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Research Article

A Randomized, Single-blind, Parallel-controlled Clinical Trial of Puze Biological V9 Probiotic as an Adjunct Therapy for Children Aged 2-7 Years with Autism Spectrum Disorder

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Abstract

Objective: To evaluate the effects of Puze Biological V9 Probiotic (Bifidobacterium animalis subsp. lactis V9) as an adjunctive intervention on core symptoms, gastrointestinal symptoms, and gut microbiota in children aged 2-7 years with Autism Spectrum Disorder (ASD). *Methods:* A randomized, single-blind, parallel-controlled design was adopted. Sixty children with ASD (aged 2-7 years) who met the DSM-5 diagnostic criteria were randomly assigned in a 1:1 ratio to two groups: the control group (n=30) received conventional rehabilitation therapy (primarily Applied Behavior Analysis, ABA); the experimental group (n=30) received conventional rehabilitation therapy plus daily oral administration of Puze Biological V9 probiotic lyophilized powder (viable count ≥1×10^10 CFU/day). The intervention period was 3 months. The primary outcome measure was the change in total score on the Childhood Autism Rating Scale (CARS). Secondary outcome measures included scores on the Gastrointestinal Symptom Rating Scale (GSRS), the Social Responsiveness Scale (SRS), the Autism Behavior Checklist (ABC), fecal microbiota metagenomic analysis, and safety indicators. *Results:* After 3 months of intervention, the decrease in the total CARS score in the experimental group (12.3 ± 3.5 points) was significantly greater than that in the control group (8.1 ± 2.9 points) ($P<0.01$). The improvement in the total GSRS score and its sub-scores (abdominal pain, bloating, constipation, etc.) in the experimental group was also significantly superior to that in the control group ($P<0.01$). Improvements in SRS and ABC scale scores were also significant in the experimental group ($P<0.05$). Metagenomic analysis showed a significant increase in the abundance of beneficial bacteria such as Bifidobacterium and Lactobacillus, and a decrease in the abundance of potential harmful bacteria such as Clostridium in the gut of children in the experimental group. There was no statistically significant difference in the incidence of adverse events between the two groups. *Conclusion:* Based on conventional rehabilitation therapy, adjunctive use of Puze Biological V9 probiotic for 3 months can safely and effectively improve core behavioral symptoms and concomitant gastrointestinal dysfunction in children aged 2-7 years with ASD. Its effect may be related to regulating the structure of gut microbiota and optimizing the function of the "gut-brain axis". This study provides clinical evidence for the application of the domestic probiotic strain V9 in the adjunctive treatment of ASD.

Keywords

Autism Spectrum Disorder, Probiotics, Bifidobacterium Animalis Subsp, Lactis V9, Gut-Brain Axis, Randomized Controlled Trial, Children, Gut Microbiota

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1. Introduction

Autism Spectrum Disorder (ASD) is a complex neurodevelopmental disorder characterized by core features of social communication impairment, narrow interests, and repetitive stereotyped behaviors, often accompanied by sensory abnormalities, emotional problems, and gastrointestinal dysfunction [1]. The prevalence of ASD is increasing worldwide, with the prevalence among children in China being approximately 1% [2]. Currently, the etiology of ASD is not fully understood, and there is a lack of specific drug treatments. Comprehensive therapy based on educational rehabilitation and behavioral interventions (such as Applied Behavior Analysis, ABA) is the core current approach, but its efficacy is limited and exhibits significant individual differences [2].

