PLOS ONE

The role of probiotics in children with autism spectrum disorders: A study protocol for a randomised controlled trial

Lingling Zhang[1ө], Yiran Xu[1ө], Hongwei Li[1], Bingbing Li[1], Guiqin Duan[2], Changlian Zhu[1,3,4]*

1 Henan Key Laboratory of Child Brain Injury and Henan Clinical Research Center for Child Neurological Disorders, Institute of Neuroscience and The Third Affiliated Hospital of Zhengzhou University, Zhengzhou, China, 2 Center for Child Behavioral Development, The Third Affiliated Hospital of Zhengzhou University, Zhengzhou, China, 3 Center for Brain Repair and Rehabilitation, Institute of Neuroscience and Physiology, University of Gothenburg, Göteborg, Sweden, 4 Department of Women's and Children's Health, Karolinska Institutet, Stockholm, Sweden

ө These authors contributed equally to this work.
* changlian.zhu@neuro.gu.se

Abstract

Background

Autism spectrum disorder (ASD) is a neurological and developmental condition that begins in infancy or earlier and lasts through the individual's lifetime. The aetiology and mechanisms of ASD are not yet fully understood, and current treatment comprises mainly education and rehabilitation, without significant improvement in the core symptoms. Recent studies suggest that microbiota change in children with ASD after the ingestion of probiotics may improve the balance of microbiota and thus ASD symptoms.

Objective

The objectives of this study are to evaluate the efficacy of probiotics on the symptoms of children with ASD and the possible mechanisms involved.

Methods

This is a prospective controlled trial. A total of 160 children with ASD will be stratified and allocated to placebo and probiotics groups randomised according to the severity of their ASD symptoms. The probiotics group will be given probiotics supplements orally twice a day for 3 months and the control group will be given a placebo at the same amount, in addition to the baseline therapy of education and rehabilitation. All the children will be evaluated systematically by using different scales, questionnaires before, during, and after 3 months' treatment, as well as 3 months after discontinuation. The potential impact of probiotics on immunity and inflammation, metabolism, and metagenome will also be investigated.

Discussion

Our previous study showed that the abundance of intestinal flora was greatly different in children with ASD, and that *Bifidobacterium* was associated with the severity of ASD. In the present study, we will investigate the impact of probiotics supplementation on the symptoms of Children with ASD, with the purpose of evaluating the possible therapeutic effects of additives on ASD and of providing a reference for clinical treatment. The results will help to disclose as yet unknown relationship between probiotics and ASD.

Trial registration

This study has been registered with Chinese Clinical Trial Registry (ChiCTR-2000037941).

Introduction

Autism spectrum disorder (ASD) is one of the most prevalent neurodevelopmental conditions. It is marked by social and communication impairment as well as limited interests and stereotypical behaviours [1]. The incidence of ASD has increased rapidly over time, from 0.31% in 2000 to 1.57% in 2009 [2, 3]. However, the prevalence of ASD varies in different countries; for example, it is currently estimated to affect 1% of the general population in Spain [4] and 1.69% in the United States [5]. A recent study in China showed that the prevalence of ASD there is similar to the estimate numbers in Western countries, that is, approximately 1% [6]. It is estimated that 62.2 million individuals globally live with ASD [7]. A longitudinal study has suggested that people with ASD have poor outcomes, and that the lifetime cost of supporting an individual is a heavy burden [8]. The significant rise of ASD is believed to be attributable to changes in the diagnostic criteria of ASD and increased awareness. Despite several extensive studies, the mechanisms and aetiology of ASD are not yet fully understood. Current studies suggest that various factors are associated with its development; these include genetic, environmental, epigenetic, and microbiome variables and their interactions [9].

Autistic spectrum disorder diagnosis depends mainly on the description of children's abnormal behaviours by parents and clinical evaluations using different scales, such as the autism diagnosis and observation scale [10]; the autism diagnosis interview scale [11]; the Childhood Autism Rating Scale (CARS) [12]; and the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) [1]. The main symptoms of ASD can be summarised as the "five no" behaviours [13], namely: no (less) look; no (less) reply; no (less) refers to; no (few) words; and inappropriate words or behaviour. The core symptoms are narrow interests and rigid behaviours. These characteristics seriously affect social interaction, study, and work. Some people with the condition cannot live independently, which places a burden on families and on society in general. There are no widely accepted effective methods to prevent or treat these core symptoms. Currently, treatment of ASD is based mainly on special education, such as applied behaviour analysis (ABA) [14], treatment and education of autistic and related communication handicapped children (TEACCH) [15], relationship development intervention [16], floor time [17], the early start Denver model [18], reciprocal imitation training [19], the preschool autism communication trial [20], and pivotal response training [21]. All these methods are used widely; however, the results show that they do not improve the core symptoms fundamentally or permanently [22].

A range of significant concurrent symptoms, such as gastrointestinal problems [23], sleep problems, anxiety, hyperactivity, and attention deficit hyperactivity disorder [24], are often neglected in targeted interventions. In fact, gastrointestinal and sleep issues are prevalent in Children with ASD [25, 26]. Gastrointestinal symptoms, such as constipation, diarrhoea, abdominal bloating, pain on evacuation, and vomiting have been frequently reported [27]. Research has shown that gastrointestinal symptoms are correlated with various maladaptive behaviours, such as self-injury, aggressive behaviours, restricted stereotypical behaviours, hyperactivity, and language regression [27–29]. However, findings on the relationship between gastrointestinal symptoms and the severity of ASD symptoms are inconsistent [30]. This could be related to differences in methodological approaches to data collection; there are no standardised definitions of gastrointestinal symptoms, and a validated instrument to ascertain gastrointestinal symptoms in individuals with ASD is lacking [23, 27].

Sleep disturbances are often an issue amongst Children with ASD. Sleep is closely related to memory, learning, mood, behaviour, immune response, metabolism, and many other physiological functions [31], and it plays a vital role in the maturation and development of the brain [32]. The most common sleep problems are insomnia, increased bedtime resistance, sleep disordered breathing, early morning wakening, and daytime sleepiness [33]. A number of studies have reported that sleep problems in Children with ASD are associated with behavioural symptoms and their severity [34–36]. Loss of sleep can lead to the accumulation of reactive oxygen species (ROS) and even death [37]. Such problems, along with the core symptoms of ASD, should be taken into account in any treatment.

Gut microbiota have attracted a good deal of attention in recent years. They maintain a stable symbiotic relationship with human beings in the intestines, which are regarded as a "second brain" because of the number of genes encoded by intestinal microbes (150 times the total genes of human cells) [38]. There has been a growing realisation that the gut-brain axis and its regulation by microbiota may play a key role in the biological and physiological basis of neurodevelopmental, age-related, and neurodegenerative disorders [39–41]. Studies have shown that intestinal flora communicates with the brain through the nervous system [42], the immune system [43–46], and the endocrine system [47, 48], which leads to shifts in cognition, social behaviour, and emotion [49, 50]. The abundance of the intestinal flora of Children with ASD undergoes great changes, whether between species or within species. It has been shown that the strains *Akkermansia*, *Coprococcus* and *Ruminococcus* are elevated in Children with ASD [51], and that *Akkermansia* increases regulatory T-cells by suppressing experimental autoimmune encephalomyelitis [52]. *Bacterial lipopolysaccharide* can induce an acute immune response by increasing serum IL-17A levels and promoting ASD-like behaviour in the offspring of mice [53, 54]. However, *Lactobacillus* and *Bifidobacterium* strains have been proven to have anti-inflammatory properties by reducing the level of inflammatory cytokines, such as IL-2, interferon-γ, IL-4, IL-13 and IL-17A, and by increasing the level of anti-inflammatory cytokine IL-10 [55, 56]. These cytokines have been noticed to change in the serum of Children with ASD [57–59].

