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Original Articles

Probiotics for Gastrointestinal Symptoms and Quality of Life in Autism: A Placebo-Controlled Pilot Trial

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Abstract

Objective: A randomized pilot trial of gastrointestinal (GI) symptoms targeting probiotic for quality of life in autism spectrum disorder (ASD).

Methods: Thirteen children, 3–12 years of age with ASD, anxiety, and GI symptoms, were randomized into a probiotic crossover trial of 8 weeks each on VISBIOME and placebo separated by a 3-week washout. VISBIOME contains eight probiotic species, mostly *Lactobacillus* and *Bifidobacterium*. Primary outcome was the Pediatric Quality of Life Inventory (PedsQL) GI module. Secondary outcomes included gut microbiota analysis, the Parent-Rated Anxiety Scale for ASD (PRAS-ASD), and parent-selected target symptoms. A mixed analysis model was applied.

Results: Thirteen children were randomized, with 10 completing the study (77% retention): 6 in probiotic/placebo sequence, 4 in placebo/probiotic sequence. Adherence to study treatment was 96%. There were no serious adverse events (AEs), and more nonserious AEs occurred with placebo than with probiotic, including those attributable to treatment. Only 6 of the 10 guessed the correct treatment at the end of week 8. Over the 19-week trial, each outcome improved from baseline and PedsQL correlated significantly with abundance of *Lactobacillus* without discernable changes to microbiota composition/diversity. Although probiotic showed more improvement than placebo, PedsQL and PRAS-ASD were not statistically significant, as expected at this sample size. PedsQL effect size was $d = 0.49$ by the general model and $d = 0.79$ by simple comparison of week 8 changes. A parent-selected target symptom showed significant improvement in GI complaints on probiotic compared with placebo ($p = 0.02$, $d = 0.79$). Probiotic effects carried over through the 3-week washout.

Conclusion: The VISBIOME formulation was safe and suggested a health benefit in children with ASD and GI symptoms who retained *Lactobacillus*. The moderate effect size compared with placebo warrants a larger trial using a parallel-group design.

Keywords: autism spectrum disorder, probiotics, gastrointestinal problems, autism, anxiety, quality of life

Introduction

This pilot trial explored the effect of a probiotic mixture on the quality of life, gastrointestinal (GI) symptoms, and anxiety in children with autism spectrum disorder (ASD), a heterogeneous developmental disorder reportedly affecting 1 in 59 children in the United States (Baio et al. 2018). Medical and mental health conditions commonly co-occur and, in combination with core ASD symptoms, negatively impact health and well-being. Commonly reported comorbidities include GI and anxiety symptoms (Ferguson et al. 2017). Controversy exists over whether these symptoms are more frequent in ASD, but in recent years, it has become clear that a diagnosis of functional GI disorders is more common in ASD subjects versus the general population (Kohane et al. 2012; Doshi-Velez et al. 2015). Mazurek et al. (2012) found that 24% of children with ASD had chronic GI symptoms, most commonly constipation

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and abdominal pain; and those with GI complaints had higher rates of anxiety and sensory overresponsivity ($p < 0.0001$). Furthermore, those with multiple GI symptoms had significantly more anxiety and overresponsivity than those with only one GI symptom ($p < 0.0001$).

Research into underlying causes of GI disorders has led to the increased awareness and understanding of the role of the gut microbiota in modulating human health and cognitive well-being. The healthy human gut contains trillions of diverse bacteria most being harmless or beneficial (Lozupone et al. 2012). Beneficial bacteria protect from pathogens, assist in metabolism and immune balance, and facilitate healthy GI homeostasis. Studies have generally found altered gut microbiota community composition in patients with GI disease, including inflammatory bowel disorders and irritable bowel syndrome (IBS), and additional studies have shown that changing the gut microbiota can positively impact GI function and symptoms (Diop et al. 2008).

Some studies even report that changes in gut microbiota can impact neurobehavioral function (Needham et al. 2018). Multiple murine models have demonstrated rescue of ASD symptoms when the normal gut microbiota was restored (Hsiao et al. 2013; de Theije et al. 2014), and emerging studies in clinical pediatric populations have begun to describe the gut microbiome in ASD (Louis 2012). De Angelis et al. (2013) compared the fecal microbiota and metabolome of children with Pervasive Developmental Disorder Not Otherwise Specified, autistic disorder, and children with neither disorder. The main bacterial phyla (Firmicutes, Bacteroidetes, Fusobacteria, and Verrucomicrobia) were significantly ($p < 0.05$) different among the three groups. Another study of gut microbiota compositional changes showed a decreased Faecalibacteria representation, with unclassified Ruminococcaceae being altered in ASD (Li et al. 2017). These bacteria are potentially able to modulate anxiety and sensory overresponsivity in preclinical models (Cussotto et al. 2019).

