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Evaluating the impact of essential amino acid-rich nutrition intervention on children with autism spectrum disorder: A randomized trial protocol ☆

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ABSTRACT

Background: Emerging evidence highlights the role of essential amino acids in brain function and behavior modulation, with deficiencies observed in children with autism. Amino acid supplementation appears to be effective in the autism management. This study aims to assess the effect of plant based amino acid intervention on plasma amino acid profile and behavior aspects in children with autism spectrum disorder.

Methods:

- The study involves parallel, randomized controlled trial that will include 68 children (age 3–6 years) with mild to moderate ASD. Children will be randomly assigned (1:1) to intervention group receiving nutritional intervention or a control group continuing standard care.
- The intervention will be carried out over 16 weeks, with a dosage based on the child's weight and dietary needs.
- Primary outcomes include changes in plasma amino acid concentrations Secondary outcomes include changes in Childhood Autism Rating Scale (CARS) and Autism Treatment Evaluation Checklist (ATEC) scores which would be measured pre- and post-intervention.

Results: Plasma amino acid levels and behavioral assessments will be compared between the groups to determine the effectiveness of the nutritional intervention in improving symptoms.

Conclusion: This trial seeks to establish a sustainable, non-pharmacological approach to managing autism symptoms. Findings would contribute to autism dietary management strategies and the role of essential amino acids in neurodevelopmental health.

☆ **Related research article:** Formulation and sensory evaluation of a plant-based essential amino acid rich food multimix" (2025) *Journal of Food Legumes*, 37(4), pp. 439–445. DOI: 10.59797/jfl.v37.i4.230. https://pub.isprd.in/index.php/jfl/article/view/1360

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Specifications table

Subject area:	Neuroscience
More specific subject area:	Food Science
Name of your protocol:	Protocol for Evaluating the Impact of Essential Amino Acid-Rich Nutrition Intervention on Children with Autism spectrum disorder
Reagents/tools:	Childhood Autism Rating Scale (CARS) Autism Treatment Evaluation Checklist questionnaire (ATEC)
Experimental design:	Parallel, randomized controlled
Trial registration:	Clinical Trial Registry of India (CTRI/2023/12/060,960).
Ethics:	On 11th September 2023, the Institutional Ethics Committee (IEC) of Sri Ramachandra Institute of Higher Education and Research, Chennai, Tamil Nadu, India, granted final approval for the study (Reference: IEC/23/JUN/179/21). Informed consent will be acquired from the parents of the children participating in the research, provided in both English and Tamil to ensure they understand the study protocol. The investigators will explain the details of the study to the parents.
Value of the Protocol:	**Innovative Nutritional Approach:** The protocol introduces a novel plant-based food multimix, combining millet and legume sources, to deliver essential amino acids — a sustainable, natural alternative to synthetic supplements. **Clinical Impact on Neurodevelopment**: By providing targeted nutritional support, this study aims to improve core symptoms of ASD, including communication, behavior, and sensory regulation, potentially enhancing overall quality of life. **Evidence-Driven and Scalable Solution:** The structured intervention design allows for systematic data collection, generating clinical evidence that can guide personalized nutrition strategies and be adapted for wider public health applications.

