Probiotics for Gastrointestinal Symptoms and Quality of Life in Autism: a placebo-controlled pilot trial

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Protocol for Pilot of Probiotic for Quality of Life in Autism

Introduction & Overview. The physical and mental/emotional health of people with autism spectrum disorder (ASD) are closely connected. The emerging data on immune abnormalities and gut microbiome differences, and interactions of the genome with these suggest a possible etiological link between physical and mental dysfunction, especially the gastrointestinal (GI) dysfunction and severe anxiety that many individuals with ASD manifest. We have preliminary clinical evidence that children with ASD & GI symptoms differ in microbiome composition and function from neurotypical children with GI symptoms. We hypothesize that altered host-microbial signals, which include altered fecal neurotransmitter gamma-aminobutyric acid (GABA) levels contribute towards anxiety and sensory over-responsivity in ASD. Our preliminary findings also show that probiotic Visbiome, formerly known as VSL#3, improves GI and pain symptoms, correlating with altered gut microbiome composition and related metabolites (the macrobiome). The proposed crossover trial will explore the possibilities of this new appreciation of the microbiome-mental/physical function connection for ASD, GI dysfunction, and anxiety. If altering the gut microbiome results in better GI and emotional function, it could improve the quality of life for children with ASD and their parents. A pilot trial with 12 children with ASD will test feasibility for a proposed three-site crossover randomized clinical trial (RCT) of probiotics (beneficial bacteria including Lactobacilli & Bifidobacteria) in 60 children 3-12 years old with ASD, GI dysfunction, & anxiety. In a balanced crossover children will be randomized 1;1 to Visbiome or placebo first, 8 weeks per condition with 3 weeks washout between. We have access to significant fecal microbiome and metabolome data from NIH-funded Human Microbiome Projects (HMP) on similar-age healthy and irritable-bowel children, with and without ASD. These will help leverage our understanding of macrobiome changes that correlate with functional improvement of GI and abdominal pain symptoms. Pilot study efficiency will also benefit from those HMPs having already collected and analyzed baseline stools for some children with ASD, thus saving significant costs for baseline stool analyses for the pilot.

Specific Aims of the Pilot:

Aim 1: To test feasibility of the planned RCT.

Hypothesis 1A: 12 children aged 3-12 years with ASD, GI dysfunction, and anxiety can be recruited from underserved and minority populations in a 15-month pilot.

Hypothesis 1B: Retention during the full crossover will be 75% or more.

Hypothesis 1C: Adherence to taking the probiotic and placebo will be 80% or more.

Hypothesis 1D: Participant satisfaction will be good (mean score matching the anchor "satisfied" on the satisfaction scale).

Aim 2: Confirm safety of Visbiome in ASD (supported by numerous published reports showing no adverse effects in patients with GI disorders).

Hypothesis 2: There will be no serious adverse events attributable to study treatment.

Aim 3. To explore effects of probiotics on quality of life (QOL) and gastrointestinal (GI) function in children age 3-12 with ASD, GI dysfunction, and anxiety.

Hypothesis 3: Children with ASD randomly assigned to receive VISBIOME will have nominally better GI QOL on the GI module of the PedsQOL scale (primary outcome) than those assigned to placebo, with average 25% or greater improvement or improvement to the normal range..

Aim 4:

To explore effects of probiotics on anxiety in 3-12 year-olds with ASD.

Hypothesis 4: Compared to baseline, children with ASD will have nominally better emotional stability (less anxiety) when taking Visbiome than when taking placebo, with average 25% or more improvement.or improvement to the normal range on the anxiety scale..

Aim 5: To confirm that the 3 week washout period is adequate for GI symptoms to return to pre-treatment baseline.

Hypothesis 5: By week 11, the score of the GI module of PedsQOL will be substantially closer to the baseline score than to the week 8 score for the group that received Visbiome first.

Exploratory Aim 6: To explore the extent to which the candidate probiotic (VISBIOME) can change the composition of gut microbiome and stool neutotransmitters in 3-12 year-olds with ASD. Responders in GI function or anxiety will be compared to nonresponders in microbiome change/retention.

If 3 of the 4 feasibility hypotheses plus Hypothesis 2 plus one of the 2 clinical outcome hypotheses are upheld, the RCT will be justified. If washout is not complete in 3 weeks, it will be adjusted to a longer period for the RCT, based on the pilot data collected monthly.

Background & Significance

The healthy human gut contains trillions of bacteria from diverse bacterial species, most being harmless or beneficial (Lozupone et al., 2012). The beneficial bacteria protect from pathogenic microbes, assist in metabolism and immune balance, and enable healthy GI functioning. Probiotics are defined by the Food and Agricultural Organization/World Health Organization, as: "Live microorganisms which when administered in adequate amounts confer a health benefit" [WHO, 2001]. "To be classified as a probiotic, the organism must have scientifically proven beneficial physiologic effects, must be safe for human consumption, must be stable in bile and acid, and must be able to adhere to the intestinal mucosa" [AACE Nutrition Guidelines Task Force, 2003]. The most common types are lactic acid bacteria and bifidobacteria.

In a meta-analysis of 5 RCTs in irritable bowel syndrome, Tiequn et al (2015) found a significant relative "risk" of improvement with Lactobacillus over placebo of 7.69 (p=0.0008), 17.62 in adults (p=0.00001) and 3.71 in children (p=0.04) without side effects. Two RCTs in neurotypical children with constipation, one in infants, found benefits (Sadeghzadeh et al, 2014; Coccorullo et al, 2010). Three other trials using a single organism compared to laxative did not.

Probiotics produce neurotransmitters, such as GABA and serotonin, which act on the brain-gut axis via the vagus, and have been dubbed "Psychobiotics" in this role (Dinan et al, 2013). The benefits reported for irritable bowel syndrome, depression, and chronic fatigue syndrome may also be related to anti-inflammatory actions (Dinan, 2013). Both GABA and serotonin are involved in actions of FDA-approved anxiolytic drugs. Rodent studies suggest that some psychobiotics (e.g., *Lactobacillus helveticus* and *Bifidobacterium longum)* can be anxiolytic (Bercik, Premysl, et al., 2011; Bercik, Park, et al., 2011; Messaoudi et al. 2011. In a 4-week trial in healthy women (Tillisch et al, 2013), fermented milk product and a probiotic (Bifidobacterium animalis subsp lactis, Streptococcus thermophiles, Lactobacillus bulgaricus, & Lactococcus lactic) affected activity of brain centers controlling emotion and sensation, documented by fMRI with an emotional faces task (p=0.004).

