Journal of Population Therapeutics & Clinical Pharmacology

RESEARCH ARTICLE
DOI: 10.53555/jptcp.v31i6.6597

ANALYSIS OF ALTERATIONS IN FECAL METAGENOMIC PROFILE IN AUTISM SPECTRUM DISORDER

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ABSTRACT
An ample repository of data suggests the connections between gut microbiota and Autism Spectrum Disorder (ASD), however, no consistent results are yet obtained as which gut bacterial genera are in lower and higher abundance in autistic and control group. Moreover, there is a lack of research concerning this issue in Pakistani population. The present study has been designed to assess the variations in gut microbial composition in autistic and control/neurotypically growing children group. For that matter, 2 autistic children and 2 normal children were enrolled in the study. Various social. demographic, and diet-related queries were taken through questionnaires. 16S rRNA sequencing was employed to assess the fecal samples to delineate the alterations in fecal metagenomic profile in autistic and normal group. Less diversity and richness was observed in ASD group as compared to the control group in calculating alpha diversity. Beta diversity exhibited differential clustering in both groups. Significant microbial differences were observed at phylum and genus level. At phylum level, the relative abundance of Proteobacteria and at the genus level, Lachnospiraceae UCG-004 was found to be lower in ASD group as compared to the control group. This research highlights the variations in gut microbial composition in autistic children and neurotypically growing ones in Pakistan.

Keywords: Autism Spectrum Disorder, Gut microbial disturbances, Fecal microbiota profile, Metagenome

INTRODUCTION
Autism Spectrum Disorder refers to a group of neurodevelopmental disorders primarily affecting social skills, behavioral domains, and verbal and non-verbal communication along with the repetitive or restricted activity or response. The prevalence of ASD has escalated in the past decade and 1 out of 59 children are being affected in United States [1]. Ideally, ASD must be diagnosed early in childhood but the diversity in symptoms, inadequacy of biomarkers, and shortcomings in diagnostic methods hinder the early diagnosis [2]. Currently, no proper treatment is available for ASD populations except the behavioral management therapies and educational interventions. This only ensures the improvement in quality of life of autistic individuals by relieving the symptoms associated with the disorder [3]. ASD imparts economic burden to the society, and the lack of proper treatment complicates the life of autistic populations [4]. Unavailability of drugs protocol and the social, economic, and emotional concerns intrigue the researchers for an in depth search to unravel the pathophysiology, diagnosis, and treatment of ASD.

ASD has an obscure etiology, with the involvement of genetic, epigenetic and environmental factors, labeling it as a multifactorial disorder. ASD individuals exhibit a wide range of phenotype of symptoms and various co-morbid health conditions are associated with them, the most common being the gastrointestinal (GI) problems including vomiting, constipation, bloating, diarrhea, and abdominal pain. Various studies report the direct connection in the severity of GI problems and the ASD behaviors. This co-morbid medical condition emphasizes the possible role of microbes residing in the human gut in the etiology of this multifactorial disorder [5]. Gut microbes have also been shown to affect the development of brain through gut-brain interaction pathways. Gut-brain axis has also been linked to the biochemical and behavioral changes in brain, primarily due to the varied composition of gut microbiota and its potential harmful effects on human brain. The varied composition of gut microbes causes gut dysbiosis which has shown strong connection with neuropathological conditions [6]. Various symptoms associated with neurodevelopmental disorders have been linked to be caused by the variations in the composition of gut microbiota. Moreover, gut microbes have been shown to possess therapeutic potentials for ASD [7].

Various studies have shown the alterations in gut microbiota composition in ASD as compared to healthy populations, and numerous species have been shown to cause microbial dysbiosis in ASD subjects. However, inconsistent results have been drawn by different studies regarding the gut microbial composition. Recently available systematic review and meta analysis concluded that higher abundance of the genera *Faecalibacterium, Bacteroides, Clostridium, Parabacteroides, and Phascolarctobacterium,* whereas *Bifidobacterium* and *Coprococcus* are found to be in lower abundance in ASD affected children as compared to healthy controls [8].Therefore. studies focusing on gut microbial composition could help researchers to provide greater insight in to the etiology of multifactorial nature of ASD.

The present study has been designed with an aim to analyze the fecal metagenomic profile of healthy and autistic children in order to ascertain the composition of microbiota in both groups. As far as we are aware, none of the studies have been conducted in Pakistani population covering this issue. 16S rRNA sequencing of fecal samples of 2 ASD affected children with ages between 2 to 9 years and 2 age-matched neurotypical children was performed in an endeavor to explicitly delineate the differences in gut microbes of ASD children and healthy controls. The specified bacteria in this study could help in the treatment opportunities and possible relief from the GI symptoms in children with ASD in Pakistani population.