Studies have revealed that *Collinsella* and *clostridium* are at higher levels in children with ASD [60, 61], and that these strains can produce short-chain fatty acids such as propionic acid and butyric acid. These have neurotoxic effects after passing through the blood-brain barrier, and cause autism-like symptoms (e.g., compulsive interests, abnormal motor movements, and atypical social interactions) in rats [62, 63]. However, probiotics can alleviate the neuroinflammation effectively induced by propionic acid in mice, such as IL-1β and IL-8, and reduce cell apoptosis [64]. Other studies found that probiotics can restore not only the biochemical parameters related to neurotransmission, but also balance the energy metabolism and oxidative stress which are associated with autism [65–67]. Researchers have also observed that some people with ASD may have abnormally porous blood-brain and intestinal barriers, so toxins

produced by certain bacteria may enter the blood and reach the brain [48]. Children with autism have been reported to have lower levels of *Bifidobacteria* and higher levels of *Lactobacillus* [68–70]. Gastrointestinal symptoms and quality of life in those with autism were improved by adding *Lactobacillus* and *Bifidobacterium* mixture to the diet [71, 72]. Intestinal microbiota may alleviate constipation through the regulation of gastrointestinal motility or the osmotic effect of metabolites and fermentation products [73], but the precise mechanisms are not clear. Long-term research results have also shown abnormal level of neurotransmitters such as serotonin, dopamine, gamma-aminobutyric acid (GABA), indole, and melatonin [74–75]. Interestingly, 5-hydroxytryptamine (5-HT) levels in the blood are associated with lower gastrointestinal symptoms in ASD [76]. Furthermore, the main neurotransmitters and hormones of GABA and melatonin are involved in sleep promotion, while serotonin, glutamate, and acetylcholine are mainly responsible for wakefulness [77]. Gamma-aminobutyric acid (GABA) is one of the major inhibitory neurotransmitters from the decarboxylation of glutamate at glutamate decarboxylase. Decreased N-methyl-D-aspartate receptor function on GABAergic neurons can lead to postsynaptic glutamate neuron disinhibition, excessive glutamate release, and neurotoxicity [78]. Disturbed GABA levels are commonly found in autistic children or rodents with neurological damage, which may be a key factor in the pathogenesis of autism [79–81]. The production of those neurotransmitters and hormones may be effected by intestinal flora such as *Streptococcus thermophilus* and indigenous spore forming bacteria (Sp) *Lachnoclostridium bolteae*,*Lachnoclostridium hathewayi*, and *Flavonifractor plautii* [69, 82]. With the deepening understanding of probiotics, research on emerging microbial strains has led to vaccines with systemic immunomodulatory activity [83] and revealed the production of neuroactive metabolites [84] and inhibitory activity against infectious microorganisms in the intestines [85]. In recent years, studies of probiotics in Children with ASD have shown great potential benefits in terms of the amelioration of gastrointestinal dysfunction [86], malnutrition, and the severity of ASD symptoms [72, 87–89]. As for non-pharmacological and relatively risk-free, probiotics may be an ideal option for ASD.

Above all, it has been shown that the intestinal flora can adjust the level of intestinal metabolites such as neurotransmitters and hormones through a change in diversity. This could have an impact on behaviour, interest, mood, the gastrointestinal tract, and sleep patterns in children. However, the precise schema and pathways are not yet fully understood. Therefore, we hypothesise that probiotic supplementation may help improve abnormal gut metabolites and immunity in Children with ASD, thus improving ASD symptoms.

Methods

Study design and participants

We will carry out a randomised, double-blind, placebo-controlled, parallel group trial to evaluate the efficacy and safety, lyophilized powder mixtures (S1 Table), contenting *Lactobacillus*, *Bifidobacteria* and *Streptococcus thermophilus*, to be taken 10 billion colony units twice a day for 3 months, followed by a 3-month washout period and then a 6-month follow up, as add-on therapy in children with ASD irrespective of any ABA and TEACCH maintenance therapy. The placebo group will be given maltodextrin. At each time point, all participants will receive the following assessments and investigations of Autism Treatment Evaluation Checklist (ATEC), CARS, social responsiveness scale second edition, children's sleep health questionnaire, survey of dietary patterns, gastrointestinal assessment questionnaires and the Bristol stool chart. The children will be required to avoid taking antibiotics during this period. If they do they will have to be excluded (Fig 1). Patients aged 3 to 12 years will be eligible if they are defined as having ASD by two child psychologists using DSM-5 [1], but if they meet one of the

	STUDY PERIOD							
	Enrolment	Allocation	Post-allocation					Close-out
TIMEPOINT	$-t_1$	0	t_1	t_2	t_3	t_4	etc.	t_x
ENROLMENT:	✓							
Eligibility screen	✓							
Informed consent	✓							
[List other procedures]	X							
Allocation		✓						
INTERVENTIONS:			✓					
[Intervention A]			◆————————◆					
[Intervention B]			X		X			
[List other study groups]	X							
ASSESSMENTS:	✓		✓	✓	✓			✓
[List baseline variables]	✓	✓						
[List outcome variables]			✓	✓	✓		etc.	✓
[List other data variables]	X							

Fig 1. The schedule of enrolment, interventions, and assessments. This list mainly includes three time points, which are the start time of intervention T0, the end time of the supplement T1 and the end time of washout period T2.

https://doi.org/10.1371/journal.pone.0263109.g001

following exclusion criteria they will not be allowed to take part: (1) they have taken antibiotics or antifungal drugs within three months; (2) they have a special diet, such as a gluten free diet, a casein diet, or a special carbohydrate diet; (3) they have taken prebiotics, probiotics, or anti-oxidants within the previous three months; (4) they have had diarrhoea, fever, or other clear inflammatory reactions within the past week; (5) they have neurological symptoms such as epilepsy; (6) they have Crohn's disease, inflammatory bowel disease, food intolerance, or any other gastrointestinal diseases; (7) they have a liver disease; (8) they have type 1 diabetes mellitus; (9) they have a confirmed diagnosis of Rett syndrome or any other genetic metabolic disease. The exit criteria are: (1) children who do not take the probiotics as required; (2) children whose blood and stool samples provide no biological information; and (3) participants cannot complete DSM-5, CARS, and the other compulsory scales for ASD evaluation. All the

participants are from the Center for Child Behavioral Development at the Third Affiliated Hospital of Zhengzhou University. This clinical trial has been registered at the Chinese Clinical Trial Registry (ChiCTR2000037941), and approved by the ethical committee of the Third Affiliated Hospital of Zhengzhou University (2020–56). All the participants will join the research on a voluntary basis. An information fact sheet and a consent form will be distributed to the front-line healthcare providers and parents after an introductory talk of the program. They understand the benefits and risks involved in the program, and their participation in the program is voluntary. They have the right to question any part of the procedure and can withdraw at any time without negative consequences. The completed consent form will be collected by the research team before the study commences.

Randomisation and masking

Randomisation was stratified according to severity, gender, age (3–6 years or 7–12 years). The ratio of male to female in the study will be about 5:1 based on the registration of in patient ASD. We will use block randomisation within sites (Fig 2). Participants will be allocated (1:1) to the probiotics or placebo group using a concealed random allocation from a computer-generated random numbers table produced by Python (a cross-platform computer programming language). The additives used for the placebo group will have the same packaging, taste, and weight as the experimental group, except for the ingredients. All participants and doctors will be blind to the allocation of treatments.
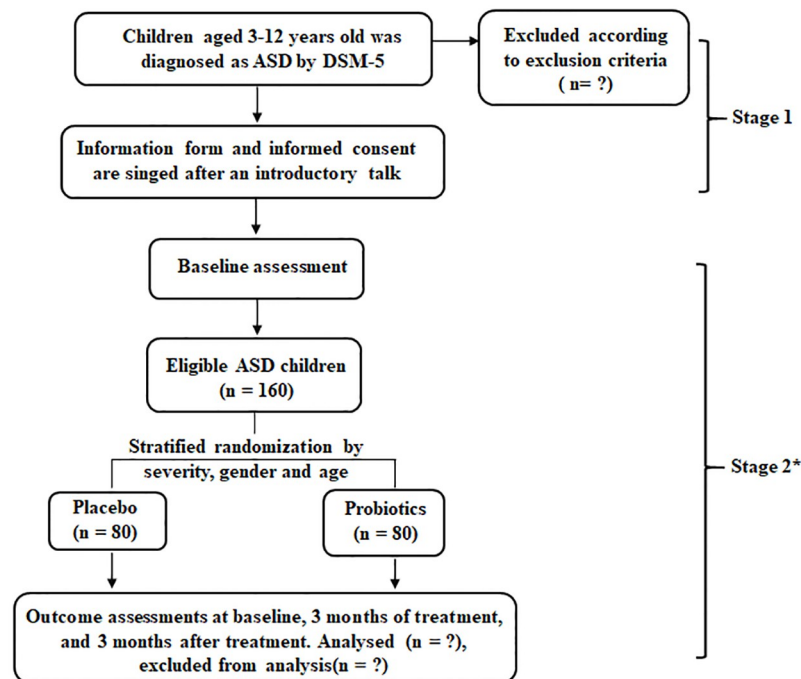
Fig 2. Schematic diagram of study design. The assessment mainly includes the severity of autism, gastrointestinal problems, sleep assessment, parental stress assessment, eating habits and diet structure. The experimental results mainly include the results of the metabolism of blood, urine and stool, and the changes in the intestinal flora, as well as the changes in immune cells and cytokines; Entire experiment includes 3-month medication and 3-month elimination. *Adverse effects are monitored throughout the period.