In a neurotypical human study, Diop (2008) found that following a stressful event, *Lactobacillus helveticus* Rosell-52 and *Bifidobacterium longum* Rosell-175 resulted in more improvement in abdominal pain and nausea/vomiting than placebo, but with no difference on psychiatric symptoms. In another study, healthy volunteers randomly assigned to probiotic experienced significant improvement on global distress, somatization, depression, anger-hostility, and problem solving, and had decreased urinary free cortisol levels compared to controls [Messaoudi, et al. 2011]. In a pilot study, patients with chronic fatigue syndrome randomly assigned to a Probiotic (*Lactobacillus casei* strain Shirota, LcS) reported significant decreases in anxiety symptoms compared to controls (Rao et al., 2009). In a study in children, Christian et al (2015) found an association between child temperament and gut microbiome. In a RCT of the 8-strain formulation (VSL#3/Visbiome) to be used in this study, Kim et al (2005) found significant reduction of bloating in adults with irritable bowel syndrome. Using the same formulation in a placebo-controlled crossover trial (6 weeks each condition, 2-week washout) in 59 neurotypical children with irritable bowel syndrome, Guandalini et al (2010) found significant benefit for abdominal pain/discomfort, bloating/gassiness, and life disruption.

Studies in ASD

In an ATN registry study, Mazurek et al (2012) found that 24% of children with ASD had chronic GI problems, most commonly constipation and abdominal pain; and those with GI problems had higher rates of anxiety and sensory over-responsivity (p<.0001). Further, those

with multiple GI problems had significantly more anxiety and over-responsivity than those with only one (p<.0001). De Angelis et al (2013) compared the fecal microbiota and metabolome of children with Pervasive Developmental Disorder Not Otherwise Specified (PDD-NOS), autistic disorder (AD), and healthy children (HC). The main bacterial phyla (Firmicutes, Bacteroidetes, Fusobacteria, and Verrucomicrobia) were significantly (*P*<0.05) different among the three groups

Our own preliminary data (Fig. 1) are supportive of these findings, with a decreased Faecalibacteria representation in ASD subjects. Our preliminary studies also show that Visbiome, formerly branded VSL#3, the probiotic to be used in this study, restores Faecalibacteria abundance in patients with GI disease and pain who respond favorably to this probiotic. Changes in unclassified Ruminococcaceae are also evident in ASD children, especially in mucosa-associated communities (Fig. 1). We have demonstrated that these types of bacteria are potent GABA producers using a novel decarboxylation conversion of dietary glutamate, modulating anxiety and sensory over-responsivity in preclinical models. In further support of microbial neurotransmitter production being associated with GI symptoms and pain in ASD, we performed a 14 day case-controlled trial (including a healthy sibling) and demonstrated that stool GABA levels strongly correlated with GI symptoms and pain episodes in an ASD child (Fig. 2). These findings build on earlier reports that fecal free amino acids and volatile organic compounds are markedly affected in PDD-NOS and especially AD. Tomova et al (2015) found a significant decrease of the Bacteroidetes/Firmicutes ratio and elevation of the amount of Lactobacillus in autistic disorder, potentially associated with altered content of GABA-producing bacteria, but this was not investigated. West et al (2013) reported a 20% improvement in autistic symptoms in an open trial of a 4-bacteria probiotic formulation.

Thus, there are **4 encouraging literature themes**: 1) Probiotics appear to improve GI function and emotional symptoms such as anxiety and depression in rodent models and in neurotypical humans; 2) Children with ASD have a high rate of GI dysfunction and a gut microbiome/macrobiome that differs from healthy controls; 3) GI problems were significantly associated with anxiety and sensory over-responsivity in ASD; 4) An open trial of probiotic in ASD suggested mild improvement in autistic symptoms. Taken together, ***these suggest the need for a randomized, placebo-controlled trial in ASD focusing on quality of life*** as affected by GI function & the emotional instability/anxiety that most children with ASD have trouble with, but also exploring possible benefit for socialization, communication, and interests.

Preliminary Work

Collaborators in this proposal have studied stool and mucosal microbiome communities in ASD children and have also investigated the effect of a specific probiotic (VSL#3, now called VISBIOME) in greating irritable bowel syndrome (IBS). VISBIOME (ExeGi Pharma LLC, Gaithersburg, MD) is made up of 4 strains of lactobacilli (*L. casei*, *L. plantarum*, *L. acidophilus*, and *L. delbrueckii* subsp. *bulgaricus*), 3 strains of bifidobacteria (*B. longum, B. infantis*, and *B. breve*), 1 strain of *Streptococcus thermophiles*, and starch. Although previously described as separate species, *B. infantis* and *B. longum* are now considered to be biotypes or subspecies of the same organism (*B. longum*). *Lactobacillus bulgaricus* has been renamed *L. delbrueckii.* Subjects took 2 packets daily (900 billion bacteria) for either 4 or 8 weeks, mixed in a cold food or noncarbonated beverage. See figures 1-3. Safety data included all 21 subjects. No deaths, infections with component bacteria, unscheduled IBS-related health care visits, change in frequency or severity of events, or reported causality occurred in subjects while taking VSL#3. There were 88 AEs reported; all were rated as not serious according to the FDA rating scale. The AEs were deemed not related to VSL#3 as the symptoms reported as AEs are common symptoms in IBS. Bloating was the most common AE reported, followed by having ≥ 4 stools in a 24-hour period.

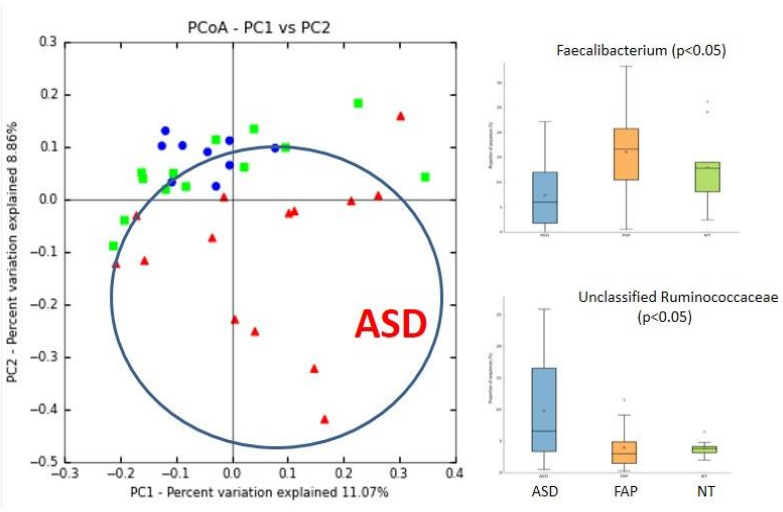
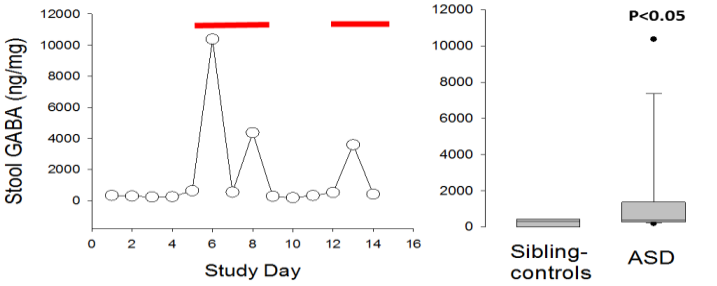


Figure 1: The colonic mucosa-associated microbiome shows distinct community-associated shifts in ASD. In a 16S rDNA deep sequencing study of mucosal biopsies from 14 children with ASD (red triangles), 12 with functional abdominal pain (FAP, green squares) and 9 healthy (blue circles) children, principal coordinate component plots demonstrated significantly different community composition in ASD children (left) including a depletion of Faecalibacteria and enrichment of Ruminococcaceae (right).