MATERIALS AND METHODS

Statement Pertaining to Ethics

The protocol encompassing the research was consented by the ethical review committee at Capital University of Science and Technology, Islamabad. The study participants showed their acceptance for the project and written informed consent was also taken.

Study Participants Recruitment

The tool employed for the diagnosis of ASD was Diagnostic and Statistical Manual for Mental Disorders (Fifth Edition (DSM-5). Participants' metadata including prenatal and postnatal factors, background history, age, gender, eating habits, growth patterns, and GI symptoms was obtained through questionnaires. The inclusion criteria was non-syndromic autism, no underlying disease except ASD diagnosed, not any previous trauma or accident or any other disease like meningitis that may play a role in brain dysfunction. All participants were not taking any antibiotics, prebiotics, probiotics, and anti-inflammatory drugs for the past three months. Healthy children were neurotypically growing and lacking any ASD core symptoms. Table 1 expresses the top notch characteristics for participants under study.

Table 1: Summary of study characteristics.

Characteristics	ASD (1)	ASD (2)	NT (1)	NT (2)
Gender	Male	Male	Female	Male
Age (Year)	2.9	7.5	8.6	5.9
Method	Metagenome	Metagenome	Metagenome	Metagenome
Paternal educational level	Graduate	Post graduate	Post graduate	Post graduate
Maternal educational level	Post graduate	Graduate	Post graduate	Post graduate
Are the parents relative	No	Yes	No	No
ASD affected individuals in family	No	No	No	No
Any gestational disease/infection ?	No	Urine infection, candida infection	No	No
Medicines taken during gestation	Supplements	Supplements	Supplements	Supplements
Food intolerances not present previously?	No	No	No	No
Alcohol consumption?	No	No	No	No
Smoking?	No	No	No	No
Preterm / full term birth	Full term	Full term	Full term	Full term
Mode of delivery	C.section	Natural birth	Natural birth	Natural birth
Age of mother at the time of birth?	20-25 years	25-35 years	25-35 years	25-35 years
Weight of the child at the time of birth?	3 kg	3.3 kg	3 kg	3.1 kg
First feed?	Formula milk	Mother feed	Honey	Mother feed
Feeding pattern	Formula milk	Mix feed	Mix feed	Mix feed
Weaning Age	8 months	6 months	6 months	7 months
Difficulty accepting new taste during weaning	Yes	Yes	No	No
Any currently used special diet	Gluten free diet	NA	NA	NA
Age of diagnosis of ASD	2-3 years	1-2 years	NA	NA
Observations at the time of diagnosis?	Speech delay	Non-responsive	NA	NA
Severity level	Level 1	Level 2	NA	NA
Cry characteristics	More than normal	Normal	Normal	Normal
Sleep characteristics	Normal	Less than normal	Normal	Normal
Physical growth trend	Satisfactory	Satisfactory	Satisfactory	Satisfactory
Cognitive growth trend	Unsatisfactory	Unsatisfactory	Satisfactory	Satisfactory
Picky Eater	Yes	Yes	No	No
Dysphagia	Yes	No	No	No
Recurrent abdominal pain	No	Yes	No	No
Constipation	No	Yes	No	No
Diarrhea	No	Yes	No	No

ASD 1 refers to autistic child 1, ASD 2 autistic child 2, NT denotes neurotypically growing child 1, and NT 2 denotes neurotypically growing 2.
Flowchart of methodology is mentioned in figure1.

