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Abstract

Gastrointestinal (GI) symptoms often affect children with autism spectrum disorders (ASD) and GI symptoms have been associated with an abnormal fecal microbiome. There is limited evidence of *Candida* species being more prevalent in children with ASD. We enrolled 20 children with ASD and GI symptoms (ASD + GI), 10 children with ASD but no GI symptoms (ASD – GI), and 20 from typically developing (TD) children in this pilot study. Fecal mycobiome taxa were analyzed by Internal Transcribed Spacer sequencing. GI symptoms (GI Severity Index [GSI]), behavioral symptoms (Social Responsiveness Scale -2 [SRS-2]), inflammation and fungal immunity (fecal calprotectin and serum dectin-1 [ELISA]) were evaluated. We observed no changes in the abundance of total fungal species (alpha diversity) between groups. Samples with identifiable *Candida* spp. were present in 4 of 19 (21%) ASD + GI, in 5 of 9 (56%) ASD – GI, and in 4 of 16 (25%) TD children (overall $P = 0.18$). The presence of *Candida* spp. did not correlate with behavioral or GI symptoms ($P = 0.38$, $P = 0.5$, respectively). Fecal calprotectin was normal in all but one child. Finally, there was no significance in serum dectin-1 levels, suggesting no increased fungal immunity in children with ASD. Our data suggest that fungi are present at normal levels in the stool of children with ASD and are not associated with gut inflammation.

Keywords: autism, *Candida*, children, fungi, gastrointestinal symptoms, yeast

What Is Known

- About 40% of children with autism spectrum disorders (ASD) have gastrointestinal (GI) symptoms.
- Children with ASD have been shown to have dysbiosis with respect to the fecal microbial community.
- Many children with ASD are treated with antifungals because of a few papers suggesting increased Candida in the stools.
- The mycobiota (fungi) in children who do not have ASD has not been compared to that of children with ASD (with and without GI symptoms).

What Is New

- Using ITS-2 sequencing of fecal samples, we were unable to identify major differences in the number of fungi or the fungal composition in children with ASD.
- These children also did not have increased gut inflammation (as measured by fecal calprotectin) or in antifungal antibodies (as measured by serum dectin-1).

Gastrointestinal (GI) symptoms are common in children with autism spectrum disorders (ASD), affecting up to 42% of children with ASD compared to 12% of typically developing (TD) children (1). Strong correlations have been made between GI symptoms and ASD disease severity (1,2). The present study has concentrated on the microbiota as part of the gut microbiome (microbes, their genes, and gene products) in ASD (3). There are few studies evaluating the fungal species known as the mycobiome—in ASD (4,5). Fungi typically constitute 0.001–0.01% of the total microbiota, and many fungal species are transiently present from the diet and oral/nasal cavity, rather than being true colonizers (6); however, *Candida* spp. can interact cooperatively with specific microbial species to produce mixed-species biofilms with potentially proinflammatory characteristics (7).

The widespread use of antifungal treatment in children with ASD became more common after a commercial laboratory at Great Plains, Lenexa, KS reported unvalidated claims of increased levels of urinary organic acids putatively originating from fungi in children with ASD (8). A survey by the Autism Research Institute (*www.autism.org*) has reported that many parents find antifungals to be an effective medication for improving behavior (9); however, there is little evidence for antifungal treatments.

When the mycobiome was evaluated in healthy individuals as a part of the Human Microbiome Project, investigators found lower fungal diversity compared to bacterial diversity (10). In children with ASD, there is controversy regarding the relative abundance of *Candida* species. Iovene *et al.* (4) identified *Candida* spp. in 57% of children with ASD and in none of their TD children. Kantarcioglu *et al.* (11) identified increased *Candid*a spp. in children with ASD using standard culture techniques, but Adams *et al.* (2) found no differences. Hughes *et al.* (12) reported a significantly higher percentage of plasma samples containing anti-*Candida albicans* antibodies in children with ASD compared to TD children. Horvath *et al.* (13) also reported that 43% of children with ASD undergoing endoscopy had a positive fungal culture from duodenal fluid, compared to only 23% of age-matched TD children. Strati *et al.* (5) used fecal Internal Transcribed Spac (ITS) sequencing to identified a 2-fold (insignificant) greater abundance of *Candida* spp. in children with ASD.

This study's primary aim was to compare the gut mycobiome of children with ASD with that of otherwise healthy children. Our secondary

objective was to evaluate whether the presence of *Candida* species in stool correlates with increased GI symptoms, behavioral problems, and/or gut inflammation (13).

