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HUMAN MICROBIOME

Gut Microbial Dysbiosis in Indian Children with Autism Spectrum Disorders

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Abstract

Autism spectrum disorder (ASD) is a term associated with a group of neurodevelopmental disorders. The etiology of ASD is not yet completely understood; however, a disorder in the gut-brain axis is emerging as a prominent factor leading to autism. To identify the taxonomic composition and markers associated with ASD, we compared the fecal microbiota of 30 ASD children diagnosed using Childhood Autism Rating Scale (CARS) score, DSM-5 approved AIIMS-modified INCLEN Diagnostic Tool for Autism Spectrum Disorder (INDT-ASD), and Indian Scale for Assessment of Autism (ISAA) tool, with family-matched 24 healthy children from Indian population using next-generation sequencing (NGS) of 16S rRNA gene amplicon. Our study showed prominent dysbiosis in the gut microbiome of ASD children, with higher relative abundances of families Lactobacillaceae, Bifidobacteraceae, and Veillonellaceae, whereas the gut microbiome of healthy children was dominated by the family Prevotellaceae. Comparative meta-analysis with a publicly available dataset from the US population consisting of 20 ASD and 20 healthy control samples from children of similar age, revealed a significantly high abundance of genus *Lactobacillus* in ASD children from both the populations. The results reveal the microbial dysbiosis and an association of selected *Lactobacillus* species with the gut microbiome of ASD children.

Keywords Autism spectrum disorder (ASD) · Gut microbial dysbiosis · Indian children · Gut-brain axis · Gastrointestinal symptoms

Introduction

Autism is a spectrum of developmental disorders, also known as autism spectrum disorders (ASDs), associated with a deficit in social-emotional reciprocity, and social interaction with restricted and repetitive behavior in early childhood [1]. The recent developments in diagnostic tools, such as ADOS-T (Autism Diagnostic Observation Schedule–Toddler Module) and ADI-R (Autism Diagnostic Interview-Revised) have helped in the early detection of ASD in the age of 12–18 months [2–5]. Similarly, hyper- or hypo-reactivity to sensory input and sensation seeking are among the new criteria for the detection of ASD as per the DSM-5 (Diagnostic and Statistical Manual of Mental Disorder, Fifth Edition) [6]. Earlier studies estimated the prevalence of ASD in five out of thousand cases, but recent reports suggest up to 12.5 cases per thousand cases [7], with boys more affected than girls [8]. Numerous theories have been proposed regarding the etiology of ASD; however, the pathophysiology of the disorder remains largely unknown. Gastrointestinal (GI) complications

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and behavioral symptoms are also observed commonly in ASD children [9]. Though the underlying link between GI complications and ASD remains poorly understood, food intolerance is proposed to play a major role in ASD [10, 11]. Interestingly, studies have reported that diet restriction (casein or gluten-free diet) shows a reduction in GI disorders leading to an improvement in the behavior of ASD individuals [12].

Recent reports have suggested the role of gut microbes in influencing brain function, and a balanced gut microbiota has been proposed to promote mental health [13]. Furthermore, several efforts have been carried out recently to understand the role and association of gut microbiota with ASD. It is suggested that a consortium of microbial communities helps in maintaining homeostasis, whereas a dysbiosis of the microbiome often leads to a negative effect on human health [14, 15]. Recent studies have also highlighted the impact of multiple pathways, maternal obesity, and gut microbiome in neurodevelopment and social behavior [15, 16].

The term gut-brain axis has been defined as the biochemical signaling communication between the gastrointestinal tract and the central nervous system. Recent reports have proposed a potential role of gut microbial dysbiosis in influencing the brain functioning due to the changes in gut metabolites, which are essential for a variety of cytokines and neuroactive compounds leading to several psychiatric disorders [17, 18]. ASD children also show a similar dysbiosis in the gut microbiome as mentioned above [19]. The influence of an altered gut microbial composition has been shown on neurodevelopment and social behavior using animal models also [20, 21].

In India, approximately 1.7–2 million ASD cases exist presently, and their number is increasing gradually with the improvements in methods for early diagnosis [22]. Colonization of gut by microbes begins at birth, but the succession and composition of the microbial community of an individual depends on a number of factors including the age, diet, genetic composition, gender, geographic location, and health status. The composition and role of gut microbiota in human health is well-studied in the western population; however, only a few attempts have been made so far to assess the gut microbiome from the Indian subcontinent [23]. The key observation from these studies was the predominance of genus *Prevotella* and *Megasphaera* in Indian adults and children [23, 24], which was attributed to the typical Indian carbohydrate-rich diet [23]. The gut microbiome in Indian population is plausibly different from the gut microbiome across the world due to the differences in dietary habits, culture, environment, and ethnicity, which makes it important to examine the dysbiosis in gut microbiome due to the major disorders or diseases in the Indian population [23].

In this work, we determined the gut microbiota of 30 ASD children to analyze the alterations in gut microbial communities by comparing with 24 family-matched healthy children from the Indian population by sequencing the V3 hypervariable region of the 16S rRNA gene. One unique aspect of this study was that both ASD and healthy children were on their native (same) diet, unlike the previous studies where the ASD children were consuming a gluten-free diet. The phylogenetic diversity and taxonomic abundance of the microbial communities were examined using phylotype profiling. Furthermore, a comparative meta-analysis was carried out using another ASD dataset from the USA population to identify the key marker taxa significantly associated with the ASD children [25].

Materials and Methods

Study Design and Subject Enrolment

The study cohort comprised of two groups. The first group consisted of 30 ASD children aged between 3 and 16 years (Table 1). They were recruited from the Sunrise Hospital in Kerala, which is located in the southern part of India. Diagnosis of all the cases was carried out using a CARS (Childhood Autism Rating Scale) score, DSM-5 approved AIIMS-modified INDT-ASD (INCLEN Diagnostic Tool for Autism Spectrum Disorder), and the ISAA (Indian Scale for Assessment of Autism) tool, with the help of experienced pediatric neurologists, psychologists and specialized nurses (Supplementary Table S1) [26–28]. The latest diagnostic tools such as ADOS/ADI-R could not be used since they are very recent and not yet commonly available in India. In our study, the ratings were based not only on the frequency of the behavior in question, but also on its intensity, peculiarity, and duration. Specifically, we included only severely affected children to understand the disorder at its extremity. The second group consisted of 24 healthy children with matched age and mostly siblings or blood relatives to the ASD children, which were diagnosed to have no gastric problem or autism spectrum disorder by pediatrician and physician. We could find the family-matched healthy children for 24 out of the total 30 ASD children and could not find the best-matched controls from the same family for rest of the six ASD cases.