In recent years, extensive research has revealed the important role of the bidirectional communication system between the gut microbiota and brain function—the "Gut-Brain Axis"—in the pathogenesis of ASD [3]. Children with ASD commonly exhibit gut microbiota dysbiosis, characterized by reduced microbial diversity, decreased beneficial bacteria such as Bifidobacterium and Lactobacillus, and increased potential harmful bacteria such as Clostridium [4]. This microbial disturbance is closely related to the severity of gastrointestinal symptoms (such as constipation, diarrhea, abdominal pain) and may exacerbate or induce neurobehavioral abnormalities through pathways affecting immune regulation, neurotransmitter metabolism (e.g., serotonin, γ-aminobutyric acid), short-chain fatty acid production, and intestinal barrier function [5]. This provides a new theoretical basis for intervening in ASD symptoms by modulating the gut microecology. Probiotics, as live microorganisms, can confer health benefits on the host when administered in adequate amounts through colonization, competition, and metabolites [6]. Several clinical studies have preliminarily confirmed that specific probiotic strains (e.g., Lactobacillus reuteri, Bifidobacterium animalis subsp. lactis Probio-M8, Bacteroides fragilis BF839) as adjunctive measures can improve social function, stereotyped behaviors, and gastrointestinal symptoms in children with ASD [7]. For example, a randomized double-blind trial in children with ASD aged 2-8 years found that Lactobacillus reuteri significantly improved scores in the social communication subdomain of the Social Responsiveness Scale (SRS) [8]. Another study showed that intervention with the domestically developed strain Bacteroides fragilis BF839 for 16 weeks significantly reduced ABC and CARS scores in children with ASD. Bifidobacterium animalis subsp. lactis Probio-M8 from Keto Biological combined with dietary intervention has also been confirmed to improve clinical symptoms and gastrointestinal health in ASD [9].

Puze Biological V9 Probiotic (Bifidobacterium animalis subsp. lactis V9) is a domestic star strain with independent intellectual property rights, isolated from the intestines of healthy Mongolian children [10]. This strain has excellent gastrointestinal tolerance, with a survival rate as high as 92.4% after 3 hours of digestion in artificial gastric juice at pH 2.0, and can tolerate 0.3% ox bile salts. Basic research confirms that the V9 strain has good functions in regulating gut microbiota balance, antagonizing pathogenic bacteria, and enhancing immunity, and has been safely used in drugs such as "Jin Shuang Qi" for over ten years [11]. Its fermentation broth is rich in organic acids, amino acids, and vitamins, possessing potential nutritional and metabolic regulatory value. However, there is currently a lack of clinical efficacy research on V9 probiotic for young children with ASD [12].

Based on the above background, we hypothesized that Puze Biological V9 probiotic can safely and effectively improve core symptoms and comorbid gastrointestinal problems in children with ASD by modulating the "gut-brain axis". Therefore, we designed and implemented this randomized, single-blind, parallel-controlled clinical trial to evaluate the efficacy and safety of V9 probiotic as an adjunct to conventional rehabilitation therapy in children aged 2-7 years with ASD, and to preliminarily explore its relationship with changes in gut microbiota.

2. Materials and Methods

2.1. Trial Design

This study was a single-center, randomized, single-blind, parallel-controlled clinical trial. The study protocol followed the principles of the Declaration of Helsinki and was approved by the Ethics Committee of the Inner Mongolia Autonomous Region Disabled Children's Rehabilitation Center. The trial was registered in the Chinese Clinical Trial Registry. The report follows the CONSORT 2025 statement.

2.2. Study Subjects

2.2.1. Source and Recruitment of Subjects

Children with ASD were recruited from the Autism Children's Rehabilitation Department of the Inner Mongolia Autonomous Region Disabled Children's Rehabilitation Center from October 2025 to February 2026.

2.2.2. Inclusion Criteria

(1) Aged 2-7 years; (2) Meeting the diagnostic criteria for ASD in the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5); (3) Childhood Autism Rating Scale (CARS) total score ≥30 points (moderate to severe autism); (4) No use of antibiotics, probiotics, or prebiotics within 1 month prior to enrollment; (5) Legal guardian signed written informed consent.

2.3. Exclusion Criteria

(1) Suffering from severe organic diseases (e.g., congenital heart disease, liver or kidney dysfunction), genetic metabolic diseases (e.g., phenylketonuria), immunodeficiency diseases, or neurological diseases (e.g., epilepsy); (2) Allergy to dairy products or any component of probiotics; (3) Participating in other clinical studies that may affect the results of this trial; (4) Guardian unable to cooperate with follow-up and assessments.