Methods

This study was approved by the Human Subjects Institutional Review Board of the University of Texas Health Science Center at Houston (HSC MS-17-1015), with patients enrolled from June 2016 to February 2019. Participants were recruited from an ongoing prospective study in children with ASD (HSC-MS-15-0751). All families signed parental consent and, when possible, child assent forms. TD children were recruited from the University of Texas-Houston's pediatrics clinics. We screened all children with the social communication questionnaire (SCQ). The diagnosis of ASD was based on testing (DSM-5: 299.00) by a psychologist, with confirmation by the Autism Diagnostic Interview-Revised (ADI-R). We accepted children of ages 4–16 years with ASD and assessed GI symptoms using the GI Severity Index (GSI) (14). Patients designated positive for GI symptoms were required to have a GSI score of >7. TD children were required to have a SCQ <9 and a GSI <4.

Exclusion Criteria

We excluded children with severe sensory impairment, brain injury, genetic syndromes, or major psychiatric illness. The characteristics of the study participants are listed in **Table 1**. Participants in the three groups were of similar age and gender. There was greater severity of GI symptoms in the ASD + GI group compared to ASD – GI and TD children. There were more Asian and fewer African-American children in the groups with ASD. SCQ and SRS raw scores were more severe in the children with ASD and GI symptoms.

Study Protocol and Sample Collections

After giving consent, parents answered the GSI and completed the Social Responsiveness Scale, 2nd edition (SRS-2) (14). Plasma was isolated from whole blood by centrifugation, aliquoted, and stored at –80°C.

Maladaptive behaviors associated with ASD were quantified by the Social Responsiveness Scale-2 (SRS-2), a 65-item rating scale measuring deficits in social behavior associated with ASD (14).

Mycobiome Analysis

DNA was extracted using the MO BIO PowerMag Microbiome DNA Isolation Kit. The ITS2 region was PCR amplified using primers ITS3 and ITS4 and analyzed as described previously (10). Sequences were stepwise clustered into operational taxonomic units (OTUs) at a similarity cutoff of 99%. There was a median of 1958 fungal reads/sample. To calculate the presence of *Candida* spp., samples with <100 total fungal ITS2 reads were excluded, and to avoid false positives due to sequence bleedover, only samples with >2 reads of a *Candida* spp. were considered to contain *Candida* spp. Fungal load was determined by qPCR. Note that there were no missing data, but there were 16 samples with <870 reads, the cutoff for diversity calculation, which agreed with the qPCR data.

Gastrointestinal Inflammation

GI inflammation was assessed by fecal calprotectin analyzed by using an enzyme-linked immunosorbent assay (ELISA) kit (Eagle Biosciences, Nashua, New Hampshire), with a polyclonal antibody against calprotectin. Normal values are generally defined to be <100μg/g stool (15).

Antifungal Immunity

Antifungal immunity was assessed by serum dectin-1 level. Dectin-1 is a sentinel receptor for fungal infections that recognizes β-1,3-glucans found in the cell walls of nearly all fungi. Deficiencies in dectin-1 or CARD9 result in enhanced susceptibility to fungal infections (16). We measured plasma levels of dectin-1 by ELISA kit (Sigma-Aldrich, Saint Louis, MO).

Data Collection, Management and Analysis

The REDCap (Research Electronic Data Capture) database was used for collecting and managing data. Sequence data was deposited in the

National Center for Biotechnology Information (NCBI)'s Sequence Read Archive (SRA) (BioProject PRJNA639900).

Statistical Analysis

We compared various characteristics among the three groups using the chi-square or extended Fisher exact tests for categorical variables, and analysis of variance or Kruskal-Wallis test for continuous variables. We compared the behavioral scores between patients with and without the presence of each specific species of fungi by the two-sample *t*-test or Wilcoxon rank sum test. General linear model (GLM) evaluated whether the presence of fungi was associated with behavioral problems. Statistical analyses used SAS 9.4 (SAS Institute. Inc., Cary, NC).