Other information about the individuals such as gender, age, and diet were recorded for seven consecutive days before the collection of fecal samples (Supplementary Table S2). All the subjects included in this study were consuming a normal omnivore native diet similar to the healthy subjects and were not on the gluten-free diet (GFD). Thus, this is the first gut microbiome study of ASD children from India, which were on a normal native diet. Informed consent was obtained from the parents/guardians of the 54 children before their enrolment in the study. All the children did not undergo any antibiotic, anti-inflammatory, or antioxidant treatment for 1 month prior to the sample collection. The protocols for sample collection, sequencing, and analysis as described in the "Materials and Methods" have been conducted in accordance with the

Table 1 Summary of ASD and healthy samples included in the study

Parameters	Autism ($n = 30$), median (range)	Healthy ($n = 24$), median (range)	P value (using Student's t test)
Age	9.5 (3–16)	9.5 (3.5–16)	$P = 0.61$
Gender ratio (M/F)	28:2	15:9	$P = 0.0063$*
BMI	14.78 (6.94–20.4)	15.79 (13.43–31)	*$P = 0.02$**

*P value significant

approved guidelines by the Institutional Ethical Committee of IISER Bhopal, India. Fecal samples were collected from each individual after the morning breakfast and were stored at − 80 °C within 2 h of collection until further processing.

DNA Extraction

DNA from the 54 fecal samples was extracted using QIAamp Stool Mini Kit (Qiagen, CA, USA) following the manufacturer's instructions. DNA concentration and quality were estimated by Qubit-HS DNA assay kit (Invitrogen, CA, USA) and agarose gel electrophoresis, respectively. The DNA samples were stored at − 80 °C until sequencing.

PCR Amplification of 16S rRNA V3 Region and Sequencing

Extracted DNA (~ 5 ng) was PCR amplified with three different 5′-end custom modified adaptor-ligated primers of the 341F and 534R (Supplementary Table S3), targeting the V3 hypervariable region of the 16S rRNA gene. A few nucleotide bases were introduced at the 5′-end to increase the overall sequence diversity of the samples, thus improving the quality of the sequenced data [29]. The quality of the PCR products was assessed using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA.). The amplicon libraries were prepared using Illumina 16S metagenomic sequencing library preparation guide. The libraries were then quantified, and 150-bp paired-end sequencing was performed using NextSeq500 (Illumina, CA, USA) at the NGS Facility, IISER Bhopal, India.

Processing of Reads and Data Analysis

The 16S rRNA V3 sequences were demultiplexed using the barcodes. After the removal of barcodes and primers, the paired-end sequences were assembled to form a single read using FLASH [30], and further quality-filtered with ≥ 80% bases in a read above Q30. A total of 34,164,683 high-quality V3 amplicon reads with a median of 413,406 reads per sample were used for the analyses. The high-quality reads were clustered into species-level OTUs (operational taxonomic units) at ≥ 97% identity with closed reference OTU picking protocol using QIIME Suite of Software and Tools (QIIME v1.5.0) [31]. The taxonomic assignment of the OTUs was carried out using the Greengenes Database v13_5 [32]. Random forest (RF) analysis was performed using the RF package in R with 500 trees and default settings on rarefied OTU tables [33]. A total of ten rarefactions were carried out at equal depth, and average importance values were calculated. Boruta, a feature selection tool using random forest, was used in R to identify the genus important for the classification of samples [34]. For the meta-analysis, an autism dataset (SRA053656) consisting of 20 ASD and 20 healthy children from the US population was downloaded from NCBI. The ASD children from the US study were of similar age as the Indian subjects but were consuming a gluten-free diet. The reads were clustered into species-level phylotypes at ≥ 97% identity using closed-reference OTU picking protocol of QIIME v1.5.0. The reads from both the studies (the USA and the Indian cohort) were combined, and genus abundance was calculated using the combined OTU table.

Statistical Analyses

All statistical analyses were performed using R software. The α-diversity metrics (observed species, Shannon, and phylogenetic diversity) and β-diversity (unweighted UniFrac distances) were calculated using QIIME on rarefied OTU counts at equal depths. All the reads were used for normalization, and the relative abundances were calculated by dividing the taxa abundance with the total number of reads in each sample. The genus abundance tables were analyzed for the identification of discriminating genera using LEfSe and Boruta. The identification of significantly different phylum, families, and genus between ASD and healthy children was performed using Wilcoxon rank sum test. The P values were corrected for multiple comparisons using false discovery rates (FDRs). Differentially abundant taxa were highlighted using GraPhlan [35]. Spearman's rank correlation coefficients were calculated for each genus with FDR-adjusted $P < 0.05$ to obtain significant correlations between the genera. Weighted and unweighted UniFrac distances were calculated between the ASD and non-ASD samples. Though, autism did not show a significant correlation with PC1 (with a $P > 0.05$), but among all the three covariates, it showed the maximum correlation. The same observation was made from Bray-Curtis distance (polyserial correlation $P = 0.11$), where the abundance of each OTU in all samples was considered rather than the phylogenetic distances

(Supplementary Tables S4 and S5). PERMANOVA (permutational multivariate analysis of variance) was applied to assess the effect of covariates such as age, BMI, and autism on the UniFrac distances, using Adonis in the vegan package in R. However, significant results were not obtained with any of the covariates (Supplementary Table S6). Random forest analysis was performed to identify the significantly differentiating OTUs separating the healthy controls from ASD samples. The random forest classifiers were applied on the labeled data using mtry = 20 and ntree = 1000. The mean decrease in accuracy, which represents the decrease in accuracy of prediction of classifier on removal of each OTU, was calculated for each OTU. The OTUs with mean decrease in accuracy ≥ 0.01 were considered as important for discriminating the samples into healthy controls and ASD groups.

Data availability DNA sequences have been deposited in NCBI-SRA under the accession number SRP093968.

