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Microbiota Revelations: First-time Prevotella spp. Identification in Iraq Pediatric Autism

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Abstract:

Autism Spectrum Disorder (ASD) often co-occurs with gastrointestinal (GI) dysfunction, suggesting gut microbiota involvement. current study, the first in Iraq, investigated fecal microbiota in autistic children (n=18, aged 2-9 years) and neurotypical controls (n=27) in Basrah. Culture techniques detected facultative or aerobic bacteria, like E. *coli*, *Enterococcus faecium*, *Lactococcus garvieae*, *Enterococcus faecalis* ,*Brevibacillus parabrevis*, *Lactobacillus salivarius*, and *Klebsiella pneumonia* while *Prevotella* spp. in ASD were found to be lower by RT-qPCR. Which was statistical insignificance. These preliminary results point to possible changes in gut flora in Iraqi children diagnosed with ASD. Larger sample sizes and further study are required.

Keyword: Autism, Gut microbiota, *Prevotella* spp., Enterotype, Iraq, Basra

Introduction:

Autism Spectrum Disorder (ASD) is defined by limitations in social contact as well as repetitive and restricted activities that occur during childhood. (American Psychiatric Association, 2013). This diseases is characterized by a variety of symptoms, such as poor verbal communication, social skills of complete withdrawal, consistency in daily routine, repetition of activities, and increased sensitivity to external stimuli(Rosenfeld, 2015),

The recent global burden study revealed that a significant proportion of young children with developmental disabilities reside in low- and middle-income countries (Olusanya *et al*., 2018). However, (Sun *et al*., 2019) reported that the majority of these children remain undiagnosed. The lack of representation of these countries in the broader ASD literature is due to the limited research originating from these regions(Franz et al., 2017).The low diagnostic rates in these countries are likely attributed to

the absence of dedicated infrastructure to assist individuals with ASD, as well as the difficulty in obtaining referrals to meet with the limited number of specialists, and low levels of parental literacy, which limit a parent's ability to understand the disorder and locate services. Families often have to manage the care of an individual with ASD on their own, with the help of extended family and community members (Divan *et al*., 2012). The quality and quantity of relevant developmental information that may be communicated with the expert is restricted when non-parent individuals bring the target children to the clinic. This is true even for the lucky families who find a suitable and accessible evaluation facility. Consequently, children who ultimately receive ASD diagnoses typically have the most significant impairments and complex phenotypic profiles which refer to the diverse and unique characteristics exhibited by different mutants (Kommu *et al*., 2017). Highly diverse etiology of ASD in which genetic and environmental factors are equally important that causing ASD patient and their families facing problematic treatment approach (Kang *et al*., 2013). The most important environmental factor is an unbalanced gastrointestinal tract (GIT) microbiota (i.e., GIT dysbiosis). For over a decade, there has been speculation about the potential engagement of gut microbes in the etiology of autism spectrum disorder (ASD). A large percentage of autistic children suffer from gastrointestinal (GI)problems, solid positive correlation was observed between GI problems and ASD severity(Adams *et al*., 2011). Although *Prevotella* spp. encompasses over 50 characterized species that can be found in diverse natural environments, the majority of *Prevotella* spp. are associated with the human population. In the human microbiota, *Prevotella* spp. are highly prevalent in various regions of the body, where they play a crucial role in maintaining the equilibrium between well-being and ailment. Factors pertaining to the host, including diet, lifestyle, and geographical location, have a fundamental impact on the diversity and prevalence of *Prevotella* species and strains within the human microbiota. These factors, in addition to the ecological relationship that *Prevotella* shares with other members of the microbiota, are likely to determine the degree to which *Prevotella* contributes to human metabolism and overall health(Tett et al., 2021). ASD prevalence in West Asia was 0.35% with the ratio of males to females (4–5: 1), and the prevalence of ASD ranged from 0.14 ‰ to 2.9 ‰ among Arab Gulf countries (Saudi, UAE, Oman, Kuwait, Qatar, and Bahrain) (Qiu *et al*., 2020). Although microbiota-level studies on autism are limited in Iraq, beside there is no one study about *Prevotella* spp. by using RT-PCR or other technique in both culture or genetic methods. The current study goal is to investigate the relation between the presence of *Prevotella* spp. and ASD in Iraqi children and establish a data record of ASD children to encourage the researchers to focusing more at ASD children in Iraq.