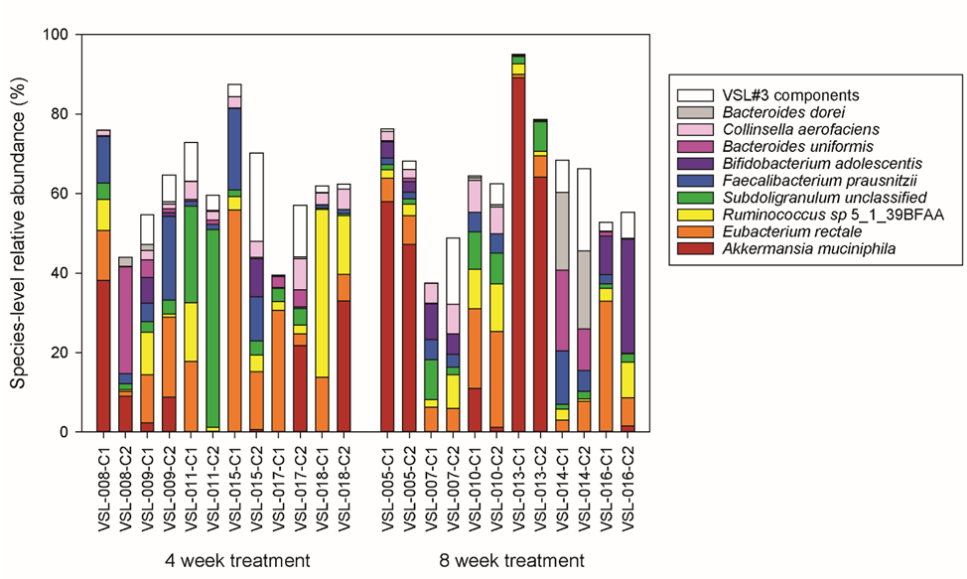


Figure 3. Changes in the composition of the most abundant members of the gut microbiome and the VSL#3 component species as a function of treatment duration. Bacterial community profiles were generated from shotgun metagenomic sequence data and Metaphlan2 profiling. Subjects are organized by treatment and pre- and post-probiotic samples are adjacent to one another (i.e., C1 vs C2). Other taxa (not specifically shown) make up the balance of each specimen's community (i.e., contribute the remaining balance, wherein the sum of each sample is 100%). In the majority of subjects (10 of 12), VSL#3 species were detected in the post-probiotic sample,

and in all subjects, the relative abundances of the most abundant community members were altered following VSL#3 consumption.

Innovation: This will be the first study to our knowledge of probiotic effect on quality of life and anxiety as well as GI function in ASD. It will include cutting-edge comprehensive stool analyses.

Research design & Method for Pilot
In a balanced crossover randomized feasibility pilot trial, 12 children aged 3-12 years with ASD, GI dysfunction, and anxiety will be randomly assigned 1:1 to probiotics first or placebo matched to probiotic first for 8 weeks then a 3-week washout followed by the opposite condition..

Inclusion Criteria: Participants (both sexes) must: 1. have DSM-5 ASD on clinical evaluation by a doctoral-level diagnostician, confirmed by Autism Diagnostic Interview-Revised or Autism Diagnostic Observation Schedule; 2. be between 3 and 12 years old; 3. have ≥2 mo. abdominal pain, constipation, diarrhea, and/or vomiting, with an item-mean score ≥2 on at least one scale of the GI module of the PedsQL scale; 4. have clinical anxiety symptoms with an item mean of ≥1.0 (0-3 scale) on the new Autism Anxiety Scale. They will be recruited from minority, poor, inner city, or rural populations.
Exclusion Criteria: 1. Antibiotics in 2 months prior to enrolling; 2. Prior bowel surgery; 3.. Chronic serious medical condition (e.g., diabetes); 4. Weight or height < 3rd %ile for age; 5. Chronic anti-inflammatory use within 2 months prior to enrolling; 6. History of inflammatory bowel disease, Celiac disease, or eosinophilic disorders (e.g., eosinophilic esophagitis); 7. Already taking probiotics within the previous 6 months.

Recruitment: For efficiency in the pilot we will take advantage of screening and initial GI & ASD assessment of an ongoing Autism Speaks study of ASD microbiome (Williams & Savidge). The baseline stool samples for a considerable number in that study have already been collected and analyzed, saving that expense for the pilot. We will aim recruitment efforts to underserved minority, inner-city, and rural populations. The Catchment area for the Nisonger Center UCEDD and NCH includes the Appalachian SE quadrant of OH and adjacent parts of WV and KY. Columbus has 45,000 Somali-Americans with a high rate of autism. We will check the race/ethnicity, zip codes, and income of the patients in the genome studies with already documented GI problems and a stool sample done and, with IRB approval, contact those who are eligible as underserved, to offer them participation in the study.

Family Engagement: Thomas Hess, leader of the NCH Family Advisory Committee, provided input to the protocol. Families are partners in the research. Parents are recognized as the experts on their own child's behavior and feelings; their frank appraisals of effects are necessary for successful results. Parent stress changes will be measured as an outcome. Their perceptions of the experience on a satisfaction survey at the end will inform implementation of the RCT. They will be given the results and will be asked to help disseminate the findings.

Diagnostic Measures: One or the other will be done by a research-reliable clinician (if not already done by a reliable administrator) at screen in addition to clinical diagnosis**:**
Autism Diagnostic Observation Schedule (ADOS) (Rutter et al., 2004; Lord, Rutter et al, 2012) is an investigator-based assessment that places the child in naturalistic social situations demanding specific responses. Behaviors are coded for social communication, social relatedness, play, imagination, and repetitive behaviors.
Autism Diagnostic Interview-Revised (ADI-R).(Rutter, et al, 2003): The ADI-R "short version" (40-item algorithm) is a highly-structured method of eliciting information from a parent to confirm a clinical impression of autism in children and adults.