Results

Sample Characterization

A total of 54 individuals consisting of 30 ASD and 24 healthy children were included in the study, and the details on the metadata and sequencing statistics are provided in Supplementary Tables S2 and S7, respectively. Fecal samples were collected from all the 54 individuals. The age of ASD and healthy children ranged between 3 and 16 years, and the median age was 9.5 years in both the groups. The ASD children were predominantly male (2 females out of 30 children), whereas the healthy children included 15 males and 9 females. The ASD children manifested GI-related problems such as maldigestion, constipation, and diarrhea, whereas the healthy children did not show any such problems. Age-matched blood relatives were included as a healthy control group, which did not show any disease symptoms and were assessed using criteria as mentioned in the "Materials and Methods" section. CARS score was used for the diagnosis of ASD children. It is a widely used rating scale for the detection and diagnosis of ASD, where high scores are associated with a higher level of impairment [36]. In this study, ASD children with CARS score > 36.5, which indicate a high level of severity, were included. The ASD children had a significantly lower BMI ($P = 0.02$) and a significantly higher male proportion ($P = 0.0063$) compared to the healthy children (Table 1).

Microbiota Comparison Between ASD and Healthy Children

The high-quality (Q > 30) 34,164,683 reads (72.74% of total raw sequences) consisting of 13,129,855 reads from 24 healthy samples and 21,043,828 reads from 30 ASD samples, with an average of 413,406 reads per sample were considered for the taxonomic and comparative analyses. To normalize the sequencing depth, subsets of 168,000 reads (equal depths) per sample were randomly picked for alpha diversity comparison. The 16S rRNA reads were clustered into species-level phylotypes at ≥ 97% identity resulting in 3627 OTUs with at least 100 sequences per OTU across all the samples. The α-diversity metrics, such as Shannon index and observed species (S_{obs}), indicated that the fecal microbial diversity of ASD group was similar to that of the healthy children (Table 2). Furthermore, the phylogenetic diversity, which is based on the taxonomic branch lengths shared within samples, showed no significant difference (P value = 0.8). Also, the comparison of S_{obs} showed no significant difference in diversity between ASD children and healthy children (Supplementary Fig. S1).

To examine the effect of covariates such as autism, BMI, and age on the taxonomic profiles, we analyzed the effect of each of these covariates on principal components (PCs) using principal component analysis (PCA) calculated from unweighted UniFrac distances. It was observed that autism correlated significantly with PC3 (Pearson's correlation coefficient with FDR adjusted $P < 0.05$), which explains the observed gut microbial compositional differences between the ASD and healthy samples (Fig. 1). Though BMI was found to be significantly different between ASD and healthy children, it did not show a significant correlation with any of the principal components. A separate clustering of the healthy and ASD samples was also observed from the PCA, which indicates differences in phylotypes between the two groups. To further assess the effect of these covariates on microbiome profiles, PERMANOVA was performed using UniFrac distances and the covariate values. The variation in UniFrac distances due to autism was observed to be marginally higher as compared to age and BMI, but the difference was statistically insignificant ($P = 0.19$) (Supplementary Table S6).

Correlation analysis of families with principal components showed that the high PC3 values (Spearman's rank correlation, FDR adjusted $P < 0.1$) were positively correlated with higher abundances of Lactobacillaceae, Mogibacteraceae, and Enterococcaceae families and were negatively correlated with a higher abundance of Prevotellaceae family (Supplementary Fig. S2). Further, Prevotellaceae, Lactobacillaceae, and Mogibacteraceae were also observed as the key families in differentiating the ASD and healthy samples.

Taxonomic Comparison of Major Taxa in ASD and Healthy Children

The three most abundant phyla identified in individuals of both the groups were Firmicutes, Bacteroidetes, and Proteobacteria, and the remaining phyla contributed less than

Table 2 Comparison of alpha diversity between ASD and healthy children

Metrics	Autism ($n = 30$), mean ± SD	Healthy ($n = 24$), mean ± SD	P value (using Student's t test)
Observed species	1392 ± 53.72	1469 ± 50.52	0.3
Shannon index	5.052 ± 0.156	5.023 ± 0.154	0.9
Phylogenetic diversity (PD)	19.82 ± 0.69	19.60 ± 0.79	0.8

3% of the total proportion in all the samples. The proportions of Bacteroidetes and Firmicutes was nearly equal in healthy children (46 ± 24%; mean ~ 46%; $n = 24$), whereas the proportion of Firmicutes (56 ± 37%; mean = 56%; $n = 30$) was higher in ASD children as compared to healthy children (37 ± 28%; mean = 37%; $n = 24$) (Fig. 2a, b). Earlier reports have also shown an increase of Firmicutes in the gut microbiota of ASD children, which may result in increased intestinal permeability [37]. A comparison of major bacterial families in ASD and healthy children revealed a higher relative abundance of Prevotellaceae (37.8%) in healthy children as compared to ASD children (28.04%), whereas a higher abundance of Veillonelleaceae was observed in the ASD children (11.38%) as compared to the healthy children (6.31%). Wilcoxon rank sum test revealed a significantly higher (FDR adjusted $P < 0.05$) relative abundance of Lactobacillaceae ($P = 0.018$), Bifidobacteraceae ($P = 0.0054$), and Veillonellaceae ($P = 0.008$) in ASD children compared to healthy children (Fig. 3a). In addition to the abundant (mean proportion > 0.001) families, a few low abundant families were also observed to be significantly different, which included Erysipelotrichaceae ($P = 0.0005$), Enterococcaceae ($P = 0.0127$), and Desulfovibrionaceae ($P = 0.03$) (Supplementary Table S8).

A comparison of major genera between ASD and healthy groups revealed a significantly higher relative abundance of *Bifidobacterium* ($P = 0.005$), *Lactobacillus* ($P = 0.018$), *Megasphaera* ($P = 0.0008$), and *Mitsuokella* ($P = 0.007$) in ASD children as compared to healthy children (Fig. 3b, Supplementary Table S9). The most prominent variation was shown by *Lactobacillus* genus, which was observed to be ~32-folds higher in ASD children as compared to healthy children. Furthermore, *L. ruminis* was found to be the most abundant species constituting 99% of all observed species in