https://doi.org/10.1371/journal.pone.0263109.g002

Sample size calculation

The sample size will be calculated using Epitools (https://epitools.ausvet.com.au/onemean). The expected difference between the probiotic and placebo groups is based on a previous study on ASD, in which the standard deviation value of behaviour score was 8.32 in the ASD group [89]. To detect a clinically significant difference in the outcome measures with the condition of 90% power ($\alpha = 0.05$; two-sided), 67 children are required for each group. Based on clinical study experience [90], we presume a drop-out rate of 19% through infection, efficacy, compliance, use of antibiotics, or other unforeseeable factors, implying a minimum of 80 children for each group.

Procedures

Before the trial, all the children will be evaluated carefully, and the research topic will be explained to the parents of the Children with ASD who meet the study's selection criteria. If they are then willing to participate in the trial, they will receive the supplements free of charge for three months and carry out the relevant inspections and tests. They will sign an informed consent agreement. They will be able to consult with the doctors if they have any questions when they are administering the supplements, but they must follow the requirements of the agreement. If they are not willing to continue the study for some reason, they can withdraw. All the potential side effects will be monitored and all the participants will be recalled at 3 months after intervention for the systematic physical examination and liver as well as kidney function analysis. The potential costs will be covered by indemnity for negligent harm. The participants will be asked to provide a one tube faeces sample 0, 1, 2, and 3 months after taking the supplements, as well as at the end of 3 months of wash out, and a tube of blood with heparin anticoagulation at 0 and 3 months. To guarantee quality all samples must be transported within 12 hours at low temperatures or they will be rejected. Other than the samples used for flow cytometry detection, which has to be completed within 12 hours, all samples will be aliquots and frozen in a freezer at -80°C.

Quality control

The trial datasets will be housed on the project files of Henan Key Laboratory of Child Brain Injury. All the principal investigators are given to access to the cleaned datasets without identifying participant information. Safety and interim analyses will be done when half of the patients are recruited, and these will be reviewed by the Safety and Data Monitoring Committee. The validity of results from the intervention can only be verified by project manager who have full access to the complete final dataset. To ensure the quality of the sample and the reliability of the data, we will set up a We chat communication group to remind the parents or guardians to carry out the required treatments and examinations regularly. Furthermore, if the children have any doubts or problems during the treatment, they can contact the doctors at any time. We will provide information about probiotics to the group to increase their understanding of the trial; this may help reduce the drop-out rate. All patient information will be confidential and will be managed by a specific researcher.

Primary outcome measure

   a) Assessment of core symptoms.   All parents/guardians will be asked to complete an ATEC [91] to evaluate changes in core symptoms in Children with ASD at the time of the baseline, the third month, and the sixth month respectively. The scale consists of four subscales: speech, perception, social interaction, and behaviour. The total score is 0–179; the

higher the score, the more serious the condition. The reduction rate of ATEC score before (S_1) and after (S_2) treatment will be used as the efficacy index (N), $N = (S_1 - S_2)/S1 \times 100\%$. Markedly effective: $N_M \geq 50\%$, Effective: N_E: 20%–50%, Ineffective: $N_I < 20\%$. Total effective rate (N_T) = ($N_M + N_E$) / total cases $\times 100\%$.

b) Assessment of severity. CARS [12] and social responsiveness scale [92] will be used. CARS scale consists of 15 items with a total score of 60 points. A score of less than 30 points means no autism is present, and a score of 30–60 points means autism is present; 30–36 points signifies moderate autism, and 37–60 points with at least five items and a score more than three points signifies severe autism. However, the social responsiveness scale mainly shows the abnormalities in the interactive social behavior of subjects and the difficulties in daily social behavior. This scale has a total of 65 items, which are divided into a 5-point scale. The score is calculated and converted into a total score, which is divided into normal, mild, moderate and moderate.

Secondary outcome measures

a) Assessment of gastrointestinal symptoms. Gastrointestinal symptoms will be assessed by gastrointestinal assessment questionnaires [93] and the Bristol Stool Chart [94]. First, in accordance with the Bristol stool chart, we will classify the stool into seven levels, namely, type I: a dry nut-like ball-shaped stool, which is difficult to discharge; type II: sausage-like but very hard; type III: sausage-like surface cracks; type IV: sausage-like or snake-like, smooth and soft; type V: soft lump; clear-cut edge (and easy to discharge); type VI: loose fragments, broken edges, or mushy stool; type VII: watery stool, with no solid parts. Type IV is an ideal healthy state. We will use the questionnaires scale to conduct a further survey of the gastrointestinal condition of the participants. The scale is composed of 15 items. Each item uses a four-level method of 0–3 points, The symptoms for the corresponding scores are: (0) asymptomatic, indicating that the patient does not have this feeling; (1) mild, i.e., occasional symptoms; (2) moderate, i.e., frequent symptoms; and (3) severe, i.e., persistent symptoms.

b) Assessment of sleep. The Children's Sleep Habits Questionnaire is based on the International Classification of Sleep Disorders [95], which is used for children aged 4–12 years. The scale is filled in by the parents, who monitor their child's sleep patterns over the previous four weeks and choose a typical week. Each item uses a 3-level score based on the frequency of sleep-related behaviours, from low to high from 1 to 3 points, respectively. There is a total of 52 items; the higher the number, the greater the children's sleep disorders. The questionnaire comprises eight common sleep disorders in children, namely: 1) sleep impedance; 2) delay in falling asleep; 3) sleep duration; 4) sleep anxiety; 5) waking at night; 6) parasomnia; 7) sleep breathing disorders; and 8) daytime sleepiness. The questionnaire uses the total score > 41 points as the standard for evaluating sleep disorders, and defines the standard for sleep disorders as a frequency of more than two nights a week in each item [35].

c) Assessment of parenting stress. The Parenting Stress Index-Short Form [96] contains 36 items with three dimensions: parental distress, parent-child dysfunction, and the difficult child. Each dimension contains 12 items. The scale uses a 5-point scale, from 1 to 5 respectively: *strongly disagree*, *disagree*, *unsure*, *agree*, and *strongly agree*. The total score can range from 36 to 180 points. The higher the score, the greater the pressure. The parenting distress dimension reflects the parents' abilities in raising the children, handling conflicts with their spouse, social support, and life pressures. The personal interaction disorder dimension evaluates the parents' attitude towards the child's failure to meet expectations and the child's inability to interact well. The child's difficulty dimension is to investigate parents' reactions to children's emotions, resistance, and non-cooperation.

d) Assessment of eating habits. The Children's Eating Behaviour Questionnaire [97] is a valid and reliable 35-item measure of children's eating behaviours. It has eight subscales: food-responsiveness, food fussiness, emotional over-eating, enjoyment of food, desire to drink, satiety-responsiveness, slowness in eating, and emotional under-eating. Higher scores reflect more extreme eating behaviours.

e) The survey of dietary patterns. The structure of food has a great impact on the gastrointestinal tract. The scale used concerns the consumption of staple foods, vegetables, fruits, eggs, meat, water, and antibiotics, and related sleep and exercise conditions (S2 Table). The "OKOK" mobile phone health management software is used to record the daily diet.

f) Metabolomics of faeces and blood. Metabolic profiling of samples will be performed on an Agilent 1290 Infinity LC system (Agilent Technologies, Santa-Clara, California, USA) coupled with an AB SCIEX Triple TOF 6600 System (AB SCIEX, Framingham, MA, USA). Chromatographic separation will be implemented on ACQUITY UPLC BEH Amide 1.7 μm (2.1 × 100 mm) columns for both positive and negative models. The column temperature will be set at 25°C.

