Brain, Behavior, & Immunity - Health 15 (2021) 100269

Contents lists available at ScienceDirect

Brain, Behavior, & Immunity - Health

journal homepage: www.editorialmanager.com/bbih/default.aspx

Mucosa-associated specific bacterial species disrupt the intestinal epithelial barrier in the autism phenome

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ARTICLE INFO

Keywords:
Autism phenome
Gut microbiome
Behavior reversal
meta-Analysis
16S rRNA sequencing
Operational taxonomic units (OTUs)

ABSTRACT

Gut-Brain Axis provides a bidirectional communicational route, an imbalance of which can have pathophysiological consequences. Differential gut microbiome studies have become a frontier in autism research, affecting 85% of autistic children. The present study aims to understand how gut microbiota of autism subjects differ from their neurotypical counterparts. This study would help to identify the abundance of bacterial signature species in autism and their associated metabolites.

16S rRNA metagenomic sequence datasets of 30 out of 206 autism subjects were selected from the American Gut Project Archive. First, the taxonomic assignment was inferred by similarity-based methods using the Quantitative Insights into Microbial Ecology (QIIME) toolkit. Next, species abundance was characterized, and a co-occurrence network was built to infer species interaction using measures of diversity. Thirdly, statistical parameters were incorporated to validate the findings. Finally, the identification of metabolites associated with these bacterial signature species connects with biological processes in the host through pathway analysis.

Gut microbiome data revealed *Akkermansia* sp. and *Faecalibacterium prausnitzii* to be statistically lower in abundance in autistic children than their neurotypical peers with a five and two-fold decrease, respectively. While *Prevotella* sp. and *Sutterella* sp. showed a five and a two-fold increase in cases, respectively. The constructed pathway revealed succinate and butyrate as the significant metabolites for the bacterial signature species identified.

The present study throws light on the role of mucosa-associated bacterial species: *Veillonella* sp., *Prevotella* sp., *Akkermansia* sp., *Sutterella* sp., *Faecalibacterium prausnitzii, Lactobacillus* sp., which can act as diagnostic criteria for detection of gut dysbiosis in autism.

1. Introduction

Autism is a neurodevelopment disorder with a prevalence rate of 1 in 54 children in the United States of America (Centers for Disease Control and Prevention, 2019). Characterized by social communication deficits and interaction with restricted, repetitive behavioral patterns, it arises early in the developmental stage and persists throughout the lifetime (Eapen et al., 2019). These deficits result in heterogeneity in autism with added severity, making diagnosis and treatment a challenging task. Genetics and environmental factors share a close association with autism. Studies ranging from linkage to present-day next-generation sequencing have identified multiple genetic factors for autism manifestation (Girirajan et al., 2013; Leblond et al., 2012). However, autism is heavily dependent on one on one therapy and behavioral-based approaches. Right from birth, after the initial inoculum is received from the mother, an individual harbors complex, diverse and unique pattern of gut microbial community owing to various environmental conditions (Rodríguez et al., 2015). Gut bacteria are in a commensal relationship with the intestinal tract, depending on the host for nutrition and space. Such interactions provide multiple benefits to the host in life processes and better sustainability. Under normal conditions, microorganisms help maintain a blood-brain barrier in the human host with a crucial role in digestion assistance, as antagonists to potential pathogens and increased immunity by promoting cell differentiation and stimulation/alteration of the immune system (Galland, 2014). This gut-brain axis monitors and integrates the central nervous system and intestinal functionalities as one unit in a bidirectional manner. This axis links the emotional and cognitive centers in the brain with gastrointestinal homeostasis (Rutsch et al., 2020). Proper maintenance of these functionalities across this axis renders stable processes to operate from gut to brain and vice versa. Any

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https://doi.org/10.1016/j.bbih.2021.100269
Received 23 March 2021; Received in revised form 6 May 2021; Accepted 10 May 2021
Available online 14 May 2021

kind of dysregulation due to pile up of neurotransmitters, altered enzyme activity, compromised immunity, increased mucus layer can lead to a leaky and dysfunctional gut-brain axis and altered abundance of gut microorganisms (Carabotti et al., 2015). These altered microorganisms can render this blood-brain barrier permeable, which impacts brain development and functionality (Kadry et al., 2020; Logsdon et al., 2018; Segarra et al., 2021). Over the last two years, many studies have confirmed a connecting link between the disrupted gut microbiome and disease severity in humans (Dhar and Mohanty, 2020; Kho and Lal, 2018; Ma et al., 2019; Zheng et al., 2020; Zhu et al., 2020). In accordance, most autistic subjects encounter genetic abnormalities, compromised immune responses, inflammatory and gastrointestinal (GI) problems (Rose et al., 2018; Van Sadelhoff et al., 2019). For instance, inflammation in the gut is induced with the help of several mediators such as cytokines with promotion and regulation as key functions. The balance of cytokine and anti-inflammatory molecules determines whether inappropriate mucosal inflammation will occur or not. Under normal conditions, only when there is a perturbational change, the neurotransmitters are released, which initiate the production and travel of metabolites to the brain through the bloodstream as a response (Rose et al., 2018). Once the metabolites reach the site of action, the signal is turned off, and the production and transport halt the cycle. However, due to the absence of stimuli to turn it off during a prolonged perturbation, a pile-up of neurotransmitters and cytokines happens in the brain, leading to lesions and inflammation in the brain and gut, respectively (Mittal et al., 2017). This initiates a systemic enteric inflammation, leading to nerve dysfunction due to a compromised gut blood-brain barrier, as evident in autism (Eshraghi et al., 2020). Studies have pinpointed autism-associated GI problems such as diarrhea, constipation, and abdominal pain to result in altered cytokine levels and neurotrophins in autistic subjects (Chaidez et al., 2014). For instance, gut dysbiosis can result in the accumulation of neurotransmitters leading to behavioral severity in autistic subjects (Ghaisas et al., 2016). A case study of a sib-pair showed that the mutant *NLGN3* gene, resulting in autism in them, was responsible for esophagitis and diarrhea. Similarly, numerous studies have shown a direct connection of bacterial signature species and perturbations in the gut with cognitive functions and the development process in the brain-vital to autism (Al-Asmakh et al., 2012; Hasan Mohajeri et al., 2018; Ma et al., 2019). This was evident from knock-out mice experiments for the mutant genes, which showed faster food movement from the small intestine (Leembruggen et al., 2020). The pattern of autism microbiome varies depending on diet, environment, age, and ethnicity, which are indicative based on differential bacterial signature species present in the autism gut compared to neurotypical peers (Fattorusso et al., 2019). Recent studies account for the lower abundance of *Bifidobacteria* sp and the mucolytic bacterium *Akkermansia muciniphila* in autistic children (Wang et al., 2011). Moreover, less diverse gut microbial composition comprises lower *Prevotella, Coprococcus,* and unclassified *Veillonellaceae* (Kang et al., 2013). A significant increment in several mucosa-associated bacteria *Clostridiales* was seen, alongside a decrease in *Dorea, Blautia,* and *Sutterella* in the autism gut (Luna et al., 2017). The study of autism and the gut-brain axis is of great importance to therapeutic research in autism. Hence, it is vital to understand the microbial makeup of the gut of an autistic subject as compared to an age-matched healthy subject, influencing autistic behavioral severity. In view of this, here, the authors have attempted to explore the differential abundance of bacterial signature species in the gut of autistic subjects.