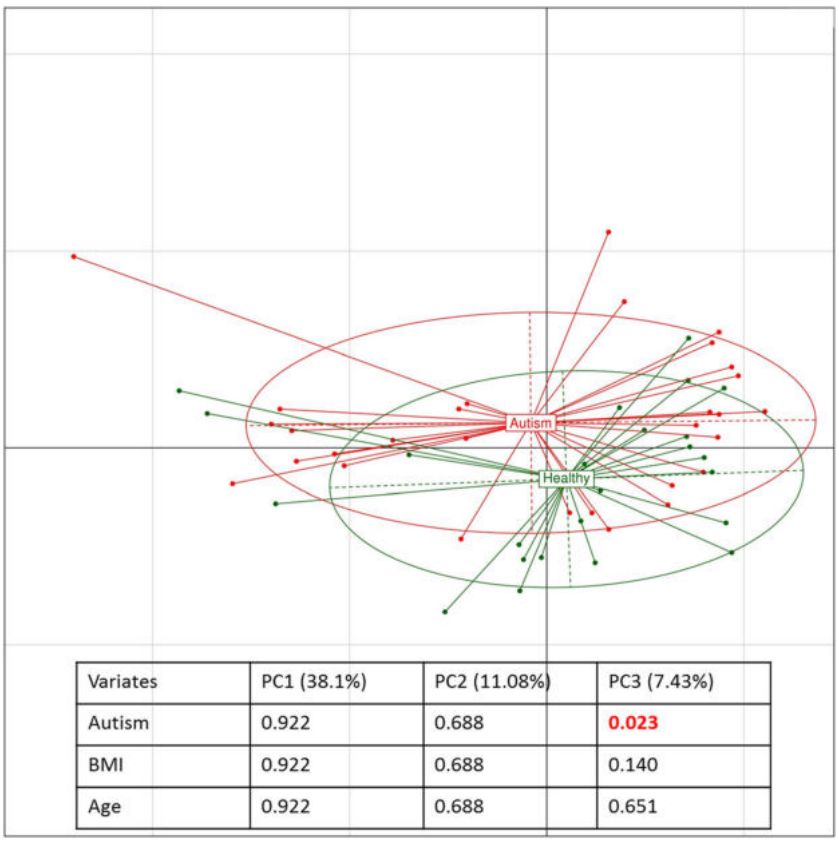

Fig. 1 Unweighted UniFrac distances were showing the difference between ASD and healthy samples phylotypes. Principal component analysis was performed using unweighted UniFrac distances (rarefied at an equal depth of 168,000 sequences/sample) between the samples. The correlations were performed between each principal component (PC) and the covariates such as age, BMI, and disease state (autism) using Pearson's correlation coefficient along with their FDR adjusted P values. Autism (at PC 3) was found to be a significant factor (FDR-adjusted P value < 0.05) to explain the differences in the composition of gut phylotypes. The samples were clustered with autism as centroids, where the ASD samples are shown in "red" and healthy samples in "green"

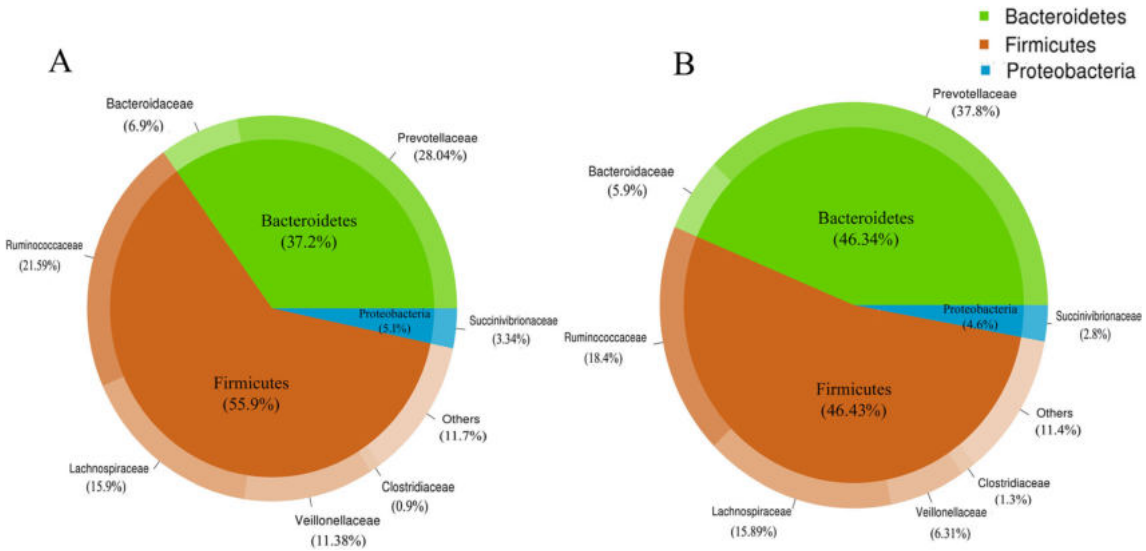

Fig. 2 Relative abundance of phylum and family in ASD and healthy children. Pie charts showing a mean relative abundance of the most abundant (> 1% mean abundance) families (outer) and phylum (inner) in **a** ASD children and **b** healthy children

the *Lactobacillus* genus in ASD children, which was confirmed by alignment (at 97% identity cutoff) against Greengenes database (version 13_5) [32].

Identification of Differentiating OTUs Using Random Forest Approach

To identify the discriminatory OTUs in ASD and healthy groups of samples, an unsupervised learning algorithm "random forest (RF)" was used as a powerful classifier to identify discriminatory OTUs based on their importance scores calculated by an increase in classification error on removing that particular OTU from the dataset. The importance values of each OTU identified as discriminatory (importance score > 0.001) were used for the analysis of species-level OTUs (Supplementary Table S10). A total of 121 OTUs were found to be discriminatory, out of which 26 OTUs belonged to genus *Prevotella* and all of these were abundant in healthy children. Whereas, 57 OTUs out of the 121 OTUs belonged to family Ruminoccocaceae, of which 56 were abundant in

