

RESEARCH ARTICLE

Attention Deficit Hyperactivity Disorder (ADHD) and the gut microbiome: An ecological perspective

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Data Availability Statement: The dataset is publicly available in the NCBI database (Accession Number: PRJNA656791). Our entire downstream analysis pipeline has been built using Plugin-Based Microbiome Analysis (PluMA, (76)) and is available for download within its publicly available pipeline pool (<http://biorg.cs.fiu.edu/pluma/pipelines.html>).

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Abstract

Attention Deficit Hyperactivity Disorder (ADHD) is an increasingly prevalent neuropsychiatric disorder characterized by hyperactivity, inattention, and impulsivity. Symptoms emerge from underlying deficiencies in neurocircuitry, and recent research has suggested a role played by the gut microbiome. The gut microbiome is an ecosystem of interdependent taxa involved in an exponentially complex web of interactions, plus host gene and reaction pathways, some of which involve neurotransmitters with roles in ADHD neurocircuitry. Studies have analyzed the ADHD gut microbiome using macroscale metrics such as diversity and differential abundance, and have proposed several taxa as elevated or reduced in ADHD compared to Control. Few studies have delved into the complex underlying dynamics ultimately responsible for the emergence of such metrics, leaving a largely incomplete, sometimes contradictory, and ultimately inconclusive picture. We aim to help complete this picture by venturing beyond taxa *abundances* and into taxa *relationships* (i.e. cooperation and competition), using a publicly available gut microbiome dataset (targeted 16S, v3-4 region, qPCR) from an observational, case-control study of 30 Control (15 female, 15 male) and 28 ADHD (15 female, 13 male) undergraduate students. We first perform the same macroscale analyses prevalent in ADHD gut microbiome literature (diversity, differential abundance, and composition) to observe the degree of correspondence, or any new trends. We then estimate two-way ecological relationships by producing Control and ADHD Microbial Co-occurrence Networks (MCNs), using SparCC correlations ($p \leq 0.01$). We perform community detection to find clusters of taxa estimated to mutually cooperate along with their centroids, and centrality calculations to estimate taxa most vital to overall gut ecology. We finally summarize our results, providing conjectures on how they can guide future experiments, some methods for improving our experiments, and general implications for the field.

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Introduction

Attention Deficit Hyperactivity Disorder (ADHD) is a significant mental health problem with a current 3.4% prevalence worldwide [1]. In the United States, ADHD affects one in 10 children (a 43% increase over the last 15 years) [2], and 3–16% of adults [3] with that percentage increasing over the past 20 years. Individuals with ADHD face many practical challenges, including risk for low academic achievement, lower employment status, and incarceration [4]. Symptoms of hyperactivity, impulsivity, and inattention characterize ADHD [5]. Underlying ADHD behavioral symptoms are deficits in the neurocognitive mechanisms of both executive function (EF) and emotional regulation (ER) [6]. EF refers to a set of cognitive control processes, including attention (specifically, one's ability to focus on relevant information while suppressing irrelevant distractors), memory, and motor skills [7]. ER generally ascribes to one's ability to effectively cope with emotionally charged circumstances (both negative and positive). These deficits include and extend beyond prefrontal-striatal networks [8]. As just one example, during attention-based tasks, reduced activation in the right dorsal attention network (including the right dorsolateral prefrontal cortex, the basal ganglia and thalamus, parietal lobe, and precuneus) has been reported [9]. Many medications have been developed to combat the disorder by influencing the underlying neurocircuitry [10].

The pathogenesis of ADHD is thought to be multifactorial, with heritability estimates at roughly 70–90% [11]. These genetic connections suggest some dependency on underlying metabolic reactions, directly or indirectly involving gene products. In the meantime, the new and exciting field of microbiome research has made its way into the mental health domain. Our gut is home to a plethora of bacteria, fungi, and other microbial organisms, whose collective genomes comprise our gut *microbiome*. Studies estimate that the average number of bacterial cells in humans matches or exceeds that of host cells [12, 13]. Each bacterium has unique genetic material that produces different sets of metabolites, which interact with each other and host metabolites downstream [14], creating a complex host-microbiome web of interactions. It has become increasingly important to pay attention to the symbiotic relationship between the gut microbiome and brain development and function, often referred to as the *gut-brain-microbiome axis* [15]. This axis is a bidirectional communication network, providing gut microbiota and metabolites an avenue for influencing brain development and function [16–20].

The fact that individuals with ADHD suffer from gastrointestinal (GI) dysfunction, including childhood digestive difficulties and low-grade inflammation [21] as well as constipation [22, 23], suggests a potential role of the gut microbiome in this disorder. For example, the plasma levels of the cytokine TNF- α were found to be significantly lower in ADHD children compared to healthy controls, and these levels were also found to be negatively correlated with gut microbiome diversity in these same samples [24]. Plasma short-chain fatty acid (SCFA) levels were also found as deficient in ADHD patients (both children and adults, [25]). This is particularly interesting because SCFAs are produced during bacterial fermentation and have been hypothesized to improve neuro-immunoendocrine functionality [26], and speculated to have a mediational role in microbiota-gut-brain crosstalk [27].

It has also been proposed that gut microbiota may affect our neurobiology by directly or indirectly altering the levels of *neurotransmitters*, including dopamine and serotonin (5-HT) [28], which fuel brain regions that mediate cognition and emotion. Although serotonin is also produced in the brain, up to 90% of serotonin is synthesized in the gut [29]. Connections between the gut microbiome and neurotransmitters, EF/ER, and *neuropsychiatric disorders* (NPDs) are already well-established. In rodents, anxiety and social behavior have been linked to the gut microbiome that can be attributed to altered neurotransmission in the hippocampus and amygdala [30]. In humans, associations between microbiome composition and ER have

been shown [20]. It has also been established that the gut microbiome can release dopamine and 5-HT, impacting ER [31, 32]. Connections on the cognitive axis related to EF are less well-established in humans, though some theories are beginning to emerge [33]. In humans, dopamine influences EF [34]. In rodents, the gut microbiome is linked to dopamine [35], and EF-like behavior [36]. The Autism Spectrum Disorder (ASD) [37], which is associated with impaired EF [38], has been linked to the gut microbiome [39]. In animal studies, the gut microbiome has been associated with anxiety-related disorders such as depression [40–45]. People with stress-related diseases have responded positively to probiotics [46, 47]. Connections between the gut microbiome and another neuropsychiatric disorder (NPD) characterized by EF/ER dysfunction such as ADHD would further support the impact of the gut microbiome on EF/ER. It could also help to explain the large amount of symptomatic overlap that exists between ADHD with other NPDs, particularly ASD [48–50], and could even provide differentiating factors [51] to help address the current diagnosis challenges due to this overlap [52], and new potential options for treatment [53].

There are limited studies that implicate the gut microbiome on clinically diagnosed ADHD, and recent efforts have been made to survey and summarize their results [54–57]. Two in particular [55, 56] contained findings from multiple published studies involving ADHD and the gut microbiome. Based on this, we make the following observations about the current state of ADHD and gut microbiome research:

1. Diversity results are contradictory and inconclusive

Table 1 shows that even closely age-matched gut microbiome studies using the same Shannon index [58] to measure alpha-diversity produce contradictory results when comparing Control and ADHD cohorts. With respect to Beta-Diversity in these same studies, some report a difference between Control and ADHD samples while others do not.

2. Results that estimate overall degree of differentiation between ADHD microbiome datasets compared to Control are inconclusive

One ADHD gut microbiome study [59] attempted the unsupervised method non-parametric multi-dimensional scaling (NMDS, [64]), but could not differentiate the two groups. Limited studies have further decomposed ADHD samples by subscale but these focus on diversity and distinguishing taxa, noting Inattention (elevated genus *Dialister* and reduced genus *Phascolarctobacterium* [63]) and Hyperactivity (lower alpha-diversity and elevated genus *Parabacteroides* [59]) properties.

3. Many microbial taxa have been proposed as elevated or reduced in ADHD compared to healthy controls, some contradictory, others mixed depending on taxonomic level, and others inconclusive

Table 2 summarizes these results. Taxa proposed as elevated in Control are colored orange, those proposed as elevated in ADHD samples are colored purple, and those with contradictory results are colored grey with bold text. Each includes corresponding literature citations. Note taxa have been grouped by the next highest taxonomic level (for example, genera *Ruminococcus*, *Coprococcus*, and *Lachnospiraceae* are all of the *Lachnospiraceae* family). Taxa in bold and italics had conflicting results over member taxa, in just italics (i.e., with family *Veillonellaceae*, member genus *Dialister* was reported elevated in Control, member species *V. parvula* was reported elevated in ADHD).

Table 1. Closely age-matched alpha- and beta-diversity results.

Age	Alpha-Diversity (Shannon Index [58])	Beta-Diversity
Mean: 11.9 [59]	Higher in Control	Difference
Mean: 9.3 [60]	Higher in ADHD	No Difference
6–10 [61]	No Change	No Difference
10 and 15 [62]	Higher in ADHD for 15, No Change for 10	No Difference
20.2 [63]	No Change	Difference (depends on metric)

Alpha- and beta-diversity results from ADHD gut microbiome studies of similar age groups.

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Table 2. Taxa proposed as elevated/reduced in ADHD.

Phylum	Class	Order	Family	Genus	Species	
Actinobacteria	Actinobacteria	Bifidobacteriales	Bifidobacteriaceae	Bifidobacterium [65], [66]		
	Coriobacteria	Coriobacteriales	Coriobacteriaceae	Eggerthella [66]		
Fusobacteria	Fusobacteria	Fusobacteriales	Fusobacteriaceae	Fusobacterium [60]		
Firmicutes	Clostridia	Clostridiales [66, 67]	Lachnospiraceae [68]	Ruminococcus	R. gnavus [68]	
			Coprococcus [63]	Coprococcus [63]	C. eutactus [66]	
			Lachnospiraceae [68]	Lachnospiraceae [68]		
			Ruminococcaceae [68]	Ruminococcaceae [68]	Faecalibacterium [61, 68]	F. prausnitzii [68]
			Clostridiaceae	Clostridiaceae	Intestinibacter [63]	
			Catabacteriaceae [59]	Catabacteriaceae [59]		
			Veillonellaceae [68]	Veillonellaceae [68]	Veillonella	V. parvula [68]
			Dialister [61]	Dialister [61]		
			Peptostreptococcaceae [61]	Peptostreptococcaceae [61]		
			Peptococcaceae [61]	Peptococcaceae [61]		
	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus [60]		
		Enterococcaceae [68]	Enterococcus [68]			
Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonadaceae [59, 66]	Porphyromonadaceae [59, 66]		
			Prevotellaceae [59]	Prevotellaceae [59]	Prevotella [59, 63]	
			Paraprevotella	Paraprevotella	P. xylaniphila [68]	
			Odoribacteriaceae [68]	Odoribacteriaceae [68]	Odoribacter [68]	O. splanchnicus [68]
			Rikenellaceae [66]	Rikenellaceae [66]		
			Bacteroidaceae [59]	Bacteroidaceae [59]	Bacteroides [59, 65]	B. uniformis [60]
			B. ovatus [60]	B. ovatus [60]		
B. caccae [68]	B. caccae [68]					
B. coprocola [60]	B. coprocola [60]					
		Parabacteroides [59]				
Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae [59]	Neisseria [59]		
		Burkholderiales	Sutterellaceae	Sutterella	S. stercoricanis [60]	
			Alcaligenaceae [61]			
	Deltaproteobacteria	Desulfobacteriales	Desulfobacteriaceae	Desulfobacter [67]		
	Gammaproteobacteria	Pseudomonadales	Moraxellaceae [61]			
	Xanthomonadales	Xanthomonadaceae [61]				

Taxa that have been proposed as elevated in Control (orange) and ADHD (purple). Taxa labelled grey with bold text had conflicting results over multiple studies. Taxa in bold and italics had mixed results over member sub-classifications (in normal italics).

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Table 2 shows several taxa families with mixed results over member genera and species, including *Bacteroidaceae*, *Prevotellaceae*, and *Veillonellaceae* (these mixed results were actually within the same study). Conflicting results were reported for the following taxa:

Order clostridiales (Phylum: Firmicutes). This order was reported as reduced in ADHD samples compared to Controls in a study with average ages of 19.5 and 27.1 in the respective sample sets [66], but a later Genome-Wide Association (GWAS, [69]) study found Clostridiales as elevated in ADHD samples compared to Controls [67].

Family Porphyromonadaceae (Phylum: Bacteroidetes). The same study (with average ages of 19.5 and 27.1 [66]) reported elevated *Porphyromonadaceae* in ADHD samples compared to Controls, but a second gender-matched study [59] with similar mean respective ages (11.9 and 13.7) reported taxa of this family as reduced in ADHD samples compared to Controls.

Family Bacteroides (Phylum: Bacteroidetes). The same study (mean ages 11.9 and 13.7, [59]) found members of this genus as elevated in ADHD samples compared to Controls. Another involving 18 to 24 month olds [65] found members of this genus as lower in ADHD samples compared to Controls (though it should be noted, these children did undergo probiotic intervention).

Genus Bifidobacterium (Phylum: Actinobacteria). Perhaps no greater mystery currently exists than the role of genus *Bifidobacterium*. One Dutch study found a nominal increase in *Bifidobacterium* in ADHD with average ADHD and Control subject ages of 19.5 and 27.1 years, respectively [66]. A longitudinal study (3 months, six months and 13 years) made a somewhat contradictory observation of reduced *Bifidobacterium* in ADHD samples during infancy, but not at age 13 [65]. A third study [70] reported reduced *Bifidobacterium* (specifically species *B. longum* and *B. adolescentis*) in ADHD children (mean age: 9.3) that actually reversed after micro-nutrient treatment, where elevated *Bifidobacterium* was observed at high ADHD-Rating Scale IV (ADHD-RS-IV, [71]) scores.

The current picture of the role played by the gut microbiome in ADHD is therefore still unclear. Most of the effort to connect ADHD to the gut microbiome has involved (1) macro-scale population metrics such as diversity, and/or (2) taxa abundances. These properties are in reality emergent from a complex and interdependent interaction web involving taxa, their gene products, and those of the host [72]. Diversity and abundance therefore ignore many underlying details behind their measurements, helping to explain the current incomplete picture. Venturing deeper into this web is critical to completing more of this picture. Two studies have attempted this task, both using multi-omics. One [68] reported differences ADHD neurotransmitter pathways. A second [66] uncovered a connection between *Bifidobacterium* and cyclohexadienyl dehydratase (CDT) abundances.

We have thus far only scratched the surface of this large and exponentially complex interaction web, and every completed piece has value. Multi-omics will continue to be critical, bridging an important gap between taxa, products, and metabolic reactions. We aim to complete another piece, that involves *ecological relationships* between taxa. Microbial taxa have been shown to demonstrate a wide variety of ecological relationships, including cooperation [73, 74] and competition [75], that ultimately impact collective functionality of the ecosystem and macroscale properties [72]. We estimate these relationships for Control and ADHD datasets and report results; including relationships, communities, driver taxa (or 'centroids') of these communities, and taxa central to overall gut ecology. Results can offer guidance on potential taxa to target for further multi-omics or laboratory experiments. The ultimate goal is to increase depth of knowledge about connections between the influence of the gut microbiome on an NPD that impacts millions of individuals worldwide.

This work involves two parts, conducted on a publicly available, gender-matched dataset of 16S gut microbiome sequences. The first involves performing the same macroscale analyses currently prevalent in ADHD gut microbiome literature, to note how this dataset compares, as well as any new and interesting trends. Metrics will include alpha- and beta-diversity, Sparse Partial Least Squares Discriminant Analysis (sPLS-DA, [76]) to estimate Control and ADHD differentiation degree, various methods for differential abundance analysis [77–80] and Qiime [81] normalized abundance compositional profiles.

In the second part we estimate ecological relationships [82] within Control and ADHD gut microbiomes. We first use Microbial Co-occurrence Networks (MCNs, [83]) to estimate these relationships [84], and then perform cluster analysis using the Affinity Propagation (AP, [85]) algorithm to discover communities of mutually supporting taxa, as well as driver or ‘centroid’ nodes of these communities. Finally we perform centrality analysis using the Ablatio Triadum (ATria, [86]) algorithm, to estimate taxa most significant to the overall ecosystem.

Materials and methods

We provide more details on the methods we use for analysis. Our entire downstream analysis pipeline has been built using Plugin-Based Microbiome Analysis (PluMA, [87]) version 2.0 and is available for download within its publicly available pipeline pool, (pipeline ADHD, available open-source at <http://biorg.cs.fiu.edu/pluma/pipelines.html>), along with processed sequence data. Source code for PluMA is available open source under the MIT software License at <http://biorg.cs.fiu.edu/pluma>. Supplementary material, including all alpha- and beta-diversity output, taxa plots, differential abundance output, networks, clusters, and central nodes are available in computer-readable TXT and CSV formats at <http://biorg.cs.fiu.edu/pluma/ADHD.html>.

Cohort

Our study starts from a publicly available dataset (Accession Number: PRJNA656791) of raw gut microbiome samples (observational, case-control) from an undergraduate student population. Members of this population completed the Adult ADHD Self Report Scale (ASRS) and were classified as “Control” or “ADHD” following the published practice [88] of scoring at least 17 on one of the two subscales (inattention or hyperactivity). A subset of patients were then randomly selected from each group (32 Control, 29 ADHD) for gut microbiome sampling. Full sequencing details are provided in the BioProject description; 16S rRNA (V3-V4 region) sequencing was used, following steps corresponding to standard Illumina protocols [89]. We include details of the experiment that we were able to gather from both the BioProject and its referenced protocols [90–92] using STORMS [93] format in [S1 Table](#). Each deidentified sample provides gender (NCIT:C17357, self-reported) and ADHD assessment (EFO:0007860) based on ASRS score (Control, ADHD Combined, ADHD Inattentive, or ADHD Hyperactivity) in its title. The project released 61 samples, but recommended excluding 3 based on outlier analysis (Mahalanobis distance, confidence level = 97.5%) leaving 58 samples: 30 Control and 28 ADHD, with 15 females in both groups. We summarize statistics in [Table 3](#). Tetachoric correlation [94] revealed little to no association (-0.05) between gender and group classification (Control or ADHD).

The study used ASRS as its method for case-control classification, and much of our analyses apply protocols which assume discrete classification schemes for samples. It is important to note however, that while the ASRS can be a useful screening tool for ADHD [95] and has been shown to achieve accuracy rates above 90% [96], it in the end does not offer an official diagnosis. Therefore, an immediate next step must involve a transition to continuous analysis

Table 3. Cohort analysis.