Tomova et al. (2015) found a significant decrease of the Bacteroidetes/Firmicutes ratio and elevation of the amount of *Lactobacillus* in ASD cases. These findings build on earlier reports that fecal free amino acids and volatile organic compounds are markedly affected in ASD. In an investigational study of mucosal-associated microbiota in children with ASD and GI symptoms, we identified correlations between serotonergic immune pathways and gut microbiome in idiopathic ASD (Luna et al. 2016). Our findings indicated that aberrant microbe/neuroimmune signaling may contribute to the manifestation of GI symptoms and core features associated with the ASD phenotype. Moreover, these findings indicate that rational microbial therapy, including probiotic supplements, are potential options to treat GI comorbidities that may contribute to ASD core symptoms.

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). The most common types are lactic acid bacteria and bifidobacteria. In a meta-analysis of five randomized clinical trials (RCTs) in IBS, 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 ($p = 0.04$). Two RCTs in neurotypical children with constipation, one in infants, found benefits (Coccorullo et al. 2010; Sadeghzadeh et al. 2014). None of these studies reported adverse side effects associated with probiotic administration.

Probiotics can produce and/or modulate tissue neurotransmitter levels, which act on the brain/gut axis and have been dubbed ''Psychobiotics'' in this role (Dinan et al. 2013; Burnet and Cowen 2013). The benefits reported for IBS, depression, and chronic fatigue syndrome may also be related to anti-inflammatory actions (Dinan 2013). Rodent studies suggest that some psychobiotics (e.g., *Lactobacillus helveticus* and *Bifidobacterium longum*) can be anxiolytic (Bercik et al. 2011a, 2011b; Messaoudi et al. 2011). In a 4-week trial in healthy women (Tillisch et al. 2013), fermented milk product and a probiotic containing *Bifidobacterium animalis* subsp *lactis*, *Streptococcus thermophilus*, *Lactobacillus bulgaricus*, and *Lactococcus lactis* affected the activity of brain centers controlling emotion and sensation, documented by functional magnetic resonance imaging with an emotional faces task ($p = 0.004$). 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 with controls (Messaoudi et al. 2011). In a pilot study, patients with chronic fatigue syndrome randomly assigned to the probiotic *Lactobacillus casei* strain Shirota, LcS reported significant decreases in anxiety symptoms compared with controls (Rao et al. 2009). In a pediatric study, Christian et al. (2015) found an association between child temperament and gut microbiota. In a RCT of the eight-strain formulation, VISBIOME, (VSL#3) used in the present study, Kim et al. (2005) found significant reduction of bloating in adults with IBS. Using the same formulation in a placebo-controlled crossover trial (6 weeks each condition, 2-week washout) in 59 neurotypical children with IBS, Guandalini et al. (2010) found significant benefit for abdominal pain/discomfort, bloating/gassiness, and life disruption. West et al. (2013) reported a 20% improvement in autistic symptoms in an open trial of a four-bacteria probiotic formulation. VISBIOME, formerly branded VSL#3, the probiotic used in this study, also restored Faecalibacteria abundance in patients with GI disease and pain who responded favorably

Thus, there are four encouraging literature themes: (1) probiotics appear to improve GI 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 symptoms and gut microbiota compositional differences that distinguish them from healthy controls; (3) GI symptoms are significantly associated with anxiety and sensory overresponsivity in ASD; (4) an open trial of probiotic in ASD suggested mild improvement in autistic symptoms. Taken together, these findings suggest the need for a randomized, placebo-controlled trial in ASD focusing on quality of life as affected by GI symptoms and emotional instability/anxiety, but also exploring possible benefit for socialization, communication, and expanded interests. In this study, we report a pilot crossover trial to test feasibility of a RCT (recruitment, retention, adherence, satisfaction), confirm safety of VISBIOME in ASD, explore effects on quality of life, GI symptoms and anxiety, test feasibility of a crossover design with a 3-week washout, and explore the extent to which the probiotic VISBIOME can change the gut microbiota composition in 3- to 12-year-olds with ASD. Our rational probiotic therapy targets GI symptoms as a significant contributor of core symptoms in children with ASD.

Trial Design and Methods

In a randomized crossover feasibility pilot trial, children 3–12 years of age with ASD, GI symptoms, and anxiety were randomly assigned 1:1 to probiotic or placebo for 8 weeks, followed by a 3-week washout and an 8-week crossover treatment (19 weeks total). All parents gave informed consent and children who were able gave informed assent using procedures and documents approved by the local IRB.

Inclusion criteria

Participants: (1) had DSM-5 ASD on clinical evaluation by a doctoral-level diagnostician, confirmed by Autism Diagnostic Interview-Revised (ADI-R) or Autism Diagnostic Observation Schedule (ADOS); (2) were between 3 and 12 years old; (3) had ≥2 months abdominal pain, constipation, diarrhea, and/or vomiting, with an item-mean score ≥2 on at least one scale of the GI module of the Pediatric Quality of Life Inventory (PedsQL) scale; (4) had clinical anxiety symptoms with an item mean of ≥1.0 (0–3 scale) on the Parent-Rated Anxiety Scale for ASD (PRAS-ASD); and (5) were English speaking (both child and at least one parent or caregiver).

