Probiotic Therapy for Treating Behavioral and Gastrointestinal Symptoms in Autism Spectrum Disorder: A Systematic Review of Clinical Trials

Jun LIU[1], Guo-bing WAN[2], Ming-shi HUANG[2], George Agyapong[1], Tian-le ZOU[3], Xue-ying ZHANG[4], Yen-Wenn LIU[5], Yi-qing SONG[6], Ying-Chieh Tsai[7], Xue-jun KONG[8]

[1]Harvard Medical School, Boston, MA, 02115, USA

[2]Women and Children's Hospital, Shenzhen 518052, China

[3]New York University, Buffalo, NY, USA, 14214

[4]Icahn School of Medicine at Mount Sinai, New York City, NY, USA, 10029

[5]National Yang Ming University, Taipei, Taiwan, 112

[6]Indiana University, Indianapolis, IN, 46202

[7]National Yang Ming University, Taipei, Taiwan, 112

[8]Massachusetts General Hospital, Charlestown, MA, USA, 02129

First authors: Jun Liu jun_liu@hms.harvard.edu, Guo-bing Wan email gbw1978@aliyun.com

Corresponding author: Xue-jun Kong xkong1@mgh.harvard.edu

Funding: None

Liu, J., Wan, G., Huang, M., Agyapong, G., Zou, T., Zhang, X., Liu, Y.-W., Song, Y., Tsai, Y.-C., & Kong, X. (2019). Probiotic Therapy for Treating Behavioral and Gastrointestinal Symptoms in Autism Spectrum Disorder: A Systematic Review of Clinical Trials. Current Medical Science, 39(2), 173–184. https://doi.org/10.1007/s11596-019-2016-4

Summary: The therapeutic potentials of probiotics in autism spectrum disorder (ASD) remains controversial, with the only existing systematic review on this topic published in 2015. Results from new trials have become available in recent years, we therefore conducted an updated systematic review, to assess the efficacy of probiotics in relieving behavioral symptoms of ASD and gastrointestinal comorbidities. Our review includes two randomized controlled trials, which showed improvement of ASD behaviors, and three open trials, all which exhibited a trend of improvement. Four of these trials concluded from subjective measures that gastrointestinal function indices showed a trend of improvement with probiotic therapy. Additional rigorous trials are needed to evaluate the effects of probiotic supplements in ASD.

Key words: autism spectrum disorder; probiotics; systematic review

Autism spectrum disorder (ASD) is a complex neurological and developmental disorder characterized by impaired communication and social interaction skills, as well as stereotypical repetitive behavioral patterns[1]. The prevalence of ASD has more than doubled from 1 in 150 as of 2000 to 1 in 59 as of 2018 in the United States of America[1], with a similar trend world-wide[2, 3]. Its etiology remains elusive, and it is thought to involve a combination of genetic changes and environmental factors[4, 5]. Among these pathogenic factors, microbiome dysbiosis of the gastrointestinal (GI) system appears to be an important player[6, 7]. In ASD patients, GI symptoms are more prevalent than their neurotypical counterparts[8, 9]. The degree of GI dysfunction is closely associated with alterations of gut microbiome, including changes in quantity, diversity, and enrichment or deficiency of certain taxonomic groups[8, 10]. Further, the severities of neurological and behavioral symptom in ASD correlate with gut microbiome profiles in some subgroups of patients[8, 10, 11]. These findings highlight the exciting possibility that microbiome manipulation may be an attractive target for therapeutic interventions for ASD.

Current management strategies for ASD focus on behavioral therapies, psychiatric medications and specific treatments for individual comorbidities, all with limited success. Parents, clinicians and scientists have explored alternative therapies, including strategies to modify gut microbiome[12, 13]. These include prebiotics, probiotics, antibiotics, special diets, fecal microbial transplantation and even helminth therapies[11, 12, 14, 15].

Probiotic supplementation is one of the most popular approaches due to ease of use, wide availability and good safety profiles. Probiotics are a concoction of living microbial strains that are ingested and believed to colonize the gut to benefit host health. One *Escherichia coli* strain, several lactic acid producing *Lactobacillus* strains, and a number of *Bifidobacteria* comprise the primary microorganisms classified as probiotic strains[11, 14, 16]. It has emerged as an emerging adjunctive therapy for several inflammatory conditions, such as inflammatory bowel disease[17] and antibiotics associated diarrhea[18]. Probiotics may aid in ASD rehabilitation by promoting leaky gut healing, modulating neuronal functions via a vagus nerve-mediated "gut-brain axis", or reducing inflammation of the central nervous system by bacterial metabolites[11, 14, 19, 20].

However, the therapeutic potentials of probiotics in ASD is still inconclusive[16]. It is unclear whether and to what extent probiotics can alleviate patients' GI symptoms, behavioral symptoms, or both. The formulation and dosage of probiotics, and suitable target ASD subtypes also remain poorly defined[16]. Despite promising murine studies showing direct evidence of behavioral modification after probiotic supplementation[21, 22], clinical correlates in human studies are less convincing. The current literature consists of mostly case reports, small scaled observational studies and cohort studies. Results in clinical trials are mixed, possibly due to the differences in species or strain of probiotic used, or methodological and population variations among the studies. High quality, large scale randomized controlled trials (RCTs) are lacking due

to ASD's status as a vulnerable population.

The only existing and the most recent systematic review published in 2015 included 4 studies with mixed study designs (including one RCT) and did not address probiotics' effect on ASD behaviors[16]. All recent review articles since 2015 are qualitative. In the past three years, a number of new studies have become available, which all focused on probiotic therapy's effect on behavioral modifications. We therefore conducted an updated systematic review of clinical trials, including RCTs and non-randomized (or quasi-experimental) controlled trials (non-RCTs), to comprehensively assess the efficacy of probiotics in behavioral symptoms of ASD, and where possible, GI comorbidities, adverse effects, and other objective changes measured by biomarkers and stool microbiome compositions.

1 MATERIALS AND METHODS

This systematic review/meta-analysis was performed and reported following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (S2 Table).

1.1 Search Strategy and Study Selection

We conducted a medical literature search using PUBMED, EMBASE, ProQuest thesis database and US National Library of Medicine clinical trial registry up to January 2018. The eligibility criteria were defined a priori, which include (1) the study includes a probiotic intervention targeting ASD population; (2) the study includes neurobehavioral assessment before and after intervention (studies with GI-only outcome measures are excluded); (3) the study is a confirmed RCT or non-RCT. We also included unpublished but completed studies in the above categories for which authors granted the right to include in the present study. The following

types of articles are excluded: anecdotal case reports of single patients, case series and any other retrospective study designs, prospective observational studies, analysis of electronic medical record or registries databases, narrative review articles, commentaries, mechanism of action research articles, or meta-analysis or systematic reviews.