Background

Autism is a complex neurodevelopmental disorder encompassing a broad spectrum of neuropsychiatric disorders that impact brain development. Symptoms typically become apparent by the age of three. DSM-V classifies several conditions under the ASD umbrella, including autistic disorder, Asperger's syndrome, pervasive developmental disorder not otherwise specified (PDD-NOS), childhood disintegrative disorder, and Rett syndrome [1]. In India, an estimated 23 out of every 10,000 children are diagnosed with autism, based on the country's first comprehensive study on autism prevalence [1]. It is assessed that worldwide, nearly one in 100 children was diagnosed to have ASD (WHO 2022). The occurrence of ASD among children (2–5 years) visiting the Anganwadi centers in Maduranthakam block, Kancheepuram district, Tamil Nadu was estimated to be 0.6 % (5/773) [2]. Children with autism often experience feeding challenges, gastrointestinal issues, and sensitivity to food taste and appearance. Consequently, lower levels of essential amino acids in autistic children may partly result from inadequate intake or restrictive eating habits [3]. Amino acids play a critical role in metabolism and immunoregulation, and amino acid metabolic imbalances are accompanying the incidence and development of numerous central nervous system diseases [4]. The observation of amino acid deficiency-linked autism, which is significantly improved via supplementation, confirms the significance of amino acids in ASD [5]. Several plasma amino acids function directly as neurotransmitters, such as glutamate and aspartate, or serve as precursors, like tryptophan and tyrosine, for key neurotransmitters including serotonin and dopamine. Disruptions or deficiencies in these amino acid systems are commonly observed in children with autism and are known to significantly contribute to the manifestation of autistic symptoms [6]. Xing Yu et al., (2021) studied the serum levels of many essential amino acids in the ASD group, which were found to be significantly reduced [7]. Xion Chen et al., (2023) found Elevated neuroactive amino acids (glutamate) and decreased essential amino acids were mostly distinct characteristics of plasma amino acids of autistic children [8]. Nutrients and dietary supplements play a vital part in maintaining an individual's health, and there is substantial research demonstrating the influence of dietary factors on the development and pathogenesis of ASD. Amino acid supplements have been shown to alleviate symptoms by serving as precursors to neurotransmitters, which may help mitigate mental disorders [9]. A pilot study (2015–2018) involving 55 children (ages 6–18) tested daily Branched Chain Amino Acid (BCAA) supplementation at a dosage of 0.4 g/kg body weight using a mixture of leucine, isoleucine, and valine. After 10 weeks, 47 % showed improved social behavior, speech, cooperation, and reduced hyperactivity in the Childhood Autism Rating Scale (CARS) [10] In a double-blind, placebo-controlled trial, 37 children aged 3 to 11 years diagnosed with autism or pervasive developmental disorder were randomly divided into two groups. One group received a placebo, while the other was given N,N-dimethylglycine for a duration of four weeks. Behavioral assessments were performed using the Vineland Maladaptive Behavior Domain and the Aberrant Behavior Checklist. Although both groups improved overall, there was no statistically significant difference between the dimethylglycine and placebo groups [11]. Pesco et al. (2020) studied the effects of N-acetylcysteine (NAC) in four teenagers (ages 14–17) with ASD and high irritability, as measured by the Aberrant Behaviour Checklist-Irritability subscale (scores > 20). NAC was given in addition to current psychotropic drugs, resulting in behavioral changes such as decreased irritability and lower antipsychotic doses. The trial revealed good tolerance of NAC; nevertheless, the specific contribution of NAC versus the combined treatment is unknown [12]. Castejon et al. (2021) conducted a double-blind, randomized, placebo-controlled study on 46 preschool children (ages 3–5) with ASD to assess the effects of a 90-day cysteine-rich whey protein (CRWP) intervention. Children received either CRWP (0.5 g/kg or 10 g) or a placebo (rice protein). Among the 40 who completed the study, those receiving CRWP showed significant improvements in glutathione levels ($p = 0.04$) and VABS-II composite scores (effect size 0.98, $p = 0.03$), along with positive changes in adaptive behavior, socialization, and maladaptive behaviors [13]. A study assessed the effects of tryptophan-enriched cereal on sleep in children with ASD, cerebral palsy, and ADHD using a double-blind design. Participants alternated between control and tryptophan-enriched cereals for five weeks, with sleep activity monitored via wrist actimeter. Results showed improved sleep