Groups	Total No	Gender	Total No	Inattentiveness	Hyperactivity	Combined
				ASRS Scores \pm SD		
Control	30	Female	15	11.2 \pm 4.3	9.6 \pm 4.9	20.8 \pm 7.2
		Male	15	9.8 \pm 3.4	9.9 \pm 4.4	19.7 \pm 6.8
ADHD	28	Female	15	22.6 \pm 6.8	19.5 \pm 5.3	42.1 \pm 10.1
		Male	13	18.2 \pm 3.8	17.3 \pm 4.7	35.5 \pm 6.1

Cohort analysis. Samples are from an undergraduate student population.

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protocols, operating on the ASRS scores (and possibly subscales as well) as purely continuous data. We present these results as preliminary work, with the expectation of an immediate segue into this continuous analysis as future work. We elaborate on these points in the discussion.

Sequences were trimmed using Trimmomatic-0.33 [97] then assembled into a complete amplicon sequence using SeqPrep [98]. Chimeric amplicon sequences were removed using USearch 6.1 [99] in reference mode against a curated RDP database [100], along with all sequences with length under 320bp. Finally for each sample, a set of 50000 sequences were randomly sampled to reduce uneven sampling bias. To establish an initial set of Operational Taxonomic Units (OTUs) we took these sequences and compiled, clustered, and analyzed them using Qiime 1.9.1 [81] open reference clustering (UClust, [99]) with a similarity threshold of 97% (GreenGenes version 13.8 reference database [101]). Each of these packages are available as PluMA plugins. Finally, we removed all singletons. S2 Table shows the average number of sequences retained in each sample after each preprocessing step.

Ethics Statement

As mentioned, we commenced this study from a publicly available dataset that was released in fully deidentified form and no master key was provided, blocking any possible route for tracing a sample back to an individual. IRB consent was therefore verbally waived for our study, by the FIU Office of Research Integrity.

Part I. Traditional macroscale analyses

We first perform macroscale analyses on this ADHD dataset that have been performed on other ADHD datasets, compare and contrast our results with those in the literature, and take note of any new and interesting observations.

Diversity analysis. Alpha- and beta-diversity plots were constructed using Qiime (version 1.9.1). Input was provided in the form of raw abundances, sampled at a depth of 15000. For alpha-diversity, we used default metrics: unique taxa count, Chao1 [102], and PD_whole_tree (phylogenetic diversity), with default parameters. We confirm results using a non-parametric *t*-test (999 Monte Carlo permutations and Bonferroni [103] correction for p-values). For beta-diversity, we plot unweighted and weighted Unifrac distance [104], confirming results through an ADONIS [105] statistical test on their respective distance matrices (999 permutations).

Differential abundance analysis. We conduct this using two methods, that produce different types of classification. The first uses Sparse Partial Least Squares Discriminant Analysis (sPLS-DA, [76]), a sparse version of the Partial Least Squares (PLS, [106]) method (confidence = 95%), as a supervised dimension reduction method for determining differentiation degree between Control and ADHD samples with respect to taxa abundance [107]. We use

centered log-ratio (CLR, [108]) transformed abundances to map to an unbound space and reduce potential compositional effects. The second attempts to uncover specific microbial taxa that are elevated/reduced in Control and ADHD samples. For this, we take a consensus approach [109] using four methods: (1) Linear discriminant analysis Effect Size (LEfSe, [77]) ($p \leq 0.05$, LDA effect size > 2) with Bonferroni [103] correction, (2) DESeq2 [78] with Benjamini-Hochberg [110] False Discovery Rate (FDR) correction ($p \leq 0.05$), ANOVA-like Differential Gene Expression Analysis of Single-Organism and Meta-RNA-Seq (ALDEx2, [79]) ($p \leq 0.05$, also with Benjamini-Hochberg correction), and Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC, [80]) ($p \leq 0.05$, Holm-Bonferroni [111] correction). Taxa were classified at the lowest possible taxonomic level.

Taxa plots. We use Qiime 1.9.1 [81] to generate taxa bar graphs, producing one bar per sample broken down by taxa percentages (this directly corresponds to our normalized data). We produce taxa plots at all levels of the taxonomic tree (from phylum to genus), plus one at the lowest possible level of classification.

Part II: Ecological relationships

Co-occurrence network analysis. We computed correlations based on log-ratio transformed taxa absolute abundances using SparCC [112] ($p \leq 0.01$) using 100 permutations and 5 iterations, and built Microbial Co-occurrence Networks (MCNs, [83]) using taxa as nodes and correlations as edges. MCNs were visualized using Cytoscape (version 3.9.1, [113]) with layout produced by Fruchterman-Reingold [114].

Clustering. MCNs were clustered using Affinity Propagation (AP, [85]) with a damping coefficient of 0.5 and 200 convergence iterations. AP has been shown to operate efficiently and successfully on signed and weighted biological networks without requiring an initial cluster count estimate, and additionally computes the most representative or *centroid* node for each cluster.

Centrality analysis. We use Ablatio Triadium (ATria, [86]) for evaluating the importance, or *centrality*, of taxa in our MCNs. ATria computes centrality for signed and weighted networks through a modified economic payment model [115] that calculates the influence of a node on all other nodes. ATria provides an alternative perspective by considering relationships (not relative abundance) when computing centrality, and unlike LEfSe does not compare sample sets. ATria produces a ranked list of important taxa and runs iteratively; once a taxon is found as central, ATria removes this taxon and its dependencies using social network theory [116]. Then it runs again to produce the next most important taxon, repeating until no edges are left. Taxa not found as important are simply not ranked.

We analyze these ecological relationships at all taxonomic levels starting from phylum. We first observe the upper three levels (phylum, class, and order) for an overview of relationships between consistently abundant taxa and an observation of general trends and behaviors. We then move to the lower three levels (family, genus and lowest possible classification level) which provide a finer level of granularity and enough taxa to perform meaningful community analyses, facilitating a vision of the key players within these dynamics. The lowest possible classification level will attempt to classify at a species level, but because of the lack of variability in the 16S gene at the species level only a limited number of taxa will be classifiable at an appropriate level of confidence. When species-level classification is not possible, the next highest level (genus or family) will be used. To be clear, these networks are based on correlations, which does not imply causation. Results from our analyses should therefore be interpreted as providing guidance and potential targets for future downstream (i.e. pathway or multi-omics) analysis, that ultimately will require lab experimentation for full verification.

Prevalence filtering. Once we venture past macroscale community metrics (alpha- and beta-diversity), we apply a prevalence filter to remove scarce taxa and restrict our analysis to core microbiome members of both groups (Control and ADHD) by adopting a prevalence threshold of 50% [117, 118]. By restricting our analysis to taxa that are not scarce, our sample size (30 and 28, 1.07 control to case ratio) achieves a power size of 88% given a two-sided confidence interval of 95% when comparing specific taxa between Control and ADHD sets (computed using EpiInfo version 7.2.5, STATCALC package), critical especially for differential abundance analysis. Additionally for co-occurrence networks, a prevalence threshold is important for avoiding spurious clusters of correlated scarce taxa that are simply not appearing across most samples, and 50% is estimated to be a moderate to conservative threshold [119]. A survey across multiple correlation algorithms (including SparCC) also found a performance decline (defined as the ratio of true to false positives) once sparsity (the percentage of zeroes in correlated taxa) exceeded 50% [120]. S2 Table shows that this prevalence filter still maintains 91% of sequence counts on average per sample.

With this prevalence filter the final set of taxa will differ slightly for Control and ADHD, as some taxa will meet the prevalence threshold in only one of the two groups. This will create bias in differential abundance calculations, as these taxa will have an artificial zero abundance in one group but not the other. To avoid this bias, we keep taxa that meet the prevalence threshold in one of the two groups in both groups for all differential abundance calculations (SPLS-DA, LefSe, DESeq, ALDEx2 and ANCOM-BC). For all other methods that do not perform differential abundance calculations between the two groups (taxa plots and co-occurrence networks), we use the prevalence filter as defined above.

Results

Part I. Traditional macroscale analyses

Diversity. Qiime [81] alpha- and beta-diversity results produced no conclusive differences between ADHD and Control. Fig 1 shows no Alpha diversity difference within error bars for ADHD using all three metrics: unique taxa count, Chao1 [102], and PD_whole_tree (phylogenetic diversity), which was later confirmed by a non-parametric *t*-test (respective $t = -1.14, -1.17, -0.967$; p -values: 0.2664, 0.246, 0.309). Beta-diversity with unweighted and weighted Unifrac [104] distance also shows no separation (Fig 2), confirmed by p -values computed from an ADONIS [105] statistical test (respective $F = 0.847, 0.581$; p -values = 0.707, 0.671 respectively). This lack of alpha- and beta- diversity differences matches several results from other datasets [61, 62, 66].

Differential abundance. One differential abundance technique involves dimensionality reduction, attempting to determine differentiation degree between datasets, by accounting for

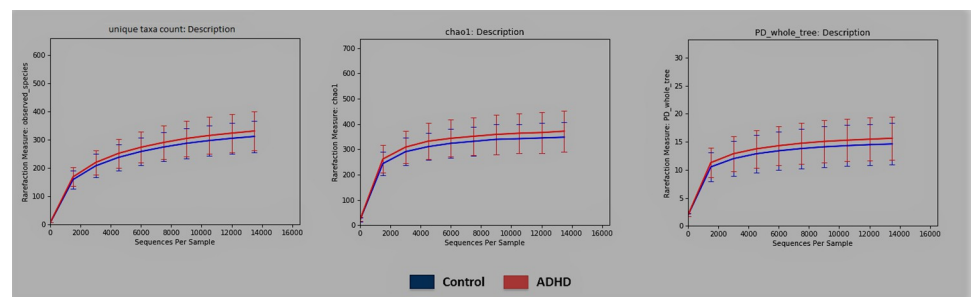


Fig 1. Alpha-diversity. Alpha-diversity of Control (blue) and ADHD (red) samples using (in order) the count of unique taxa, Chao1 richness [102], and phylogenetic diversity, with error bars.

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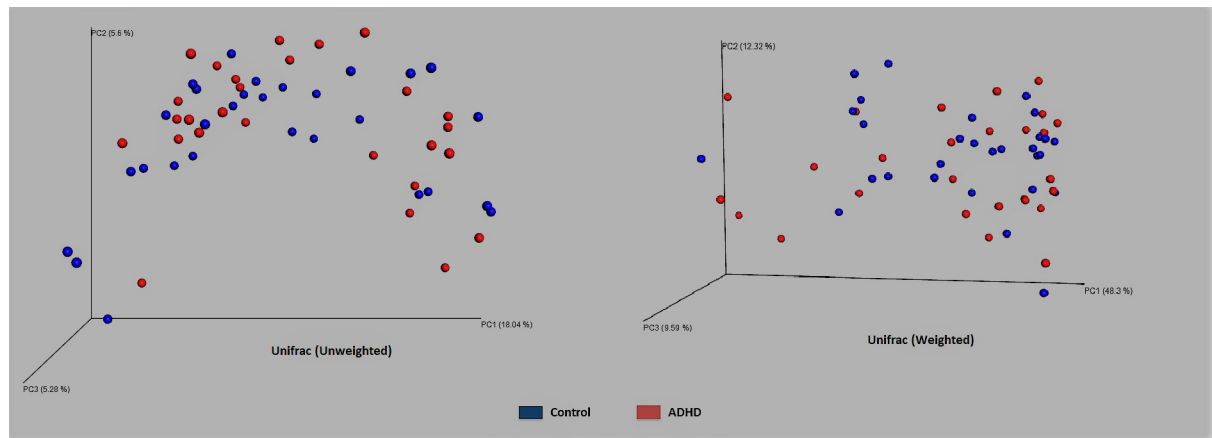


Fig 2. Beta-diversity. Beta-diversity of Control (blue) and ADHD (red) samples computed using unweighted and weighted Unifrac [104] distance.

<https://doi.org/10.1371/journal.pone.0273890.g002>

all variables in each set [121] but displaying in a reduced dimensional space using principal component analysis. Unsupervised and supervised approaches can be used, with supervised having prior sample classification knowledge (i.e., Control or ADHD). When applying a CLR transform to our data in Fig 3 (ellipse confidence level = 95%), we see that even our supervised method Sparse Partial Least Squares Discriminant Analysis (sPLS-DA, [76]) is unable to convincingly differentiate Control vs. ADHD. Table 4 confirms this, as classification error rates of the two principal components for general Control vs. ADHD range from 40 to 50%. This is even with a supervised method, which needs to be taken with a grain of salt anyway, as supervised methods have *a priori* sample category knowledge and have sometimes been shown to differentiate completely random data [107].

Techniques for dimensionality reduction like sPLS-DA estimate the degree of differentiation between two sets (e.g., Control and ADHD). For more targeted future lab studies, it may also be useful to estimate specific taxa that are elevated in one set of samples (e.g., Control) compared to another set of samples (e.g., ADHD). As differential abundance techniques have been shown to vary widely in their results [109], we use four techniques (LEfSe [77], DESeq2 [78], ALDEx2 [79] and ANCOM-BC [80]) with a range of different correction mechanisms for their *p*-values (Bonferroni [103], Benjamini-Hochberg [110], and Holm-Bonferroni [111]) though keep the same *p*-value threshold of 0.05.

We first show results for LEfSe ($p \leq 0.05$, Bonferroni correction) both as a cladogram (Fig 4) and a bar graph (Fig 5). LEfSe has identified orange taxa as elevated for Control, and purple taxa as elevated for ADHD. Taxa produced by LEfSe will be classified at the lowest taxonomic level possible.

The cladogram (Fig 4) shows these taxa on the phylogenetic tree, highlighting those closely related. Fig 4 shows one Bacteroidetes family (*Odoribacteriaceae*) distinguishing ADHD, while Firmicutes (family *Turicibacteriaceae* and its order *Turicibacterales*) distinguishing Control. The bar graph (Fig 5) uses Linear Discriminant Analysis (LDA, [122]) to order by differentiation degree, expanding to include genera and species. ADHD continues to be predominated by Bacteroidetes taxa, again including *Odoribacteriaceae* and its genera *Odoribacter* and *Butyrivimonas*, supporting earlier claims of *Odoribacteriaceae* as ADHD-elevated [68]. *Veillonella* and member *V. dispar* were also reported as elevated in ADHD. Control continues to be predominated by Firmicutes taxa (now including genus *Turicibacter*).

We also run three other methods: DESeq2 [78] and ALDEx2 [79] (both with Benjamini-Hochberg [110] corrected $p \leq 0.05$, and ANCOM-BC [80] with a Holm-Bonferroni corrected

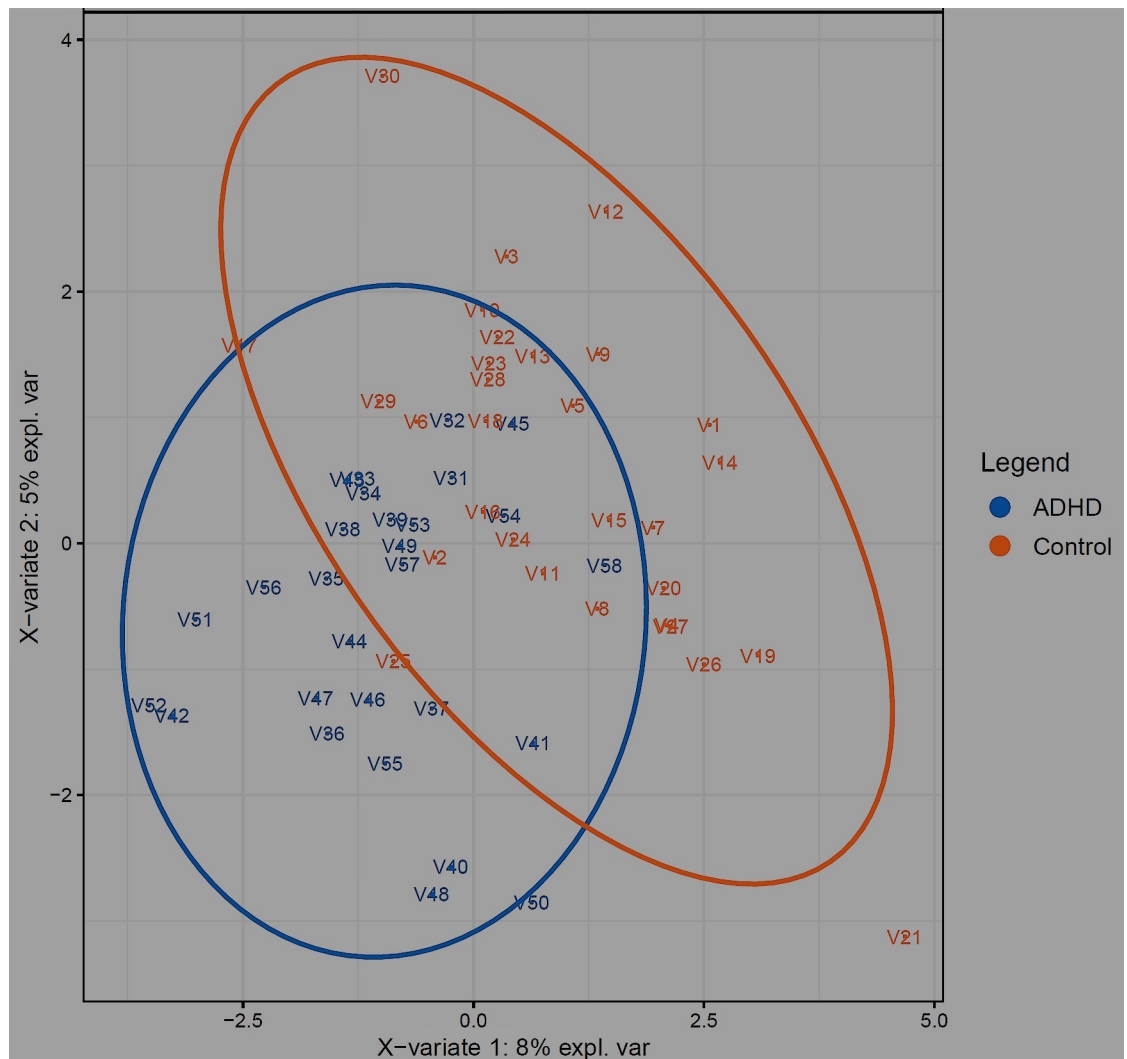


Fig 3. Differential analysis. Results of running sPLS-DA [76] on microbiome abundance data (ellipse confidence level 95%), comparing Control (orange) and ADHD (blue) groups.

