Gut metagenomics discriminates unique microbial signatures in diverse symptomatic profiles with attention-deficit/hyperactivity disorder

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Research

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Abstract

Background

Attention-deficit/hyperactivity disorder (ADHD) is a highly heterogeneous psychiatric disorder that can be divided into inattentive (I-ADHD), hyperactive-impulsive (HI-ADHD), and combined (C-ADHD) subtypes. Different early life events and environmental factors correlated with the gut microbiota community have been implicated in the development of ADHD. However, whether different ADHD symptomatic presentations are associated with distinct microbiota composition and function still unknown. Therefore, we carried out metagenomic analysis from 207 subjects to characterize the gut microbial profiles in ADHD and subgroup patients.

Results

The current study revealed that the gut microbiota composition (beta diversity) can be effectively distinguished between C-ADHD patients and HCs, but not I-ADHD patients and HCs, nor general ADHD patients and HCs. Features include underrepresentation of 8 species belonging to the genus *Bacteroides* and enrichment of 5 species of *Bifidobacterium* and *Prevotella* in general ADHD patients (all $p < 0.05$). Eight of the above species became progressively reduced (*ovatus*, *thetaiotaomicron*, *intestinalis*, *cellulosilyticus*, and *fluxus* belonging to the genus *Bacteroides*) or enriched (*Prevotella_copri*, *Prevotella_buccae* and *Bifidobacterium_breve*) from healthy controls (HCs) to I-ADHD and C-ADHD patients. Predicted metabolic functions from these distinguished gut microbial markers described a certain compensatory host metabolism in ADHD and subgroup patients. Particularly, pyridoxal 5'-phosphate (a dominant vitamin B6 active type) biosynthesis pathways were significantly reduced in C-ADHD patients, because serum vitamin B6 deficiency in ADHD patients was found previously. Of note, we identified diverse virulence factor and antibiotic resistance from the gut microbiota of ADHD patients. The abundance of antibiotic resistance ontology ANT(9)-Ia positively correlated with the abundance of *Prevotella_amnii*, which was enriched in ADHD patients. Moreover, species-based bacterial markers were used to construct classifiers and achieved a higher AUC of 0.87 in C-ADHD vs. HC than that in ADHD vs. HC (AUC = 0.84).

Conclusions

These findings uncover alterations in microbial composition in subgroup patients and provide potential biomarkers for diagnosis different symptomatic presentations for ADHD.

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Background

Attention deficit/hyperactivity disorder (ADHD), a childhood-onset psychiatric disorder, has a worldwide prevalence of approximately 5% [1, 2]. According to its syndromic profiles, this neurodevelopmental disorder can be divided into inattentive (I-ADHD), hyperactive-impulsive (HI-ADHD), and combined (C-ADHD) presentations [1, 3]. ADHD is associated with highly heterogeneous impairment in cognitive and social functions and may result in future poor lifetime outcomes such as academic failure and mental illness [1, 4]. It is important to highlight that the different ADHD symptomatic profiles are associated with diverse types and levels of negative outcomes [3]. Although gene–environment interactions are implicated in ADHD development [5, 6], the underlying pathophysiological mechanisms remain largely unknown.

Recent studies have provided growing evidences that gut microbiota dysbiosis in childhood or adulthood may increase the risk of psychiatric disorders, such as major depressive disorder [7], bipolar disorder [8], schizophrenia [9], autism spectrum disorder [10], and ADHD [11]. These results suggest a role of the gut microbiota in brain function and behavior and support communication between the gut and the brain (microbiota-gut-brain axis) [12, 13]. Of note, many environmental risk factors of ADHD development, such as cesarean delivery [14], formula feeding [15], antibiotic use [16], and diet style[17] are also associated with gut bacterial compositions.

Studies based on 16S rRNA sequencing have preliminarily distinguished different gut microbiota between ADHD patients and healthy controls (HCs) from several small size cohorts [18–22]. However, no studies have been carried out to compare bacterial variation between patients with different ADHD symptomatic profiles and HCs. Since there are highly heterogeneous neurobehavioral deficits among patients with different ADHD symptomatic profiles, we might lose relevant specificities when trying to distinguish the bacterial taxa between a mixed and heterogeneous group of ADHD patients and HCs. In addition, the 16S rRNA sequencing approach may omit some key information owing to the limited taxonomic and functional resolution level.

Therefore, in this study, metagenomics sequencing was performed to discriminate microbial composition and function between subgroups of patients with ADHD. The identified gut microbial biomarkers require further evaluation for its diagnostic performance for classifying different symptomatic profiles of patients with ADHD.

Results

Demographic and clinical characteristics of the recruited participants

A total of 207 children and adolescents were recruited, including 98 ADHD patients (38 I-ADHD, 53 C-ADHD, 7 HI-ADHD) and 109 HCs. As the number of subjects with HI-ADHD was too low to compare with other subgroups and HC, ultimately, 38 I-ADHD, 53 C-ADHD and 109 HC were included in the subgroup analysis. The general demographic characteristics from the recruited subjects are displayed in Table 1 (I-

ADHD, C-ADHD and HC), Table S1 (Supporting Information, ADHD and HC), and Table S11 (Supporting Information). There were no significant differences among the three groups in age, body mass index (BMI), premature birth, maternal pregnancy with metabolic disease, or antibiotic use during pregnancy or infancy. The proportion of male subjects among C-ADHD patients was higher than that among HCs (98.1% vs 81.7%, $p = 0.007$). The proportion of only children among C-ADHD patients was lower than that among HCs (39.6% vs 65.1%, $p = 0.007$). The percent of cesarean deliveries among I-ADHD patients was lower than that among HCs (36.8% vs 60.6%, $p = 0.023$). Furthermore, low birth weight occurred more often in C-ADHD patients than in HCs (20.8% vs 5.5%, $p = 0.012$). Patients in I-ADHD and C-ADHD had lower scores on the intelligence quotient (IQ) ($p < 0.001$). Predictably, all ADHD diagnostic indices, including the Diagnostic and Statistical Manual of Mental Disorders_Attention Deficits (DSM_AD) scores, DSM_Hyperactivity/Impulsivity Deficits (DSM_HD) scores, and total Conners Parent Rating Scales (CPRS) scores, were higher in I-ADHD and C-ADHD patients.

Table 1
The baseline characteristics of ADHD subgroup patients (I-ADHD = 38; C-ADHD = 53) and HCs (n = 109) in the study cohort.

Characteristics	I-ADHD (n = 38)	C-ADHD (n = 53)	HC (n = 109)	*p value
Age, years, mean ± S.D.	9.4 ± 2.1	8.8 ± 1.9	8.9 ± 1.8	0.372
BMI, kg/m^2, mean ± S.D.	17.3 ± 3.9	16.9 ± 2.7	17.1 ± 4.3	0.737
Male, No. (%)	33 (86.8)	52 (98.1)	89 (81.7)	0.007[b]
IQ, mean ± S.D.	103.0 ± 14.5	101.9 ± 13.5	112.0 ± 13.5	4.399E-05[ab]
Only child, No. (%)	19 (50.0)	21 (39.6)	71 (65.1)	0.007[b]
Maternal pregnancy with metabolic disease, No. (%)	0 (0)	2 (3.8)	2 (1.8)	0.645
Cesarean section, No. (%)	14 (36.8)	24 (45.3)	66 (60.6)	0.023[a]
Premature birth, < 37 weeks, No. (%)	2 (5.3)	4 (7.5)	6 (5.5)	0.920
Low birth weight, < 2.5 kg, No. (%)	5 (13.2)	11 (20.8)	6 (5.5)	0.012[b]
Maternal antibiotic use during pregnancy, No. (%)	2 (5.3)	0 (0)	1 (0.9)	0.151
Antibiotic use during infancy, No. (%)	5 (13.2)	4 (7.5)	8 (7.3)	0.510
DSM_AD scores, mean ± S.D.	6.6 ± 0.9	7.5 ± 1.2	1.6 ± 1.1	4.728E-34[abc]
DSM_HD scores, mean ± S.D.	1.9 ± 1.1	7.0 ± 1.6	0.9 ± 0.9	2.010E-29[abc]
Total CPRS scores	4.2 ± 1.7	6.4 ± 2.5	1.7 ± 1.1	6.902E-28[abc]

S.D., standard deviation; BMI, body mass index; IQ, intelligence quotient; DSM, the diagnostic and statistical manual of mental disorders; DSM_AD, DSM attention deficits; DSM_HD, DSM hyperactivity/impulsivity deficits; CPRS, Conners' Parent Rating Scales. *p value based on the Kruskal-Wallis test (continuous variables, or the Wilcoxon rank-sum test for two groups) or Fisher's exact test (categorical variables) for all groups. [a]$p < 0.05$ for I-ADHD and HC; [b]$p < 0.05$ for C-ADHD and HC; [c]$p < 0.05$ for I-ADHD and C-ADHD.

Characteristics	I-ADHD (n = 38)	C-ADHD (n = 53)	HC (n = 109)	*p value
Rutter types, No. (%)	9 (23.7)	34 (64.2)	2 (1.8)	1.464E-06[bc]
A	12 (31.6)	3 (5.7)	10 (9.2)	
N	5 (13.2)	7 (13.2)	4 (3.7)	
M				

S.D., standard deviation; BMI, body mass index; IQ, intelligence quotient; DSM, the diagnostic and statistical manual of mental disorders; DSM_AD, DSM attention deficits; DSM_HD, DSM hyperactivity/impulsivity deficits; CPRS, Conners' Parent Rating Scales. *p value based on the Kruskal-Wallis test (continuous variables, or the Wilcoxon rank-sum test for two groups) or Fisher's exact test (categorical variables) for all groups. [a]$p < 0.05$ for I-ADHD and HC; [b]$p < 0.05$ for C-ADHD and HC; [c]$p < 0.05$ for I-ADHD and C-ADHD.