Outcome Measures (see also Table 1, Schedule of Measures):

The primary outcome measure is the Gastrointestinal (GI) Module of the *Pediatric Quality of Life Inventory (PedsQL*) (Varni et al., 2001; *(Varni, Burwinkle, & Seid, 2006;* Varni et al, 2014*).* It is a 74-item survey with 14 scales (# of items): stomach pain & hurt (6 items), discomfort when eating (5), food & drink limits (6), trouble swallowing (3), heartburn/reflux (4), nausea/vomiting (4), gas & bloating (7), constipation (14), blood in poop (2), diarrhea (7), worry about going poop (5), worry about stomachaches (2), medicines (4), and communication (5). Report forms for specific age ranges assess the parent's perception of the child's GI function and/or symptoms during the last month on a 5-point scale from 0 (never a problem) to 4 (almost always a problem). Items are reverse-scored and transformed to a 0-100 scale so lower scores reflect worse GI dysfunction Response choices are in Likert-scale format ranging from 0 to 4 (0=Never, 1=Almost Never, 2=Sometimes, 3=Often, 4=Almost Always). There are 4 versions, for ages 2-4 years, 5-7 years, 8-12 years, and 13-18 years. We will use the age-normed scale appropriate for each child; once selected, the same scale will be used throughout that child's participation. *Clear written instructions will be reviewed with the parent for all scales.*

An important secondary measure is *Target Symptom Rating* (Arnold et al, 2003), for which parents are asked to name the 2 problems of most concern to them at baseline; a clinician helps the parent quantify and describe the problem (frequency, duration, severity, interference with daily life) at baseline. At subsequent visits the clinician reminds the parent of the previous description and helps them again quantify/describe the current state. A panel of blind clinicians reviews the descriptions and rates each on a 9-point scale relative to baseline, from remission to disastrously worse, with 5=no change. These ratings are averaged, capturing the issues of most concern to parents across families. For purposes of this study, one of the 2 problems will be required to pertain to GI function, and will be analyzed separately as well as being averaged into the overall symptom rating.

The main measure of *emotional stability/anxiety* is the new *Parent Anxiety Checklist--ASD* (Scahill, Lecavalier, Bears, & Aman, *2015)* developed by the NIMH-funded consortium, "Toward outcome measurement of anxiety in youth with autism spectrum disorders": Using the Child and Adolescent Symptom Inventory's anxiety items as its nucleus, investigators have expanded the scale's content following a review of the literature and after conducting 6 focus groups with parents of children with ASD. This scale will likely become the standard for use in randomized clinical trials. We are fortunate to have the most recent (41-item) version available.

The Aberrant Behavior Checklist (ABC) (Aman et al., 1985a, 1985b) is a 58-item parent rating on a 0-3 scale with five subscales: 1) Irritability (includes agitation, aggression, and self-injury, 15 items); 2) Social Withdrawal (16 items); 3) Stereotypies (7 items); 4) Hyperactivity (16 items); and 5) Inappropriate Speech (4 items), (Aman et al., 1985a; Aman et al. 1985b; Aman et al., 1987, Brown, Aman, & Havercamp, 2002). The ABC is commonly used in ASD RCTs.

Social Responsiveness Scale (SRS) (Costantino et al., 2003). This 65-item rating scale measures the severity of autism spectrum symptoms as they occur in natural social settings. Completed by a parent or teacher in 15 to 20 minutes, the SRS provides a clear picture of a child's social impairments, assessing social awareness, social information processing, capacity for reciprocal social communication, social anxiety/avoidance, and autistic preoccupations and traits. It is appropriate for use with children from 4 to 18 years of age and will detect changes in core ASD symptoms.

The abbreviated *Children's Sleep Habits Questionnaire (CSHQ) (Owens, Spirito, & Mcguinn, 2000)* will be a secondary outcome measure. It includes 33 items rated retrospectively over the previous week by parents. Eight subscales include: (1) bedtime resistance (2) sleep onset latency, (3) sleep duration, (4) anxiety around sleep, (5) night awakenings, (6) sleep disordered breathing, (7) parasomnias and (8) morning waking/daytime sleepiness. In a recent study of the Autism Treatment Network, 75% of the participants analyzed had a CSHQ score $\geq$41, the clinical threshold for sleep problems (Hollway, Aman, and Butter, 2013). Sleep greatly affects quality of life for both children and parents, and it is important to detect any changes in this important vegetative function.

The Parenting Stress Index Short Form (PSI) (Abidin,1995) will be given at baseline & 8 wk. The PSI is used to evaluate the degree of stress in the parent-child relationship. The Short

Form has 36 items from the full length PSI, rated on a 5-point scale from 1 = strongly disagree, to 5 = strongly agree. It is completed in 10-15 minutes. The PSI may be used for parents of children up to 12 years. It yields a Total Score and three domain scores. This will detect effect on parental stress and QOL.

Vital signs, concomitant treatments, & adverse events will be collected each visit. Fecal bacterial DNA and metabolites will be measured at baseline and end of each condition. Microbiome will be characterized by sequencing the 16S rRNA gene and extensive bioinformatics analysis of bacterial composition, diversity, and community structure. PCR will assist 16S sequencing efforts to quantify probiotic loads in stool specimens. LC- and GC-mass spectrometry will identify metabolites of interest in anxiety, as well as a panel of microbial-produced neurotransmitters (GABA, serotonin, indoles). Complete multi-omic analysis of a subset of children in each order of condition, integrating microbiome, metabolome, and clinical metadata, will allow the identification of correlative profiles with anxiety in ASD.

Treatment: The probiotic mix (VISBIOME) will be mainly Bifidobacteria and Lactobacilli (8 species listed under "Preliminary work"), in view of the previously reported encouraging clinical studies and safety data. Probiotic and matched placebo will be supplied by the company in powder packets containing 900 billion organisms, a half packert to be taken twice daily mixed with food.

Adherence will be measured by packet counts of returned probiotic and placebo containers. An additional check on adherence for a subset of children will be change in specific VISBIOME bacterial content in gut microbiome composition.

Statistical Considerations: During the pilot phase, the focus will be on assessment of feasibility (meeting at least 3 of the specific items for Aim 1), safety (Aim 2; defined as no serious AEs attributed to the study treatment) and some suggestion of improvement in the PedsQOL GI scale, the primary outcome of the study, or anxiety, the chief secondary outcome. Sample size is limited by the pilot budget. If we confirm that the 3 week washout period is reasonable (Aim X) then the intent is to pool these results with the larger randomized study for the final analysis.

See RCT section for **protection of human subjects**. Briefly, the study uses a commercially available product with safety evidence; locks & passwords insure confidentiality.

Specific Aims for Stage 2, Randomized ClinicalTrial:
Aim 1 (Primary Aim): To test effects of probiotics (beneficial bacteria contained in VISBIOME: Bifidobacterium breve, Bifidobacterium longum, Bifidobacterium infantis, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus paracasei, Lactobacillus bulgaricus, & Streptococcus thermophilus) on gastrointestinal quality of life (GI-QOL) in 3-12 year-olds with ASD, GI dysfunction, and anxiety.

Hypothesis 1: Children with ASD will improve significantly more on the GI module of the PedsQOL when receiving probiotics than when receiving placebo.

Aim 2 (Secondary);
To test effects of probiotics on emotional stability in 3-12 year-olds with ASD & anxiety.

Hypothesis 2: Children with ASD will improve significantly more in anxiety when taking probiotics than when taking placebo.

Aim 3 (Exploratory): To examine moderator & mediator effects of gut macrobiome and related GI function.