2.4. Randomization and Blinding

A computer-generated block randomization sequence (block size of 4) was completed by an independent statistician not involved in subject recruitment and assessment. Sixty eligible children were randomly assigned in a 1:1 ratio to the experimental or control group. The allocation scheme was concealed using sealed, opaque, sequentially numbered envelopes (SNOSE). Due to the different nature of the interventions (oral probiotic powder vs. no additional supplement), this study adopted a single-blind design, meaning outcome assessors and data analysts were blinded to group assignment. The children and their guardians were aware of the group assignment but were instructed not to disclose it to the assessors.

3. Interventions

3.1. Control Group

Received conventional structured rehabilitation training, mainly including Applied Behavior Analysis (ABA), Treatment and Education of Autistic and related Communication-handicapped Children (TEACCH), speech therapy, occupational therapy, etc. Training intensity was no less than 20 hours per week, lasting for 3 months. No probiotic or prebiotic products were taken during this period.

3.2. Experimental Group

On the basis of conventional rehabilitation training (same as the control group), took V9 probiotic lyophilized powder provided by Inner Mongolia Puze Biological Products Co., Ltd. orally after breakfast daily. Each sachet contained Bifidobacterium animalis subsp. lactis V9 with a viable count ≥1×10^10 CFU. One sachet per day was taken, dissolved in water or milk ≤35°C, continuously for 3 months.

Both groups of children maintained their original dietary habits during the study and were prohibited from taking other probiotics, prebiotics, or antibiotics (if antibiotics were necessary due to infection, it was required to be recorded and reported).

4. Assessment Indicators and Measurement Tools

All assessments were completed at baseline (before intervention, T0) and at the end of the 3-month intervention (T1) by uniformly trained professional assessors who were blinded to group assignment.

4.1. Primary Outcome Measure

Childhood Autism Rating Scale (CARS): Contains 15 items assessing interpersonal relationships, imitation, emotional response, body use, adaptation to environmental changes, visual response, auditory response, near receptor response, anxiety response, verbal communication, non-verbal communication, activity level, intellectual functioning, and general impression. Total score 15-60, ≥30 indicates autism, higher scores indicate more severe symptoms.

4.2. Secondary Outcome Measures

Gastrointestinal Symptom Rating Scale (GSRS): Contains 15 questions divided into 5 domains: diarrhea, constipation, abdominal pain, reflux, and indigestion, using a 7-point Likert scale (1=no symptoms, 7=most severe symptoms). Higher total score indicates more severe gastrointestinal symptoms.

Social Responsiveness Scale (SRS): Contains 65 items assessing 5 dimensions: social awareness, social cognition, social communication, social motivation, and autistic mannerisms. Total score 0-195, higher scores indicate more severe social impairment.

Autism Behavior Checklist (ABC): Contains 57 items divided into 5 factors: sensory, relating, body and object use, language, and social and self-help. Higher total score indicates more severe abnormal behaviors.

Gut Microbiota Analysis: At T0 and T1 time points, approximately 5g of fresh morning stool samples were collected from the children, placed in sterile fecal collection tubes, and immediately stored in a -80 ℃ ultra-low temperature freezer. All samples were sent for metagenomic sequencing to analyze the composition and diversity changes of gut microbiota.

Safety Assessment: All adverse events (such as diarrhea, abdominal pain, rash, fever, etc.) during the intervention period were recorded, and their causal relationship with the intervention was assessed.

5. Sample Size Calculation

Based on previous similar studies and pilot data, it was assumed that the CARS score in the experimental group would decrease by 4.0 points more than in the control group (standard deviation 5.0 points). Setting the significance level $\alpha=0.05$ (two-sided) and test power $1-\beta=0.80$. Using PASS 15.0 software for calculation, the required sample size per group was approximately 26 cases. Considering an approximately 15%

dropout rate, it was finally determined to include 30 cases per group, totaling 60 cases.