Exclusion criteria

(1) Antibiotics in 2 months before enrolling; (2) prior bowel surgery; (3) chronic serious medical condition (e.g., diabetes); (4) weight or height less than the third percentile for age; (5) chronic anti-inflammatory use within 2 months before enrolling; (6) history of inflammatory bowel disease, Celiac disease, or eosinophilic disorders (e.g., eosinophilic esophagitis); and (7) already taking probiotics within the previous 6 months.

Diagnostic measures

One of the following measures was performed by a research-reliable clinician in addition to clinical diagnosis.

Autism Diagnostic Observation Schedule. ADOS (Rutter et al. 2004; Lord et al. 2012) 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. 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 (Rutter et al. 2003).

Outcome measures

Unless otherwise noted, all measures were done at baseline, week 4, week 8 (end of first condition), week 11 (end of washout), week 15, and week 19 (end of second condition).

The primary outcome measure was the *GI Module of the PedsQL* (Varni et al. 2001, 2006, 2014). This is a 74-item survey with 14 scales (No. of items): stomach pain and hurt (6 items), discomfort when eating (5), food and drink limits (6), trouble swallowing (3), heartburn/reflux (4), nausea/vomiting (4), gas and 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 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 symptoms. There are four versions, for ages 2–4, 5–7, 8–12, and 13–18 years. We used the age-normed scale appropriate for each child; once selected, the same scale was used throughout that child's participation, assessed at baseline.

The main measure of emotional stability/anxiety was the 25-item single-factor *PRAS-ASD* (Scahill 2017; Scahill et al. 2018) developed by the multisite NIMH-funded project ''Toward outcome measurement of anxiety in youth with autism spectrum disorders.'' The mean PRAS-ASD sum for the online ASD sample was 29.04 ± 14.92. Coefficient alpha and item response theory marginal reliability were 0.93 and 0.92, respectively. In a clinical sample ($N = 116$) selected for at least mild anxiety, the mean on the PRAS-ASD was 31.0 ± 15.6 (range 1–65), an item mean of 1.24 ± 0.62. Pearson correlations ranged from 0.33 to 0.66 with parent ratings of ASD symptom severity and repetitive and disruptive behavior, supporting divergent validity. The Pearson correlation of 0.83 with a parent-rated measure of anxiety commonly used in the general pediatric population supports convergent validity. Test/retest reliabilities were 0.88 at 12 days and 0.86 at 24 days. An item mean of 1.0 was required for study entry.

An important secondary measure was *Target Symptom Rating* (Arnold et al. 2003), for which parents are asked to name the two 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 blinded clinicians reviews the descriptions and rates each on a 9-point scale relative to baseline, from remission (1) to disastrously worse (9), 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 two problems was required to pertain to GI function and was analyzed separately as well as being averaged into the overall symptoms rating. As ''no change'' on this scale is scored 5, we subtracted 5 from values reported for the Target Symptom Scale, so that a negative number indicates improvement from baseline and a positive number indicates deterioration.

The Aberrant Behavior Checklist. The Aberrant Behavior Checklist (ABC) (Aman et al. 1985a, 1985b) is a 58-item parent rating on a 0–3 scale with 5 subscales: (1) irritability (includes agitation, aggression, and self-injury, 15 items); (2) social withdrawal (16 items); (3) stereotypes (7 items); (4) hyperactivity (16 items); and (5) inappropriate speech (4 items) (Aman et al. 1985a, 1985b, 1987; Brown et al. 2002; Lam and Aman 2007). The ABC is commonly used in ASD RCTs.

Social Responsiveness Scale. This 65-item rating scale measures the severity of autism spectrum symptoms as they occur in natural social settings (Constantino et al. 2003; Constantino 2012). Completed by a parent or teacher in 15–20 minutes, the Social Responsiveness Scale (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 aims to detect changes in core ASD symptoms.

Children's Sleep Habits Questionnaire. The abbreviated Children's Sleep Habits Questionnaire (CSHQ) (Owens et al. 2000) was 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 ≥41, the clinical threshold for sleep problems (Hollway et al. 2013).

The Parenting Stress Index Short Form. The Parenting Stress Index (PSI) Short Form (Abidin 1995, 2012) was completed at baseline and end of each 8-week treatment phase. The PSI Short Form is used to evaluate the degree of stress in the parent/child relationship. The Short Form has 36 items from the full-length PSI Short Form, rated on a 5-point scale from 1 = strongly disagree, to 5 = strongly agree. It is completed in 10–15 minutes. The PSI Short Form may be used for parents of children up to 12 years. It yields a Total Score and three domain scores.