Exploratory Hypothesis 3A: Children with more severe baseline GI complaints will have more improvement in GI function and greater placebo-controlled anxiety improvement with probiotics than those with fewer GI complaints.

Exploratory Hypothesis 3B: Children with ASD whose GI function improves more will have greater improvement of anxiety than those with less GI improvement.

Exploratory Hypothesis 3C: Children with ASD whose fecal analysis shows a greater enrichment in probiotic, especially Faecalibacteria, with reduced stool GABA levels will have greater improvement of GI function, and anxiety than those with less probiotic bacteria.

Exploratory Aim 4: To explore effects of probiotics and prebiotics on social communication, social responsiveness, and scope of interests).

As part of several independent NIH-funded Human Microbiome Projects (HMP) on healthy and IBS children aged 7-12 years, we already have access to significant fecal microbiome and metabolome data to leverage our understanding of macrobiome changes that correlate with functional improvement of GI and abdominal pain symptoms associated with constipation (the major GI complaint in children with ASD).

Research design & Methods
In a crossover design, 60 children age 3-12 years with ASD and impairing emotional instability will be randomly assigned 1:1 to probiotics or probiotic-matched placebo first for 8 weeks, with a 3-week washout followed by the opposite condition. Randomization will be stratified by site, and blocked over time with blocks varying in size.

Inclusion/exclusion criteria, treatment, and measures will be the same as in the pilot. See the ***schedule of measures below***.

Treatment will be identical with the pilot except that if the pilot data indicate that 3 weeks washout is not sufficient, the washout period will be lengthened as indicated by pilot data. In the unexpected event that the washout takes more than 7 weeks, the design will be changed to parallel groups and the sample expanded. We have a commitment from the ExeGI company to provide active 8-strain probiotic and matched placebo ***We have initiated conversation about an IND with Paul David at the FDA so that this can be wrapped up by the project start date.***

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Recruitment: All 3 clinical sites have established recruitment sources and strategies, being the main referral centers for respective metropolitan areas. Local ATN family advisory committees will assist with recruitment. All sites are tertiary referral centers for a large low-income inner city population, large numbers of minorities, and some rural patients. Although restricted to underserved minorities, lower SES, and rural populations, we anticipate a broader pool of potential subjects than usual because this trial does not require withdrawing from medication. The OSU site will recruit through the NCH gastroenterology clinic, where co-PI Kent Williams

sees 4-6 new children with ASD and GI complaints each week. NCH (and OSU's Nisonger Center UCEDD) draw from the southeastern third of Ohio, north and west West Virginia, and Eastern Kentudky, a large Appalachian rural area, as well as inner city Columbus.
The University of Pittsburgh site will recruit study participants primarily from the Merck Child Outpatient Clinic. In accordance with ATN recommendations, children served at the Merck Program are regularly screened for both GI and Sleep problems. This allows program MDs and clinicians to readily identify children who may require intervention for GI symptoms. In addition, we will work with the Department of Gastroenterology at Children's Hospital of Pittsburgh to also identify potential study participants. The aim will be to recruit underserved minority, inner-city and rural populations. The catchment area for the program includes western PA, northern WVA, and eastern Ohio. This regions includes metropolitan, suburban and rural areas.
The Children's Hospital of Philadelphia (CHOP) site will recruit primarily from established patients at the institution's Regional Autism Center (RAC) or from a registry housed at the Center for Autism Research (AutismMatch). Children evaluated and treated at RAC are routinely screened for GI problems and behavioral challenges, allowing the clinical team to easily identify and refer potential study participants. Families enrolled in AutismMatch have expressed interest in participating in research studies related to ASD and can be prescreened for comorbid GI problems. Finally, the AS-ATN Philadelphia site has two actively engaged pediatric gastroenterologists who will aid in identifying and referring potential participants. CHOP's catchment area spans Southeastern PA, New Jersey, and Delaware, with patients representing urban, suburban, and rural communities.

Family Engagement: Thomas Hess, leader of the NCH ATN family advisory committee (FAC), has already provided input, and other sites' FAC input will be incorporated before the RCT. Families are partners in the research and will be given a plain-language summary of the study. Parents are experts on their own child's behavior and feelings; their frank appraisals of effects are essential for successful results. Parent stress changes will be measured. Their perceptions of overall usefulness on a satisfaction survey will inform future research.

Schedule of Measures

Measure	Screen	Baseline	4 Wk	8 Wk	11 Wk	15 Wk	19 Wk	Minutes
ADOS or ADI-R	X							45-150
Medical Hx, PE	X							20
Demographics		X						10
Vital Signs	X	X	X	X	X	X	X	5
Concomitant Meds	X	X	X	X	X	X	X	2-5
Adverse Events		X	X	X	X	X	X	2-10
Parent Anxiety Checklist-ASD	X	X	X	X	X	X	X	10
GI module of PedsQL	X	X	X	X	X	X	X	10-15
Target Symptoms		X	X	X	X	X	X	10
Aberrant Behavior Checklist		X	X	X	X	X	X	10
Social Responsiveness Scale		X		X	X		X	15-20
Sleep Questionnaire (CSHQ)		X		X	X		X	15
Stool Sample		X		X	X		X	
Parent Stress Index		X		X	X		X	10-15
Adherence packet counts			X	X		X	X	
Satisfaction Questionnaire							X	5-10

Statistical Considerations:

Analysis. With our cross-over design we will have (in children completing the study), a total of 6 measurements, three each for period 1 and period 2, plus a screening value. We intend to model the results using a generalized mixed models analysis of variance, with the average of the screen (if available) and baseline of period 1 included as a predictor of the outcome, and subject as a random factor. There are several specific effects which will be tested: (a): is there a significant change for period 2 baseline from period 1, which determines whether we need to incorporate a term for treatment order in the analysis; and (b) is treatment and time x treatment (together) significant predictors of outcome? We hypothesize that the two terms together (treatment and time x treatment) together will provide a significant improvement, and speculate that the time x treatment interaction will not be significant, which would imply that the treatment benefit has (largely) occurred with 4 weeks of treatment, based on pilot data presented above in IBD. As suggested by the reviewer, time will be treated as a categorical factor in the primary analysis, and a continuous factor in a sensitivity analysis. Site will be included as a random effect, and either a site x treatment or a site x treatment x time interaction will be considered depending on the results of the time and time x treatment interactions in the analysis of the primary and major secondary endpoints. We intend to combine data from both the pilot phase and the larger randomized study in this analysis if the washout period is confirmed in the pilot phase. The primary outcome will be the level of the PedsQOL GI module Important secondary outcomes will be the change for anxiety and Target symptom ratings, . Other secondary outcomes include the sleep scale SRS, and ABC.

A major secondary analysis will assess the impact of the underlying GI complaint. The precise complaints will be determined based on the data availability, but based on prior data we expect to be using two variables: constipation (yes/no) and pain (yes/no). These analyses will focus on an interaction of the GI complaint flags with the primary predictor of outcome (treatment or time x treatment, as determined in the initial analysis).