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$p \leq 0.05$. Table 5 shows our results. In addition to p -values we include the applicable effect size—Linear Discriminant Analysis (LDA) for LEfSe, Log-Fold Change (LFC) for DESeq2, and Effect Size (EFF) for ALDEx2, computed as the ratio between the group difference and the larger of the two internal group variations. Note ANCOM-BC did not report any differentially abundant taxa. *Turicibacter* and *Butyricimonas* (in **bold**) are the only genera reported more than one of these methods, and *Turicibacter* was actually reported by three (LEfSe, DESeq2 and ALDEx2).

Table 4. Principal component error rates.

Principal Component	Largest Predicted Score	Distance to Centroid (Euclidean)	Distance to Centroid (Mahalanobis)
1	0.476	0.481	0.481
2	0.453	0.457	0.452

Classification error rates of the top two principal components in Fig 3.

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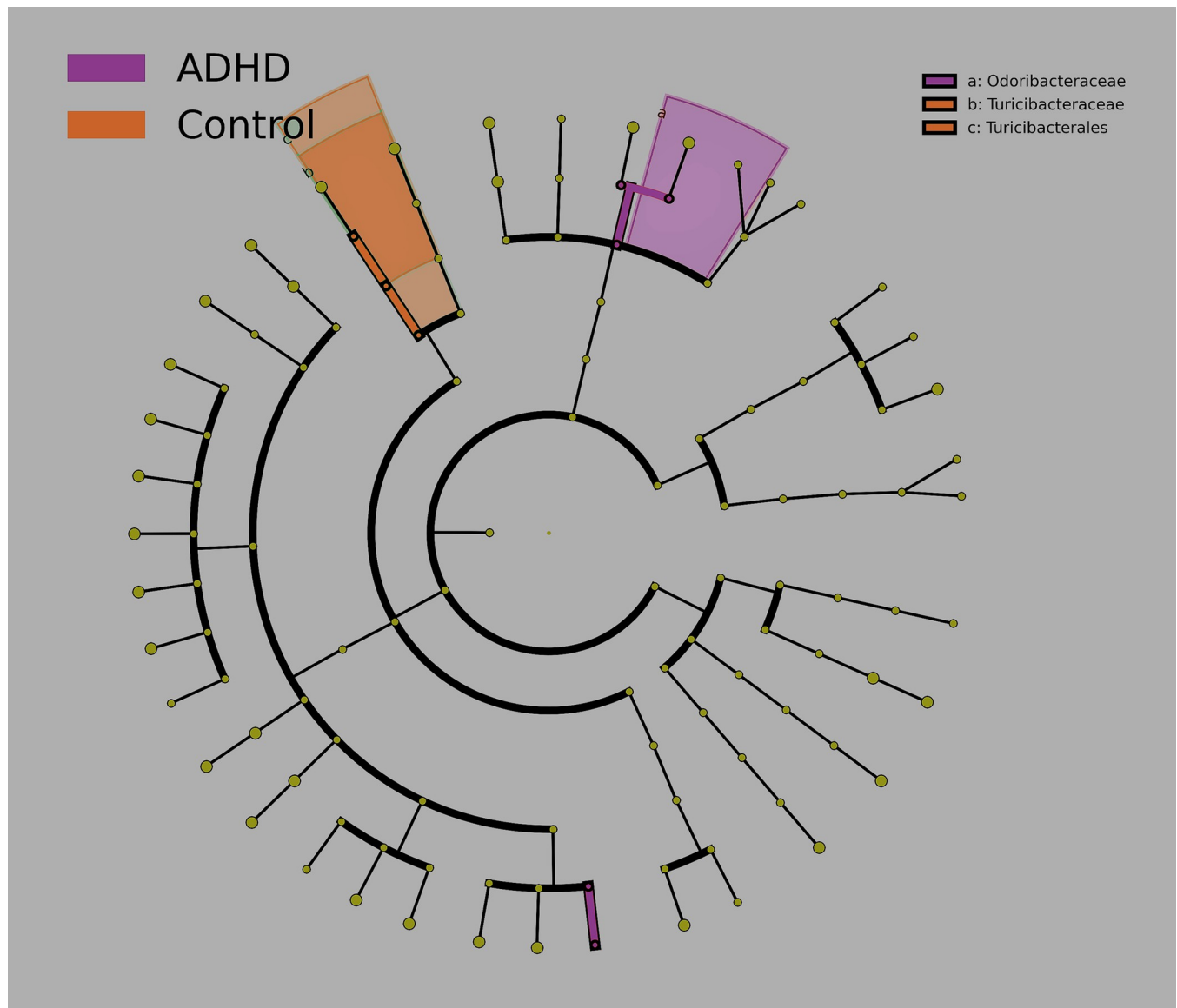


Fig 4. Differential abundance (Cladogram). Taxa reported by LEfSe as elevated for Control (orange) and ADHD (purple) groups, produced by LEfSe [77] ($p \leq 0.05$, Bonferroni correction). LEfSe-reported taxa are plotted on a cladogram, with each concentric circle representing a phylogenetic classification level (innermost = phylum). Shared areas represent distinctive regions of the phylogenetic tree.

<https://doi.org/10.1371/journal.pone.0273890.g004>

Genus *Odoribacter* has also been previously reported as ADHD-elevated [68]. Although species *H. parainfluenzae* has not been observed as Control-elevated in any ADHD gut microbiome studies, its genus (*Haemophilus*) has [63]. This same study [68] found family *Veillonellaceae* as Control-elevated and member species *V. parvula* as differentially abundant in ADHD. Our LEfSe analysis found *Veillonellaceae* members *Veillonella* and *V. dispar* as differentially abundant in ADHD samples. Although *Turicibacter* has never previously reported as an elevated or reduced taxon in any ADHD gut microbiome study, it has been reported in one involving depression in mice [42]. Metabolically in mice, *Turicibacter* signals the gut to produce serotonin (5-HT) [123], which influences ER [124]. Both ADHD and depression are characterized by ER neurocircuitry deficiencies. LEfSe did not report any EF-associated taxa. This may be

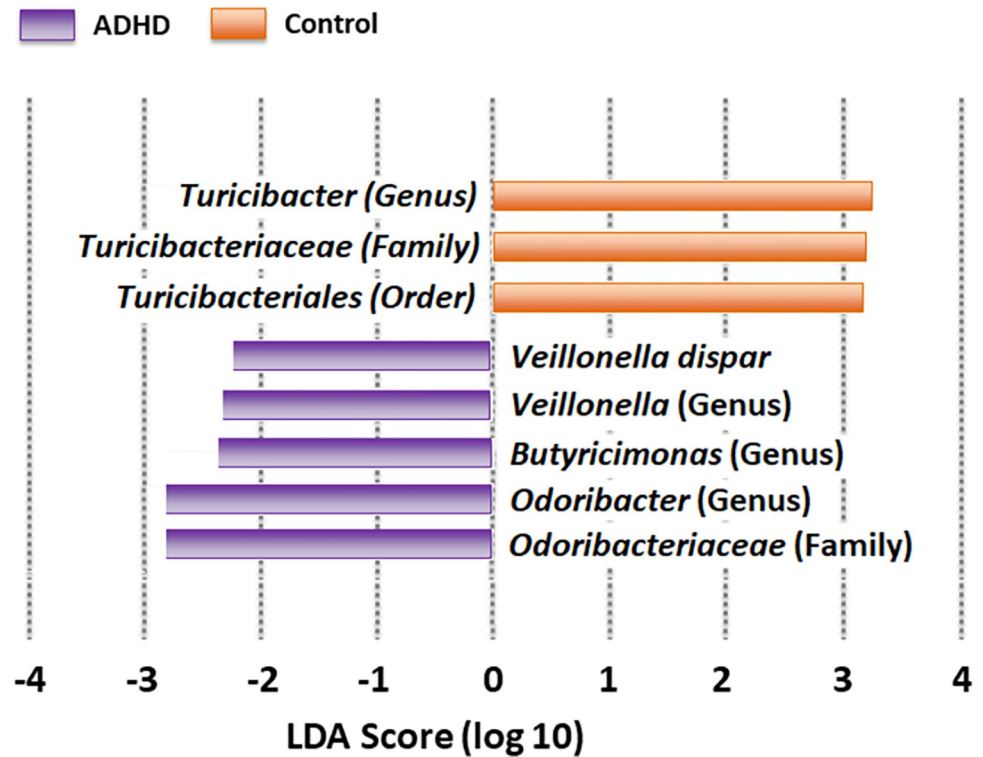


Fig 5. Differential abundance (Bar graph). LEfSe-reported taxa ordered by Linear Discriminant Analysis (LDA, [122]). A higher magnitude indicates more reliable differentiation.

<https://doi.org/10.1371/journal.pone.0273890.g005>

largely because EF is more strongly regulated by dopamine [124], for which the gut only produces roughly 50% [125], compared to 90% of 5-HT [29].

Taxa plots. Taxa bar plots visualize taxa relative abundances [126]. We generated these at all phylogenetic tree levels beginning with phylum (Fig 6). Samples on the x-axis are ordered by increasing ASRS score, and the y-axis represents relative abundance.

A typical gut microbiome profile [127] is observed, dominated by Firmicutes and Bacteroidetes phyla, followed by Actinobacteria and Proteobacteria. Control has slightly elevated

Table 5. Taxa reported by various differential abundance methods throughout our analysis.

Taxonomic Level	Taxon	Algorithm	Control/ADHD	Effect Size and p-value
Family	<i>Clostridiaceae 1</i>	ALDEx2	Control	EFF = 7.90, p = 0.016
Genus	<i>Coprobacillus</i>	DESeq2	ADHD	LFC = 2.00, p = 0.044
Genus	<i>Odoribacter</i>	LEfSe	ADHD	LDA = 2.68, p = 0.027
Genus	<i>Turicibacter</i>	LEfSe	Control	LDA = 3.04, p = 0.028
		DESeq2		LFC = 2.12, p = 0.007
		ALDEx2		EFF = 1.75, p = 0.033
Genus	<i>Haemophilus parainfluenzae</i>	DESeq2	Control	LFC = 0.572, p = 0.042
Genus	<i>Butyricimonas</i>	LEfSe	ADHD	LDA = 2.37, p = 0.019
		ALDEx2		EFF = 3.15, p = 0.032
Lowest Possible	<i>V. dispar</i>	LEfSe	ADHD	LDA = 2.31, p = 0.039

Taxa reported as elevated by various differential abundance methods, when comparing Control vs ADHD, and further broken down by subscale. LDA = Linear Discriminant Analysis effect size (for LEfSe), LFC = Log Fold Change (for DESeq2), EFF = Effect Size (for ALDEx2).

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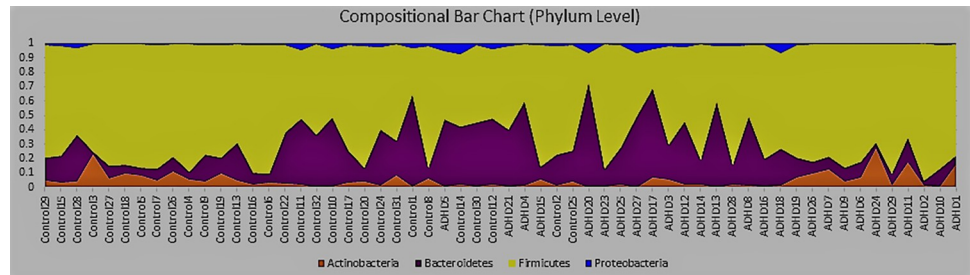


Fig 6. Taxa plots, phylum level. Plots of taxa relative abundance for each subject, generated using Qiime [81], conducted at the phylum level. Subjects are ordered by increasing Adult ADHD Self Report Scale (ASRS) score, with the y-axis representing relative abundance.

<https://doi.org/10.1371/journal.pone.0273890.g006>

Firmicutes (70–66%), mirroring an earlier study [66] that importantly [128] also sequenced the same 16S V3-V4 region. Slightly contrary to this same study, which reported this difference to be largely occupied by an ADHD Actinobacteria increase, ours was mostly occupied by an ADHD Bacteroidetes increase (from 22% to 25%). Yet Actinobacteria remains mysterious in Fig 6, elevated at very high ASRS scores, but also at very low scores. Bacteroidetes and Proteobacteria also appear reduced at these same extremes. These seemingly contradictory results create challenges in drawing meaningful conclusions with respect to role(s) played by these phyla. Yet they capture our interest, especially given the earlier reported anomalous behavior of an Actinobacteria genus, *Bifidobacterium*, at high and low ASRS-IV scores [70].

Class and order levels produced bar charts similar to Fig 6; we include these as S1 and S2 Figs. Levels below order often had too many taxa to clearly view dynamics. We include the genus level (Fig 7), family and species as S3 and S4 Figs, as the genus level includes *Bifidobacterium*. And indeed, it turns out, *Bifidobacterium* (blue, bottom) has elevated abundances high and low ASRS scores, appearing most responsible for this same behavior in its phylum

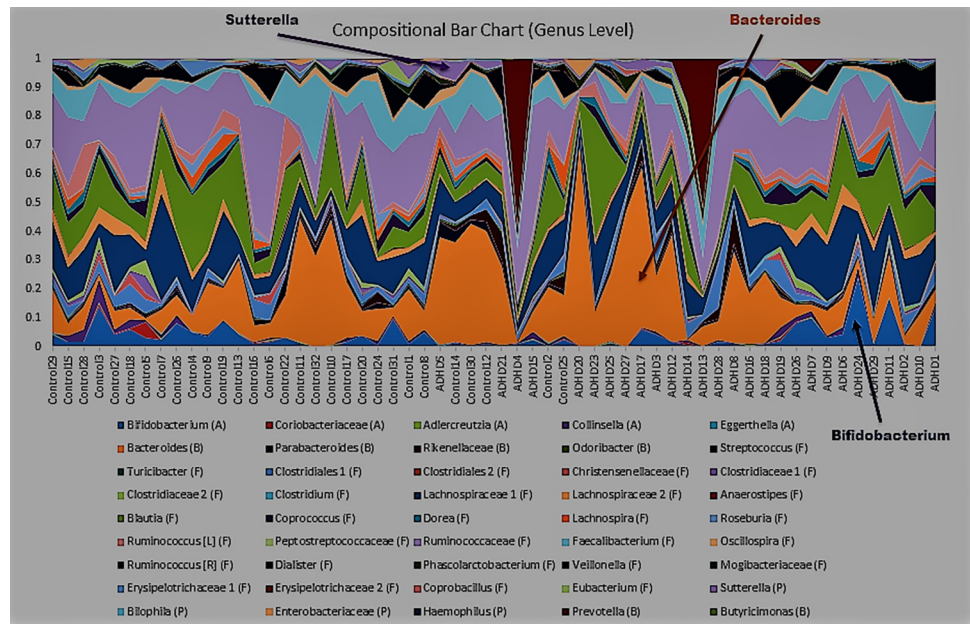


Fig 7. Taxa plots, genus level. Plots of taxa relative abundance for each subject, generated using Qiime [81], conducted at the genus level. Subjects are ordered by increasing Adult ADHD Self Report Scale (ASRS) score, with the y-axis representing relative abundance.

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Actinobacteria (Fig 6). *Bacteroides* (orange, middle) is a highly abundant genus that also mirrors the behavior of its phylum (Bacteroidetes, Fig 6), increasing in the middle and decreasing at extremes. Proteobacteria is more difficult to observe given its low relative abundance (1–2%), though genus *Sutterella* (lilac, top) also appears to follow this trend. These three observations are verified in S5–S7 Figs.

This is not the first time these taxa have generated interest. Many Actinobacteria, and especially *Bifidobacterium*, have been used as probiotics and are considered elements of a healthy gut [129–134]. As discussed, genus *Bacteroides* and its family *Bacteroidaceae*, as well as several member species, have been reported differentially abundant in ADHD [59, 60, 65, 68]; some elevated, others reduced. Some have argued *Bacteroides* to be the most important "window" to understanding the human gut [135]. Species *Sutterella stercoricanis* was also reported as ADHD elevated [60]. These same taxa make multiple appearances in studies involving other NPDs as well. *Bacteroidaceae* was reported as the most differentially abundant LEfSe Major Depressive Disorder (MDD) taxon in one study [45]. Another reported elevated *Bacteroides* and reduced *Bifidobacterium* in anxiety [136]. *Sutterella* is elevated in Autism Spectrum Disorder (ASD) [137], a condition with so much symptomatic overlap with ADHD that an ASD +ADHD phenotype has been established [138].

Discussion. These analyses produced a few interesting preliminary observations, but once again their birds-eye view limited the depth we could pursue. Our taxa plots showed a perfect example: even though there was a visible trend between ASRS score and *Bifidobacterium*, *Bacteroides*, and *Sutterella* abundances, no definitive conclusions could be observed. Fundamentally macroscale behaviors emerge from microscale interactions. We attempt to unlock some of these mysteries by now exploring *ecological relationships*.

Microbial ecological relationships take many forms. They can be positive or negative, mutual (cooperation [73, 74] or competition [75]) or one-way (commensalism [139] or amensalism [140]). In particular, two-way relationships (cooperation and competition) can be approximated using correlations [84]. We use SparCC [112] to compute correlations, which has advantages in reducing compositional effects within relative abundances. We build Microbial Co-occurrence Networks (MCNs, [83]) using taxa as nodes and SparCC correlations as edges, and perform community detection on these networks using the clustering algorithm Affinity Propagation (AP, [85]). Finally, we use Ablatio Triadium (ATria, [86]) as a centrality algorithm to produce a ranked list of important taxa in each MCN. ATria is specifically designed for signed and weighted networks, incorporating both social network [117] and economic theory [116] in its calculations. It is also iterative, removing dependencies of a central node before computing the next most central.

When computing correlations, we use a p -value threshold of 0.01, the generally recommended value for disease studies [141], to increase the confidence of our results, as the historically accepted threshold of 0.05 has come under recent question [142, 143]. Because our study is exploratory and meant to guide future experimentation, we chose to err on the side of false positives as opposed to false negatives, and increase the strength of the threshold as opposed to correcting the p -value. Our p -value threshold of 0.01 produced correlations with magnitude moderate (0.4, [144]) or stronger more than 95% of the time (279 out of 293 total over all MCNs). We therefore report all of these candidate relationships in our MCNs, each of which can be subsequently experimentally verified in future studies.

During our analyses we sometimes use "cooperation" to refer to a positive SparCC correlation and "competition" when referring to a negative. We emphasize, however, that correlations are an *estimate* of ecological relationships, that ultimately require further downstream analysis (multi-omics and experimental verification) before establishing official conclusions. With the underlying web of interactions being exponentially complex and large-scale laboratory

experiments potentially costly, our results can provide guidance regarding target taxa and avenues to pursue.