The dietary and defecation habits among I-ADHD, C-ADHD and HC are listed in Table S2 and Table S3 (Supporting Information). We found no significant differences among the three groups in all dietary and defecation habits, including infant feeding, the preference of side dishes, staple foods, yogurt and other fermented food, and defecation frequency, smoothing, and shape.

Fecal microbiome diversity in ADHD and subgroup patients

The alpha diversity of gut microbiota was estimated between ADHD or subgroup patients and HCs. We found significantly lower gene numbers in ADHD patients than in HCs ($p = 0.042$), though the gut microbiota richness was similar between the two groups (Shannon index, $p = 0.076$, Fig. 1a). Subgroup gene numbers demonstrated that there was lower dissimilarity between I-ADHD and HC ($p = 0.11$) than between C-ADHD and HC ($p = 0.017$, Fig. 1b). However, alpha diversity analysis revealed no significant difference between any two groups among C-ADHD, I-ADHD and HC.

To assess whether the gut microbiota can be effectively distinguished between the groups, supervised analysis with spare PLS-DA (sPLS-DA) and permutational multivariate analysis of variance (PERMANOVA) were performed at the gene family level. From the sPLS-DA results, samples of ADHD patients and HCs were clustered into two groups, though the PERMANOVA results suggested that the microbial composition was not significantly different between the two groups ($p = 0.227$, Fig. 1c). However, there was a significant dissimilarity between C-ADHD and HC ($p = 0.020$, Fig. 1d). No significant findings were obtained between I-ADHD vs. HC ($p = 0.134$) and I-ADHD vs. C-ADHD ($p = 0.519$). Together, these results suggest greater gut microbiota variation between C-ADHD and HC than between ADHD and HC.

PERMANOVA was performed to explore the influence of other host characteristics on gut microbiota. In addition to the ADHD subgroups, sex and IQ were also significantly associated with gut microbial composition (Table S4, Supporting Information). Of note, there was a higher proportion of male subjects among C-ADHD subgroup than that among HCs. To address whether the gut microbiota differences

found in Fig. 1 just translate sex difference between two groups, we further performed analyses stratified for males. The gut microbiota richness (Shannon index, $p = 0.076$) in male subjects between ADHD and HCs was the same as mixed sex analysis. The gene numbers ($p = 0.057$) and gut microbiota richness (Shannon index, $p = 0.076$) was similar in male subjects between ADHD patients and HCs (Figure S1a). The gene numbers ($p = 0.017$) and gut microbiota richness (Shannon index, $p = 0.076$) in male subjects between C-ADHD and HC were similar to mixed sex analyses (Figure S1b). PERMANOVA results showed a more significant difference in male samples between C-ADHD and HC ($p < 0.001$, Figure S1d), compared with ADHD and HC ($p = 0.028$, Figure S1c). Therefore, unmatched sex here didn't alter the outcome that greater gut microbiota variation between C-ADHD and HC than between ADHD and HC.

ADHD and subgroups are associated with distinct microbial compositions

Metagenomic sequencing analysis (linear discriminant analysis effect size (LEfSe)) identified 12 bacterial taxa enriched in ADHD patients and 33 enriched in HCs (Fig. 2a). At the bacterial phylum level, *Bacteroidete*s, *Firmicutes*, *Proteobacteria* and *Actinobacteria* were the most abundant taxa in both groups (Table S5, Supporting Information). The HCs displayed a higher abundance of *Fusobacteria* than the ADHD patients. At the order and family levels, the relative abundances of members of the orders *Fusobacteriales*, *Flavobacteriales*, *Rhodocyclales* and *Sphigomonadales* and of the families *Fusobacteriaceae*, *Flavobacteriaceae*, *Rhodocyclaceae*, *Sphigomonadaceae* and *Bacillales_noname* were lower in the ADHD patients than in the HCs, whereas the relative abundance of members of the family *Prevotellaceae* was higher in the ADHD patients. At the species level, eight of the top 18 HC-enriched species (*ovatus*, *fragilis*, *thetaiotaomicron*, *intestinalis*, *cellulosilyticus*, *salyersiae*, *fluxus*, and *nordii*) belonged to the genus *Bacteroides*. Species in *Bifidobacterium* (*breve* and *bifidum*) and *Prevotella* (*amnii*, *buccae* and *copri*) were more abundant in the ADHD patients than in the HCs. A cladogram of significantly different taxa is shown in Fig. 2b, and an overview of the relative abundance of different bacteria at the genus level between ADHD patients and HCs is shown in Table S6 (Supporting Information).

We next compared the gut microbial compositions in subgroups of patients with ADHD and HCs. Detailed information on the significantly different bacterial taxa among I-ADHD, C-ADHD and HC is shown in Table S7 and Table S8 in the Supporting Information. We compared the numbers of significantly different bacterial taxa among the groups, which were, from more to less in proper order, C-ADHD vs. HC (70), I-ADHD vs. C-ADHD (34), and I-ADHD vs. HC (24) (Fig. 3a). Moreover, we found that the bacterial difference patterns in C-ADHD vs. HC and C-ADHD vs. I-ADHD were largely shared (Fig. 3a, b). Thirteen gut bacterial taxa abundant in HC compared with C-ADHD were also enriched in I-ADHD compared with C-ADHD. These shared abundances in I-ADHD and HC were *Rhizobiales*, *Oscillospiraceae*, *Bilophila*, *Oscillibacter*, *Subdoligranulum*, *Bacteroides_cellulosilyticus*, *Bacteroides_fluxus*, *Bacteroides_nordii*, *Bacteroides_ovatus*, *Lachnospiraceae_bacterium*, *Bilophila_wadsworthia*, *Oscillibacter_unclassified*, and *Subdoligranulum_unclassified*. The other 5 bacterial taxa (*Listeriaceae*, *Prevotellaceae*, *Veillonellaceae*, *Listeria*, *Listeria_marthii*) were highly enriched in C-ADHD compared with I-ADHD or HC. The specific

different bacterial taxa between I-ADHD vs. HC, C-ADHD vs. HC and I-ADHD vs. C-ADHD are shown in Figure S2 (Supporting Information). Together, these data provided initiatory evidence of greater dissimilarity in gut microbial composition between C-ADHD vs. HC than between I-ADHD vs. HC. Notably, C-ADHD patients can be distinguished from I-ADHD patients by different gut microbiota profiles.

In addition, the Jonckheere-Terpstra test identified a progressive scarcity or prevalence from HCs to I-ADHD and C-ADHD patients (Fig. 3c). We found that *Prevotella_copri*, *Prevotella_buccae* and *Bifidobacterium_breve* were progressively enriched from HC to I-ADHD and C-ADHD, while progressively reduced enrichment of the species *ovatus*, *thetaiotaomicron*, *intestinalis*, *cellulosilyticus*, and *fluxus* belonging to the genus *Bacteroides* were also identified. Therefore, these results suggest that the progressively increased or reduced enrichment of microbial taxa may be associated with the severity of ADHD development.

Distinct bacterial functional profiles in ADHD and subgroups

Microbial metagenomic sequencing data were used to predict discrepancy in functional metabolic pathways in ADHD and subgroup patients. Altogether, 362 pathways from the MetaCyc metabolic pathway database that presented in more than 10% of samples were identified and analyzed. A total of 9 pathways were significantly different between ADHD patients and HCs ($p < 0.05$, LDA score > 2; Fig. 4a). LEfSe revealed that only bacterial xylose degradation IV was significantly enriched in the ADHD patients, while bacterial pathways for inosine-5'-phosphate biosynthesis II, flavin biosynthesis III (fungi), L-phenylalanine degradation IV, adenine and adenosine salvage III, starch degradation V, hydrogen production VIII, purine ribonucleoside degradation and L-rhamnose degradation I were significantly reduced in the ADHD patients.

Subsequently, the diversity of functional pathways in ADHD subgroups was identified. The C-ADHD subgroup had predicted enrichment in bacterial pathways for S-adenosyl-L-methionine cycle I and xylose degradation and reduction in GDP-mannose-derived O-antigen building blocks biosynthesis, pantothenate and coenzyme A biosynthesis III, L-histidine biosynthesis, L-arginine biosynthesis III, L-rhamnose degradation I, flavin biosynthesis III, inosine-5'-phosphate biosynthesis I, pyridoxal 5'-phosphate biosynthesis and salvage and NAD salvage pathway I, compared to HC (Fig. 4b). In the I-ADHD subgroup, the predicted bacterial pathways of L-lysine, L-threonine and L-methionine biosynthesis I, anhydromuropeptide recycling, tetrahydrofolate biosynthesis and salvage and heme biosynthesis from glycine were increased, whereas pyrimidine ribonucleotide de novo biosynthesis, purine ribonucleoside degradation, GDP-mannose biosynthesis and stachyose degradation were decreased compared to those in the HC. Of note, the Venn diagram showed zero overlap in diverse pathway profiles in C-ADHD vs. HC and I-ADHD vs. HC (Fig. 4c), suggesting distinct gut bacterial functions in ADHD subgroups. The specific different bacterial functional pathways between I-ADHD vs. HC, C-ADHD vs. HC and I-ADHD vs. C-ADHD are shown in Figure S3 (Supporting Information).