Results

Fifty children participated. Twenty children (17 males and 3 females), with a mean age of 9.3±3.8 years, were enrolled in the ASD þ GI group; 20 children (15 males and 5 females) ages 9.8±3.8 years were enrolled in the TD group; and 10 children (10 males and no females) ages 8.1±3.1 years were enrolled in the ASD – GI group (**Table 1**). The severity of ASD symptoms was assessed with SRS *T*-scores interpreted per protocol as: 60–65: mild range; 66–75: moderate range; and 76 or higher: severe range. The median SRS *T*-score was higher among the ASD + GI group 82.5 (73, 87), indicating severe impairment compared to the ASD – GI group median SRS *T*-score of 72.5 (70, 76), $P = 0.03$ (Table 1).

For analysis of diversity, all samples were rarefied to 870 reads/sample, leaving 34 samples for analysis. The leading genera identified in fecal samples were *Saccharomyces* (in 30, or 61% of the 49 children with good quality fecal ITS2 DNA), *Penicillium* (in 27, or 55%), *Aspergillus* (in 14, or 29%), and *Candida* (in 12, or 24%). There were no significant differences in total fungal DNA copies among the three groups (**Fig. 1A**). The alpha diversity was not significantly different among the three groups (observed OTUs: ASD + GI/TD $P = 0.12$, ASD – GI/ TD $P = 0.06$, ASD + GI/ASD – GI $P = 0.55$; Shannon diversity index: ASD + GI/TD $P = 0.42$, ASD – GI/TD $P = 0.14$, ASD + GI/ASD – GI $P = 0.75$; Fig. 1B-a and b). Note that there were no missing data, but there were 16 samples with <870

Table 1. Children characteristics between population groups typically developing (TD), ASD without gastrointestinal symptoms (ASD – GI) and ASD with gastrointestinal symptoms (ASD + GI)

Variable	*TD (N = 20) (group a)*	*ASD – GI (N = 10) (group b)*	*ASD + GI (N = 20) (group c)*	*P value**	*All (N = 50)*
Age, y, mean (SD) (95% CI for mean)	9.8 (3.8) (8.0, 11.6)	8.1 (3.1) (5.9, 10.3)	9.3 (3.8) (7.5, 11.0)	0.48†	9.3 (3.66) (8.2, 10.3)
Male, n (%) (95% CI for percentage)	15 (75) (50, 91)	10 (100) (69, 100)	17 (85) (62, 97)	0.20§	42 (84) (74, 94)
Hispanic, n (%) (95% CI for percentage)	6 (30) (12, 54)	1 (10) [c] (0, 45)	10 (50) [b] (27, 73)	0.08§	17 (34) (21, 47)
Race, n (%)					
Caucasian (95% CI for percentage)	11 (55) (32, 77)	6 (60) (26, 88)	13 (65) (41, 85)	0.03§	30 (60) (46, 74)
African American (95% CI for percentage)	3 (15) (3, 38)	0 (0) NA	1 (5) (0, 25)		4 (8) (0, 16)
Asian (95% CI for percentage)	0 (0) [b] NA	4 (40) [a] (12, 74)	4 (20) (6, 44)		8 (16) (6, 26)
Mix/other (95% CI for percentage)	6 (30) (12, 54)	0 (0) NA	2 (10) (1, 32)		8 (16) (6, 26)
Candida spp, n (%) (95% CI for percentage)	4/16 (25) (7, 52)	5/9 (56) (21, 86)	4/19 (21) (6, 46)	0.18§	13/44 (30) (16, 43)
GSI, median (Q1, Q3) (95% CI for median)	0 (0, 0)[bc] (0, 0)	2.5 (1, 4)[ac] (0, 5)	9 (8, 10)[ab] (8, 10)	< 0.01 ¶	2.5 (0, 8) (0, 7)
SCQ					
Mean (SD) (95% CI for mean)	1.1 (1.7)[bc] (0.3, 1.9)	18.9 (2.3)[ac] (17.2, 20.6)	24.9 (6.0)[ab] (22.1, 27.7)	< 0.01†	14.2 (11.71) (10.9, 17.5)
Median (Q1, Q3) (95% CI for median)	0 (0, 2.5)[bc] (0, 2)	18.5 (18, 2)[ac] (16, 21)	24.5 (21, 29.5)[ab] (21, 29)	< 0.01 ¶	17 (1, 23) (3, 21)
SRS raw score					
Mean (SD)) (95% CI for mean	NA	91.6 (12.9)[c] (82.4, 100.8)	106.8 (21.2)[b] (96.8, 116.7)	0.04 **	101.7 (19.98) (94.2, 109.2)
Median (Q1, Q3) (95% CI for median)	NA	90.5 (83, 100)[c] (75, 103)	111.5 (90.5, 124.5)[b] (93, 124)	0.03‡	99.5 (86, 120) (90, 117)
SRS *T*-score					
Mean (SD) (95% CI for mean)	NA	73.0 (5.1)[c] (69.3, 76.7)	79.6 (8.2)[b] (75.8, 83.4)	0.02 **	77.4 (7.87) (74.5, 80.3)
Median (Q1, Q3) (95% CI for median)	NA	72.5 (70, 76) [c] (66, 78)	82.5 (73, 87)[b] (74, 86)	0.03‡	76 (71, 84) (72, 83)