Part II. Ecological relationships

Upper levels: Phylum, class, and order. Fig 8 shows MCNs at the phylum (Fig 8(A) and 8(B)), class (Fig 8(C) and 8(D)), and order (Fig 8(E) and 8(F)) levels. Taxa (nodes) in all MCNs are colored by phylum (legend at the bottom of Fig 8). Node size is proportional to relative abundance (larger = higher). Correlation (edge) color represents sign; green indicates positive (est. cooperation) and red indicates negative (est. competition). Edge thickness is proportional to correlation magnitude (thicker = stronger). Networks are visualized using the Fruchterman-Reingold algorithm [114], which spatially orients nodes based on edge weight (closer = more positive). Nodes are labeled with their taxon and provided with ATria centrality ranking if found important (format: #rank, T = Tie). At the phylum level only (Fig 8(A) and 8(B)), we label each edge with its correlation value. Phylum-level MCNs (Fig 8(A) and 8(B)) show SparCC appears to handle compositional effects well, as despite collectively encompassing about 95% of both populations, Firmicutes and Bacteroidetes are only weakly negatively correlated.

Table 6 shows every correlation in all three MCNs, and its sign, + (green) or–(red). Correlations that appear only in Control are highlighted orange, only in ADHD highlighted purple, and in both highlighted grey. Correlations at each taxonomic level are grouped by their next highest level classification; for example in row 1: phyla Actinobacteria and Bacteroidetes were negatively correlated in both phylum-level MCNs (Fig 8(A) and 8(B)), member classes Actinobacteria and Bacteroidia were negatively correlated only in Control (Fig 8(C)), as were member orders Bifidobacteriales and Bacteroidales (Fig 8(E)). White, italicized correlations were not present in either MCN, but a correlation among descendants was; for example in row 3: phyla Actinobacteria and Firmicutes were not correlated in either MCN, nor were member classes Actinobacteria and Clostridia, but member orders Bifidobacteriales and Clostridiales were positively correlated in Control (Fig 8(E)).

Table 7 shows collective ATria results, similarly grouped. At each level, taxa found equally important in both MCNs are highlighted grey; taxa found more important in Control light orange, and only important in Control dark orange (analogous case for ADHD and purple). Taxa ranked as first or tied for first in either MCN are **bold**.

Taxa bar plots are mirrored here: ADHD showed elevated Bacteroidetes at the expense of Firmicutes, and these taxa are negatively correlated in both MCNs (Fig 8(A) and 8(B)). But while Firmicutes and Bacteroidetes dominate both populations (largest nodes, Fig 8(A) and 8(B)) as is typical in the gut microbiome [127], SparCC and ATria estimate a far less abundant phylum, Actinobacteria (roughly 4% of both populations), as most important to their overall gut ecology. In both MCNs (Fig 8(A) and 8(B)), phylum Actinobacteria has the strongest negative correlations and ATria ranks it first (Table 7).

We make three more observations at these upper taxonomic levels, that we keep in mind when moving to the lower:

(A) A core *Proteobacteria-Bacteroidetes* positive correlation (est. cooperation) forms. Table 6 shows this, with *Proteobacteria* and *Bacteroidetes* (the only positive correlation in either phylum-level MCN), member classes *Betaproteobacteria* and *Bacteroidia*, and member orders *Burkholderiales* and *Bacteroidiales*.

(B) In Control, taxa in (A) have more negative edges with *Actinobacteria* (est. competition), especially order *Bifidobacteriales*. The highest magnitude negative edges in both phylum-level MCNs (Fig 8(A) and 8(B)) involve *Actinobacteria* with *Proteobacteria* and *Bacteroidetes*. Yet

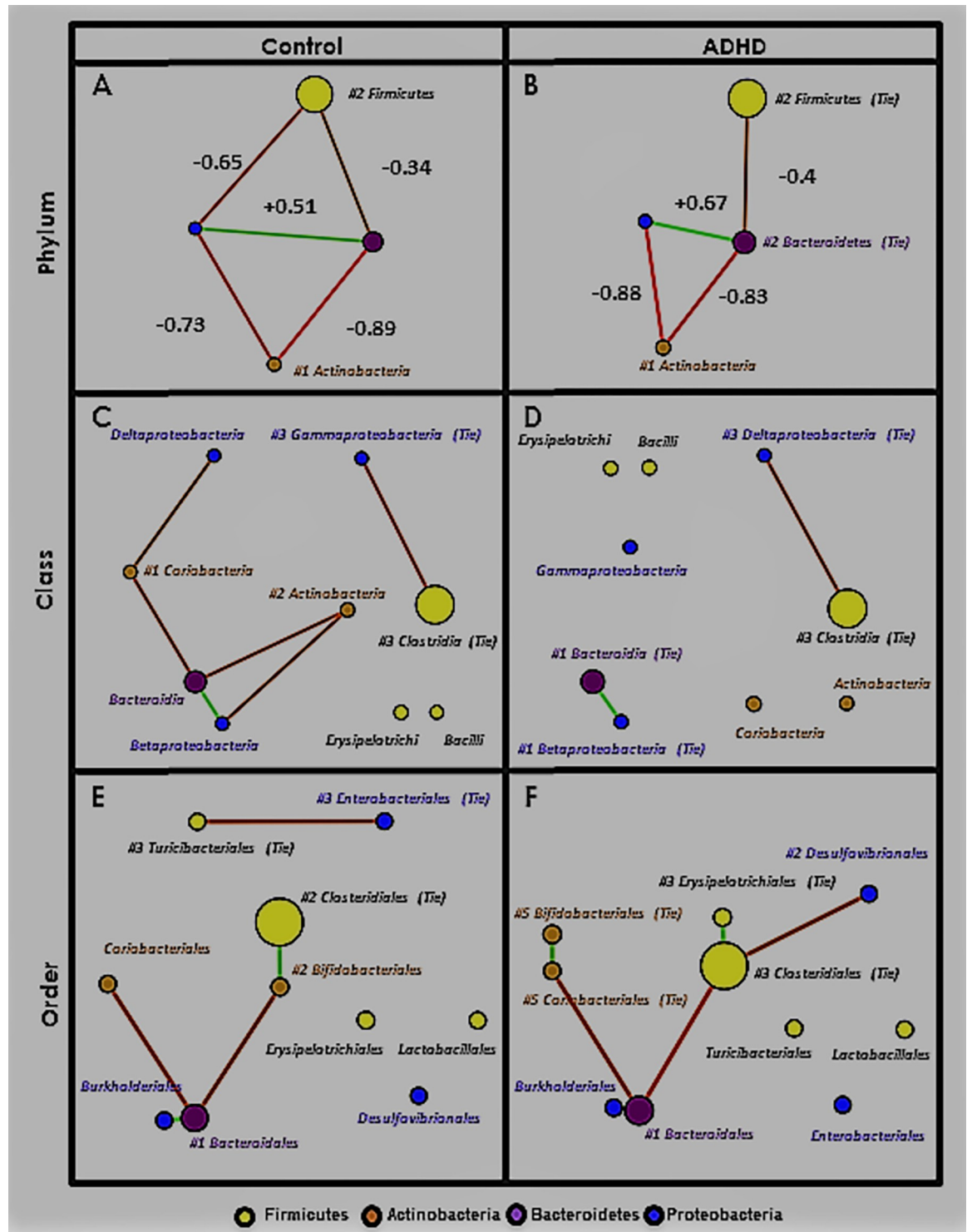


Fig 8. Upper-level Microbial Co-occurrence Networks (MCNs). MCNs at the phylum (A), class (B), and order (C) taxonomic levels, visualized using Cytoscape [57], and oriented by Fruchterman-Reingold [58]. Nodes represent taxa, colored by phylum (yellow = Firmicutes, purple = Bacteroidetes, brown = Actinobacteria, blue = Proteobacteria) with size directionally proportional to abundance. The co-occurrences are distinguished by those that co-habit (green edges) and co-avoid (red edges). SparCC [112] correlation ($p \leq 0.01$) was used as edge weight and also the parameter for Fruchterman-Reingold when determining edge length (larger = closer). SparCC correlations are shown at the phylum level. All taxa found as important by ATria are denoted by a pound sign (#) followed by its rank (ties indicated).

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Table 6. Upper-level taxa correlations.

Phylum		Class		Order	
Actinobacteria-Bacteroidetes	-	Actinobacteria-Bacteroidia	-	Bifidobacteriales-Bacteroidales	-
		Coriobacteria-Bacteroidia	-	Coriobacteriales-Bacteroidales	-
Actinobacteria-Proteobacteria	-	Actinobacteria-Betaproteobacteria	-		
		Coriobacteria-Deltaproteobacteria	-		
Actinobacteria-Firmicutes		Actinobacteria-Clostridia		Bifidobacteriales-Clostridiales	+
Proteobacteria-Firmicutes	-	Gammaproteobacteria-Bacilli		Enterobacteriales-Turicibacteriales	-
		Gammaproteobacteria-Clostridia	-		
		Deltaproteobacteria-Clostridia	-	Desulfovibrionales-Clostridiales	-
Proteobacteria-Bacteroidetes	+	Betaproteobacteria-Bacteroidia	+	Burkholderiales-Bacteroidales	+
Bacteroidetes-Firmicutes	-	Bacteroida-Clostridia		Bacteroidales-Clostridiales	-

Upper level taxa correlations ($p \leq 0.01$), grouped by taxonomic classification. Orange = only found in Control, purple = only found in ADHD, grey = found in both. +(green) = positive correlation, -(red) = negative correlation.

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while the two consistently dependent Actinobacteria classes (Actinobacteria and Coriobacteria) continue this same dynamic with Bacteroidia (Bacteroidetes) and Betaproteobacteria (Proteobacteria) in Control (Fig 8(C) and Table 6), they are completely disconnected in ADHD (Fig 8(D)). Worth noting, this is despite their average relative abundance being nearly the same in Control/ADHD: Coriobacteria 1.5/1.1%, and Actinobacteria 3.2/3.7%. Further, ATria ranks Actinobacteria and Coriobacteria as the top two Control taxa (Table 7). In ADHD, Bacteroidia and Betaproteobacteria are the top two (Table 7), and the MCN shows no negative edges (est. competition) at all involving these taxa (Fig 8(D)).

The order level reveals Bifidobacteriales (Actinobacteria) may be more responsible for this difference than Coriobacteriales (Coriobacteria). While Bifidobacteriales and Coriobacteriales both continue their negative correlations with Bacteroidales (Bacteroidia) in Control, only Coriobacteriales does in ADHD. Table 6 actually shows all edges involving Bifidobacteriales to be exclusive to Control, now including a positive correlation with Clostridiales (the most abundant Firmicute). An increased participation of order Bifidobacteriales thus emerges as a distinguishing feature of Control, which is further supported by ATria (Table 7), which ranks Bifidobacteriales higher (tied for second) in Control, and Coriobacteriales only in ADHD.

Table 7. Upper-level ATria rankings.

Phylum	Class	Order
Actinobacteria (#1/#1)	Actinobacteria (#2/NR)	Bifidobacteriales (#T2/T5)
	Coriobacteria (#1/NR)	Coriobacteriales (NR/#T5)
Bacteroidetes (NR/#T2)	Bacteroidia (NR/#T1)	Bacteroidales (#1/#1)
Firmicutes (#2/#T2)	Bacilli (NR/NR)	Turicibacteriales (#T3/NR)
	Clostridia (#T3/#T3)	Clostridiales (#T2/#T3)
	Erysipelotrichia (NR/NR)	Erysipelotrichiales (NR/#T3)
Proteobacteria (NR/NR)	Betaproteobacteria (NR/#T1)	
	Deltaproteobacteria (NR/#T3)	Desulfovibrionales (NR/#2)
	Gammaproteobacteria (#T3/NR)	Enterobacteriales (#T3/NR)

Upper-level ATria results, grouped by taxonomic classification. Dark orange = only ranked in Control, dark purple = only ranked in ADHD, light orange = higher ranked in Control, light purple = higher ranked in ADHD, grey = evenly ranked. **Bold** taxa are ranked #1.

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(C) *A shift in Firmicutes-Proteobacteria dynamics.* This begins immediately at the phylum level (Fig 8(A)) with Control having a negative correlation (-0.65) that is absent in ADHD (Fig 8(B)). The most abundant Firmicute class (Clostridia) is negatively correlated with different Proteobacteria classes; Gammaproteobacteria in Control (Fig 8(C)), Deltaproteobacteria in ADHD (Fig 8(D)), and the latter continues at the order level (Fig 8(F)) with Clostridiales (Clostridia) and Desulfovibrionales (Deltaproteobacteria). In Control (Fig 8(E)), a negative correlation emerges between order Enterobacteriales (class Gammaproteobacteria) and LEfSe-reported order Turicibacteriales (class Bacilli).

Summary. Upper-level analysis revealed increased Actinobacteria participation in Control gut ecology, especially order Bifidobacteriales. Much of this involved negative correlations with a core of positively correlated Bacteroidetes (order Bacteroidales) and Proteobacteria (order Burkholderiales). Recalling our taxa plots and anomalous behavior involving genera *Bifidobacterium* (Bifidobacteriales), *Bacteroides* (Bacteroidales), and *Sutterella* (Burkholderiales), we are now interested in exploring these dynamics at lower taxonomic levels. We will continue to observe Firmicutes-Proteobacteria dynamics, as despite a still unclear picture, a clear distinction is shown between Control and ADHD.

Lower levels: Family, genus, and lowest possible. Fig 9 shows Control and ADHD MCNs at family (Fig 9(A) and 9(B)), genus (Fig 9(C) and 9(D)), and lowest possible taxonomic classification levels (Fig 9(E) and 9(F)). In this latter MCN each taxon is classified at the species level if possible (rare with 16S), otherwise more commonly the genus level is used. Schemes regarding color, node size, and edge thickness are the same as Fig 8. Since the MCNs are now larger we do not label every node, only those that we reference in our analyses. We also extend Table 6 to include correlations from every taxonomic level, but as this is also very large we include it as S3 Table and extract only relevant portions to our discussion. We perform a similar task with ATria, and S4 Table.

Fig 9 shows taxa separating into a group of primarily Bacteroidetes (dark purple, lower left), and another of primarily Firmicutes (yellow, upper right). Enough taxa are also now present to perform meaningful community analysis. Fig 10 shows the same MCNs as Fig 9, after running Affinity Propagation (AP, [112]) and coloring by cluster. At the family level (Fig 10(A) and 10(B)) four clusters form. One is dominated by Bacteroidetes, family *Bacteroidaceae* (BB, magenta). Two are dominated by Firmicutes, one family *Lachnospiraceae* (FL, gold), and the other family *Ruminococcaceae* (FR, green). In Control (Fig 10(A)) the fourth cluster consists of three mixed-family Firmicutes (FM, dark teal). In ADHD (Fig 10(B)) two of these are absent and the Proteobacteria family *Enterobacteriaceae* is present, leaving it no longer Firmicutes-dominant (M, grey).

Clusters BB and FR remain at the genus level (Fig 10(C) and 10(D)). Several Firmicutes, family *Lachnospiraceae*-dominant clusters emerge, referred to as FL1, FL2, etc. (gold shades). A mixed-family Actinobacteria cluster of genera *Bifidobacterium* and *Collinsella* forms in both MCNs (AM, brown), and an Actinobacteria, family *Coriobacteriaceae*-dominated cluster forms in ADHD (Fig 10(D), AC, burnt sienna). A small group of two *Clostridiaceae* family composes cluster FC in Control (Fig 10(C), aqua). In ADHD (Fig 10(D)), a cluster (orange) emerges as the only Firmicutes-dominant cluster with positive correlations to cluster BB. This eventually becomes present in both lowest-level MCNs (Fig 10(E) and 10(F)) with core member Control-elevated genus *Turicibacter*, so we call this cluster FT.

At the lowest level we kept cluster names as consistent as possible with genus-level membership (for example, a cluster mostly comprised of FL2 genus-level taxa would also be named FL2 at the lowest level). Both MCNs (Fig 10(E) and 10(F)) now include a mixed-family, Bacteroidetes-dominant cluster BMI (pink), and Control includes a second (BM2, orchid). S5–S7 Tables list all clusters, members, and centroids at all levels. As with earlier tables, we will extract portions relevant to our discussion.

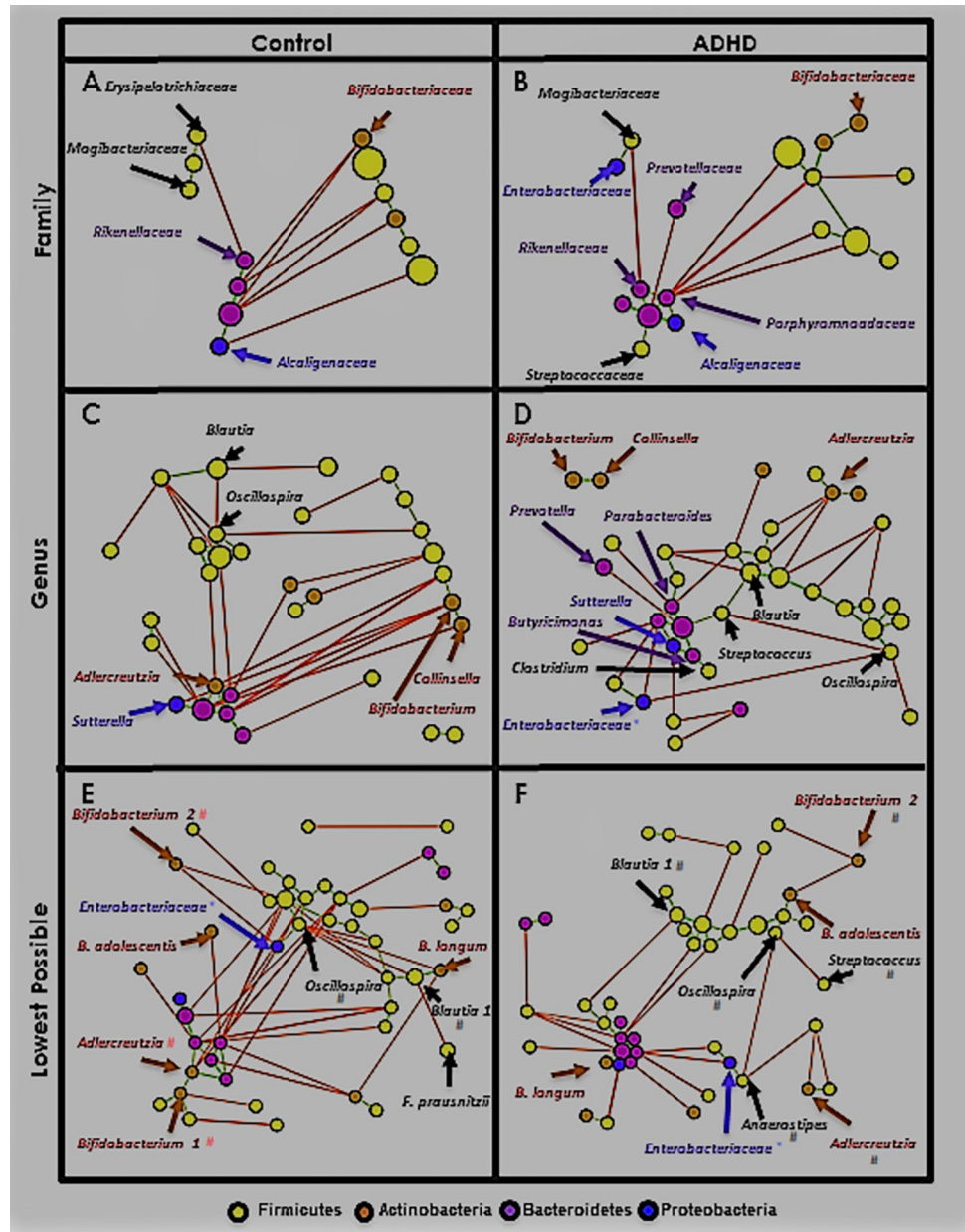


Fig 9. Lower-level MCNs. MCNs at the family (A), genus (B), and species (C) taxonomic levels. Network visual properties, including node and edge size, color, and orientation, are the same as Fig 8. Taxa noted throughout our analyses are labeled (* = family-level taxon, # = genus-level taxon).