g) Changes in inflammatory profiles. Inflammatory profiles will be detected by flow cytometry. All FACES processes will be completed within 12 hours by using the antibodies of surface and intracellular markers of peripheral blood mononuclear cells (PBMC). PBMCs will be isolated by density gradient centrifugation as described previously [98]. Flow cytometry will be used to detect cytokines IL-17A, IL-10, IFN-γ, IL-6, IL-21, IL-2, IL-1β, IL-8 produced by $CD4^+$ and $CD8^+$ cells and IL-1β, IL-10, IL-6, IL-8 produced by monocytes. Briefly, PBMCs will be stimulated with PMA/ionomycin for 4 h in the presence of BD GolgiStop, as previously described [99]. Then PBMCs will be washed and stained for $CD4^+$ and $CD8^+$ cells and monocytes. Cytokine staining was performed with fixed permeability method for anti-IL-17A, anti-IL-10, anti-IFN-γ, anti-IL-6, anti-IL-21, anti-IL-2, anti-IL-1β, anti-IL-8. Samples will be taken and analysed by BD FACSCelesta flow cytometer and Flowjo software.

h) Diversity and richness of flora. A total of 1μg DNA per sample was used as input material for the DNA sample preparations. Sequencing libraries were generated using NEBNext® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA) following the manufacturer's recommendations, and index codes will be added to attribute sequences to each sample. The DNA sample will be fragmented by sonication to a size of 350bp, then DNA fragments will be end-polished, A-tailed, and ligated with the full-length adaptor for Illumina sequencing with further PCR amplification. Finally, PCR products will be purified (AMPure XP system) and libraries will be analysed for size distribution by Agilent2100 Bioanalyzer and quantified using real-time PCR. The clustering of the index-coded samples will be performed on a cBot Cluster Generation System in accordance with the manufacturer's instructions. After cluster generation, the library preparations will be sequenced on an Illumina HiSeq platform and paired-end reads generated.

Statistical analyses and power

The severity and ASD before and after administration of probiotics will be analysed in accordance with the requirements of the scale previously described [66]. Relevant questionnaires will be analysed by Chi-square test. Statistical significance will be set at $p < 0.05$. All raw data can be available at the Website (http://beta.fairsharing.org/3752) after we finish the study.

The entire study has multiple omics measurements that are taken at different points. The metagenome sequencing data will be analysed using meta-seq metagenome pipeline. The specific process is as follows. First, the raw reads will be extracted from the sequencing results, and fast quality control (version 0.11.9) with default parameters will be used to evaluate their quality. Adaptor sequences

in the reads will be trimmed using Trimmo matic (Version 0.38). Braken2 (version 2.0.8b), Kraken, and Kaiju (version 1.6.3) will be used for taxonomic classification of the metagenome short reads. The meta-seq pipeline will involve the use of meta SPAdes (version 3.13.2) and MEGAHIT (version 1.2.9) for short read assembly. Meta Prodigal (version: 2.6.3) will be employed for gene prediction of the assembled metagenomic contigs. The abundance of each ORF (measured with TPM) will be quantified with Salmon (version 1.1.0). The functional information of the ORFs will be described with KEGG, AMR and COG. R package DESseq2 will detect the differential abundance ontology or taxonomy catalogue. Assembled contigs with more than 1500bp will be used to reconstruct the metagenome-assembled Genome using Meta BAT (version 2.12.1). CheckM (version 1.0.7) will enable evaluation of the quality and completeness of the reconstructed draft genome. GTDB-tk (version 1.0.2) will be used to identify the lineage classification of the obtained metagenome-assembled Genome.

The raw UPLC-Q-TOF/MS data will be converted to mzXML files using ProteoWizard msConverte tool and then processed using XCMS online software. The heat plot of metabolites will be obtained through Multi Experiment Viewer software 4.9.0 after unit variance scaling for each metabolite. The online KEGG database (http://www.genome.jp/kegg/) will be used for the identification of metabolic pathways.

Discussion

The prevalence of ASD continues to increase worldwide, but valid therapeutic strategies to improve core symptoms are lacking. This places additional demands on affected families and on society as a whole. Therefore, there is an urgent need to develop such strategies. The present study aims to explore whether probiotics supplementation has the potential to improve the symptoms of ASD and the possible mechanisms by which they may work.

Our previous study showed that the diversity of gut microbiota in Children with ASD changed significantly, and the changes in *Bifidobacterium* was correlated with the severity of ASD [60]. This suggests that the gut is associated with the occurrence and development of ASD, so probiotics may have potential value in clinical applications. It has been reported that gut microbiota from ASD donors have been transplanted to sterile mice that exhibit autistic behaviour. When the ASD mice were treated with candidate microbial metabolites, their abnormal behaviour improved significantly, and the excitability of brain neurons were regulated [100]. A Systematic review found that altered gut microbiota in autistic children may trigger intestinal leakage, of which short-chain fatty acids, lipopolysaccharide, calprotectin, lysozyme and lysozyme can be used as biomarkers for early detection of intestinal leakage [101]. This indicates that gut microbiota could be a target for ASD treatment.

Probiotics supplements can protect the intestinal barrier and reduce effectively the occurrence of intestinal diseases [102, 103]. Oral Bif195 can reduce the risk of small intestinal disease caused by acetylsalicylic acid [104]. In addition, probiotics have certain effects on the immune system. Nasal administration of *Rhamnose* GG prevents the development of birch pollen-induced allergic asthma in a strain-specific manner [105]. Furthermore, in the current consensus, the role of probiotics is not considered to be solely mediated by microflora; in fact, their metabolic function may go beyond their effect on colonial microflora, and this may open the door to a wider range of probiotic possibilities. A meta-analysis found that polyunsaturated fatty acids (especially eicosapentaenoic acid) can assist in the treatment of depression and improve attention deficit/hyperactivity disorder [106].

Previous studies have shown that probiotics or their metabolites regulate changes in immune cells, cytokines, and emotional behaviour, and have great potential therapeutic effect. Therefore, it is necessary to carried out more comprehensive studies on the pathogenesis of

autism to find further effective treatment methods. This protocol describes a multi-level, multi-faceted, independent, and closely related randomised controlled study. All the training, assessment, and testing will be completed by specially trained and experienced child psychologists or rehabilitation trainers and nurses. They will carry out the training according to the requirements of the agreement. In the meanwhile, all parents or guardians of the children will receive professional training to learn about the relevant precautions before they participate in the trial. The specific information collected will be recorded in detail, and those who do not meet the requirements will be excluded. Given the influence of subjective factors, the questionnaire is not only scored by parents, but also evaluated by the doctors who participate in the intervention or related tests.

In sum, the object of this study is to clarify whether probiotics mixing is an effective treatment for children with ASD in daily clinical practice. Meanwhile, it will also help us to clarify the changes in the immune and intestinal systems of the condition, laying a foundation for discovering its pathogenesis. In addition, probiotics are much more risk-free than drugs; some ingredients are common in infant dairy products. Therefore, the Children with ASD or parents are likely to be more willing to accept them and to cooperate in the research.

Supporting information

S1 Checklist. SPIRIT 2013 checklist: Recommended items to address in a clinical trial protocol and related documents[*].
(DOC)

S1 Table. List of probiotic mixtures.
(DOCX)

S2 Table. Eating habits questionnaire.
(DOCX)

S1 File.
(DOCX)

S2 File.
(DOCX)

Acknowledgments

Author Contributions

Conceptualization: Changlian Zhu.

Data curation: Lingling Zhang, Hongwei Li.

Formal analysis: Yiran Xu, Bingbing Li.

Funding acquisition: Changlian Zhu.

Methodology: Hongwei Li, Bingbing Li, Changlian Zhu.

Project administration: Yiran Xu.

Resources: Guiqin Duan, Changlian Zhu.

Supervision: Guiqin Duan, Changlian Zhu.

Validation: Yiran Xu, Changlian Zhu.

Visualization: Guiqin Duan.

Writing – original draft: Lingling Zhang, Yiran Xu, Changlian Zhu.