SRS *T*-scores of 66 through 75 are interpreted as indicating moderate deficiencies in reciprocal social behavior. Confidence interval for means are obtained from *t*-test. Confidence interval for percentage of categorical variables are obtained from exact method for Binomial distribution and when number of samples are ≥30 obtained from Normal approximation of Binomial distribution. Confidence intervals for medians (50th percentile) are obtained using PROC UNIVARIATE in SAS. ASD = autism spectrum disorders; CI = confidence interval; GSI = Gastrointestinal Symptom Severity Index; SCQ = social communication questionnaire; SD = standard deviation; SRS = Social Responsiveness Scale-2 (SRS-2).

* *P*-values are calculated between three groups of patients: neurotypical (NT), ASD without GI symptoms (ASD – GI) and ASD with GI symptoms (ASD + GI).

† Denotes *P* values obtained by analysis of variance.

§ Denotes *P* values obtained by Fisher exact test.

¶ Denotes *P* values obtained by Kruskal-Wallis test.

‡ Denotes *P* values obtained by Wilcoxon rank sum test.

** Denotes *P* values obtained by *t*-test. Note that a higher SRS score indicates greater impairment.

a,b,c Indicates the significant differences in pair-wise comparisons for the estimates. Values without superscripts indicate no significant difference with values in other groups. Values with superscripts indicate significant difference with values in other groups.

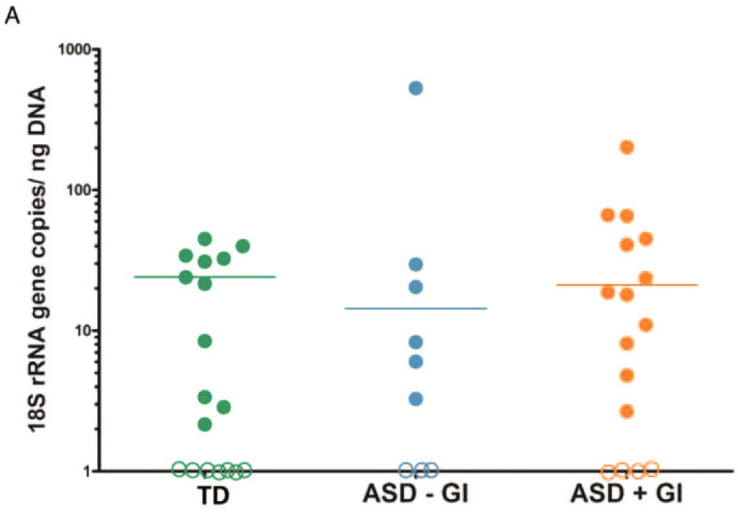
reads, the cutoff for diversity calculation, which agreed with the qPCR data. There were also no significant differences among the three groups when looking at mycobiome principal coordinates analysis (PCoA) using the Bray-Curtis distance metric ($P = 0.13$; Fig. 1B-c). Of the samples with identifiable fungi, *Candida* spp. were present in 4 of 19 (21%) ASD + GI, in 5 of 9 (56%) ASD – GI, and in 4 of 16 (25%) TD children ($P = 0.18$) (Table 1). No significant change in behavioral scores or GI symptoms were found when correlating to the presence of *Candida* spp. (which included *Candida* spp. *albicans*, *tropicalis*, *bracansis*, *mesenterica*, *oleophila*, *parapsilosis*, and *sake*) in stool ($P = 0.38$).