2. Materials and methods

2.1. Sample cohort

The datasets for the study: 16S rRNA V4 semi-conserved hypervariable region sequences were obtained from the American Gut Project (crowd-funded project by Rob Knight) with study accession PRJEB11419 at European Nucleotide Archive (ENA). The research group chose the V4 region for sequencing mostly because of its fine resolution to identify phylum level information accurately as the whole 16S rRNA gene. A total of 15,096 sample sequences were initially screened for autism subjects, based on provided attributes and detailed case histories. Primary investigators and the authors of this study obtained informed written consent from all participants for experimentation related to human subjects. The authors identified 206 samples to be autistic with comorbidities such as epilepsy, Alzheimer's, seizure, and schizophrenia in them. Out of which, 30 classical autism samples without any comorbidities with 30 age-matched controls were selected based on inclusion-exclusion criteria: mode of sequencing, age, diet, exercise, microbial health, and lifestyle diet. Sample case histories were referenced for uniformity in dietary habits of the case controls datasets. Although the sample size is small, the homogeneous nature of the sample cohort holds the study significant. Demultiplexed raw sample data files were retrieved in FASTQ format from the ENA archive. The detailed workflow has been included (Fig. 1).

2.2. Metagenomic data analysis

Comparative metagenomic data analysis was carried out using Quantitative Insights into Microbial Ecology (QIIME 1.8.0) (Kuczynski et al., 2012) to assess microbial profiling analysis. QIIME was used to perform sequence alignment, identification of operational taxonomic units (OTUs), elaboration of phylogenetic trees, and phylogenetic and taxon based analysis of measures of diversity, both alpha diversity (within samples) and beta diversity (between samples).

Bioinformatics analysis for the gut microbiome study included preprocessing, 16S rRNA detection, OTU clustering and identification, taxonomical classification of bacterial sequences, and diversity analysis (Supplementary Material 1).

Preprocessing step included converting FASTQ files into FASTA and qual files, truncation at the specific base position, and filtering with demultiplexing and removing primers and quality filters. Quality filtering parameters were set for QIIME: (i) a minimum average quality Phred score of 33 in reads as a threshold; (ii) a minimum and maximum sequence length in the range of 200–1000 nucleotides. To have stringency, no primer mismatches were allowed (setting the parameter: primer mismatches = 0). Maximum error counts of only 1.5 in barcodes were permitted. Removal of adapters, PCR primers, and low-quality reads was performed for effective analysis. Low reading bases were truncated through statistic result files in the form of quality bins and quality score plots beyond 140 nucleotide bases. Chimeric sequences generated by the incomplete 16S region of interest were detected and removed using the USEARCH tool. The mapping file was generated as a unique sample identifier with information about barcode and primer used per sample. This mapping file is used for downstream analysis to demultiplex the high throughput sequence data.

OTU clustering analysis was performed for the processed microbiome datasets using the uclust algorithm, identified at $\geq$97% sequence similarity. Uclust is the best-suited algorithm for closed reference OTU clustering, performed by grouping raw sequence reads based on sequence similarity threshold into OTUs. These clusters represented a taxonomic unit of bacterial genus or species, with a 97% similarity threshold at genus level while 98% or 99% identity for species separation. Reads with no sequence hits in the reference sequence collection were discarded from downstream analysis.

