Analysis of Yeast and Fungi in Children with ASD vs. Neurotypical Controls

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ABSTRACT

The gastrointestinal (GI) tract is home to a complex and diverse microbial ecosystem that contributes to health or disease in many aspects. While bacterial species are the majority in the GI tract, their cohabitants, fungal species, should not be forgotten. Children with autism spectrum disorder (ASD) often suffer from GI disorders and associated symptoms, implying a role the bacterial and fungal gut microbiota play in maintaining human health. The irregularities in GI symptoms can negatively affect the overall quality of life or even worsen behavioral symptoms the children present. Even with the increase in the availability of next-generation sequencing technologies, the composition and diversities of fungal microbiotas are understudied, especially in the context of ASD. We therefore aimed to investigate the gut mycobiota of 36 neurotypical children and 38 children with ASD. We obtained stool samples from all participants, as well as autism severity and GI symptom scores to help us understand the effect the mycobiome has on these symptoms. By targeting the fungal internal transcribed spacer (ITS) and bacterial 16S rRNA V4 regions, we obtained fungal and bacterial amplicon sequences, from which we investigated the diversities, composition, and potential link between two different ecological clades. From fungal amplicon sequencing results, we observed a significant decrease in the observed fungal OTUs in children with ASD, implying a lack of potentially beneficial fungi in ASD subjects. We performed Bray-Curtis principal coordinates analysis and observed significant differences in fungal microbiota composition between the two groups. Taxonomic analysis showed higher relative abundances of *Candida*, *Pichia, Penicillium*, and *Exophiala* in ASD subjects, yet due to a large dispersion of data, the differences were not statistically significant. Interestingly, we observed a bimodal distribution of *Candida* abundances within children with ASD. *Candida's* relative abundance was not significantly correlated with GI scores, but children with high *Candida* relative abundances presented significantly higher Autism Treatment Evaluation Checklist (ATEC) scores, suggesting a role of *Candida* on ASD behavioral symptoms. Regarding the bacterial gut microbiota, we found marginally lower observed OTUs and significantly lower relative abundance of *Prevotella* in the ASD group, which was consistent with previous studies. Taken together, we demonstrated that autism is closely linked with a distinct gut mycobiota, characterized by a loss of fungal and bacterial diversity and an altered fungal and bacterial composition.

Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by impaired social interactions, communication, and stereotyped/repetitive behaviors. In addition to the behavioral abnormalities seen in the disorder, several comorbidities have also been documented. Anxiety, sleep problems, immune disorders, sensory sensitivities, intellectual disabilities, and gastrointestinal symptoms are common comorbidities in individuals with ASD. The causes of ASD are not well-known, but are believed to involve a combination of genetic and environmental factors. The Centers for Disease Control and Prevention (CDC) released new data in 2014, identifying every 1 in 68 children as having ASD [1]. With such a large percentage of children being diagnosed on the spectrum, studies aiming to understand the mechanism of ASD and its comorbidities have gained interest.

Gastrointestinal (GI) disorders are among the most common comorbidities children with ASD face. Recent studies have suggested that children with ASD are more likely to present GI symptoms compared to neurotypical children [2-5]. These GI symptoms have also been linked to greater severity of ASD related symptoms [6]. It can be concluded that GI symptoms decrease the overall quality of life in children with ASD. There are many possible ways that GI disorders can affect ASD symptoms, including pain/discomfort, decreased nutrient absorption, decreased production of vitamins by gut bacteria, decreased production of butyrate for energy for intestinal epithelial cells, intestinal permeability leading to increased absorption of allergens, inflammation leading to altered immune response and neuroinflammation, altered production of neurotransmitters, and toxins produced by bacteria or fungi. The microbiota of the gut in children with autism has been studied and compared to neurotypical children, showing an alteration in the gut microbiota composition in children with ASD [7-12], although the studies are somewhat inconsistent.

In a survey conducted by the Autism Research Institute (ARI) that collected parent ratings of behavioral effects of various biomedical interventions, antifungal medications were among the highest rated. The highly rated effectiveness of antifungal medications further strengthens the importance of understanding the role of fungi within the gut in children with ASD. While there have been several studies focusing on the role gut bacteria plays in GI comorbidities in ASD, there have been few studies focusing on the mycobiome, or fungal composition, of the gut. Culture-dependent studies have been previously used to classify the presence of certain fungi and yeast. A study by Horvath and Perman reported 43% of children

with ASD undergoing endoscopy had positive cultures of yeast in duodenal juice compared to 23% of same aged controls [13]. Another study by Adams et al. observed somewhat higher levels of *Candida albicans* and other yeast by culture in children with autism and neurotypical controls, but the differences between the groups were not significant [14]. Culture-dependent techniques do not provide the robustness and sequencing depth that next generation sequencing (NGS) technologies do. With major improvements in sequencing technology, high throughput surveys of genomes can be conducted within fractions of the traditional method costs and time. NGS has enabled metagenomic studies and has been used to study the gut fungal flora in children with autism by Strati et al. [7]. They observed no significant difference in fungal alpha diversity between ASD and NT groups. However, they observed a significant difference in beta diversity between the two groups and also observed a nonsignificant two-fold increase in the relative abundance of *Candida* in the ASD group [7]. In our study, the gut yeast/fungi composition of 35 children with autism and 37 neurotypical children was investigated. The first goal of this study was to investigate and compare the taxonomic fungal composition and alpha/beta diversity between the two groups. The second goal of this study was to determine if the yeast/fungi abundance correlated with GI symptoms and autism related behavioral symptoms. The third and final goal was to determine if the yeast/fungi diversity and composition had any correlations with the bacterial diversity and composition from the same samples.