References

1. Kocsis RN. Book Review: Diagnostic and Statistical Manual of Mental Disorders: Fifth Edition (DSM-5). Int J Offender Ther Comp Criminol. 2013; 57(12):1546–8. https://doi.org/https%3A//doi.org/10.1177/0306624x13511040

2. Baron-Cohen S, Wheelwright S, Cox A, Baird G, Charman T, Swettenham J, et al. Early identification of autism by the CHecklist for Autism in Toddlers (CHAT). J R Soc Med. 2000; 93(10):521–5. https://doi.org/10.1177/014107680009301007 PMID: 11064690.

3. Baron-Cohen S, Scott FJ, Allison C, Williams J, Bolton P, Matthews FE, et al. Prevalence of autism-spectrum conditions: UK school-based population study. Br J Psychiatry. 2009; 194(6):500–9. https://doi.org/10.1192/bjp.bp.108.059345 PMID: 19478287.

4. Catala-Lopez F, Ridao M, Hurtado I, Nunez-Beltran A, Genova-Maleras R, Alonso-Arroyo A, et al. Prevalence and comorbidity of autism spectrum disorder in Spain: study protocol for a systematic review and meta-analysis of observational studies. Syst Rev. 2019; 8(1):141. https://doi.org/10.1186/s13643-019-1061-1 PMID: 31200773.

5. Baio J, Wiggins L, Christensen DL, Maenner MJ, Daniels J, Warren Z, et al. Prevalence of autism spectrum disorder among children aged 8 years—Autism and Developmental Disabilities Monitoring Network, 11 Sites, United States, 2014. MMWR Surveill Summ. 2018; 67(6):1–23. https://doi.org/10.15585/mmwr.ss6706a1 PMID: 29701730.

6. Sun X, Allison C, Wei L, Matthews FE, Auyeung B, Wu YY, et al. Autism prevalence in China is comparable to Western prevalence. Mol Autism. 2019; 10:7. https://doi.org/10.1186/s13229-018-0246-0 PMID: 30858963.

7. Gakidou E GBD 2016 Eiak Factors Collaborators. Global, regional, and national comparative risk assessment of 84 behavioural, environmental and occupational, and metabolic risks or clusters of risks, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. Lancet. 2017; 390(10100):1345–422. https://doi.org/10.1016/S0140-6736(17)32366-8 PMID: 28919119.

8. Benevides T, Carretta H, Rust G, Shea L. Racial and ethnic disparities in benefits eligibility and spending among adults on the autism spectrum: A cohort study using the Medicare Medicaid Linked Enrollees Analytic Data Source. PloS one. 2021; 16(5):e0251353. https://doi.org/10.1371/journal.pone.0251353 PMID: 34032811.

9. Abdellatif B, McVeigh C, Bendriss G, Chaari A. The promising role of probiotics in managing the altered gut in autism spectrum disorders. Int J Mol Sci. 2020; 21(11). https://doi.org/10.3390/ijms21114159 PMID: 32532137.

10. Park HS, Yi SY, Yoon SA, Hong SB. Comparison of the autism diagnostic observation schedule and childhood autism rating scale in the diagnosis of autism spectrum disorder: a preliminary study. Soa Chongsonyon Chongsin Uihak. 2018; 29(4):172–7. https://doi.org/10.5765/jkacap.180015 PMID: 32595311.

11. Lord CRM, Le Couteur A. Autism Diagnostic Interview-Revised: a revised version of a diagnostic interview for caregivers of individuals with possible pervasive developmental disorders. J Autism Dev Disord. 1994; 24(5):659–85. https://doi.org/10.1007/BF02172145 PMID: 7814313.

12. Moon SJ, Hwang JS, Shin AL, Kim JY, Bae SM, Sheehy-Knight J, et al. Accuracy of the Childhood Autism Rating Scale: a systematic review and meta-analysis. Dev Med Child Neurol. 2019; 61(9):1030–8. https://doi.org/10.1111/dmcn.14246 PMID: 30977125.

13. Zwaigenbaum L, Bauman ML, Stone WL, Yirmiya N, Estes A, Hansen RL, et al. Early identification of autism spectrum disorder: recommendations for practice and research. Pediatrics. 2015; 136 Suppl 1: S10–40. https://doi.org/10.1542/peds.2014-3667C PMID: 26430168.

14. Myers SM, Johnson CP. Management of children with autism spectrum disorders. Pediatrics. 2007; 120(5):1162–82. https://doi.org/10.1542/peds.2007-2362 PMID: 17967921.

15. Siaperas P, Higgins S, Proios P. Challenging behaviours on people with autism: A case study on the effect of a residential training programme based on structured teaching and TEACCH method. Psychiatriki. 2007. PMID: 22466678.

16. Hobson JA, Tarver L, Beurkens N, Hobson RP. The relation between severity of autism and care-giver-child interaction: a study in the context of relationship development intervention. J Abnorm Child Psychol. 2016; 44(4):745–55. https://doi.org/10.1007/s10802-015-0067-y PMID: 26298470.

17. Dionne M, Martini R. Floor time play with a child with autism: a single-subject study. Can J Occup Ther. 2011; 78(3):196–203. https://doi.org/10.2182/cjot.2011.78.3.8 PMID: 21699014.

18. Dawson G, Rogers S, Munson J, Smith M, Winter J, Greenson J, et al. Randomized, controlled trial of an intervention for toddlers with autism: the Early Start Denver Model. Pediatrics. 2010; 125(1):e17–23. https://doi.org/10.1542/peds.2009-0958 PMID: 19948568.

19. Ingersoll B. Pilot randomized controlled trial of Reciprocal Imitation Training for teaching elicited and spontaneous imitation to children with autism. J Autism Dev Disord. 2010; 40(9):1154–60. https://doi.org/10.1007/s10803-010-0966-2 PMID: 20155309.

20. Green J, Charman T, McConachie H, Aldred C, Slonims V, Howlin P, et al. Parent-mediated communication-focused treatment in children with autism (PACT): a randomised controlled trial. The Lancet. 2010; 375(9732):2152–60. https://doi.org/10.1016/S0140-6736(10)60587-9 PMID: 20494434.

21. Gengoux GW, Abrams DA, Schuck R, Millan ME, Libove R, Ardel CM, et al. A pivotal response treatment package for children with autism spectrum disorder: An RCT. Pediatrics. 2019; 144(3). https://doi.org/10.1542/peds.2019-0178 PMID: 31387868.

22. Medavarapu S, Marella LL, Sangem A, Kairam R. Where is the evidence? a narrative literature review of the treatment modalities for autism spectrum disorders. Cureus. 2019; 11(1):e3901. https://doi.org/10.7759/cureus.3901 PMID: 30911457.

23. Holingue C, Newill C, Lee LC, Pasricha PJ, Fallin DM. Gastrointestinal symptoms in autism spectrum disorder: A review of the literature on ascertainment and prevalence. Autism Res. 2018; 11(1):24–36. https://doi.org/10.1002/aur.1854 PMID: 28856868.

24. Lai M-C, Kassee C, Besney R, Bonato S, Hull L, Mandy W, et al. Prevalence of co-occurring mental health diagnoses in the autism population: a systematic review and meta-analysis. Lancet Psychiatry. 2019; 6(10):819–29. https://doi.org/10.1016/S2215-0366(19)30289-5 PMID: 31447415.

25. Penzol MJ, de Pablo GS, Llorente C, Moreno C, Hernandez P, Dorado ML, et al. Functional gastrointestinal disease in autism spectrum disorder: a retrospective descriptive study in a clinical sample. Front Psychiatry. 2019; 10:179. https://doi.org/10.3389/fpsyt.2019.00179 PMID: 31024351.

26. McCue LM, Flick LH, Twyman KA, Xian H. Gastrointestinal dysfunctions as a risk factor for sleep disorders in children with idiopathic autism spectrum disorder: A retrospective cohort study. Autism. 2017; 21(8):1010–20. https://doi.org/10.1177/1362361316667061 PMID: 28954536.

27. Restrepo B, Angkustsiri K, Taylor SL, Rogers SJ, Cabral J, Heath B, et al. Developmental-behavioral profiles in children with autism spectrum disorder and co-occurring gastrointestinal symptoms. Autism Res. 2020. https://doi.org/10.1002/aur.2354 PMID: 32767543.

28. Ferguson BJ, Dovgan K, Takahashi N, Beversdorf DQ. The relationship among gastrointestinal symptoms, problem behaviors, and internalizing symptoms in children and adolescents with autism spectrum disorder. Front Psychiatry. 2019; 10:194. https://doi.org/10.3389/fpsyt.2019.00194 PMID: 31024357.

