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Investigating the impact of probiotic on neurological outcomes in Rett syndrome: A randomized, double-blind, and placebo-controlled pilot study

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Abstract

This pilot study investigates the feasibility and assesses the impact of *Lactobacillus plantarum* PS128 probiotics on the neurological function in Rett syndrome. We conducted a randomized, double-blind, and placebo-controlled trial on Rett syndrome with *MECP2* mutation aged between 1 and 50 years in Taiwan. In this pilot study, twice-daily *L. plantarum* PS128 or placebo was administered for 16 weeks. In addition to feasibility, we also assessed the changes utilizing the Mullen Scales of Early Learning. In total, 36 participants were finally randomized into *L. plantarum* PS128 (n = 18) or placebo (n = 18) groups. At the end of intervention, the retention rates were 100% for *L. plantarum* PS128 and 94.44% for placebo, with withdrawal rates of 5.56% for the placebo group. Both groups tolerated well, except for one *L. plantarum* PS128 participant who reported loose stool. The probiotic group showed a change of 2.19 ± 3.76, while the placebo group had -0.85 ± 5.09 ($p = 0.051$) in the total age-equivalent scores of Mullen Scales of Early Learning. There was a significant difference in the change of the total score on the Burke–Fahn–Marsden Movement Scale between probiotc group and placebo group (-12.19 ± 12.12 vs -4.59 ± 4.20, $p = 0.020$). In leg dystonia, the probiotic group exhibited a change of -4.11 ± 5.11 compared with -0.38 ± 1.50 in the placebo group ($p = 0.008$). Our findings affirm the feasibility of *L. plantarum* PS128 in Rett syndrome. Future clinical trials are mandatory to further explore its long-term impact on Rett syndrome.

Lay abstract

Rett syndrome often involves gastrointestinal symptoms and gut microbiota imbalances. We conducted a study to explore the feasibility of probiotic *Lactobacillus plantarum* PS128 and the impact on neurological functions in Rett syndrome. The results of our investigation demonstrated that the supplementation of probiotic *L. plantarum* PS128 was feasible and well tolerated, with 100% retention rate and 0% withdrawal rate. In addition, there was only one participant who had loose stool after taking *L. plantarum* PS128. Further, there was a tendency to enhance overall cognitive developmental level, as assessed using Mullen Scales of Early Learning. In addition, it significantly improved dystonia, as assessed using the

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Burke–Fahn–Marsden Movement Scale, in comparison with the placebo group. This study provides a strong foundation for future research and clinical trials exploring the potential of *L. plantarum* PS128 probiotics as a complementary therapy for individuals with Rett syndrome.

Keywords
dystonia, microbiota–gut–brain axis, probiotics, Rett syndrome

Background

Rett syndrome (RTT) is a neurodevelopmental disorder caused by a genetic mutation in the *MECP2* gene. The condition is primarily characterized by prominent neurological regression, seizures, gastrointestinal (GI) manifestations, and motor impairments such as stereotypies and dystonia. In addition, certain individuals with RTT may exhibit clinical traits overlapping with the autistic spectrum. To date, trofinetide is the sole Food and Drug Administration (FDA)-approved treatment for RTT in children aged 2 years and older. Clinical studies by Neul et al. (2023) demonstrated a significant improvement in RTT behavioral scores with the use of this drug.

The gut microbiota are believed to play a crucial role in brain development and neurological function, facilitated by the bidirectional communication of the microbiota–gut–brain axis, involving immunological, hormonal, and neuronal signals. Dysfunction of this axis has been linked to the pathogenesis of various neurological diseases, including autism, Parkinson's disease, and Alzheimer's disease (Cryan et al., 2019). In RTT, gut microbiome analysis reveals a proinflammatory status, reduced microbiota richness, and altered profiles of short-chain fatty acids (SCFAs). These dysbiotic changes may be correlated with the severity of the disease (Borghi et al., 2017; Strati et al., 2016), indicating that malfunction of the microbiota–gut–brain axis might be part of the complex pathogenesis of RTT.

In addition, previous studies have shown decreased brain-derived neurotrophic factor (BDNF) levels (Abuhatzira et al., 2007; Chang et al., 2006; H. Wang et al., 2006) and alterations in monoaminergic levels, including decreased dopamine, norepinephrine, and serotonin levels (Samaco et al., 2009; Taneja et al., 2009) in the brain. These changes may contribute to the pathogenesis of RTT. Moreover, systemic oxidative stress has been identified in RTT individuals, with a strong correlation with the patient's clinical status (Filosa et al., 2015; Leoncini et al., 2011). Therefore, treatments aimed at improving dopaminergic functions and BDNF expression could potentially alleviate cognitive and neurological impairments in individuals with RTT.

Lactobacillus plantarum PS128 (PS128), isolated from the traditional Taiwanese fermented vegetable food product fu-tsai, has been studied extensively. Research has demonstrated that PS128 ameliorated behavioral deficits in germ-free mice by modulating the expression of brain neurotransmitters (W. H. Liu, Chuang, et al., 2016 Y. W. Liu, Liu, et al., 2016. In addition, PS128 was found to modulate the gut microbiota, increase BDNF expression in the central nervous system (CNS) of a mouse model of Parkinson's disease induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, and alleviate motor deficits(Liao et al., 2020). In addition, in a recent clinical study, PS128 ingestion significantly improved neurological behaviors in individuals with autism (Y. W. Liu et al., 2019). These findings suggest that PS128 probiotic supplementation holds promise as a potential clinical therapy for neurological disorders. Based on this evidence, our present pilot study aimed to investigate the feasibility of utilizing PS128 probiotics in RTT and its potential in enhancing neurological function in RTT. Through the collection of comprehensive preliminary data, we seek to establish a robust foundation for the subsequent comprehensive and definitive clinical trial.

Methods

This randomized, double-blind, and placebo-controlled pilot study was performed in a tertiary referral center in Taiwan.

Ethics

The institutional review board approved this study. Informed consent was obtained from the parent/care giver of each participant. The full protocol is available, on request, from the corresponding author.

Participants

Participants diagnosed with RTT and *MECP2* mutation and aged between 1 and 50 years were included for this study. Participants were recruited from the RTT joint clinic and under the assistant of Taiwan Rett association. Participants who had consumed antibiotics and yogurt or probiotic products 4 weeks prior to enrollment were excluded for this study. Participants were required to refrain from consuming yogurt or probiotic products during the study period. They were allowed to continue their regular medications and treatments. Nonetheless, those who were prescribed antibiotics during the study period were excluded from analysis after the study.

Intervention

In this study, participants were allocated randomly to receive either a placebo or PS128, following a permuted block design within a stratum of four assignments. The PS128 and placebo treatments were administered in a 1:1 ratio. Physicians, study staff, and participants were blinded to the group assignment. The identity of supplements was concealed by virtue of a placebo that matched the PS128 in terms of identity of capsule, packaging, labeling, and administration schedule or prescription by Bened Biomedical Co., Ltd.