<https://doi.org/10.1371/journal.pone.0273890.g009>

Finally, to measure cluster size, tightness, and interactions, we produce a heatmap of taxa correlations (Fig 11) with taxa ordered on the x- and y-axes by Fig 10 cluster. Green/red intensity at each point (x, y) denotes the degree of positive/negative correlation between taxa x and y (symmetric, by definition). Clusters appear as rough squares of positive (green) correlations on the diagonal. We outline each box with the same color as its corresponding Fig 10 cluster.

We first continue to pursue observations (A)-(C) from the upper taxonomic levels. Afterwards, we discuss any new and interesting trends.

(A) A core Proteobacteria-Bacteroidetes positive correlation (est. cooperation) forms. Recall the orders involved in this correlation were Burkholderiales (Proteobacteria) and Bacteroidales

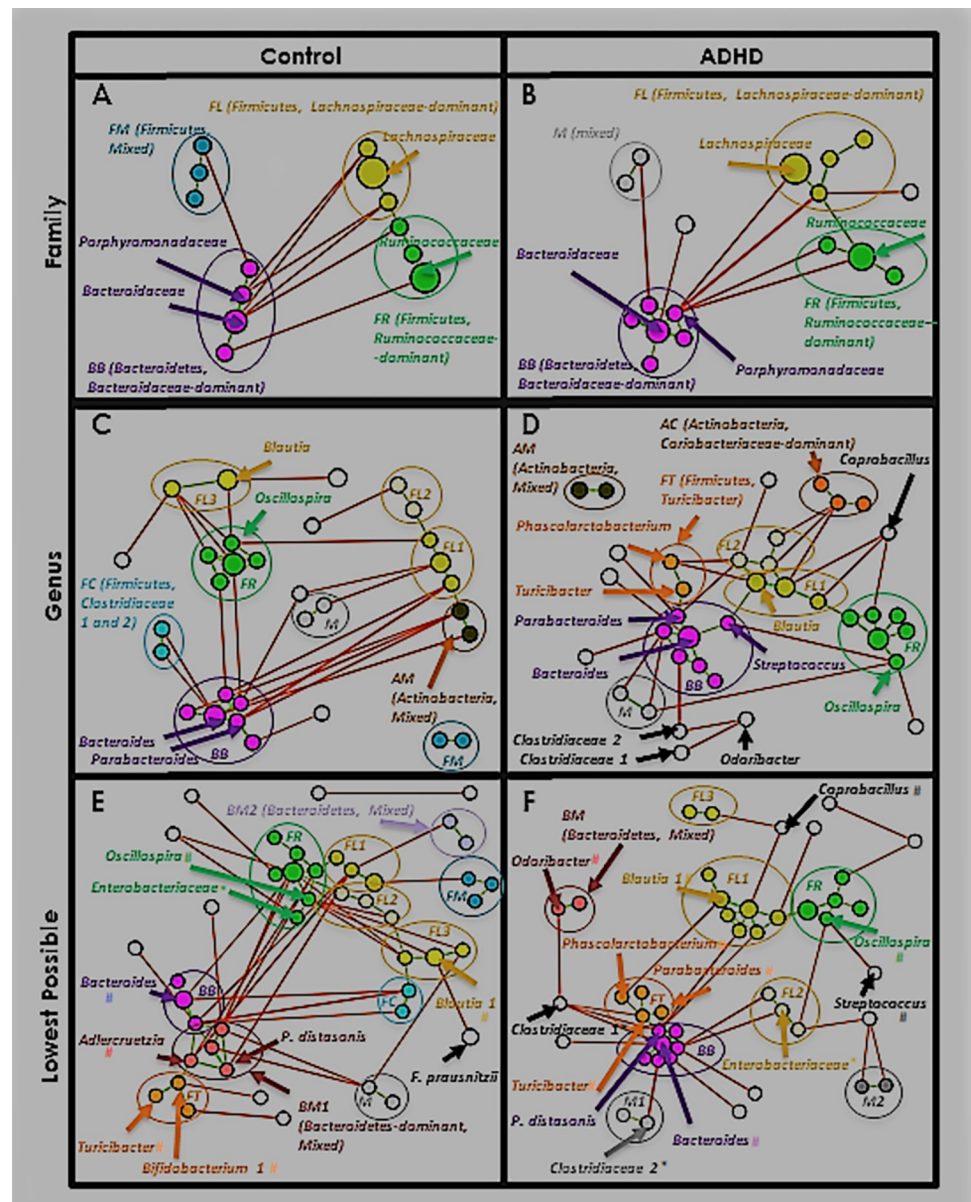


Fig 10. Clusters. Same MCNs as Fig 9, after clustering with the affinity propagation (AP) algorithm [85]. Family-level clusters are each given a unique color, and labeled with their dominant phylum and member family. New clusters that form at each lower taxonomic level are labeled, colored with shades corresponding to their dominant phylum/family when applicable—i.e. at the genus level FL1-FL3 are different shades of gold (family-level FL). Taxa noted throughout our analyses are labeled (* = family-level taxon, # = genus-level taxon).

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(Bacteroidetes). This corresponds to cluster BB, with genus *Sutterella* and multiple *Bacteroidales* taxa. In ADHD this cluster is larger and includes more *Bacteroidales* plus some Firmicutes, and nearly all members are positively correlated with its centroid *Bacteroides*. Additionally it has fewer negative correlations (est. competition) with other clusters.

Cluster BB is the only cluster with order Burkholderiales and Bacteroidales descendants. Table 8 shows all correlations involving Burkholderiales and Bacteroidales lineages, organized and shaded using the same scheme as Table 6. One core positive correlation survives all six taxonomic levels in Control and ADHD (12 MCNs total, the only correlation in our entire dataset

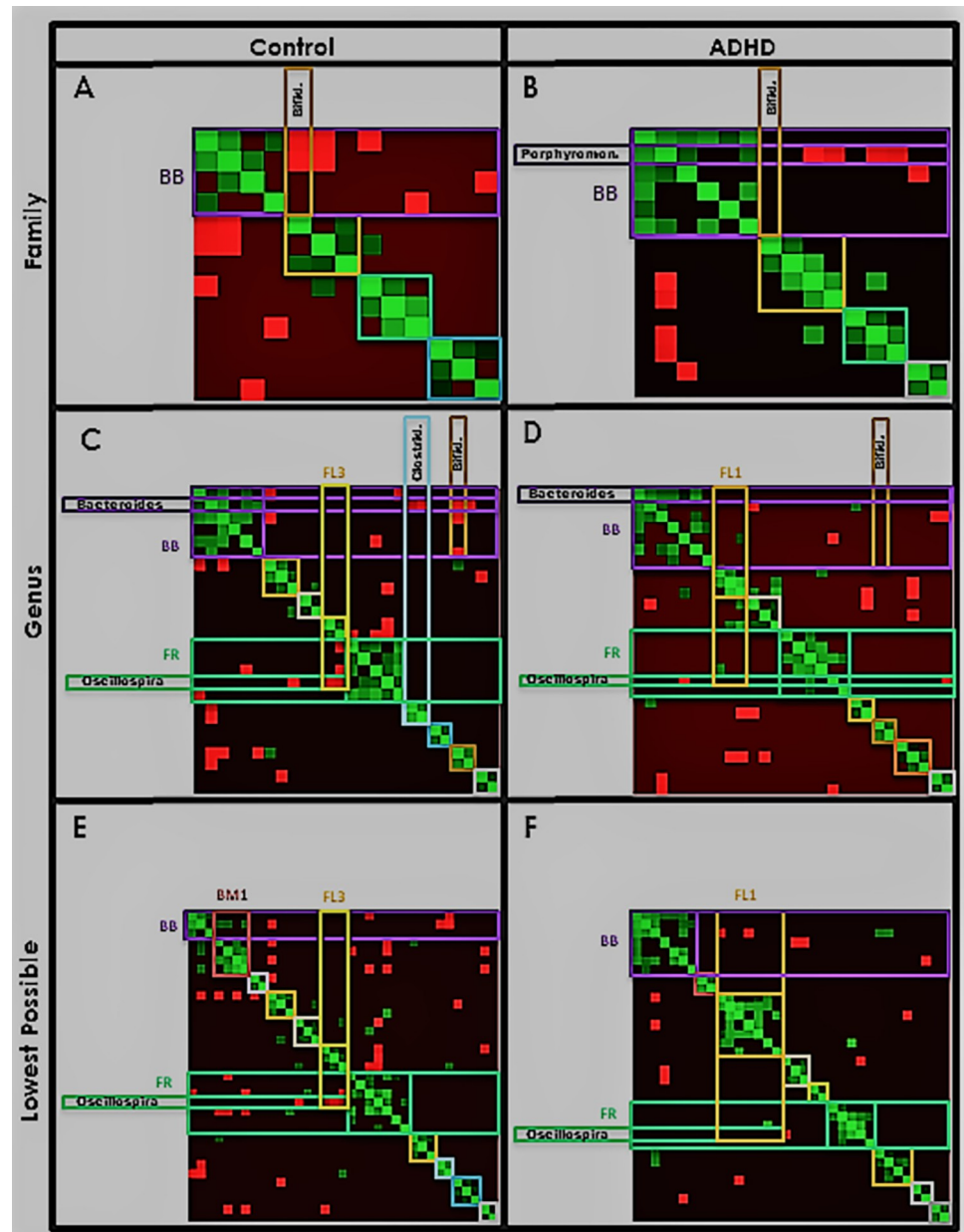


Fig 11. Heatmaps. Heatmap representation of taxa correlations (green = positive, red = negative), with taxa organized on each axis by cluster (symmetric matrix). The area corresponding to the intersection of each cluster with itself is outlined with a box using the corresponding cluster color in Fig 10. Taxa and clusters noted throughout our analyses are labeled on the axes.

<https://doi.org/10.1371/journal.pone.0273890.g011>

with this property). This occurs between genera *Sutterella* and *Bacteroides*. Several others involving *Sutterella* and its family *Alcaligenaceae* with cluster *BB* members are present only in ADHD—support for a larger cluster *BB* in ADHD. *Alcaligenaceae/Sutterella* are immediately visible in Fig 9, as the only Proteobacteria (royal blue) among a slew of Bacteroidetes (dark purple).

Fig 10 also illustrates the increase in ADHD cluster *BB* size, as do the heatmaps (Fig 11, magenta square). Table 9 quantifies differences in node and edge count.

Table 8. Burkholderiales-Bacteroidales correlations.

Phylum	Class	Order	Family	Genus	Species		
Proteobacteria-Bacteroidetes	+ Betaproteobacteria-Bacteroidia	+ Burkholderiales-Bacteroidales	+ Alcaligenaceae-Bacteroidaceae	+ Sutterella-Bacteroides	+ Sutterella-Bacteroides		
					+ Sutterella-B. uniformis		
					+ Alcaligenaceae-Porphyromonadaceae	+ Sutterella-Parabacteroides	+ Sutterella-P. distasonis
					+ Alcaligenaceae-Odoribacteriaceae	+ Sutterella-Butyricimonas	+ Sutterella-Butyricimonas
					Alcaligenaceae-Rikenellaceae	Sutterella-Rikenellaceae	+

Correlations between order Burkholderiales-Bacteroidales lineages, shaded using the same scheme as Table 6 (grey present in both MCNs, purple only ADHD).

<https://doi.org/10.1371/journal.pone.0273890.t008>

Table 9 shows cluster BB size to mysteriously drop in Control from the genus to the lowest level, from six taxa down to three. A closer look at Fig 10(C) and 10(E) shows several genus-level BB members may be joining a mixed-family, Bacteroidetes-dominant cluster (BM1, pink) at the lowest level. Table 10, which shows BB and Control BM1 members, confirms this. Core BB members are shown in bold, while italicized members are unique to Control or ADHD. Taxa of genus-level Control cluster BB members *Odoribacter*, *Adlercruetzia*, *Parabacteroides* (*P. distasonis*) and *Bacteroides* (*B. ovatus*) compose Control cluster BM1 at the lowest level.

Table 11 supports weakened connections between BB and BM1 taxa in Control, showing higher intra-correlation values (0.61 and 0.62) relative to inter-correlation (0.44).

Table 10 also shows cluster BB members that differ between the MCNs. Cluster BB gains a different Actinobacteria–species *B. longum* (ADHD) and genus *Adlercruetzia* (Control, eventually joining BM1). The presence of Firmicutes (yellow) is exclusive to ADHD, including family *Streptococcaceae* and member genus *Streptococcus*, plus genus *Clostridium*. Family *Odoribacteriaceae* (reported by LefSe as ADHD-elevated) joins cluster BB only in ADHD, and the sole *Clostridium* connection to cluster BB is with genus *Butyricimonas* (Fig 9(D)), a taxon reported as ADHD differentially abundant by two methods (LefSe and ALDEx2).

Table 10 also indicates BB/BM1 centroids, which we see across the board for ADHD are genus *Bacteroides* and its family *Bacteroidaceae*. In Control this belongs to *Porphyromonadaceae* (family) and descendant *Parabacteroides* (genus), until the BB-BM1 “split” where genus *Bacteroides* becomes centroid of BB and species *P. distasonis* of BM1. Table 12 shows connectivity of each of these taxa within their corresponding cluster. Percentage-wise, in ADHD *Bacteroidaceae/Bacteroides* is a much stronger centroid; in fact over all levels only one cluster BB taxon was not positively correlated (*Clostridium*, genus level). Particularly given the ADHD cluster BB size increase, this could imply a significant role of *Bacteroidaceae/Bacteroides* in stabilizing a large ADHD Bacteroidetes-dominant community (would require additional experiments to verify).

Table 9. Cluster BB size.

Taxonomic Level	Family		Genus		Lowest	
	Control	ADHD	Control	ADHD	Control	ADHD
Cluster BB size: Taxa (+ Edges)	4 (3)	6 (7)	6 (7)	7 (9)	3 (2)	7 (11)

Control and ADHD cluster BB size. Notation: Taxa (edges).

<https://doi.org/10.1371/journal.pone.0273890.t009>

Table 10. *Bacteroidaceae/Bacteroides* dominant clusters.

Level	Community	Cluster Type	Control		ADHD	
			Taxon	Phylum	Taxon	Phylum
Family	Bacteroidetes-dominant (B)	Bacteroidaceae-dominant (BB)	Bacteroidaceae		Bacteroidaceae*	
			<i>Porphyromonadaceae*</i>		Porphyromonadaceae	
			Alcaligenaceae		Alcaligenaceae	
			Rikenellaceae		Rikenellaceae	
					<i>Odoribacteraceae</i>	
				<i>Streptococcaceae</i>		
Level	Community	Cluster Type	Taxon	Phylum	Taxon	Phylum
Genus	Bacteroidetes-dominant (B)	Bacteroidaceae-dominant (BB)	Bacteroides		Bacteroides*	
			Parabacteroides*		Parabacteroides	
			Sutterella		Sutterella	
			Rikenellaceae		Rikenellaceae	
			<i>Odoribacter</i>		<i>Butyricimonas</i>	
			<i>Adlercruetzia</i>		<i>Streptococcus</i>	
				<i>Clostridium</i>		
Level	Community	Cluster Type	Taxon	Phylum	Taxon	Phylum
Species	Bacteroidetes-dominant (B)	Bacteroidaceae-dominant (BB)	Bacteroides*		Bacteroides*	
			Bacteroides uniformis		Bacteroides uniformis	
			Sutterella		Sutterella	
					<i>Parabacteroides distasonis</i>	
				<i>Rikenellaceae</i>		
				<i>Butyricimonas</i>		
				<i>Bifidobacterium longum</i>		
		Bacteroidetes, Mixed (BM1)		<i>Odoribacter</i>		
		<i>Bacteroides ovatus</i>				
		<i>Parabacteroides distasonis*</i>				
		<i>Adlercruetzia</i>				

Bacteroides, *Bacteroidaceae* dominant clusters (BB) and Bacteroidetes, Mixed family (BM1) cluster in Control. Core taxa are bold, taxa exclusive to one MCN (Control or ADHD) are italicized, and centroids are marked with an asterisk (*). Phylum: purple = Bacteroidetes, yellow = Firmicutes, brown-Actinobacteria, blue = Proteobacteria.

<https://doi.org/10.1371/journal.pone.0273890.t010>

Interestingly ATria (Table 13) shows *Bacteroidaceae/Bacteroides* and lineages to nearly always have higher importance in Control, supporting a more “global” importance to overall gut ecology as opposed to a more local importance (cluster BB) in ADHD. MCNs agree, as in ADHD *Bacteroidaceae/Bacteroides* have few connections outside cluster BB (Fig 10(B), 10(D) and 10(F)). In Control (Fig 10(A), 10(C) and 10(E)) *Bacteroidaceae/Bacteroides* have many external connections, mostly negative (est. competition).

Control MCNs (Fig 10(A), 10(C) and 10(E) and heatmaps) (Fig 11(A), 11(C) and 11(E), magenta rectangle) show negative correlations (red) to be fairly evenly distributed among

Table 11. *Bacteroidaceae*-dominant community intra- and inter-correlations.