Methods

Ethics Statement

The Institutional Review Board (IRB) at Arizona State University approved the study (ASU IRB Protocol #: 1206007979, 1004005109). The study was advertised in Arizona, USA. People who expressed interest in participating were mailed a consent form with a preliminary questionnaire regarding GI severity index scoring and general participation questions. Sample collection kits were mailed to the prospective participants upon receiving a parent/guardian signed informed consent form and completed questionnaires.

Study participants and sample collection

For this study, 36 neurotypical children and 38 children with ASD were recruited. The subjects had an age range of 3-17 years. The Autism Treatment Evaluation Checklist (ATEC), Pervasive Developmental

Disorder Behavior Inventory (PDD-BI), and Autism Diagnostic Observation Schedule (ADOS) were used to assess the children with autism. A GI symptom assessment was done for all subjects using the 6-item GI severity index (6-GSI). Enrolled subjects did not use any antifungal or antibiotic medications within one month prior to their enrollment in this study.

Sample Collection and DNA extraction

Stool samples were collected from each subject and transported to Arizona State University with dry ice. Upon arrival, they were stored at -80ºC until extraction. Microbial genomic DNA was isolated using a PowerSoil DNA extraction kit (Mobio Carlsbad, CA).

Sequencing of the Fungal ITS region and bacterial 16S rRNA region

For fungal sequencing, the extracted DNA was further processed using the ITS Illumina Amplicon Protocol obtained from the Earth Microbiome Project (http://www.earthmicrobiome.org/protocols-and-standards/its/). For each DNA sample, the internal transcribed spacer (ITS) region library was constructed for the Illumina MiSeq platform, using the ITS1f-ITS2 primer set (Forward primer 5'-CTTGGTCATTTAGAGGAAGTAA-3' and Reverse primer 5'-GCTGCGTTCTTCATCGATGC-3'). Samples were amplified in triplicates and pooled. Amplicons were run on an agarose gel to verify the presence of PCR product. Upon verification, amplicons were quantified using the Quant-iT PicoGreen dsDNA assay kit. Amplicons were then pooled into a sterile tube and cleaned using the MoBio UltraClean PCR Clean-Up Kit. Concentrations and A260/280 ratios of the final cleaned pools were taken to verify quality. Aliquots were then sequenced using Illumina MiSeq sequencer. For bacterial sequencing, Illumina MiSeq sequencing technology was used as described in Kang et al. [15]. The 16S rRNA V4 region was targeted using the primer set of 515f-806r. The library construction and bacterial and fungal sequencing were conducted at the Microbiome Analysis Laboratory in the Biodesign Institute of Arizona State University (http://krajmalnik.enviornmentalbiotechnology.org/microbiome-lab.html).

Data Analysis and Statistical tests

Since high-throughput sequencing allows pooling of samples into a single sequencing run, the samples must be demultiplexed. Quantitative Insights Into Microbial Ecology (QIIME1 version 1.6.0) was used to pre-process the fungal demultiplexed samples [16]. Pre-processing involved removing the PCR primers, DNA barcodes, and filtering the sequence reads for low quality nucleotides. A quality filter score of Q20

was used. The preprocessed sequences were then clustered into Operational Taxonomic Units (OTUs). OTUs were clustered by performing open-reference OTU-picking against the UNITE database [17] using BLAST v 2.2.2 [18]. The sequences were clustered at a 97% similarity against the UNITE database, which roughly equates to a genus level taxonomic classification. To eradicate any downstream biases originating from a variance in sequence depths within the samples, the OTU table was rarefied to a depth of 2081 sequences per sample. The core diversity analysis command was used in QIIME to perform alpha and beta diversity tests. Observed OTUs, and Bray-Curtis principal coordinate analysis metrics were chosen to represent the alpha and beta diversities of the samples. QIIME2 (version 2018.2) [19] was used to carry out the bacterial 16S rRNA analysis from the same subjects. The raw single-end sequencing data was converted to a .qza file and imported into QIIME2. A visualization file was created for the raw data and the quality of the sequence reads were visualized to determine an adequate left and right read length. The sequences were denoised to better discriminate between true sequence reads and sequencing errors using DADA2 [20], and truncated from 4-151 base pairs. A phylogenetic tree was constructed to allow investigation of phylogenetically based alpha diversity metrics. Alpha and beta diversity metrics were investigated using the core diversity metrics command and limiting the sampling depth of all the samples to 6492. Next, the taxonomic analysis was carried out using a Naive Bayes classifier trained on Silva 119 99% OTUs from 515F/806R region of sequences. A table with relative abundance of bacteria at the genus level was achieved.