Studies in ASD were searched with the terms “autistic disorder”, “autism spectrum disorder”, or “autism”, as medical subject headings. The term “autism” or “asperger” were used as free text terms. These terms were combined using the set operator AND with articles searched with the following terms: “probiotics” or “probiotic”, “Saccharomyces”, “Lactobacillus”, “Bifidobacterium”, or “Escherichia coli”, both as medical subject heading terms and as free text items. We did not impose language restrictions. Titles and abstracts of the papers identified by this initial search strategy were evaluated by two independent reviewers (JL and MH). We obtained potentially relevant papers and conducted subsequent full text screen. We also conducted a recursive search of the literature based on the bibliographies of the relevant full text articles. Two independent reviewers conducted full text screen using pre-designed eligibility criteria. Any disagreement between investigators was resolved by consensus.

1.2 Outcome Assessment

1.2.1 ASD Behavioral Outcome(s) The outcomes included improvements in behaviors, such as overall assessment, speech/language/communication, sociability, sensory/cognitive awareness, and health/physical/behavior disruptive behavior. All available psychiatric comorbidities such as anxiety, aggressive behavior, inattention and opposition/defiance, were also extracted. Data were available in the following formats: (1) ASD behavioral scores (total and subcategories), determined using validated clinician evaluation tools, such as Clinical Global Impression

Severity (CGI-S) and Clinical Global Impression Improvement (CGI-I) forms. Data were extracted as continuous variables (mean, standard deviation [SD], total sample size [N]), in the format of treatment vs. baseline or treatment vs. placebo control. (2) ASD behavioral scores (total and subcategories), determined using validated parent/care-giver assessment questionnaires, such as the Autism Treatment Evaluation Checklist (ATEC), Development Behavior Checklist–primary caregiver version(DBC-P), Aberrant Behavior Checklist-Taiwan version (ABC-T), Child Behavior Checklist (CBCL), Social Responsiveness Scale (SRS), and Swanson, Nolan and Pelham Questionnaire-IV-Taiwan version (SNAP-IV-T) questionnaires. Data were extracted as continuous variables (mean, SD, N), in the format of treatment vs. baseline or treatment vs. placebo control. (3) Patient improvement in ASD behavioral scores, data were extracted as dichotomous variables (either as number of patients who showed improvement/number of patients who did not show improvement; or as percentage of patients who showed improvement and N), in the format of treatment vs. baseline or treatment vs. placebo.

1.2.2 ASD GI Function Outcome(s) Data for the following GI symptoms/problems were extracted from the included articles: overall assessment, constipation, diarrhea, stool consistency, stool smell, flatulence, flatus, abdominal pain, stool frequency, wherever available. Data were available in the following formats: (1) GI function scores (total and subcategories), determined using validated tools such as GSI (GI severity index) or ATEC subcategory. Data were extracted as continuous variables (mean, SD, N), in the format of treatment vs. baseline or treatment vs. placebo control. (2) Patient improvement in GI scores (as above) or GI symptoms according to qualitative GI diary or stool card. Data were extracted as dichotomous variables (either as number of patients who showed improvement/number of patients who did not show improvement; or as percentage of patients who showed improvement and N), in the format of

treatment vs. baseline or treatment vs. placebo.

In addition, we collected information on other outcomes, including other adverse events, study withdrawals, including all-cause withdrawals and withdrawals from adverse events, as well as serum or stool biomarker changes, if available.

1.3 Data Extraction

One reviewer extracted data from each study into a pre-piloted Microsoft Excel spreadsheet (XP professional edition; Microsoft Corp, Redmond, WA, USA). A second reviewer cross-checked each data variable extracted against the original publication. Disagreement was resolved via discussion. In addition, the following information was extracted wherever available (including RCT, non-RCTs): age of trial participants, gender of trial participants, country of origin, setting of the study (primary, secondary or tertiary care-based), probiotic species, dosage and schedule of probiotics, dosage and schedule of control therapy if applicable, duration of therapy, number of individuals incurring any adverse events. When necessary, we contacted the authors to obtain original data via e-mail. Authors of Parracho*et al*[13], Kałużna-Czaplińska *et al*[23], and West *et al*[24] were contacted but no additional original data was returned.

1.4 Assessment of Risk of Bias

This was conducted by two investigators according to guidance published in the Cochrane Handbook[25]. For RCTs, risk of bias was assessed by recording the Cochrane Collaboration's risk of bias method used to assess randomization generation, concealment of treatment allocation, and whether blinding was implemented for participants, personnel, and outcomes assessors (Table 1). We also examined evidence of incomplete outcomes data, and whether there was

evidence of selective reporting of outcomes. Each domain was rated as low, unclear and high. For non-RCTs and before-and-after studies, level of evidence was graded based on the methodological index for non-randomized studies (MINORS) scale (Table 2)[26]. Study authors were contacted to obtain further information (e.g. details on blinding) if necessary. Risk of bias was assessed for each outcome. Any disagreement in risk of bias assessment was resolved by discussion.

1.5 Qualitative Data Synthesis

Due to small number of studies, the variability in the assessment tools used and limited availability of data from included studies, we predominantly adopted qualitative approach for data synthesis. All relevant statistically significant changes, missing data, changes that are not significant or have no discussion of statistical significance, were reported and summarized in Tables 1, 2, and 4-6.

Table 1
Table 2

2 RESULTS

Initial literature searches identified 1260 articles (fig. 1). Three additional papers were published and identified through hand-search of systematic review reference lists. We additionally identified 7 ongoing/recently completed studies including one published RCT protocol that met inclusion criteria. Authors of one of the recently completed studies gave permission to include their results in the present systematic review, but results are not available for the other studies. After removing duplicates, 727 articles were excluded based on title and abstract screening (162 further duplicates, 31 retrospective study design, 16 mechanism research, 37 animal research, 6 analysis of clinical database or registries, 156 review articles, 190 not

about ASD and/or probiotics, 3 case series, 6 case report, 7 study designs that satisfy the inclusion criteria but do not involve behavioral assessment, 8 book chapter, conference abstract, 105 no abstract or full text available). After title and abstract screening, 19 articles were selected for full-text screening. In total, 5 papers, including two RCTs and three non-RCTs, met inclusion criteria for the present systematic review and meta-analysis[13, 23, 24, 27, 28]. An overview of the reviewed studies is presented in Table 3. The other 14 articles were excluded (4 duplicates, 1 RCT protocol only, 1 inappropriate patient population, 3 inappropriate study design, 3 inappropriate outcomes, 1 inappropriate intervention, 1 review). Of the two RCTs, one was a randomized parallel double-blind placebo-controlled trial whereas one was a randomized double-blind cross-over study. The other three studies were non-randomized, open-label, single arm, before-and-after interventional trials.

2.1 Characteristics of Study Participants

As shown in tables 4 and 5, the two RCTs included a total of 142 patients (age range: 4-16 years), all of whom were diagnosed with ASD. None of the studies included information regarding ASD subtypes. Approximately 98% were male and approximately 2% were female.