efficiency in children with ASD, enhanced sleep efficiency and reduced wake bouts in those with cerebral palsy, and enhancements in assumed sleep, actual sleep duration, sleep efficiency, and immobile time in children with ADHD when consumed at both dinner and breakfast [14]. Abraham et al. (2022) performed a randomized controlled trial with 63 children (ages 3–6) with mild to moderate ASD, evaluating l-carnosine as an adjunct therapy. Despite the intervention, no significant differences were found in any outcome measures, suggesting that l-carnosine did not improve ASD symptoms [15]. Early nutrition plays a crucial role in neurodevelopment. Understanding the recent trends in nutritional status and feeding behaviors in children with ASD is essential to gain insight into the challenges they face and to emphasize the need for early, comprehensive interventions [16]. Existing research primarily focuses on synthetic supplements, leaving a gap in understanding the potential benefits of plant-based bioactives for cognitive health and emotional well-being. Shanmugam et al. (2021) highlighted the role of bioactive peptides from plant proteins, produced through gut microbial action, in improving memory, behavior, and cognition. These peptides, found in pulses, wheat, soy, and oats, may help balance gut microbiota and alleviate autism spectrum disorder (ASD) symptoms [17]. Plant-based interventions are essential to bridge this gap. This parallel randomized controlled trial is proposed with the following objectives:

- To assess the effect of plant-based essential amino acid intervention on plasma essential amino acid levels in children with ASD.
- To compare the changes in behavioral and biochemical outcomes between the intervention and control groups.
- To analyze the potential correlation between plasma essential amino acid levels and behavioral improvements in children receiving supplementation.

The study protocol was designed following the guidelines of the Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT) 2013. These guidelines provide a comprehensive outline to warrant transparency, quality, and consistency in the design of clinical trials and reporting. Following the SPIRIT 2013 checklist helps enhance the reliability and reproducibility of the study outcomes [18]. The trial is designed as a parallel, randomized controlled study with two groups. an intervention arm receiving plant-derived essential amino acid intervention and a control arm, with participants allocated in a 1:1 ratio. The study adopts an explanatory framework, aiming to evaluate the efficacy of the supplement under precise conditions to determine its impact on behavioral, social, communication, and language domains, as well as plasma essential amino acid profiles in children with ASD.

Description of protocol

Study setting

The trial will be conducted at the Karthikeyan Child Development Unit (KCDU), an early childhood development assessment outpatient department (OPD), and Vidya Sudha, an early intervention school. Both facilities are part of the Sri Ramachandra Institute of Higher Education and Research, located in Chennai, India.

Eligibility criteria

Inclusion criteria

- ➢ Children of both genders diagnosed with autism (mild to moderate CARS score between 30 and 36.5) between 3 and 6 years of age who are undergoing standard care processes of occupational therapy, speech therapy, and special education. The CARS assessment will be done by the certified clinical psychologists, and the diagnosis will be confirmed by the head of the developmental pediatrics.
- ➢ Parents who are willing to give consent for participation.

Exclusion criteria

- ➢ Children with severe ASD symptoms (CARS score from 37 to 60) on medications.
- ➢ Children who are allergic to gluten, casein, and soy.
- ➢ Children who are deficient or excess in two are more amino essential amino acids (His 68 to 120μmol/L, Ile 37 to 140μmol/L, Leu 70 to 170μmol/L, Lys120 to 290μmol/L, Met13 to 30μmol/L, Phe26 to 86μmol/L, Thr 67 to 150μmol/L, Try 26 to 110μmol/L, Val 160 to 350μmol/L (NIH 2022).
- ➢ Children with abnormal amino acid profile values, including glutamine, glycine, ornithine, and arginine.
- ➢ Children who are undergoing treatment for chronic metabolic disorders, genetic and chromosomal abnormalities, or severe intellectual disability.

Interventions

After initial screening and acquiring informed consent, children will be randomly assigned to the 2 groups (the control or the intervention group).

Intervention arm: Participants will receive food multimix sachets in eco-friendly zip-lock pouches labeled with specific days. The supplement will meet 60–70 % of essential amino acid requirements, with the remaining 30–40 % obtained through regular dietary intake. Initially, 10 g/day will be provided for the first 7 days. If no side effects are reported, the dosage will be increased to 25–35 g/day, calculated based on the child's height and weight using the ICMR 2017 recommendations for essential amino acids. The detailed methodology of the formulation of the food multimix was published under the title of Formulation and sensory evaluation of plant-based essential amino acid-rich food multimix in the Journal of Food Legumes. The product invention was registered and

published in the Intellectual Property Rights, India (application no 202,441,035,439, publication no 44/2024). Parents will receive instructions on how to administer the multimix and will maintain a log of daily consumption in a calendar diary provided to them.