The result of association analysis between metabolic pathways and gut microbial composition demonstrated that there were 14 significantly different species associated with metabolic pathways. We

found that *Prevotella_copri*, which was enriched in C-ADHD, was positively associated with enrichment in the bacterial S-adenosyl-L-methionine cycle I pathway compared with HC. There was an overrepresentation of *Bifidobacterium_longum*, *Bifidobacterium_pseudocatenulatum* and *Bifidobacterium_catenulatum* in I-ADHD, which was positively correlated with the increased superpathway of L-lysine, L-threonine and L-methionine biosynthesis I and the superpathway of pyrimidine ribonucleoside degradation compared with C-ADHD or HC. *Bacteroides_ovatus*, *Bacteroides_nordii*, *Lachnospiraceae_bacterium*_5_1_57FAA and *Lachnospiraceae_bacterium*_7_15_8FAA enriched in both I-ADHD and HC were positively associated with enrichment of the superpathway of GDP-mannose-derived O-antigen building blocks biosynthesis in these patients (Fig. 5). These findings suggest that there was a certain agreement in the variation tendency between intestinal species abundance and its metabolic functions.

ADHD patients show diverse virulence factor and antibiotic resistance in their gut microbiota

Metagenomic sequencing analysis provides the chance to study the gut bacterial virulence factor and antibiotic resistance in the patients with ADHD on a large size. We did not find significantly differences in the numbers of virulence factor ($p = 0.064$) and antibiotic resistance ontology (ARO) ($p = 0.9$) between ADHD patients and HCs (Figure S4a, Supporting Information). Subgroup virulence factor and ARO numbers demonstrated that there was also no significant difference between any two groups among C-ADHD, I-ADHD and HC (Figure S4b, Supporting Information). Moreover, we found the increased abundance of 7 and 2 virulence factors in ADHD patients and HCs, respectively (Figure S4c and Table S9, Supporting Information). However, none of these virulence factors was associated with gut microbial taxonomy abundance.

We also identified the overrepresentation of 3 and 4 ARO in ADHD patients and HCs, respectively (Fig. 6a and Table S10, Supporting Information). Interestingly, the abundance of ARO CcrA correlated with the abundance of *Bacteroides_ fragilis*, which was enriched in HCs. The enrichment of ARO ANT(9)-Ia correlated with the abundance of *Prevotella_amnii*, which was overrepresented in ADHD patients (Fig. 6b).

Gut microbial taxa associated with ADHD clinical characteristics

The associations of gut microbial compositions and clinical symptoms in ADHD were also assessed. Spearman's rank correlation analyses showed significant correlations of bacterial species with symptom severity scores (Fig. 7). The abundant species in C-ADHD compared with HC, *Prevotella_buccae*, *Bifidobacterium_breve*, and *Bifidobacterium_bifidum* were enriched and positively associated with the scores of both total CPRS and DSM. Interestingly, several species belonging to the genus *Bacteroides* that were abundant in HCs were negatively correlated with DSM_HD and/or DSM_AD scores. We found that increased relative abundances of *Bacteroides_nordii, Bacteroides_cellulosilyticus* and *Bacteroides_intestinalis* were associated with fewer symptoms in both hyperactivity/impulsivity

(DSM_HD scores) and inattention (DSM_AD scores). While *Bacteroides_thetaiotaomicron* and *Bacteroides_ovatus* were negatively associated only with DSM_AD scores.

ADHD classification based on gut microbiota profiles

Random forest (RF) classifications were constructed based on the species level. The relative abundance of 6 bacterial species (Figure S5a, Supporting Information) distinguished the ADHD patients from the HCs (Fig. 8a; AUC = 0.84, 95% CI 0.78–0.89). For ADHD subgroup classifications, 8 selected species (Figure S5b, Supporting Information) distinguished the C-ADHD patients from HCs (Fig. 8b) with an AUC of 0.87 (95% CI 0.80–0.93), while the relative abundance of 35 bacterial species (Figure S5c, Supporting Information) distinguished the I-ADHD and C-ADHD subgroups (Fig. 8c; AUC = 0.76, 95% CI 0.65–0.86). However, no species was found that could be used as marker to distinguish I-ADHD patients and HCs.

These results suggest that the classifier was able to differentiate ADHD or C-ADHD patients from HCs with good performance. Differentiation effect of the outcome, in turn, was C-ADHD vs. HC, ADHD vs. HC, I-ADHD vs. C-ADHD, and I-ADHD vs. HC. Therefore, the gut microbial variation in C-ADHD subgroup patients was greater than that in ADHD patients compared with HCs. In addition, the bacterial difference between two ADHD subgroups, C-ADHD and I-ADHD, was striking.

Discussion

In the present study, we characterized the gut bacterial metagenomic profiles in ADHD as a general group and its two major symptomatic presentations. We also identified several microbial taxa that were associated with the clinical parameters and severity of ADHD. Moreover, we built a bacterial species-based classification, which exhibits a higher AUC in C-ADHD versus HC than that in general ADHD patients vs. HCs. These findings not only confirm alterations in the gut microbiome composition of ADHD patients [18–21] but more importantly provide new microbial biomarkers to discriminate subgroups of ADHD.

The metagenomic data here may not directly compare with those results from 16S rRNA sequencing analysis [18–22]. Nevertheless, our findings demonstrated gut dysbiosis in patients with ADHD compared with HCs, which was consistent with previous studies. We found a significantly lower gene number in ADHD or C-ADHD patients than in HCs, though the gut microbiota richness, namely, alpha diversity, was similar between any two groups. There were also no significant differences in alpha diversity between ADHD patients and HCs in the Dutch [18] and Chinese (Zhejiang and Beijing) cohorts [19, 23]. However, two other cohorts from Germany [20] and China (Taiwan) [21], showed reduced or increased alpha diversity in ADHD patients, respectively. Regarding beta diversity, the current study revealed that the gut microbiota can be effectively distinguished between C-ADHD patients and HCs, but not between ADHD patients and HCs. We further found much dissimilarity between C-ADHD patients and HCs than comparing patients with ADHD as whole group and HCs. Of note, these results indicated that the analysis between ADHD subgroups and HC could reveal more significant bacterial diversity, which may be attenuated by comparing total ADHD patients and HCs.

Notably, the reported distinct bacterial taxa between ADHD patients and HCs were highly inconsistent in previous studies [18–23]. The disturbed bacterial taxa in one cohort may never be replicated or may be opposite in abundance in another cohort. This diversity might reflect the cohort differences in age, sex, region, diet, medication use, early life environment, maternal health, and cesarean delivery, since all these factors could affect gut microbial composition. Here, we found underrepresentation of 8 species (*ovatus*, *fragilis*, *thetaiotaomicron*, *intestinalis*, *cellulosilyticus*, *salyersiae*, *fluxus*, and *nordii*) belonging to the genus *Bacteroides* in ADHD patients. Members of the genus *Bacteroides* are usually beneficial for gut function and are correlated with neurodevelopment [24, 25]. In addition, species in *Bifidobacterium* (*breve* and *bifidum*) and *Prevotella* (*amnii*, *buccae* and *copri*) were more abundant in the ADHD patients than that in the HCs. Although we did not find considerable overlap with the previously reported microbial signature of ADHD, the increased *Bifidobacterium_breve* and *Bifidobacterium_bifidum* agreed with the results from the Dutch cohort study [18]. The genus *Bifidobacterium* has been reported to affect the level of enzymes associated with the synthesis of phenylalanine, the precursor of dopamine [18, 26]. Dopamine, an important monoaminergic neurotransmitter, has been implicated in the pathophysiology of ADHD [27, 28].

In terms of ADHD subgroups, the numbers of distinct bacterial taxa from more to less in proper order were C-ADHD vs. HC, I-ADHD vs. C-ADHD, and I-ADHD vs. HC. The bacterial difference patterns between C-ADHD vs. HC and I-ADHD vs. C-ADHD were largely shared. We found a slight bacterial difference between I-ADHD patients and HCs, and we speculate that relatively slight symptoms in I-ADHD may be involved in this difference. Thus, we identified several progressively enriched microbial taxa from HC to I-ADHD and C-ADHD, which is consistent with the clinical severity of ADHD [3, 29]. Interestingly, the enrichment of the species *Prevotella_copri*, *Prevotella_buccae* and *Bifidobacterium_breve* progressively increased, while *ovatus*, *thetaiotaomicron*, *intestinalis*, *cellulosilyticus* and *fluxus* belonging to the genus *Bacteroides* progressively decreased from HCs to I-ADHD and C-ADHD patients. Moreover, these species in *Prevotella*, *Bifidobacterium* and *Bacteroides* were also associated with hyperactivity/impulsivity and/or inattention symptoms. Taken together, these results further suggest a more significant dissimilarity in C-ADHD vs. HC compared with ADHD vs. HC. Moreover, there were distinguished gut microbial patterns in the I-ADHD and C-ADHD subgroup patients. We can obtain more accurate gut microbial information from subgroup analysis, which is potentially helpful in the diagnosis of ADHD subtypes.

The functional metabolic pathways predicted from fecal metagenomic analysis exhibited additional divergence in ADHD and subgroup patients. We found that functions correlated with energy regulation in host metabolism, including inosine-5'-phosphate biosynthesis, flavin biosynthesis, adenine and adenosine salvage and purine ribonucleoside degradation, were reduced in ADHD patients. However, it is unclear whether these alterations contribute to abnormal host symptoms, though aberrant brain energy metabolism is involved in some psychiatric disorders [30]. The starch degradation, hydrogen production and rhamnose degradation pathways were also predicted to be decreased in ADHD patients, and these metabolic pathways were associated with host gut functions [31–33]. Moreover, relatively enriched xylose degradation and reduced phenylalanine degradation pathways in ADHD patients are particularly striking. The disturbance of xylose metabolism is implicated in *Drosophila* hyperactivity behavior [34].