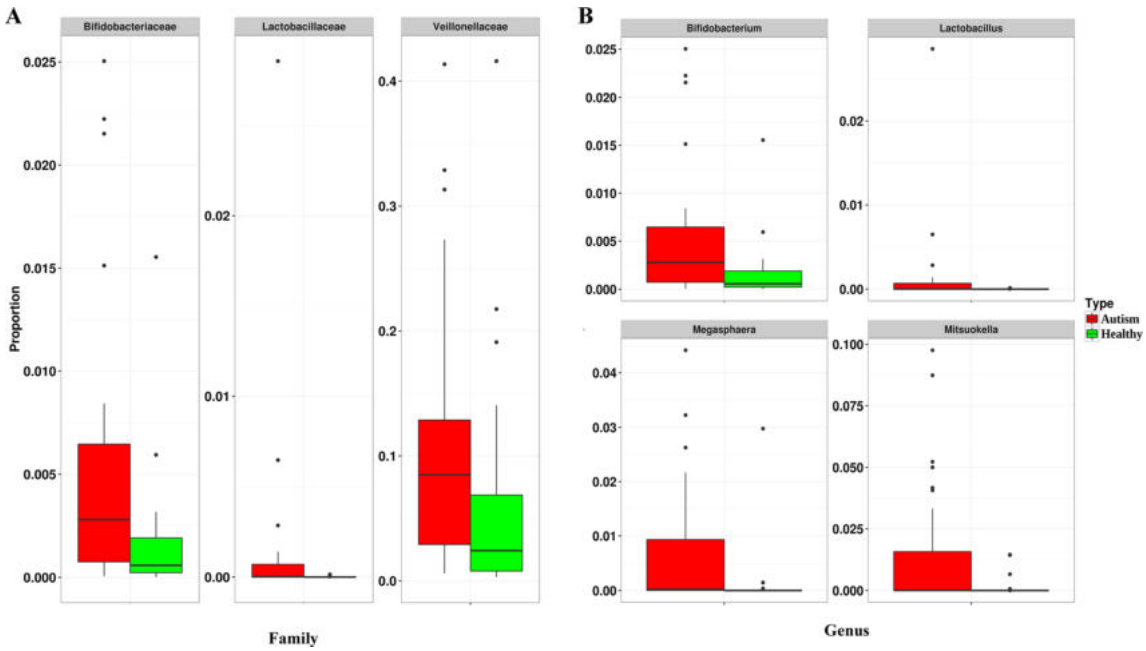

Fig. 3 Families and genera showing significant variations between ASD and healthy children identified using Wilcoxon rank sum test. The **a** families and **b** genera that were significantly different (FDR-adjusted *P* value < 0.05 using Wilcoxon rank sum test) between ASD and healthy children are shown as box plots. The boxes represent the Interquartile range (IQR) between first quartile (25th percentile) and the third quartile (75th percentile)

ASD children. Additionally, 28 OTUs belonged to the family Lachnospiraceae and were also observed to be abundant in ASD children.

Phylogenetic plot of the taxa of discriminatory OTUs displayed an abundance of *Prevotella* from family Prevotellaceae, *Faecalibacterium* from family Clostridiaceae, and *Roseburia* from family Lachnospiraceae in healthy children, whereas *Ruminococcus* from family Ruminococcaceae, *Coprococcus*, and *Butyrivibrio* from family Lachnospiraceae, and *Klebsiella* from family Enterococcaceae were found abundant in ASD children (Supplementary Fig. S3). To identify the most discriminatory genera, a feature selection tool Boruta was used [34, 38]. The classification error rates resulting from the prediction of data using genus proportions were found to be significantly lower than the error rates due to the random guess (Fig. 4). The Boruta algorithm revealed five genera (green boxplots in Fig. 4) to be significantly discriminatory for classifying the samples between the two groups (ASD and healthy). In addition, four genera (yellow box plots in Fig. 4) were selected by Boruta to be near significantly discriminatory in the classification of samples. The *Lactobacillus* (Lactobacillaceae family), *Bifidobacterium* (Bifidobacteriaceae family), *Megasphaera* and *Mitsuokella* (Veillonellaceae family), and *Odoribacter* (Odoribacteraceae family) were observed to be highly predictive, and four of these were also found to be significantly different ($P < 0.05$) using Wilcoxon rank sum test. Similarly, LEfSe, which is a linear discriminant analysis based method, was used to identify the differentially enriched genera between ASD and healthy, and also revealed *Lactobacillus* ($P = 0.011$), *Bifidobacterium* ($P = 0.005$), *Mitsuokella* ($P = 0.008$), and *Megasphaera* ($P = 0.0008$) to be enriched in ASD group compared to healthy children (Supplementary Table S11 and Supplementary Fig. S4) [39].

Comparative Meta-Analysis of Two Different Populations Revealed *Lactobacillus* to Be Significantly Associated with Autism

To identify autism-specific marker taxa (irrespective of diet and geographical location), a meta-analysis approach was carried out by comparing our dataset with a similar dataset of ASD children from the US population (SRA053656 data). A total of 6156 OTUs (≥ 10 abundance) were obtained from SRA053656 dataset [25]. The OTUs of ASD and healthy samples from the Indian (this study) and US datasets were combined to construct a combined OTU table using the closed-reference OTU picking protocol of QIIME. The combined dataset from the Indian and US population contained 9031 OTUs. The abundance of different genera was calculated from these OTUs. The bias arising due to the differences in sequencing depths was nullified by using normalized abundance from each sample. Among the commonly present genera in both (USA and India) the populations, Wilcoxon rank sum test revealed genus *Lactobacillus* to be significantly higher (FDR adjusted $P < 0.01$) in ASD children as compared to healthy children in both the populations (Fig. 5).

Determination of Relative Abundance of *Lactobacillus* in Healthy and ASD Children

The fold change and levels of *Lactobacillus* in individuals were compared between healthy and ASD children. *Lactobacillus* was found to be ~ 32-fold higher in abundance (calculated after removing an outlier sample A35) in ASD children as compared to healthy children (Fig. 6a). Furthermore, a higher level of *Lactobacillus* was also seen in most of the ASD children as compared to the healthy children, which shows that the observed abundance of *Lactobacillus* is not an artifact due to their high abundance in a few ASD cases but is a commonly abundant genus across most of the ASD children in this study (Fig. 6b).

Discussion

Several large-scale metagenomic studies have shown the role of the human gut microbiome in human health. Significant variations due to the differences in lifestyle, diet, and geographical location have been observed in the composition of the human gut microflora [18, 40–42]. Recently, the associations between human gut microbiota and human physiological and immunological diseases are also emerging [40, 43, 44]. Furthermore, the gut microbiota is also found to influence human behavior, and the term "microbiome-gut-brain" axis has been coined to describe it [14]. The etiology of ASD is now better understood and the characteristics symptoms such as behavioral changes and gastrointestinal complications are common and well-established features of this disorder [45–47]. 16S rRNA gene sequence-based studies have shown dysbiosis of the gut microbiome in ASD children in the American and European populations [48, 49]. The alteration of gut flora in ASD was also associated with significant dysbiosis of certain bacterial taxa such as *Sutterella*, *Akkermansia*, and *Fusobacterium* [50, 51]. However, consistent results were not obtained due to the variable factors such as population, age, gender, diet, and autism severity [37, 49–52].