PS128 was isolated, and deposited under DSMZ Accession No. DSM 28632. The genome architecture of PS128 has been previously reported (Liu et al., 2015). The PS128 product was supplied by Bened Biomedical Co., Ltd. in the final form of capsules containing creamy white powders. The probiotic capsules weighed 425 ± 25 mg and contained 3×10^{10} colony-forming unit (CFU)/capsule of PS128, with microcrystalline cellulose as the carrier, whereas the placebo capsules only contained microcrystalline cellulose. All capsules were identical in taste and appearance. They were stored at a refrigerated temperature of 4°C–8°C. Oral administrations of one capsule of PS128 or placebo were given on a 12-h time interval daily to participants in the treatment and placebo groups, respectively.

Assessments

Neuropsychological assessment

The cognitive developmental level was assessed using the Mullen Scales of Early Learning (MSEL) (Mullen, 1995). The measurements were obtained at baseline and end of study. The methodology and procedure employed were according to those described in a previous study (Yang et al., 2019). The examiners were blinded to the identity of the treatment groups. Two examiners with bachelor's degree in pediatric physical therapy were blinded to the identity of the treatment groups. They were trained in the materials and procedures of the MSEL and rehearsed assessments with senior examiners with a doctoral degree and had >10 years of experience in using the MSEL in clinical practice or research assessment. The training continued until $\geqslant 95\%$ of the items in each examiner's result in the practice sessions agreed with the senior examiner's assessments. MSEL contains five subscales: visual reception (VR), expressive language (EL), receptive language (RL), fine motor (FM), and gross motor (GM). It is a comprehensive norm-referenced developmental test for children aged from 0 to 68 months.

As the girls participating in this study fell outside the typical age range for the test's administration, their cognitive developmental level was reported in age equivalents, which had been utilized in another study investigating RTT (Smith-Hicks et al., 2017). Each subscale's raw score can be transformed into age-equivalent scores, allowing for a comparison with data from a normative sample of American children. To determine the overall cognitive developmental level, the age-equivalent scores of the four MSEL subscales (VR, EL, RL, and FM) are combined to create the total age-equivalent scores. Because the functional hand usage and verbal responses required by the assessments are abilities often lost in the RTT population, we made several modifications to the scoring method through discussions with pediatric neurologists and physical therapists (S. Y. Wang et al., 2022). The modified procedures of administration have been examined and validated for Taiwanese children with Rett aged 2–18 years (S. Y. Wang et al., 2022). While the MSEL has not been further validated for individuals beyond 18 years in RTT, we applied it in this study due to the consideration of severe neurological impairment (MSEL age equivalent less than 36 months (S. Y. Wang et al., 2022)) and the plateauing of neurological function before 18 years, and the potential for regression in RTT for the participants (Leonard et al., 2017).

Dystonia assessment

The Burke–Fahn–Marsden movement scale (BFMMS) (Burke et al., 1985) and Unified Dystonia Rating Scale (UDRS) (Krystkowiak et al., 2007) were used to assess the severity of dystonia in each participant at baseline and end of study. The evaluations were performed by two pediatric neurologists who were blinded to the identity of the treatment groups. The measurements were obtained at baseline and end of the study. The BFMMS assesses dystonia in nine body regions, which encompass the eyes, mouth, speech and swallowing, neck, trunk, arms, and legs. The scoring scale ranges from 0 (indicating the minimum severity) to 120 (representing the maximum severity) for each region. The total possible score on the BFMMS is between 0 and 120, with higher scores indicating a more severe dystonic condition.

The UDRS provides a comprehensive evaluation of individual body areas, encompassing 14 regions. Dystonia severity and duration are rated for each region, including separate ratings for the proximal and distal limbs. The scores for all body regions are then aggregated to derive an overall rating of dystonia severity, which falls within the range of 0–112. A higher UDRS score indicates a more pronounced level of dystonia.

Clinical severity

Rett Syndrome Severity Scale (RSSS) was introduced by Kaufmann et al. (2012) to evaluate the clinical severity of RTT. This scale encompasses seven distinct parameters, as outlined in our previous research (Wong et al., 2017). The RSSS includes the following parameters: (1) epilepsy condition, (2) respiratory irregularities, (3) scoliosis, (4)

walking ability, (5) hand function, (6) language, and (7) sleep condition. Each parameter is assessed using a scale ranging from 0 (absent) to 3 (severe), resulting in a total score that ranges from 0 to 21.

Social-behavioral and daily adaptive functioning

Daily adaptive functioning was measured using the Chinese version of Vineland Adaptive Behavior Scales (VABS) (Sparrow & Cicchetti, 1989) (The VABS-C (Wu et al., 2004)). This scale is a questionnaire completed by caregivers to assess adaptive behavioral functioning in children between the ages of 3 and 12 years, or in children older than 12 years with intellectual or developmental disabilities. It measures the child's proficiency in various daily activities across five subscales: Communication, which includes three subdomains: receptive communication, expressive communication, and reading and writing skills; daily living skills, which consists of three subdomains: personal living skills, domestic living skills, and community living skills; socialization, encompassing three subdomains: interpersonal relationships, play and leisure time, and coping skills; and motor skills, comprising two subdomains: FM and GM skills. Each item on the VABS-C is scored on a scale from 0 to 2, with higher scores indicating greater ability in the evaluated areas. The total score is derived from the summation of the scores across the five subscales.

We utilized the Anxiety, Depression, and Mood Scale (ADAMS) (Esbensen et al., 2003) as a means to assess anxiety and depression levels among participants. The ADAMS is a parent-completed questionnaire which includes 28 items. The scale encompasses five subscales: manic/hyperactive behavior, depressed mood, social avoidance, general anxiety, and compulsive behavior. This widely used instrument is particularly valuable for screening anxiety, depression, and mood disorders in individuals with cognitive dysfunction. Higher scores indicate higher levels of anxiety-, depression-, or mood-related symptoms.

The Ghuman–Folstein Screen for Social Interaction (GF-SSI) (Ghuman et al., 1998) is a parent/caregiver-report screening tool comprising 54 items. It primarily focuses on assessing reciprocal social interaction, including joint attention skills. The items are positive (prosocial) and are rated on a 4-point frequency scale, where a score of 0 indicates the behavior is displayed "almost never" and a score of 3 indicates the behavior is displayed "almost all the time." Therefore, lower scores on the SSI suggest a slower or delayed development in social interaction, while higher scores indicate more normative development in this area.

Early Social-Communication Scales (ESCS) (Mundy et al., 2003) were utilized to assess both proto-declarative and proto-imperative joint attention behaviors. ESCS is a structured observational measurement scale that employs standardized testing procedures. It aims to measure the nonverbal socio-communicative skills of children younger than 30 months. We applied it in this study due to the consideration of severe neurological impairment in RTT. During the assessment, children's behaviors were categorized into three groups: (1) initiating joint attention and responding to joint attention; (2) initiating behavior requests and responding to behavior requests; and (3) initiating social interactions and responding to social interactions. The total scores for each behavior were obtained by summing up the frequencies of occurrences. The overall total score was then determined by adding the individual scores from each subscale.