Phenylalanine is the precursor of dopamine, which is well studied and known as a dominant neurotransmitter deficit in ADHD pathophysiology. Although very few functional analyses predicted bacterial profiles in previous studies, the increased levels of predicted cyclohexadienyl dehydratase, responsible for phenylalanine synthesis, from the study of the Dutch cohort [18] and the current data together suggest a critical role of abnormal phenylalanine metabolism in ADHD patients. We hypothesized that insufficient dopamine signals in the brain induce potentially compensated precursor production through the gut microbiota.

The functional analysis in subgroups showed some shared and distinct differential metabolic pathways in C-ADHD patients compared with ADHD patients. Notably, arginine and pyridoxal 5'-phosphate biosynthesis pathways were specifically reduced in C-ADHD patients. Arginine, a precursor of nitric oxide, is related to better memory [35] and improved intestinal inflammation [36]. Pyridoxal 5'-phosphate is a dominant vitamin B6 active type, and serum vitamin B6 was decreased in ADHD patients [37]. Moreover, there was no overlap in diverse pathways between C-ADHD vs. HC and I-ADHD vs. HC, suggesting distinct gut bacterial functions in ADHD subgroups.

Growing number of studies describe gastrointestinal issues and early life antibiotic exposure in ADHD patients [16, 38], elements that are associated with gut bacterial community. Therefore, we further analyzed the virulence factor and antibiotic resistance in ADHD patients' gut microbiota. The abundance of ARO ANT(9)-Ia correlated with the abundance of *Prevotella_amnii*, which was enriched in ADHD patients. Virulence factor and antibiotic resistance analyses could provide more integrated information for gut microbiota functions [39] although their significance validation was required in further studies.

Of note, the current study is the first to characterize the gut microbial community not only in ADHD patients but also in the I-ADHD and C-ADHD patient subgroups. We obtained a satisfactory classification with robust efficacy for distinguishing ADHD or C-ADHD patients from HCs. Here, we used species-based bacterial markers to calculate the AUC and achieved ideal values between ADHD vs. HC (0.84) and C-ADHD vs. HC (0.87). However, the AUC between I-ADHD and C-ADHD (0.76) indicated weak discriminatory validity. Therefore, these data further suggest that gut bacterial composition analysis in subgroup patients would obtain better differentiation. The similar bacterial patterns between I-ADHD patients and HCs may underline the smaller difference in ADHD vs. HCs than in C-ADHD vs. HCs. Together, the species used in the classification were potential microbiota markers for ADHD diagnosis, although further validation was required in larger cohorts.

The main strengths of the present study include metagenomic sequencing, subgroup analyses and larger sample size. Secondly, gut microbial virulence factor and antibiotic resistance were analyzed. Moreover, only medication-naïve patients with ADHD were recruited in the current study to exclude the effects of medication on gut microbiota. The same applies to psychiatric comorbidity that was also excluded. Given that the gut microbiome composition is highly correlated with diet pattern, a questionnaire including diet and defecation habits was collected from individual participants to assess dietary differences between groups. There are also some limitations in this study. First, our study could not obtain distinct gut profile

information for HI-ADHD patients, due to the lack of enough HI-ADHD cases. Second, in line with a real-world scenario, the groups were not completely matched by sample size, sex, only child status, percentage of cesarean section and low birth weight. Although we found unmatched sex didn't alter the outcome that greater gut microbiota variation between C-ADHD and HC than between ADHD and HC. Third, we performed a cross-sectional study, and further longitudinal work should be conducted to further assess age- or medication-associated gut microbiota variation.

Conclusions

Our study characterized the distinct gut microbiota panel in ADHD and its subgroups of patients. We found more gut microbial alterations in C-ADHD patients than that in I-ADHD patients. We also identified several progressively enriched or decreased microbial taxa from HC to I-ADHD and C-ADHD, which is consistent with the clinical severity of ADHD. The predicted functional metabolic pathways according to these distinct bacterial taxa suggest compensatory host metabolism in ADHD patients. The current study provides new evidence to support that the microbiota-gut-brain axis is associated with ADHD. Further studies are required to evaluate these microbiota biomarkers in both ADHD and its phenotypically different clinical presentations.

Methods

Subjects

A total of 207 Chinese children and adolescents were recruited, including 98 ADHD patients (Y = 9.0 years, SD = 2.0) and 109 HCs (Y = 8.9 years, SD = 1.8). All case and control samples were collected at Xijing Hospital, Shaanxi, China, between March 20, 2018, and February 27, 2020. ADHD patients were diagnosed and grouped using integrated visual and auditory continuous performance test (IVA-CPT) and structured diagnostic interview conducted according to the clinical Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV) by two experienced child psychiatrists. The numbers of the three different subgroups were 38 I-ADHD, 53 C-ADHD and 7 HI-ADHD, respectively. ADHD patients and HCs had to meet the following criteria: (1) age between 6 and 15 years old; (2) no use of any antibiotic treatment for at least three months before sample collection; and (3) no history of treatment with any medication for ADHD. The participants who had other psychiatric or neurological diseases were excluded. Children and adolescents whose IQs were below 70 according to the Wechsler Intelligence Scale for Children (WISC-V, http://wiscv.com/) were also excluded. In particular, in this study, a questionnaire including diet and defecation habits was collected from individual participants to assess dietary differences between the three groups. All of the participants provided written informed consent, and the study was approved by the ethics committee of Xijing Hospital, Fourth Military Medical University (ID: KY20182002-1). The present study was registered at ClinicalTrials.gov (ID: NCT03447223).

DNA extraction and library construction

Fresh feces (approximately 0.5 g) were immediately moved into a sterilized collection tube by a sterilized wood stick from a clean toilet (MGI, China) by the parents of ADHD patients and HCs. The preservation method includes a reagent containing imidazolium-based ionic liquid [40]. After transport on dry ice, fecal samples were stored at -80 °C until DNA extraction. Total bacterial DNA from each fecal sample was extracted from ~ 200 mg of stool with the NucleoSpin® Soil kit (Macherey-Nagel, Germany) according the manufacturer's instructions. Qubit (Invitrogen, USA) and 1% agarose gel electrophoresis were used to analyze the quality of DNA. The details of DNA library construction were as follows: 1 μg genomic DNA was randomly fragmented by Covaris (Covaris, USA), and the fragmented DNA samples were tested by gel electrophotometry and then purified by a kit. The fragmented DNAs were combined with End Repair Mix, incubated at 20 °C for 30 min and then purified by a kit. The repaired DNAs were combined with A-Tailing Mix and incubated at 37 ℃ for 30 min. Illumina adaptors were ligated to the Adenylated 3'Ends DNA, incubated at 16 °C for 16 h, and then purified with a kit. After selecting the correct insert size DNA fragments, several rounds of PCR amplification with PCR Primer Cocktail and PCR Master Mix were performed to enrich the adapter-ligated DNA fragments. The AxyPrePTM Mag PCR Clean-up kit (Axygen, USA) was used to purify DNA in all steps of DNA library construction. The final DNA libraries were assessed for the average insert size using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA) and quantified by an ABI StepOnePlus Real-Time PCR system (Applied Biosystems, USA).

Metagenomic sequencing

Samples were sequenced on the Illumina HiSeq X Ten platform with an insert size of 300 bp (paired end, 150 base pairs). Before further bioinformatic analysis, raw reads containing adaptor sequence, low quality (lower Q-score 20 rate more than 50%) and ambiguous bases (N base rate more than 5%) were filtered out with SOAPnuke [41], and 1,486.5 Gb of high-quality PE reads for the 207 samples were acquired with an average of 7.18 Gb per sample (Table S12, Supporting Information). To remove human host DNA contamination, reads were aligned to the human genome reference (hg19) by SOAPaligner [42] (v2.22, parameters: -m 280 -x 420 -r 1 -l 32 -s 75 -c 0.9), and the mapping reads were discarded. The average rate of host contamination was 1.25 ± 5.23%.

Metagenomic microbial community and functional profiling

The profile of microbial composition for each sample was calculated using MetaPhlAn2 (v2.0) [43], which uses ~ 1 M unique clade-specific marker genes (including bacterial, archaeal, viral and eukaryotic) to estimate the relative abundance of bacterial taxa. The parameters of MetaPhlAn2 were set as '–nproc 10 --stat avg_g –ignore_viruses –ignore_eukaryotes –ignore_archaea'. Then, all sample profiles were merged using merge_metaphlan_tables.py.

Functional profiling for each sample was performed using HUMAnN2 [44] (v0.11.2). In brief, HUMAnN2 rapidly identified known microbial species in samples with MetaPhlAn2 and then constructed a customized pangenome database in which all genomes have been preconstructed and functionally annotated. Sample reads were mapped to this database with Bowtie2 [45], with the unmapped reads translated and mapped to a protein database (UniRef90) [46] with Diamond [47]. Finally, all mapping

reads were used to estimate gene family abundance and then annotated to metabolic enzymes to reconstruct and quantify metabolic pathways (MetaCyc) [48]. HUMAnN2 was run by the default parameters. All sample profiles were merged and renormalized using humann2_join_tables and humann2_renorm_table, respectively.