In comparison, three non-RCT studies included 85 patients with ASD (age range: 3–16 years). Gender proportions cannot be calculated because one of the studies did not report patient gender. For the two studies where gender proportions are reported, 75% pooled total subjects were male and 25% were female. None of the studies included information regarding ASD subtypes.

Figure 1
Table 3

Table 4
Table 5

2.2 Probiotics Used and Features of Treatment

The RCTs all studied the used of probiotics with a placebo comparator (table 6). Liu *et al*[27] and Parracho*et al*[13] both used *Lactobacillus plantarum* as intervention with similar dosage (3 vs 4.5 x 10^10 CFU/capsule/D), and similar duration of treatment (4 vs 3 weeks), respectively. Table 6

Probiotic strains used in the open trials varied, including *Lactobacillus acidophilus*[23], a mixture of *Lactobacillus acidophilus*, *Lactobacillus rhamnosus* and *Bifidobacterialongum*[28], and a mixture of *Lactocillus acidophilus*, *Lactobacillus casei, Lactobacillus delbruecki, Bifidobacterialongum* and *Bifidobacteriabifidum*[24]. Interestingly, intervention in Shaaban *et al*[28]also contains prebiotic ingredients (carrot powder), whereas intervention in West *et al*[24] contained postbiotic derivatives *from L rhamnosus* V strain including peptidoglycan, muramyl peptides, and nucleotide-containing components or DNA motifs. The three studies had variable durations of therapy (2 to 6 months), with a variable range of dosages. In Shaaban *et al*, the cohort received behavioral therapy during probiotics administration. Although patients with use of psychiatric medications within the preceding 3 months were excluded, it was not clear whether they received additional medications for behavioral symptoms during the probiotics intervention. In Kałużna-Czaplińska *et al*[23], it is unclear whether patients received therapy or medication for ASD (author contacted, no reply). In West *et al*, 14.25% patients were receiving concurrent prescription medications, but the nature of the medications was not specified and there is no information regarding ongoing behavioral therapies (author contacted, replied but no additional information was provided).

2.3 Risk of Bias and Quality of Evidence

Tables 1 and 2 outline risk of bias and quality assessment ratings for each of the included studies. For RCTs, Liu *et al*[27] was deemed to have overall high quality of study design, and Parracho *et al*[13] is downgraded to moderate quality because of higher risks of bias. Open trials were mostly judged to be at serious to critical risk of bias, due to lack of randomization, blinding and control groups. Their results are at most indicators of clinical experience instead of evidence for the effectiveness of the intervention. Apart from Shaaban *et al*[28] methodological quality for these studies were graded as low to very low. Shaaban *et al* received moderate grade of quality rating due to its superior study design, including larger sample size, appropriate baseline characterizations, use of appropriate statistical methods assessment tools.

2.4 Qualitative Data Synthesis

2.4.1 Behavioral **M**odification As shown in Table 4, the two RCTs used different tools to assess ASD behavioral symptoms. Parracho *et al*[13] used a care-giver questionnaire, DBC-P, which is a ranking score test consisting of 96 items. It assesses five major categories of behavioral characteristics including Disruptive/Antisocial behavior, Self-absorbedness, Communication disturbances, Anxiety, and Social Relating. Liu *et al* adopted both clinician assessment tools and care-giver questionnaires to evaluate ASD-related behavioral symptoms. They cover a more comprehensive range of psychiatric, behavioral and social symptoms of ASD. Lower score indicates less symptom in every test.

The two RCTs were unable to provide conclusive evidence for improvement of ASD behavior indices using either subjective or objective measurements, due to suboptimal statistical analysis methodologies used in both studies. Parracho *et al*[13] claimed statistically significant

improvement of behavioral scores for disruptive antisocial behavior, anxiety problems, and communication disturbances compared to baseline following probiotics treatment ($p<0.05$), with no simultaneous improvement in the placebo arm. Although the overall indicator of behavioral/emotional disturbances calculated by the total DBC-P score was lower (better) after treatment than baseline, the placebo group also showed improvement in this indicator from baseline. However, authors did not provide adequate statistical analysis between [end point-baseline difference in placebo group] and [end point-baseline difference in treatment group], rending their analysis effectively that of an open trial. In Liu *et al*, no statistically significant differences in behavioral scores detected between probiotics and placebo control groups after 4 weeks. Similar to Parracho *et al*, authors did not conduct statistical testing of the difference (score of week 4 – score of baseline) between PS128 (the strain *L. plantarum* used) and Placebo groups in the overall analysis.

In open trials, both West *et al*[24] and Shaaban *et al*[28] both used ATEC, whereas Kałużna-Czaplińska *et al*[23] used an unspecified tool to assess ASD related behaviors. All three studies showed a trend of behavioral improvement with probiotics. All assessments were performed by care-givers. West *et al* showed mean ATEC score significantly decreased ($p<0.05$) following initiation of treatment, in all four of the ATEC categories including speech/language communication, sociability, sensory cognitive awareness, health/physical behavior. Shaaban *et al* showed that the total ATEC scores (p value=0.0001) and scores in all four categories ($p<0.05$) significantly decreased following probiotic supplementation. Kałużna-Czaplińska *et al* assessed patients' ability of concentration, eye contact, follow out orders and reaction to other people's emotions before and after treatment. They showed that a portion of the subjects showed improvement in each of the four domains of autistic behaviors, especially for "ability of

concentration" (60%) and "follow out orders" (68%). However, no statistical method was reported, and no concrete numbers or P values were attached to the proportions.

2.4.2 GI Symptom Modification Only one of the two RCTs, Parracho *et al*[13] assessed bowel functions (bowel movement and stool consistency) and GI symptoms (abdominal pain, intestinal bloating and flatulence) using a self-diary. Probiotic feeding resulted in a statistically significant ($P<0.01$) higher percentage of 'formed' stool (73.3%) compared to the placebo (64.8%), whilst the percentage of 'hard' stool samples was lower (8.1% vs 15.9%). On the other hand, no significant difference was detected for bowel movements and any GI symptoms between treatment and placebo groups compared to baseline.