The Rett Syndrome Behavioral Questionnaire (RSBQ) (Mount et al., 2002) was employed in this study. This questionnaire consists of 45 items and serves to evaluate the behavioral and emotional characteristics of individuals with RTT. It is further categorized into eight subscales: (1) general mood, (2) breathing problems, (3) hand behavior, (4) face movements, (5) body rocking and expressionless face, (6) night-time behavior, (7) fear/anxiety, and (8) walking/standing. Each item is scored on a scale of 0–2, with a maximum total score of 90, indicating the presence of significant symptoms.

The Chinese version of the Pediatric Evaluation of Disability Inventory (PEDI-C) (Tseng & Chen, 2012) is a parent-report questionnaire that has been translated into from the PEDI (Haley et al., 1992) into Chinese. Its purpose is to assess the daily functional skills of children aged $\geqslant$6 months who have developmental disabilities. The questionnaire focuses on three domains: (1) self-care, (2) mobility, and (3) social function. To evaluate each domain, two scales are used: the Functional Skills Scale (FSS), which identifies the child's daily functional abilities, and the Caregiver Assistance Scale (CAS), which measures the level of independence and the amount of assistance required for functional activities. Higher scores on both the FSS and CAS indicate greater independence in daily living skills. The PEDI-C has undergone psychometric validation, demonstrating excellent internal consistency and interrater reliability. It has also shown good construct validity in Taiwanese children (Chen et al., 2009, 2010).

GI assessment

The stool forms and constipation were assessed at baseline and end of study. The stool form of each sample was graded according to the modified Bristol Stool Form Scale for Children (mBSFS-C) (Chumpitazi et al., 2010), which ranges from type 1 to 5 (hard to watery), with type 3 graded as normal stool form.

Stool microbiota assessment

The stool of the participants was collected once defecated and kept at 4°C during transportation and then stored at −80°C until use. Extraction of fecal DNA was carried out following standard protocols (Costea et al., 2017). For library

construction, the prokaryotic rRNA V3-V4 hypervariable region was amplified using region-specific primer set (forward: 5′-CCTACGGRRBGCASCAGKVRVGAAT-3′ and reverse: 5′-GGACTACNVGGGTWTCTAATCC-3′). At the same time, indexed adapters were added to the ends of the amplified DNA to generate an indexed library for next-generation sequencing.

Endpoints

The primary focus of this study was to examine the key metrics, including recruitment rate, retention rate, withdrawal rate, and participant tolerance to adverse effects. In addition, we also assessed the changes from baseline to week 16, utilizing the MSEL. The secondary endpoints in this study were the changes from baseline to week 16 in the BFMMS, UDRS, VABS-C, ADAMS, GF-SSI, ESCS, RSBQ, PEDI-C, RSSS, GI function (mBSFS-C, constipation condition), and stool microbiota, which analyzed through 16S rRNA sequencing.

Statistical analysis

Baseline demographic data

Descriptive statistics were used to summarize the baseline demographics of the participants. The difference of discrete variables between placebo and probiotic groups was analyzed by chi-square/Fisher's exact test. The difference between continuous variables was analyzed by independent samples t test.

Outcome analysis

Retention and withdrawal rates

The retention and withdrawal rates at the end of the 16-week intervention were collected, and the difference of these variables between placebo and probiotic groups was analyzed by chi-square/Fisher's exact test.

Cognitive, dystonia, clinical severity, and social-behavioral and daily adaptive functioning

The independent samples t test was used to analyze outcome variables in the participants regarding the change between baseline and the end of the 16-week intervention. The change of score between baseline and week 16 was then modeled using a multivariable regression analysis. Age, treatment group, and baseline score were included as covariates in the regression model. This approach allowed us to assess the association between the change of score between baseline and week 16 and the independent variables while accounting for potential confounding factors. To provide a range of plausible values for the treatment effect estimate, 95% confidence intervals (CIs) were calculated.

The statistical analysis was performed hierarchically for dystonia, clinical severity, and social-behavior functioning; if the total score analysis yielded statistical significance, then further analysis would be conducted on the subscales.

Statistical significance was defined as $p < 0.05$.

Further, we utilized a multivariable regression analysis, factored in age, treatment group, and baseline score as covariates within the regression model, allowing us to assess the relationship between changes in scores between baseline and week 16 and independent variables while considering potential confounding factors.

GI measure

Chi-square/Fisher's exact test was used to analyze the difference in stool characteristics and presence of constipation between groups after intervention. Statistical significance was defined as $p < 0.05$.

16S metagenome sequencing and data analysis

For raw data collection, 2×250 paired-end (PE) sequencing was performed with Illumina MiSeq instrument according to the manufacturer's instructions. The adaptor sequences were removed using Cutadapt (v1.9.1) (Martin, 2011) and QIIME analysis package (v1.9.1) was used for 16S rRNA data analysis. PE reads were joined using overlapping sequences. The joined sequences were then trimmed to remove low-quality bases (Q_{phred} score < 20) from the 5′ and 3′ ends. Raw reads with overlapping regions less than 20 bps or a total length shorter than 200 basepairs (bps) were discarded. SILVA 132 (Quast et al., 2013) was used as the reference database and chimera sequences were removed using UCHIME (Edgar et al., 2011). The resulting clean reads were subjected to operational taxonomic units (OTU) clustering using VSEARCH (1.9.6) (Rognes et al., 2016) at a 97% threshold. For beta diversity analysis, beta distances between each sample were calculated based on Bray-Curtis and UniFrac distance matrix (Lozupone & Knight, 2005). Intergroup differences were explored by Principal Co-ordinates Analysis (PCoA) analysis and statistical significances were calculated by Adonis and Anosim analysis using R package "*vegan*." Differential analysis of community composition was calculated using linear discriminant analysis effect size (LEfSe) method (Segata et al., 2011).

Community involvement. The study was conducted after close discussion with the parents of RTT individuals. The Taiwan Rett Syndrome Association also aided in recruiting participants. The parents of RTT individuals also actively participated in the study, and provided information about the treatment results.

Table 1. The clinical characteristics of the participants at baseline.