Statistical significance was reported using the rank-based Mann-Whitney U test because the data was not normally distributed. Pearson and Spearman correlation tests were performed using Python (Scipy/Statlib states library).

Results

Subject Characteristics

Stool samples were collected from 41 neurotypical children and 42 children with ASD (termed NT and ASD, respectively). After quality filtering and removing nine samples that were from revisiting subjects, there were a total of 72 stool samples used for analysis: 37 from unique NT subjects and 35 from unique ASD subjects. The neurotypical subjects had an average age (±SD) of 8.4 (± 3.8) and the ASD subjects had an average age of 8.6 (±3.9), with average GI scores of 1.4 (±1.9) and 4.2 (±2.4), respectively. Rank-based Mann-Whitney statistical testing did show a significant difference in the GI scores between

the NT and ASD subjects (3x higher in ASD, 2-tailed $p<0.05$). Table 1 summarizes the subject characteristics used in the gut fungal diversity and composition analysis.

Table 1. Study participant characteristics.

Subject Characteristics	Neurotypical	Autistic
Total subjects (n)	37	35
Male/Female	28/9	32/3
Age (years)	8.4 ± 3.8	8.6 ± 3.9
ATEC	--	66.3 ± 24.7
PDD-BI	--	-53.5 ± 56.0
6-GSI	1.4 ± 1.9	4.2 ± 2.4

Autistic Subjects Present an altered Gut Mycobiome: alpha and beta diversity

We obtained a total of 2,277,416 qualified sequence reads (31,631 ± 58,003 per sample in average). QIIME1 was used to process the fungal ITS sequencing data and further analyze the gut mycobiome of the subjects in this study. Of the 83 stool samples collected, 72 unique, high-quality samples were used to assess the alpha diversity, beta diversity, and taxonomic composition of the gut mycobiome. To assess alpha diversity among the neurotypical and autistic controls, the observed OTUs metric was used. A significantly higher number of observed OTUs were present in the NT group compared to the ASD group (2-tailed Mann-Whitney U test, $p<0.05$); figure 1 A). A median value (±MAD) of OTUs in ASD and NT samples were 250.6 (±138.4) and 514.3 (± 60.6), respectively. Beta diversity was assessed using the Bray-Curtis principal coordinate analysis (PCoA), and a significant difference between the PC1 values of NT and ASD samples was also observed (2-tailed Mann-Whitney U test, $p<0.05$; figure 1 B).