All three open trials assessed GI function, to varying degrees, and all studies showed a trend of improvement of GI function indices using subjective measures. West *et al*[24] assessed GI function with both stool frequency diary and ATEC, which is completed by care-giver and has built-in assessment of constipation and diarrhea in the health/physical/behavior sub-category of the questionnaire. West *et al* showed the majority of participants in this study reported severe constipation (84%) or diarrhea (56%) at baseline; 48 % reported decreases in diarrhea severity and 52% reported decreases in constipation severity after treatment, although this is no discussion of statistical significance. Similarly, the study found reduction in ATEC-diarrhea (2.4 vs 1.6) and constipation (1.0 vs 0.4) scores after treatment, but it is unclear whether the results are statistically significant. Parents also reported increased stool frequency although this was not statistically significant. Shaaban *et al* used modified six-item Gastrointestinal Severity Index (6-GSI), another care-giver questionnaire including questions on constipation, diarrhea, stool consistency, stool smell, flatulence, and abdominal pain. Kałużna-Czaplińska *et al*[23] used an unspecified assessment tool. Shaaban *et al*[28] showed significant reduction in the total GSI score

(7.23 vs 3.57, $p<0.0001$), and in all sub-categories ($p<0.05$), including scores of constipation (1.27 vs 0.73), stool consistency (1.2 vs 0.87), flatulence (1.17 vs 0.87), and abdominal pain (1.17 vs 0.73) following probiotic supplementation. They also reported that 60% showed improvement in abdominal pain, 57% in flatulence, 42.5% in constipation, 37.5% in diarrhea, 25% in stool smell, and 16.6% in stool consistency. The author did not discuss statistical significance associated with the changes in proportions. Kałużna-Czaplińska *et al*[23] reported that 100% participants had GI symptoms at baseline. It did not report changes in GI function following treatment.

2.4.3 Subgroup Analysis Liu *et al*[27] is the only study that performed analysis by stratification. They divided the subjects treated with probiotics into ages of 7-12 and 13-15 years for questionnaire items that were identified as significantly different between treatment and baseline by the initial pooled analysis. The differences in scores (week 4 − baseline) for the questionnaires were examined. However, the subgroup analysis was done post-hoc (stratification was not described as part of the initial study design) and the authors did not comment on the statistical power for performing subgroup analysis.

Liu *et al*'s post-hoc subgroup analysis showed a small but statistically significant improvement in SNAP-IV-T total score (P=0.02) and opposition/defiance sub-scale score (P=0.03) in probiotics groups. Difference (score of week 4 – score of baseline) between PS128 and Placebo groups was compared by student's t test[27].

2.4.4 Correlations between GI and behavioral symptoms improvement Shaaban *et al*[28] shows improvements in gastrointestinal symptoms (assessed by the 6-GSI) were strongly correlated

with the improvements of the severity of autism (assessed by the ATEC) after probiotic supplementation, (r = 0. 674, p value= 0.0001).

2.4.5 Adverse Effects Overall, adverse effects of probiotic therapy are infrequent and non-severe, although only 40% included studies reported adverse effects. Only two studies reported adverse effects in detail. Shaaban *et al* monitored adverse effects throughout the study without providing details of assessment. No serious adverse effects were reported. In total 6 mild and transient incidences (out of N=30) were reported: diarrhea (1 patient), bloating (2 patients), abdominal cramps (2 patients) and skin rash (1 patient). Importantly, none of the patients reported cessation of the intervention. Parracho *et al*[13] is the only study that provided details on adverse effect monitoring (description of event, date and duration recorded throughout the study, on separate forms). They found that 4 subjects (out of N=62) withdrew from study due to adverse effects, including skin rash (1 patient), diarrhea (2 patients), and weight loss (1 patient).

Liu *et al*, West *et al*, and Kałużna-Czaplińska *et al* did not perform or performed only limited assessment of adverse effects. Liu *et al* included assessment of adverse effects though there was no detail on how this was done. They reported diarrhea as the only adverse effect. However, it was not clear what proportion of patients experienced this adverse effect, and whether the proportion of subjects suffering from diarrhea differ in the treatment and placebo groups. West *et al* did not design their study to monitor adverse events, but authors noted that none of the respondents reported cessation of intervention due to adverse effects. There was no mentioning of adverse effect assessment in Kałużna-Czaplińska *et al*.

2.4.6 Changes in Stool/Urine Markers and Other Clinical Indices Parracho *et al*[13] and Shaaban *et al*[28] assessed changes in the stool microbiota composition. Parracho *et al* found that probiotic supplementation resulted in significantly higher Lab158 (*Lactobacilli* and *Enterococci* group) counts, and significantly lower Erec482 (*Clostridium* cluster XIVa) compared to the placebo group. In Shaaban *et al*, the stool samples were tested to compare the levels of beneficial bacteria (mainly *Bifidobacteria* and *Lactobacillus* species) between patients with ASD and healthy controls (note: the study only included healthy controls for stool assessment and not for probiotics treatment). They found that the levels of *Bifidobacteria* were significantly lower in stool of ASD children compared to control group (p value=0.0001) at baseline. After probiotics treatment, there was a significant increase in the colony counts of *Bifidobacteria* and *Lactobacillus* in stool of autistic children using PCR method (p value <0.0001).

Shaaban *et al* also showed that (n=18) overweight patients had a statistically significant decrease in the body weight (p< 0.014) and BMI (p value < 0.01) after probiotic treatment for 3 months.

Kałużna-Czaplińska *et al*[23] assessed the level of urine D-arabinitol (DA, a metabolite of pathogenic candida species shown to be elevated in urine of ASD patients) and the ratio of urine D-/L-arabinitolin the urine of children with autism before and after probiotic treatment using capillary gas chromatography/mass spectrometry. The probiotic supplementation let to a significant decrease in D-arabinitol and the D-/L-arabinitolratio (P<0.05)

3 DISCUSSION

We conducted a systematic review to qualitatively synthesize the available evidence on the efficacy and safety of probiotic supplementation as an adjunctive treatment for ASD. In contrast

to the 2015 systematic review that included retrospective studies, we only included prospective RCTs and open-label trials in order to minimize bias, confounding, and difficulty making causal inferences. In summary, all studies (both RCT and non-RCTs) pointed towards a similar trend of improvement in both care-giver reported ASD symptoms and GI symptoms after probiotic therapies. Unfortunately, due to flaws in statistical analyses, the paucity of RCTs, inherent study limitations in non-randomized trials, and significant between-trial heterogeneity, our study provide only suggestive but not conclusive evidence regarding the efficacy of probiotics on GI and behavioral symptoms among ASD patients.

3.1 Effect of Probiotics on ASD Symptoms: RCT Evidence

Among the two RCTs, Liu *et al*[27] offers superior study design, but both studies suffered from suboptimal statistical analysis. Although Parracho *et al*[13] designed their study as a placebo-controlled trial, the authors only performed data analysis comparing baseline and post treatment behavioral scores without statistical analysis between delta value in the probiotics and placebo groups. Thus, the inadequate data analysis effectively rendered the study as an open-label trial, undermining the strength of their conclusion and rendering their claim of statistical significance invalid. Liu *et al* appeared to have made similar errors in their overall analysis, but they conducted statistical comparison of the difference (score of week 4 – score of baseline) between PS128 and Placebo groups in the subsequent subgroup analysis.