Analysis for 16S ribosomal DNA (rDNA) amplicon sequences was done. Stools were collected at baseline, end of each condition, and end of washout. Fecal bacterial DNA was prepared and sequenced at baseline and at the end of each treatment condition as previously reported by us (Luna et al. 2016). Microbiota community composition was characterized by sequencing the 16S rRNA gene and bioinformatics analysis of bacterial composition, diversity, and community structure. Briefly, Illumina paired-end sequence reads (16S V4 region) were demultiplexed with bcl2fastq version 2.17; 16S sequencing primers were removed using the fastx_trimmer script in FASTX-toolkit version 0.0.13; the phiX sequence controls and human reads were removed by Bowtie version 2.3.4 (Langmead and Salzberg 2012) with prebuilt phiX index and human reference genome index (version hg19); paired-end reads were merged by PEAR version 0.9.10 (Zhang et al. 2014) with the options (-t 136 and -q 19) for 16S V4 region; sliding window-based quality filtering for the merged reads was performed using LotuS version 1.561 (Hildebrand et al. 2014) with its default sdm option file (sdm_miSeq.txt); all the filtered reads were then concatenated into one file for chimera filtering with VSEARCH version 2.4.2 (Rognes et al. 2016) against the reference database (SILVA release 128). All the downstream analysis was carried out with QIIME version 1.9.1 (Caporaso et al. 2010): clean reads mapped to SILVA release 128 were clustered into operational taxonomic units (OTUs) using the UCLUST algorithm; representative sequence of each OTU was picked by using pick_rep_set.py script with the option (-m most_abundant); assign_taxonomy.py script was used for assigning taxonomy to representative sequences with RDP classifier and SILVA release 128, and a final OTU table was made using make_otu_table.py script; rarefaction at 20,000 reads was performed by subsampling processing the OTU table;

Table 1. Baseline Demographics and Baseline Clinical Characteristics by Treatment Sequence

		Treatment group		
Variable	*Level*	*Probiotic, then placebo (N = 6)*	*Placebo, then probiotic (N = 4)*	p
Age at randomization (years)	Mean (SD)	8.83 (2.80)	8.76 (1.18)	0.97
Gender	Female	16.7% (1)	75.0% (3)	0.19
Ethnicity	Non-Hispanic or Non-Latino origin	100% (6)	100% (4)	.
Race	Black or African	33.3% (2)	75.0% (3)	0.71
	Caucasian/White	33.3% (2)	25.0% (1)	
	Other/multiracial	33.3% (2)	0.0% (0)	
Education group	Regular private/parochial preschool	16.7% (1)	0.0% (0)	0.47
	Regular public grade school	50.0% (3)	100% (4)	
	Special school for children with emotional/behavioral/learning problems	33.3% (2)	0.0% (0)	
Income level	$20,001–$40,000	16.7% (1)	75.0% (3)	0.10
	$40,001–$60,000	0.0% (0)	25.0% (1)	
	$60,001–$90,000	50.0% (3)	0.0% (0)	
	More than $90,000	33.3% (2)	0.0% (0)	
Primary caregiver	Biological father	16.7% (1)	0.0% (0)	0.67
	Biological mother	83.3% (5)	75.0% (3)	
	Grandmother	0.0% (0)	25.0% (1)	
Primary caregiver education level	Advanced graduate or professional degree	16.7% (1)	0.0% (0)	0.71
	College graduate	66.7% (4)	50.0% (2)	
	Some college or post-high school	16.7% (1)	50.0% (2)	
ADOS2 total score	Mean (SD)	8.00 (6.38)	17.67 (6.66)	0.11
ADOS2 comparison score	Mean (SD)	4.25 (3.40)	8.67 (2.31)	0.11
PedsQL GI total	Mean (SD)	66.73 (20.193)	59.60 (17.214)	0.58
PRAS-ASD	Mean (SD)	40.00 (15.582)	41.25 (10.012)	0.89
ABC (1) irritability	Mean (SD)	18.50 (12.661)	25.00 (10.801)	0.43
ABC (2) lethargy	Mean (SD)	18.83 (12.336)	21.25 (13.598)	0.78
ABC (3) stereotypy	Mean (SD)	9.33 (4.803)	10.00 (8.287)	0.87
ABC (4) hyperactivity	Mean (SD)	22.67 (11.639)	35.00 (10.132)	0.12
ABC inappropriate speech	Mean (SD)	5.33 (3.327)	8.25 (4.500)	0.27
SRS total	Mean (SD)	82.17 (7.859)	87.00 (4.761)	0.31
CSHQ total score	Mean (SD)	43.50 (8.02)	59.75 (13.00)	0.03
PSI total stress	Mean (SD)	108.17 (20.731)	129.50 (17.767)	0.13

ABC, Aberrant Behavior Checklist; ADOS2, Autism Diagnostic Observation Schedule-2; CSHQ, Children's Sleep Habits Questionnaire; PedsQL GI, Pediatric Quality of Life Gastrointestinal; PRAS-ASD, Parent-Rated Anxiety Scale for autism spectrum disorders; PSI, Parenting Stress Index; SD, standard deviation; SRS, Social Responsiveness Scale.

alpha-diversity, beta-diversity, and per-sample or per-group taxa abundance were also measured using QIIME's scripts. Correlation analyses were carried out using in-house R scripts incorporating multiple testing correction with the Benjamini–Hochberg method.

Treatment

The probiotic mix (VISBIOME, formerly identified as VSL#3) is made up of four strains of lactobacilli (*L. casei*, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, and *Lactobacillus delbrueckii* subsp. *bulgaricus*), three strains of bifidobacteria (*B. longum*, *Bifidobacterium infantis*, and *Bifidobacterium breve*), one strain of *S. 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*). *L. bulgaricus* has been renamed *L. delbrueckii*. VISBIOME is available commercially from ExeGi Pharma, LLC, Gaithersburg, MD. Probiotic and matched placebo were supplied by the company in powder packets each containing 900 billion bacteria; starting dose the first 4 weeks of each condition was a half packet twice daily mixed with food; at 4-week and 15-week visit, there was an option to increase to a full packet twice daily if no effect was noted.