MATERIALS AND METHODS:

Sample collection:

Stool samples were collected from 45 child in a sterile plastic container, kept with ice under anaerobic conditions, and immediately taken to the Basrah university, Biology department laboratory, ASD samples were collected from different parties like autism private sector center (Ausrti center, private Physician clinic, volunteers) .HC samples coming from volunteers in Basrah province south of Iraq at the period from the beginning of October 2022 to the end July 2023. The recruited participants (n=45), who ranged in age from two to nine years for both groups (ASD and HC), had not taken any

kind of antibiotic or antifungal treatment. Local physician evaluated children with ASD using the Diagnostic and Statistical Manual of Mental Disorders (DSM-5).

Bacterial isolation and identification:

About 1 gm of stool sample was placed in thioglycolate broth with gas flushing (CO_2 + N_2) with 80% and 20% ratio respectively and incubated at 37 ºC for 24-48h or until the appearing of turbidity. Firstly, 20 sample were cultured by streaking on Columbia agar, chocolate agar with vitamin K and horse serum as supplement for fastidious bacteria growth with flushing gas and incubating anaerobically at anaerobic jar on 37 ºC for 24-48h. the same sample was cultured on blood agar aerobically, subculture was performed on Nutrient agar**.** The growing colonies were stained with Gram stain and examined by microscope, oxidase and catalase assays were performed, re-cultured on nutrient agar plates for subsequent experiments and in nutrient agar stocks (Roberts & Greenwood, 2008).

Molecular study:

Identification of bacterial species:

DNA Extraction:

DNA extraction begin after a single colony of bacterial isolate activated in 5 ml of sterilized Brain Heart Infusion Broth (BHIB) and incubated at 37°C for 24 h according to the procedure of Presto™ Mini g DNA bacteria kit from Geneaid company.

Detection of genomic DNA by agarose gel electrophoresis:

The extracted bacterial DNA and other PCR product in the current study were detected using agarose gel electrophoresis with 0.8% or 1.5% and visualized under ultra violet transilluminator(Cleaver company) according to Sambrook & Russell, (2001).

Amplifying of 16S rRNA by PCR:

16 S rRNA of the bacterial isolate was amplified by PCR with universal primers 27 F `5-AGAGTTTGATCCTGGCTCAG-3`, 1492 R 5`-GGTTACCTTGTTACGACTT-3` according to Miyoshi et al. (2005).The reagents were used in 25 µl of go Taq green master mix, and 2 µl DNA template, 2 µl of both the forward, and reverse primer, the mixture was completed to total volume of 50 µl with 19 µl nuclease free water (NFW). The mixture then was vortexed and centrifuged for 1 min, the reaction program for PCR was; Initial denaturation at 95 ° C for 5 min., 1cycle, denaturation at 95° C for 30 sec. and annealing at 55° C for 30 sec. and extension at 72° C for 1 min, the last three steps were repeated for 35 cycle, Final extension 72° C for 5 min. 1 cycle. The size of product was approximately1500 bp.

Detection of *16S rRNA* product:

The ampliphied*16S rRNA* was detected by the gel electrophoresis with construction 1.5 % agarose and visualized by UV transilluminator as mentioned previously. The size of the product was compared with 100bp ladder (promega). The result of the product was photographed using private camera.

Sample preparation for sequencing:

Fifteen µl of PCR product for *16S rRNA* was sent to Macrogen Company for sequencing. PCR product of each sample was labeled with a number identical to the number of excel sheet which is given by Macrogen company, the products purification was accomplished by the company for sequencing.

Bacterial species:

The National Centre for Biotechnology Information (NCBI) and the Basic Local Alignment search tool (BLAST) were used to identify the different bacterial species according to Kerbauy *et al*.(2011). The nucleotide sequence was copied and entered into the "BLAST" program after it had been proofread. This tool compared the sequences of the discovered species with those of other species.