The human gut microbiome of children from the Indian subcontinent is not comprehensively characterized [24]. In fact, the Indian population is a lesser explored population in terms of gut metagenomic profiling with cohorts exhibiting vast diversity in culture, food habits, ethnicity, and geographical locations. Therefore, to understand the association of gut microflora with ASD, comprehensive profiling and comparisons of gut microflora in healthy and ASD individuals is much needed. It also becomes more relevant since the ASD children

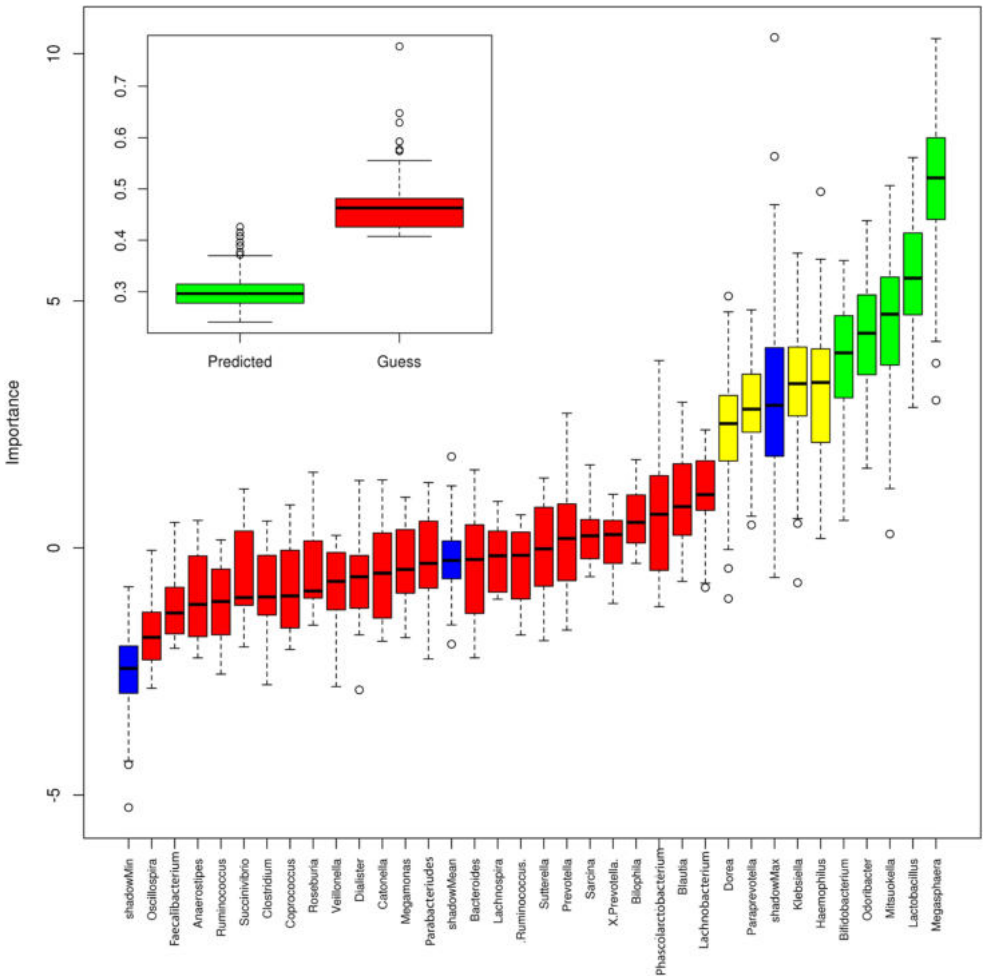

Fig. 4 The model used for the classification of ASD and healthy samples based on genus abundance profiles across the samples using random forest (RF). The boxplots colored in "green" were selected as highly discriminatory by Boruta algorithm, and the boxplots in "yellow" were selected as near discriminatory between the datasets. The boxplots in "red" were found as nondiscriminatory. The "blue" boxplot showed minimum, median, and maximum z-scores of shadow genera

in India are generally on their native diet, as observed in this study. In contrast, previous studies carried out so far had explored the microbial diversity of ASD children, which were on a gluten-free diet. With this objective, we investigated the gut microbiota of 30 ASD children from the Indian population and compared with 24 family and age-matched healthy children to nullify the variations arising due to differences in food habits and the environment. Thus, the novelty of our study lies in the inclusion of age- and family-matched children forming a reasonable-sized Indian cohort, along with a comprehensive comparative analysis with another population, which resulted in the identification of bacterial taxa associated with autism. Several reports and hypothesis have shown the dominance of ASD in males, and in this study, we also observed an identical trend of a higher male proportion in ASD children [8, 53]. Further, no influence of gender on the phylogenetic profiles was found, which was assessed using correlation analysis by PCoA scores. This suggests that gender did not influence the phylogenetic profiles in this study (Supplementary Table S12). Further, the observed lower BMI in ASD children could be a consequence of associated GI disorders in ASD, causing most of them to be underweight [54].

The first key observation from the study was that the diversity of fecal microbiota was not significantly (α- and β-diversity) different in ASD and healthy children. Furthermore, using the PCA (Fig. 1), the correlations of the covariates showed autism to be a significant component in differentiating the gut microbiome of ASD and healthy samples at PC3. The proportion of phylum Firmicutes was found to be significantly higher in ASD cases, which corroborates with the earlier reports where a higher proportion of Firmicutes as compared to Bacteroidetes was linked with dysbiosis in gut microbiome and gut-related diseases [55–57]. Several recent reports have also suggested that the gut microbial dysbiosis can modulate human behavior and can also play a role in the development or presentation of ASD-associated symptoms [15, 58], which corroborates with our findings. Prevotellaceae emerged as the most abundant family in the fecal microbiota of healthy children in this study, which is similar to the observations made in a recent report from the Indian population [23]. In contrast, the cohorts from USA and Europe were dominated by family Bacteroidaceae [59, 60]. These differences appear to be mostly attributable to the differences in geographical location and diet between the two populations [61].