Variables	Baseline		p value
	PS128	Placebo	
	N = 18	N = 17	
Age, in years	18.39 ± 9.73	16.25 ± 8.57	0.495
Sex (female/male)	18/0	16/1	0.296
Cognition			
MSEL, in age equivalent (month)			
Overall cognitive developmental level	28.00 ± 23.36	27.65 ± 19.05	0.961
Gross motor	9.33 ± 5.74	10.06 ± 6.03	0.711
Visual reception	7.33 ± 5.73	7.29 ± 4.63	0.982
Fine motor	6.67 ± 6.08	5.65 ± 6.76	0.643
Receptive language	8.06 ± 6.18	7.71 ± 4.00	0.843
Expressive language	5.94 ± 7.85	7.00 ± 5.20	0.641
Dystonia			
BFMMS	56.58 ± 22.07	52.88 ± 21.17	0.616
UDRS	61.14 ± 13.88	56.41 ± 15.24	0.314
RSSS	11.39 ± 3.33	10.94 ± 3.72	0.709
Social-behavioral and daily adaptive functioning			
VABS-C	22.89 ± 27.26	19.47 ± 24.57	0.699
RSBQ	43.44 ± 12.08	43.18 ± 12.91	0.950
ADAMS	27.11 ± 9.93	25.24 ± 10.19	0.585
GF-SSI	21.50 ± 8.46	22.59 ± 9.02	0.716
ESCS	23.50 ± 22.14	17.35 ± 15.42	0.350
PEDI-C			
FSS	36.44 ± 36.53	32.53 ± 30.78	0.735
CAS	10.56 ± 14.95	10.06 ± 14.88	0.922
Gastrointestinal evaluation			
Stool character (%)			
Normal	6 (33.33)	6 (35.29)	0.903
Abnormal	12 (66.67)	11 (64.71)	
Constipation (%)			
Presence	9 (50.00)	11 (64.71)	0.591
Absence	9 (50.00)	6 (35.29)	

ADAMS: Anxiety, Depression, and Mood Scale; BFMMS: Burke–Fahn–Marsden movement scale; CAS: Caregiver Assistance Scale; FSS: Functional Skills Scale; MSEL: Mullen Scales of Early Learning; PEDI-C: Chinese version of the Pediatric Evaluation of Disability Inventory, PS128: *Lactobacillus plantarum* PS128; RSBQ: Rett Syndrome Behavioral Questionnaire; RSSS: Rett Syndrome Severity Scale; GF-SSI: Ghuman–Folstein Screen for Social Interaction; UDRS: Unified Dystonia Rating Scale; VABS-C: Chinese version of Vineland Adaptive Behavior Scales; ESCS: Early Social-Communication Scales.

Results

Demographic, baseline characteristics, recruitment rate, retention rate, withdrawal rate, and adverse effects

Enrollment occurred between August 2017 and December 2017. A total of 50 individuals were assessed for eligibility and, of this total, 14 individuals were excluded. Also, of this total, 13 individuals did not meet the inclusion criteria and 1 individual declined to participate. A total of 36 individuals were randomized to PS128 or placebo groups. One participant had to discontinue the study due to peritonitis after 11 weeks on placebo. Thirty-five participants completed the study, in which 17 participants were administered placebo and 18 participants were administered PS128 (Table 1). The flow diagram (Figure 1) summarizes the study enrollment through analysis. The retention rates at the study's end were 100% (18/18) in the PS128 group and 94.44% (17/18) in the placebo group, with withdrawal rates of 0% (0/18) and 5.56% (1/18), respectively (p = 1.000). Regarding tolerability, only one participant was reported to have loose stool after taking PS128. This was considered as mild, and the participant tolerated very well. All the other participants also tolerated very well without any other adverse effects.

At the final assessment, there were a total of 35 participants divided into two groups: the probiotic group, which comprised 18 individuals (18 females) with a mean age of 18.39 ± 9.73 years (ranging from 2.24 to 35.88 years), and the placebo group, which consisted of 17 individuals (16

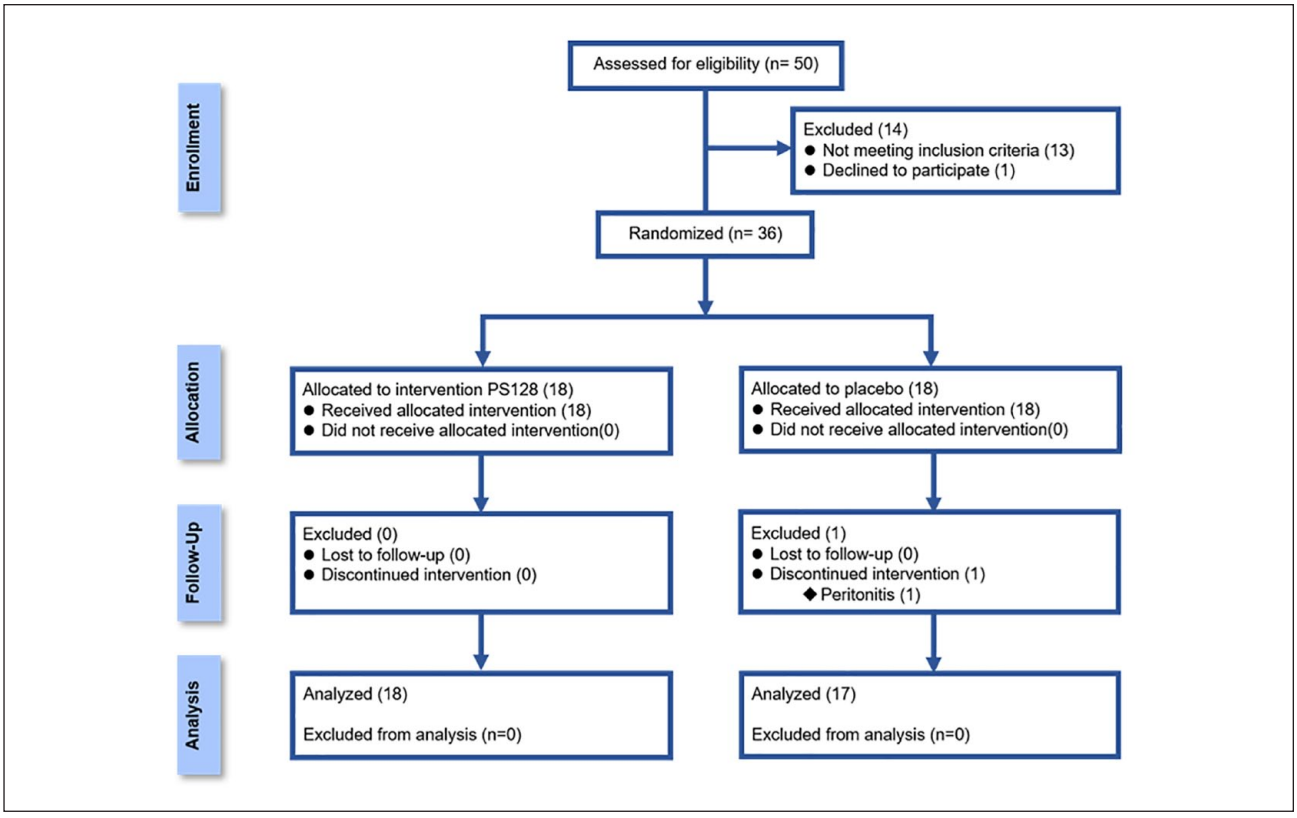

Figure 1. Flowchart of participant disposition, which follows the CONSORT 2010 statement: extension to randomized pilot and feasibility trial (Eldridge et al., 2016).
This flow chart depicts the randomization process for the pilot study investigating the impact of *Lactobacillus plantarum* PS128 (PS128) probiotic, using a placebo-controlled design, in individuals with Rett syndrome. Total 50 individuals were assessed for eligibility and, of this total, 14 individuals were excluded. A total of 36 participants were finally randomized to PS128 or placebo groups. Total 35 participants completed the study. One was excluded from the study due to peritonitis which prevented her from completing the trial after 3 weeks on PS128.

females and 1 male) with a mean age of 16.25 ± 8.57 years (ranging from 3.07 to 32.52 years). Table 1 presents the baseline demographics of the participants, revealing no significant differences between the placebo and PS128 groups regarding baseline characteristics. Regarding genotypes, the 35 participants (as presented in Table S1) demonstrated a total of 20 distinct genotypes. Nevertheless, due to the limited number of cases and the considerable variability in genotypes, we did not conduct a statistical analysis.

