**Effect of probiotic supplementation on fecal microbiota in children with autism spectrum disorder-a pilot randomized controlled trial**

Autism or autism spectrum disorder (ASD) is a set of heterogeneous neurodevelopmental conditions, characterised by early-onset difficulties in social communication and unusually restricted, repetitive behaviour and interests.1 The prevalence of ASD has increased considerably over the past 30 years, and it is now diagnosed in 1.46% of individuals.2The estimated lifelong cost of supporting an individual with ASD is $2.4 million in USA and £1.5 million in UK.3 A study from Western Australia reported the median ASD-related annual cost to the family as $34,900 with an extra cost of $1,400/year for each additional symptom.4 Genetic predisposition and environmental factors (e.g. intestinal dysbiosis, excessive inflammation, altered intestinal permeability, oxidative stress, perinatal nutritional deficiency, and immune imbalance) play an important role in the pathogenesis of ASD.5 Current options for improving developmental outcomes of children with ASD are limited. Behavioural interventions and drugs only partially improve ASD symptoms, and drugs are often associated with adverse effects.6, 7A systematic review reported no conclusive evidence supporting complementary and alternative therapies for improving ASD symptoms.8

Emerging evidence suggests that modulating the gut microbiota-brain axis by probiotic supplementation may be a novel therapy for improving developmental outcomes in ASD.9, 10 Gut symptoms are common in children with ASD.  The median (range) prevalence of constipation, diarrhea and of any or ≥ one symptom is 22% (4.3-45.5%), 13.0% (2.3-75.6%) and 46.8% (4.2-96.8%) respectively.11

A systematic review of gut microbiome showed that gut dysbiosis is common in children with ASD.12-15 Other studies show that disruption of the gut-brain axis 16 and elevated short chain fatty acids (SCFA), especially propionic acid 17, 18 may play an important role in the pathogenesis of ASD. Fiorentino et al reported increased expression of genes and proteins associated with blood brain barrier dysfunction, neuro-inflammation, and intestinal permeability in post-mortem brain and duodenal samples from children with ASD compared with children without ASD or Schizophrenia.19 Furthermore, the promising results of fecal microbiota transplant support the involvement of gut microbiota in ASD.20

Probiotics are a promising strategy for improving the gastrointestinal (GI) and behavioural symptoms in children with ASD considering their ability to modulate the gut-microbiota-brain axis by various pathways.21, 22 Probiotics correct dysbiosis, inhibit gut colonization with pathogenic bacteria23 , enhance gut barrier function24 25, and enteric nervous system maturation26, and exert an anti-inflammatory effect.27 Studies in mouse model show that probiotics ameliorate defects in communicative, stereotypic, anxiety-like and sensorimotor behaviours.28, 29 Even, single strain of gut bacteria (*Lactobacillus (L). reuteri*) has been shown to reverse autism-related social behavior in mice.30 Neurodevelopmental conditions such as ASD have a strong genetic etiology. Tabouy et al identified gut dysbiosis in Shank3 KO mice. Decreased relative abundance of *L. reuteri* positively correlated with brain expression of gamma-Aminobutyric acid (GABA) receptor subunits. Supplementation with *L. reuteri* attenuated unsocial behavior specifically in male Shank3 mice, decreased repetitive behaviors in both male and female Shank3 KO mice, and affected GABA receptor gene expression and protein levels in multiple brain regions, supporting the potential of probiotics in improving behavioural symptoms of ASD**. 31** El-Ansary et al reported that probiotic treatment reduces the autistic-like excitation/inhibition imbalance in juvenile hamsters induced by orally administered propionic acid and clindamycin.32

