firmicutes, Verrucomicrobiota, and Actinobacteriota (Figures 4A and B). At the genus level, Akkermansia was significantly enriched in the ASD FECAL SAMPLE group (Figure 4C).

Mice Harboring ASD Microbiota Exhibit Unique Transcriptional Profiles

Analysis of gene set variation revealed notable discrepancies in metabolic pathways, secondary metabolite biosynthesis, amino acid synthesis, and microbial metabolism in various environments, including carbon metabolism, were identified in the ORAL SAMPLE group, as illustrated in Figure 5A. In the FECAL SAMPLE group, significant GO pathways encompassed metabolic processes, the synthesis of secondary metabolites, microbial metabolism across different environments, ABC transporter activities, and amino acid biosynthesis (Figure 5B).

DISCUSSION

The present study delves into the impact of VPA on the behavioral characteristics and microbial composition of mice. Notably, significant behavioral alterations, including impairments in social interaction, heightened anxiety-like behaviors, and repetitive actions in VPA-treated mice, were observed. These findings corroborate previous studies on the behavioral effects of VPA and underscore the potential role of this compound in elucidating the pathophysiology of ASD.[20]

In the TCST, VPA-treated mice displayed a diminished preference for social novelty, indicating a disruption in social behavior. This is consistent with previous findings suggesting that VPA exposure during early development may perturb social behavior, a hallmark symptom of ASD.[21] Furthermore, the VPA group exhibited reduced exploration and fewer entries into the central area of the OFT, indicative of decreased exploratory behavior. These observations suggest that VPA may exert a regulatory influence on neural circuits mediating both social and exploratory behaviors. The EPM and LDBT provided further

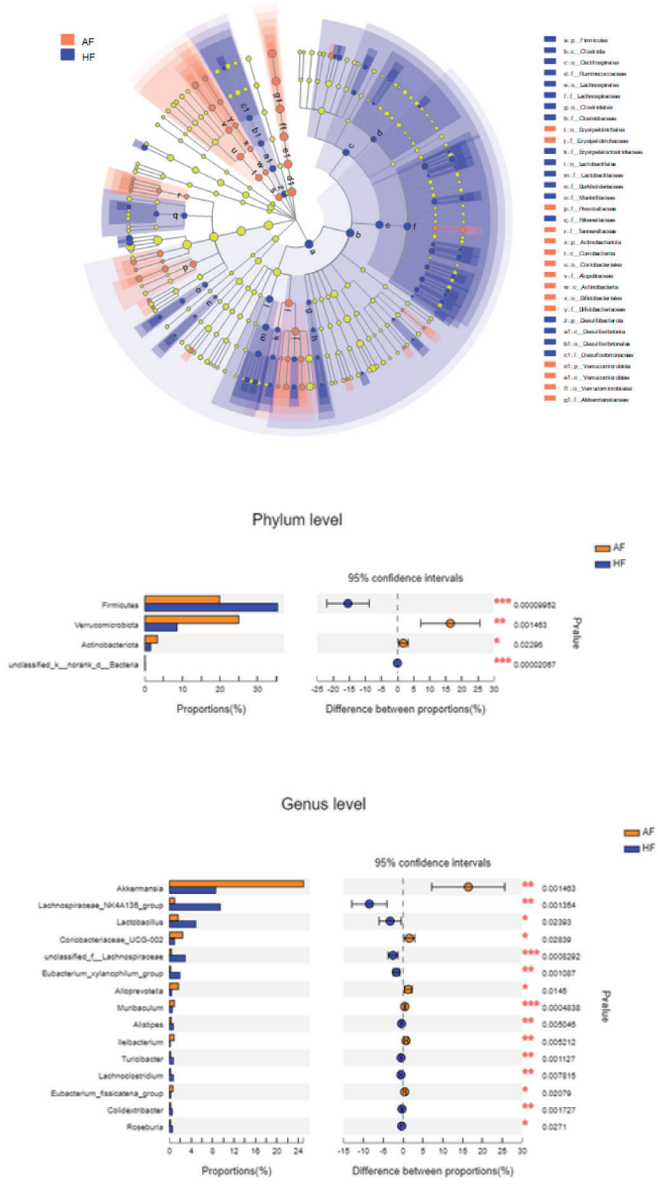

Figure 4. Examination of Microbial Disparities Across Mouse Cohorts of Fecal Samples. Distinctive (A) cladogram, (B) phyla and (C) genera were identified in the fecal samples from control and VPA group mice.

evidence of anxiety-like behaviors in VPA-treated mice. These mice exhibited a decreased duration of time spent in open arms and fewer entries into the light compartment, respectively, compared to control mice. Such behaviors are reminiscent of those observed in individuals with ASD and anxiety disorders,[22,23] thus highlighting the potential utility of VPA as a model to study these co-occurring conditions.