The effect sizes of behavioral improvement measured in both trials were small, if any, so the question remains whether such improvement should be considered clinically relevant. In addition, Liu *et al*[27] is the only study that adopted clinician assessment, yet it did not observe statistically significant ASD improvement using clinician assessment tools. Whereas Liu *et al* reported power calculation for determining sample sizes to assess anticipated effects on primary

outcomes, Parracho *et al*[13] did not include power calculation. Both trials also suffered from relatively short trial duration. Previous trials investigating the effect of probiotic therapies in inflammatory bowel diseases typically followed patients for 6-24 months of therapy[17]. Liu *et al* and Parracho *et al* both assessed the effect of the probiotics for less than 1 month. Since microbiome modification is a slow process and establishment of a new gut eco-system may take time, it is possible that the marginal clinical benefits observed in both studies can be partially attributable to the short durations of treatments.

Additionally, there are other factors that may contribute to between-trial inconsistencies, including small sample sizes, questionable allocation concealment/incomplete randomization, variable duration of treatments, high rates of dropouts (especially in Parracho *et al*), differences in the probiotic strains, and different study populations. Other issues that affect the quality of both studies include incomplete characterization of baseline characteristics, poorly defined primary and secondary end points, less comprehensive raw data reporting and adverse event reporting, and inadequate statistical modeling. Both RCTs observed very high levels of placebo effect, and Parracho *et al* noted high inter-subject variability.

3.2 Effect of Probiotics on ASD Symptoms: Non-RCT Evidence

Non-RCT evidence was mostly consistent with the RCT results, which suggests that probiotics treatment may be beneficial in improving behavioral symptoms. However, these studies provide only lower quality evidence compared to RCTs due to potential for selection bias, confounding, or any residual sources of bias in the study design and statistical analysis. Among them, Shaaban *et al* appears to have higher-quality study design and lower risk of bias[28]. However, there may be serious flaws associated with their data analysis, which could further undermine their conclusion. For example, the author reported patients' weight before and after probiotics

treatment to be 25.91 +/- 5.32 kg to 25.79 +/- 5.16 kg, respectively. This change was associated with a p-value of 0.014, which seems unlikely given the small sample size and small magnitude of change. We contacted the authors regarding this concern and requested original data, but authors did not reply to our inquiry. Therefore, it is not clear whether there are systematic errors associated with their data analysis related to other parts of the analysis, including primary outcomes. West *et al* carries the highest risk of bias and poorest study design and statistical methodology[24], whereas Kałużna-Czaplińska*et al* had limited analysis of GI symptoms and behavioral symptoms despite having relatively low risk of bias[23].

3.3 Probiotics and GI Symptoms

Both RCT and non-RCT suggest that probiotics may improve GI symptoms, but high-quality evidence is lacking. Although most studies attempted to assess probiotics' effect on GI symptoms, the majority of the trials were not adequately designed to do so. The study design of Parracho *et al*[13], West *et al*[24], and Shaaban *et al*[28] included children with GI symptoms as well as those without GI symptoms. However, none of the studies performed either stratification by GI symptoms initially or post-hoc subgroup analysis focusing on those with GI symptoms at baseline, which would be a more logical approach to assess probiotics' effect on GI symptoms. Notably, neither of the two placebo-controlled trials were designed to assess GI symptoms. Liu *et al* did not assess GI symptoms, which is a major drawback of the study. Although Parracho *et al* compared GI scores between treatment and placebo group after the intervention, their analysis did not take into account the baseline GI symptoms. The inadequate statistical analysis seriously undermines their conclusion regarding GI symptom improvement.

3.4 Adverse Effects of Probiotics

Most studies were not designed to assess adverse reactions, including the only properly designed RCT, Liu *et al*[27] (Table 6). However, among the studies that monitored adverse effects, the reported adverse reactions appear to be mild and infrequent. Only Parracho *et al*[13] described study withdraw (4 out of 62) due to adverse effects.

3.5 Dose-response Relationship

The five studies included in the current systematic review span a variety of treatment characteristics, including different probiotic strains, single vs multi-species, different treatment durations. Due to the paucity of data and poor inter-comparability between studies, there is currently no evidence to suggest that any treatment regimen is superior and whether single species is better than multiple species. In 3 out of the 5 studies examined, probiotics were used as an adjunctive therapy while patients continued to receive their regular medical management and behavioral therapies. Two studies did not specify medication and therapy use.

A probiotics dose-response relationship for treatment of GI symptoms and behavioral symptoms cannot be extrapolated based on the cumulative data from the existing trials due to the large heterogeneity between studies. According to recent meta-analyses, clinical studies investigating probiotic therapies for most other diseases such as *Clostridium Difficile*-associated diarrhea, necrotizing enterocolitis, prevention of atopic dermatitis, slow intestinal transit, prophylaxis in colorectal cancer and relief of irritable bowel syndrome, demonstrated no clear dose-response relationships[18]. On the other hand, both meta-analyses and dedicated dose-response studies have observed a positive correlation between dose and antibiotic-associated diarrhea risk[18]. For inflammatory bowel disease, although most studies have not investigated dose-relationships[17] , one study examined several different doses of the probiotic and per protocol analysis indicated a statistically significant difference in dose-responsiveness; however,

they failed to show statistical significance in the intent-to-treat analysis[29]. In general, the dose concentration of probiotics needed to achieve clinical effect has been quoted as>10^6 cfu/ml in the small bowel and>10^8 cfu/g in the colon[30]. None of the studies included in this systematic review provided information regarding dose concentration. None of the studies included in this systematic review performed dose-response analysis.

3.6 Differential Response to Probiotic by Subgroups of ASD Patients

ASD is a heterogeneous condition with tremendous individual variability and existing evidence is insufficient to reach any conclusion regarding which subgroup of patients may benefit the most from probiotics. The key question to parents, care-givers and clinicians is, if probiotic therapy works (which has yet to be proven by high quality RCTs), and what subtypes of ASD patients may benefit more from it. Despite relatively insufficient power, sub-group analysis from this systematic review and meta-analysis suggest that ASD symptom improvement appeared to be associated with improvement of GI symptoms[28], and that the younger age group appeared to benefit more from probiotic therapy compared to older children[27]. There is insufficient data to compare treatment responses in different gender. Future studies may be able to show a greater impact of probiotics by limiting the cohorts to younger patients, and those with more severe baseline GI functions.

3.7 Limitations

Limitations to this systematic review include the lack of robust empirical research on probiotic intervention for ASD in the published literature and hence small number of eligible studies. Even in studies that are included, most have small sample sizes that are not adequately powered, which

further limit the quality of this systematic review. The wide range of probiotic regimen together with the limited number of studies makes it unfeasible to compare effectiveness between studies using quantitative methods. In addition, there may be publication bias in this field, and clinical trials with only negative results may not have been published in the first place, therefore skewing the conclusion towards a more positive outlook of probiotic efficacy.