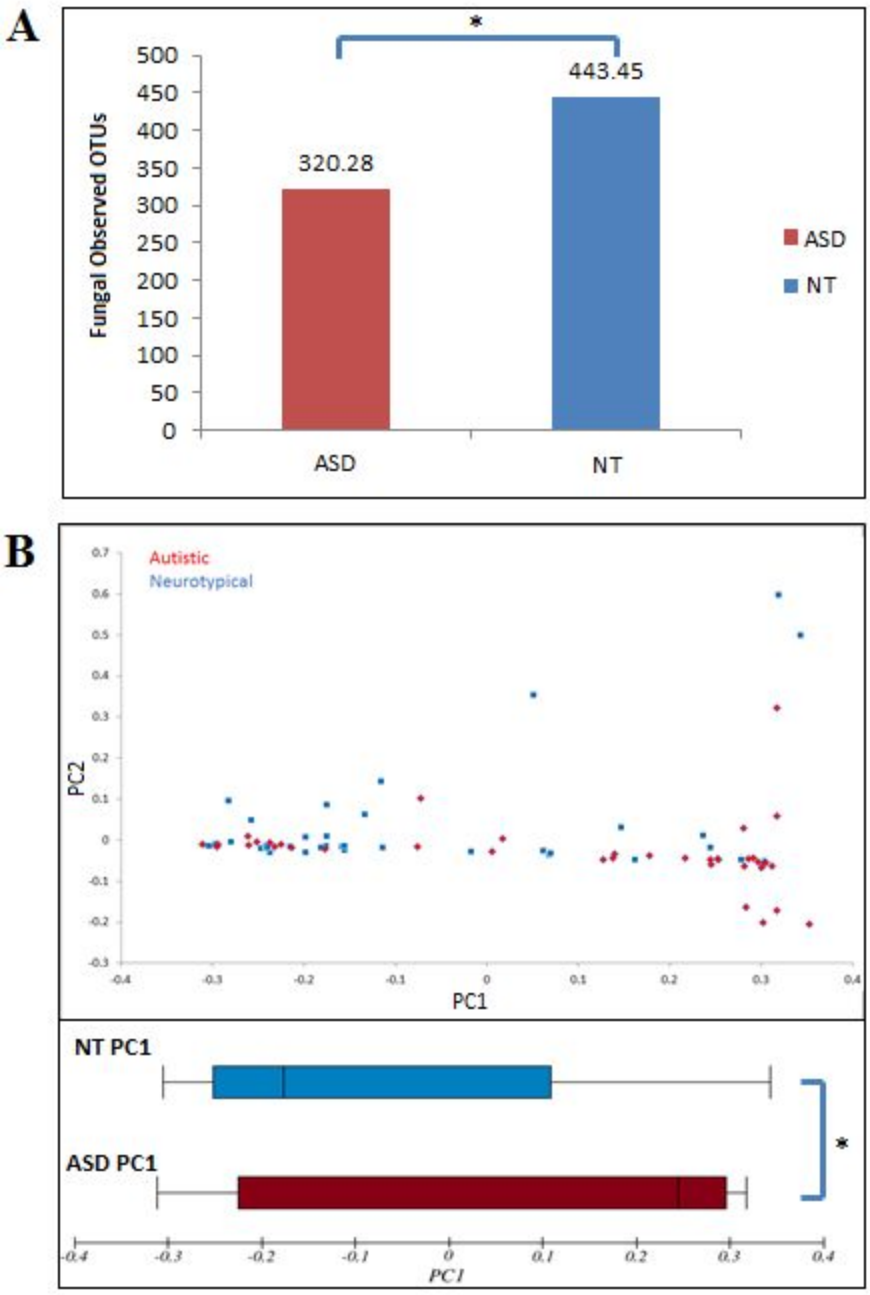

Figure 1. Diversity of gut mycobiota in neurotypical and autistic children.(**A**) Average number of fungal observed OTUs present in ASD and NT samples. (**B**) Bray-Curtis Principal Coordinate Analysis (PCoA) plot showing PC1 vs. PC2 values of each samples in the NT (blue-colored box) and ASD (red-colored box) groups. (*: $p<0.05$ by two-tailed Mann-Whitney U test). For the bottom figure, the lines represent the median, -0.175 and 0.244, top to bottom respectively.

Autistic Subjects Present a Bimodal distribution of Candida abundance

Taxonomic analysis lead to the identification of 68 fungal taxa fully classified to the genus level and 13 partially classified (classified to a level higher than the genus level) fungal taxa. Further classification of the 81 fungal taxa into yeast or fungi revealed the ASD samples had a taxonomic composition of 40.6% non-yeast fungi, 6.9% non-*Candida* yeast, 20.2% *Candida*, and 32.3% unclassified. The NT samples had a taxonomic composition of 34.9% non-yeast fungi, 3.9% non-*Candida* yeast, 8.9% *Candida*, and 52.3% unclassified (figure 2 A). Genus level classification showed *Candida* (20.2% ASD; 8.86% NT), *Pichia* (5.1% ASD; 0.03% NT), *Penicillium* (2.5% ASD; 1.70% NT), *Exophiala* (2.2% ASD; 2.03% NT), *Saccharomyces* (1.74% ASD; 2.88% NT), and *Eurotium* (1.96% ASD; 3.82% NT) as the most abundant genera in our subjects (Table 2). We observed the relative abundance of *Candida* to be roughly twice as much in ASD subjects than in NT, but given a large variance within each cohort, there was no statistical significance between the distributions as a whole (2-tailed Mann-Whitney U test, FDR-corrected $p>0.05$, uncorrected value $p>0.05$). Upon further analysis of the relative abundance of *Candida* among the ASD and NT samples, we observed a bimodal distribution of *Candida* in the ASD cohort (figure 2 B).

Table 2. Top 6 most abundant genera of fungi/yeast observed in ASD and NT samples. Mean, standard deviation (SD), median, and median absolute deviation (MAD) reported from left to right.

	ASD (%) n=35				NT (%) n=37			
Genus	**Mean**	**SD**	**Median**	**MAD**	**Mean**	**SD**	**Median**	**MAD**
Candida	20.229	37.374	0.144	0.118	8.866	20.710	0.252	0.160
Pichia	5.105	20.805	0.003	0.003	0.032	0.092	0.007	0.007
Penicillium	2.504	12.381	0.008	0.008	1.707	7.318	0.022	0.022
Exophiala	2.156	12.631	0.003	0.003	2.030	7.453	0.000	0.000
Eurotium	1.963	11.531	0.003	0.003	3.821	12.725	0.022	0.020
Saccharomyces	1.742	3.493	0.058	0.058	2.886	5.183	1.221	1.219