MSEL evaluation

The mean change in MSEL scores from baseline to week 16 was 2.19 ± 3.76 in the group that received PS128, whereas the placebo group had a score of −0.85 ± 5.09 (p = 0.051) (Table 2). However, the multivariable regression analysis model estimated a significant improvement in the overall cognitive developmental level change at the follow-up by 3.09 months (p = 0.033, 95% CI = (0.28, 5.91)) in the group that received PS128, as compared to the placebo group, after adjusting for baseline scores and age (Table 3).

When examining each subscale individually, no significant differences were found in the changes from baseline to week 16 (Table 2). The multivariable regression analysis also showed no statistically significant differences when compared to the placebo group after adjusting for age and baseline scores for each scale (Table S2). Nevertheless, there appeared to be an improvement in visual function change by 1.65 months (p = 0.069, 95% CI = (−0.14, 3.43)) in the group that received PS128, compared to the placebo group, after adjusting for baseline scores and age.

The above data indicated the feasibility of PS128 for further randomized control trial in the future.

Secondary outcome

Dystonia. The mean change in BFMMS total scores from baseline to week 16 was −12.19 ± 12.12 in the group that received PS128, whereas the placebo group had a score of −4.59 ± 4.20 (p = 0.020) (Table 2). When examining the subscales, in the leg dystonia scores within BFMMS, the probiotic group exhibited a change of −4.11 ± 5.11 compared to −0.38 ± 1.50 in the placebo group (p = 0.008).

Table 2. The change of covariate after intervention, expressed in mean and standard deviation.

Variables	PS128	Placebo	p value
	N = 18	N = 17	
Cognition			
MSEL, in age equivalent (month)			
Overall cognitive developmental level	2.19 ± 3.76	−0.85 ± 5.09	0.051
Gross motor	0.78 ± 2.13	1.59 ± 3.64	0.432
Visual reception	0.50 ± 2.28	−1.12 ± 3.35	0.103
Fine motor	0.56 ± 1.98	0.06 ± 2.79	0.546
Receptive language	1.14 ± 2.13	0.79 ± 2.20	0.641
Expressive language	0 ± 4.13	−0.59 ± 2.67	0.622
Dystonia			
BFMMS			
Total score	−12.19 ± 12.12	−4.59 ± 4.20	0.020
Eye	−0.22 ± 0.62	−0.34 ± 0.68	0.589
Mouth	−0.22 ± 0.96	−0.06 ± 0.91	0.623
Speech and swallow	−0.67 ± 1.24	−0.19 ± 1.11	0.245
Neck	−0.19 ± 0.57	−0.28 ± 0.58	0.663
Arms (bilateral)	−6.22 ± 7.73	−2.81 ± 3.10	0.099
Legs (bilateral)	−4.11 ± 5.11	−0.38 ± 1.50	0.008
Trunk	−0.56 ± 1.65	−0.56 ± 1.26	0.989
UDRS	−8.03 ± 8.60	−3.88 ± 7.55	0.140
RSSS	−0.28 ± 0.96	−0.12 ± 0.78	0.593
Social-behavioral and daily adaptive functioning			
VABS-C	1.00 ± 1.88	0.94 ± 3.25	0.948
RSBQ	−3.72 ± 6.29	−2.76 ± 5.43	0.634
ADAMS	−0.78 ± 4.70	−2.35 ± 4.82	0.335
GF-SSI	0.89 ± 3.08	−0.53 ± 3.78	0.231
ESCS	0.06 ± 12.75	−1.47 ± 10.12	0.699
PEDI-C			
FSS	−0.89 ± 4.69	1.88 ± 12.33	0.381
CAS	0.72 ± 3.61	−0.06 ± 3.75	0.534
Gastrointestinal evaluation at 16th week			
Stool character (%)			
Normal	12 (66.67)	8 (47.06)	0.241
Abnormal	6 (33.33)	9 (52.94)	
Constipation (%)			
Presence	6 (33.33)	9 (52.94)	0.241
Absence	12 (66.67)	8 (47.06)	

ADAMS: Anxiety, Depression, and Mood Scale; BFMMS: Burke–Fahn–Marsden movement scale; CAS: Caregiver Assistance Scale; FSS: Functional Skills Scale; MSEL: Mullen Scales of Early Learning; PEDI-C: Chinese version of the Pediatric Evaluation of Disability Inventory; PS128: *Lactobacillus plantarum* PS128; RSBQ: Rett Syndrome Behavioral Questionnaire; RSSS: Rett Syndrome Severity Scale; GF-SSI: Ghuman–Folstein Screen for Social Interaction; UDRS: Unified Dystonia Rating Scale; VABS-C: Chinese version of Vineland Adaptive Behavior Scales; ESCS: Early Social-Communication Scales.
Post-intervention scores could be assessed in Supplemental data (Table S2).

Further, the multivariable regression analysis model estimated a significant improvement in total BFMMS change at the follow-up by 8.05 points months (p = 0.004, 95% CI = (−13.30, −2.80)) in the group that received PS128, as compared to the placebo group. This improvement was observed after adjusting for baseline scores and age (Table 3). The analysis also estimated a significant decrease in the leg dystonia score by 4.12 points (p = 0.001, 95% CI = (−6.52, −1.73)) and arm dystonia score by 3.90 (p = 0.019, 95% CI = (−7.10, −0.69)) in the PS128 group, after adjusting for age and baseline score.

Regarding UDSR, no significant differences were found in the changes from baseline to week 16 (Table 2). The multivariable regression showed no statistically significant difference compared to the placebo group after adjusting for age and baseline score (Table S2).

Clinical severity. Clinical severity was assessed using the RSSS. No significant differences were found in the changes from baseline to week 16 (Table 2). The multivariable

Table 3. The multivariable regression analysis model, which adjusts for the respective baseline score and age, explores the relationship between the total score (representing overall cognitive developmental level) on the MSEL and the total score as well as the arm, leg, and arm subscales on the BFMMS.