**Kaluzne** et al reported significant improvement in core symptoms of autism (e.g. eye contact, correct recognition of human emotion) accompanied by significant modification in urinary arabinol in 22 children with ASD supplemented with *L. acidophilus* for two months.33 **Parracho** et al studied the effect of *L. plantarum* WCSF1 (4.5x1010 CFU/day) on gut microbiota in children with ASD (3-16 years) in a double-blind, placebo-controlled, crossover-trial over 12-weeks. Probiotic supplementation increased the lactobacilli and enterococci counts, and reduced the *Clostridium* cluster XIVa counts significantly compared to placebo. It also improved the stool consistency and total behaviour problem scores significantly from the baseline values compared with placebo.34 In a surveyof ASD children with GI symptoms receiving a multistrain probiotic, caregivers assessed ASD signs and symptoms before and after 21 days of treatment using the autism treatment evaluation checklist (ATEC). Severity of diarrhea and constipation was reduced in 48% and 52% of the children respectively. Total ATEC score was reduced in 88% of children, suggesting improvement in ASD symptoms.35 Recently, **Shaaban** et al assessed the gut microbiota (q-PCR), and GI and autistic symptoms (ATEC) in 30 autistic children (5-9 years) in an open label study of probiotics (Total 100×106 cfu of *L. acidophilus,* *L. rhamnosus* and *Bifidobacterium (B.) longum* per gram). After 3 months’ of supplementation faecal bifidobacteria and lactobacilli counts increased and were accompanied by significant improvement in GI and autism symptoms compared with baseline values.36 None of the clinical studies reported adverse effects related to probiotic supplementation.

A recent updated systematic review, and expert recommendations have emphasised the need for rigorous RCTs of probiotics in children with ASD.37,38,39 Considering the encouraging evidence from preclinical and clinical studies, we aim to evaluate the role of probiotics in children with ASD.

**Aims and hypotheses**

We aim to assess the feasibility of a conducting a definitive RCT assessing effects of probiotics in community based young children with ASD.

*Primary hypothesis:* Children with ASD receiving probiotic supplementation will have significant compositional changes in gut microbiome compared to those receiving placebo.

*Secondary hypothesis:* The compositional changes in gut microbiome will be accompanied by changes in fecal SCFA, GI and core behavioural symptomsof ASD.

**Methods and participants**

We propose a pilot RCT to assess feasibility of recruiting community based young children with ASD, assuring their follow up at TKI for the comprehensive assessments, and collecting stool samples from home, followed by safe transfer, storage, analysis. However, this pilot trial will have adequate power (80%) to test the primary hypothesis i.e. probiotics (compared with placebo) will lead to significant changes in the composition of gut microbiome.

Children (age 2 to 5 years) with confirmed diagnosis of ASD will be randomised to receive oral supplementation with either probiotic or placebo in a 1:1 ratio after obtaining a written informed consent from a parent/legal guardian.

**Ethics:** Institutional ethics committee approval will be obtained before commencing the recruitment.

**Randomisation, allocation concealment, and blinding:** Randomisation (using computer generated random numbers) will be stratified based on Mullen’s Early learning composite score as <85, or ≥85. This approach is selected because consideration of the intellectual/developmental ability is important as it reflects severity of ASD, and influences the ability for proper neurodevelopmental assessment. (Figure 1) Allocation concealment will be optimised by using serially numbered, sealed, coded, opaque envelopes and recording demographic data before allocation. The probiotic and placebo sachets will be equal volume, identical in appearance, to ensure masking of investigators, participants, and outcome assessors. Randomisation will be initiated once a consented and screened participant has met all eligibility criteria and baseline assessments have been completed. The trial manager will randomise the participants and allocate a set of sachets containing either the probiotic or placebo (labelled with codes to maintain blinding).

Environmental conditions for sachet storage will be monitored in a securely located fridge using an electronic temperature logging system (records temperature every two minutes and sends an alarm when temperature exceeds an acceptable threshold outside of the 2-8 degree range). Temperature logs will be monitored weekly to ensure maintenance of environmental conditions. Any temperature excursion greater than 8 degrees will be notified to the lead investigator within 24 hours and documented in temperature logs. Any study medication affected by temperature excursions will be kept separate from unaffected medication until approved for use by the lead investigator. At the end of the trial, the parents/caretakers of the recruited children will return any unused medications to the research team.

**Pre-stated subgroup:** This will be based on the GI Severity Index (GSI).40 Children with a total score of ≥4 (with at least 3 score points from the first six items of the scale) will be considered as having significant GI symptoms (Appendix 1).