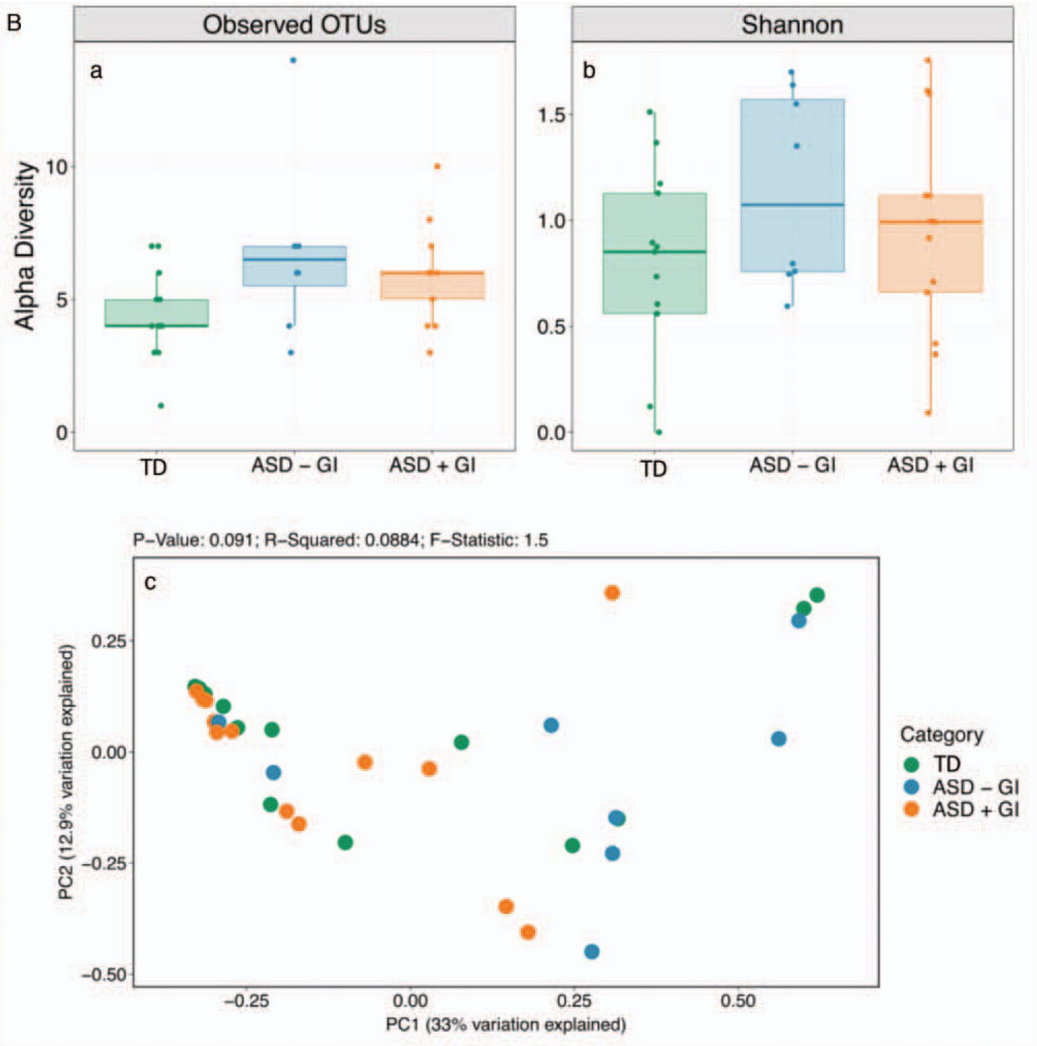
Fecal calprotectin levels were within the normal reference range comparing the three groups, with the exception of one individual with ASD without GI symptoms (Supplemental Figure 1A). There was no significant difference in serum dectin-1 level-measured anti-fungal immunity in children with ASD, in this case compared to the ASD + GI group ($P = 0.44$) (Supplemental Figure 1B).

Figure 1. Fungal DNA concentration in stools of patients and fecal mycobiota analysis. All samples with DNA remaining following ITS2 sequencing were measured by quantitative PCR targeting the 18S rRNA gene. The three groups of patients were: typically developing (TD) children (TD, n = 20), ASD without gastrointestinal symptoms (ASD – GI, n = 10), and ASD with GI symptoms (ASD + GI, n = 20).

(A) Solid circles represent samples with detectable fungal DNA copies. Open circles represent samples where no fungi were detected. For qPCR analysis, there were 18 samples, 9 samples, and 16 samples in the three groups with adequate DNA copies. Samples with open circles had no identifiable fungal DNA.