3.8 Conclusion and Future Directions

Our systematic review of available trial data provided suggestive evidence regarding the potential beneficial role of microbiome dysbiosis in improving behavioral and GI symptoms among ASD patients. However, available RCTs and nonrandomized trials suffer from potential biases due to poorly designed trials with small sample sizes. Specifically, to promote comparability of future research, we call for ASD clinical investigators to standardize assessment tools for both behavioral symptoms and other symptoms of interest (e.g. using ATEC for ASD symptoms and GSI for GI functions). Due to the high placebo effect observed in the existing studies, it is also important for future research to consistently implement objective, clinician assessment rather than relying on care-giver report. Other tools, such as urine and serum markers, and stool microbiome (as done in Parracho*et al*[13] and Shaaban *et al*[28]), can assist in the interpretation of the results. We noted that the majority of the current studies have not taken advantage of these tools. Several ongoing and new studies are in the horizon (S1 Table), and the field remains hopeful as more reliable data from rigorously designed RCTs become available in the near future.

Acknowledgements

We thank Clara Hobbie for manuscript editing.

Conflict of Interest Statement

Authors declare no conflicts of interests.

REFERENCES

1 Baio J, Wiggins L, Christensen DL, *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

2 Ouellette-Kuntz H, Coo H, Lam M, *et al*. The changing prevalence of autism in three regions of Canada. J Autism Dev Disord, 2014,44(1):120-136

3 Taylor B, Jick H, Maclaughlin D. Prevalence and incidence rates of autism in the UK: time trend from 2004-2010 in children aged 8 years. BMJ Open, 2013,3(10):e003219

4 Karimi P, Kamali E, Mousavi SM, *et al*. Environmental factors influencing the risk of autism. J Res Med Sci, 2017,22:27

5 Matelski L, Van de Water J. Risk factors in autism: Thinking outside the brain. J Autoimmun, 2016,67:1-7

6 Berding K, Donovan SM. Microbiome and nutrition in autism spectrum disorder: current knowledge and research needs. Nutr Rev, 2016,74(12):723-36

7 Rosenfeld CS. Microbiome Disturbances and Autism Spectrum Disorders. Drug Metab Dispos, 2015,43(10):1557-71

8 Adams JB, Johansen LJ, Powell LD, *et al*. Gastrointestinal flora and gastrointestinal status in children with autism--comparisons to typical children and correlation with autism severity. BMC Gastroenterol, 2011,11:22

9 Kang V, Wagner GC, Ming X. Gastrointestinal dysfunction in children with autism spectrum disorders. Autism Res, 2014,7(4):501-6

10 Chaidez V, Hansen RL, Hertz-Picciotto I. Gastrointestinal problems in children with autism, developmental delays or typical development. J Autism Dev Disord, 2014,44(5):1117-27

11 Liu J., Zhang, M., Kong, X. Gut Microbiome and Autism: Recent Advances and Future Perspectives. North American Journal of Medicine and Science, 2016,9:1-12

12 Kang DW, Adams JB, Gregory AC,*et al*. Microbiota Transfer Therapy alters gut ecosystem and improves gastrointestinal and autism symptoms: an open-label study. Microbiome, 2017,5(1):10

13 Parracho H., Gibson, G. R., Knott, F., *et al*. A double-blind, placebo-controlled, crossover-designed probiotic feeding study in children diagnosed with autistic spectrum disorders. International Journal of Probiotics and Prebiotics, 2010,5(2):69-74

14 Navarro F, Liu Y, Rhoads JM. Can probiotics benefit children with autism spectrum disorders? World J Gastroenterol, 2016,22(46):10093-102

15 Sathe N, Andrews JC, McPheeters ML, *et al*. Nutritional and Dietary Interventions for Autism Spectrum Disorder: A Systematic Review. Pediatrics, 2017,139(6)

16 Srinivasjois R, Rao S, Patole S. Probiotic supplementation in children with autism spectrum disorder. Arch Dis Child, 2015,100(5):505-6

17 Ghouri YA, Richards DM, Rahimi EF, *et al*. Systematic review of randomized controlled trials of probiotics, prebiotics, and synbiotics in inflammatory bowel disease. Clin Exp Gastroenterol, 2014,7:473-87

18 Ouwehand AC. A review of dose-responses of probiotics in human studies. Benef Microbes, 2017,8(2):143-51

19 Dolan KE, Finley HJ, Burns CM, *et al*. Probiotics and Disease: A Comprehensive Summary-Part 1, Mental and Neurological Health. Integr Med (Encinitas), 2016,15(5):46-58

20 Kaur IP, Kuhad A, Garg A, *et al*. Probiotics: delineation of prophylactic and therapeutic benefits. J Med Food, 2009,12(2):219-35

21 Buffington SA, Di Prisco GV, Auchtung TA, *et al*. Microbial Reconstitution Reverses Maternal Diet-Induced Social and Synaptic Deficits in Offspring. Cell, 2016,165(7):1762-75

22 Hsiao EY, McBride SW, Hsien S, *et al*. Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. Cell, 2013,155(7):1451-63

23 Kaluzna-Czaplinska J, Blaszczyk S. The level of arabinitol in autistic children after probiotic therapy. Nutrition, 2012,28(2):124-6

24 West NP, Horn PL, Pyne DB, *et al*. Probiotic supplementation for respiratory and gastrointestinal illness symptoms in healthy physically active individuals. Clin Nutr, 2014,33(4):581-7

25 Higgins JPT GS, editor. Cochrane Handbook for Systematic Reviews of Interventions Version 5.1.0, 2011.

26 Slim K, Nini E, Forestier D, *et al*. Methodological index for non-randomized studies (minors): development and validation of a new instrument. ANZ J Surg, 2003,73(9):712-6

27 Liu Y-W, Liong, M. T., Chung, Y.-C. E., *et al*. Lactobacillus plantarum PS128 alleviates symptoms of autism spectrum disorder in Taiwanese children: A randomized, double-blind, placebo-controlled trial. Under review in Scientific Reports, 2018.

28 Shaaban SY, El Gendy YG, Mehanna NS, *et al*. The role of probiotics in children with autism spectrum disorder: A prospective, open-label study. Nutr Neurosci, 2018,21(9):676-81

29 Matthes H, Krummenerl T, Giensch M, *et al*. Clinical trial: probiotic treatment of acute distal ulcerative colitis with rectally administered Escherichia coli Nissle 1917 (EcN). BMC Complement Altern Med, 2010,10:13

30 Minelli E, Benini A. Relationship between number of bacteria and their probiotic effects. Microb Ecol Health Dis, 2008,20(4):180-183

(Received Jan. 7, 2019; revised)

Tables

1. Cochrane risk of bias assessment for RCTs.

Cochrane risk of bias assessment

	Allocation concealment	Blinding of outcome assessors for all outcomes	Blinding of participants and personnel for All outcomes	Incomplete outcome data for all outcomes	Selective outcome reporting	Sequence Generation	Other sources of bias	**Grade of quality of overall study design**
Liu 2018	Low	Low	Low	Low	High	Low	Unclear	**High**
Parracho 2010	Unclear	Unclear	Unclear	High	High	Low	Low	**Mod-erate**