6. Statistical Analysis

SPSS 26.0 software was used for data analysis. Measurement data conforming to normal distribution are expressed as mean ± standard deviation ($\bar{x} \pm s$), otherwise expressed as median (interquartile range); count data are expressed as frequency (percentage). The primary analysis followed the intention-to-treat (ITT) principle. Baseline comparisons between groups used independent samples t-test or Mann-Whitney U test (measurement data) and chi-square test or Fisher's exact test (count data). The primary outcome measure (change in CARS total score) was analyzed using analysis of covariance (ANCOVA), with baseline score as a covariate. Secondary outcome measures were analyzed using repeated measures analysis of variance or non-parametric tests. Gut microbiota α-diversity (e.g., Shannon index, Chao1 index) was analyzed using t-test or Mann-Whitney U test, β-diversity was analyzed using principal coordinate analysis (PCoA) and PERMANOVA test. Differences in microbial species composition were analyzed using LEfSe analysis. All tests were two-sided, with $P<0.05$ considered statistically significant.

7. Ethics

This study was approved by the Ethics Committee of the Inner Mongolia Autonomous Region Disabled Children's Rehabilitation Center. All subjects' guardians signed informed consent forms. De-identified individual participant data will be made available to qualified researchers upon reasonable request after the study results are published. The authors declare that they have no competing interests.

Table 1. *Comparison of Baseline Characteristics of Study Subjects.*

Characteristic	Experimental Group (n=30)	Control Group (n=30)	Statistic	P-value
Demographic Data				
Age (years), $\bar{x} \pm s$	4.5 ± 1.2	4.7 ± 1.3	t = -0.65	0.518
Sex (Male/Female), n	24 / 6	22 / 8	$\chi^2 = 0.31$	0.577
Disease-Related Characteristics				
CARS Total Score, $\bar{x} \pm s$	42.3 ± 5.1	41.8 ± 4.9	t = 0.40	0.689
ABC Total Score, $\bar{x} \pm s$	78.5 ± 15.2	76.9 ± 14.6	t = 0.43	0.667
SRS Total Score, $\bar{x} \pm s$	125.4 ± 20.3	122.8 ± 19.7	t = 0.52	0.603
GSRS Total Score, $\bar{x} \pm s$	28.6 ± 6.8	27.9 ± 7.1	t = 0.39	0.696
Concomitant GI Symptoms, n (%)				
Constipation	18 (60.0%)	16 (53.3%)	$\chi^2 = 0.27$	0.600
Diarrhea	8 (26.7%)	10 (33.3%)	$\chi^2 = 0.34$	0.560
Abdominal Pain/Bloating	15 (50.0%)	13 (43.3%)	$\chi^2 = 0.27$	0.600

8. Results

8.1. Subject Flow and Compliance

During the study, 1 child in the experimental group was lost to follow-up due to family relocation, and 1 child in the control group withdrew due to inability to tolerate rehabilitation training. Ultimately, 58 children (29 in the experimental group, 29 in the control group) completed the 3-month intervention and all assessments and were included in the per-protocol (PP) analysis. The ITT analysis included all 60 randomized children. Intervention compliance was good in both groups, with probiotic intake compliance >95% in the experimental group.

8.2. Primary Outcome Measure: Change in CARS Score

After 3 months of intervention, the total CARS scores of children in both groups decreased significantly compared to baseline (all $P<0.01$). Analysis of covariance (with baseline CARS score as a covariate) showed that the decrease in the total CARS score in the experimental group (12.3 ± 3.5 points) was significantly greater than that in the control group (8.1 ±

2.9 points), with a statistically significant between-group difference (F=21.56, P<0.001). Subgroup analysis showed that the experimental group had significantly greater improvement than the control group in dimensions such as "Interpersonal Relationships", "Imitation", "Emotional Response", and "Verbal Communication" (all P<0.05).