Control BB (Intra)			Control BM1 (Intra)			Control BB-BM1 (Inter)			ADHD BB (Intra)		
Taxa	Edges	Mean Correlation	Taxa	Edges	Mean Correlation	Taxa	Edges	Mean Correlation	Taxa	Edges	Mean Correlation
3	2	0.61 ± 0.15	4	4	0.62 ± 0.1	4	3	0.44 ± 0.02	7	11	0.56 ± 0.12

Intra-correlations between cluster BB members, and BB-BM1 inter-correlations in Control.

<https://doi.org/10.1371/journal.pone.0273890.t011>

Table 12. Cluster BB centroid connectivity.

Level	Family		Genus		Lowest	
	Control	ADHD	Control	ADHD	Control	ADHD
MCN						
<i>Bacteroidaceae/Bacteroides</i>	2/3 (66%)	5/5 (100%)	3/5 (60%)	5/6 (84%)	2/2 (100%)	6/6 (100%)
<i>Porphyromonadaceae/Parabacteroides/P. distasonis*</i>	2/3 (66%)	2/5 (40%)	4/5 (80%)	3/6 (50%)	3/3* (100%)	3/6 (50%)

Connectivity between the centroid of cluster BB (* = BM1) and the rest of its cluster members.

<https://doi.org/10.1371/journal.pone.0273890.t012>

cluster BB taxa. By contrast in ADHD (Figs 10–11(B)), nearly all cluster BB negative correlations are localized to family *Porphyromonadaceae* (ranked #1 by ATria). Fig 9(B) shows *Porphyromonadaceae* to be the sole cluster BB member negatively correlated with the Firmicutes-dominant portion (Fig 9(B), upper right, collectively more than 70% of the population).

Table 14 shows that for all MCNs, in Control more than two-thirds of cluster BB had negative correlations with members of other clusters, compared to less than half in ADHD. Negative edge count was also almost always higher for Control, despite a smaller cluster BB. Collectively these results show that in Control cluster BB is smaller, and more connected to other clusters, primarily through negative correlations (est. competition). In ADHD cluster BB is larger, and more isolated.

Table 15 provides a few final interesting observations for various Bacteroidetes taxa.

(B) In Control, taxa in (A) have more negative edges with Actinobacteria (est. competition), especially Bifidobacteriales. We now know taxa from (A) to correspond to cluster BB, which in both MCNs contained one core Proteobacteria (family *Alcaligenaceae*/genus *Sutterella*) and otherwise primarily Bacteroidetes. We also observed cluster BB taxa to have far more negative correlations (est. competition) with other clusters in Control. We now see if this is also true with order Bifidobacteriales lineages, including genus *Bifidobacterium*. Our analysis in fact reveals that **negative correlations between *Bifidobacterium* or any parent/descendant with any Bacteroidetes or Proteobacteria are exclusive to Control and absent in ADHD.**

Table 16 shows all correlations involving *Bifidobacterium* and its lineages, grouped and colored as in previous tables. Not only are negative Bacteroidetes correlations exclusive to Control (orange), but these taxa include the most abundant Bacteroidetes family/genus *Bacteroidaceae/Bacteroides* (ADHD cluster BB centroid), as well as family/genus *Porphyromonadaceae/Parabacteroides* (Control cluster BB centroid). Another appears at the lowest level between soecues *B. adolescentis* and *B. ovatus*. With Proteobacteria, negative *Bifidobacterium* correlations are

Table 13. Bacteroidetes rankings.

Phylum	Class	Order	Family	Genus	Lowest Possible
Bacteroidetes (NR/#T2)	Bacteroidia (NR/#1)	Bacteroidales (#1/#1)	Bacteroidaceae (#T9/#T12)	Bacteroides (#10/#T23)	B. uniformis (#12/#7)
					B. ovatus (#2/#T20)
					Bacteroides (#T23/#T28)
			Odoribacteriaceae (NR/#2)	Odoribacter (#9/#T21)	Odoribacter (#3/#T20)
				Butyricimonas (NR/#4)	Butyricimonas (NR/#17)
			Porphyromonadaceae (#T7/#1)	Parabacteroides (#T12/NR)	Parabacteroides (#T19/#T24)
					P. distasonis (NR/#T24)
Prevotellaceae (NR/#5)	Prevotella (NR/#18)				
Rikenellaceae (#T7/#3)	Rikenellaceae (#T12/#8)	Rikenellaceae (#T19/#8)			

ATria rankings of Bacteroidetes taxa. Dark orange = only ranked in Control, dark purple = only ranked in ADHD, light orange = higher ranked in Control, light purple = higher ranked in ADHD, grey = evenly ranked. **Bold** taxa are ranked #1.

<https://doi.org/10.1371/journal.pone.0273890.t013>

Table 14. Negative correlations between cluster BB and other clusters.

Level	Family		Genus		Lowest	
	Control	ADHD	Control	ADHD	Control	ADHD
MCN						
Cluster BB (-) Edges with Other Clusters (Participation Rate)	7 (100%)	5 (33%)	10 (88%)	4 (43%)	5 (67%)	5 (43%)

Amount of negative correlations between members of *Bacteroidaceae*-dominant cluster BB and other clusters. Notation: Number (participation rate).

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also observed with genus *Sutterella* (core cluster BB member) and family *Enterobacteriaceae*, also only in Control. Heatmaps confirm family/genus *Bifidobacteriaceae/Bifidobacterium* to be negatively correlated with cluster BB taxa only in Control (Fig 11(A)–11(D), intersection of brown and magenta rectangles). By contrast, the only ADHD correlation is positive and within cluster BB (species *B. longum* with species *B. uniformis*).

Table 16 also shows *Bifidobacterium* to even have far more Firmicutes connections (positive and negative) in Control. Collectively 24 correlations were observed in Control, compared to 9 in ADHD, supporting an overall increase in *Bifidobacterium* participation in Control. ATria (Table 17) also almost uniformly ranks *Bifidobacterium* and its lineages higher in Control. Again, this is despite *Bifidobacterium* average abundances being relatively the same (slightly higher in ADHD in fact, 3.6% to 3.2%).

(C) *A Shift in Firmicutes-Proteobacteria dynamics.* Only two Proteobacteria families/genera were consistently present. One was genus *Sutterella* (family *Alcaligenaceae*), already noted as a core cluster BB member. The other is family *Enterobacteriaceae*, which our analysis supports being mostly responsible for this shift.

Table 18 shows all Proteobacteria-Firmicutes correlations. A couple of negative correlations can be seen involving family/genus *Alcaligenaceae/Sutterella*, with Firmicutes families *Ruminococcaceae* (Control) and *Clostridiaceae* (both). Far more significant are the differences involving family *Enterobacteriaceae*. One is its negative correlation with genus *Oscillospira* in ADHD (genus level), that becomes a positive correlation with *Oscillospira* in Control (lowest level). This is the only time, over all twelve MCNs, where a correlation sign changed between the same two taxa in Control vs. ADHD.

Interesting shifts involving family *Enterobacteriaceae* and various Firmicutes occur even at the family level, however. A small mixed-family, Firmicutes-dominant cluster FM forms (Fig 10(A), upper left), consisting of families *Mogibacteriaceae*, *Christensenellaceae*, and *Erysipelotrichiaceae* (Table 19). In ADHD, *Enterobacteriaceae* instead joins family *Mogibacteriaceae* to form a small two-taxon mixed cluster M (Fig 10(B), upper left, and Table 19).

Dynamics of FM and M taxa change between the MCNs. Fig 10(A) and 10(B) shows a distinguishing core FM/M feature is the negative correlation with family *Rikenellaceae* of cluster BB, but the taxon involved changes from family *Erysipelotrichiaceae* in Control to family *Mogibacteriaceae* in ADHD. Table 20 (ATria) shows the two taxa from Control cluster FM

Table 15. Additional Bacteroidetes observations.

Taxa	Observation
Family <i>Odoribacteriaceae</i>	ADHD-elevated, ADHD cluster BB member, only ranked in ADHD.
Family <i>Prevotellaceae</i> and member genus <i>Prevotella</i>	ADHD negative correlation with <i>Bacteroidaceae/Bacteroides</i> is the only negative correlation between two Bacteroidetes taxa in any MCN. Only ranked in ADHD,
Family <i>Rikenellaceae</i>	Ranked in every MCN at every level, and always higher for ADHD.

Additional observations we make for some other Bacteroidetes taxa.

<https://doi.org/10.1371/journal.pone.0273890.t015>

Table 16. *Bifidobacterium* correlations.

Phylum	Class	Order	Family	Genus	Lowest Possible
Actinobacteria- Bacteroidetes	Actinobacteria- Bacteroidia	Bifidobacteriales- Bacteroidales	Bifidobacteriaceae- Bacteroidaceae	Bifidobacterium -Bacteroides	B. adolescentis-B. ovatus B. longum-B.uniformis
			Bifidobacteriaceae- Porphyromonadaceae	Bifidobacterium— Parabacteroides	
Actinobacteria Proteobacteria	Actinobacteria -Betaproteobacteria	Bifidobacteriales- Burkholderiales	Bifidobacteriaceae- Alcaligenaceae	Bifidobacterium- Sutterella	
	Actinobacteria- Gammaproteobacteria	Bifidobacteriales- Enterobacteriales	Bifidobacteriaceae- Enterobacteriaceae	Bifidobacterium- Enterobacteriaceae	Bifidobacterium 2 – Enterobacteriaceae
Actinobacteria- Firmicutes	Actinobacteria- Clostridia	Bifidobacteriales -Clostridiales	Bifidobacteriaceae- Turicibacteriaceae	Bifidobacterium- Turicibacter	Bifidobacterium 1 – Turicibacter
			Bifidobacteriaceae- Lachnospiraceae	Bifidobacterium -Lachnospiraceae 2	Bifidobacterium 1 – Blautia 2 Bifidobacterium 1 – Ruminococcus [L] B. longum-Blautia 1
			Bifidobacteriales- Clostridiales	Bifidobacterium- Clostridiales	B. adolescentis- Clostridiales 1
			Bifidobacteriaceae- Veillonellaceae	Bifidobacterium- Veillonella	Bifidobacterium 2 – Ruminococcaceae B. longum-Oscillospira
			Bifidobacteriaceae- Veillonellaceae	Bifidobacterium- Veillonella	Bifidobacterium 2 – Dialister
Actinobacteria- Actinobacteria	Actinobacteria- Actinobacteria	Bifidobacteriales- Bifidobacteriales	Bifidobacteriaceae- Bifidobacteriaceae	Bifidobacterium- Bifidobacterium	Bifidobacterium 2 –B. adolescentis
	Actinobacteria- Coriobacteria	Bifidobacteriales- Coriobacteriales	Bifidobacteriaceae- Coriobacteriaceae	Bifidobacterium- Adlercruetzia Bifidobacterium- Collinsella Bifidobacterium- Eggerthella	Bifidobacterium 1 -Adlercruetzia B. longum-E.lenta

Correlations involving *Bifidobacterium* and its lineages. Orange = only found in Control, purple = only found in ADHD, grey = found in both. +(green) = positive correlation, -(red) = negative correlation.

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“replaced” by family *Enterobacteriaceae* in ADHD cluster *M*, families *Christensenellaceae* and *Erysipelotrichiaceae*, are only ranked in Control, and family *Mogibacteriaceae* only ranked in ADHD. This applied across all descendants, with the one notable exception being genus *Coprobacillus* (family *Erysipelotrichiaceae*), ranked #1 for ADHD at the genus and lowest levels (the only taxon to be ranked #1 in two MCNs). Recall *Coprobacillus* was also reported by

Table 17. *Bifidobacterium* rankings.

Phylum	Class	Order	Family	Genus	Lowest Possible
Actinobacteria (#1/ #1)	Actinobacteria (#2/ NR)	Bifidobacteriales (#T2/ #T5)	Bifidobacteriaceae (#5/ NR)	Bifidobacterium (NR/ #T19)	Bifidobacterium 1 (#10/ NR) Bifidobacterium 2 (#1/11) B. longum (#15/NR)

ATria rankings of *Bifidobacterium* and lineages. Dark orange = only ranked in Control, dark purple = only ranked in ADHD, light orange = higher ranked in Control, light purple = higher ranked in ADHD, grey = evenly ranked. **Bold** taxa are ranked #1.

<https://doi.org/10.1371/journal.pone.0273890.t017>

Table 18. Proteobacteria-Firmicutes correlations.

Phylum	Class	Order	Family	Genus	Lowest Possible			
Proteobacteria-Firmicutes	Betaproteobacteria—Clostridia	Burkholderiales-Clostridiales	Alcaligenaceae—Ruminococcaceae	-				
			Alcaligenaceae—Clostridiaceae	-	Sutterella—Clostridiaceae 2	-	Sutterella—Clostridiaceae 2	-
	Gammaaproteobacteria—Bacilli	Enterobacteriales—Turicibacteriales	-					
	Gammaaproteobacteria—Clostridia	Enterobacteriales-Clostridiales	Enterobacteriaceae—Mogibacteriaceae	+				
			Enterobacteriaceae—Lachnospiraceae		Enterobacteriaceae—Anaerostipes		Enterobacteriaceae—Anaerostipes	+
			Ruminococcaceae—Enterobacteriaceae		Enterobacteriaceae—Oscillospira	-	Enterobacteriaceae—Oscillospira	+
	Gammaaproteobacteria—Erysipelotrichi	Enterobacteriales-Erysipelotrichiales		Enterobacteriaceae-Erysipelotrichiaceae	+	Enterobacteriaceae-Erysipelotrichiaceae 2	+	
Deltaproteobacteria—Clostridia	Desulfobvibrionales—Clostridiales	-						

Correlations between Proteobacteria and Firmicutes taxa. Orange = only found in Control, purple = only found in ADHD, grey = found in both. +(green) = positive correlation, -(red) = negative correlation.

<https://doi.org/10.1371/journal.pone.0273890.t018>

DESeq2 as elevated in ADHD compared to Control. Other members of the *Erysipelotrichaceae* family (including the family itself) were only ranked by ATria in the case of Control. We label it in Fig 10(D) and 10(F), noting its negative correlations with multiple Firmicutes-dominant clusters.

Family *Enterobacteriaceae* was also only ranked in ADHD, across all three lower levels. Its *Oscillospira* positive correlation (Table 18) is the only Control correlation involving *Enterobacteriaceae*, and *Enterobacteriaceae* actually joins genus *Oscillospira*'s cluster (FR, Fig 10(E)) in Control. The sign change takes place at the genus level in ADHD (Fig 9(D)), where *Oscillospira* and *Enterobacteriaceae* are negatively correlated. Although this correlation did not persist to the lowest level (Fig 9(F)), *Enterobacteriaceae* is still positively correlated with genus *Anaerostipes*, a taxon negatively correlated with *Oscillospira* across the board. We therefore observe *Enterobacteriaceae* dynamics to shift from a state that favors *Oscillospira* cooperation in Control, to *Oscillospira* competition in ADHD. The role of *Enterobacteriaceae* in gut ecology has historically been controversial [145], with both beneficial [146] and pathogenic [147] properties emerging. Gut dysbiosis has actually been shown to trigger horizontal gene transfer between the two types [148].

Table 19. Mixed-family clusters.

Community	Cluster Type	Control			ADHD		
		Cluster	Taxon	Phy	Cluster	Taxon	Phy
Firmicutes-dominant (F)	Mixed (FM)	FM	<i>Mogibacteriaceae</i>				
		FM	<i>Christensellaceae*</i>				
		FM	<i>Erysipelotrichiaceae</i>				
Mixed (M)	N/A				M	<i>Mogibacteriaceae</i>	
					M	<i>Enterobacteriaceae</i>	

Control and ADHD clusters consisting of taxa from multiple families. Phylum: purple = Bacteroidetes, yellow = Firmicutes, brown-Actinobacteria, blue = Proteobacteria.

<https://doi.org/10.1371/journal.pone.0273890.t019>

Table 20. Mixed-family cluster member rankings.

Phylum	Class	Order	Family	Genus	Lowest Possible
Firmicutes (#2/#T2)	Clostridia (#T3/#T3)	Clostridiales (#T2/#3)	Christensenellaceae (#2/NR)	<i>Christensenellaceae</i> (NR/NR)	Christensenellaceae (#18/NR)
			Mogibacteriaceae (NR/#T6)	Mogibacteriaceae (NR/#6)	
	<i>Erysipelotrichia</i> (NR/NR)	Erysipelotrichales (NR/#T3)	Erysipelotrichaceae (#3/NR)	Coprobacillus (NR/#1)	Coprobacillus (#T31/#1)
				Erysipelotrichaceae 1 (#6/NR)	Erysipelotrichaceae 1 (#16/NR)
				Erysipelotrichaceae 2 (#11/NR)	
Eubacterium (#T14/NR)	<i>E. dolicum</i> (#T29/NR)				
Proteobacteria (NR/NR)	Gammaproteobacteria (#T3/NR)	Enterobacteriales (#T3/NR)	Enterobacteriaceae (NR/#T6)	Enterobacteriaceae (NR/#7)	Enterobacteriaceae (NR/#T26)

Members of mixed family clusters from Table 19, their lineages, and ATria rankings. Dark orange = only ranked in Control, dark purple = only ranked in ADHD, light orange = higher ranked in Control, light purple = higher ranked in ADHD, grey = evenly ranked. **Bold** taxa are ranked #1.

<https://doi.org/10.1371/journal.pone.0273890.t020>

New observations. We make the following new observations at the lower levels.

(D) *LEfSe* reported taxa: *Genera Turicibacter and Odoribacter*. Earlier we noted ADHD-elevated family *Odoribacteriaceae* as an ADHD cluster *BB* member (Table 15). We now observe two remaining *LEfSe*-reported genera: *Turicibacter* (Control) and *Odoribacter* (ADHD).

Cluster *FT* (Fig 10, orange) was the only Firmicutes-dominant cluster with members positively correlated with any Bacteroidetes-dominant cluster (*BB* in ADHD, *BMI* in Control). We named this cluster *FT* because of core member genus *Turicibacter*. *Turicibacter* (Firmicutes, Control-elevated), which joins genus *Phascolarctobacterium* (Firmicutes, reduced in Inattention, [63]) to form *FT* at the genus level in ADHD (Fig 10(D)), where it is not present in Control. At the lowest level, *FT* is slightly larger (by one taxon) in ADHD. Supplementing the earlier trend of less cluster *BB* negative correlations (est. competition) in ADHD, this also supports the presence of a larger cluster with positive correlations (est. cooperation) as well, with *Turicibacter* as its centroid (Table 21).

In ADHD *Turicibacter* provides the sole genus-level (Fig 10(D)) *FT-BB* positive correlation, with genus *Parabacteroides* (Bacteroidetes, previously reported elevated in Hyperactivity, [59]). At the lowest level (Fig 10(F)) *Parabacteroides* joins *FT*, and along with *Turicibacter* forms *FT-BB* positive correlations, with member species *P. distasonis*. Interestingly in Control (Fig 10(E)), the *FT-BB* positive correlation does not involve Firmicutes or Bacteroidetes taxa at

Table 21. FT members.