2. MINORS risk of bias assessments for non-RCTs.

MINORS Criteria for non-comparative/non-randomized studies

	Risk of bias	A clearly stated aim	Inclusion of consecutive patients	Prospective collection of data	Endpoints appropriate to the aim of the study	Unbiased assessment of the study endpoint	Follow-up period appropriate to the aim of the study	Loss to follow up less than 5%	Prospective calculation of the study size	An adequate control group	Contemporary groups	Baseline equivalence of groups	Adequate statistical analyses	Score/(total possible)	**Grade of quality of overall study design**
Shaaban 2017	Serious	2	2	2	2	1	2	2	0	NA	NA	NA	2	15/18	**Moderate**

West 2013	Critical	2	0	2	1	0	1	0	0	NA	NA	NA	1	7/18	**Very low**
Kałużna-Czaplińska 2012	Serious	2	2	2	2	0	2	2	0	NA	NA	NA	1	11/18	**Low**

3. Basic characteristics of included studies.

Study	**Study design**	**Intervention group subjects (total,analyzed); control group subjects (total,analyzed)**	**Intervention age (range, mean); Control age (range, mean)**	**Intervention gender; control gender**	**Inclusion criteria**	**Baseline characteristics**	**Included in Srinivasjois et al 2015 systematic review?**
Parracho et al 2010 [13]	a double-blind, placebo-controlled, crossover-designed feeding study	62,17; 62,17	4-16, mean unknown; 4-16, mean unknown	59 M 3 F; 59 M 3 F	diagnosis of ASD	The baseline TIPS scores were significantly higher ($P<0.05$) than those during both probiotic and placebo period.	Y
Liu et al 2018 [27]	a double-blind, placebo-controlled, parallel feeding study	40,38; 40,34	7-15, 10.08; 7-15, 9.91	all male; all male	confirmed diagnosis of ASD based on DSM-V; all subjects were confirmed to have ASD using ADI-R	no major baseline differences in demographic and clinical characteristics between treatment and control group. Although two elements of the questionnaires (sensory from ABC-T and social awareness from SRS) differed between the PS128 and placebo groups at baseline (week 0), these elements were not parts of the subject	N

						selection and randomization criteria	
Kałużna-Czaplińska et al 2012 [23]	open trial with self control	22,22; NA	4-10, 5.6; NA	20 M 2 F; NA	ASD diagnosis based on DSM-IV	100% GI symptoms. Dietary patterns: Varied 12 Restricted 10	Y
West et al 2013 [24]	open trial with self control	33,25 (ATEC)/21(GI); NA	3-16, 7.92; NA	not reported	voluntary participation ; ASD diagnosis	high prevalence GI symptoms (constipation and diarrhea)	N
Shaaban et al 2017 [28]	open trial with self control and neurotypical control	30,30; NA	5-9 mean unknown; NA	19 M 11 F; NA	The diagnosis of ASD as established by 2 senior child psychologists	Sixty percent of our patients were overweight (+1SD < ZBMI A<+2SD) and forty percent had a ZBMI A within the normal range (-1SD < ZBMIA<+1 SD).	N

4. Characteristics of probiotic therapies of included studies.

Study	**Probiotic therapy**					**Comparator**	**Concurrent medications or therapies**
	Bacteria strains	**Bacteria quantity per capsule/dose**	**Frequency of therapy**	**Duration of therapy**	**Other ingredients**		
Parracho et al 2010 [13]	*Lacto bacillus plantarum* WCFS1	4.5x10^10 CFU	unclear	Group I received placebo during the first feeding period (3 weeks) and probiotic during the second feeding period (3 weeks)	NA	Maltodextrin, 110mg per capsule	Not specified
Liu et al 2018 [27]	*Lactobacillus plantarum* PS128	3x10^10 CFU	1 capsule daily	4 weeks	microcrystalline cellulose	Empty capsule of microcrystalline cellulose	Participants were allowed to continue their regular medications

Kałużna-Czaplińska et al 2012 [23]	*Lactobacillus acidophilus* (strain Rosell-11)	5x10^9 CFU	2 doses daily	2 month	none	NA	children were on sugar free diet throughout the study
West et al 2013 [24]	*Lactocillus acidophilus, Lactobacillus casei, Lactobacillus delbruecki, Bifidobacteria longum, Bifidobacteria bifidum*	10^10 CFU	1 capsule 3 times daily	6 months	Del-Immune V® powder which is a lysed lyophilized powder	NA	14 were receiving concurrent prescription medications, 6 reported no concurrent prescription medication, and 5 did not report concurrent prescription medications
Shaaban et al 2017 [28]	*Lactobacillus acidophilus, Lactobacillus rhamnosus and Bifidobacteria longum*	5x10^8 CFU	1 dose daily	3 months	dry carrot powder	NA	No other microbiome-altering medications; however cohort received behavioral therapy during the probiotics administration

5. Outcomes summary: ASD behaviors and GI symptoms.

Study	ASD behaviors		GI symptoms	
	Assessment tool(s)	**Results**	**Assessment tool(s)**	**Results**
Parracho et al, 2010 [13]	DBC-P	1). Authors claimed statistically significant ($p<0.05$) improvement of behavioral scores for disruptive antisocial behavior, anxiety problems and communication disturbances, but data analysis is suboptimal (see results and discussion).	Assessed bowel functions (bowel movement and stool consistency) and GI symptoms (abdominal pain, intestinal bloating and flatulence) using a self-diary.	1). Authors claimed that probiotic feeding resulted in a statistically significant ($P<0.01$) higher percentage of 'formed' stool (73.3%) compared to the placebo (64.8%), whilst the percentage of 'hard' stool samples was lower (8.1% vs 15.9%), but data analysis is suboptimal (see results and discussion) 2). No significant difference was detected for bowel movements and any GI symptoms between treatment and placebo groups compared to baseline.
Liu et al, 2018 [27]	both clinician assessment tools and care-giver questionnaires: CGI-S, CGI-I, ADI-R, ABC-T, CBCL, SRS, SNAP-IV-T	1). No statistically significant differences in behavioral scores detected between probiotics and placebo control groups after 4 weeks. Authors did not conduct statistical testing of the difference (score of week 4 – score of baseline) between PS128 and Placebo groups in the overall analysis. 2). Post-hoc subgroup analysis showed a small but statistically significant improvement in SNAP-IV-T total score ($P=0.02$) and opposition/defiance sub-scale score ($P=0.03$) in probiotics groups. Difference (score of week 4 – score of baseline) between PS128 and Placebo groups was compared by student's t test.	NA	NA
Kałużna-Czaplińska 2012 [23]	Unspecified tool that assessed patients' ability of concentration, eye contact, follow out orders and reaction to other peoples' emotions before and after treatment.	1). A portion of the subjects showed trend of improvement in each of the four domains of autistic behaviors, especially for "ability of concentration" (60%) and "follow out orders" (68%).(no P values)	unspecified tool	100% participants had GI symptoms at baseline. Authors did not report changes in GI function following treatment.