Virulence factor proteins and AROs were analyzed according to methods previously reported [39]. In brief, protein reference databases were downloaded from the Virulence Factors Database (VFDB, v201806) [49] and Comprehensive Antibiotic Resistance Database (CARD, v2.0.1) [50], respectively. Then, high quality sequencing reads were aligned to protein reference databases using DIAMOND [47] and the parameters were set as '–threads 10 –max-target-seqs 1 –outfmt 6'. If the paired-end reads aligned to the same protein and the identity was more than 70%, E value lower than 1e-05, they were considered to be vailed alignment. Finally, quantified as counts per million (CMP) was used to calculate the proteins abundances: the raw valid counts (number of valid alignments) divided by the library sizes (total high quality sequencing reads of each sample) and multiplied by one million.

Taxa, gene families, pathways, virulence factor proteins and AROs which presented in less than 10% samples were discarded in further analysis.

Statistical analysis

Alpha diversity of samples was estimated by Shannon diversity at the gene family level. Beta diversity between samples was estimated by Bray-Curtis distance at the gene family level via the 'vegdist' function in the vegan R package. PERMANOVA based on the Bray-Curtis distance matrix was performed via the 'adonis' function from the R package vegan, and the permuted *p* value was obtained by 9,999 permutations. Supervised analysis with spare PLS-DA was performed using the mixOmics package in R. Differential relative abundances of taxa and pathways were detected by the Wilcoxon rank-sum test and LEfSe [51], and the significance levels were p value < 0.05 and LDA > 2. The correlations between the relative abundance of species and metabolic pathways or host characteristics were calculated by Spearman's rank correlation coefficient and visualized by heatmap in R using the ComplexHeatmap package. Because of the high correlation between species and metabolic pathways, a method reported previously [52] was used to determine the significance p value ($0.05/(25 \times 399) = 5.01 \times 10^{-6}$). Virulence factor proteins and AROs were considered significant different between ADHD patients and HCs if p value of Fisher's exact test and Wilcoxon rank sum test both less than 0.05. Odds ratio (OR) and 95% were calculated by logistic regression in R and adjusted age, sex and BMI. Biomarkers used to discriminate ADHD patients and HCs were identified based on a random forest model (randomForest 4.6–14 package) using the relative abundance profile at the species level [53], and the result was assessed by receiver operating characteristic (ROC) curves using the pROC package in R. The AUC is a convenient tool for comparing and validating classifiers, and values of 0.90-1.00 are excellent, 0.80–0.89 are good, 0.70–0.79 are fair, and < 0.70 are poor [54].

Abbreviations

ADHD:Attention-deficit/hyperactivity disorder; HCs:healthy controls; BMI:body mass index; IQ:intelligence quotient; DSM:Diagnostic and Statistical Manual of Mental Disorders; PERMANOVA:permutational multivariate analysis of variance; ARO:antibiotic resistance ontology; RF:Random forest; VFDB:Virulence Factors Database; CARD:Comprehensive Antibiotic Resistance Database; CMP:counts per million

Declarations

Availability of data and materials

The metagenomic shotgun sequencing data for all samples have been deposited in the CNGB Nucleotide Sequence Archive (CNSA) under accession code CNP0000729. Other data that support the findings of this study are available within the paper and its Supplementary Information files or from the corresponding author upon reasonable request.

Ethics approval and consent to participate

This study was approved by the ethics committee of Xijing Hospital, Fourth Military Medical University (ID: KY20182002-1). All participants were informed about the purpose of this study and provided written informed consent.

Consent for publication

Not applicable.

Competing interests

Luis Augusto Rohde has received grant or research support from, served as a consultant to, and served on the speakers' bureau of Medice, Novartis/Sandoz and Shire/Takeda in the last three years. The ADHD and Juvenile Bipolar Disorder Outpatient Programs chaired by Dr Rohde have received unrestricted educational and research support from the following pharmaceutical companies in the last three years: Novartis/Sandoz and Shire/Takeda. Dr Rohde has received authorship royalties from Oxford Press and ArtMed and travel grants from Shire to take part in the 2018 APA annual meeting and from Novartis to take part of the 2017 AACAP annual meeting.

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Authors' contributions

Y.L., H.T.S., Y.F.H., and A.Q.Y. contributed equally to this study. L.Z.X., Y.L., X.S., and X.F.X. conceived, designed, and supervised the study. H.T.S., P.W., M.T., C.W., F.F.J., A.Q.Y., and X.S. recruited the subjects and collected the fecal samples. Y.F.H., X.F.X., J.Y.Z., and S.L.S. participated in the data interpretation and statistical analysis. Y.L. and Y.F.H. drafted the manuscript. L.Z.X., L.A.R., J.H., D.D.Q., and L.X.Z. checked and revised the manuscript. All authors read and approved the final manuscript.

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Figures

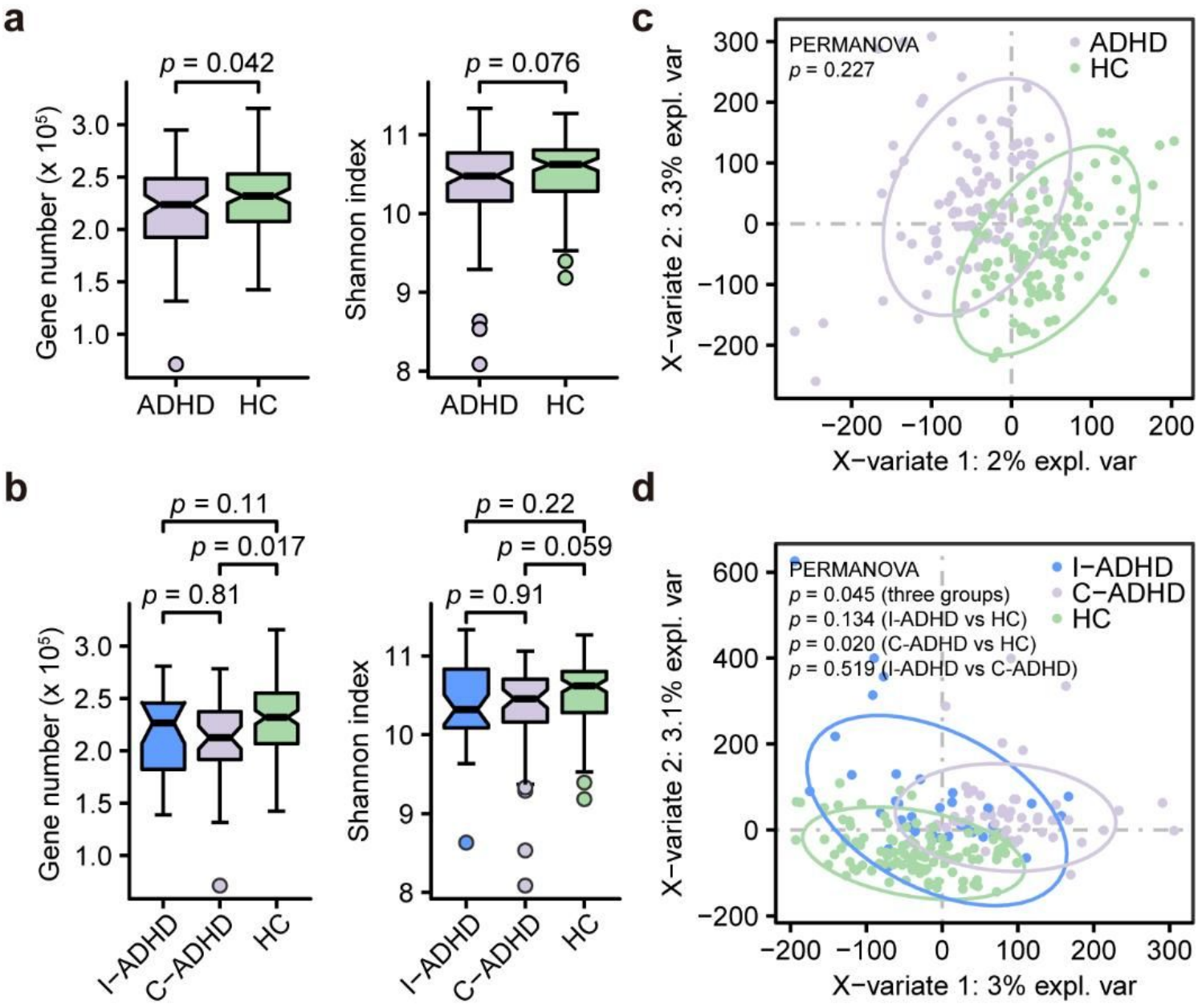

Figure 1

Alpha and beta diversity analyses in ADHD patients and HCs. (a, b) Gene count and alpha diversity (Shannon index) in ADHD (a) or subgroup patients (b) and HCs. The Wilcoxon rank-sum test was used to determine significance. (c, d) Supervised analysis with spare PLS-DA in ADHD (c) or subgroup (d) patients at the gene level. PERMANOVA calculation based on the Bray Curtis distance at the gene level.