Additional analyses will study the relationship of baseline GI complaints, change in GI complaints, or in the subset of patients with microbiome data, change in GI flora with changes in GI function, anxiety, and other variableds. Treatment will not be included as a predictor in these analyses. Instead we will focus solely on the underlying biological variables as predictors. As an illustration of the approach, we would use the change in GI complaints at 4 weeks as a predictor of overall pediatric QOL at 4 weeks and change in GI complaints at 8 weeks as predictor of pediatric QOL at 8 weeks in the generalized linear model framework, taking into account that these are repeated measures.

Sample Size Considerations

We will recruit a total of 60 participants to the second stage study. We plan to pool the 12 participants from the first stage with these 60 participants, assuming that the designs are compatible, and provide power calculations both with (and without) such pooling. Assuming a sample size of 72 (60) and approximately 85% completion of the study, we would have a total of 61 (51) participants with complete data. As a very simplified approach to estimating the power of the study, a paired t-test would have 90% power to detect a difference of 0.43 (0.47) population standard deviations (alpha=0.05, two-tailed). We expect that the power for the proposed analysis proposed would be somewhat higher (i.e. smaller differences could be detected with the same power) because of the additional measurements available for

participants completing the study and because we would expect to have partial data even from participants dropping-out of the study. Importantly, we would be able to detect relatively moderate effect even in subgroups of size 30 (25) of 0.53 (0.61) such as when the effects are considered only in participants with pain or constipation as the major complaint.

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Microbial Composition:

Processing and Analysis of 16S Sequence Data and targeted metabolite analysis.

Bacterial DNA will be analyzed through the Illumina pyrosequencing pipeline. Amplification will target the 16S rDNA gene of the bacterial genome, specifically a ~500 bp amplicon spanning the V1-V3 region of the gene. Each sample will be amplified using a barcoded primer, which yields a unique sequence identifier tagged onto each individual sample library. Processing for next generation sequencing will be performed, including library generation, amplicon purification, and emulsion PCR. Sequencing by synthesis utilizing the Illumina MiSeq sequencer will yield roughly >50,000 reads per sample. All generated sequences will be quality filtered to remove mismatched sequences (to primer or molecular identifier), short reads, and low quality sequences (including ambiguous base calls). The remaining quality-checked sequence set will be analyzed via QIIME using a reference database. Multiple analyses will be performed on the 16S data, including determination of bacterial diversity, evenness, richness, and relative abundance of the bacteria identified in each sample. QIIME and supervised machine learning will be employed to characterize changes in relative abundance of specific organisms and overall bacterial diversity based on disease classification.

For targeted metabolite analysis of stool GABA and serotonin, selective reaction monitoring (SRM) by Liquid Chromatography/Mass Spectrometry (LC/MS/MS) will be used as established in the Savidge laboratory.

Analysis and integration of microbiome and clinical metadata:

Metadata collected on all patients is listed above Our project goal is to contrast 16S rDNA metagenome and targeted metabolites with clinical metadata, including responsiveness to probiotics in the crossover trial. The TCMC has been instrumental in developing 16S rDNA gene and whole genome shotgun sequencing as part of the Human Microbiome Project (HMP) since its inception. Correlation of clinical metadata with multi-omic data will be assessed by univariate analysis and multivariate regression analyses using STATA or SAS software. Univariate statistics will include the Mann-Whitney U test for nonparametric continuous data and $\chi 2$ for comparisons of proportions among categorical data. Normality of data will be assessed using the Shapiro-Wilk test. A multivariate logistic regression model will be constructed using a forward stepwise process with all variables associated with a p-value < 0.20 on univariate analysis included in the final model. The goodness of fit of the final model will be assessed using the Hosmer-Lemeshow test. All tests will use a p-value <0.05 to be statistically significant.

Human Subjects Considerations

Subjects will be boys and girls age 3-12 with ASD, chronic or recurrent GI dysfunction (constipation, abdominal pain, diarrhea, or bloating) and anxiety symptoms. Per RFA requirements, they will be recruited exclusively from underserved populations (minority, poor, inner-city, or rural) at each site. Children are involved because that is where we expect the most benefit of the treatment.

The treatment, assessments, and procedures are minimal risk, using a commercially available dietary supplement that we have found safe in preliminary studies, including in children with ASD. In 21 subjects there were no serious adverse events, including no infections with component bacteria, unscheduled IBS-related health care visits, change in frequency or severity of events, or reported causality while taking VSL#3 (Visbiome). There were 88 total AEs reported; they were deemed not related to VSL#3 because the symptoms reported as AEs are

common symptoms in IBS. Bloating was the most common, followed by having ≥ 4 stools in a 24-hour period.

There could be some initial bloating or other GI discomfort, but one reason the children are trying this treatment is that they already have GI dysfunction. Two RCTs of irritable bowel syndrome, one in adults and a crossover study in children, both showed improvement of bloating.The risk of discomfort will be minimized by giving it with food. The only physical specimens collected are stools.

The risk to confidentiality is minimized by the usual data and source document security of locked paper files and password-protected electronic files. The parental nuisance and time cost of multiple assessments at 7 visits to the center are ameliorated by reimbursement for transportation and time. Possible psychological distress from a question is ameliorated by informing that if a question is distressing, it can be skipped. Weekly teleconferences with a clinical panel will review possible adverse events. The value of breakthrough knowledge to be gained and possible individual participant benefit far outweigh the risk.

Resources and Environment forTwo-Phase Study of Probiotics for Quality of Life through Gastrointestinal and Emotional Stability in Youth with Autism Spectrum Disorders, GI Problems, & Anxiety: Single-Site Pilot Trial Followed by 3-Site Randomized Clinical Trial

The lead co-PIs are L. Eugene Arnold, M.D., Professor Emeritus of Psychiatry at the Nisonger Center UCEDD, Ohio State University, and Kent Williams, M.D., Assistant Professor of Pediatrics and Director of Endoscopy, in the Department of Pediatric Gastroenterology, Hepatology, and Nutrition at Nationwide Children's Hospital (NCH), both in Columbus. For the follow-on RCT, the PIs and sites will be Benjamin Handen, Ph.D., Center for Autism and Developmental Disorders, University of Pittsburgh, and Amanda Bennett M.D., M.P.H., Division of Developmental and Behavioral Pediatrics, Children's Hospital of Philadelphia. An important collaborator for both the pilot and RCT will be Tor Savidge, Ph.D., Texas Children's Microbiome Center, Baylor College of Medicine, Houston. Thus the disciplines represented include psychiatry, gastroenterology, psychology, developmental/behavioral pediatrics, & microbiomics

The OSU/NCH site in Columbus has a University Center of Excellence in Developmental Disorders (UCEDD, Nisonger Center) as well as an ATN site and is a tertiary referral center for about a third of the state of OH. NCH is the only children's hospital in central OH. Nisonger Center has a well-established clinical trials program with successful execution of both federal and industry trials, both pharmacologic and complementary/alternative treatments. It is one of the Research Units in Pediatric Psychopharmacology (RUPP) Autism Network sites and has all the requisite space, exam rooms, equipment, computers, etc. needed. Nisonger Center has special expertise in assessment in ASD, having developed the ABC, NCBRF, C-SHARP, etc.