West et al, 2013 [24]	ATEC score	1). Mean ATEC score significantly decreased ($p<0.05$) following initiation of treatment, in all four of the ATEC categories including speech/language communication, sociability, sensory cognitive awareness, health/physical behavior.	both stool frequency diary and ATEC, which has built-in assessment of constipation and diarrhea	1).The majority of participants in this study reported severe constipation (84%) or diarrhea (56%) at baseline; 48 % reported decreases in diarrhea severity and 52% reported decreases in constipation severity after treatment, although this is no discussion of statistical significance. 2).Study found reduction in ATEC-diarrhea (2.4 vs 1.6) and constipation (1.0 vs 0.4) scores after treatment, but it is unclear whether the results are statistically significant. 3).Parents also reported increased stool frequency although this was not statistically significant.
Shaaban et al, 2017 [28]	ATEC score	1). The total ATEC scores (p value=0.0001) and scores in all four categories ($p<0.05$) significantly decreased following probiotic supplementation. 2). improvements in gastrointestinal symptoms were strongly correlated with the improvements of the severity of autism (assessed by the ATEC) after probiotic supplementation (p value= 0.0001).	6-GSI care-giver questionnaire	1). Significant reduction in the total GSI score ($p<0.0001$), and in all sub-categories ($p<0.05$), including constipation (1.27 vs 0.73), stool consistency (1.2 vs 0.87), flatulence (1.17 vs 0.87), and abdominal pain (1.17 vs 0.73) with probiotics. 2). 60% showed trend of improvement in abdominal pain, 57% in flatulence, 42.5% in constipation, 37.5% in diarrhea, 25% in stool smell, and 16.6% in stool consistency. (No P value)

6. Other outcomes, adverse effects and comments.

Study	**Adverse effects**	**Other outcomes**	**comments**
Paraccho et al, 2010 [13]	4 subjects (out of N=62) withdrew from study due to adverse effects, including skin rash (1 patient), diarrhea (2 patients), and weight loss (1 patient),	Probiotic supplementation resulted in significantly higher ($p<0.05$) Lab158 and significantly lower Erec482 counts compared to the placebo group.	1). High dropout rate 2). Didn't use ITT (intention -to-treat) analysis 3). High inter-individual variability 4). significant placebo effect 5). Data analysis for primary outcome is inadequate
Liu et al, 2018 [27]	Study included assessment of adverse effects though there was no detail on how this was done. Diarrhea was noted in some patients	NA	1). 2 authors are members of the company that develops the probiotic therapy 2). Some analysis not ITT 3). strong placebo effects 4). No GI function assessment.

			5). No life style factors, microbiome changes, or other clinical indices collected
Kałużna-Czaplińska 2012 [23]	Unclear whether adverse effect was assessed. None reported	Probiotic supplementation let to a significant decrease in urine D-arabinitol and D-arabinitol / L-arabinitol ratio ($P<0.05$)	1). Poor overall statistical reporting 2). Assessment tools unspecified 3). Study did not track GI symptom changes after treatment
West et al, 2013 [24]	Study was not designed to monitor adverse events	NA	1). 2 authors are members of the company that develops Delpro 2). Poor statistical methods and no P values provided for changes in GI symptoms/ATEC-GI scores after treatment
Shaaban et al, 2017 [28]	1). Side effects include diarrhea (1), bloating (2), abdominal cramps (2) and skin rash (1). 2). All adverse effects were mild and transient, no patients reported cessation of the intervention.	1) The levels of *Bifidobacteria* were significantly lower in stool of ASD children compared to healthy control group (p value=0.0001) at baseline. After probiotics treatment, there was a significant increase in the colony counts of *Bifidobacteria* and *Lactobacillus* (p value <0.0001). 2) overweight ASD patients showed a statistically significant decrease in the body weight (p value < 0.014) with a significant decrease in BMI (p value < 0.01) after probiotic treatment.	1). No financial conflict of interest 2). P values and SDs are provided for each measurement 3). important limitation is that cohort received behavioral therapy during the probiotics administration

Descriptions of Supplementary Information

S1 Table. Ongoing clinical trials not included in this review.

This table contains the characteristics of ongoing or completed but unpublished clinical trials that are researching the interaction of probiotics with Autism Spectrum Disorder symptoms and behavior.

File Format: Microsoft Word Document

S2 Table. PRISMA checklist

This table contains the specifications of a review following the PRISMA guidelines, as this manuscript followed. PRISMA is a minimum list of items that must be completed when undergoing a review or meta-analysis and is a tool to evaluate these reviews.

File Format: Microsoft Word Document

Supplementary Table 1. Ongoing clinical trials not included in this review.

Study	Author	Source	Location	Status	Study design	Intervention and comparator
Gut to brain interaction in Autism Spectrum Disorders: a randomized controlled trial on the role of probiotics on clinical, biochemical and neurophysiological parameters	Elisa Santocchi et al	BMC Psychiatry	IRCCS Fondazione Stella Maris	Ongoing	Parallel design, double-blind RCT(1:1)	Drug: Vivomixx vs. Placebo
Probiotics and Oxytocin Nasal Spray on Social Behaviors of Autism Spectrum Disorder (ASD) Children	Xuejun Kong et al	Clinicaltrial .gov	Massachusetts General Hospital	Not yet recruiting	Cross-over, double-blinded, controlled trial	Drug: intranasal oxytocin and oral probiotics vs. Vitamin C
Probiotics for Quality of Life in Autism Spectrum Disorders	NA	Clinicaltrial .gov	Ohio State University	Completed	Randomized cross-over trial	Drug: Visbiome Extra Strength vs. Maltose
Road to Discovery for Combination Probiotic BB-12 With LGG in Treating Autism Spectrum Disorder	J. Marc Rhoads et al	Clinicaltrial .gov	The University of Texas Health Science Center, Houston	Recruiting	Randomized parallel design	Drug: BB-12 with LGG vs. Placebo
Efficacy of Vivomixx on Behaviour and Gut Function in Autism Spectrum Disorder	NA	Clinicaltrial .gov	University College, London	Recruiting	Randomized controlled cross-over design	Drug: Vivomixx vs. Placebo
Effect of Milk Oligosaccharides and Bifidobacteria on the Intestinal Microflora of Children With Autism	NA	Clinicaltrial .gov	University of California, Davis	Completed	Randomized cross-over trial	Drug: Synbiotic vs. Prebiotic

Supplementary Table 2. PRISMA checklist

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	3-5
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	5-8
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	NA
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	5-6
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	5-6
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	5-6
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	5-6, 34
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	7-9
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	7-9
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	9
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	NA
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	NA
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	22
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	16
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	10, 34
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	11, 28, 29
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	12, 26, 27
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	30-32
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	13-18
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	NA
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	16
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	18-19
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	18-23
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	22-23
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	1