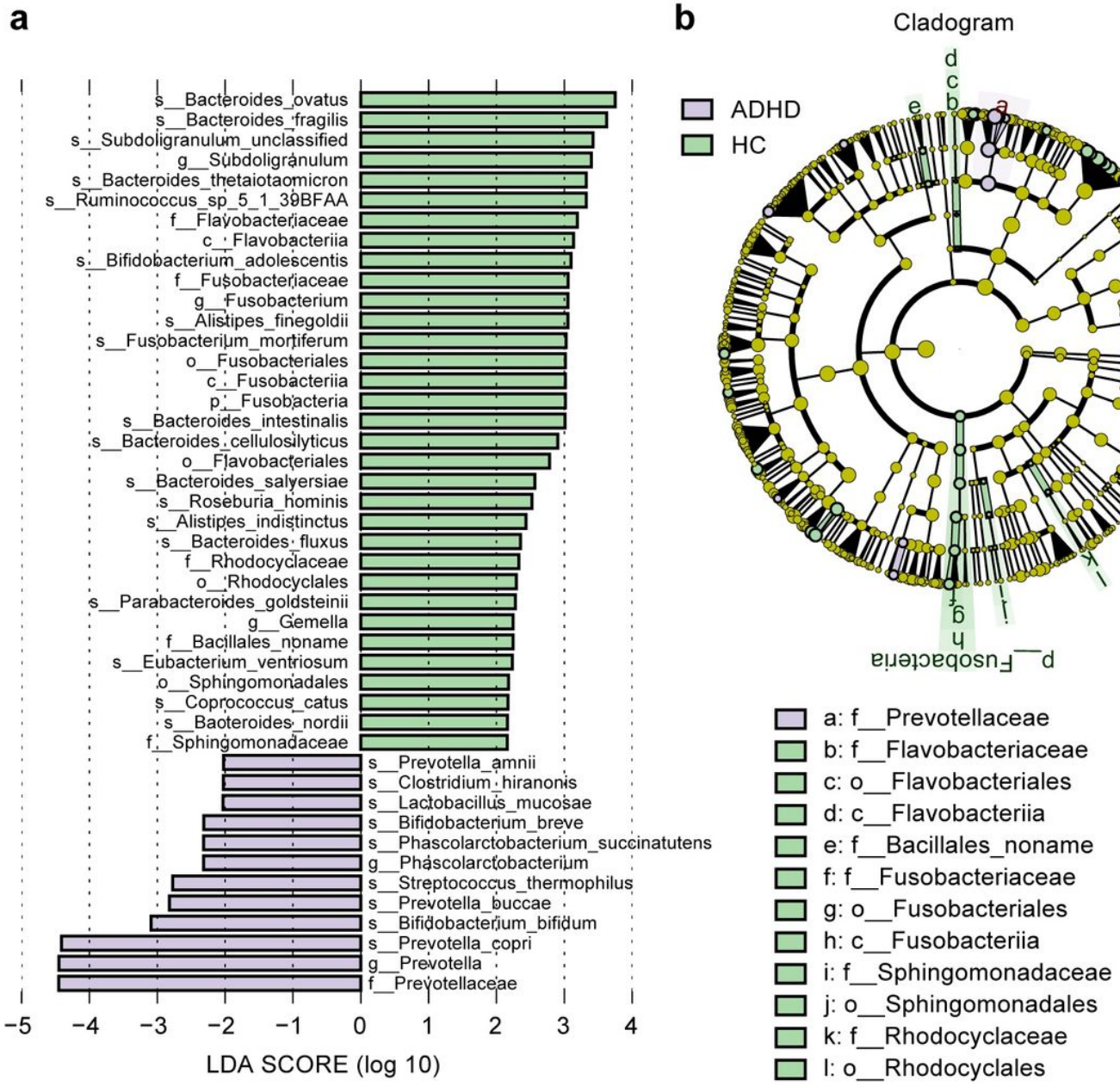

Figure 2

Significantly different taxa between ADHD patients and HCs. (a) LDA effect size analysis identified significantly different taxa between ADHD patients and HCs. The LDA scores (log 10) > 2 and $p < 0.05$ are shown. A negative LDA score indicated enrichment in ADHD patients (purple), while a positive LDA score indicated enrichment in HCs (green). Bar length indicates the effect size of each taxon. (b) Taxonomic cladogram obtained from LEfSe analysis. The circles from inside to outside represent different classification levels, and the size of each dot is proportional to its relative abundance. The colored taxa

represent significantly different taxa between ADHD patients and HCs. Purple, ADHD-enriched; Green, HC-enriched.

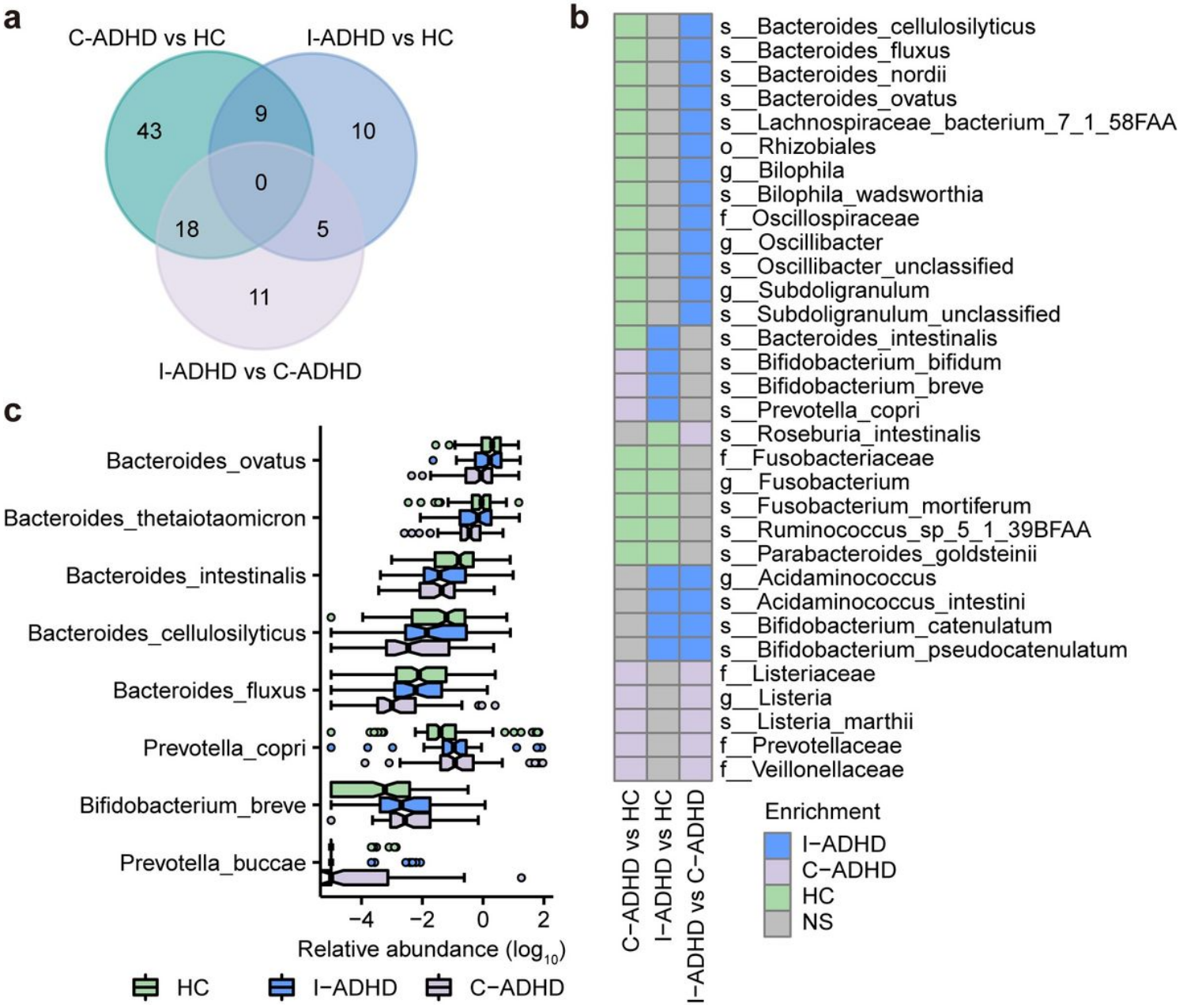

Figure 3

The gut microbial composition of C-ADHD is different from that of I-ADHD and HC. (a) Venn diagram of significantly different taxa among different comparisons. (b) Heat map of sharing significantly different taxa among different comparisons. LEfSe analysis was used to detect significantly different taxa in (a) and (b). (c) Boxplot of significantly different species among HC, I-ADHD, and C-ADHD. Significant differences among groups were evaluated using the Jonckheere-Terpstra test and adjusted by the Benjamin-Hochberg (BH) method ($p < 0.05$).