Tor Savidge at the Baylor Texas Children's Microbiome Center has already worked productively with co-PI Kent Williams and has all equipment, software, and other resources needed for analysis and interpretation of stool samples for microbiome and metabolome.

The other two clinical sites are well established centers for study of autism. Pittsburgh and Ben Handen have done numerous clinical trials, both pharmacological and behavioral. University of Pittsburgh is an ATN site and is well-known for psychiatric research. It is the tertiary referral center for western PA and northern WV, and has all the necessary clinical, equipment, and personnel resources. CHOP has the largest pediatric health care network in the U.S., is tertiary referral center for much of Eastern PA, and has all necessary resources for carrying out the RCT. The PI there is a leader in investigating anxiety in autism.

All sites have an ATN family advisory committee to help with recruitment.

References

Abidin, RR (1995). Parenting Stress Index - Short Form (3rd edition). Odessa, FL: Psychological Assessment Resources Inc.

Abidin R (2012). Parenting Stress Index Manula (4th ed). Odessa FL: Psychological Assessment Resources.

Aman, M.G., Singh, N. N., Stewart, A. W., Field, C. J. (1985a). The Aberrant Behavior Checklist: A behavior rating scale for the assessment of treatment effects. American Journal of Mental Deficiency, 89, 485-491.

Aman MG, Singh NN, Stewart AW, Field CJ. Psychometric characteristics of the Aberrant Behavior Checklist. Am J Ment Def. 1985b;489:492-502.

Aman MG1, Singh NN, Turbott SH. Reliability of the Aberrant Behavior Checklist and the effect of variations in instructions. Am J Ment Defic. 1987 Sep;92(2):237-40.

Arnold LE, Vitiello B, McDougle CJ, Scahill L, Shah B, Gonzalez NM, Chuang S, Davies M, Hollway J, Aman MG, Cronin P, Koenig K, Kohn AE, McMahon DJ, Tierney E. Parent-defined Target Symptoms Respond to Risperidone in RUPP Autism Study: Customer Approach to Clinical Trials. J. Amer. Acad. Child & Adolesc Psychiatry, 2003 Dec.; 42(12):1443-1450.

AACE Nutrition Guidelines Task Force, AACE Guidelines", American Association of Clinical Endocrinologists Medical Guidelines for the Clinical Use of Dietary Supplements and Nutraceuticals, Endocrine Practice, Vol 9 No. 5 September/October 2003 417

Bercik, P, A J Park, D Sinclair, A Khoshdel, J Lu, X Huang, Y Deng, et al. "The Anxiolytic Effect of Bifidobacterium Longum NCC3001 Involves Vagal Pathways for Gut-brain Communication." *Neurogastroenterology and Motility: The Official Journal of the European Gastrointestinal Motility Society* 23, no. 12 (December 2011): 1132–1139.

Bercik, Premysl, Emmanuel Denou, Josh Collins, Wendy Jackson, Jun Lu, Jennifer Jury, Yikang Deng, et al. "The Intestinal Microbiota Affect Central Levels of Brain-derived Neurotropic Factor and Behavior in Mice." *Gastroenterology* 141, no. 2 (August 2011): 599–609, 609.e1–3.

Bodfish, J. W., Symons, F. W., & Lewis, M. H. (1999). The Repetitive Behavior Scale. Western Carolina Center Research Reports.

Brown, E. C., Aman, M. G., & Havercamp, S. M. (2002). Factor analysis and norms on parent ratings with the Aberrant Behavior Checklist Community for young people in special education. *Research in Developmental Disabilities, 23*, 45-60.

Burnet PWJ, Cowen PJ. Psychobiotics highlight the pathways to happiness. Biological Psychiatry 2013;74:708–709

Christian Lisa M., Jeffrey D. Galley, Erinn M. Hade, Sarah Schoppe-Sullivan, Claire Kamp Dush[g], Michael T. Bailey. Gut microbiome composition is associated with temperament during early childhood. *Brain, Behavior, and Immunity* Volume 45, March 2015, Pages 118–127

Coccorullo, P., Strisciuglio, C., Martinelli, M., Miele, E., Greco, G., Staiano, A. Lactobacillus reuteri (DSM 17938) in Infants with Functional Chronic Constipation: A Double-Blind, Randomized, Placebo-Controlled Study. J Pediatr 2010;157:598-602

Constantino, J.N., Davis, S.A., Todd, R.D., Schindler, M.K., Gross, M.M., Brophy, S.L., Metzger, L.M., Shoushtari, C.S., Splinter, R., Reich, W. (2003). Validation of a brief quantitative measure of autistic traits: Comparison of the Social Responsiveness Scale with the Autism Diagnostic Interview – Revised. *Journal of Autism and Developmental Disorders, 33* (4), 427-433.

Constantino J N. Social Responsiveness Scale. (2012). Western Psychological Services. Los Angels, CA.

De Angelis M, Piccolo M, Vannini L, Siragusa S, De Giacomo A, Serrazzennetti DI, Cristofori F, Guerezoni, ME, Gobbetti M, Francavilla R. (2013) Fecal Microbiota and Metabolome of Children with Autism and Pervasive Developmental Disorder Not Otherwise Specified . PLoS ONE 8(10): e76993. doi:10.1371/journal.pone.0076993

Dinan TG, Stanton C, Cryan JF. Psychobiotics: a novel class of psychotropic. BIOL PSYCHIATRY 2013;74:720–726.

Diop, Laurent, Sonia Guillou, and Henri Durand. "Probiotic Food Supplement Reduces Stress-induced Gastrointestinal Symptoms in Volunteers: a Double-blind, Placebo-controlled, Randomized Trial." *Nutrition Research (New York, N.Y.)* 28, no. 1 (January 2008): 1–5.

Gadow, K. G., & Sprafkin, J. (2002). Child Symptom Inventory 4: Screening and Norms Manual. StonyBrook, NY: Checkmate Plus, LTD.

Gondalia et al 2012. Molecular Characterisation of Gastrointestinal Microbiota of Children With Autism (With and Without Gastrointestinal Dysfunction) and Their Neurotypical Siblings, Autism Research, 5 (6).

Guandalini S, Magazzu G, Chiaro A, et al (2010), VSL#3 improves symptoms in children with irritable bowel syndrome: randomize placebo-controlled double-blind crossover. *JPGN* (2010) 51:24-30.

Gur TL, Worly BL and Bailey MT(2015) Stress and the Commensal Microbiota: Importance in parturition and infant neurodevelopment. *Front. Psychiatry* 6:5. doi:10.3389/fpsyt.2015.00005

Hollway, J.A., Aman, M.G., Butter, E. (2013). Correlates and risk markers for sleep disturbance in participants of the Autism Treatment Network. *Journal of Autism and Developmental Disorders.* DOI 10.1007/s10803-013-1830-y.