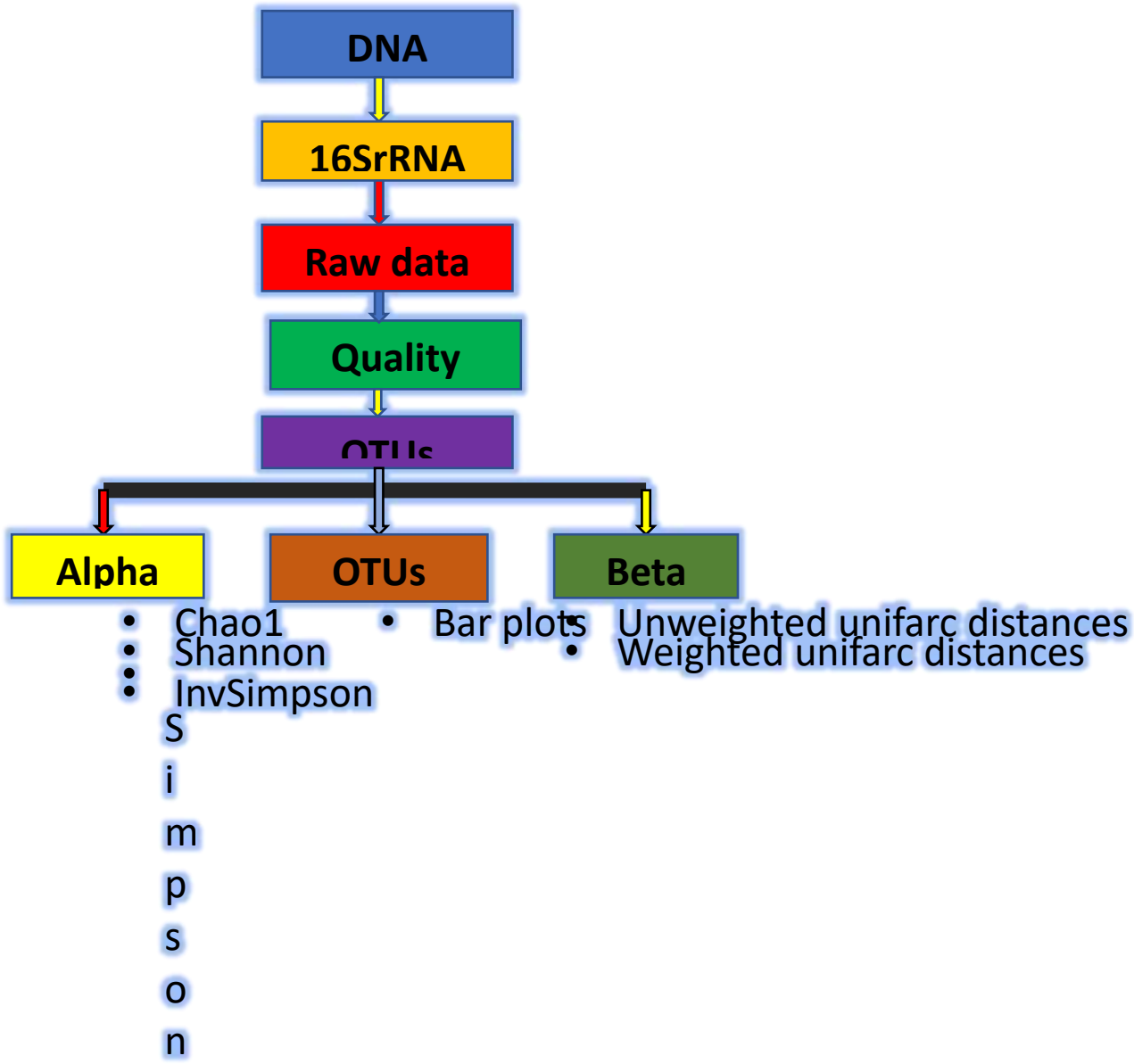

Figure1: Flowchart of the methodology for determination of fecal metagenomic profile in ASD and NT (neurotypically) growing children. OTU stands for Operational Taxonomic Units.

Fecal Sample Extraction and Collection

Fecal samples of 4 children with ages 5-10 years were taken. 2 samples were of ASD patients and 2 of healthy controls/neurotypically (NT) growing ones. The samples were taken in 500ml falcon tubes each containing 6ml of Phosphate-Buffered Saline (PBS). 10gram of samples was added to each falcon tube. The PBS was made by adding 1 tablet of PBS saline tablet (0.96mg) to 30ml distilled water.100ml was made by adding 70ml of distilled water. The samples were stored overnight at -20°C and then transported to the laboratory where they were stored at-80°C before analysis.

Extraction of DNA

Bacterial genomic DNA was extracted through standard procedure of phenol-chloroform method [9].

Amplification of PCR

The extracted DNA was run on agarose gel and DNA quantification was measured using Thermo scientific Multi Skan Go Instrument.

16S rRNA Gene Sequencing

Illumina MiSeq Sequencing

200 ng genomic DNA was taken to amplify V3–V4 regions of the 16S rDNA gene. The purified PCR amplicons were sequenced on Illumina Mi-Seq platform. Fragment library was constructed by using paired-end method to carry out paired-end sequencing. The base quality scores of Illumina HiSeq (TM) /MiSeq platforms are expressed in Q Phred.

Sequence Data Processing

Raw data import

Raw paired-end reads (FASTQ) from the original DNA fragments were imported in Quantitative Insights into Microbial Ecology (QIIME) version 2 2021.4 software, a very fast and accurate Metagenomic analysis tool. Manifest file method was employed to import the samples from paired end reads.

Quality checking and removal of chimeric sequence

DADA2 denoising method was used to perform various functions including quality filtering, denoising and removal of chimeric sequences. In order to have constant read length pertaining to all reads, read truncation method was used with the following criteria: (i) truncation length of upto245 bp (ii) minimum abundance of 8 counts.