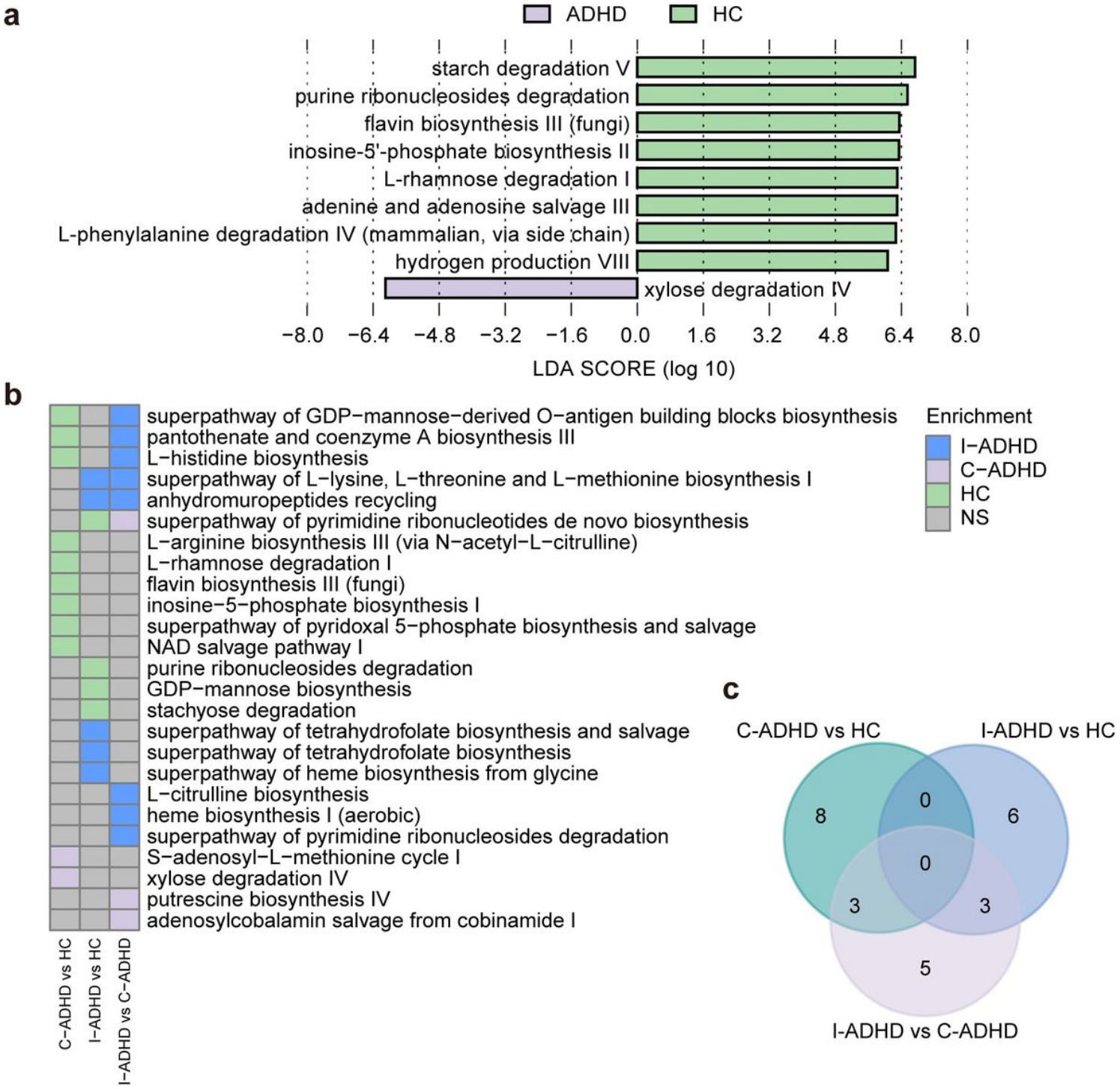

Figure 4

Gut microbial functions of C-ADHD are different from those of I-ADHD and HC. (a) Significantly different pathways between ADHD subgroup patients and HCs. The LDA scores (log 10) > 2 and $p < 0.05$ are shown. Bar length indicates the effect size of each species. (b) Heat map of sharing significantly different pathways among different comparisons by LEfSe analysis. (c) Venn diagram of significantly different pathways among different comparisons.

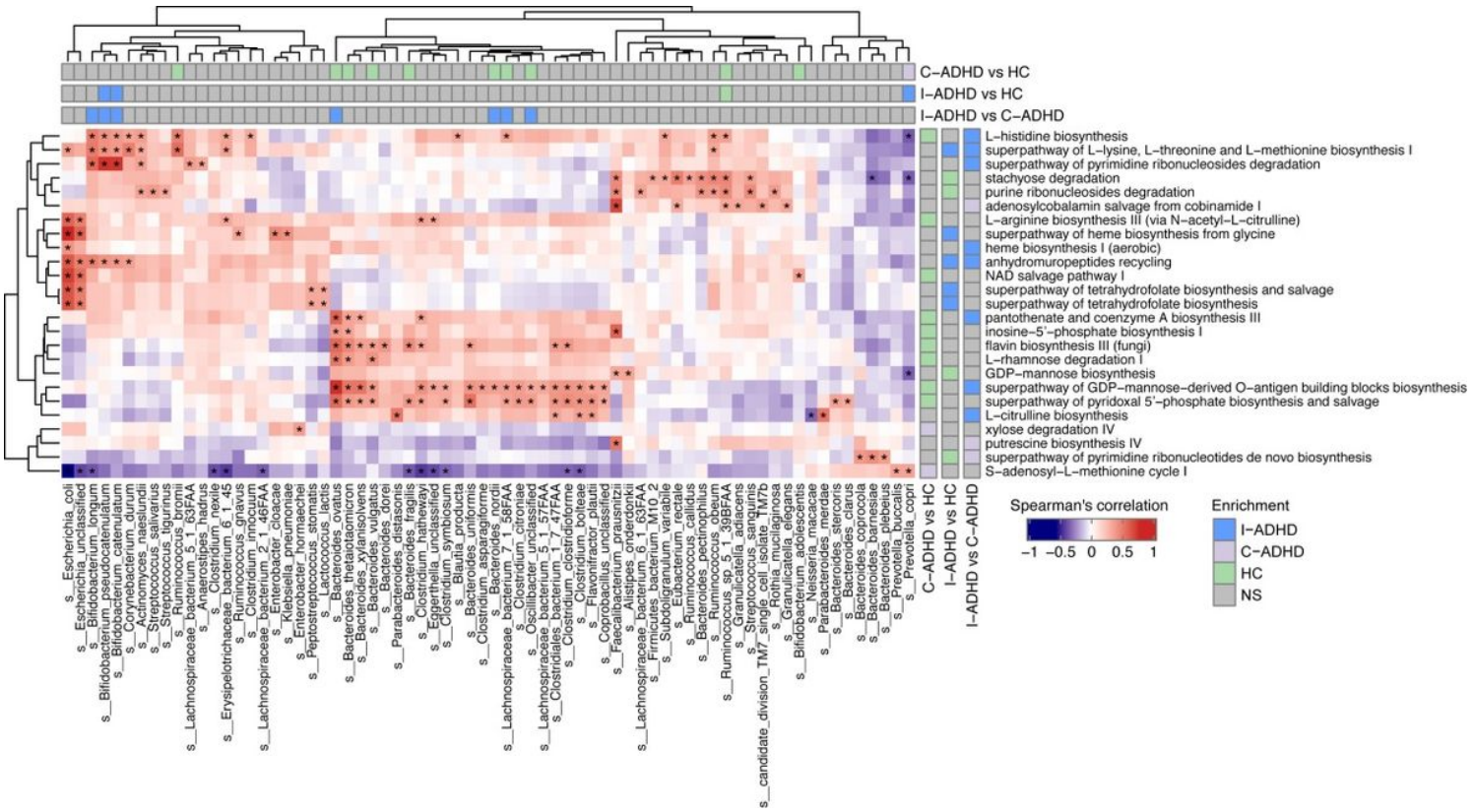

Figure 5

Correlation between gut microbial composition and function. Heat map of Spearman's correlation coefficient between the relative abundance of species and pathways (red and purple for positive and negative correlation, respectively). Species and pathway enrichment direction in different comparisons are shown on the top and left, respectively. Blue, I-ADHD-enriched; purple, C-ADHD-enriched; green, HC-enriched; gray, no significant difference. '*' denotes $p < 0.001$.