Control arm: Participants will continue their standard care process. After the intervention period, parents in the control arm will be provided nutritional counseling on essential amino acid-rich food sources to support their child's dietary needs. Parents are advised to discontinue the supplement if gastrointestinal discomfort or allergic reactions persist.

To ensure compliance and adherence, the following strategies will be implemented:

- Parents in both arms will be provided with a calendar diary to track the consumption of ingredients used in the preparation of the food multimix. This will assist the researcher in identifying and addressing any potential biases related to food consumption.
- WhatsApp groups will be used for reminders and addressing queries related to the intervention.
- Parents in the intervention arm will be asked to return unused and used sachets during follow-ups.
- A 3-day dietary record including 2 weekdays and one weekend day will be conducted every 15 days to assess amino acid intake from regular meals.

Relevant concomitant care and interventions:

- Concomitant care such as routine therapy sessions such as occupational, behavior, speech, art, music, and yoga therapy will be permitted during the trial for both arms.
- Parents will be advised to give any additional protein or amino acid supplementation outside of the study intervention to their children.
- Any deviations in concomitant care will be documented and considered during data analysis.

Outcomes

Primary Outcome: The main outcome of this study is the plasma amino acid concentrations, which will be measured at pre and post-intervention (16 weeks). These levels include 48 amino acids, comprising the 20 basic amino acids that are essential for protein synthesis and all neuroactive amino acids. The change in plasma amino acid levels will be analyzed using the mean difference and compared between the intervention and control arms to assess the efficacy of the plant-derived intervention. Monitoring plasma amino acid levels offers valuable insights into the intervention's effectiveness in addressing amino acid imbalances and metabolic disturbances in children with autism. This statement is confirmed by the findings of Randozzo et al. (2023), who concluded that analyzing amino acid profiles in ASD patients enhances the understanding of metabolic pathways that can be targeted for therapeutic interventions [19].

Secondary Outcomes: Childhood Autism Rating Scale (CARS) scores will be assessed at the beginning and at the end of the research to determine variations in autism symptom severity [20]. The mean score differences will be analyzed to measure the treatment effect. Autism Treatment Evaluation Checklist (ATEC) scores will be assessed pre and post-intervention to evaluate improvements in speech/language, sociability, sensory/cognitive awareness, and health/physical behavior. The changes in ATEC scores will indicate the overall effectiveness of the intervention. Anthropometric data, including age, gender, height, weight, and mid-upper arm circumference, will be collected at the beginning and end of the study. These metrics will be analyzed to monitor physical growth and ensure the intervention's nutritional adequacy.

The rationale for analyzing plasma amino acids as the primary outcome is that blood parameters serve as a reliable and objective indicator of intervention efficacy. Unlike subjective assessments such as CARS and ATEC, plasma amino acid analysis provides quantifiable data. Additionally, numerous studies have concluded that amino acid profiling is one of the most effective indicators for identifying underlying issues. The treatment effect will be determined by comparing changes in plasma amino acid levels, CARS scores, and ATEC scores between the two groups over the study period.

Participant timeline

Fig. 1 displays the participant timeline at different time points throughout the study.

Sample size

Design: Independent groups

$$n = \left(\frac{r+1}{r}\right)\frac{\sigma^2(Z_\beta + Z_{\alpha/2})^2}{(d\text{ifference})^2}$$

$$n = \left(\frac{1+1}{1}\right) \times \left(\frac{(1^2) \times ((0.95 + 1.96)^2)}{0.5^2}\right)$$

r = **ratio** of controls to cases = 1

$\boldsymbol{\sigma = 1}$, $\mathbf{Z}_{\boldsymbol{\beta}}$= 0.95, $\mathbf{Z}_{\boldsymbol{\alpha/2}}$ = 1.96, $\boldsymbol{d}$ = **Effect Size** (the difference in means) = 0.5

n = 68 (34 for each arm)

The effect size was derived from the article titled "Effect of l-Carnosine as adjunctive therapy in the management of children with autism spectrum disorder: a randomized controlled study" (Abraham et al. [15]).