Taxonomic inference for the observed OTU was assigned using the consolidated comprehensive Greengenes (DeSantis et al., 2006)and SILVA reference databases (Pruesse et al., 2007) for sequence annotation of bacterial metagenomes. The OTU list was used to build the OTU table in Biological Observation Matrix (BIOM) format, required for downstream analysis. This BIOM file helped in the visualization of the different bacteria present in samples and their relative abundances. Summarization of taxa in communities was performed using pie charts and plots at seven different taxonomic levels (L) of summaries (L1 L7)**:** L1: Kingdom,

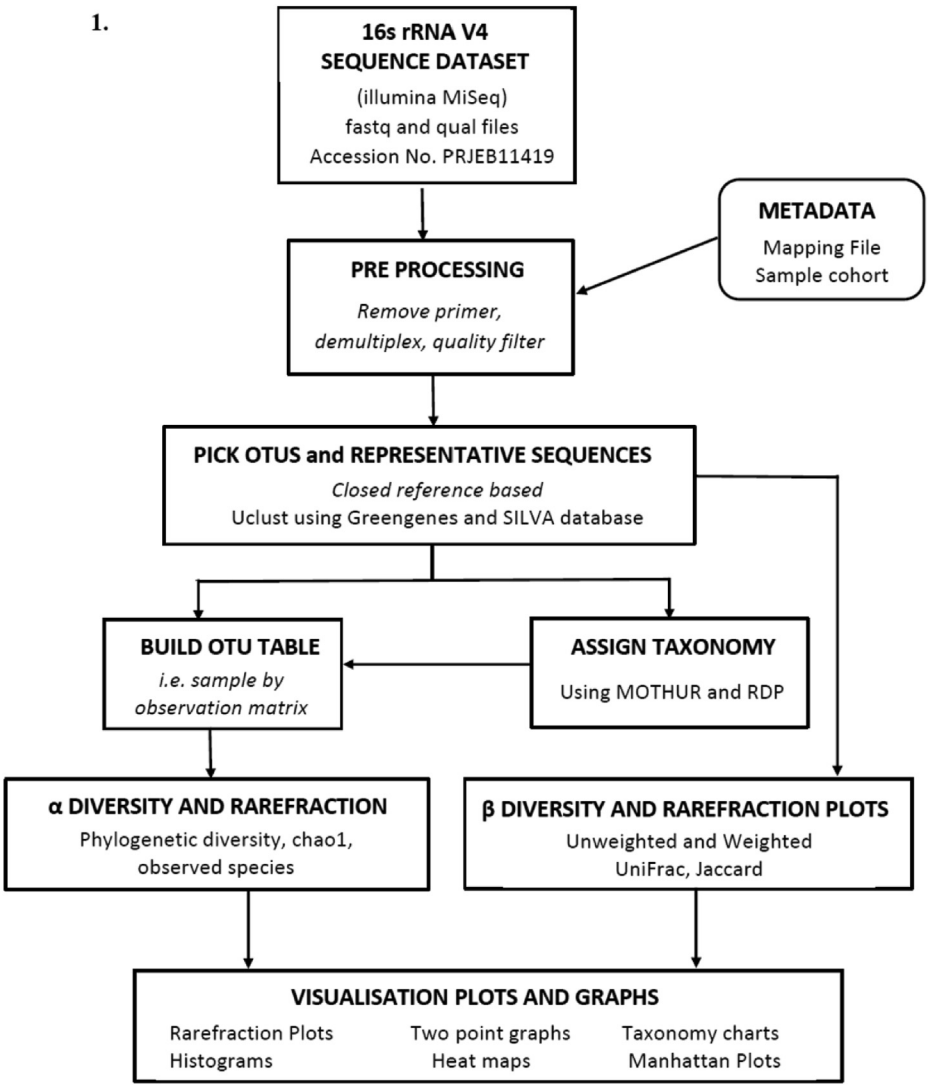

Fig. 1. Schematic flowchart of the 16S rRNA sequence analysis pipeline for the study.

L2: Phylum, L3: Class, L4: Order, L5: Family, L6: Genus, and L7: Species levels.

2.3. Statistical analysis

Comprehensive diversity analysis was performed through various diversity indices for subsequent comparative and statistical evaluation. First, a rarefaction study for the samples was performed to maintain even depth reads to avoid false positive outcomes. Minimum rarefaction was chosen depending on the minimum number of sequences per sample. Plateau structured rarefaction plot was indicative of proper sequence coverage and sampling depth. Second, measures of diversity alpha diversity (within-sample diversity) and beta diversity (between-sample diversity) were calculated for the diversity of microbiome structure in the sample cohort. For each rarefied OTU Table, alpha diversity was calculated using estimates of species richness and diversity indices like phylogenetic distance/diversity (PD_whole_tree), chao1, observed_otus, observed_species, and Shannon and Simpson Index, respectively. Beta diversity was calculated based on weighted and unweighted UniFrac analysis variants revealing differential OTU distribution among case-control samples. Weighted UniFrac detected community differences arising from differences in relative taxa abundance, while Unweighted UniFrac identified the absence or presence of the OTUs from each community and detected community differences arising from the differential presence of specific taxa. Various statistical analysis parameters like P-value, Standard Deviation, Mean and Standard Error were calculated to ensure statistical significance. All information about statistical analysis is tabulated in Supplementary Figure 1.

2.4. Critical bacterial species analysis

Taxonomic level of summary for bacterial species obtained was used to filter out bacterial species based on their relative abundance and relatedness to autism pathophysiology using curated literature on autism gut microbiome since its inception. The top five critical bacterial species were selected for downstream analysis based on inclusion criteria: bacteria to be selected should have: significant fold change and relative abundance of the bacteria with a P-value $\geq$0.05. Exclusion criteria were to exclude bacterial species with nonsignificant P-value, no fold change, and negligible difference in abundance in cases versus controls.

2.5. Pathway analysis

A network pathway was created using Ingenuity Pathway Analysis (IPA) to establish a relationship between the identified bacterial signature species (Krämer et al., 2014). IPA contains a repository of metabolomics data against which bacterial species and their biological functions were used as input. Once the identified bacterial species were

added to the pathway designer, relevant disease and functions with valid P-value were added and connected to relevant bacterial species in a step-by-step manner. Each identified species was subjected to expand for two direct relationships, which were then connected to the rest of the pathway. Similarly, metabolites were connected to the bacterial species and the functions identified. Finally, upstream and downstream of the bacterial metabolites and genes present were overlaid. These rendered gene networks responsible for the bacterial species, which were cross-validation against the current literature. The pathway enrichment was performed using P-value, Z score, and Jaccard similarity testing to identify enriched genes and bacteria and their correlations (Gholipoorfeshkecheh et al., 2020).