Table 2. *Comparison of Changes in CARS and Subscale Scores Before and After Intervention in Both Groups of Children ($\bar{x} \pm s$).*

Assessment Item	Group	Baseline (T0)	Post-Intervention (T1)	Change Value (T1-T0)	Between-Group Difference P-value
CARS Total Score	Experimental	42.3 ±5.1	30.0 ±4.8*	-12.3 ±3.5	<0.001
	Control	41.8 ±4.9	33.7 ±5.2*	-8.1 ±2.9	
Interpersonal Relationships	Experimental	3.5 ±0.6	2.2 ±0.5*	-1.3 ±0.4	0.012
	Control	3.4 ±0.7	2.7 ±0.6*	-0.7 ±0.3	
Imitation	Experimental	3.2 ±0.8	2.0 ±0.7*	-1.2 ±0.5	0.008
	Control	3.1 ±0.7	2.4 ±0.6*	-0.7 ±0.4	
Emotional Response	Experimental	3.0 ±0.5	1.9 ±0.4*	-1.1 ±0.3	0.003
	Control	2.9 ±0.6	2.3 ±0.5*	-0.6 ±0.3	
Verbal Communication	Experimental	3.8 ±0.9	2.5 ±0.8*	-1.3 ±0.5	0.019
	Control	3.7 ±0.8	2.9 ±0.7*	-0.8 ±0.4	

Note: Compared with baseline in the same group, P < 0.01.

8.3. Secondary Outcome Measures

8.3.1. Gastrointestinal Symptoms (GSRS)

After intervention, the total GSRS scores of both groups decreased, but the improvement in the experimental group was more significant. The decrease in the total GSRS score in the experimental group (-10.2 ± 3.1 points) was significantly greater than that in the control group (-5.8 ± 2.7 points) (P<0.001). The experimental group also showed significantly better improvement than the control group in sub-items such as abdominal pain, bloating, and constipation (all P<0.01).

8.3.2. Social Function (SRS) and Behavior (ABC)

After intervention, the total SRS and ABC scores in the experimental group decreased significantly (P<0.01), and the magnitude of decrease was significantly greater than that in the control group (P<0.05). The experimental group also showed a better improvement trend in SRS sub-dimensions such as social communication and social motivation.

8.3.3. Gut Microbiota Changes

Metagenomic analysis showed that the α-diversity (Shannon index) of the gut microbiota in the experimental group children had an increasing trend after intervention compared to baseline, but did not reach statistical significance (P=0.052). β-diversity analysis (PCoA based on Bray-Curtis distance) showed that the microbiota structure of the experimental group after intervention was significantly separated from that at baseline and from the control group after intervention (PERMANOVA, P<0.05). LEfSe analysis found that the relative abundance of Bifidobacterium, Lactobacillus, and Akkermansia significantly increased after intervention in the experimental group, while the relative abundance of Clostridium and some members of Enterobacteriaceae significantly decreased (LDA score > 2.0, P<0.05).

Table 3. *Comparison of Changes in Secondary Outcome Indicator Scores Before and After Intervention in Both Groups of Children ($\bar{x} \pm s$).*

Assessment Scale	Group	Baseline (T0)	Post-Intervention (T1)	Change Value (T1-T0)	Between-Group Difference P-value
GSRS Total Score	Experimental	28.6 ±6.8	18.4 ±5.2*	-10.2 ±3.1	<0.001

Assessment Scale	Group	Baseline (T0)	Post-Intervention (T1)	Change Value (T1-T0)	Between-Group Difference P-value
SRS Total Score	Control	27.9 ±7.1	22.1 ±6.0*	-5.8 ±2.7	0.023
	Experimental	125.4 ±20.3	108.7 ±18.5*	-16.7 ±8.2	
ABC Total Score	Control	122.8 ±19.7	113.2 ±17.9*	-9.6 ±7.1	0.035
	Experimental	78.5 ±15.2	65.3 ±13.8*	-13.2 ±6.5	
	Control	76.9 ±14.6	69.1 ±12.9*	-7.8 ±5.7	

Note: Compared with baseline in the same group, P < 0.01.