	MSEL: Total score (overall cognitive developmental level)			
	Coefficient	SE	95% CI	p value
PS128[a]	3.09	1.38	0.28, 5.91	0.033
Baseline score	−0.10	0.03	−0.17, −0.03	0.007
Age	−0.01	0.08	−0.17, 0.16	0.948
Constant	1.95	1.99	−2.11, 6.01	0.334

	BFMMS: Total score				BFMMS: Arm				BFMMS: Leg			
	Coefficient	SE	95% CI	p value	Coefficient	SE	95% CI	p value	Coefficient	SE	95% CI	p value
PS128[a]	−8.05	2.58	−13.30, −2.80	0.004	−3.90	1.57	−7.10, −0.69	0.019	−4.12	1.17	−6.52, −1.73	0.001
Baseline score	−0.26	0.07	−0.40, −0.11	0.001	−0.50	0.11	−0.71, −0.28	<0.001	−0.13	0.07	−0.27, 0.01	0.067
Age	0.65	0.17	0.30, 1.00	0.001	0.37	0.10	0.18, 0.57	0.001	0.22	0.07	0.09, 0.36	0.003
Constant	−1.56	3.77	−9.25, 6.12	0.681	0.60	2.24	−3.96, 5.17	0.789	−1.72	1.59	−4.96, 1.52	0.286

BFMMS: The Burke–Fahn–Marsden movement scale; CI: confidence interval; MSEL: Mullen Scales of Early Learning; PS128: *Lactobacillus plantarum* PS128; SE: standard error.
[a]Placebo as reference.

regression showed no statistically significant difference compared to the placebo group after adjusting for age and baseline score (Table S2).

Social-behavioral and daily adaptive functioning. VABS, RSBQ, ADAMS, SSI, ESCS, and PEDI were used to assess social-behavioral and daily adaptive functioning in the participants. No significant differences were found in the changes from baseline to week 16 (Table 2). The multivariable regression showed no statistically significant difference compared to the placebo group after adjusting for age and baseline score (Table S2).

GI and gut microbiota

Regarding constipation and stool consistency, no statistically significant changes were found after the intervention in either of the groups (Table 2).

To evaluate the potential alteration of gut microbiota after PS128 intervention and delineate the underlying mechanism, we characterized the bacterial gut microbiota of the participants. Fecal samples from all the participants were collected before (V0) and after (V1) 16 weeks of PS128 or placebo intervention. 16S rRNA sequencing was carried out with good quality. An average of 90,000 PE reads were obtained for each sample and about 90% of the raw sequences reached a Phred *quality* score of 30 (Q score). After quality filtering, trimming, and chimera removal, an average of 70,000 clean reads were retained, which provide sufficient read depth for further analysis (Table S3 and Figure S1).

To delineate the microbiota diversity of each participant, we calculated several alpha diversity indexes, such as Chao1, Ace, Shannon, and Simpson, for either observed species diversity (Figure 2(a) and (b)) or major species diversity (Figure 2(c) and (d)). No statistically significant difference was observed for all four indexes between all four groups. The result suggests that PS128 treatment did not affect the alpha diversity of the participants. The microbiota composition between the four groups was further compared to verify their beta diversity (Table S4). An increase of OTU13 in the PS128 V1 group was observed in the heat map analysis (Figure 3(a)). After searching the representative sequence of OTU13 on National Center for Biotechnology Information (NCBI) database using BLAST, we found that it perfectly matched the 16S rRNA sequence of *L. plantarum/pentosus*. Since PS128 is an *L. plantarum*, we postulate that OTU13 is our probiotic strain (PS128). This observation confirmed the successful intervention of PS128. Despite the difference of OTU13, we did not observe an apparent pattern from the heat map and there is no distinct distribution of each group in PCoA analysis (Figure 3(b)). To further compare the microbiota composition of each sample, we applied UniFrac distance (Figure S2) and differential analysis between the groups

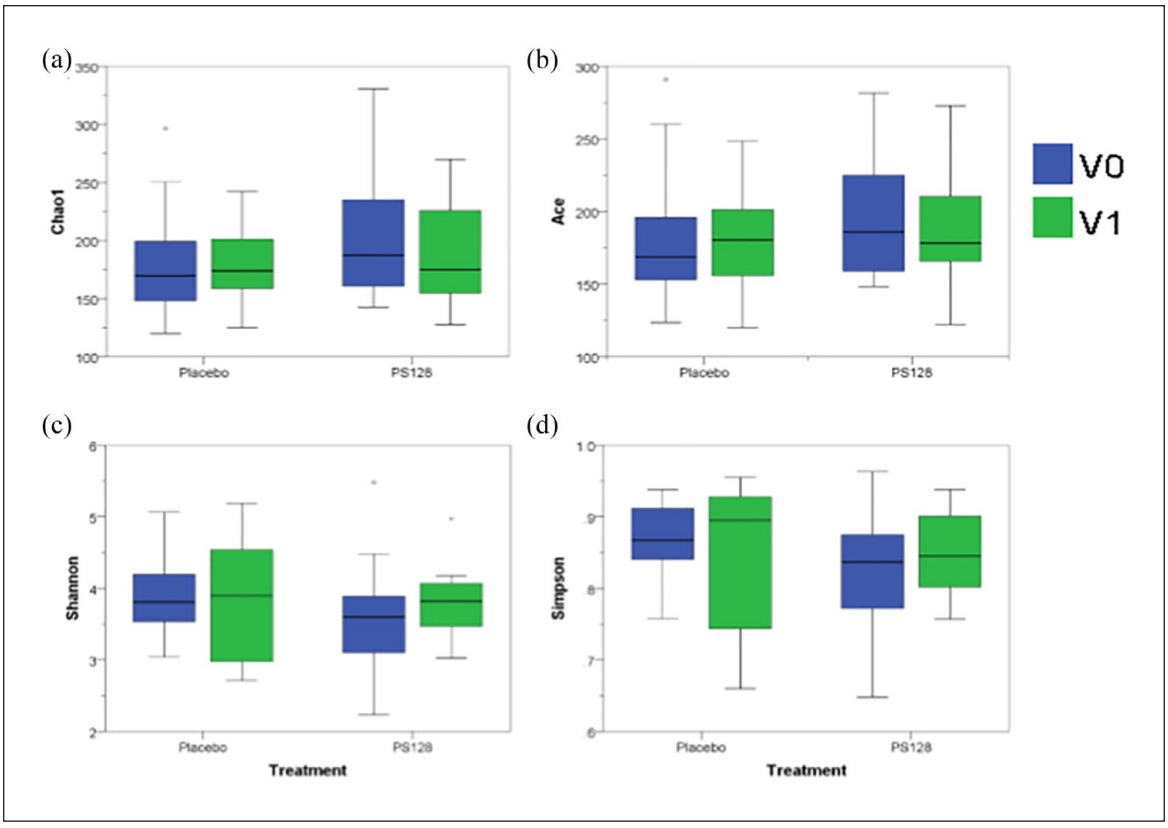

Figure 2. Alpha diversity index of the gut microbiota of both PS128 and placebo groups. Clustered OTU data were used to calculate (a) Chao1 and (b) Ace index for observed species diversity. On the other hand, (c) Shannon and (d) Simpson index were calculated for major species diversity. No statistical significance was observed in each group using the Kruskal–Wallis test. (V0 = baseline; V1 = 16 weeks after intervention).

using Adonis and Anosim analysis (Figure 3(c)). We also performed LEfSe analysis to characterize the differential presented microbes between placebo V1 and PS128 V1 at the various taxonomic hierarchy. In consonant with our previous observation, in the heat map analysis, *Lactobacillus* was the only enriched genus in PS128 V1 (Figure S3). Therefore, from the change of microbiota and the efficacy of PS128 treatment on cognition and dystonia, our results showed the feasibility of PS128 trial for longer duration and larger sample size.