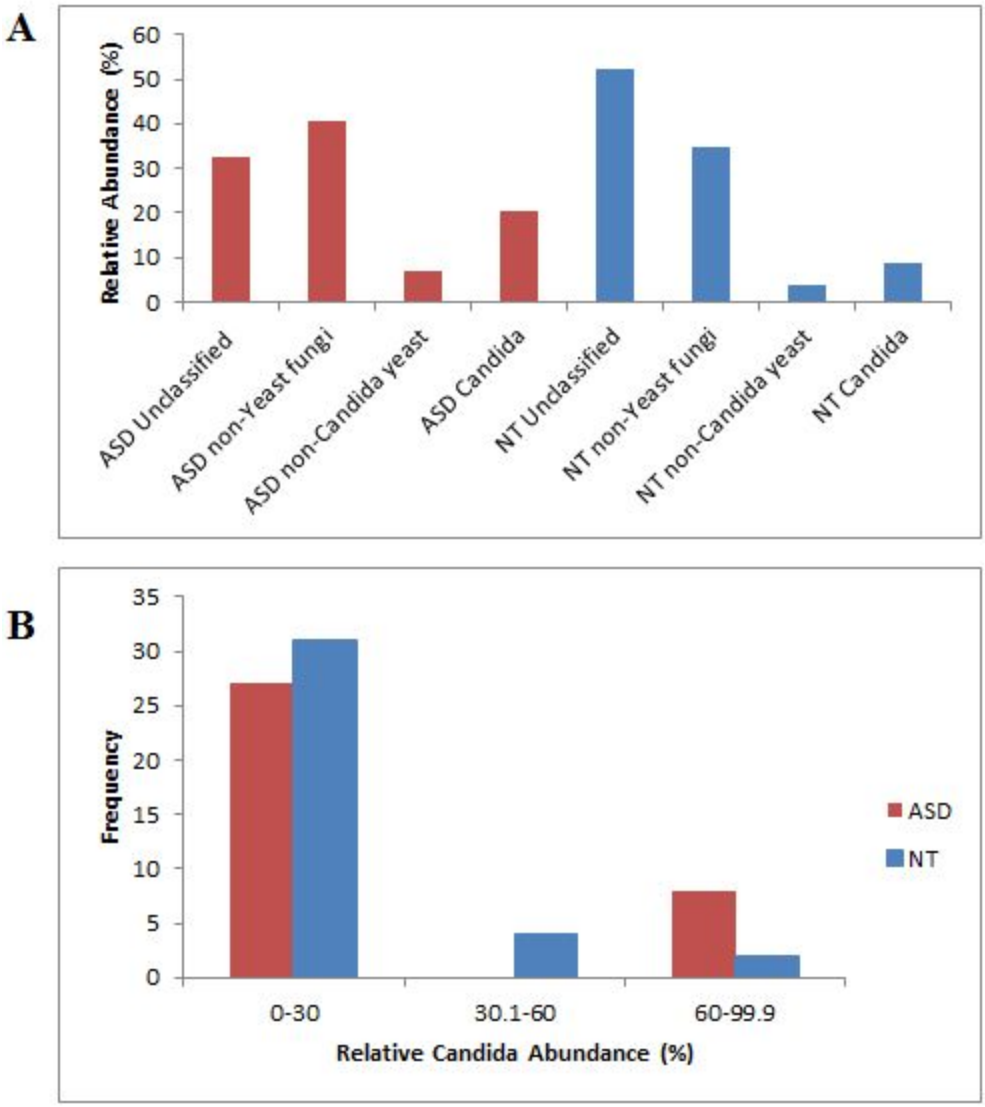

Figure 2. Fungal Taxonomic analysis at the genus level for ASD and NT samples. (**A**) Bar chart showing the composition of fungi, yeast, *Candida*, and unclassified genera in ASD (red) and NT (blue) samples. (**B**) Distribution of *Candida* abundance (%) within the ASD (red) and NT (blue) samples. A bimodal distribution of *Candida* is observed in the ASD samples.

GI symptoms and Autism related Behavioral Symptoms vary with Candida levels in ASD subjects

Gastrointestinal symptoms, primarily chronic constipation and diarrhea (often alternating), are among the most prevalent comorbidities in subjects with ASD [2-5]. Correlational analysis was done to determine if levels of yeast/fungi abundance correlate with 6-item GI severity index scores (GI) and Autism Treatment Evaluation Checklist (ATEC) scores. ATEC scores were correlated against *Candida* levels and a marginally significant positive correlation was observed (Pearson's correlation $r = 0.27$, $p = 0.09$, figure 3

A). GI scores were correlated with *Candida* levels in both ASD and NT groups. No significant correlation was observed between GI scores and the level of *Candida* present in the two cohorts (Spearman's and Pearson's $p>0.05$, figure 3 B and C). However, we did notice a distinct separation within the ASD group based on the relative abundance of *Candida*, such as low (<70%) and high (>70%) *Candida* subgroups (figure 3 B). We compared GI scores between high and low *Candida* groups within ASD subjects, but did not find any significant difference (2-tailed Mann-Whitney U test, $p>0.05$). Intriguingly, we observed subjects with high *Candida* levels (>70%) presented 40% higher ATEC scores (high *Candida*; 84.9 ± 23.9, low *Candida*; 60.6 ± 22.4, 2-tailed Mann-Whitney U test, $p<0.05$, figure 3 D).