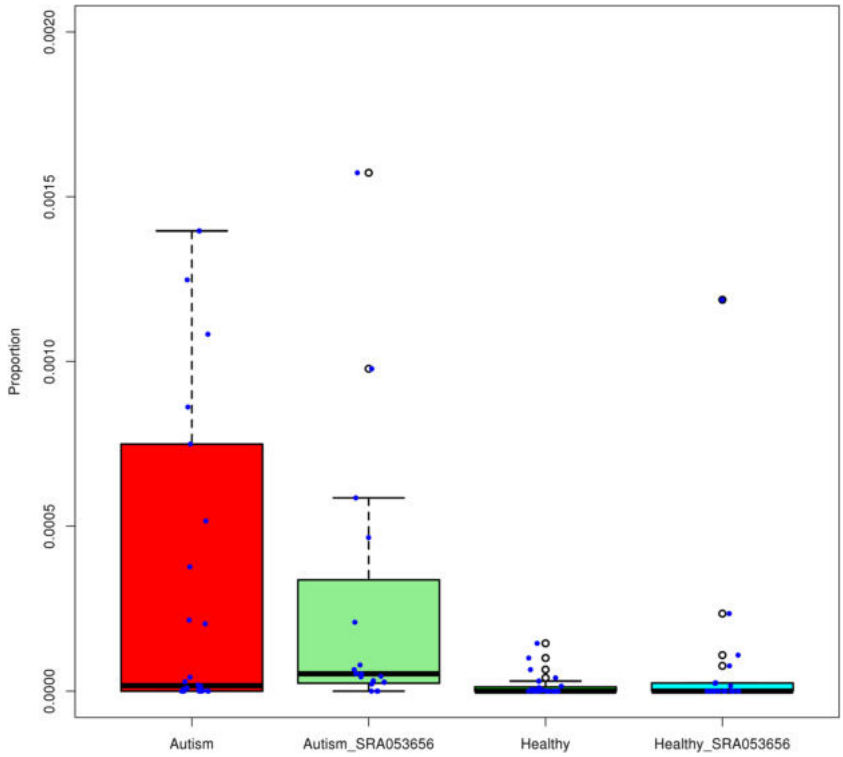

Fig. 5 The meta-analysis identified *Lactobacillus* to be significantly associated with autism in Indian and US populations. The comparison of genera in ASD and healthy children from combined Indian and USA population using Wilcoxon rank sum test identified *Lactobacillus* to be significantly abundant in ASD children as compared to healthy samples in both the dataset with FDR-adjusted *P* value < 0.01. The boxplots represent the interquartile range with the upper and lower ends of boxes showing 75th percentile and 25th percentile of the data distribution, respectively. The dark "black" lines between the boxes represent the median values. The whiskers extending on both sides of the plot are 1.5 * IQR. The open circles outside the whiskers represent the outliers. The "blue" dots in the plot show individual sample proportion values of *Lactobacillus* in different groups of samples

Interesting revelations appeared from the comparison of microbial taxa between the ASD and healthy groups. Genus *Lactobacillus* (family Lactobacillaceae), *Bifidobacterium* (family Bifidobacteraceae), *Megasphaera*, and *Mitsuokella*

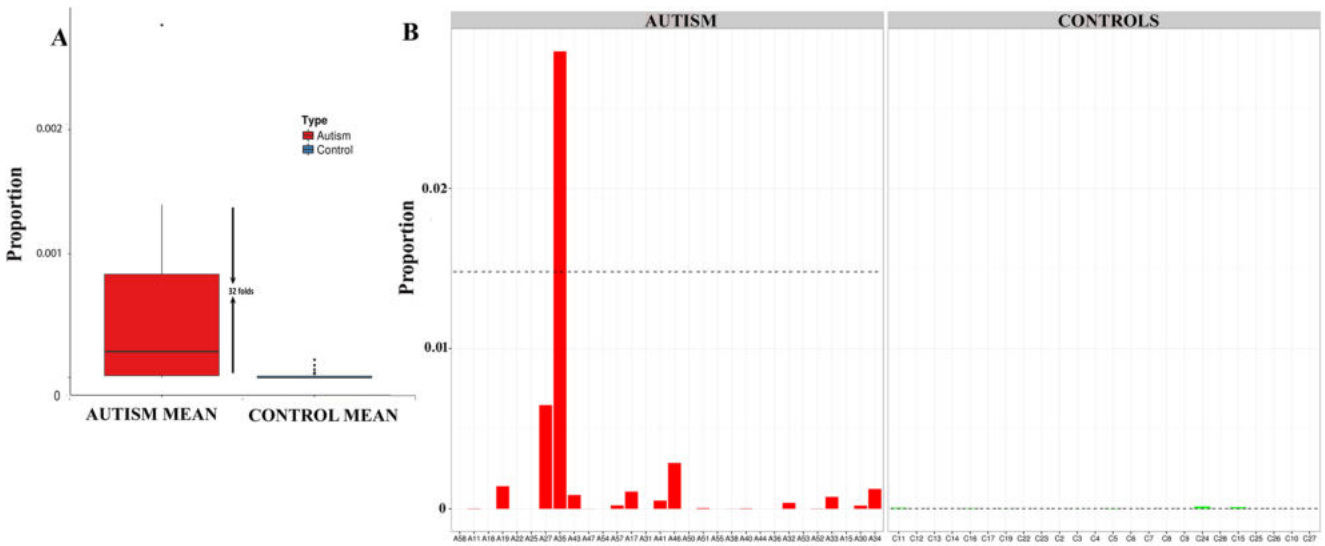

Fig. 6 Relative abundance of *Lactobacillus* in ASD and healthy children and their fold difference. **a** The fold difference of *Lactobacillus* genus between ASD samples and healthy children, which showed a very high proportion of *Lactobacillus*, are shown here. The fold difference was calculated after removing the outlier A35. **b** Bar plots are showing the relative abundance of *Lactobacillus* between ASD and healthy children with mean values (as a dashed line)

(Veillonellaceae) were significantly abundant in ASD children as compared to healthy children. The Veillonellaceae family has been associated with carbohydrate-rich diet, which also contains gluten [62]. In this study, both ASD and healthy children were on a similar native diet, which is omnivorous but is also carbohydrate-rich; thus, the abundance of Veillonellaceae family in both ASD and healthy cases with a higher abundance in ASD seems to be due to the consumption of carbohydrate-rich diet [63]. The RF-based analysis further confirmed these results showing a high importance score for genus *Lactobacillus* in association with ASD.