Discussion

This pilot study represents the first clinical trial investigating the feasibility and effects of probiotic supplementation on neurological and GI profiles in individuals with RTT. Our findings indicate that supplementation with PS128 is feasible in RTT, with 100% of the retention rate in the PS128 group and 94.44% in the placebo group. There was no withdrawal in the PS128 group, while there was only 5.56% of withdrawal in the placebo group. The PS128 was also well tolerated, with only one participant reporting loose stool under the PS128 supplement. There was also a tendency to improve cognitive developmental level and significantly reduce the severity of dystonia, particularly in the upper and lower extremities of individuals with RTT. Of note, regarding the cognition, as the multivariable regression analysis model revealed a noteworthy improvement in the change of total score of MSEL (overall cognitive developmental level) at follow-up in the group administered PS128 compared to the placebo group, with an estimated increase of 3.09 months, taking into account that the mean overall cognitive developmental level at baseline for both groups was around 28 months, the observed difference of 3.09 months in the cognitive developmental level represents approximately 10% of the baseline score. This substantial difference is considered to be of clinical significance. In addition, in the PS128 group, the multivariable regression showed there was a significant decrease in the change of BFMMS total scores at follow-up, amounting to 8.05 points ($p = 0.004$, 95% CI = (−13.30, −2.80)), when compared to the placebo group. After adjusting for baseline score and age, the decrease in arm dystonia scores was 3.90 points ($p = 0.019$, 95% CI = (−7.10, −0.69)) and in leg dystonia score was 4.12 points ($p = 0.001$, 95% CI = (−6.52, −1.73)). Considering that the total mean score of PS128 at baseline was 56.58, the observed difference of approximately 14% in the total score is noteworthy.

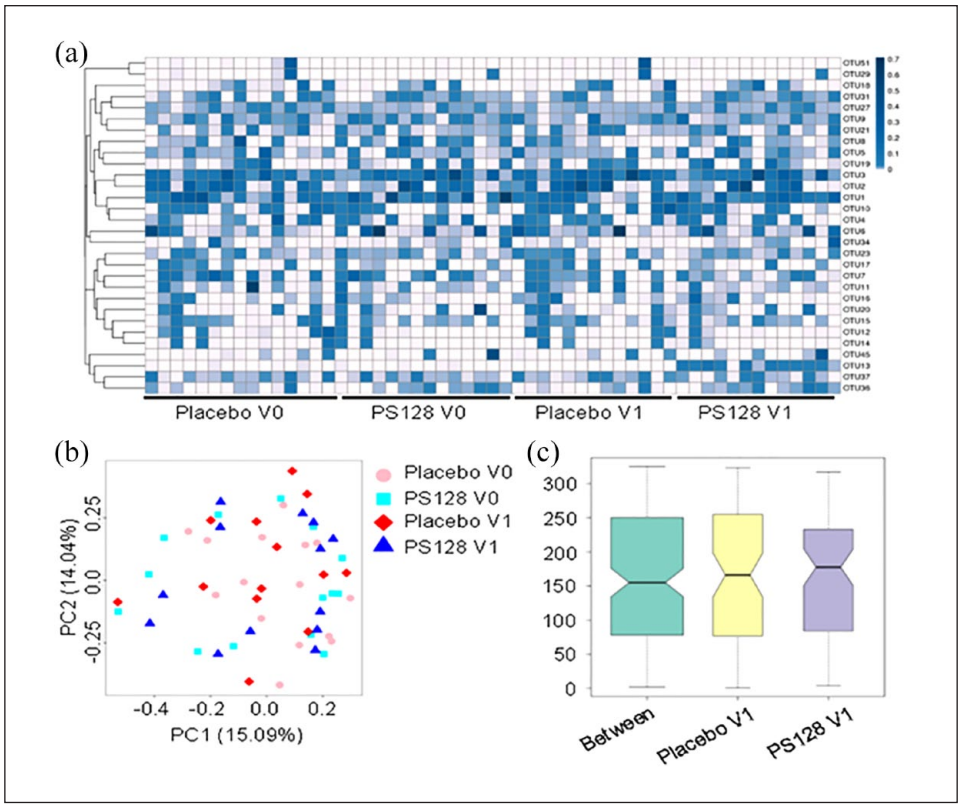

Figure 3. Microbiota comparison between each group. (a) Unclustered OTU heat map. Top 30 OTUs with the most relative abundance from each individual were shown. (b) PCoA analysis of all four groups. (c) Adonis analysis of placebo V1 and PS128 V1 (V0 = baseline; V1 = 16 weeks after intervention).

Similarly, the mean baseline scores for arm and leg dystonia were 19.61 and 18, respectively, and the reductions of 3.90 and 4.12 points represent approximately 19.89% and 22% of the baseline scoring for arm and leg dystonia, respectively. These findings indicate significant improvements in BFMMS total scores, arm dystonia, and leg dystonia in the PS128 group when compared to the placebo group. The magnitudes of these changes, as a percentage of the baseline scores, highlight the clinical importance of these observed differences.

Stool microbiota analysis confirmed the successful intervention of PS128, as it showed an increase in *L. plantarum/pentosus* in the PS128 group at the end of the study.

This study was based on previous research indicating GI dysfunction and dysbiosis of gut microbiota in RTT compared to normal controls (Borghi et al., 2017; Strati et al., 2016). Our results also revealed dysbiosis of gut microbiota in RTT individuals. In addition, previous studies have shown decreased BDNF levels and alterations in monoaminergic neurotransmitters in the brains of RTT individuals, contributing to the pathogenesis of the condition. BDNF plays a crucial role in cognitive function, and lower levels have been observed in autopsy brain samples from RTT individuals (Abuhatzira et al., 2007; Chang et al., 2006; H. Wang et al., 2006). Furthermore, modulation of the monoaminergic system may influence the motor deficits and dystonia observed in RTT (Samaco et al., 2009; Taneja et al., 2009). Systemic oxidative stress has also been identified in RTT individuals, strongly correlating with the patients' clinical status (Filosa et al., 2015; Leoncini et al., 2011).

The beneficial neurological effects of PS128 in RTT individuals are hypothesized to be mediated through the microbiota-brain axis (Figure 4). This axis, involving the gastrointestinal tract (GIT) and the CNS, provides bidirectional homeostatic communication through immunological, hormonal, and neuronal signals. Dysfunction of this axis has been associated with the pathogenesis of various neurological diseases (Cryan et al., 2019). In RTT, gut microbiome analysis has shown a proinflammatory status, reduced microbial richness, and altered SCFA profiles, suggesting a potential link between gut dysbiosis and disease severity (Borghi et al., 2017).