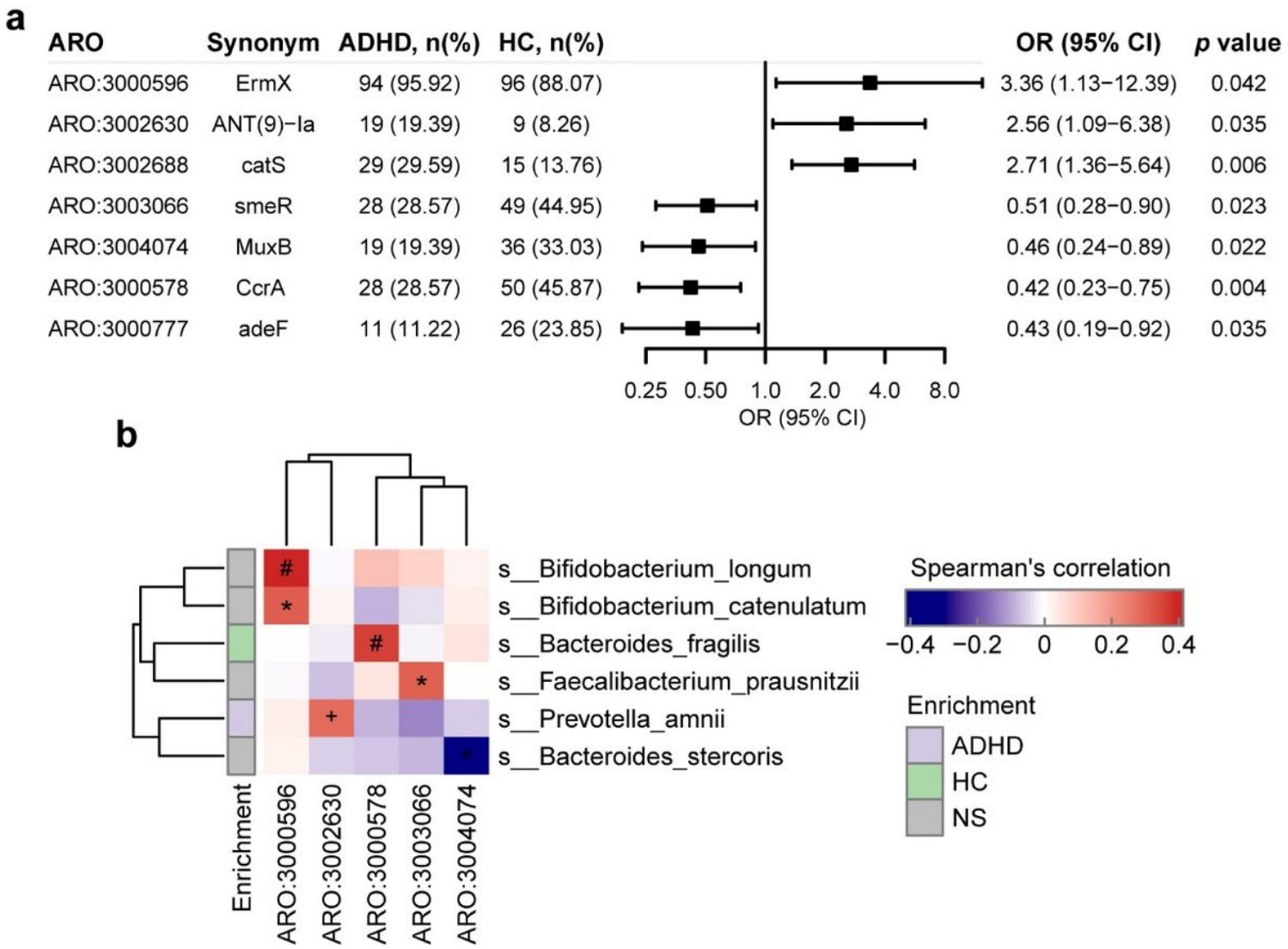

Figure 6

Antibiotic resistance ontology (ARO) between ADHD and HC. (a) Odd ratios of ARO in ADHD patients and HCs. Black squares and bars represent odds ratios and 95% CI, respectively, and were adjusted age, sex and BMI by logistic regression. (b) Correlation between gut microbial composition and ARO. Heat map of spearman's correlation coefficient between relative abundance of species and ARO (red and purple for positive and negative correlation, respectively). Species enrichment direction was shown on left. Purple, ADHD-enriched; green, HC-enriched; gray, no significant difference. '+' denotes P < 0.05; '*' denotes P < 0.01, '#' denotes P < 0.001.

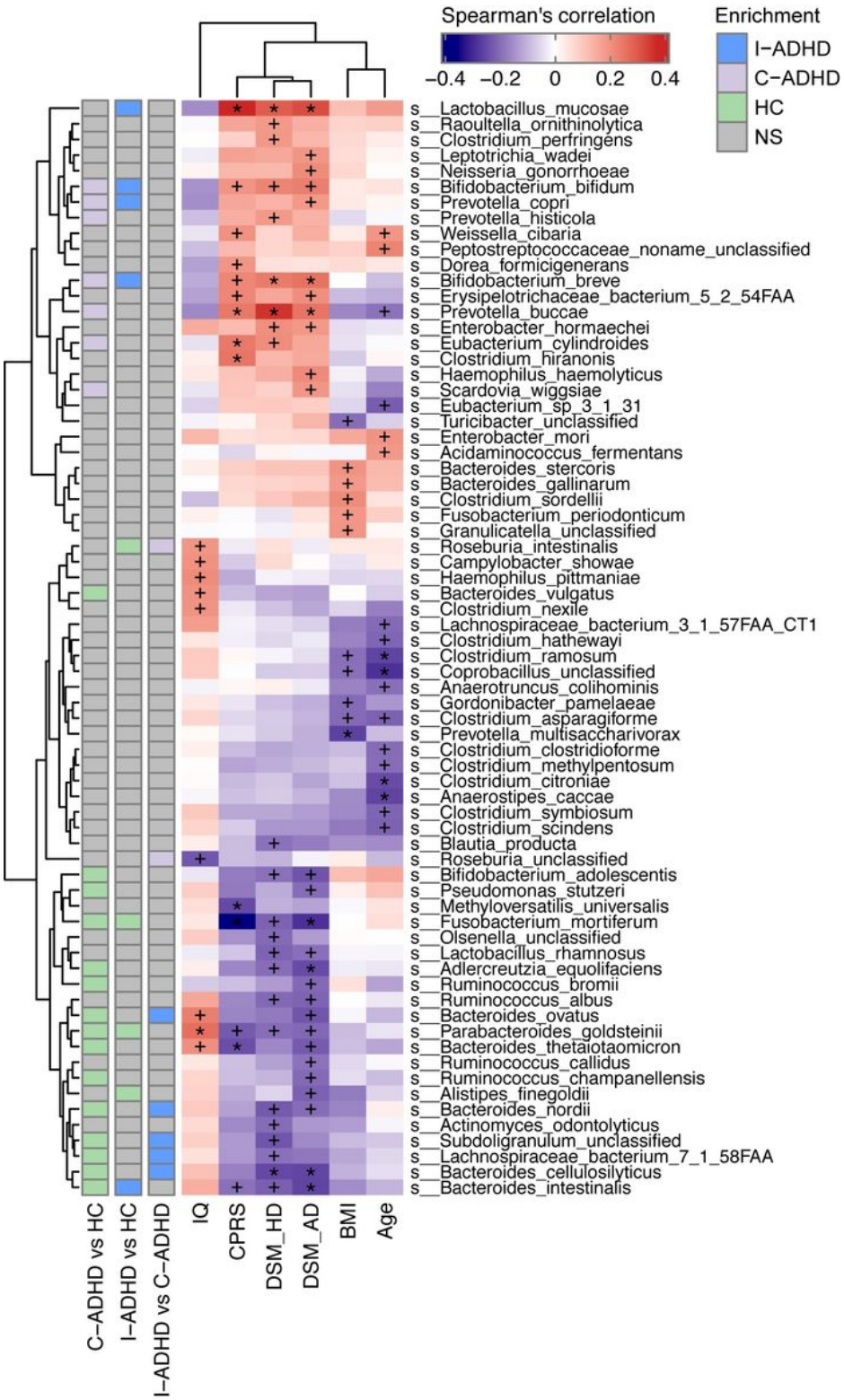

Figure 7

Correlation between gut microbiota species and ADHD clinical characteristics. Heat map of Spearman's correlation coefficient between the relative abundance of species and ADHD clinical characteristics (red and purple for positive and negative correlation, respectively). Species enrichment direction in different comparisons is shown on the left. Blue, I-ADHD-enriched; purple, C-ADHD-enriched; green, HC-enriched; gray, no significant difference. '+' denotes $p < 0.01$; '*' denotes $p < 0.001$.

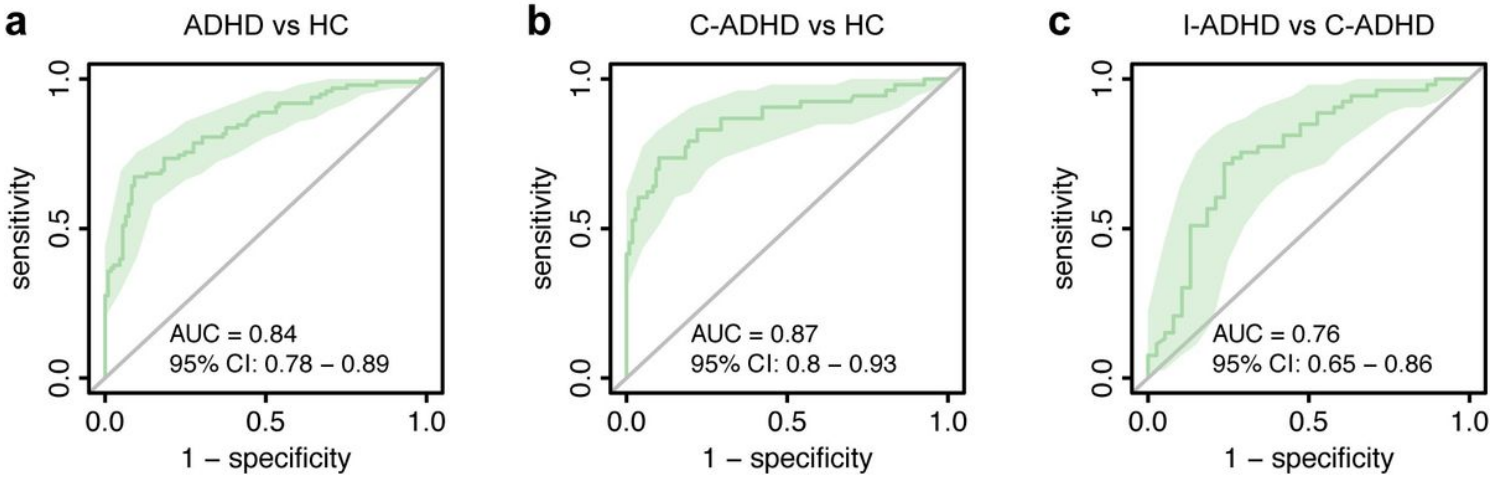

Figure 8

Classification of samples among different groups by relative abundance at the species level. (a) ROC generated between ADHD patients and HCs by 6 microbial markers selected by the random forest model. The AUC was 0.84, and the 95% CI was 0.78-0.89 (green area). (b) ROC generated between C-ADHD patients and HCs by 8 microbial markers selected from the random forest model. The AUC was 0.87, and the 95% CI was 0.8-0.93 (green area). (c) ROC generated between I-ADHD and C-ADHD patients by 35 microbial markers selected by the random forest model. The AUC was 0.76, and the 95% CI was 0.65-0.86 (green area).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- ADHDsupportinginformation120200514.docx
- ADHDsupportinginformation220200514.xlsx