On comparison of our data with a similar autism microbiome study dataset from the USA, the gut microbiome of the Indian ASD children was abundant in genus *Megasphaera*, *Bifidobacterium*, and *Mitsuokella*, whereas the gut microbiome of the US ASD children was abundant in genus *Akkermansia*, *Ruminococcus*, and *Lactobacillus*, which could be attributed the different dietary habits and the consumption of gluten-free diet by ASD children in the former study. Interestingly, *Prevotella* was depleted in the ASD children from both populations. Most notably, *Lactobacillus* emerged as the only common genus in the two populations, which showed significantly higher abundance in ASD children as compared to healthy children. However, in the study by Kang et al., though *Lactobacillus* appeared as a significantly abundant genus, the authors did not conclude any association of *Lactobacillus* with autism (Supplementary Table S13) [25]. In this study also, a ~ 32-fold higher relative mean abundance of *Lactobacillus* in ASD samples was observed as compared to the healthy samples. The individual ASD samples also showed a higher abundance of this genus. These observations point towards an association of higher abundance of genus *Lactobacillus* with autism. However, if the reason for the overabundance is a cause or a consequence of the disorder requires further analysis.

The bacterial species belonging to genus Lactobacillus are lactic acid producers commonly known as lactic acid bacteria (LAB) and have gained much attention due to their probiotic nature. *Lactobacillus* is a common inhabitant of our gut and is considered as a prominent member of the healthy gut microbiota in infants, which is primarily because their diet is rich in lactate from the milk. The proportion of this genus decreases with an increase in age since the diet becomes more diverse as they grow. Interestingly in this study, which included samples from young children in the age group of 3–16 years, a contrasting pattern of gradual increase in Lactobacillus proportion with an increase in age was observed in the ASD children (Supplementary Table S14). Both *Lactobacillus* and *Bifidobacterium* are considered as probiotic bacteria with beneficial roles in human health. The immunomodulatory properties of LABs, including *Bifidobacterium*, are a topic of recent interest [64, 65]. A study by Cvijin et al. showed that a higher proportion of *Lactobacillus* leads to the inhibition of an immune modulator enzyme IDO1 (indoleamine 2,3-dioxygenase), which ultimately affects the Th17 cell functioning and leads to the atopic dermatitis condition [66, 67]. Another study reports the higher proportion of IL-6, IL-10, TNF-α (pro-inflammatory cytokine), and certain chemokine production by NF-κβ pathways in the intestinal epithelium cells with a *Lactobacillus* strain [68]. The elevated levels of certain pro-inflammatory cytokines (IL-6, IL-12p40) are associated with impaired communication, aberrant behaviors, and atopic dermatitis situation by disrupting the blood-brain barrier in ASD [69, 70]. A differential influence on function due to the strain or species variants of a taxa could also be noted in the cases of *L. reuteri* and *L. ruminis*, where the former has shown to revert the ASD-like behavior in model-based studies [71], whereas the latter is known to have immunomodulatory activity and was also observed to be the most abundant species in ASD cases in this study [72].

In the context of gut microbial dysbiosis observed in ASD, an important aspect is the role of gut microbiota in the regulation of short-chain fatty acid (SCFA) production [73]. SCFAs are gut microbial-derived bacterial metabolites which play a critical role in the immune system, brain functioning, and behavior [43, 58]. It has been suggested that dysbiosis in gut microbiota leads to an imbalance in the SCFAs levels in the circulatory system, which may lead to an increased gut permeability detrimental to the ASD children [74]. Also, a high abundance of Lactobacillaceae and Bifidobacteriaceae families has been associated with a decrease in SCFA-producing bacteria [75]. Previous metabolomics studies have also shown lower levels of SCFAs in ASD children [76]. In this study, we also observed a reduction of certain SCFA producing microbes such as *Faecalibacterium* and *Roseburia* in ASD children, which supports the hypothesis that the lower levels of SCFA in ASD leads to an imbalance in brain functioning and behavior, and thus, the proposed introduction of these strains as a probiotic to the affected children could help in alleviation of the common gastrointestinal problems [77]. Our study also revealed certain other microbial families such as Veillonellaceae and Enterococcaceae to be higher in ASD children [77]. These were not reported in previous ASD studies but corroborate with an earlier report by Oberc et al., which suggests their association with other GI disorders such as Crohn's disease [78]. The plausible reason for the presence of these species appears to be the native omnivorous diet of ASD children, unlike the gluten-free diet in the other similar studies [25, 49].

To conclude, the microbial profiling of age and family-matched ASD and healthy children and the inclusion of a modest number of samples in this study provide useful insights on diversity and taxa changes associated with autism. The study highlights the differences in microbial community structure between the ASD and healthy children. The key novel finding is the high abundance of genus *Lactobacillus* among ASD children in Indian and US population, which

point towards the association of this genus with autism. This association draws support from the fact that despite the differences in diet in the Indian (normal native diet) ASD and US (gluten-free diet) ASD children, *Lactobacillus* species were the most dominant in both the populations. However, it remains unclear whether the abundance of *Lactobacillus* is a cause or an effect of ASD. Also, there could be several confounding factors that might possibly affect the observed associations. Among these factors, the influence of diet, habitat, and age was examined in this study and did not show any significant affect. We anticipate that the future studies including more samples from multiple populations will help to validate the findings of the present study. Furthermore, to gain functional insights into the gut microbiome of ASD individuals, metagenomics and metabolomics studies are needed to better comprehend the association and role of *Lactobacillus* and other bacterial markers with ASD in Indian and other populations. This study provides the largest dataset of gut flora from Indian children and is also the first study, which has shown the association of gut microbiome with autism in the Indian population. The novel insights on the significant differences in gut flora between ASD and healthy children are expected to aid in developing an improved understanding and potential role of microbial community in ASD pathophysiology. The results also provide leads for further research to identify the therapeutic and diagnostic potential of the gut microbiome in ASD.

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Author Contributions JP and BM collected the samples. DBD carried out the metagenomic data analysis and all computational and statistical analysis. RS and AM carried out the library preparation and sequencing work. AM, DBD, JP, RS, TG, and VKS drafted the manuscript. MMA and NA performed the diagnosis of all the cases. TG and VKS conceived the work and participated in the design of the study. All authors read and approved the final manuscript.

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Compliance with Ethical Standards

The protocols for sample collection, sequencing, and analysis as described in the "Materials and Methods" was conducted in accordance with the approved guidelines by the Institutional Ethical Committee of IISER Bhopal, India.

Competing Interests The authors declare that they have no competing interests.

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