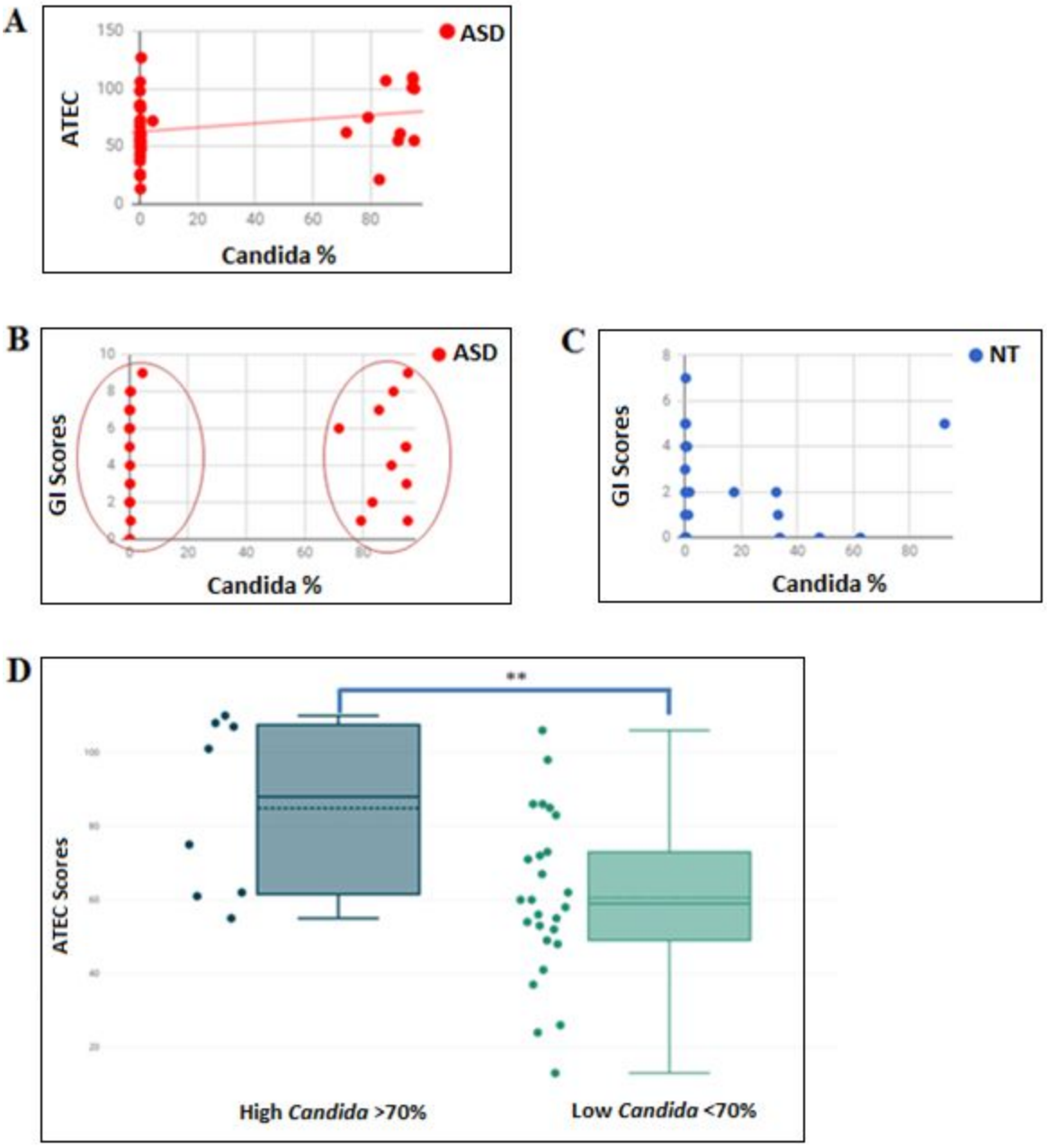

Figure 3. Taxonomic analysis at the genus level between ASD and NT samples. (**A**) Correlation between *Candida* relative abundance (%) and ATEC scores. Marginally significant positive correlation was observed (Pearson's correlation r = 0.27, p = 0.09) (**B**) Correlation between *Candida* relative abundance (%) and GI scores in ASD subjects. A separation, or bimodal distribution in this data was observed. No significant correlation was observed (Spearman's and Pearson's p>0.05) (**C**) Correlation between *Candida* relative abundance (%) and GI scores in NT subjects. No significant correlation was observed (Spearman's and Pearson's p>0.05) **(D)** ATEC scores within ASD and NT groups with high and low *Candida* relative abundances. (**: p<0.05 by two-tailed Mann-Whitney U test)

ASD and NT subjects show no correlation between gut bacterial microbiome and fungal mycobiome

We also performed bacterial 16S rRNA amplicon gene sequencing of the stool samples and investigated whether bacterial diversity and composition were different between NT and ASD groups. Similar to the fungal mycobiome, we also observed marginally higher (1.1x) bacterial alpha diversity in NT group compared with ASD group (one-tailed Mann-Whitney U test, $p=0.09$) (figure 4 A), which is consistent with what we observed in previous studies [8, 15]. We also confirmed that relative abundances of *Prevotella* was significantly lower (3.9x) in ASD group compared with NT group [8] (two-tailed Mann-Whitney U test $p<0.05$, figure 4 B). We, then, investigated any potential correlations between fungal and bacterial diversity and their compositions. Stool samples were collected from 73 subjects, and during bacterial sequencing analysis, 1 sample from the ASD group was screened during quality filtering, leaving a total of 72 samples for further analysis. To allow for an unbiased comparison, filtered out sequences from the fungal analysis were removed from the bacterial analysis and vice versa. Comparisons between fungal and bacterial data were done from 71 samples (34 ASD, 37 NT). Correlational tests were done to the observed OTUs from the bacterial and fungal data. We observed no significant correlation between observed OTUs in both groups from bacterial and fungal data analysis (Spearman's correlation $r = 0.15$, $p>0.05$; Pearson's correlation $r = 0.12$, $p>0.05$; figure 4 C). Furthermore, correlational analysis between the fungal genus *Candida* and the bacterial genus *Prevotella* revealed no significant correlations among ASD and NT subjects (Spearman's correlation $r = -0.03$, $p>0.05$; Pearson's correlation $r = 0.18$, $p>0.05$; figure 4 D).