3. Results

16s rRNA gut microbiome analysis of 30 cases controls sample cohort showed 17,000 high quality, enriched filtered, and classifiable reads with a mean average (±SD) of 24464.00 ± 15146.0 sequences per sample in each category. The total number of observations was 5462 with a table count of 1467860 with a density of 0.084. On further filtration and downstream analysis, a total of 4372 and 4370 OTUs were identified in the autism and control cohorts, respectively.

Phylum *Firmicutes* were harbored significantly higher in cases (45.6%) than control samples (38.6%). In contrast, the Phylum *Proteobacteria, Actinobacteria*, and *Verrucomicrobia* were in higher abundance in control (31.4%) than that of cases (20.6%). *Synergistetes* were present only in control samples, while *Euryarchaeota, Cyanobacteria, Fusobacteria* are exclusively present in autism samples with 0.1%, 0.1%, and 0.7% abundance, respectively (Table 1, Fig. 2). Phylum-wise, multiple overlaps were observed across cases and controls alike. On further slice down at the species level, differential species abundance was found across cases and control samples. Following *Firmicutes* trend in Phylum, significantly higher abundance was harbored by *Streptococcus* belonging to Firmicutes in cases. Genus *Bacteroides* were present across all the samples (Fig. 2). Although the V4 sequencing could not imply bacterial species at the genus level with much confidence, there were 240 genera identified for the sample set.

Single and multiple rarefactions of the sample data for measures of diversity indices were performed. Based on the OTU sequence depth of 10, the OTU counts have been equalized at 140 and 40 sequences per sample for a rarefaction measure of 40 and 15 for cases and controls, respectively. It depicts the diversity across cases and controls (Fig. 3). Two-point Manhattan plot shows lower P values with higher peaks with logarithmically transformed P values. The horizontal lines represent P values of 0.10, 0.05, and 0.01, inclusive of $P = 0.10$, highlighting taxa *Prevotella* sp. approaching significance in the analysis. In the Manhattan plot, the first significant peak (position 380) corresponds to *Prevotella* with higher proportions and relative abundance in the cases (Fig. 3). A mixture of red and black could be seen on the heat map suggesting that the species abundance is similar across controls than across cases versus controls. Black indicated that the samples constituted similar taxonomy patterns, and red indicated differential taxonomy in the samples (Fig. 4). Heat map depicting Morisita Horn beta diversity using OTU table for *Prevotella* sp. *was* revealed with a varying gradient of species abundance. This map ranged from conserved to unique abundance blocks as the coloration scale lightens (Fig. 4).

Taxa summary gave ~230 genera present in case and control samples. Five were selected for the downstream study considering their relative abundance and their relatedness to autism. *Akkermansia* sp. was in lower abundance by 0.6% in the autism sample. *Prevotella* sp. and *Veillonella* sp. were in higher abundance in autism samples by 4.9% and 2.7%, respectively. *Sutterella* sp. have a twofold change in cases than in controls. *Faecalibacterium* sp. showed two-fold reduced abundance in cases compared to controls (Table 1).

Pathway analysis revealed three significant hubs for metabolites: succinate, butyrate, and pyruvate. These were interconnected through functions such as pyruvate and butyrate shared cell death of cortical neurons as a common functionality. Butyrate was enriched for numerous autism-relevant functions, namely, associative and motor learning, long-term potentiation of collateral synapses, and long-term memory and contextual conditioning. Butyrate was shown to be responsible for inflammation of cerebellar granule cell and astrocytes, inhibition of Salmonella, cell death of cortical and cortico-striatal neurons, and lesioning of the striatum. In addition, butyrate was shown to be interacting with *SLC26A5*-known to be responsible for hearing loss or deafness in autism as a phenotype. Succinate is involved in cell apoptosis via the TGF beta signaling pathway, glutamate degradation, and valproic acid and calcium ions (Fig. 5).

Table 1
Relative abundance of enriched bacterial genera in the fecal microbiota of autism cases and control samples with fold change.

Sl. No.	Enriched bacterial genera	Relative Abundance (in %)		Fold Change
		Case	Control	
1.	*Veillonella*	3.4	0.1	Five Fold
2.	*Prevotella*	5.4	0.6	
3.	*Akkermansia*	0.2	1.2	
4.	*Citrobacter*	0.1	0.6	
5.	*Bifidobacterium*	1.0	3.8	
6.	*Fusobacterium*	0.6	0	
7.	*Sarcina*	0.3	0.1	Two Fold
8.	*Sutterella*	0.4	0.2	
9.	*Faecalibacterium*	4.7	8.1	
10.	*Lachnospira*	0.7	1.1	
11.	*Dialister*	1.1	1.7	
12.	*Bacteroides*	14.9	18.6	
13.	*Streptococcus*	2.9	3.2	
14.	*Lactobacillus*	0.1	0.5	No Fold Change
15.	*Enterococcus*	0.1	0.5	
16.	*Haemophilus*	1.3	0.6	
17.	*Collinsella*	0.2	0.1	
18.	*Corynebacterium*	0.2	0.1	
19.	*Ruminococcus*	0.1	0.2	
20.	*Roseburia*	0.1	0.2	
21.	*Phascolarctobacterium*	0.2	0.3	
22.	*Parabacteroides*	1.9	1.6	
23.	*Blautia*	1.8	1.9	