**Participants and recruitment:** We aim to enrol 40 children (age 2 to 5 years) with confirmed ASD, from the community using the comprehensive database at the CliniKids (Telethon Kids Institute) - an internationally recognised premier Australian research institute focused on improving the health and wellbeing of children. CliniKids runs a dedicated research program for children with ASD headed by one of the principle investigators (AW).

**Inclusion criteria:** (1) Age: 2–5 years. We have selected this as the age of the target population considering early intervention improves outcomes due to plasticity of the developing brain and potentially modifiable abnormalities in brain circuity.41-43 (2) Confirmed diagnosis of ASD based on DSM-5 criteria.44

**Exclusion criteria:** (1) Major congenital anomalies (2) Epilepsy syndromes, significant sensory impairment (e.g., blindness, deafness), neonatal hypoxic ischemic encephalopathy requiring therapeutic cooling (3) Coeliac disease, inflammatory bowel disease (4) Use of probiotics for ≥4 weeks in the 90 days before enrolment (5) Current or recent (within 4 weeks before enrolment) exposure to antibiotics, chemotherapy or immunosuppressant agents, or laxatives. (6) Prosthetic devices including heart valves (7) Confirmed HIV, Hepatitis B, and/or Hepatitis C. (8) Known allergy to probiotics (9) Special diets(10) Cows’ milk protein allergy, food allergy, or conditions such as atopic dermatitis, or eczema.

**Intervention:** The probiotic selected for this study is Vivomixx® Italy, with each sachet containing total 450 billion (450x10^9) lyophilized bacterial cells of eight probiotic strains: *Streptococcus thermophilus* DSM 24731*, B. breve* DSM 24732, *B. longum* DSM 24736, *B. infantis* DSM 24737), and *L. acidophilus* DSM 24735, *L. plantarum* DSM 24730, *L. paracasei* DSM 24733*, L.* *delbrueckii subsp. bulgaricus* DSM 24734). All strains in Vivomixx work in synergy (http://www.vivomixx.eu/en/2017/09/06/product-facts-why-so-many-different-strains/) Vivomixx® is currently being studied for various conditions in children (non-alcoholic steatohepatitis, infantile colic and ASD) 45-47 and adults (HIV) 48, 49, and pregnant women. 50It is available as water-soluble powder and administered orally. It can be dissolved directly in the mouth or mixed in a cold, but not carbonated drink.

**Probiotic protocol:** Our probiotic selection and protocol is based on ongoing *(ClinicalTrials.gov Identifier: NCT03369431,* *NCT02708901,* *NCT02903030)* and previous *51-54* studiesincluding a survey reporting that multi-strain high dose probiotics may be more effective in conditions associated with severe and chronic gut dysbiosis55 , and the recommendations of an expert group for probiotic RCTs in ASD. 37

The participants will receive 450 billion CFU of probiotics (*or an equivalent volume of placebo)* twice a day for one month followed by 450 billion CFU (*or an equivalent volume of placebo)* once a day for three months.47 The placebo sachets will contain 4.4 g of maltose and silicon dioxide. The trial supplement will be administered to children at home by their parents/legal guardian. The duration of supplementation ensures adequate time for gut colonization, which requires 2–3 weeks on an average 56, and for influencing clinical outcomes.

To ensure compliance or for any adverse effect, the research team will contact the parents/caregivers weekly basis. Participants will be allowed to stop the trial supplement in case of intolerance (vomiting, diarrhoea) or if requested by their parents/legal guardians. Parents/legal guardians will be provided contact details of the research team for round the clock communication.

**Stool sample collection and analysis:** Fecal microbiota will be assessed using the 2 samples collected from each participant (before and after completing 4 months of study supplementation). Metagenomics studies will be performed by extracting the fecal DNA and creating an amplicon 16s rRNA library for analysis by high throughput 454 pyrosequencing to allow calculation of diversity indices and rarefaction curves. (Appendix)

**Primary outcome**

This will be the “Compositional differences” in gut microbiome. The selected markers of ‘compositional differences’ will include alpha-diversity, richness, and abundance of particular species/pathways, or clusters based on unsupervised clustering of gut microbiome.