(B) Fecal mycobiota diversity analysis. Data from fungal ITS2 sequences rarefied to 870 reads/sample. Note that there was one sample with no ITS sequencing reads and 16 samples that had <870 reads, which is the cutoff used for plotting diversity. Lines represent the median value for each group. There were no significant differences among the group comparisons. (a) Alpha diversity of mycobiota with observed operational taxonomic units (OTUs), non-adjusted P value = 0.42, for three group comparisons. (b) Alpha diversity of mycobiota with Shannon Diversity Index, P value = 0.44 among three groups. The displayed are the interquartile range (IQR; boxes), median (line), and 1.5 IQR (whiskers). (c) Principal coordinates analysis (PCoA). Ordination was performed in R by PCoA using the Bray-Curtis distance metric. No differences among the groups were significant. ASD = autism spectrum disorders; GI = gastrointestinal.


A
18S rRNA gene copies/ ng DNA
1000
100
10
1
TD
ASD - GI
ASD + GI


B
Observed OTUs
a
Alpha Diversity
10
5
0
TD
ASD – GI
ASD + GI
Shannon
b
1.5
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0.5
0.0
TD
ASD – GI
ASD + GI
P–Value: 0.091; R–Squared: 0.0884; F–Statistic: 1.5
c
PC2 (12.9% variation explained)
0.25
0.00
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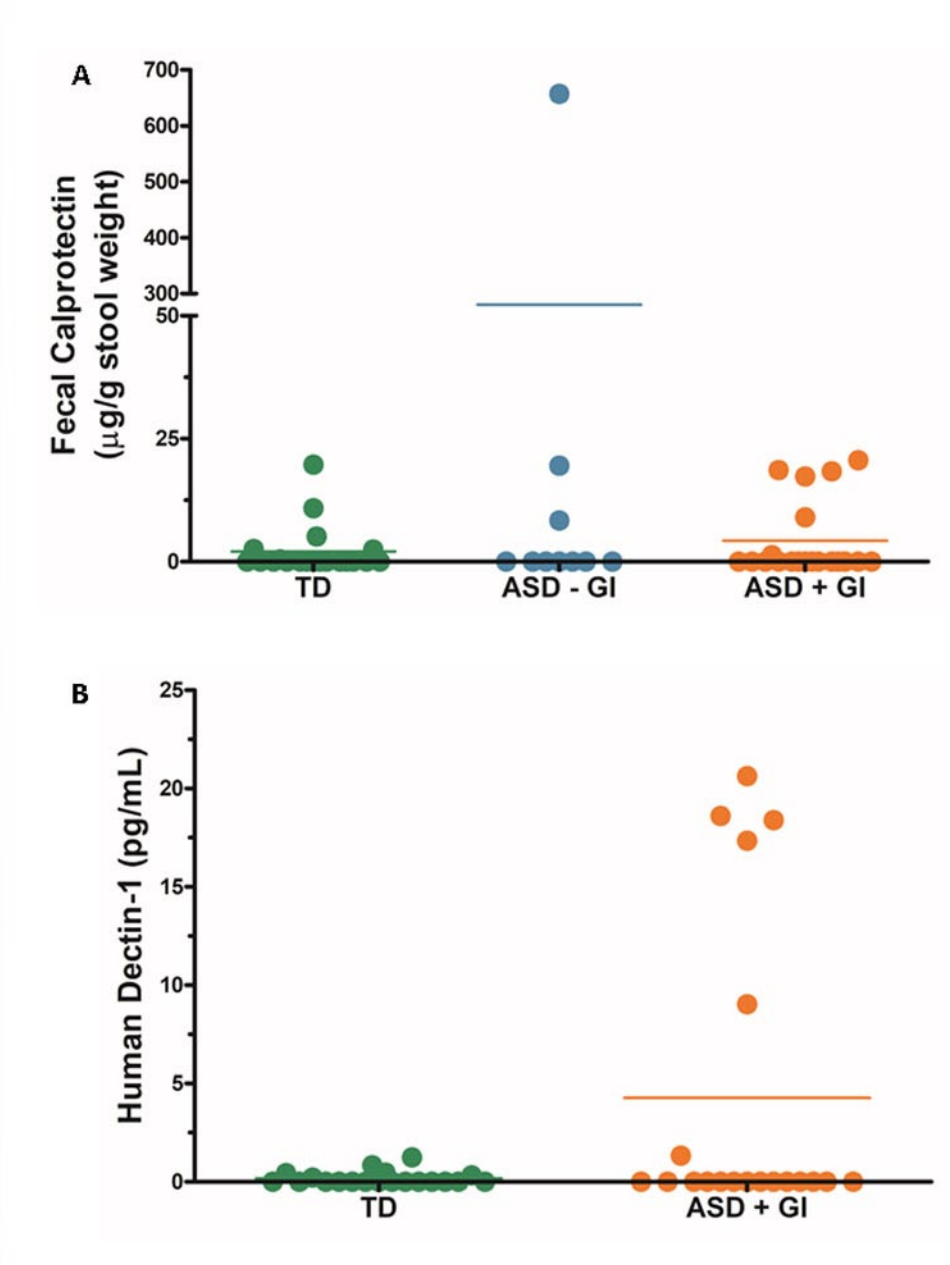
0.25
0.50
PC1 (33% variation explained)
Category
TD
ASD – GI
ASD + GI