Table 4. Changes in Relative Abundance of Major Gut Microbiota at Genus Level in Children of Both Groups After Intervention (Top 10).

Genus	Experimental Group (Baseline)	Experimental Group (Post-Intervention)	Control Group (Baseline)	Control Group (Post-Intervention)	Group-Time Interaction P-value
Bacteroides	25.3%	26.1%	24.8%	25.5%	0.712
Prevotella	18.7%	19.5%	19.2%	18.9%	0.845
Bifidobacterium	5.2%	9.8%*	5.0%	5.5%	0.002
Faecalibacterium	7.1%	8.3%	7.4%	7.6%	0.154
Lactobacillus	1.5%	3.2%*	1.6%	1.8%	0.008
Ruminococcus	4.3%	4.8%	4.5%	4.4%	0.332
Akkermansia	0.8%	2.1%*	0.9%	1.0%	0.015
Clostridium	6.5%	3.9%*	6.3%	6.0%	0.004
Escherichia/Shigella	3.2%	2.0%	3.0%	2.8%	0.089
Alistipes	2.8%	3.0%	2.9%	2.9%	0.901

*Note: * indicates comparison with baseline in the same group, P < 0.05. Bold indicates genera with statistically significant group-time interaction.*

8.4. Safety Analysis

During the intervention period, 2 cases (6.7%) in the experimental group experienced mild diarrhea, and 1 case (3.3%) had transient loss of appetite. Symptoms resolved spontaneously within 1 week, and none withdrew from the study. In the control group, 1 case (3.3%) developed a mild rash (considered related to contact materials in the rehabilitation training environment). There was no statistically significant difference in the incidence of adverse events between the two groups (P>0.05). No serious adverse events related to the study intervention occurred.

Table 5. Record and Analysis of Adverse Events.

Adverse Event Type	Experimental Group (n=30)	Control Group (n=30)	P-value
Gastrointestinal Related	2 (6.7%)	0 (0%)	0.492
Diarrhea	2	0	
Abdominal Pain/Bloating	0	0	
Infection	0 (0%)	0 (0%)	-

Adverse Event Type	Experimental Group (n=30)	Control Group (n=30)	P-value
Upper Respiratory Tract Infection	0	0	
Other	1 (3.3%)	1 (3.3%)	1.000
Rash	0	1	
Loss of Appetite	1	0	
Total	3 (10.0%)	1 (3.3%)	0.614
Adverse Events Leading to Withdrawal	0	0	-
Serious Adverse Events	0	0	-

9. Discussion

This study is the first to evaluate the adjunctive therapeutic effect of the domestic probiotic strain—Puze Biological Bifidobacterium animalis subsp. lactis V9—on young children with ASD. The results show that based on 3 months of conventional rehabilitation training, daily supplementation with V9 probiotic can more significantly improve core behavioral symptoms (CARS, ABC, SRS scores) and comorbid gastrointestinal symptoms (GSRS score) in children aged 2-7 years with ASD, with good safety. This finding is consistent with the results of several clinical trials on probiotic intervention for ASD in recent years, further supporting the feasibility of intervening in ASD by modulating the "gut-brain axis" [13].

In this study, the improvement in the total CARS score in the experimental group was 4.2 points greater than that in the control group, an improvement magnitude of clinical significance. It is particularly noteworthy that V9 probiotic showed a clear synergistic effect on core social-emotional dimensions such as "Interpersonal Relationships" and "Emotional Response". This is similar to the results of a study using Lactobacillus reuteri, which also found that probiotics mainly improved social communication ability in children with ASD, rather than overall severity [14]. This may suggest that probiotics have a relatively specific regulatory effect on certain specific neural circuits in ASD (such as the social reward circuit).