Taxonomy Assignment

After the detection of chimeric sequences, comparison was carried out by using SILVA (https://www.arb silva.de/download/archive/qiime) for 16S rRNA as reference database.

Naïve Bayes classifier and q2-feature classifier plugin in QIIME 2 was used to assign likely taxonomic features (Operational taxonomic units (OTUs), genus, specie, phylum) to each read. The classifiers used were working over 97% similarity OTUs sequences as compared to the database that was used as reference.

The estimate of richness index or alpha diversity was calculated by Shannon index through phyloseq in R. Weighted UniFrac and unweighted UniFrac algorithms were used to measure beta diversity, according to principal coordinate analysis (PCA) through phyloseq in R.

Statistical Analysis

The statistical analysis was carried out through R (http://cran.r-project.org/). P values that were lower than 0.05 were considered as significant while conducting data analysis.

RESULTS

Extraction of DNA, Amplification of PCR, and 16S rRNA Gene Sequencing

260/280 ratio of nucleic acid showed the quality whereas concentration is shown in ng/ul and is represented in Table 2.

Table 2: Summary of DNA quantification in 4 samples.

Sr.No	Sample ID	Nucleic Acid 260/280	Nucleic Acid Concentration in (ng/ul)
1	AH	1.89	770
2	AI	1.83	780
3	NF	1.79	720
4	NU	1.81	773

AH refers to austistic sample 1, AI refers to austistic sample 2, NF refers to normal sample 1, and NU refers to normal sample 2.

Illumina MiSeq Sequencing

The statistics of raw data of each sample are in the table 3.

Table 3: Raw data statistics.

Sample ID	Index	Reads	Yields (Gbases)	Q30 (%)
AI	TCGTAGTA+TTAAGGAG	150,800	0.0754	90.19
NF	TCGTAGTA+TCTGCATA	157,412	0.07871	90.03
NU	TCGTAGTA+CTCCTTCC	161,987	0.08099	89.82
AH	TCGTAGTA+CAATCCTC	148,931	0.07447	89.77

Q30 (%) refers to the percentage of bases with Phred value greater than 30.

Microbiota Alterations

The study reveals the perturbations in gut microbial composition in ASD children and normal ones, both at phylum and genus level. A total of 8 phylum and 95 genera were shown to be differentially abundant in both groups (Supplementary material S1. S2). At the phylum level, Proteobacteria was significantly higher in normal children as opposed to the autistic ones (Table 4, Figure 2). *Lachnospiraceae-UCG-OO4* was significantly higher in normal children than the ones with ASD (Table 4, Figure 3) at the genus level.

Table 4: Gut microbial alterations in ASD and NT (neurotypical) children at phylum and genus level with significant differences (p value <0.05).

Variables	ASD	NT	P value (T test)
Phylum level			
Proteobacteria	10.518	12.303	0.04969
Genus level			
Lachnospiraceae-UCG-OO4	0.143	0.156	0.02766