Supplemental Figure 1. Stool and plasma biomarkers.

A. Fecal calprotectin measured by ELISA, normalized by stool weight. Three-group (TD (n = 20), ASD-GI (n = 10), and ASD+GI (n = 20)) comparisons revealed no significant differences.

B. Plasma dectin-1 measured by ELISA with a two-group (TD vs. ASD+GI) comparison. No significant differences were found. Lines represent the mean value for each group.

Discussion

Fungal communities are present in varying abundance in the human body including the skin, oral mucosa, and intestinal tract. Fungi may be linked to the exacerbation of several GI diseases (7,17), including inflammatory bowel disease, celiac disease, and Hirschsprung disease-associated enterocolitis. *Candida* spp. are considered to be commensal yeasts but do have the capacity under certain conditions to become pathogenic and to cause invasive disease (18).

In our study, we found that fungi were present at similar levels with respect to both diversity and abundance in children with or without ASD. The number of fungal taxa was similar to but even lower in children compared to those reported in healthy adults and those with inflammatory bowel disease (17). Even though we excluded children consuming yogurt or antibiotics, other factors, including diet, may have influenced the number of *Candida* spp. Another study noted that the fecal bacterial population was impacted by long-term diets, with *Candida* spp. abundance being strongly associated with recent consumption of carbohydrate (19). We found that GI symptoms did not correlate with the presence of *Candida* spp. in stool. Similar findings seen in Iovene *et al.* and Hughes et al. who found that GI symptoms were not associated with *Candida* immunoglobulin (IgG) level (3,4,12).

One group reported that antifungal (nystatin) treatment of children with severe ASD improved symptoms and reduced abnormal urinary metabolites such as tartaric acid and arabinose, thought to be byproducts of yeast (11); however, antifungals can affect more than just the fungal population, altering the function of the microbiota as well (20). Although the severity of behavioral symptoms did not correlate with presence of *Candida* spp., we note that behavioral symptom severity was higher in children with ASD + GI symptoms compared to the ASD without GI symptoms; however, notably, there was a low incidence of GI inflammation as measured by fecal calprotectin, confirming prior studies (5,15).

We recognize the limitations of our study, including a small sample size and lack of a standardized diet or itemized evaluation of recent oral intake at the time of evaluation. Additionally, serum dectin-1 is not specific for *Candida* spp.; and fecal calprotectin has widely variable sensitivity for diagnosing small bowel Crohn disease (42–100%) and colonic Crohn (66–100%) (reviewed in (15)).

Conclusion

In this pilot study, we were unable to identify major differences in the number of fungi or the fungal composition in children with ASD, and the children did not show evidence of increased gut inflammation or anti-fungal antibodies. Our findings raise concerns about long-term anti-fungal treatment for children with ASD. Prolonged anti-fungal therapy is not benign, with potentially toxic effects including compromise of liver function, diarrheal symptoms, and increased risk for anti-fungal resistance. Further studies are needed to substantiate our findings.

* * * * *

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Data sharing statement Five years after enrollment of the final subject in this study, the complete study data files with sufficient data documentation for proper analysis, will be made available to the public on a secure website requiring password identification for access. The final datasets will be stripped of identifiers before release for sharing, to ensure that the confidentiality of all subjects is maintained and that the data

is used only for research. We will also require a data sharing agreement from all who wish to make use of the data, which provides for a commitment to using the data only for research purposes and not to identify any individual participant; using best statistical and ethical practices in analyzing and reporting finding; securing the data using appropriate information technology; crediting the source and the funding agencies of the original project in all publications and presentations; and destroying or returning the data after analyses are completed.

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