Adherence was measured by packet counts of returned probiotic and placebo containers. Vital signs, concomitant treatments, and adverse events (AEs) were collected at each visit. Diet logs were completed by parents for 3 days before each stool sample collection to determine whether the child's diet changed significantly since the baseline stool sample was collected, thus allowing us to adjust for dietary changes as a potential confounding factor in stool sample variation.

Statistical methods

Subject characteristics were compared at baseline among probiotic supplement (S) first then placebo (P) sequence (SP) and placebo first then supplement sequence (PS) groups. In this pilot feasibility study, the main emphasis was on effect size rather than statistical significance. Nevertheless, probiotic effect over the entire study was assessed using a mixed model with outcome measures post-baseline as the outcome and with fixed effects for treatment (probiotic versus placebo), categorical time (week 4, week 8), baseline value for the outcome measure, and an interaction between treatment and time. This model accounts for the crossover design and repeated measurements by including a random effect for subjects within sequence group and phase of study and with errors for subjects within treatment and study week modeled with a variance components only correlation structure. Effect size for the group difference is given alongside model estimates and is calculated as the estimated mean difference between groups divided by the standard error times the square root of the number of unique subjects included in the model. AEs are summarized by number of events and number of subjects in the MedDRA system organ class and preferred term with a p-value comparing the number of subjects across drug groups from an exact McNemar test.

Results

Thirteen patients were randomized to order, but 3 dropped out because of distance and transportation without providing any follow-up data, leaving 10 who progressed through to study completion: 6 in SP and 4 in PS crossover groups. The baseline characteristics for those continuing in the study show well-balanced sequences on age, sex, other demographics, and clinical characteristics (Table 1). All of the children had at least rudimentary language. The median adherence to study treatment was 96% (97% for probiotic, 95% for placebo). Participant parental satisfaction had a mean score of 26 points on a 10–30-point scale (higher score better). Only 6 of the 10 guessed the correct treatment at the end of week 8 (5 would be expected by chance); 2 said they could not tell; and 2 guessed incorrectly. Stool collections were 97% complete.

Table 2. Adverse Events by Condition and Relationship to Study Treatment

System organ class	*Preferred term*	*AE related to study*	*Probiotic*	*Placebo*
Gastrointestinal disorders	Abdominal distension	Unlikely related	1 (10%, events = 1)	1 (10%, events = 1)
	Abdominal pain	Unrelated	0	1 (10%, events = 1)
		Unlikely related	1 (10%, events = 1)	1 (10%, events = 1)
	Diarrhea	Possibly related	0	1 (10%, events = 2)
	Flatulence	Unrelated	2 (20%, events = 2)	0
		Unlikely related	0	1 (10%, events = 1)
		Possibly related	1 (10%, events = 1)	1 (10%, events = 1)
	Nausea	Unrelated	1 (10%, events = 1)	2 (20%, events = 2)
	Vomiting	Possibly related	1 (10%, events = 1)	0
General disorders and administration site conditions	Pyrexia	Possibly related	0	1 (10%, events = 1)
Immune system disorders	Multiple allergies	Unrelated	0	1 (10%, events = 1)
Infections and infestations	Nasopharyngitis	Unrelated	2 (20%, events = 2)	0
	Upper respiratory tract infection	Unrelated	2 (20%, events = 2)	0
Metabolism and nutrition disorders	Decreased appetite	Possibly related	0	1 (10%, events = 1)
		Probably related	1 (10%, events = 1)	0
Nervous system disorders	Headache	Unrelated	0	1 (10%, events = 1)
Psychiatric disorders	Depressed mood	Unlikely related	1 (10%, events = 1)	0
	Encopresis	Unlikely related	0	1 (10%, events = 1)
		Possibly related	0	1 (10%, events = 1)
	Enuresis	Unlikely related	1 (10%, events = 1)	0
		Possibly related	0	1 (10%, events = 1)

AE, adverse event.

Table 3. Concomitant Medication

Medication	*No. of children taking*	*Primary indication*
Miralax	2	Constipation
Zofran	Other	Nausea
Tums	1	Acid reflux
Flintstone vitamin	2	Nutrition
Lamotrigine	1	Behavior
Guanfacine	2	Behavior
Risperidone	1	Behavior
Vyvanse	1	Hyperactivity
Quillivant	1	ADHD symptoms
Sertraline HCL	2	Anxiety
Azithromycin	1	Cough, runny nose
Ibuprofen childrens	1	Cold, fever
Melatonin	2	Sleep
Benadryl	1	Sleep
Albuterol	1	Asthma
Singulair	1	Asthma
Tylenol Jr	1	Headache
Zyrtec	3	Allergies
Clonidine	1	Vocal tics

ADHD, attention-deficit/hyperactivity disorder.