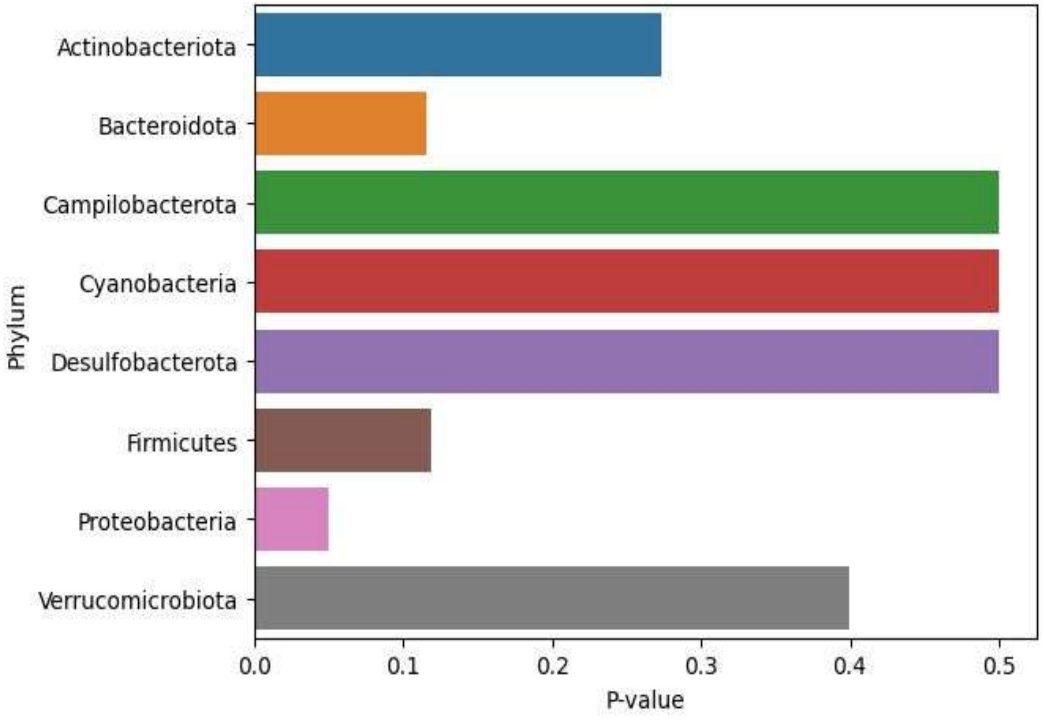

Figure2: Bar plot of 8 phylum showing differential abundance in ASD and NT children. Proteobacteria shows significant decrease (p value 0.04) in ASD children as compared to the NT ones.

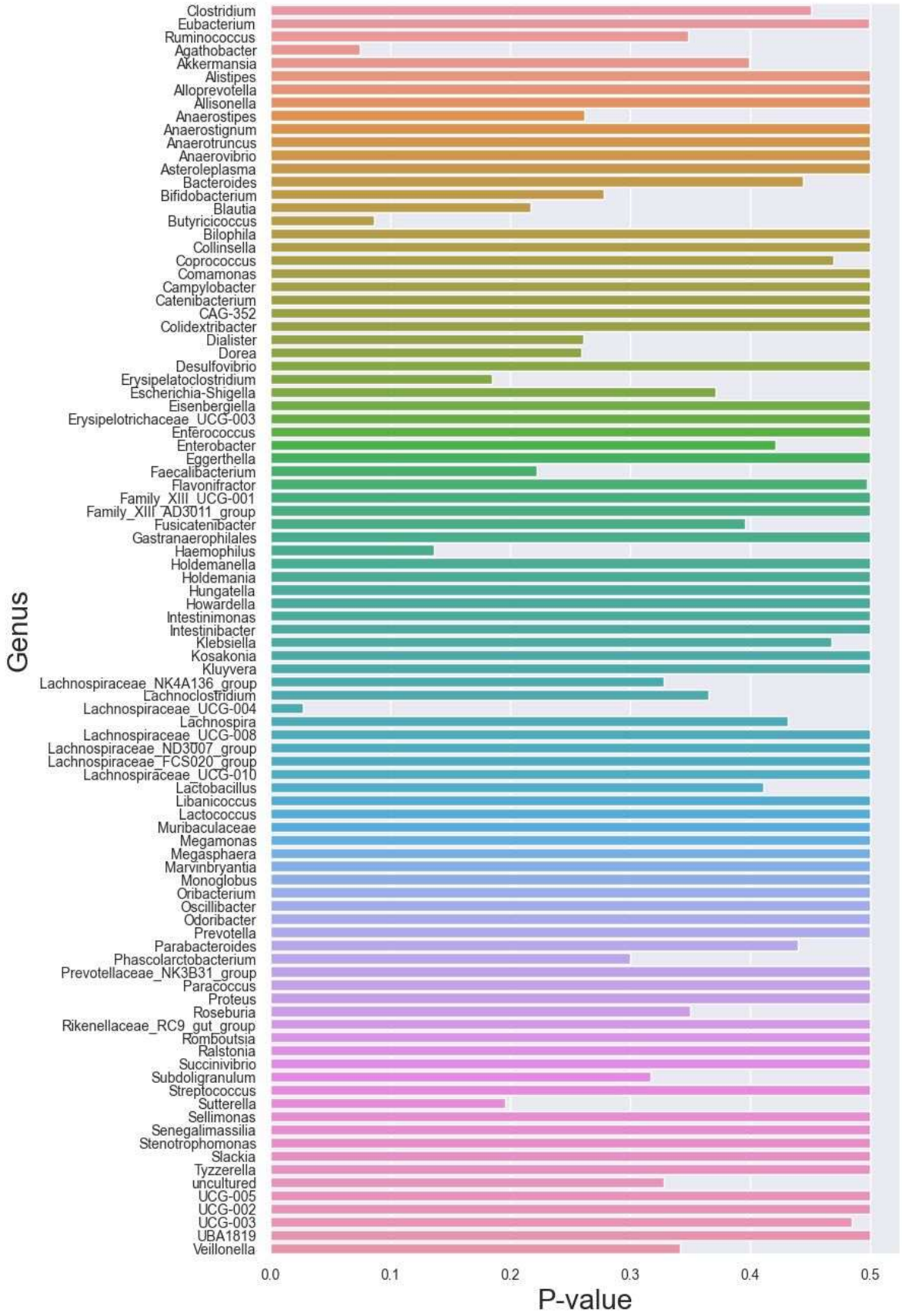

Figure 3: Bar plot of 95 genus showing differential abundance in ASD and NT children. Lachnospiraceae-UCG-004 shows significant decrease (p value 0.02) in ASD children as compared to the NT ones.