29. Bresnahan M, Hornig M, Schultz AF, Gunnes N, Hirtz D, Lie KK, et al. Association of maternal report of infant and toddler gastrointestinal symptoms with autism: evidence from a prospective birth cohort. JAMA Psychiatry. 2015; 72(5):466–74. https://doi.org/10.1001/jamapsychiatry.2014.3034 PMID: 25806498.

30. Prosperi M, Santocchi E, Balboni G, Narzisi A, Bozza M, Fulceri F, et al. Behavioral phenotype of asd preschoolers with gastrointestinal symptoms or food selectivity. J Autism Dev Disord. 2017; 47 (11):3574–88. https://doi.org/10.1007/s10803-017-3271-5 PMID: 28861653.

31. Acosta MT. Sleep, memory and learning. Medicina (B Aires). 2019; 79 Suppl 3:29–32. PMID: 31603840.

32. Peirano PD, Algarin CR. Sleep in brain development. Biol Res. 2008; 40(4):471–8. https://doi.org/https%3A//doi.org/10.4067/S0716-97602007000500008 PMID: 18575679.

33. Kose S, Yilmaz H, Ocakoglu FT, Ozbaran NB. Sleep problems in children with autism spectrum disorder and intellectual disability without autism spectrum disorder. Sleep Med. 2017; 40:69–77. https://doi.org/10.1016/j.sleep.2017.09.021 PMID: 29221782.

34. Yang XL, Liang S, Zou MY, Sun CH, Han PP, Jiang XT, et al. Are gastrointestinal and sleep problems associated with behavioral symptoms of autism spectrum disorder? Psychiatry Res. 2018; 259:229–35. https://doi.org/10.1016/j.psychres.2017.10.040 PMID: 29091821.

35. Johnson CR, Smith T, DeMand A, Lecavalier L, Evans V, Gurka M, et al. Exploring sleep quality of young children with autism spectrum disorder and disruptive behaviors. Sleep Med. 2018; 44:61–6. https://doi.org/10.1016/j.sleep.2018.01.008 PMID: 29530371.

36.   Souders MC, Zavodny S, Eriksen W, Sinko R, Connell J, Kerns C, et al. Sleep in children with autism spectrum disorder. Curr Psychiatry Rep. 2017; 19(6):34. https://doi.org/10.1007/s11920-017-0782-x PMID: 28502070.

37.   Vaccaro A, Kaplan Dor Y, Nambara K, Pollina EA, Lin C, Greenberg ME, et al. Sleep loss can cause death through accumulation of reactive oxygen species in the gut. Cell. 2020. https://doi.org/https%3A//doi.org/10.1016/j.cell.2020.04.049 PMID: 32502393.

38.   Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, et al. A human gut microbial gene catalogue established by metagenomic sequencing. Nature. 2010; 464(7285):59–65. https://doi.org/10.1038/nature08821 PMID: 20203603.

39.   Sharon G, Sampson TR, Geschwind DH, Mazmanian SK. The central nervous system and the gut microbiome. Cell. 2016; 167(4):915–32. https://doi.org/10.1016/j.cell.2016.10.027 PMID: 27814521.

40.   Forssberg H. Microbiome programming of brain development: implications for neurodevelopmental disorders. Dev Med Child Neurol. 2019; 61(7):744–9. https://doi.org/10.1111/dmcn.14208 PMID: 30868564.

41.   Srikantha P, Mohajeri MH. The possible role of the microbiota-gut-brain-axis in autism spectrum disorder. Int J Mol Sci. 2019; 20(9). https://doi.org/10.3390/ijms20092115 PMID: 31035684.

42.   Han W, Tellez LA, Perkins MH, Perez IO, Qu T, Ferreira J, et al. A neural circuit for gut-induced reward. Cell. 2018; 175(3):665–78 e23. https://doi.org/10.1016/j.cell.2018.08.049 PMID: 30245012.

43.   Lamas B, Richard ML, Leducq V, Pham HP, Michel ML, Da Costa G, et al. CARD9 impacts colitis by altering gut microbiota metabolism of tryptophan into aryl hydrocarbon receptor ligands. Nat Med. 2016; 22(6):598–605. https://doi.org/10.1038/nm.4102 PMID: 27158904.

44.   Mukherji A, Kobiita A, Ye T, Chambon P. Homeostasis in intestinal epithelium is orchestrated by the circadian clock and microbiota cues transduced by TLRs. Cell. 2013; 153(4):812–27. https://doi.org/10.1016/j.cell.2013.04.020 PMID: 23663780.

45.   Tsilioni I, Patel AB, Pantazopoulos H, Berretta S, Conti P, Leeman SE, et al. IL-37 is increased in brains of children with autism spectrum disorder and inhibits human microglia stimulated by neurotensin. Proc Natl Acad Sci U S A. 2019; 116(43):21659–65. https://doi.org/10.1073/pnas.1906817116 PMID: 31591201.

46.   Erny D, Hrabe de Angelis AL, Jaitin D, Wieghofer P, Staszewski O, David E, et al. Host microbiota constantly control maturation and function of microglia in the CNS. Nat Neurosci. 2015; 18(7):965–77. https://doi.org/10.1038/nn.4030 PMID: 26030851.

47.   Abdel-Haq R, Schlachetzki JCM, Glass CK, Mazmanian SK. Microbiome-microglia connections via the gut-brain axis. J Exp Med. 2019; 216(1):41–59. https://doi.org/10.1084/jem.20180794 PMID: 30385457.

48.   Fiorentino M, Sapone A, Senger S, Camhi SS, Kadzielski SM, Buie TM, et al. Blood-brain barrier and intestinal epithelial barrier alterations in autism spectrum disorders. Mol Autism. 2016; 7:49. https://doi.org/10.1186/s13229-016-0110-z PMID: 27957319.

49.   Yano JM, Yu K, Donaldson GP, Shastri GG, Ann P, Ma L, et al. Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis. Cell. 2015; 161(2):264–76. https://doi.org/10.1016/j.cell.2015.02.047 PMID: 25860609.

50.   Jastrzebska-Wiesek M, Partyka A, Rychtyk J, Sniecikowska J, Kolaczkowski M, Wesolowska A, et al. Activity of serotonin 5-HT1A receptor biased agonists in rat: anxiolytic and antidepressant-like properties. ACS Chem Neurosci. 2018; 9(5):1040–50. https://doi.org/10.1021/acschemneuro.7b00443 PMID: 29266914.

51.   Zurita MF, Cardenas PA, Sandoval ME, Pena MC, Fornasini M, Flores N, et al. Analysis of gut microbiome, nutrition and immune status in autism spectrum disorder: a case-control study in Ecuador. Gut Microbes. 2020; 11(3):453–64. https://doi.org/10.1080/19490976.2019.1662260 PMID: 31530087.

52.   Liu S, Rezende RM, Moreira TG, Tankou SK, Cox LM, Wu M, et al. Oral Administration of miR-30d from feces of MS Patients suppresses MS-like symptoms in mice by expanding Akkermansia muciniphila. Cell Host Microbe. 2019; 26(6):779–94 e8. https://doi.org/10.1016/j.chom.2019.10.008 PMID: 31784260.

53.   Kugelberg E. Neuroimmunology: IL-17A mediates a path to autism. Nat Rev Immunol. 2016; 16 (4):205. https://doi.org/10.1038/nri.2016.35 PMID: 26972722.

54.   Kim S, Kim H, Yim YS, Ha S, Atarashi K, Tan TG, et al. Maternal gut bacteria promote neurodevelopmental abnormalities in mouse offspring. Nature. 2017; 549(7673):528–32. https://doi.org/10.1038/nature23910 PMID: 28902840.

55.   Jang YJ, Kim WK, Han DH, Lee K, Ko G. Lactobacillus fermentum species ameliorate dextran sulfate sodium-induced colitis by regulating the immune response and altering gut microbiota. Gut Microbes. 2019; 10(6):696–711. https://doi.org/10.1080/19490976.2019.1589281 PMID: 30939976.

56. Sichetti M, De Marco S, Pagiotti R, Traina G, Pietrella D. Anti-inflammatory effect of multistrain probiotic formulation (L. rhamnosus, B. lactis, and B. longum). Nutrition. 2018; 53:95–102. https://doi.org/10.1016/j.nut.2018.02.005 PMID: 29674267.