Level	Community	Cluster Type	Control		ADHD	
			Taxon	Phy	Taxon	Phy
Family	Firmicutes-dominant (F)	Turicibacter-core (FT)	Not present.		<i>Turicibacter</i>	
					<i>Phascolarctobacterium</i>	
Level	Community	Sub-Community	Taxon	Phy	Taxon	Phy
Genus	Firmicutes-dominant (F)	Turicibacter-core (FT)	Turicibacter		Turicibacter*	
			<i>Ruminococcus [L]</i>		<i>Phascolarctobacterium</i>	
			<i>Bifidobacterium 2*</i>		<i>Clostridiales 2</i>	
					<i>Parabacteroides</i>	

Cluster *FT* (Firmicutes-dominant, *Turicibacter*-core) members. Phylum: purple = Bacteroidetes, yellow = Firmicutes, brown-Actinobacteria, blue = Proteobacteria.

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all, but rather two Actinobacteria–*Bifidobacterium 1* (*FT* centroid), and *Adlercruetzia* (*BB*). This continues our observed increases in Actinobacteria and particularly *Bifidobacterium* involvement in Control gut ecology.

Cluster *FC* forms in Control (Fig 10(C) and 10(E), aqua) and contains two family *Clostridiaceae* taxa. In both MCNs these taxa negatively correlate with multiple cluster *BB* members, and in ADHD (Fig 10(F)) family *Clostridiaceae 1* has negative correlations with *BB* centroid genus *Bacteroides* plus taxa involved in *FT*-*BB* cooperation: species *P. distasonis*, and *FT* centroid genus *Turicibacter*. In both MCNs, they participate in correlations that favor cluster *BB* competition (especially the more abundant *Clostridiaceae 1*).

Exclusive to ADHD is a negative correlation (est. competition) between these family *Clostridiaceae* taxa and ADHD-elevated genus *Odoribacter*—both at the genus level (Fig 10(D)), and family *Clostridiaceae 1* at the lowest level (Fig 10(F)). Genus *Odoribacter* was reported by LEfSe as elevated in ADHD, and this negative correlation implies that an increase in *Odoribacter* abundance will decrease family *Clostridiaceae 1*. ALDEx2 reported *Clostridiaceae 1* as differentially abundant in Control, and upon further inspection *Clostridiaceae 1* average relative abundance is indeed reduced by a factor of two in ADHD vs. Control. Cooperation between families *Clostridiaceae 1* and 2 in Control (forming *FC*) is also absent in ADHD.

(E) *Changes in the role of genus Adlercruetzia* (Actinobacteria). In contrast to genus *Bifidobacterium* (*Bifidobacteriaceae*), *Adlercruetzia* is a member of the other consistently present Actinobacteria family, *Coriobacteriaceae*. While the distinguishing feature of family/genus *Bifidobacteriaceae/Bifidobacterium* was increased Control participation, the distinguishing feature of family *Coriobacteriaceae* appears to be changes in cluster membership. In fact over all *Coriobacteriaceae* descendants, only once (*Collinsella*, genus level, cluster AM, Fig 10(C) and 10(D)) were any in the same Control and ADHD cluster. Table 22 also shows ATria results to be more mixed for family *Coriobacteriaceae*, compared to family *Bifidobacteriaceae* (Table 19).

We earlier noted genus *Adlercruetzia* as the Actinobacteria member of cluster *BB/BM1* in Control, and (along with genus *Bifidobacterium 1*) connecting clusters *FT* and *BB*. Table 23 shows that outside of *Bifidobacterium 1*, its positive correlations in Control were entirely with Bacteroidetes taxa (all *BB/BM1* members). By contrast in ADHD, *Adlercruetzia* relationships mostly occur with Firmicutes, including a cluster membership with genus/species *Eubacterium/E. dolicum*. Several negative correlations are seen between genus *Adlercruetzia* and different Firmicutes, with no overlap between Control and ADHD. This suggests genus *Adlercruetzia* may play a significantly different role in Control and ADHD gut ecologies.

(F) *Bacteroidetes-Firmicutes positive correlations (est. cooperation) are entirely exclusive to ADHD, and absent in Control.* Table 24 shows all Bacteroidetes–Firmicutes positive correlations. They are entirely limited to ADHD, and with one exception (genus *Clostridium*) involve class Bacilli descendants.

We have already seen most of these, including genera *Clostridium* and *Butyricimonas*, and the ADHD *FT*-*BB* connections involving genera *Turicibacter* and *Parabacteroides*, and species

Table 22. *Coriobacteriaceae* rankings.

Phylum	Class	Order	Family	Genus	Lowest Possible
Actinobacteria (#1/#1)	Coriobacteria (#1/NR)	Coriobacteriales (NR/#T5)	Coriobacteriaceae (NR/#4)	Adlercruetzia (NR/#17)	Adlercruetzia (#14/NR)
				Collinsella (NR/#T19)	C. aerofaciens (NR/#15)
				Coriobacteriaceae (#18/NR)	
				Eggerthella (#4/NR)	E. lenta (#T25/NR)

ATria rankings of *Coriobacteriaceae* and its lineages. Dark orange = only ranked in Control, dark purple = only ranked in ADHD, light orange = higher ranked in Control, light purple = higher ranked in ADHD, grey = evenly ranked. **Bold** taxa are ranked #1.

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Table 23. *Adlercreutzia* correlations.

Phylum	Class	Order	Family	Genus	Lowest Possible
Actinobacteria- Bacteroidetes	Coriobacteria- Bacteroidia	Coriobacteriales- Bacteroidales	Coriobacteriaceae- Bacteroidaceae	Adlercreutzia- Bacteroides	+ Adlercreutzia- B. uniformis
				Adlercreutzia- Parabacteroides	+ Adlercreutzia- P. distasonis
				Adlercreutzia- Odoribacter	+
Actinobacteria- Firmicutes	Actinobacteria- Clostridia	Coriobacteriales- Costridiales	Coriobacteriaceae- Erysipelotrichaceae-	Adlercreutzia- Eubacterium	+ Adlercreutzia- E. dolicum
			Coriobacteriaceae- Lachnospiraceae	Adlercreutzia -Blautia	-
				Adlercreutzia- Lachnospira	- Adlercreutzia— Lachnospira
				Adlercreutzia— Lachnospiraceae 1	-
			Adlercreutzia— Lachnospiraceae 2	Adlercreutzia— Lachnospiraceae 2	
Coriobacteriaceae- Ruminococcaceae	Adlercreutzia- Ruminococcus	- Adlercreutzia- Ruminococcus			
Actinobacteria- Actinobacteria	Actinobacteria- Coriobacteria	Bifidobacteriales- Coriobacteriales	+ Bifidobacteriaceae- Coriobacteriaceae	+ Bifidobacterium- Adlercreutzia	Bifidobacterium 1 -Adlercreutzia
	Coriobacteria— Coriobacteria	Coriobacteriales— Coriobacteriales	Coriobacteriaceae- Coriobacteriaceae	Adlercreutzia- Coriobacteriaceae	+

Correlations involving genus *Adlercreutzia* and its lineages. Orange = only found in Control, purple = only found in ADHD, grey = found in both. +(green) = positive correlation, -(red) = negative correlation.

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P.distasonis. We now analyze the remaining top row, between genera *Bacteroides* (family *Bacteroidaceae*) and *Streptococcus* (family *Streptococcaceae*).

Firmicutes taxa were only ever present in cluster *BB* in ADHD, and we earlier noted family *Streptococcaceae* and its genus *Streptococcus* as two of those taxa. Their cluster *BB* positive correlation was with centroid family/genus *Bacteroidaceae/Bacteroides*. Additionally cluster *BB* had almost no negative correlations (est. competition) with *FL/FR* (collectively 70% of the population) in ADHD, compared to a significant amount in Control.

What makes genus *Streptococcus* interesting for ADHD is that across all MCNs, it forms the only positive correlation between cluster *BB* and *FL/FR* (Fig 10(D)). In other words, in addition to estimating significantly less *BB*-(*FL/FR*) competition in ADHD, our MCNs also estimate cooperation only in ADHD, between genera *Streptococcus* (*BB*) and *Blautia* (*FL1*).

Table 24. Bacteroidetes-Firmicutes positive correlations.

Phylum	Class	Order	Family	Genus	Lowest Possible
Bacteroidetes- Firmicutes	Bacteroidia -Bacilli	Bacteroidales Lactobacillales	Bacteroidaceae	+ Bacteroides	+
			Streptococcaceae	-Streptococcus	+
			Porphyromonadaceae Turicibacteriaceae-	Parabacteroides -Turicibacter	+ Parabacteroides- Turicibacter
		Parabacteroides -Turicibacter	+ P.distasonis -Turicibacter		
Bacteroidales- Clostridiales	-	Odoribacteriaceae- Clostridiaceae	Butyricimonas -Clostridium	+	

Bacteroidetes-Firmicutes positive correlations, over all MCNs. Orange = only found in Control, purple = only found in ADHD, grey = found in both. +(green) = positive correlation, -(red) = negative correlation.

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Table 25. *Streptococcaceae/Streptococcus* rankings.

Phylum	Class	Order	Family	Genus	Lowest Possible
Firmicutes (#2/#T2)	Bacilli (NR/NR)	Lactobacillales (NR/NR)	Streptococcaceae (NR/#T12)	Streptococcus (NR/#T23)	

ATria rankings of family/genus *Streptococcaceae/Streptococcus*. Dark orange = only ranked in Control, dark purple = only ranked in ADHD, light orange = higher ranked in Control, light purple = higher ranked in ADHD, grey = evenly ranked. **Bold** taxa are ranked #1.

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Fig 10(D) and 10(F) also show *Streptococcus* to be negatively correlated with genus *Oscillospira* in ADHD, a taxon we noted earlier its correlation sign change with family *Enterobacteriaceae*. ATria (Table 25) also only ranks family/genus *Streptococcaceae/Streptococcus* as important in ADHD.

(G) A shift in genera *Blautia*-*Oscillospira* dynamics, and their respective clusters. Thus far genus *Oscillospira* has been noted for two ADHD-exclusive negative correlations, with taxa only ranked in ADHD: family *Enterobacteriaceae* and genus *Streptococcus*. *Enterobacteriaceae*-*Oscillospira* was the only correlation to ever change sign from Control (positive) to ADHD (negative). *Streptococcus* was noted for its correlation with genus *Blautia*, the sole positive correlation between the largest Bacteroidetes-dominant cluster (BB) and Firmicutes-dominant clusters (FL/FR) in any MCN.

Previous studies have indicated butyrate-producing *Oscillospira* as a healthy gut taxon [149], specifically associated with leanness [150]. *Blautia* is actually a taxon that has been associated with obesity [151]. And interestingly in the Control MCN (Fig 10(C) and 10(E)) *Blautia* and *Oscillospira* are negatively correlated, but not in ADHD (Fig 10(D) and 10(F)).

Since obesity has been associated with ADHD [152], the shift in *Enterobacteriaceae* (*Oscillospira* cooperation in Control, competition in ADHD) and *Streptococcus* (*Blautia* cooperation and *Oscillospira* competition in ADHD) correlations become interesting, favoring *Blautia* cooperation and *Oscillospira* competition. Indeed correlation can never imply causation and further experimental verification is required. But ATria results (Table 26) also support this, ranking *Blautia* higher in ADHD and *Oscillospira* in Control.

In fact our heatmap (Fig 11(C)–11(F)) shows by intersecting *Oscillospira*'s row (small green rectangle) with the columns of *Blautia*'s cluster (gold rectangles, Control FL3, ADHD FL1) that *Oscillospira* is negatively correlated with *Blautia*'s entire cluster in Control, and these correlations are completely absent in ADHD.

The lowest level MCNs (Fig 10(E) and 10(F)) also show *Blautia*'s cluster as larger in ADHD, and *Oscillospira*'s cluster as larger in Control. Table 27 contains members of these clusters. *Blautia* and *Oscillospira* each belong to a cluster dominated by its respective family: *Lachnospiraceae* (FL), and *Ruminococcaceae* (FR). *Oscillospira* is a core FR member and at the lowest level, we see the Control FR cluster (with family *Enterobacteriaceae* now a member). *Blautia* is consistently a member of the same cluster as both family *Lachnospiraceae* taxa in ADHD, comparably larger than its FL3 Control cluster.

Table 26. *Blautia* and *Oscillospira* rankings.

Phylum	Class	Order	Family	Genus	Lowest Possible
Firmicutes (#2/#T2)	Clostridia (#T3/#T3)	Clostridiales (#T2/#3)	Lachnospiraceae (NR/NR)	Blautia (#T16/#13)	Blautia 1* (NR/#19)
			Ruminococcaceae (#4/#T8)	<i>Oscillospira</i> (#3/#10)	<i>Oscillospira</i> (#5/#9)

Blautia and *Oscillospira* ATria rankings (plus lineages). Dark orange = only ranked in Control, dark purple = only ranked in ADHD, light orange = higher ranked in Control, light purple = higher ranked in ADHD, grey = evenly ranked. **Bold** taxa are ranked #1. * = The lowest level had two *Blautia* taxa; we assumed the more abundant (*Blautia* 1, overall 9.3% relative abundance vs 0.6%, composing 93% of the *Blautia* population).

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Table 27. *Blautia* and *Oscillospira* clusters.

Level	Community	Cluster Type	Control			ADHD		
			Cluster	Taxon	Phy	Cluster	Taxon	Phy
Lowest	Firmicutes-dominant,	Lachnospiraceae-dominant (FL)	FL3	<i>Blautia</i> 1*		FL1	Lachnospiraceae 1	
			FL3	Dorea 2		FL1	Lachnospiraceae 2*	
			FL3	<i>Bifidobacterium longum</i>		FL1	Coprococcus	
						FL1	<i>Ruminococcus [L]</i>	
						FL1	<i>Blautia</i> 1	
						FL1	Dorea 2	
						FL1	<i>Faecalibacterium prausnitzii</i>	
		Ruminococcaceae-dominant (FR)	FR	Ruminococcaceae*		FR	Ruminococcaceae	
			FR	<i>Ruminococcus [R]</i>		FR	<i>Ruminococcus [R]</i>	
			FR	<i>Oscillospira</i>		FR	<i>Oscillospira</i>	
			FR	Clostridiales 1		FR	Clostridiales 1*	
			FR	<i>Coprobacillus</i>		FR	<i>Bifidobacterium adolescentis</i>	
FR	<i>Enterobacteriaceae</i>							
		<i>Lachnospira</i>						

Clusters involving *Blautia* and *Oscillospira*. Phylum: purple = Bacteroidetes, yellow = Firmicutes, brown = Actinobacteria, blue = Proteobacteria.

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Heatmaps also indicate increased participation of *Oscillospira*'s cluster (FR) in Control (large green rectangle, Fig 11(C)–11(F)), including negative correlations with cluster BB that are absent in ADHD, yet another example of reduced ADHD cluster BB competition. In the MCNs, Fruchterman-Reingold places cluster FR (green) in a much more central position in Control (Fig 10(C) vs 10(D), and 10(E) vs. 10(F)). The negative correlations between *Oscillospira* and *Blautia*'s entire cluster FL3 (Figs 10(C) and 11(E)) are also evident, almost separating FL3 from the MCN. In ADHD *Blautia*'s cluster FL1 (gold) occupies a much more central

Table 28. *Lachnospiraceae* and *Ruminococcaceae* rankings.

Phylum	Class	Order	Family	Genus	Lowest Possible
Firmicutes (#2/#T2)	Clostridia (#T3/#T3)	Clostridiales (#T2/#3)	<i>Lachnospiraceae</i> (NR/NR)	Anaerostipes (#T19/NR)	Anaerostipes (#6/#T26)
				Blautia (#T16/#13)	Blautia 1 (NR/#19)
					Blautia 2 (#9/#T22)
				Coprococcus (NR/#5)	Coprococcus (#T21/#6)
				Dorea (#T14/#12)	Dorea 2 (#13/NR)
				Lachnospira (NR/#16)	Lachnospira (#T27/#5)
				Lachnospiraceae 1 (#19/#15)	Lachnospiraceae 1 (#17/#2)
				Lachnospiraceae 2 (#7/NR)	Lachnospiraceae 2 (#8/#14)
				Roseburia (NR/NR)	Roseburia 1 (NR/#T28)
					Roseburia 2(#T25/#T22)
				Ruminococcus (#1/#11)	R. gnavus (NR/#12)
				Ruminococcaceae (#4/#T8)	Faecalibacterium (#T16/#2)
				F. prausnitzii (#11/NR)	
				Oscillospira (#3/#10)	
				Oscillospira (#5/#9)	
	Ruminococcaceae (#8/#9)				
	Ruminococcaceae (#4/#18)				
	Ruminococcus (#2/NR)				
	Ruminococcus (#T21/NR)				

ATria rankings of *Lachnospiraceae* and *Ruminococcaceae* taxa. Dark orange = only ranked in Control, dark purple = only ranked in ADHD, light orange = higher ranked in Control, light purple = higher ranked in ADHD, grey = evenly ranked. **Bold** taxa are ranked #1.

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Table 29. Largest, consistently present clusters.

Cluster	Attribute	Observation
<i>BB</i>	Largest Bacteroidetes-dominant cluster	Larger in ADHD, with more internal cooperation and less external competition.
<i>FL</i> (1,2,...)	Multiple Firmicutes, family <i>Lachnospiraceae</i> -dominant clusters	One large, centrally located cluster emerges in ADHD (<i>FL1</i>). Others are small, about the same size, and more disconnected (all are this way in Control).
<i>FR</i>	Firmicutes, family <i>Ruminococcaceae</i> -dominant	Smaller and less centrally located in ADHD.
<i>FT</i>	Firmicutes, core member genus <i>Turicibacter</i>	Slightly larger in ADHD.

Clusters that we note as being consistently present in Control and ADHD MCNs, and observations.

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position (Fig 10(D) and 10(F)), with increased ADHD size particularly noticeable at the lowest level (Fig 10(F)).

ATria (Table 28) indicates a general increased importance of *Blautia*'s family (*Lachnospiraceae*) in ADHD, and *Oscillospira*'s family (*Ruminococcaceae*) in Control. A couple of noteworthy taxa follow this trend. Species *Faecalibacterium prausnitzii* (*Ruminococcaceae*), an anti-inflammatory bacterium [153] touted as a next-generation probiotic [154], is only ranked in Control. Species *Ruminococcus gnavus* (*Lachnospiraceae*), known to produce an inflammatory polysaccharide [155], is only ranked in ADHD.