4. Discussion

The present study reveals that 1.8% of the American gut population under the pressure of autism with GI problems. Few mucosa-associated bacterial species were present in abundance, while some species were present in lesser quantities in autistic subjects. These notable mucosal bacteria suggest elevated bacterial populations in autistic children, leading to augmented autistic behavior, namely anxiety, behavioral differences, and other irritational consequences. OTU analysis showed a balanced trend across cases and control samples; however, a gradient of varied abundance at the phylum level was observed. Positive correlation of abundance of Phylum Firmicutes with five behavioral domains: gross motor skills, fine motor, adaptation, personal-social, and language, have been reported with significant Pearson's $r = 0.327$ and P-value < 0.05 in autism. (Ding et al., 2020; Zhang et al., 2021). A higher abundance of Firmicutes indicates that they have not impacted the autism behavior at the phylum level. However, the abundance of butyrate quantity due to increased Firmicutes might lead to gut dysbiosis (Liu et al., 2019). In the current study, the ratio of Bacteroidetes/Firmicutes was significantly higher in autistic children, primarily due to varied environmental conditions and differential lifestyle habits, reflected in their relative abundance. This finding is in alignment with a research study on Chinese children with autism (Liu et al., 2019). This ratio can be considered as a hallmark and relevant marker for gut dysbiosis. A higher ratio indicates an increased abundance of *Bacteriodetes* sp., which results in autism phenotype severity (Coretti et al., 2018). However, previous studies have

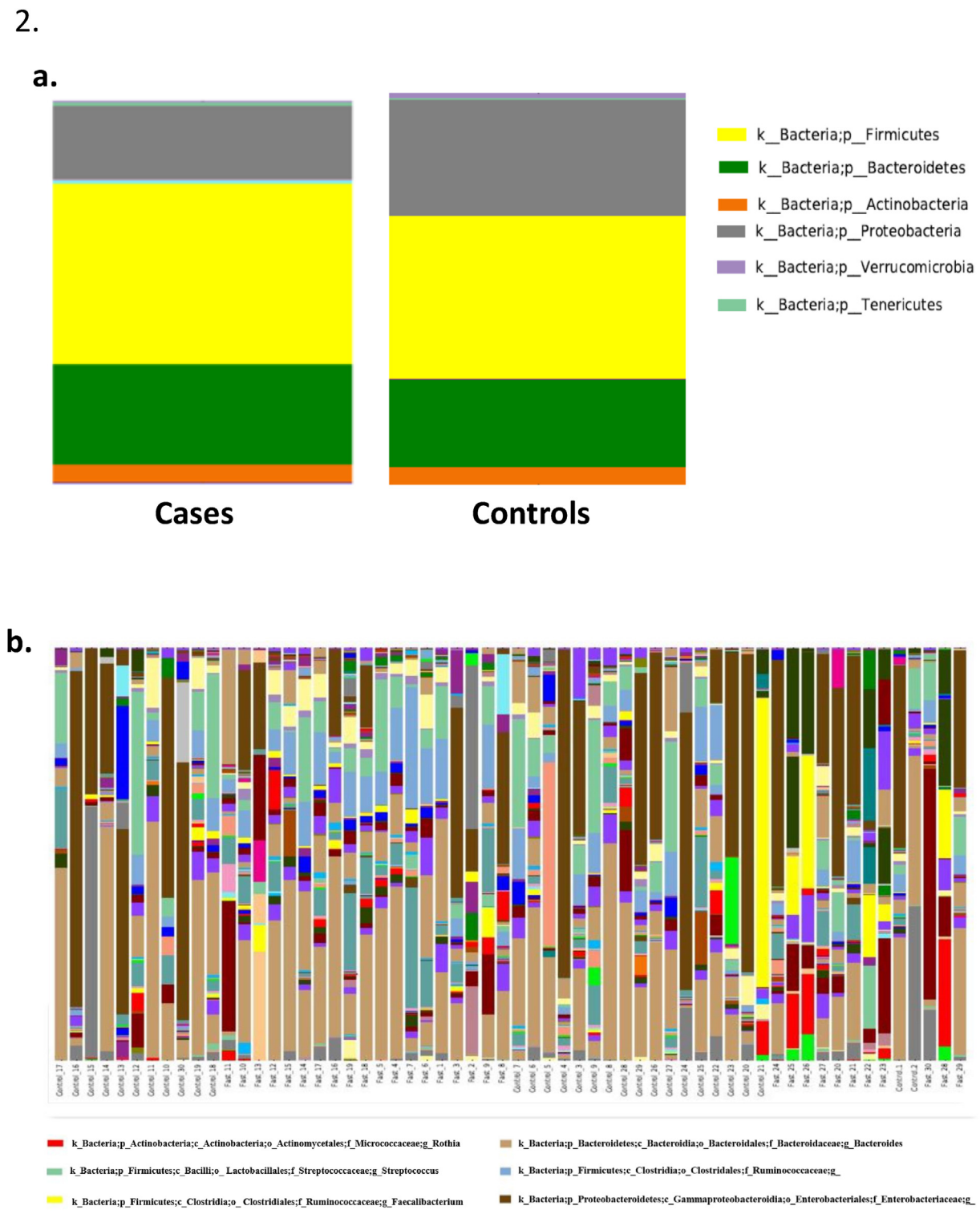

Fig. 2. Gut microbial gradient diversity of autism cases versus control at (a) phylum and (b) species level.

shown negative associations between the proportion of Bacteroidetes and cognitive disability, as evident in the current study at the phylum level (Manderino et al., 2017). Further, our analysis at the taxa levels indicated an unbalanced abundance of Firmicutes than Bacteriodetes in the autism cases. This resulted in a shift in the colonization of beneficial gut bacteria, indicating higher susceptibility to harmful and pathogenic bacteria for autistic behavioral severity. Our observations on the elevated *Proteobacteria* levels contrast with reported inclinations towards chronic psychosocial stress, persistent inflammatory response, and invasion of intestinal epithelial cells in mouse models (Langgartner et al., 2017). This suggests that level of *Proteobacteria* is a potential diagnostic criterion for autism gut dysbiosis.