There were no serious AEs. No AEs occurred significantly more often with probiotic over placebo; 14 occurred with probiotic and 17 with placebo (Tables 2 and 3). However, there were four infections (two nasopharyngitis and two upper respiratory tract infections) while on probiotics and none while on placebo ($p=0.13$), which the physician did not consider attributable to study treatment. Of AEs that were probably or possibly attributable to study treatment, three participants reported them while on probiotic and seven reported them while on placebo. Of GI AEs probably or possibly attributable to study treatment, two occurred with probiotic and two with placebo.

Over the 19-week study period, each outcome measure showed improvement over baseline, with the probiotic phase showing more improvement than the placebo phase, but the difference for PedsQL (primary outcome) and PRAS-ASD (main secondary outcome) did not meet statistical significance (Table 4 and Supplementary Table S1). Another important secondary measure was target symptoms selected by parents. On the first target symptom selected by parents, participants showed more improvement while on probiotics over placebo after 8 weeks of treatment ($p=0.02$, $d=0.79$), but the second target symptom effect was not significant. The average of the two also did not show significance.

In this pilot study, effect sizes were of more utility than statistical significance. The primary outcome, PedsQL, showed an effect size of $d=0.49$ by the general model (Table 4) and $d=0.79$ by simple comparison of week 8 changes from respective baselines (Supplementary Tables S1 and S2); the PRAS-ASD showed $d=0.07$; and average of target symptoms showed $d=0.52$, all in the direction of more improvement with probiotic over placebo. More details can be found in Supplementary Tables S1–S5.

Figure 1 and Supplementary Table S4 illustrate the carryover of the effect of the first condition through the 3 weeks of washout to week 11. The 11-week improvement from original baseline on the PedsQL GI module from the probiotic condition is greater than from the placebo condition by $d=0.67$, despite no treatment between weeks 8 and 11.

Longitudinal fecal microbiota characterization was performed in the 10 children who completed the crossover study since VISBIOME has previously been reported to alter microbiota community dynamics. α-Diversity and relative family abundances were evaluated longitudinally using 16S rDNA V4 sequencing and bioinformatics analysis that we described previously for exploring microbiota community dynamics in children with ASD and GI symptoms (Luna et al. 2016). No specific treatment-associated shifts were evident in either α-diversity or family level composition of bacterial species that could be attributed to probiotic administration (Fig. 2)—that is, probiotics did not significantly alter microbiome community complexity or composition in the stool. As highlighted in a recent report demonstrating unique host and microbiota-dependent factors that determine empiric probiotic colonization in humans (Zmora et al. 2018), analysis of individual children demonstrated microbiota heterogeneity (Supplementary Fig. S1) that requires analysis to assess probiotic correlations with improvement in clinical outcomes. In the present work, we found

Table 4. Difference in Treatment Response Between Probiotic and Placebo Conditions

Outcome measure	*Estimate*	*Standard error*	*Lower*	*Upper*	*Effect size*	p
PedsQL GI total (0–100)	5.76	3.72	−2.09	13.60	0.49	0.14
PRAS-ASD total (0–75)	−0.82	3.89	−8.99	7.35	0.07	0.84
ABC (1) irritability (0–45)	0.80	3.00	−5.49	7.10	0.08	0.79
ABC (2) lethargy (0–48)	−0.43	2.98	−6.69	5.83	0.05	0.89
ABC (3) stereotypy (0–21)	0.40	1.69	−3.14	3.95	0.08	0.81
ABC (4) hyperactivity (0–48)	3.07	3.68	−4.65	10.79	0.26	0.41
ABC (5) inappropriate speech (0–12)	−0.43	1.03	−2.59	1.73	0.13	0.68
PSI total stress (36–180)	2.65	6.42	−10.9	16.20	0.13	0.68
SRS total (T-score)	−3.07	2.68	−8.73	2.58	0.36	0.27
CSHQ total score	1.33	2.92	−4.83	7.50	0.14	0.65
Target symptom No. 1—P2RC S1 (−4 to 4)	−1.50	0.60	−2.75	−0.25	0.79	0.022
Target symptoms—P2RC mean (−4 to 4)	−0.80	0.49	−1.83	0.23	0.52	0.12

Estimate shows the change of the probiotic group minus the change of the placebo group. Higher scores are better for the PedsQL GI total. Hence a positive estimate means more improvement with probiotics compared with control. Higher scores are worse for PRAS-ASD, ABC subscales, PSI Total Stress, SRS total, and CSHQ. Hence a negative estimate means more improvement with the probiotics compared with control. Target symptom change scores are centered at 0 with a −4 for symptom completely gone and 4 for disastrously worse. Hence a negative estimate means more improvement with probiotics compared with control.

ABC, Aberrant Behavior Checklist; CSHQ, Children's Sleep Habits Questionnaire; PedsQL GI, Pediatric Quality of Life Gastrointestinal; PRAS-ASD, Parent-Rated Anxiety Scale for autism spectrum disorders; PSI, Parenting Stress Index; SRS, Social Responsiveness Scale.