**Secondary outcomes**

(1) *Developmental outcomes:* Participants will undergo following developmental assessments and parenteral questionnaire at the time of enrolment and after four months of trial supplementation performed by accredited and experienced multidisciplinary research team members (Appendix)

(a) *Psychosocial and cognitive outcomes:* The Preschool Language Scale – 5 , Repetitive Behaviour Scale – Revised (RBS-R), The Short Sensory Profile (SSP-2),Vineland Adaptive Behavioural Scales – 2nd edition (VABS-II), Mullen’s Scale of Early Learning (MSEL)

(2) *Gastrointestinal outcomes:* These will be assessed by theGSI (Appendix).

(3) *Fecal SCFA:*Fecal acetate, propionate, and butyrate levels will be analysed using gas chromatography.

**Approach to bioinformatics analysis for primary outcome**

**Sample size estimation:** Based on the simulated gamma parameter of Dirichlet-multinomial distribution57 (R package 'HMP') in the autism metagenomics data58, the frequency profile of the top 14 most abundant genera (cumulative frequency >95% of the total quantifiable reads) from 40 autism and 31 neurotypical individuals of public data was used as input. Samples from 20 children in each group (50,000 16S reads; ~50M shotgun reads) would have ~80% power to detect the compositional taxonomic difference of gut microbiome at 5% α level. Most of the parametric and non-parametric test (Wilcoxon Rank-Sum Tests) will have 80% power for individual metagenomics markers (e.g. abundance of particular species) given a non-negligible effect size (fold change or Cohen's d) at sample size 20. T-test will be preferred in our analysis by converting the raw variables into normal-distribution variables.

The power of detecting compositional difference between two groups (autism and healthy control) at a given level of type 1 error (5%) depends on two factors— the sample size and the number of reads. The reads number of 50,000 is one of the common throughputs of 16S rRNA sequencing (it corresponds to around 5Gb shotgun metagenomic sequencing). We have used this fixed number of reads in our power evaluation. The number of samples in each group will influence the type 2 error (and power) detecting the compositional differences between two groups based on metagenomic simulations. The number of Monte-Carlo experiments was set to 5000 for each simulated sample.

The approach to bioinformatics analysiswill be based on the principle of intention to treat*.*

**Type of variables**: (1) Categorical: Presence/absence of particular species or pathways; the clusters based on unsupervised clustering of gut microbial profile, etc. (2) Continuous: Alpha-diversity, richness, abundance of particular species or pathways, or the difference of these numeric variables (treatment vs baseline) etc.

**Odds ratio (OR) vs relative risk (RR):** The R package "sjstats" and “logisticRR” can be used to calculate the OR and the RR between binary outcomes (e.g. presence/absence of a particular species or a pathway) and the treatment options

**Approach to statistical analysis for** **clinical (secondary) outcomes**

Continuous variables will be compared using the t test for normally distributed data and Wilcoxon rank sum test for skewed data. Categorical variables will be compared using the fisher’s exact test. Logistic regression analysis will be used for analysis of binary outcomes (primary outcome) to derive relative risk and 95% confidence intervals (CI). Linear regression analysis will be used for continuous outcomes to derive regression coefficients and respective CI. A p value <0.05 will be considered statistically significant.

**Data and safety monitoring:** An independent data and safety monitoring committee (DSMC) will be established to monitor the safety of participants, and quality of data collection 59. The DSMC will comprise of a chair, clinician and a statistician who are not involved in the trial. Interim analyses will be done after recruiting 20 participants. We will use unadjusted chi-squared test to compare the probiotic vs. placebo group outcomes. The trial will be stopped if significantly more individuals in the probiotic group will have significant adverse events such as anaphylaxis or probiotic sepsis compared to those receiving placebo (p<0.0294, binomial test). Stopping the trial under such circumstances will be guided by the Pocock rule60 and advice from the DSMC.

**Independent assessment of trial supplements**: Dr AD Keil (Department of Microbiology, PathWest Laboratory Medicine WA, Perth Children’s Hospital) will assess the probiotic and placebo in the NATA certified laboratories, to (1) confirm the presence of lactobacilli and bifidobacteria and (2) test for contaminant viruses (adenovirus type 40/41, rotavirus and norovirus by PCR) and bacteria (*Campylobacter, Shigella, Salmonella* species and *Clostridium difficile* toxin gene by PCR).