Further, measures of diversity: alpha and beta diversity were performed to understand the species abundance within and between samples for the taxonomic classification. Alpha diversity calculation ensures even depth of the sample set through rarefaction, maintained at 140 nucleotides. Alpha-diversity was measured as the observed richness for an average sample within a species, while beta-diversity was calculated

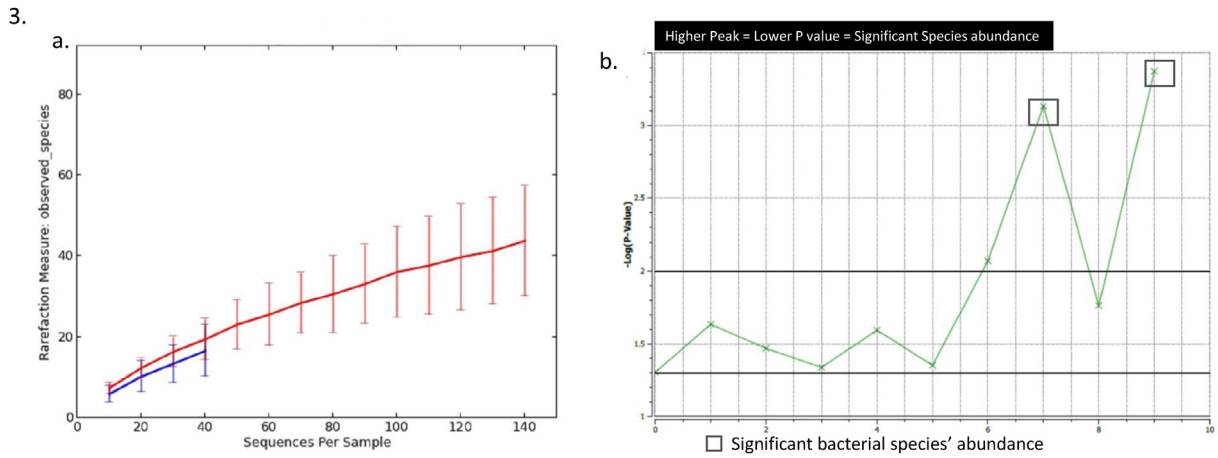

Fig. 3. a). Alpha diversity graph for case and control samples using QIIME respectively b). Two-part Manhattan test plot depicting peaks enclosed in squares corresponding to species abundance in autism samples using QIIME data.

to denote variation in community bacterial composition among samples within species (Walters and Martiny, 2020). These diversity indices hold importance for better lineage and characterization of the bacterial population, within-alpha and among-beta the bacterial population. Beta diversity with anatomical positions near to 1 with Morisita Horn values appears black, which implies that the samples have similar taxonomy patterns, while red indicates differential taxonomy patterns. Heat maps generated using OTUs indicate that the majority of the abundant species present in the samples are conserved, highlighted in green. Interestingly, a couple of blocks are unique to a few samples and shared across the rest.

The selected five genera for downstream study fall under mucosa-associated bacteria in the gut. *Akkermansia* sp. produces mucosin, necessary to maintain the gut mucosal layer in normal condition. Mucin produced by *Akkermansia* sp. in lower abundance results in thinning of the mucosal layer, increasing gut permeability (Geerlings et al., 2018; Zhang et al., 2019). Research studies suggest a correlation between higher levels of *Prevotella* sp. and dietary habits in autistic subjects (Plaza-Díaz et al., 2019). This suggests a unique link for autism and *Prevotella* sp. phylotype in the present study. A higher level of *Prevotella* sp. influences inflammation, mucosal, and systemic T cell activation, like *Akkermansia* sp (Iljazovic et al., 2021; Larsen, 2017). However, *Prevotella* sp. and *Veillonella* sp. have been known to have co-occurrence in networks with reduced abundance in autism cases. The deviation observed in the current study is indicating their importance for further study. *Sutterella* sp. is connected to the regulation of mucosal metabolism and intestinal epithelial integrity (Hiippala et al., 2016). *Sutterella* sp. with two-fold elevation in the autism samples indicate their importance as a potential diagnostic criterion for gut dysbiosis in autism in this study for the first time. *Faecalibacterium prausnitzii* is an oxygen-sensitive, butyrate-producing gut bacteria, which alters the epithelial layer's integrity with modulation to the immune system through anti-inflammatory activities (Zhang et al., 2021). All these features indicate a possible association of mucosa-associated bacteria with autism behavior severity.

Pathway enrichment helps in connecting dots from bacteria to metabolites to gene mutations in autism cases. The constructed pathway reveals succinate and butyrate as the significant metabolites for the identified significant bacterial signatures, namely *Prevotella*, *Akkermansia*, *Veillonella*, *Sutterella*, and other members of Bacteriodetes and Firmicutes majorly.

The unbalanced ratio of succinate production/consumption due to differential community niche interaction might result in intestinal disruption resulting in dysbiosis. It can impair various levels of host functionalities localized in the gut and brain. Moreover, gut bacteria *Prevotella* sp. produced succinic acid, which is known to degrade glutamate, which is a neurotransmitter and plays a crucial role in learning and memory. Glutamate influences early developmental events, some of which occur before synapse formation: processes involved in migration, differentiation, proliferation, or survival during neural development (DeSantis et al., 2006). Succinate increases the uptake of valproic acid to perfused brain. Valproic-exposed infants have greater chances of having autism and are restricted to the digestive tract due to enteric coating administered to the pregnant mother. Once the blood-brain barrier is compromised, it can easily reach the brain and inhibit GABA receptors and result in dysfunctioning of neural tubes, low verbal ability, and other features of autism (Ghodke-Puranik et al., 2013). Altered succinate levels result in dysregulated calcium homeostasis and metabolic functions in autistic subjects (Cheng et al., 2017).