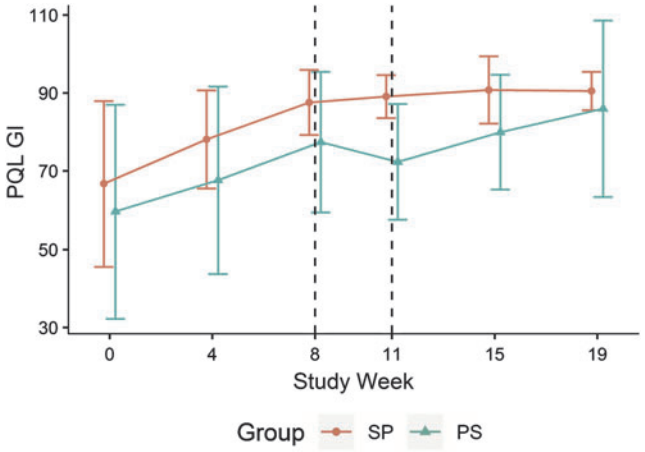

FIG. 1. Primary outcome, Pediatric Quality of Life GI Module, over the course of both conditions by sequence group. Higher score is better. Vertical dotted lines delineate the 3-week washout between conditions. Difference at 11 weeks is significant ($p = 0.006$). GI, gastrointestinal; PS, placebo first; SP, probiotic supplement first. Color images are available online.

that the relative abundance of *Lactobacillus* OTU GM884480.1.1531 correlated significantly with the PedsQL score (Fig. 2). Because the beneficial effects of VISBIOME may be mediated to the host independently of microbiota compositional shifts, deep microbiome or functional characterization was beyond the scope of the present study.

Discussion

In this 10-subject crossover study, we did not expect statistical significance and did not find it, except on the first parent-selected target symptoms. We were interested in assessing feasibility, safety (defined as no serious AEs attributed to the study treatment), and some reasonable suggestion of improvement in the PedsQL GI scale, the primary outcome, or anxiety and microbiota community composition, the chief secondary outcomes.

Feasibility

Although recruitment efforts were restricted by the funding agency to underserved populations, we were able to recruit 13 families in 10 months, with 76% retention for the whole 19-week trial, with good adherence, good tolerance of the probiotic, good satisfaction, and acceptable blinding (correct guesses about treatment assignment were about what would be expected by chance). The dropouts were for distance and transportation problems of rural Appalachian families, not side effects or other intolerance/unpalatability. With no restriction to specific demographic populations, it should be possible to recruit 20 per year, with a lower dropout rate. Stool collections were practical, with only one of possible 43 stools missing. The data suggest that a parallel-group design will be necessary for a larger RCT because of the considerable carryover to at least 3 weeks beyond the last dose of probiotic. This is both good news and bad news: it suggests a more enduring effect from 8 weeks of probiotic, but means that the crossover design used in this pilot study is not practical. Another aspect of feasibility is sensitivity of the measures. It appears that the primary measure, the PedsQL GI module, is sensitive, and target symptom assessment is also moderately sensitive. However, the insensitivity of the anxiety scale was disappointing; a different measure of anxiety should be considered for a larger prospective RCT, although it is possible that this probiotic formulation simply does not affect anxiety in ASD. Similarly, the ABC, an accepted standard in ASD RCTs, did not detect a probiotic effect, probably because it did not focus on the GI symptoms targeted by the probiotics.

Safety

There were no serious AEs, as predicted. The probiotic formulation was not associated with any more AEs than placebo, including when restricted to those attributable to treatment and when restricted to GI AEs. This confirms previous data on safety of this formulation.

Effect sizes

The primary outcome, PedsQL GI module, had a medium effect, although not significant at this sample size. Target symptom assessment also showed a respectable medium-to-large effect. These are encouraging enough to warrant a large RCT. Any such RCT will have to take into consideration the large improvement in both groups from baseline to 8 weeks and from baseline to 11 weeks. These indicate that a large part of the observed improvement is due to regression to the mean or placebo response or both. Treatments in ASD are notorious for showing a large placebo response, so this is not unexpected and can be dealt with in future power calculations.

Limitations

Limitations of this study include the small sample, partially an artifact of sponsor restrictions on eligible populations. The small sample precluded potentially informative analyses split by sex and type of GI dysfunction. Another is the too-short washout, but this served the feasibility purpose of informing the correct design for a larger trial. A third is the choice of a new anxiety scale that was presumed to be specific to ASD but was ultimately insensitive for this particular treatment. A fourth is the use of 16S sequencing without metagenome analysis coupled with metabolomics profiling. Failure to exclude obesity may have been an oversight, but no participants were particularly obese.

Conclusion

The probiotic VISBIOME is a safe treatment in children with ASD and GI symptoms, but efficacy for quality of life is unproven. Effect sizes of target symptoms and PedsQL suggest that a larger RCT is warranted, which should use a parallel-group design because of carryover of effect beyond the treatment. The sample should be large enough to allow subgroup analyses by sex, type of GI dysfunction, and other participant characteristics.