The sample size was derived using GPower v3.1.

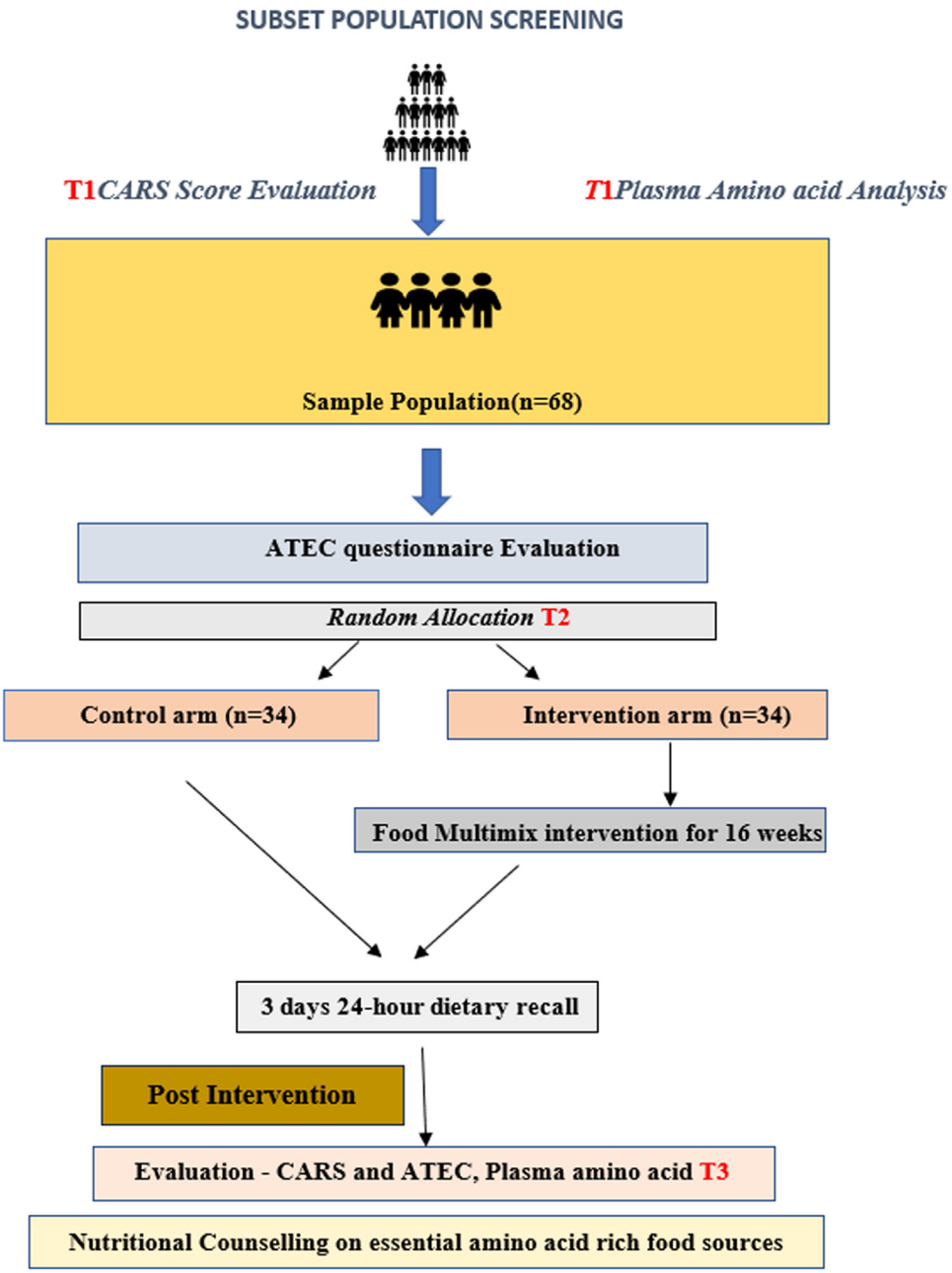

Fig. 1. illustrates the participant timeline across various time point.