Butyrate, indicated in the pathway, could be produced by gut microbiota members such as *Faecalibacterium prausnitzii, Lachnospira* sp., *and Roseburia* sp. from the family *Ruminococcaceae* and *Lachnospiraceae,* respectively. There are multiple other indirect production sources of butyrate from propionate and acetate formed from *Akkermansia muciniphila* (Phylum *Verrucomicrobia*): all of which are altered in the current investigation. Hence, due to the lower concentration of these bacterial signatures, a reduced amount of butyrate is predicted to be produced in the sample cohort. Moreover, a carbohydrate-free diet in autism, evident from the available case history of the sample cohort, is predictive of reduced butyrate production by the host. Hence, it can be suggested that the reduced amount of butyrate would alter functionalities connected to butyrate, such as disrupted oxidative state of mucosal cells, impaired modulation of neurotransmitters (De Angelis et al., 2013) and disrupts memory formation and neuronal plasticity (Hasan Mohajeri et al., 2018). Butyrate and succinate are known to exert a crucial physiological role on the brain, such as inflammation of astrocytes, and are considered key mediators in gut-brain crosstalk on dysregulation, which results in gut dysfunction (Venegas et al., 2019). Further, an association of inflammation in astrocytes with altered butyrate levels opens a new avenue to correlate autism manifestation and the blood-brain barrier. Astrocytes with functionalities, namely, modulation of synaptic transmission and maintenance of brain homeostasis, are directly correlated to early brain development. Any dysfunction in these during autism onset can result in damage as brain vulnerability is high during childhood (Eshraghi et al., 2020). Levels of butyrate determine the induction of long-term potentiation in collateral synapses, impacting synaptic plasticity and memory (Pandey et al., 2015). Differential abundance of bacteria produces altered metabolites with the potential direct effect on neural processes has been reported elsewhere through metabolomics analyses of urine and fecal metabolites in Indian and Chinese populations (Jain et al., 2019; Shaw, 2010). Although predictive, the current investigation can be a precursor analysis for large-scale studies for a significant correlation between succinate and butyrate with autism gut pathophysiology. Differences reported on microbial diversity and composition can be attributed to the fact that several microorganisms can perform the same function.