The gut microbial alpha diversity was compared between the two groups. Alpha diversity calculated through various indices is shown in table 5 and figure 4.

Table 5: Alpha diversity has been calculated through different indices. Shannon, Simpson and InvSimpson are shown to be highly significant.

Alpha diversity indexes	AH	AI	mean	NF	NU	Mean	p-value
Chao1	3776.193	3723.224	3749.708	5149.979	6624.05	5887.015	0.1389
se.chao1	3.580157	1.727755	2.653956	24.39499	47.0935	35.74424	0.4528
ACE	3776.293	3726.959	3751.626	5186.256	6604.344	5895.3	0.1392
se.ACE	30.44108	29.99825	30.21967	35.23002	39.24066	37.23534	0.06597
Shannon	7.50383	7.519959	7.511895	7.569138	7.769872	7.669505	0.006609
Simpson	0.998952	0.999052	0.999002	0.99898	0.999171	0.999076	2.36E-05
InvSimpson	953.7483	1055.137	1004.443	980.7659	1205.738	1093.252	0.02694
Fisher	775.5838	748.8164	762.2001	1087.041	1424.098	1255.569	0.1527

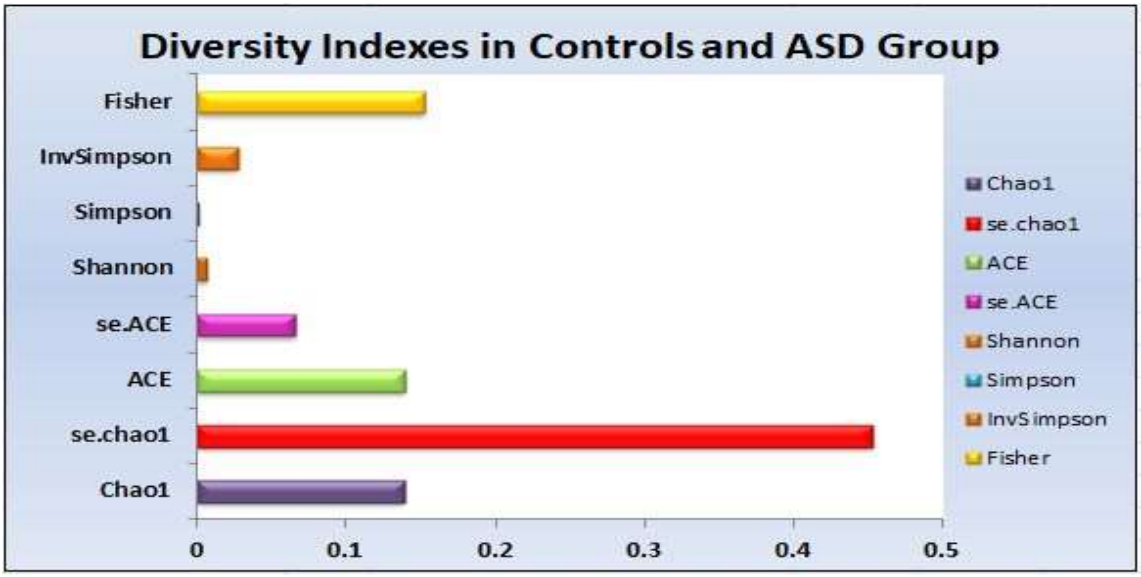

Figure4: Bar plot of various alpha diversity indices in ASD and control group. Shannon, Simpson and InvSimpson are found to be highly significant.

A lower specie richness was found in ASD children than in NT ones while assessing alpha diversity through Choa1, Shannon, Simpson and Invsimpson. Thus ASD groups shows lower biodiversity than the NT group.