Enrollment

To ensure adequate participant registration and achieve the desired sample size, the recruitment strategy will involve collaboration with the developmental pediatrician and clinical psychologists. The process will be straightforward, with accessible consent forms and assistance from the principal investigator to guide parents through each step.

Assignment of interventions

Random allocation of the 68 participants into intervention and control arms will be conducted by the RAND formula in Microsoft Excel. Each participant will randomly draw a chit from a jar containing all 68 numbers. The number drawn will correspond to the pre-generated random allocation in the Excel sheet, defining their allocation in the 2 groups (the intervention or control group). The data analysts will be the only individuals remaining blinded to the group assignments.

Data collection, management, and analysis

Plasma amino acid analysis will involve the collection of 4 mL of peripheral blood from participants, drawn by trained pediatric phlebotomists into heparinized cuvettes and sent for analysis to the central laboratory at Sri Ramachandra Medical Center. The quantification of participants plasma-free amino acids will be performed using high-performance liquid chromatography/electron ionization mass spectrometry (HPLC/ESI-MS) with pre-column derivatization [21]. The CARS (Childhood Autism Rating Scale) will be administered through direct observation of the children and structured interviews with parents, while the ATEC (Autism Treatment Evaluation Checklist) will be administered by the investigator during both pre- and post-assessments. Assessors will be trained to ensure data consistency and accuracy, with duplicate measurements conducted where feasible to enhance data quality. The study instruments, including CARS and ATEC, have demonstrated reliability and validity in similar populations for assessing autism-related behaviors and treatment responses [22,23]. In India, the National Nutrition Monitoring Bureau and the Indian Nutrition Profile Survey utilize the 24-hour dietary recall method to assess dietary intake. This open-ended survey collects detailed information on food consumption within a 24-hour period, beginning with the last consumed meal. The investigator requests the children's parents to recall the foods consumed, including quantities and leftovers, which are subtracted from the total amount of cooked food. Raw ingredients used in cooking are also recorded, and respondents are asked to point out the spoons or cups used to measure components for accurate data. To minimize biases, data collection is conducted during a week without festivities, fasting, or feasting that could influence dietary habits [25]. Calculations will be made based on the Indian Food Composition Table 2017. To promote participant retention and follow-up, regular communication with participants and caregivers will be maintained through appointment reminders, educational sessions, and flexible scheduling options. Efforts will be made to collect partial data in cases of participant discontinuation or protocol deviation, with documentation of all collected data to analyze trends and potential causes for non-adherence.

Data entry will be carried out by the principal investigator and stored in a password-protected folder. Entire data will be retained for five years after the completion of the study. Statistical analysis will be done in the SPSS 2023 version. Independent *t*-test to compare the mean plasma amino acid levels between the intervention and the control group. A paired *t*-test will be applied to analyze the changes in amino acid values within the groups. Mann-Whitney The U test is used to compare differences between groups when the data do not follow a normal distribution. Pearson's correlation/Spearman's rank correlation to assess the association between changes in plasma amino acid levels and behavioral improvements.

Monitoring

The data will be presented to the Institutional Ethics Committee for approval. The progress of the research will be reviewed once every 6 months as per the ICMR and NDCT rules, 2019. Incomplete data analysis based on the underlying mechanism. Nonignorable missing data, which depends on the missing values themselves, can be addressed using group-based models and multiparameter models. When incomplete data are considered negligible after correcting for relevant covariates, methods such as random-effect models and multiple data imputation can be applied. In cases where missing data are completely ignorable, standard approaches like complete case analysis or change from baseline methods can be used. To ensure the reliability of results, sensitivity analyses under different assumptions are performed to evaluate the potential impact of missing data [24]. In the event of any harm or side effects, the incident will be promptly reported to the IEC. Appropriate actions will be taken, including suspension from the study, re-evaluation of the intervention, dosage modification, enhanced monitoring, and referral to healthcare professionals if necessary.