Clinical Significance

This pilot probiotic trial addresses the intersection of Autism, GI dysfunction, bacteriology, and neuroimmunology. It constitutes part of the emerging interest in the gut microbiome that constitutes a significant proportion of the DNA in each human and are not only necessary for normal GI homeostasis but also influence brain function (''microbiota/gut/brain axis''). The results of this pilot study, with a medium effect on GI symptoms and quality of life, a significant effect on parent-selected target symptoms, and good safety profile, justify a larger intervention trial for children with

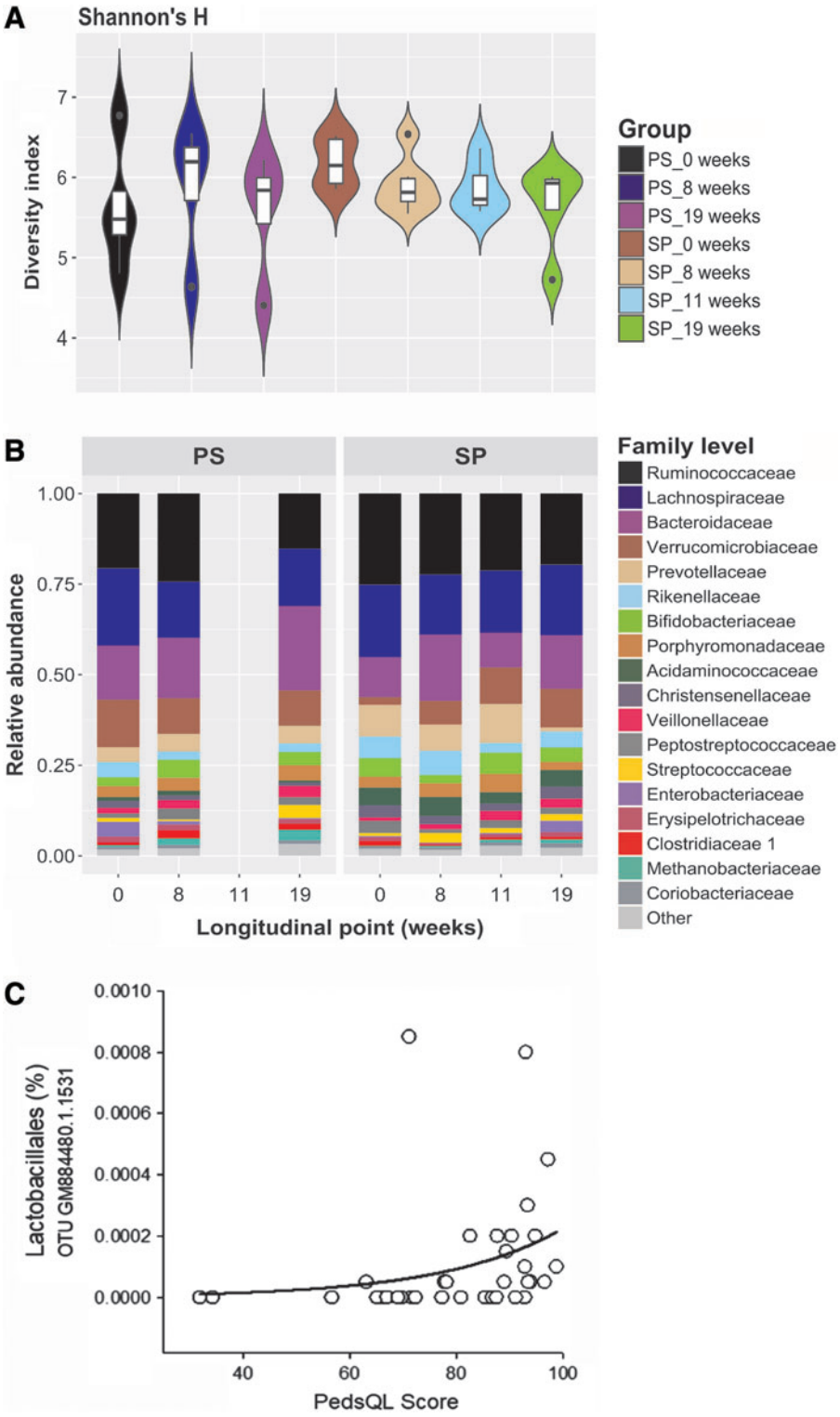

FIG. 2. Microbiota community dynamics following VISBIOME treatment. **(A)** Shannon H diversity index does not show significant alterations following probiotic or placebo. **(B)** Relative family abundance does not show a significant difference over the course of both conditions by sequence group. **(C)** *Lactobacillus* correlation with the PedsQL score with an exponential curve fit. Regression analysis shows a significant correlation; Spearman's Rho = 0.573579609, $p = 0.022$ after applying Benjamini–Hochberg multiple testing correction. PedsQL, Pediatric Quality of Life Inventory; PS, placebo first; SP, probiotic supplement first. Color images are available online.

autism and GI dysfunction. One caution is that the results may not be generalizable beyond the eight-ingredient probiotic used in this pilot study.

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Disclosures

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Supplementary Material

Supplementary Figure S1
Supplementary Table S1
Supplementary Table S2
Supplementary Table S3
Supplementary Table S4
Supplementary Table S5

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