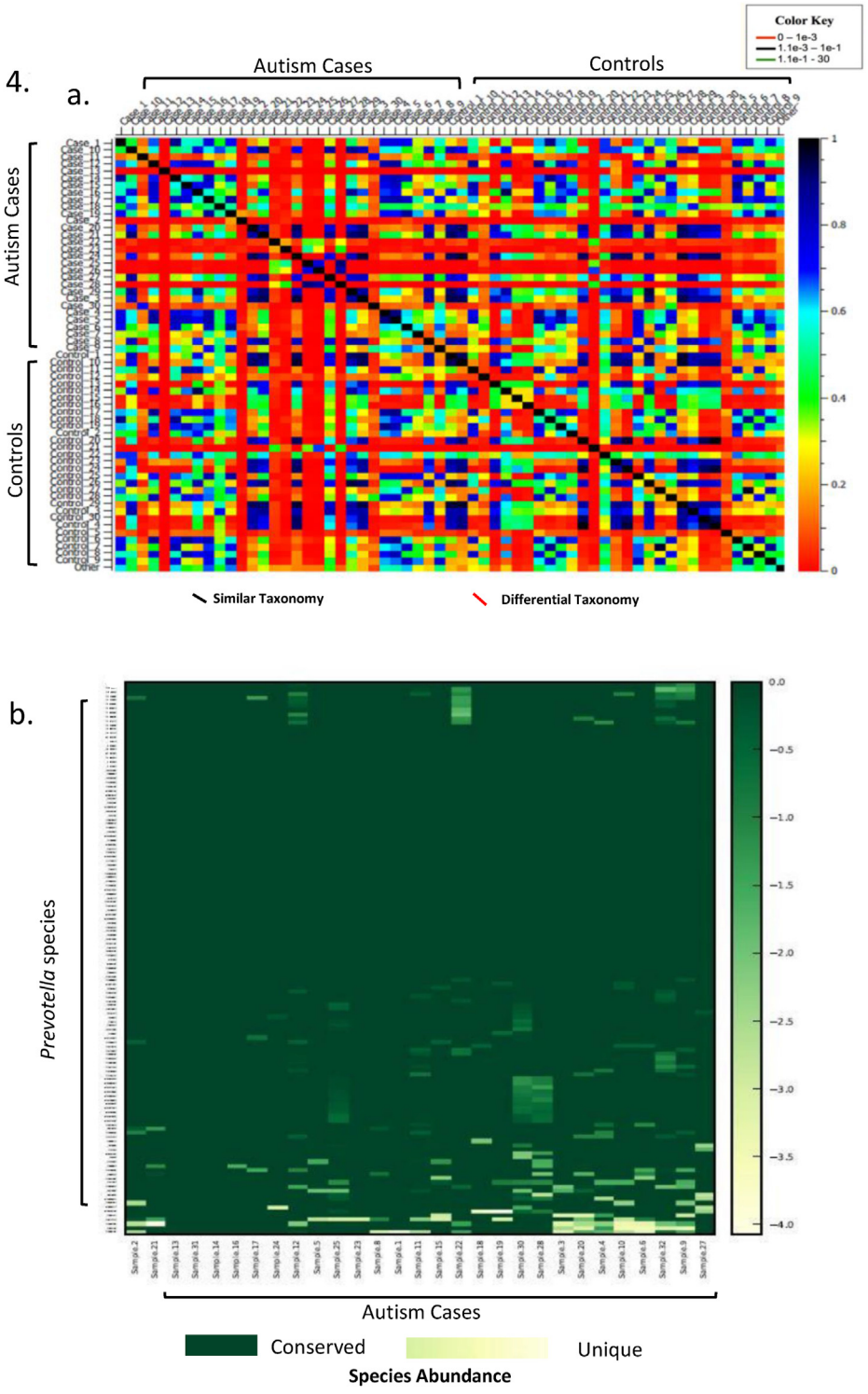

Fig. 4. Heat map of beta diversity of gut microbiota a) Overall bacterial species identified and b) Heat map of Prevotella in autism cases with blocks of the differential gradient of divergence in terms of species abundance.

5. Conclusion

The present study throws light on the identification and role of mucosal-associated bacterial species such as *Veillonella* sp. *Prevotella* sp., *Akkermansia* sp., *Sutterella* sp., *Faecalibacterium prausnitzii, Lactobacillus* sp. present differentially in autism cases versus neurotypical peers. Thus, these findings indicate that mucosa-associated bacteria can play a significant role in the autism phenome and act as diagnostic criteria for gut dysbiosis in autism. The study is suggestive of a connecting link between gut microbiome brain axis and autistic behavior. The need is to shift the focus to the poorly understood bacterial species such as *Sutterella* sp. and *Faecalibacterium* sp. to delineate their role in the mechanism for dysbiosis. The present knowledge of gut microbiome analysis is widespread and is yet to reach a universal statement relevant to understand the underlying principle for complex conditions like autism which relies primarily on therapy and management rather than treatment. This study

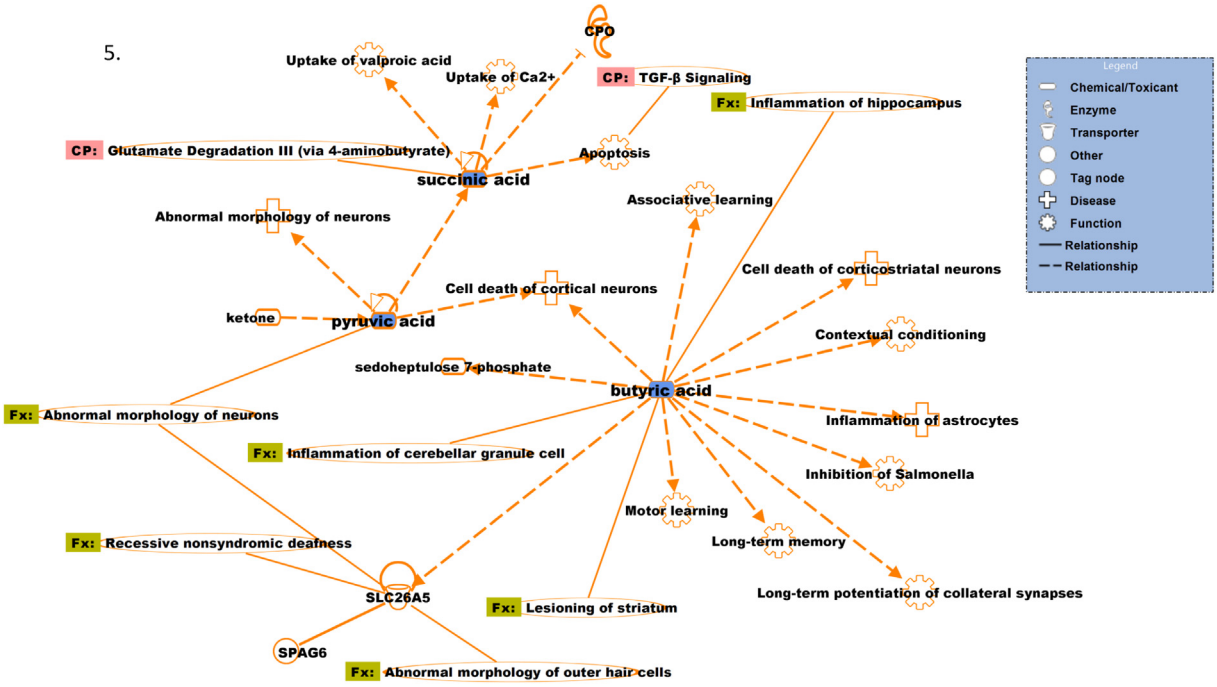

Fig. 5. Pathway showing the interaction between the gut bacteria and their metabolites.

complements existing knowledge about the gut microbiome mechanism for autism. Also, these bacterial species played a role in metabolites and their functionalities which directly impacts autism-related behaviors. The investigation is suggestive of warranting mechanistic studies in a larger sample size of homogenous autism subjects due to its small sample size. Further, experiments are required to determine whether microbiome, GI, or immune abnormalities can sufficiently cause primary behavioral features of autism. It would help identify peripheral specific targets and exact brain changes to develop novel autism therapeutics.

Declaration of competing interest

None.

Acknowledgments

We thank the subjects and their families for participating in this study; American Gut Project provided the sample data. The University of Mysore for providing the facility to conduct this work, the Research Scholars of Genetics and Genomics lab, the University of Mysore for their support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbih.2021.100269.

Abbreviations

QIIME	Quantitative Insights into Microbial Ecology
OTUs	Operational Taxonomic Units
GI	Gastro-Intestinal
ENA	European Nucleotide Archive
BIOM	Biological Observation Matrix
L	Level
IPA	Ingenuity Pathway Analysis
SCFA	Short-Chain Fatty Acid

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