Summary. Four clusters were consistently present in both Control and ADHD MCNs. Three are Firmicutes-dominant (*FL*, *FR*, *FT*) and one is Bacteroidetes-dominant (*BB*).

Table 29 shows their attributes, and summarizes observations we made about each.

Table 30 summarizes correlations between members of these clusters. Other than the one exception in ADHD involving genera *Streptococcus* and *Blautia*: *FT* is the only Firmicutes-dominant cluster with taxa positively correlated with Bacteroidetes-dominant cluster (*BB*) members, and all correlations involving *FL/FR* (largest Firmicute-dominant clusters) and *BB* taxa are negative. *FT* is completely disconnected from *FL/FR* except some ADHD competition. *FL-FR* competition only happens in Control.

Finally, we summarize taxa (Table 31) and relationships (Table 32) that we noted throughout our analyses.

Discussion

Traditional analysis methods (i.e. diversity and composition) prevalent in current ADHD gut microbiome literature provide a macroscale representation of a complex ecosystem.

Table 30. Cluster member interactions.

Cluster 1	Cluster 2	Observation
<i>BB</i>	<i>FL</i>	Always (-), with one exception in ADHD (<i>Streptococcus-Blautia</i>)
<i>BB</i>	<i>FR</i>	Always (-)
<i>BB*</i>	<i>FT</i>	Always (+)
<i>FL</i>	<i>FR</i>	Generally (+). Some (-) in Control (all involve either <i>Ruminococcus</i> (<i>FL</i>) or <i>Oscillospira</i> (<i>FR</i>))
<i>FL</i>	<i>FT</i>	Generally disconnected. Some (-) in ADHD (all involve <i>Phascolarcobacterium</i> (<i>FT</i>))
<i>FR</i>	<i>FT</i>	Always disconnected.

Interactions between taxa from Table 29 clusters (* = In Control, this took place with *BMI* after the *BB* "split").

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Table 31. Taxa noted throughout our analysis.

Level	Taxon	Observation
Genus	<i>Adlercruetzia</i>	Role change from Control (Bacteroidetes cooperation) to ADHD (<i>E. dolichum</i> cooperation). Competition with different Firmicutes (in ADHD).
Genus	<i>Bacteroides</i>	Centroid (with nearly 100% connectivity) of cluster <i>BB</i> in ADHD.
Genus	<i>Bifidobacterium</i>	Higher participation in Control (mostly competition). Competition with all Bacteroidetes or Proteobacteria taxa is entirely exclusive to Control, including multiple members and centroid of cluster <i>BB</i> . Cooperation (small amount) is entirely exclusive to ADHD. Ranked higher in Control than ADHD nearly 100% of the time, including a #1 ranking at the lowest taxonomic level.
Genus	<i>Coprobacillus</i>	DESeq2-elevated ADHD taxon, and ranked #1 for ADHD in two MCNs (genus and lowest possible). Competes with multiple <i>Lachnospiraceae</i> taxa, including the most abundant.
Family	<i>Enterobacteriaceae</i>	Involved with Firmicutes-Proteobacteria shifts. Only ranked in ADHD.
Species	<i>F. prausnitzii</i>	Probiotic species only ranked in Control
Family	<i>Lachnospiraceae</i>	Most abundant family, generally ranked higher in ADHD
Genus	<i>Phascolarctobacterium</i>	Only <i>FT</i> member connected to another Firmicute-dominant cluster (competition). Only ranked in ADHD.
Family	<i>Porphyromonadaceae</i>	#1 ADHD family, only cluster <i>BB</i> member to compete with <i>FL/FR</i> .
Family	<i>Rikenellaceae</i>	Previously reported as ADHD-elevated. Ranked by ATria as important in all six lower level MCNs, and always higher in ADHD.
Genus	<i>Ruminococcus [L]</i>	#1 Control genus, involved in <i>FL-FR</i> competition (only observed in Control).
Species	<i>R. gnavus</i>	Produces inflammatory biosaccharide, only ranked in ADHD
Family	<i>Ruminococcaceae</i>	Second-most abundant family, generally ranked higher in Control
Genus	<i>Turicibacter</i>	Reported as differentially abundant (Control) by three methods (LEfSe, DESeq and ALDEx2), core member (centroid in ADHD) of <i>FT</i> .

Summary of notable taxa throughout our ecological analyses, and observations.

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Table 32. Relationships noted throughout our analyses.

Taxonomic Level	Relationship(s)	Reason
Genus	<i>Bacteroides-Sutterella</i> (+, both)	Only core correlation consistent across both sample sets at all levels (12 MCNs).
Genus	<i>Bacteroides-Prevotella</i> (-, ADHD)	Only competition involving two Bacteroidetes taxa.
Genus	<i>Bacteroides-Streptococcus</i> (+, ADHD)	<i>Streptococcus</i> is one of only two Firmicutes genera to join cluster <i>BB</i> , through this connection.
Genus	<i>Butyricimonas-Clostridium</i> (+, ADHD)	<i>Butyricimonas</i> was only ranked by ATria in ADHD, and reported as ADHD differentially abundant by two methods (LEfSe and ALDEx2). <i>Clostridium</i> is one of only two Firmicutes to join cluster <i>BB</i> , through this connection.
Family-Genus	<i>Clostridiaceae-Odoribacter</i> (-, ADHD)	<i>Odoribacter</i> was reported as ADHD-elevated by LEfSe. In ADHD competes with <i>Clostridiaceae</i> taxa that compete with multiple cluster <i>BB</i> members (including its centroid). <i>Clostridiaceae</i> was reported as ADHD-reduced (Control-elevated) by ALDEx2.
Family-Genus	<i>Enterobacteriaceae-Oscillospira</i> (+, Control; -, ADHD)	Only correlation ever to change sign from Control to ADHD. Taxa involved are in the same cluster in Control.
Genus	<i>Blautia-Oscillospira</i> (-, Control) <i>Streptococcus-Blautia</i> (+, ADHD) <i>Streptococcus-Oscillospira</i> (-, ADHD)	<i>Blautia</i> is associated with obesity and <i>Oscillospira</i> with leanness. <i>Oscillospira</i> (<i>FR</i>) competes with every member of <i>Blautia</i> 's cluster (<i>FL3</i>) in Control. <i>FL-FR</i> competition only happens in Control. In ADHD <i>Streptococcus</i> cooperates with <i>Blautia</i> (obesity) and competes with <i>Oscillospira</i> (leanness) <i>Streptococcus-Blautia</i> is the only time a cluster <i>BB</i> member (largest Bacteroidetes-dominant) ever cooperates with taxa from <i>FL</i> or <i>FR</i> (largest Firmicutes-dominant, collectively over 70% of the population). <i>Streptococcus</i> is only ranked in ADHD, <i>Blautia</i> is ranked higher in ADHD, <i>Oscillospira</i> is ranked higher in Control. <i>Blautia</i> 's cluster (<i>FL1</i>) is larger and more central in ADHD. <i>Oscillospira</i> 's (<i>FR</i>) is larger and more central in Control.

Summary of notable relationships throughout our ecological analyses, and observations.

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Conducting some of these approaches on equal-sized, gender-balanced undergraduate Control and ADHD gut microbiome datasets produced many results that corresponded with this literature, plus a potentially new Control-elevated genus, *Turcibacter* (reported by LEfSe, DESeq2 and ALDEx2). Current literature, as well as our results, suggest this macroscale perspective leaves a largely incomplete picture due to its neglect of underlying complexity. Our goal was to complete more of this picture by venturing deeper, by analyzing two-way ecological relationships (cooperation and competition), plus community detection, and centrality.

Our results provide a deeper meaning to those from the macroscale. Previous differential abundance studies have reported collectively inconclusive results with respect to *Bifidobacterium* [65, 66] and *Bacteroides* [59, 65, 70]. We experienced similar issues with our taxa plots, where anomalous results involving elevated genus *Bifidobacterium* and reduced genera *Bacteroides* and *Sutterella* at ASRS extremes (high and low) imposed significant challenges when interpreting results. Our MCNs estimate that a Bacteroidetes-dominant community (cluster *BB*) forms in both microbiomes, with *Bacteroides* and *Sutterella* both core members, that in ADHD is larger and resides in conditions that favor its cooperation, as opposed to competition in Control. Several cluster *BB* members have previously been reported as ADHD-elevated, including *Butyricimonas* (reported by two of our differential abundance methods), *Parabacteroides* (for hyperactivity, [59]), *Bacteroides uniformis* [60], *Rikenellaceae* [66], and *Odoribacteriaceae* [68], plus two member descendants (*Bacteroides ovatus* and *Sutterella stercoricanis*, [60]), making its increased size, tightness, and more favorable environment observed in our ADHD MCN interesting. Moreover our MCNs estimate a shift in roles played by *Bacteroides* and *Bifidobacterium* between Control and ADHD microbiomes. In ADHD, *Bacteroides* was estimated as the centroid (driver) taxon for cluster *BB*, having cooperative with nearly every member. And *Bifidobacterium* shifted from exclusively competitive relationships with cluster *BB* members (including its most abundant and centroid) in Control, to exclusively a cooperative relationship in ADHD—with *Bacteroides uniformis*, one of the taxa previously reported as ADHD-elevated [60]. In addition to these relationships with cluster *BB* our MCNs estimated *Bifidobacterium* and its lineages to be much more active in Control gut ecology (24 relationships) compared to ADHD (9 relationships), with ATria ranking *Bifidobacterium* higher in Control more than 85% of the time. Overall, these results suggest potentially significant differential roles played by both *Bifidobacterium* and *Bacteroides* in the Control and ADHD gut ecosystems.

Potential roles played by taxa reported by our differential abundance analysis also became observable. Our MCNs estimated genus *Odoribacter*, reported by our analysis and another [68] as ADHD-elevated, to also compete with two family *Clostridiaceae* taxa, one of which (*Clostridiaceae 1*) was reported by ALDEx2 as ADHD-reduced (subsequently we found, by a factor of two). *Clostridiaceae 1*, in turn, was estimated to compete with multiple taxa from cluster *BB* (much larger in ADHD) in both of our MCNs. Exploring candidate metabolic reactions involved within this triangle may therefore also be important for future experimentation. *Butyricimonas*, reported by two of our differential analysis methods (LEfSe and ALDEx2) as ADHD-elevated, joined cluster *BB* in ADHD and provided the sole cooperative relationship with *Clostridium*, a taxon otherwise completely disconnected from both MCNs. *Coprobacillus*, reported by DESeq2 as ADHD-elevated, was ranked #1 twice by ATria in the ADHD MCN, which estimated competitive relationships with abundant *Lachnospiraceae* taxa, reported by another study as Control-elevated [68].

New interesting taxa and communities also emerged. Cluster *FT* (cooperative with cluster *BB*) was larger in ADHD. Cluster *FR* (Family *Ruminococcaceae*-dominant, competitive with cluster *BB*) was smaller in ADHD. *Ruminococcaceae* taxa (previously reported as ADHD-reduced [68]) were also almost universally less central in ADHD, including the probiotic *Faecalibacterium prausnitzii* (only ranked by ATria in Control), which was also reported as

ADHD-reduced in two other studies [61, 68]. Our MCNs also showed inflammatory polysaccharide *Ruminococcus gnavus* (only ranked by ATria in ADHD), to have ADHD-exclusive competition with *F. prausnitzii*. *Ruminococcaceae* member genus *Oscillospira* was also estimated to have ADHD-exclusive competition, with Proteobacteria family *Enterobacteriaceae* (cooperative and fellow *FR* member in Control), and cluster *BB* member Firmicute *Streptococcus*. In particular, the shift in relationship between *Enterobacteriaceae* and *Oscillospira* from cooperation (Control) to competition (ADHD), as well as the dynamic involved in the triangle composed of genera *Streptococcus*, *Blautia*, and *Oscillospira* (Table 32, last row), emerged as noteworthy potential targets for further exploration.

This exploration can add deeper meaning to these results through pursuit of additional studies targeting some of these taxa and relationships, including multi-omics [156] and/or physical laboratory experiments. Fundamentally, ecological relationships manifest through internal interplay within the underlying web of interactions [157]. Cooperation could take place for example if two taxa produce a nutrient that the other consumes; competition could take place if two taxa consume a nutrient that neither produces. Coupling taxa to metabolites they produce and consume and analyzing pathways can help elucidate underlying mechanisms behind these ecological relationships. These pathways can then be searched for neurotransmitters to establish ADHD connections. Clustering could also be performed by functional pathway, enabling a more direct association between microbial genes and specialized biological pathways involving neurotransmitters that influence ADHD.

While results from our analysis could be used as guidance for more in-depth analysis of any ADHD dataset (present or future), it is of course possible (and likely) that changes within attributes of the target population can impact ecological relationships. We are merely laying the groundwork, and with very few studies even attempting this level of analysis [66], an enormous breadth of knowledge remains and many future improvements to our analyses are possible. Published metadata for this dataset was somewhat limited. Future studies involving ADHD and the gut microbiome should account for factors such as BMI [158], demographics [159], ethnicity [160], use of medication/probiotics [61], use of antibiotics [161], diet [162] and gastrointestinal issues [163], age [164], and official diagnosis (beyond ASRS). Additionally although we did strengthen the threshold of our *p*-value for correlations in our MCNs, future work could incorporate multiple hypothesis corrections and sensitivity analysis for ATria for more thorough statistical relevance validation. More meaning to relationships in our MCNs can also be uncovered, through *causality* studies. Causality would give direction to edges, enabling detection of both two- and one-way (i.e. commensalism [139], amensalism [140]) relationships. This can be achieved through for example Bayesian Networks [165], which detect relationships where a taxon is conditionally dependent on another. Conditional dependence also eliminates spurious edges that can occur with correlations; for example, two entities that co-occur with a mutual entity will naturally tend to co-occur [116] (this was also a dependency removed by ATria after finding a central node). Sazal *et al.* [166] have already verified such networks as a predictor for oral microbiome colonization order. Time can also factor into ecological relationships because while sometimes these relationships are constant in microbiomes [167], they can also be transitive [168] or even time-varying [169]. DBNs that account for time have already been used to predict long-term infant gut behavior [170]. Higher-level network metrics such as modularity [171] and vulnerability [172] would provide another potential avenue for comparing and contrasting Control and ADHD MCNs. Amplicon Sequence Variants (ASVs, [173]) can be used in place of the current Operational Taxonomic Units (OTUs) that are generated by similarity-based clustering. ASVs exhibit more reliability at lower levels of the taxonomic tree and can improve the granularity of our MCNs, achieving more species- and sometimes even strain-level classifications.

Also given that ASRS does not offer an official diagnosis and this metadata did provide ASRS scores, an immediate future goal should be a migration from case-control to continuous data analysis with respect to ASRS. Our taxa plots, which observed abundances of core taxa with respect to ASRS score, have already started this process. Some of our other analysis protocols (i.e. alpha- and beta-diversity) will trivially propagate. While differential abundance analysis will ultimately be difficult without explicit sample classifications, networks have potential given some of the strategies mentioned above. For example, multi-omic network techniques such as marginal correlation analysis have already proven useful for correlating heterogeneous datasets [174], making them potentially applicable to estimating correlation between microbial abundance and ASRS scores. Applying Bayesian techniques to such networks could assist in estimating the conditional presence or absence of a microbe-microbe correlation on another dependence (i.e., high or low ASRS score).

A more complete understanding of ADHD and the gut microbiome will best equip the community to make the right decisions when administering treatment(s). Our results, coupled with those in the literature, suggest that the gut microbiota cannot afford to be ignored when it comes to ADHD, and treatments directly targeting the gut microbiome have potential. Encouraging results have been uncovered for gluten and casein-free diets [53], Microbiota Transfer Therapy (MTT, [175, 176]), and probiotics [177] with ASD. Our results also indicate that the gut microbiome is an ecosystem, and any changes to one single element will likely impact other members. Additionally since the human gut microbiome is widely varied across individuals [178], personalized medicine should be used when developing such treatments.

Supporting information

S1 Fig. Class-level taxa plot. Bar plot of taxa relative abundance, at the class level. Samples are ordered on the x -axis by increasing ASRS score.

(PNG)

S2 Fig. Order-level taxa plot. Bar plot of taxa relative abundance, at the order level. Samples are ordered on the x -axis by increasing ASRS score.

(PNG)

S3 Fig. Family-level taxa plot. Bar plot of taxa relative abundance, at the family level. Samples are ordered on the x -axis by increasing ASRS score.

(PNG)

S4 Fig. Taxa plot: lowest possible classification level. Bar plot of taxa relative abundance, using the lowest possible classification level. Samples are ordered on the x -axis by increasing ASRS score.

(PNG)

S5 Fig. *Bifidobacterium* abundances. Relative abundance plot of *Bifidobacterium*. Samples are ordered on the x -axis by increasing ASRS score.

(PNG)

S6 Fig. *Bacteroides* abundances. Relative abundance plot of *Bacteroides*. Samples are ordered on the x -axis by increasing ASRS score.

(PNG)

S7 Fig. *Sutterella* abundances. Relative abundance plot of *Sutterella*. Samples are ordered on the x -axis by increasing ASRS score.

(PNG)

S1 Table. Experimental and lab methods. Data obtained from the openly available BioProject (PRJNA656791), along with cited protocols [90–92].

(DOCX)

S2 Table. Data quality table. Average number of sequences retained after every preprocessing step.

(DOCX)

S3 Table. All correlations, all MCNs. Every correlation in all MCNs, grouped by taxonomy. Orange = only found in Control, purple = only found in ADHD, grey = found in both. +(green) = positive correlation, -(red) = negative correlation.

(DOCX)

S4 Table. All rankings, all MCNs. ATria rankings of all taxa over all MCNs, grouped by taxonomy. Dark orange = only ranked in Control, dark purple = only ranked in ADHD, light orange = higher ranked in Control, light purple = higher ranked in ADHD, grey = evenly ranked. **Bold** taxa are ranked #1.

(DOCX)

S5 Table. All family-level clusters. Communities within Control and ADHD family-level MCNs. Phylum: purple = Bacteroidetes, yellow = Firmicutes, brown = Actinobacteria, blue = Proteobacteria.

(DOCX)

S6 Table. All genus-level clusters. Communities within Control and ADHD genus-level MCNs. Phylum: purple = Bacteroidetes, yellow = Firmicutes, brown = Actinobacteria, blue = Proteobacteria.

(DOCX)

S7 Table. All clusters, lowest possible taxonomic classification. Communities within Control and ADHD MCNs using the lowest possible level of taxonomic classification. Phylum: purple = Bacteroidetes, yellow = Firmicutes, brown-Actinobacteria, blue = Proteobacteria.

(DOCX)

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