**Team and track record:** Guided by Prof Whitehouse and Prof Patole, the research team has international recognition in autism as well as probiotic research. Furthermore, the team has excellent track record in clinical trials, translational research, and evidence based practice of medicine. Considering the expertise and track record of CliniKids in research on community-based children, we will complete the recruitment in 2 years.

**Data handling, storage, confidentiality:** The NHMRC Australian guidelines will be followed for data handling, storage and protecting confidentiality 61.

**Reporting:** CONSORT check list will be followed for reporting the results62 .

**Conflict of interest:** None of the investigators have any conflict of interest of any kind;

**Sponsorship:** *TKI/CAHS will be the sponsor of the study (to be finalised ASAP)*

**Role of Manufacturer:** The manufacturer will only supply the product free and not have any role in the design, conduct, analysis, and reporting of results

**Funding:** We have internal funding to support the trial.

**Translational significance:** The novel and robust data generated from our study will help in designing an adequately powered definitive RCT assessing the effect of probiotics on clinically important core behavioural symptoms in children with ASD. The probiotic selected for the study is commercially available. Given their simplicity, availability, and low cost, translation of probiotics into clinical practice is expected to be easy. The increasing use of probiotic supplementation as standard treatment for high-risk preterm infants supports our expectation.

**Appendix**

**(1) Gastrointestinal severity index (GSI):** The GSI40 is a composite score designed to capture signs and symptoms of gastrointestinal distress reported very commonly by parents of children with ASD. It was constructed by adapting a modified Truelove and Witts Severity Index used for clinical trials in ulcerative colitis (Lichtiger et al. [1994](https://link-springer-com.pklibresources.health.wa.gov.au/article/10.1007/s10803-006-0141-y#CR24))63 to incorporate a total of nine variables relating to GI signs and symptoms.GSI score ≥7 (Total score: 0–15, with higher values corresponding to greater severity) indicates moderate to severe gastrointestinal distress.

**(2) Developmental assessments**:

***(a)* The Preschool Language Scale – 5** (PLS-5): The PLS-5 is a clinician-administered assessment tool designed to evaluate Auditory Comprehension (receptive language), Expressive Communication (expressive language), and overall language functioning in children from birth to age 7 years, 11 months 64. It has been shown to be a psychometrically valid and reliable measure of language skills. Cronbach’s alpha is fair to excellent for children under 2 years, 5 months (α = 0.80–0.96), and excellent for older children (α = 0.91–0.97).

***(b)*The Short Sensory Profile (SSP-2**): The SSP is a caregiver-report questionnaire that is applicable for children aged 3–14.65 The 34 item questionnaire uses a 5-point Likert scale for caregivers to report how frequently their child responds to sensory input in their daily activities. Internal consistency (0.79–0.86), test-retest stability (0.83–0.97) and inter-rater reliability (0.70–0.80) are all adequate.

***(c)*Repetitive Behaviour Scale – Revised (RBS-R):** The RBS-R is a 43-item caregiver-report questionnaire. Each item is scored on a four-point Likert scale ranging from 0 (behaviour does not occur) to 3 (behaviour occurs and is a severe problem).66Higher scores indicate greater levels of atypicalities.

***(d)Vineland Adaptive Behavioural Scales – 2nd edition (VABS-II):*** The VABS II is a commonly used measure of adaptive functioning in the domains of communication, social skills, daily living skills, and motor skills for individuals aged from birth through 90 years of age. The current study will use the caregiver completed ‘survey form’ of **VABS-II**, which takes ~20 minutes to complete. The scale has acceptable psychometric properties, including excellent split half reliability coefficients (r=0.94 to 0.97) and test–retest reliability (r=0.93).67 ***(e)******Mullen’s Scale of Early Learning (MSEL):*** This is a standardized developmental assessment of cognitive functioning of young children from birth to 68 months. It is based on the child’s responses to activities prepared by the examiner. 68 It takes ~40 minutes to complete and measures five skill domains, including receptive and expressive language, areas of interest for the current study. Scores from each of these scales are transformed into T-Scale scores, centred around mean of 50 (SD=10). Scores from the subtests are combined to form an ‘Early Learning Composite’, which provides a measure of the overall neurocognitive development (M=100, SD=15). Test–retest reliability is adequate (M=.90; R=.71–.96) and inter-scorer reliability is strong (R=.91–.99).