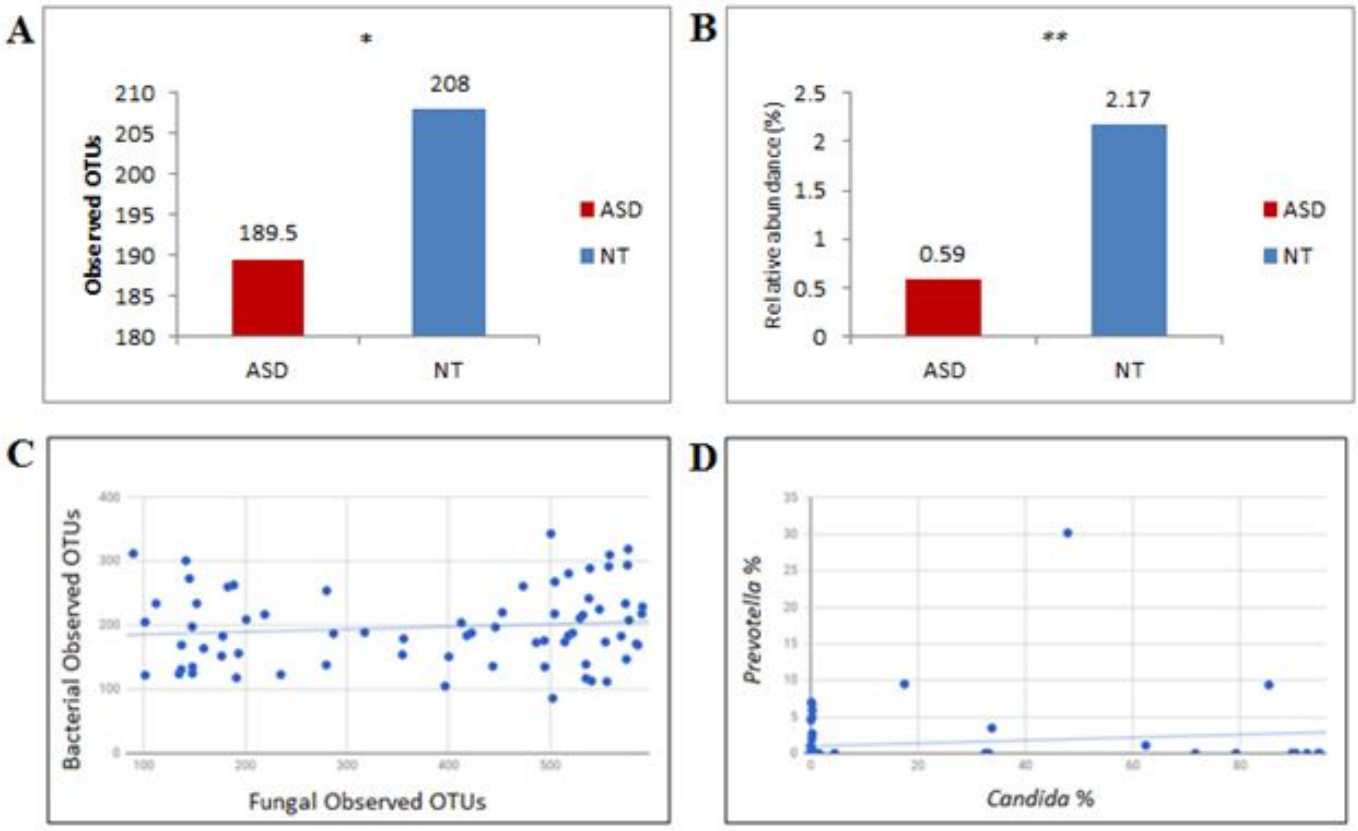

Figure 4. Correlational analysis between bacterial and fungal diversity composition. (A) Average bacterial observed OTUs in ASD and NT groups (*: 1-tailed Mann-Whitney U test p=0.08). **(B)** Relative abundance of *Prevotella* in ASD and NT groups (**: 2-tailed Mann-Whitney U test p<0.05). (**C**) Scatterplot showing fungal and bacterial observed OTUs from ASD+NT groups. No significant correlation was observed (Spearman's correlation r = 0.15, p>0.05; Pearson's correlation r = 0.12, p>0.05). (**D**) *Candida* vs. *Prevotella* relative abundances from ASD+NT groups. No significant correlation was observed (Spearman's correlation r = -0.03, p>0.05; Pearson's correlation r = 0.18, p>0.05).