57. Nadeem A, Ahmad SF, Attia SM, Al-Ayadhi LY, Al-Harbi NO, Bakheet SA. Dysregulation in IL-6 receptors is associated with upregulated IL-17A related signaling in CD4+ T cells of children with autism. Prog Neuropsychopharmacol Biol Psychiatry. 2020; 97:109783 https://doi.org/10.1016/j.pnpbp.2019.109783 PMID: 31655158.

58. Eftekharian MM, Ghafouri-Fard S, Noroozi R, Omrani MD, Arsang-Jang S, Ganji M, et al. Cytokine profile in autistic patients. Cytokine. 2018; 108:120–6. https://doi.org/10.1016/j.cyto.2018.03.034 PMID: 29602155.

59. Moaaz M, Youssry S, Elfatatry A, El Rahman MA. Th17/Treg cells imbalance and their related cytokines (IL-17, IL-10 and TGF-beta) in children with autism spectrum disorder. J Neuroimmunol. 2019; 337:577071. https://doi.org/10.1016/j.jneuroim.2019.577071 PMID: 31671361.

60. Ding X, Xu Y, Zhang X, Zhang L, Duan G, Song C, et al. Gut microbiota changes in patients with autism spectrum disorders. J Psychiatr Res. 2020; 129:149–59. https://doi.org/10.1016/j.jpsychires.2020.06.032 PMID: 32912596.

61. Strati F, Cavalieri D, Albanese D, De Felice C, Donati C, Hayek J, et al. New evidences on the altered gut microbiota in autism spectrum disorders. Microbiome. 2017; 5(1):24. https://doi.org/10.1186/s40168-017-0242-1 PMID: 28222761.

62. De Vadder F, Kovatcheva-Datchary P, Goncalves D, Vinera J, Zitoun C, Duchampt A, et al. Microbiota-generated metabolites promote metabolic benefits via gut-brain neural circuits. Cell. 2014; 156 (1–2):84–96. https://doi.org/10.1016/j.cell.2013.12.016 PMID: 24412651.

63. Svoboda E. Could the gut microbiome be linked to autism? Nature. 2020; 577(7792):S14–S5. https://doi.org/10.1038/d41586-020-00198-y PMID: 31996829.

64. Al Suhaibani A, Bacha AB, Alonazi M, Bhat RS, El-Ansary A. Testing the combined effects of probiotics and prebiotics against neurotoxic effects of propionic acid orally administered to rat pups. Food Sci Nutr. 2021; 9(8):4440–51. https://doi.org/10.1002/fsn3.2418 PMID: 34401092.

65. Ben Bacha A, Al-Orf N, Alonazi M, Bhat RS, El-Ansary A. The anti-inflammatory and antiapoptotic effects of probiotic on induced neurotoxicity in juvenile hamsters. Food Sci Nutr. 2021; 9(9):4874–82. https://doi.org/10.1002/fsn3.2435 PMID: 34531999.

66. Bjorklund G, Meguid NA, El-Bana MA, Tinkov AA, Saad K, Dadar M, et al. Oxidative stress in autism spectrum disorder. Mol Neurobiol. 2020; 57(5):2314–32. https://doi.org/10.1007/s12035-019-01742-2 PMID: 32026227.

67. Bjorklund G, Saad K, Chirumbolo S, Kern JK, Geier DA, Geier MR, et al. Immune dysfunction and neuroinflammation in autism spectrum disorder. Acta Neurobiol Exp (Wars). 2016; 76(4):257–68. https://doi.org/10.21307/ane-2017-025 PMID: 28094817.

68. Adams JB, Johansen LJ, Powell LD, Quig D, Rubin RA. Gastrointestinal flora and gastrointestinal status in children with autism—comparisons to typical children and correlation with autism severity. BMC Gastroenterol. 2011; 11:22. https://doi.org/10.1186/1471-230X-11-22 PMID: 21410934.

69. Kang DW, Ilhan ZE, Isern NG, Hoyt DW, Howsmon DP, Shaffer M, et al. Differences in fecal microbial metabolites and microbiota of children with autism spectrum disorders. Anaerobe. 2018; 49:121–31. https://doi.org/10.1016/j.anaerobe.2017.12.007 PMID: 29274915.

70. Pulikkan J, Maji A, Dhakan DB, Saxena R, Mohan B, Anto MM, et al. Gut microbial dysbiosis in indian children with autism spectrum disorders. Microb Ecol. 2018; 76(4):1102–14. https://doi.org/10.1007/s00248-018-1176-2 PMID: 29564487.

71. Arnold LE, Luna RA, Williams K, Chan J, Parker RA, Wu Q, et al. Probiotics for gastrointestinal symptoms and quality of life in autism: a placebo-controlled pilot trial. J Child Adolesc Psychopharmacol. 2019; 29(9):659–69. https://doi.org/10.1089/cap.2018.0156 PMID: 31478755.

72. Sanctuary MR, Kain JN, Chen SY, Kalanetra K, Lemay DG, Rose DR, et al. Pilot study of probiotic/colostrum supplementation on gut function in children with autism and gastrointestinal symptoms. PloS one. 2019; 14(1):e0210064. https://doi.org/10.1371/journal.pone.0210064 PMID: 30625189.

73. Vriesman MH, Koppen IJN, Camilleri M, Di Lorenzo C, Benninga MA. Management of functional constipation in children and adults. Nat Rev Gastroenterol Hepatol. 2020; 17(1):21–39. https://doi.org/10.1038/s41575-019-0222-y PMID: 31690829.

74. Liu S, Li E, Sun Z, Fu D, Duan G, Jiang M, et al. Altered gut microbiota and short chain fatty acids in Chinese children with autism spectrum disorder. Sci Rep. 2019; 9(1):287. https://doi.org/10.1038/s41598-018-36430-z PMID: 30670726.

75. Dan Z, Mao X, Liu Q, Guo M, Zhuang Y, Liu Z, et al. Altered gut microbial profile is associated with abnormal metabolism activity of Autism Spectrum Disorder. Gut Microbes. 2020; 11(5):1246–67. https://doi.org/10.1080/19490976.2020.1747329 PMID: 32312186.

76. Marler S, Ferguson BJ, Lee EB, Peters B, Williams KC, McDonnell E, et al. Brief report: whole blood serotonin levels and gastrointestinal symptoms in autism spectrum disorder. J Autism Dev Disord. 2016; 46(3):1124–30. https://doi.org/10.1007/s10803-015-2646-8 PMID: 26527110.

77. Ballester P, Richdale AL, Baker EK, Peiro AM. Sleep in autism: A biomolecular approach to aetiology and treatment. Sleep Med Rev. 2020; 54:101357. https://doi.org/10.1016/j.smrv.2020.101357 PMID: 32759030.

78. Kraguljac NV, White DM, Reid MA, Lahti AC. Increased hippocampal glutamate and volumetric deficits in unmedicated patients with schizophrenia. JAMA Psychiatry. 2013; 70(12):1294–302. https://doi.org/10.1001/jamapsychiatry.2013.2437 PMID: 24108440.

79. El-Ansary A, Zayed N, Al-Ayadhi L, Qasem H, Anwar M, Meguid NA, et al. GABA synaptopathy promotes the elevation of caspases 3 and 9 as pro-apoptotic markers in Egyptian patients with autism spectrum disorder. Acta Neurol Belg. 2021; 121(2):489–501. https://doi.org/10.1007/s13760-019-01226-z PMID: 31673995.

80. Ford AC, Lacy BE, Talley NJ. Irritable bowel syndrome. New Engl J Med. 2017; 376(26):2566–78. https://doi.org/10.1056/NEJMra1607547 PMID: 28657875.

81. Reilly J, Gallagher L, Leader G, Shen S. Coupling of autism genes to tissue-wide expression and dysfunction of synapse, calcium signalling and transcriptional regulation. PloS one. 2020; 15(12): e0242773. https://doi.org/10.1371/journal.pone.0242773 PMID: 33338084.

82. Luna RA, Oezguen N, Balderas M, Venkatachalam A, Runge JK, Versalovic J, et al. Distinct microbiome-neuroimmune signatures correlate with functional abdominal pain in children with autism spectrum disorder. Cell Mol Gastroenterol Hepatol. 2017; 3(2):218–30. https://doi.org/10.1016/j.jcmgh.2016.11.008 PMID: 28275689.