Ethics and dissemination

On 11th September 2023, the Institutional Ethics Committee (IEC) of Sri Ramachandra Institute of Higher Education and Research, Chennai, Tamil Nadu, India, granted final approval for the study (Reference: IEC/23/JUN/179/21). The trial was prospectively registered in the Clinical Trial Registry of India (CTRI/2023/12/060,960). The Four Principles of Biomedical Ethics—autonomy, beneficence, non-maleficence, and justice—serve as the foundation for ensuring ethical conduct in clinical trials, safeguarding participants' rights, safety, and dignity, while preserving scientific integrity.

Informed consent will be acquired from the parents of the children participating in the research, provided in both English and Tamil to ensure they understand the study protocol. The investigators will explain the details of the study to the parents. Contact information will be included in the consent form for reporting any side effects. Personal information will not be shared with anyone. Before statistical analysis, participants will be given a specific number instead of their names to ensure anonymity. Blood test results will be shared with the parents in PDF format via email or WhatsApp. At the conclusion of the study, the study findings will be published in a peer-reviewed open-access journal to facilitate dissemination. Participant-level datasets will be included as appendices in the journal. Additionally, partial data will be presented at conferences during the course of the study.

Protocol validation

Autism is a neurophysiological condition that emerges in initial childhood and has a lasting impact throughout life. It disturbs various developing stages of a child's life, including communication, behavior, education, social interactions, and even nutritional status. As a result, individuals with autism are at increased risk of experiencing nutritional imbalances [26]. Inadequate intake of essential amino acids can result in a range of clinical symptoms, such as vomiting, loss of appetite, depression, anxiety, insomnia, fatigue, weakness, and growth retardation in children. These signs arise primarily from the body's inability to synthesize proteins due to an absence of essential amino acids. A sufficient supply of amino acids is crucial for the production of neurotransmitters,

hormones, muscle growth, and various cellular functions. Such insufficiencies are more common in economically disadvantaged regions [27]. Animal-sourced proteins deliver all the essential amino acids essential to the human body. However, the rising obesity epidemic has sparked growing interest in plant-based protein diets and their potential effects on both health and the environment [28]. This randomized controlled trial aims to address amino acid imbalances and provide a sustainable, plant-based amino acid-rich intervention to manage ASD symptoms and behavior. The trial will respond to the growing demand from both autistic children and their parents for feasible, non-invasive, and non-pharmacological interventional approaches. It will also provide a valuable finding for ongoing international discussions about the role of nutrition and amino acids in autism management. The study will employ a stringent methodology with objective primary outcome measures to assess autism-associated characteristics and will also document developmental, cognitive, emotional, and behavioral challenges. This comprehensive approach will provide a broad assessment of the effectiveness of the plant-based amino acid intervention for the children with autism and their families.

Limitations

The study on the essential amino acid-rich supplement intervention may have certain limitations. The duration of the intervention (4 months), might not fully capture the long-term effects or improvements. Dietary variability among participants, including differences in dietary intake and lifestyle factors, could pose inconsistencies in outcomes. Additionally, adherence to the supplement might be affected by sensory sensitivities common in children with ASD, influencing compliance and overall effectiveness. Without a placebo or blinding, there is also a risk of caregiver or participant bias affecting perceived results. The choice of outcome measures could further limit the study, as behavioral and cognitive changes might be challenging to quantify objectively. Moreover, individual differences in metabolism and nutrient absorption could lead to varied responses among participants. Acknowledging these limitations is essential for interpreting the results and refining future research.

CRediT author statement

Jayashree R: Conceptualization, Methodology, Writing- Original draft preparation. **Gayathri G:** Conceptualization, Methodology, Supervision, Validation, Reviewing and Editing. **Udayakumar N:** Conceptualization, Methodology, Reviewing and Editing. **Kalpana CA:** Conceptualization, Methodology, Reviewing and Editing.

AI declaration

Chat GPT was used to enhance the language. However, the content was not generated.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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