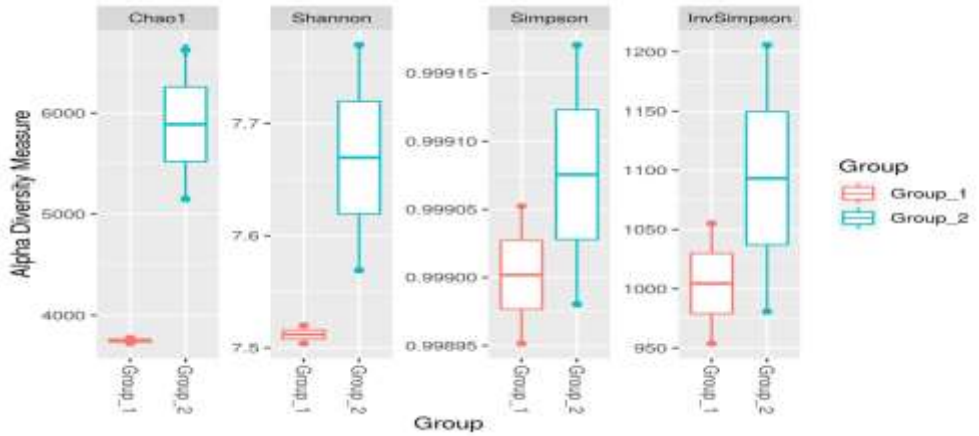

Figure 5: Alpha diversity assessed through Chao1, Shannon, Simpson, and InvSimspon. All alpha diversity measurement indexes exhibit lower values in ASD group as compared to the NT group depicting a lower specie richness in ASD group (Group 1 refers to ASD, and Group 2 refers to NT children).

The beta diversity calculated on weighted UniFrac distances and unweighted UniFrac distances showed that the ASD group was clustered apart from the NT group. The differential clustering or dissimilarity in clustering between the both groups exhibit that the both groups do not share the same gut microbial composition.

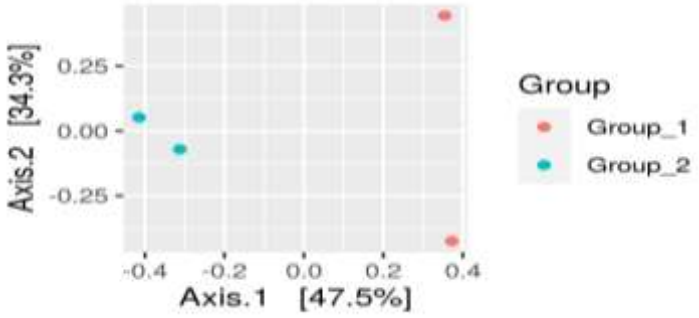

Figure 6: Unweighted unifrac distances exhibiting the differential clustering of ASD and NT group. ASD group is shown in red colour as group 1 and NT group in blue colour as group 2. ASD group is sparasely clustered from NT group.

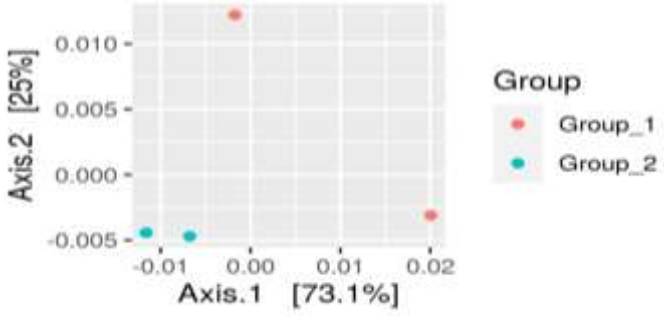

Figure 7: Weighted unifrac distances exhibiting the differential clustering of ASD and NT group. ASD group is shown in red colour as group 1 and NT group in blue colour as group 2. The dissimilarity in clustering of both groups show the differntial gut microbial composition in both groups.

The overall results obtained in the project has been summarized in figure 8.

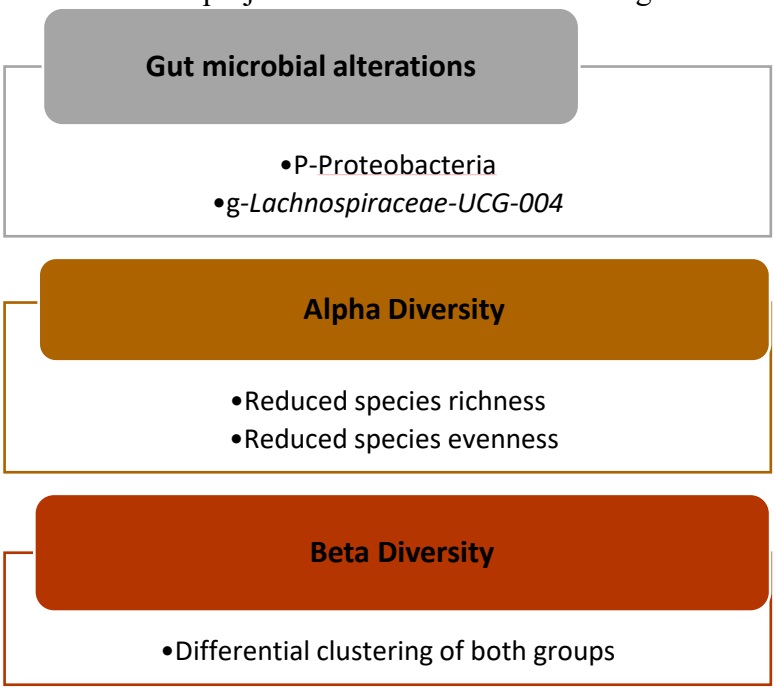

Figure 8: Figure depicts the overall result in terms of microbial alterations, alpha and beta diversity.