83. Geva-Zatorsky N, Sefik E, Kua L, Pasman L, Tan TG, Ortiz-Lopez A, et al. Mining the human gut microbiota for immunomodulatory organisms. Cell. 2017; 168(5):928–43. https://doi.org/10.1016/j.cell.2017.01.022 PMID: 28215708

84. Valles-Colomer M, Falony G, Darzi Y, Tigchelaar EF, Wang J, Tito RY, et al. The neuroactive potential of the human gut microbiota in quality of life and depression. Nat Microbiol. 2019; 4(4):623–32. https://doi.org/10.1038/s41564-018-0337-x PMID: 30718848.

85. Kau AL, Ahern PP, Griffin NW, Goodman AL, Gordon JI. Human nutrition, the gut microbiome and the immune system. Nature. 2011; 474(7351):327–36. https://doi.org/10.1038/nature10213 PMID: 21677749.

86. Sanctuary M, Kain J, Chen S, Kalanetra K, Lemay D, Rose D, et al. Pilot study of probiotic/colostrum supplementation on gut function in children with autism and gastrointestinal symptoms. PloS one. 2019; 14(1):e0210064. https://doi.org/10.1371/journal.pone.0210064 PMID: 30625189.

87. Wang Y, Li N, Yang JJ, Zhao DM, Chen B, Zhang GQ, et al. Probiotics and fructo-oligosaccharide intervention modulate the microbiota-gut brain axis to improve autism spectrum reducing also the hyper-serotonergic state and the dopamine metabolism disorder. Pharmacol Res. 2020; 157:104784. https://doi.org/10.1016/j.phrs.2020.104784 PMID: 32305492.

88. Liu YW, Liong MT, Chung YE, Huang HY, Peng WS, Cheng YF, et al. Effects of Lactobacillus plantarum PS128 on children with autism spectrum disorder in taiwan: A randomized, double-blind, placebo-controlled trial. Nutrients. 2019; 11(4). https://doi.org/10.3390/nu11040820 PMID: 30979038.

89. Shaaban SY, El Gendy YG, Mehanna NS, El-Senousy WM, El-Feki HSA, Saad K, et al. The role of probiotics in children with autism spectrum disorder: A prospective, open-label study. Nutr Neurosci. 2018; 21(9):676–81. https://doi.org/10.1080/1028415X.2017.1347746 PMID: 28686541.

90. Yuan J, Song J, Zhu D, Sun E, Xia L, Zhang X, et al. Lithium Treatment is safe in children with intellectual disability. Front Mol Neurosci. 2018; 11:425. https://doi.org/10.3389/fnmol.2018.00425 PMID: 30524233.

91. Geier DA, Kern JK, Geier MR. A comparison of the autism treatment evaluation checklist (ATEC) and the childhood autism rating scale (CARS) for the quantitative evaluation of autism. J Ment Health Res Intellect Disabil. 2013; 6(4):255–67. https://doi.org/10.1080/19315864.2012.681340 PMID: 23914277.

92. Kovacs Balint Z, Raper J, Michopoulos V, Howell L, Gunter C, Bachevalier J, et al. Validation of the Social Responsiveness Scale (SRS) to screen for atypical social behaviors in juvenile macaques. PloS one. 2021; 16(5):e0235946. https://doi.org/10.1371/journal.pone.0235946 PMID: 34014933.

93. Svedlund J, Sjödin I, Dotevall G. GSRS—a clinical rating scale for gastrointestinal symptoms in patients with irritable bowel syndrome and peptic ulcer disease. Dig Dis Sci. 1988; 33(2):129–34. https://doi.org/10.1007/BF01535722 PMID: 3123181.

94.   Lewis SJ, Heaton KW. Stool form scale as a useful guide to intestinal transit time. Scand J Gastroen-terol. 1997; 32(9):920–4. https://doi.org/10.3109/00365529709011203 PMID: 9299672.

95.   Keogh S, Bridle C, Siriwardena N, Nadkarni A, Laparidou D, Durrant S, et al. Effectiveness of non-pharmacological interventions for insomnia in children with Autism Spectrum Disorder: A systematic review and meta-analysis. PloS one. 2019; 14(8):e0221428. https://doi.org/10.1371/journal.pone.0221428 PMID: 31437211.

96.   Reitman D, Currier RO, Stickle TR. A critical evaluation of the Parenting Stress Index-Short Form (PSI-SF) in a head start population. J Clin Child Adolesc Psychol. 2002; 31(3):384–92. https://doi.org/10.1207/S15374424JCCP3103_10 PMID: 12149976.

97.   Wardle J, Guthrie CA, Sanderson S, Rapoport L. Development of the children's eating behaviour questionnaire. J Child Psychol Psychiatry, 2001; 42(7):963–70. https://doi.org/10.1111/1469-7610.00792 PMID: 11693591.

98.   Ahmad SF, Ansari MA, Nadeem A, Bakheet SA, Al-Ayadhi LY, Attia SM. Upregulation of peripheral CXC and CC chemokine receptor expression on CD4(+) T cells is associated with immune dysregula-tion in children with autism. Prog Neuropsychopharmacol Biol Psychiatry. 2018; 81:211–20. https://doi.org/10.1016/j.pnpbp.2017.10.001 PMID: 28986277.

99.   Ahmad SF, Zoheir KMA, Ansari MA, Nadeem A, Bakheet SA, Al-Ayadhi LY, et al. Dysregulation of Th1, Th2, Th17, and T regulatory cell-related transcription factor signaling in children with autism. Mol Neurobiol. 2017; 54(6):4390–400. https://doi.org/10.1007/s12035-016-9977-0 PMID: 27344332.

100.   Sharon G, Cruz NJ, Kang DW, Gandal MJ, Wang B, Kim YM, et al. Human gut microbiota from autism spectrum disorder promote behavioral symptoms in mice. Cell. 2019; 177(6):1600–18 e17. https://doi.org/10.1016/j.cell.2019.05.004 PMID: 31150625.

101.   Al-Ayadhi L, Zayed N, Bhat RS, Moubayed NMS, Al-Muammar MN, El-Ansary A. The use of biomark-ers associated with leaky gut as a diagnostic tool for early intervention in autism spectrum disorder: a systematic review. Gut Pathog. 2021; 13(1):54. https://doi.org/10.1186/s13099-021-00448-y PMID: 34517895.

102.   Sun J, Qi C, Zhu H, Zhou Q, Xiao H, Le G, et al. IgA-Targeted lactobacillus jensenii modulated gut bar-rier and microbiota in high-fat diet-fed mice. Front Microbiol. 2019; 10:1179. https://doi.org/10.3389/fmicb.2019.01179 PMID: 31178854.

103.   Krumbeck JA, Rasmussen HE, Hutkins RW, Clarke J, Shawron K, Keshavarzian A, et al. Probiotic Bifidobacterium strains and galactooligosaccharides improve intestinal barrier function in obese adults but show no synergism when used together as synbiotics. Microbiome. 2018; 6(1):121. https://doi.org/10.1186/s40168-018-0494-4 PMID: 29954454.

104.   Mortensen B, Murphy C, O'Grady J, Lucey M, Elsafi G, Barry L, et al. Bifidobacteriumbreve Bif195 pro-tects against small-intestinal damage caused by acetylsalicylic acid in healthy volunteers. Gastroen-terology. 2019; 157(3):637–46 e4. https://doi.org/10.1053/j.gastro.2019.05.008 PMID: 31095949.

105.   Spacova I, Petrova MI, Fremau A, Pollaris L, Vanoirbeek J, Ceuppens JL, et al. Intranasal administra-tion of probiotic Lactobacillus rhamnosus GG prevents birch pollen-induced allergic asthma in a murine model. Allergy. 2019; 74(1):100–10. https://doi.org/10.1111/all.13502 PMID: 29888398.

106.   Firth J, Teasdale SB, Allott K, Siskind D, Marx W, Cotter J, et al. The efficacy and safety of nutrient supplements in the treatment of mental disorders: a meta-review of meta-analyses of randomized con-trolled trials. World Psychiatry. 2019; 18(3):308–24. https://doi.org/10.1002/wps.20672 PMID: 31496103.