Regarding gastrointestinal symptoms, the improvement effect of V9 probiotic was particularly prominent, with the decrease in the total GSRS score being nearly twice that of the control group. Gastrointestinal problems are common in children with ASD, and symptom severity is often positively correlated with behavioral problems [15]. Due to its excellent acid and bile salt tolerance (survival rate >92% in gastric juice at pH 2.0), the V9 strain can effectively colonize the intestine and antagonize pathogenic bacteria through competitive inhibition, production of antimicrobial substances, etc., repairing the intestinal barrier, thereby rapidly relieving symptoms such as abdominal pain, bloating, and constipation. The improvement of gastrointestinal symptoms itself can enhance the comfort of the children and reduce emotional and behavioral problems triggered by discomfort, which may indirectly contribute to the improvement of behavioral scores [16].

This study preliminarily revealed the possible mechanism of action of V9 probiotic through metagenomic analysis. After intervention, the abundance of beneficial bacteria such as Bifidobacterium, Lactobacillus, and Akkermansia significantly increased, while the abundance of potential harmful bacteria such as Clostridium decreased in the gut of children in the experimental group. Bifidobacterium and Lactobacillus are key genera producing short-chain fatty acids (SCFAs, such as butyrate, propionate) [17]. SCFAs are not only the main energy source for intestinal epithelial cells, maintaining intestinal barrier integrity, but can also enter the brain via the vagus nerve or blood circulation, affecting the synthesis and metabolism of neurotransmitters (e.g., serotonin, γ-aminobutyric acid), and regulating neuroinflammation and synaptic plasticity. Akkermansia is closely related to improving intestinal mucosal barrier function and regulating immunity. These beneficial changes in microbial structure may be the microbiological basis for the improvement of behavioral symptoms [18]. This echoes the findings of the Probio-M8 study, which also found that after probiotic intervention, beneficial bacteria increased along with changes in specific metabolic pathways (such as amino acid, neurotransmitter metabolism).

Compared with similar studies, the advantages of this study are: First, the study subjects were young children aged 2-7 years, the golden period for intervention, with greater plasticity; Second, a randomized controlled design was adopted, and allocation concealment and assessor blinding were strictly implemented, reducing bias; Third, not only behavioral symptoms but also gastrointestinal symptoms and gut microbiota changes were systematically assessed, providing clues for mechanism exploration; Fourth, the V9 strain used is a domestic strain with complete independent intellectual property rights and long-term safety validation, making its application more practical.

The limitations of this study include: First, the sample size is relatively small, and it is a single-center study, so the generalizability of the conclusions requires verification by larger-

scale multi-center studies; Second, the intervention period was 3 months, which is relatively short for observing the long-term effects of probiotics and the stability of microbial colonization; Third, due to the nature of the intervention, blinding of subjects and intervention implementers could not be achieved (single-blind design), which may introduce performance bias; Fourth, blood biomarkers such as inflammatory factors and neurotransmitter metabolites were not detected, limiting deeper mechanistic interpretation from the "microbiota-metabolism-immune-neural" axis [19].

10. Conclusion

This randomized controlled trial indicates that, based on conventional rehabilitation training, daily supplementation with Puze Biological V9 probiotic (≥1×10^10 CFU/day) for 3 consecutive months is a safe and effective adjunctive treatment method that can significantly improve core behavioral symptoms and gastrointestinal dysfunction in children aged 2-7 years with Autism Spectrum Disorder. Its efficacy may be related to V9 probiotic regulating gut microbiota structure, increasing beneficial bacteria such as Bifidobacterium and Lactobacillus, and optimizing "gut-brain axis" function. This study provides new evidence-based medical evidence for applying domestic high-quality probiotic resources to the clinical management of ASD. Future research needs to involve larger samples, longer cycles, and integrate multi-omics (metagenomics, metabolomics, immunomics) to further elucidate the mechanism of action of V9 probiotic and explore its personalized application strategies in children with different subtypes and severities of ASD.

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Data Availability Statement

De-identified data supporting the results of this study will be made available from the corresponding author upon reasonable request after the article is published.

Conflicts of Interest

All authors declare no conflicts of interest. The probiotic preparation was provided free of charge by Inner Mongolia Puze Biological Products Co., Ltd. The company did not participate in the study design, data collection, analysis and interpretation, or writing of the paper.

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