The Phylum Proteobacteria and genus *Lachnospiraceae-UCG-004* has been found to be lower in ASD group than in NT group. Both species richness and evenness (alpha diversity) has found to be reduced in ASD group. Moreover both groups show difference in clustering in beta diversity depicting difference in microbial compostion in both groups.

DISCUSSION and CONCLUSION

ASD has emerged as a multifactorial disorder and various risk factors including genetic, epigenetic, and environmental have been speculated in the etiology of this complex spectrum disorder. This study has been designed in order to evaluate the differences between autistic children and healthy ones, mainly by comparison of their gut microbiota, as ample evidences support the putative role of gut microbiota in this multifactorial disorder even though the exact mechanism of involvement remains obscure [6].

16S rRNA sequencing of 2 autistic children and 2 nneurotypically growing children was assessed in the present study to delineate the differences in gut microbial composition in Pakistani children. The present study shows that the autistic children harbor a less diverse gut microbiota along with the reduced richness, as compared to the control group. A decrease in alpha and beta diversity in autistic children has been found as compared to the control group. The present study also reports the significant deviations and alterations in the gut microbial composition in autistic children as compared to the neurotypically growing ones. At the phylum level, relative abundance of proteobacteria was lower in ASD subjects and at genus level, the abundance of Lachnospiraceae UCG 004 was found to be significantly different between autistic children and neurotypically growing ones. The genus was found to be in lower abundance in autistic subjects as compared to the normally developing ones. Members of Lachnospiraceae are anaerobes which ferment indigestible carbohydrates of host to produce butyrate [10]. The lower abundance of Lachnospiraceae UCG 004 presumably leads to lower butyrate levels, which is a key metabolite in maintaining the integrity of microbiome-gut-brain axis [11]. The expression of tight junction-associated proteins is upregulated by Adenosine Monophosphate-activated (AMP) protein kinase which in turn is regulated by butyrate [12]. Strengthening of mucosal immunity and restoration of BBB premeability is also owed to butyrate because of its ability to upregulate the histone acetylation and expression of tight junction proteins [13]. Butyrate has also the ability to alter the expression of tyrosine hydroxylase gene, thus indirectly regulating the sysnthesis of dopamine [14]. Taken together, butyrate can regulate the gut-brain axis and the lower levels can lead to disastrous spectrum in ASD. Moreover, the proliferation of pathogenic gut microbiota and lower abundance of healthy gut microbiota leads to dysbiosis in autistic children. This dysbiosis is associated with the pathogenic condition of GI disorders. Thus it is speculated that the gut inflammation and GIS could be reversed in ASD subjects by maintaining and restoring the healthy levels of Lachnospiraceae UCG 004. Certain previous studies also report the lower abundance of members of Lachnospiraceae in autistic subjects [10, 14-17]. By considering already reported studies and findings of the present research, it could be concluded that the members of Lachnospiraceae significantly contribute in maintaining a healthy gut environment and their dysbiosis is directly correlated with GI disorders in ASD children.

The number of samples evaluated in the present study are less than the usual number of samples reported in meta genome analysis in ASD studies. The main reason behind this has been the taboo associated with this disorder in Pakistan and subsequent unwillingness of the parents to provide fecal samples. This unwillingness basically owes to the social, emotional, and cultural aspects in Pakistan, where patients with such disorders are still stigmatized and thus there is a usual denial from acceptance of such disorders. However, the results of this preliminary study are statistically significant and correlated.

Figure Legends

Figure1: Flowchart of the methodology for determination of fecal metagenomic profile in ASD and NT (neurotypically) growing children. OTU stands for Operational Taxonomic Units.

Figure2: Bar plot of 8 phylum showing differential abundance in ASD and NT children. Proteobacteria shows significant decrease (p value 0.04) in ASD children as compared to the NT ones.
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Figure 5: Alpha diversity assessed through Chao1, Shannon, Simpson, and InvSimspon.
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Declarations
Acknowledgments
Not applicable

Consent for Publication
The authors provide fully consent for publication.
Ethical Approval
Not applicable

Funding
Not applicable

Conflict of interest
The authors share no conflict of interest

Data availability
Not applicable

Authors contribution
SMB and SK conceived the study design, SK performed the research, SK analyzed the data, SK used tools and performed the analysis, SMB reanalyzed the data, SK wrote the manuscript, SMB and SK proof read the manuscript for submission.

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