**(3) Stool samples and analysis:** These will a) As soon as possible after enrolment, but *before commencing* the trial supplement b) *Within one week after completing the 4 months of supplementation*. If a child were to drop out of the study before four months, every effort will be made to collect the stool samples at that stage. Written instructions will be given to parents to collect the stool samples in the yellow specimen jars provided at enrolment. They will be asked to keep the samples in their kitchen fridge at 4-60C (not the freezer compartment). Once collected, they will call the study coordinator. An accredited courier will be sent to the patient’s home to collect the sample and bring it back to the CliniKids , where it will be stored in the deep freezer at -80-degree C in cryovials. Stool sample analysis will involve fecal metagenomics and SCFA assay69, 70 71, 72 to study the pathways of probiotic effects.

**Metagenomics** will be performed by extracting the fecal DNA and creating an amplicon 16s rRNA library for analysis by high throughput 454 pyrosequencing to allow calculation of diversity indices and rarefaction curves. Resulting sequences will be compared with published sequence libraries data for taxonomic classification. The DNA will be extracted from stool samples using the method of Matsuki et al 2003 73. Briefly, the stool samples will be thawed on ice, diluted to allow harvesting of the bacterial cells by centrifugation. Bacterial cells will be re-suspended in lysis buffer and broken using a bead beater. Harvested DNA will be purified before use in PCR reactions.Amplicon libraries targeting the 16S rRNA encoding gene will be prepared to obtain deep surveys of the microbial communities using the GS-FLX platform. PCR primers specific for the V3-V4 region of the 16S rRNA encoding gene (Escherichia coli positions 338 to 802) containing 454-specific adapter sequences as well as an 8-base pair barcode will be utilized. This barcode-based primer approach allows sequencing of multiple samples in a single 454 sequencing run without needing physical partitioning.

**Analysis of sequencing data:** Processing of the 16S rRNA-derived sequence inventories will be performed using the QIIME toolkit (QIIME 1.5.0). Briefly, OTUs will be selected at 97% sequence identity using uclust and a representative sequence will be chosen for each OTU based on the most abundant sequence. Representative sequences will then be aligned using PyNAST, and a taxonomic classification will be assigned to representative sequences using the RDP Classifier. These PyNAST-aligned sequences will be used to build a phylogenetic tree with FastTree, and unweighted UniFrac distances calculated between all samples.

**SCFA:** Fecal SCFA (acetate, propionate, and butyrate) levels will be analysed using gas chromatography.

**Questionnaire for diet and medications: During the study period ,we will check if there have been any changes in diet or medications. This will involve 2 phone calls a month apart**

**Figure 1:** **Study flow chart**

**Eligibility:** Age: 2-5 years, and ASD confirmed by DSM-V criteria

**Exclusion Criteria**

1. Congenital anomalies

2. Hypoxic ischemic encephalopathy,

3. Coeliac, Inflammatory bowel disease

4. Use of probiotics in previous 90 days

5. Current or recent (within 4 weeks before enrolment) exposure to antibiotics, chemotherapy or immunosuppressant agents of antibiotics, immunosuppressive

6. Current (or in previous 4 weeks) use of laxatives

7. Implanted prosthetic devices (e.g. heart valves)

8. Positive for HIV, Hepatitis B, and/or Hepatitis C

9. Special diets

10.. ). Cows’ milk protein allergy, food allergy, or conditions such as atopic dermatitis, or eczema.

1st contact in CliniKids

Consent

No

Yes

Baseline assessments: VABSII, MSEL, PLS-5, SSP-2, RBS-R, GSI and stool samples for metagenomics and metabolomics

No Follow up

MSEL Composite score≥85

MSEL Composite score<85

Randomisation

Randomisation

**4 months**

Probiotics

Placebo

Probiotics

Placebo

**Primary outcome:** Faecal metagenomics

**Secondary outcomes:** VABSII, MSEL, PLS-5, SSP-2, RBS-R, GSI assessments, Faecal SCFA

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