Discussion

The GI tract is home to crucial bacteria and fungi that help maintain host health. Increased frequency of GI and autism-related behavioral symptoms in children with ASD implies a possible connection between gut microorganisms and autism [21, 22]. With robust sequencing technologies more readily available, thorough analysis of gut microbiota can be conducted with greater accuracy and depth than ever before. A broader understanding of the diversity and composition of microorganisms in a diseased versus healthy human gut can potentially serve as a biomarker in targeting gut dysbiosis or even pathogenic species responsible for these observed comorbidities. This understanding would not only aid in the diagnosis of

autism, it can also benefit the development of personalized treatments for autism-related behavioral and GI symptoms.

In this study, children with ASD presented a significantly less diverse gut fungal microbiota, when considering the number of observed OTUs. While not many studies have investigated gut fungal diversity in diseased states, lower bacterial diversity has been observed in various diseased states such as inflammatory bowel syndrome (IBS) [23], obesity [24], types 1 and 2 diabetes [25][26], and ASD. Higher gut bacterial diversity in healthy states may imply that a more diverse gut microbiota can help protect against environmental perturbations. The dysbiosis, characterized by the loss of fungal diversity in children with ASD may often result in alterations in structural and functional profiles of the gut microbiota that can be responsible for the comorbidities observed. Less diverse gut microbiota in diseased states can also be a result of a pathogenic microorganism being introduced to the gut and potentially outcompeting more beneficial counterparts. Identification of such dysbiosis in gut microbiota could potentially help clinicians with earlier detection of ASD or ASD related symptoms in children and even potentially help target pathogenic species.

Taxonomic analysis in this study revealed *Candida* as one of the most abundant fungal genus in the gut mycobiota, presenting at higher abundances in children with ASD than in neurotypical children. While traditional culture-dependent studies have shown the prevalence of *Candida* in children with ASD [14] and other disease states such as IBS [27] and Crohn's Disease [28], only one study has shown similar observations of elevated *Candida* in ASD groups using NGS sequencing technologies [7]. *Candida* has gained attention since its role has been recognized as a versatile and opportunistic pathogen. Studies from animal models have shown that *Candida* colonization can inhibit the healing of inflammatory lesions and that inflammation can even promote colonization [28]. The dysbiosis, or overgrowth, of *Candida* may be partly responsible for GI symptoms that children with ASD experience, although we did not observe any difference in GI severity between the group with and without high levels of *Candida*. However, we observed a bimodal distribution of *Candida* in children with ASD. Relative abundances of *Candida* were bimodally distributed, with most children with ASD having low levels, but 23% having highly elevated levels of the relative abundance of *Candida*, versus 2.7% of the NT group. These results are similar to two previous culture studies, which found a higher abundance of yeast in children with ASD, although the difference was not significant. The high levels of yeast in a small subset of the children could be due

to multiple factors, including, diets or antibiotic use. In this study we only included subjects who had not taken any antibiotics one month prior to stool sample collections. While a long-term effect of antibiotic use has not been adequately addressed in previous studies, studies focusing on the effects of antibiotics on the human gut microbiota have shown that taxonomic composition and diversities were restored in roughly 4 weeks after taking antibiotics [29]. The possibility of long-term effects of antibiotics on gut microbiota should not be ignored. Significantly less fungal diversity was observed in the ASD group with elevated (greater than 70% relative abundance) *Candida*, implying a detrimental role of *Candida*.

Despite a growing interest in fungal sequencing analysis, molecular techniques and associated bioinformatics pipelines are not well defined. We employed next-generation sequencing technologies to target the ITS region, but the lack of a 'golden-standard' in fungal ITS sequencing data processing presents a great barrier when designing a study and comparing data between studies. Another limitation includes a lack of a standard reference database for gut fungal ITS sequencing. The reference database only captures a finite number of sequences and if a particular sequence is not included in the database, it will remain unclassified. In fact, we observed that a large portion of sequences were unclassified, possibly due to the limitations of our reference database. Further investigation by testing different reference databases is warranted. Another limitation that was previously mentioned stems from the long-term effects of antibiotic use. More detailed data from study participants on antibiotic use can help determine if antibiotic use led to an increase in commensal microbe abundance. Lastly, the presence of fungal and bacterial genera was measured in terms of relative abundance. Measuring absolute abundance could provide more accurate information as to the levels of certain genera present in the groups.
A limitation of this study is that it has focused on measurements of relative abundance. We recommend that future studies also investigate the quantitative level of yeast, as we suspect that children with ASD may also have higher absolute levels of yeast, and quantitative levels of yeast may also be strong associated with ASD severity.

In summary, we demonstrated that individuals with ASD have a distinct gut mycobiota, characterized by a loss of diversity, and an altered composition of the fungal community. We also noticed a bimodal distribution of *Candida* abundance in the ASD group, and those children with high relative abundances of *Candida* had higher ATEC scores, a measure of autism severity. Interestingly, the abundance of *Candida* was more closely linked to behavioral symptoms than GI symptoms. Consistent with previous studies, we

additionally observed marginally lower gut bacterial diversity in ASD groups, and a significantly higher abundance of *Prevotella* in neurotypical children. While we were limited with detailed data on antibiotic use, diet, or supplement/probiotic use from our cohorts, findings in this study solidifies a distinct gut fungal flora in children with ASD. Future studies should include clinical trials of antifungals in children with ASD who have elevated levels of Candida and other yeast.

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