Hsiao EY, McBride SW, Hsien S, Sharon G, Hydo ER, McCue T, Codelli JA, Chow J, Reisman SE, Petrrosino JF, Patterson PH, Mazmanian SK. Microbiota Modulate Behavioral and Physiological Abnormalities Associated with Neurodevelopmental Disorders. *Cell* 155, 1451–1463, December 19, 2013. Toward Effective Probiotics for Autism and Other

Neurodevelopmental Disorders. *Cell*, Volume 155, Issue 7, 19 December 2013, Pages 1446-1448

Kim HJ, Roque MIV, Camilleri M, et al (2005). A randomized controlled trial of a probiotic combination VSL#3 in irritable bowel syndrome with bloating. *Neurogastroenterol. Motil* 17:687-696.

Lam, K.S.L. and Aman, M.G. (2007). The Repetitive Behavior Scale-Revised: independent validation in individuals with autism spectrum disorders. *Journal of Autism and Developmental Disorders, 37(5)*: 855-866.

Lord, C., Rutter, M., DiLiavore, P. C., Risi, S., & Gotham, K. (2012).Autism Diagnostic Observation Schedule 2. LA: Western Psychological Services.ABC

Louis P. Does the Human Gut Microbiota Contribute to the Etiology of Autism Spectrum Disorders? Dig Dis Sci (2012) 57:1987–1989 DOI 10.1007/s10620-012-2286-1

Lozupone, Catherine A., Jesse I. Stombaugh, Jeffrey I. Gordon, Janet K. Jansson, and Rob Knight. "Diversity, Stability and Resilience of the Human Gut Microbiota." *Nature* 489, no. 7415 (Sep*tember 13, 2012): 220–230.*

Mazurek MO, Vasa RA, Kalb LG, Kanne SM, Rosenberg D, Keefer A, Murray DS, Freedman B, Lowery LA (2012). Anxiety, sensory over-responsivity, and gastrointestinal problems in children with autism spectrum disorders. *J. Abnom Child Psychol* 41(1), 165-176. DOI: 10.1007/s10802-012-9668-x

Messaoudi, Michaël, Robert Lalonde, Nicolas Violle, Hervé Javelot, Didier Desor, Amine Nejdi, Jean-François Bisson, et al. "Assessment of Psychotropic-like Properties of a Probiotic Formulation (Lactobacillus Helveticus R0052 and Bifidobacterium Longum R0175) in Rats and Human Subjects." *The British Journal of Nutrition* 105, no. 5 (March 2011): 755–764.

Mullen EJ (1989). Mullen Scales of Early Learning. Bloomington, MN: Pearson Assessments.

Owens, J. A., Spirito, A., & Mcguinn, M. (2000). The children's sleep habits questionnaire (CSHQ): Psychometric properties of a survey instrument for school-aged children. Sleep, 23, 1043–1051.

Rao, A Venket, Alison C Bested, Tracey M Beaulne, Martin A Katzman, Christina Iorio, John M Berardi, and Alan C Logan. "A Randomized, Double-blind, Placebo-controlled Pilot Study of a Probiotic in Emotional Symptoms of Chronic Fatigue Syndrome." *Gut Pathogens* 1, no. 1 (2009): 6.

Roid GH (2003). Stanford-Binet Intelligence Scales (5th ed.).Itasca, IL: Riverside Publishing.

Rutter M, Le Couteur A, Lord C. 2003. Autism diagnostic interview revised. Western Psychological Services. Torrance, CA.

Rutter. M., Le Couteur, A., & Lord, C. (2004). *Autism Diagnostic Observation Schedule (ADOS): Manual*. Multi-Health Systems Inc. (MHS).

Sadeghzadeh, M., Rabieefar, A., Khoshnevisasl, P., Mousavinasab, N., Eftekhari, K. The Effect of Probiotics on Childhood Constipation: A Randomized Controlled Double Blind Clinical Trial. Int. J. of Ped. (2014): 937212. DOI: 10.1155/2014/937212

Scahill, L., Lecavalier, L., Bearss, K., & Aman, M. G. (2015). Parent Anxiety Checklist – Autism Spectrum Disorder.

Tiequn B, Guanqun C, Shuo Z (2015). Therapeutic effects of Lactobacillus in treating irritable bowel syndrome: a meta-analysis. Intern Med 54:243-249, 2015. DOI: 10.2169/internalmedicine.54.2710

Tillisch K, Labus J, Kilpatrick L, Jiang Z, Stains J, Ebrat D, Legrian-Raspaud S, Trotin B, Naliboff B, Mayer EA (2013). Consumption of fermented milk product with probiotic modulates brain activity. Gastroenterology2013 June: 144(7). DOI: 10.1053/j.gastro.2013.02.043

Tomova A, Husarova V, Lakatosova S, Bakos J, Vlakova B, Babinska K, Ostatnikova D. Gastrointestinal microbiota in children with autism in Slovakia. Physiology & Behavior 138 (2015) 179–187.

Varni, J. W., Seid, M., Kurtin, P. S. (2001). PedsQL™ 4.0: reliability and validity of the Pediatric Quality of Life Inventory™ version 4.0 Generic Core Scales in healthy and patient populations. Medical Care, 39, 800-812.

Varni, J.W., Burwinkle, T.M., & Seid, M. (2006). The PedsQL™ 4.0 as a school population health measure: Feasibility, reliability, and validity. Quality of Life Research, 15, 203-215.

Varni, J.W., Bendo, C.B., Denham, J., Shulman, R.J., Self, M.M., Neigut, D.A., Nurko, S., Patel, A.S., Franciosi, J.P., Saps, M., Verga, B., Smith, A., Yeckes, A., Heinz, N., Langseder, A., Saeed, S., Zacur, G.M., Pohl J.F. PedsQL Gastrointestinal Symptoms Module: Feasibility, Reliability, and Validity. JPGN (2014): 59(3). DOI: 10.1097/MPG.0000000000000414

Walker L, Caplan-Dover A, Rasquin-Weber A. Questionnaire on Pediatric Gastrointestinal Symptoms, Rome III Version (QPGS-RIII). In: Drossman D, Corazziari E, Delvaux M, Spiller R, Talley N, Thompson W, et al., editors. Rome III: The Functional Gastrointestinal Disorders. 3rd ed. McLean, Virginia: Degnon Associates, Inc.; 2006. p. 963-90.

West R, Roberts E, Sichel LS, Sichel J (2013) Improvements in Gastrointestinal Symptoms among Children with Autism Spectrum Disorder Receiving the Delpro® Probiotic and Immunomodulator Formulation. J Prob Health 1: 102. doi:10.4172/jph.1000102

WHO | Health and Nu*tritional Properties of Probiotics in Food Including Po*wder Milk with Live Lactic Acid Bacteria, a Joint FAO/WHO Expert Consultation. Cordoba, Argentina, 1 - 4 October 2001." *WHO*, n.d. http://www.who.int/foodsafety/publications/fs_management/probiotics/en/.