The investigation of the oral and fecal microbial communities of VPA-treated mice revealed significant shifts in their composition. A reduction in alpha diversity, as indicated by the Shannon, Chao1, and Simpson indices, suggests that VPA perturbed the balance of microbial populations in both the oral cavity and the gut, which

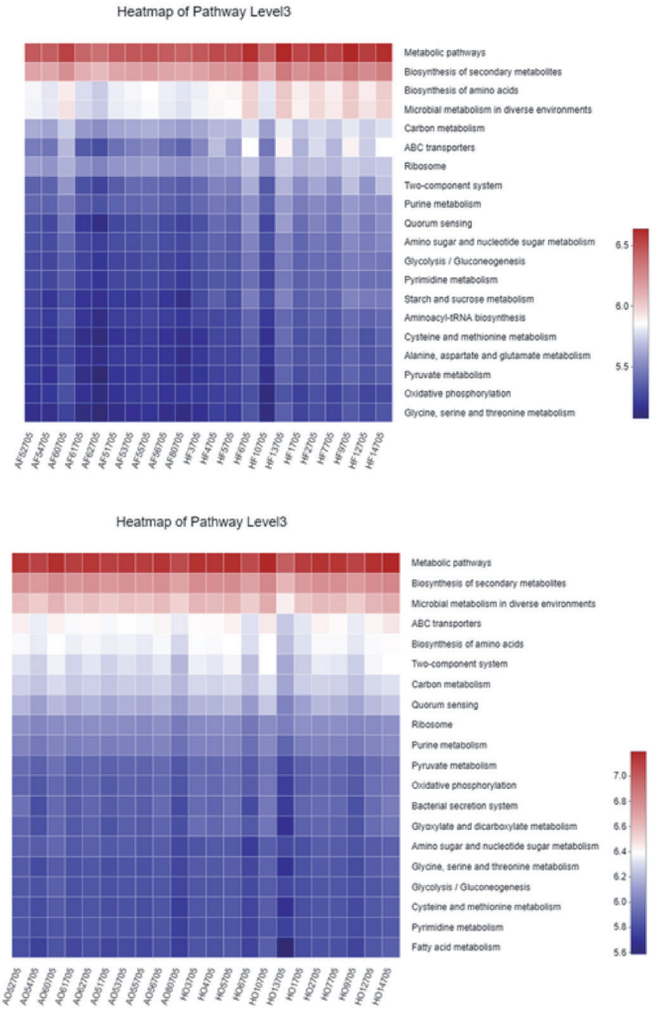

Figure 5. The heat map derived from gene set variation analysis reveals significantly different pathways between control and VPA mice of (A) oral and (B) fecal samples.

is consistent with previous studies.[24,25] Moreover, these findings point to a distinct microbial signature in VPA-treated mice, with taxa such as unclassified_k__norank_d__Bacteria and Streptococcus showing altered abundance in oral samples, while Firmicutes, Verrucomicrobiota, and Actinobacteriota, along with Akkermansia, were discriminative in fecal samples. These changes may have profound implications for host health, given the intricate interplay between the gut microbiota and the central nervous system. Interestingly, the gene set variation analysis identified significant pathway differences in the transcriptional profiles of mice harboring VPA-induced microbiota. Alterations in metabolic pathways, secondary metabolite biosynthesis, amino acid synthesis, and microbial metabolism in various environments were observed. These findings suggest that VPA-induced dysbiosis may modulate the expression of genes involved in critical metabolic processes and potentially influence behaviors associated with ASD.[26]

The present study has several limitations that merit consideration. First, the sample size was relatively small, which may have limited our ability to detect subtle differences in some behavioral and microbiota measures. Second, the study was conducted in mice, and the relevance of the findings to humans, particularly in the context of ASD, requires cautious interpretation. Third, while controlling for several variables, including litter size and maternal care, the influence of confounding factors cannot be completely ruled out. Fourth, the analysis focused on the molecular and microbiota aspects of the VPA model; future studies should also consider the neural circuitry and neurochemistry affected by VPA.

In conclusion, these findings underscore the complex interplay between environmental factors, such as VPA exposure, and the microbiota on behavior. The present study provides novel insights into the pathophysiology of ASD and suggests potential avenues for therapeutic interventions. Future research is warranted to further elucidate the mechanisms by which the microbiota modulate behavior and to explore the clinical relevance of these findings in ASD and other neuropsychiatric disorders.

Data Availability Statement: The authors confirm that the data supporting the findings of this study are available within the article.

Ethics Committee Approval: This study was approved by the Ethics Committee of Stomatological Hospital and Dental School, Tongji University (Approval Number: [2024]-SR-43; Date: February 10, 2024).

Informed Consent: N/A.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – B.S.L., Y.M.L.; Design – B.S.L., Y.M.L.; Supervision – Y.T.X.; Resources – Y.T.X.; Materials –Y.T.X.; Data Collection and/or Processing –B.S.L.; Analysis and/or Interpretation – B.S.L., Y.M.L.; Literature Search – Y.T.X.; Writing – B.S.L., Y.T.X.; Critical Review – Y.M.L.

Declaration of Interests: The authors have no conflict of interest to declare.

Funding: The authors declared that this study has received no financial support.

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