Previous studies have demonstrated that gut microbiota modulation through probiotic supplementation can impact brain function in neurological diseases, as evidenced in animal models and human studies (Hsiao et al., 2013; Y. W. Liu et al., 2019). PS128 has been found to ameliorate anxiety- and depression-like behaviors in mice by modulating brain neurotransmitters and reducing proinflammatory cytokines. It also increases BDNF levels and attenuates neuroinflammation and oxidative stress in a mouse model

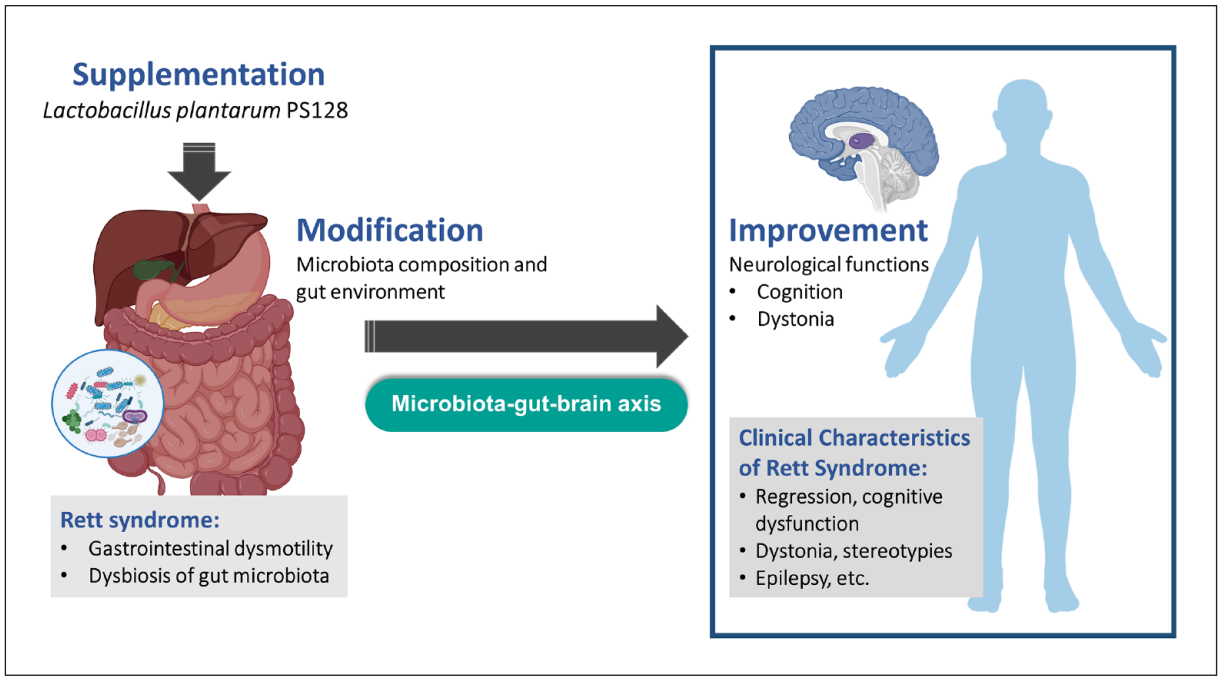

Figure 4. The schematic diagram illustrates the supplementation of PS128 and its impact on neurological function. Individuals with RTT exhibit neurological regression, cognitive dysfunction, dystonia, and stereotypies. In addition, they may experience gastrointestinal dysmotility and dysbiosis of gut microbiota. The supplementation of PS128 was shown potential to improve the neurological functions, including the overall cognitive developmental level and the dystonia of upper and lower extremities, possibly through microbiota–gut–brain axis.

of Parkinson's disease, leading to improvements in motor deficits (Liao et al., 2020). Moreover, a recent clinical trial on autism demonstrated that PS128 improved opposition/defiance behaviors in autistic spectrum disorder (ASD) children (Y. W. Liu et al., 2019). These findings suggest that PS128 can influence neurological function through multiple pathways in different models.

However, our study has some limitations. While the study provides valuable insights into the potential effects of PS128 in RTT, there are several limitations to consider. To begin, this is a pilot trial with a relatively small sample size, which may have limited the statistical power to detect significant differences in some outcomes. Future studies with larger sample sizes could provide more robust results. Second, the duration of the intervention was 16 weeks. It remains unclear whether long-term use of PS128 would yield more pronounced or sustained effects on cognition, dystonia severity, and other outcomes. A longer follow-up period could help to understand the long-term benefits and safety profile of PS128 in RTT individuals. Moreover, the assessments employed in this study, such as MSEL and the dystonia scale, have not been specifically validated for use in RTT. Although the MSEL has been utilized in other research involving RTT participants, there is currently no cognitive measure specifically validated for assessing cognition in RTT. Utilizing cognitive assessments specifically tailored and validated for RTT could improve the accuracy and relevance of the study's findings, providing a better understanding of how the probiotic affects cognitive function in this unique population. Moreover, the study did not conduct a statistical analysis of the genotypes due to the limited number of cases and considerable variability. Further research with a larger cohort of participants could explore potential genotype-related effects and responses to PS128 intervention. Finally, while PS128 improved neurological functions in our RTT cohorts, it did not alter GI function or gut microbial richness. The study did not examine gut microbiota inflammatory and metabolite profiles, suggesting that the impact of probiotics on neurological function in RTT may not be solely through microbiota composition changes but may involve alterations in metabolites, BDNF expression, or other signaling molecules. Nonetheless, considering the absence of curative medications for RTT and the oral tolerability of probiotics, our data provide valuable insights and justify further investigations into the long-term effects of PS128 supplementation in this population.

Conclusion

In conclusion, the study results suggest that PS128 intervention is feasible with a high retention rate. It may also have a positive impact on cognitive function and dystonia severity in RTT participants. The PS128 was well tolerated,

with minimal adverse effects. These improvements may be attributed to the modulation of the gut microbiota and its bidirectional communication with the brain through the microbiota–gut–brain axis. Future research with larger sample sizes and longer intervention periods is warranted to validate and extend these findings. In addition, further investigations could explore potential genotype-related effects and provide deeper insights into the underlying mechanisms of PS128 in RTT.

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Author contributions

L.C.W contributed to design and conceptualized the study, analyzed the data, and drafted the article for intellectual content and advised by W.-T.L.; C.-J.H and H.-P.W. helped in patient enrollment and interpreted the data and advised by W.-T.L.; Y.-T.W helped in the cognitive and behavioral measurement and analysis; H.-F.C contributed to the analysis of microbiota; J.-H.L. helped in statistic analysis of the data; S.-C.H and W.-C.T. helped in patient enrollment; Y.-C.T. helped in providing guidance on study design.

Availability of data and materials

The datasets used and/or analyzed during this study are available from the corresponding author on reasonable request.

Declaration of conflicting interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: Y.-C.T. owns stock in Bened Biomedical Co., Ltd. The other authors had no financial disclosure.

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Ethics approval and consent to participate

The institutional review board of National Taiwan University Hospital approved this study. All participants' families signed the informed consent to attend the study.

Consent for publication

Not applicable

Trial Registration

The study, titled "The Role of Probiotics PS128 in Movement Disorders," was reported on ClinicalTrials.gov (NCT03259971). https://clinicaltrials.gov/ct2/show/NCT03259971

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Supplemental material

Supplemental material for this article is available online.

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