RT-PCR:

Bacterial RNA extraction:

Following the activation of about 1g of sample faeces in thioglycolate broth (TM, India), RNA extraction was initiated in compliance with the GENEzolTM TriRNA Pure kit (Geneaid, Taiwan) methodology. Following that, the extraction was incubated for eighteen hours at 37°C.

cDNA synthesis by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR):

cDNA is synthesised using the reverse transcriptase polymerase chain reaction (RT-PCR). After standardising the RNase free blank, the A 260/A 280 ratio was calculated. Using a Nanodrop spectrophotometer (Avans Biotechnology Inc., Taiwan) (Abd Al-Wahid, et al., 2023), the concentration and purity of the RNA in 45 samples were evaluated. From every sample, cDNA was generated using the DiaStarTM RT kit (SolGent, Korea). The manufacturing process had been followed. Total RNA and nuclease-free water were added to the cDNA master mix tubes (Bioneer, Korea). After thoroughly combining the materials with a microvortex, they were placed in a thermal cycler (Bioneer, Korea) and heated to 37 ºC for 10 minutes, 60 ºC for an hour, and 95 ºC for five minutes.

Detection of *Prevotella* spp:

In addition to the culture method that used for other type of aerobic and anaerobic Bacteria, molecular method specifically Real time-qPCR was also applied for detecting the *Prevotella* spp. Target gen was amplified by SYPR green dye as (Matsuki, Watanabe, Fujimoto, Takada, et al., 2004). Each sample were used in the present study had three technical repeats and contains reagents as following: 10 µl of Go Taq qPCR master mix (Promega, USA), 1 µl from each forward and reverse primer g-prevo-F CACRGTAAACGATGGATGCC and g-prevo-R GGTCGGGTTGCAGACC (513 bp (Matsuki, et al., 2004) (microgene, Korea), 1 µl of cDNA and 7 µl of Nuclease free water (Bioneer, Korea) that is mixed well and subjected to analytikjena thermal cycler (An Endress+Hauser, Germany). The program in the beginning used to amplify Prevotella gene was included one cycle at 94◦C for 5 min. 40 cycles at 95◦C for 20 sec, 55◦C for 20 sec and 72◦C for 50 sec. gradient PCR to the cDNAs was optimized and gel electrophoresis for several annealing temperature (52.9-58 ◦C)to determine the exact annulling temperature (54.4◦C) Specific Real time-PCR was also applied to make sure the melting curve was one peak as showed in figure-4. The obtained qPCR data were analyzed using the CT values to compare between ASD and HC groups. Rephrase first run and second run optimized.

Result:

Isolation of bacteria:

From 45 samples were collected from male and female ,18 sample autistic children and 27 sample controls during the current study, One gram of stool sample enrchied in thioglycolate broth, 20 sample were flushed with(CO_2 + N_2) gas 80%:20% ratio respectvily, to be cultured anaerobiclly. However, the result showed that the developing colonies were for only aerobic bacteria. The following idetification using Gram stain showed that 95% were Gram positive and 5% was Gram negative . Oxidase and catalase tests were also conducted, Catalase positive were 90%. Whereas the other 10% was Catalase negative. The Oxidase result was the same to Catalase. The above twenty bacterial isolates were subjected to more identification using the molucular approch. As the first step, DNA extraction was made to these isolates.

Identification of bacterial species by *16S rRNA* gene:

***16S rRNA* gene amplification of bacterial isolates**:

The *16S rRNA* of 20 bacterial isolates were amplified using conventional PCR the amplified gene were visualized as has been noticed in agarose gel electrophoresis agarose and their position was approximately 1500 bp comparing with a molecular DNA ladder.

Identification of bacterial species by *16 S rRNA* sequencing:

All amplicons of the *16 S rRNA* gene were subjected to sequence and identified using BLAST website and Chromas programm to remove incorrect baess. The result of the sequance showed that the species were: *Enterococcus faecium* (n=2 of 20/ 10%), *Escherichia coli* (n=12of 20/ 60%), *Lactococcus garvieae* (n=2/ 10%), *Enterococcus faecalis* (n=1/5%) and *Brevibacillus parabrevis* (n=1/5%), *Lactobacillus salivarius* (n=1/5%), and *Klebsiella pneumonia* (n=1/ 5%). However, *Prevotella* spp. and other anaerobic microbiota were not identified.

Detection of *Prevotella* spp. using RT-PCR:

In the current study, *Prevotella* spp. were also identified using a molecular method known as real-time PCR. SYPR green dye was used to amplify the target gene, according to Mashiki, Watanabe, Fujimoto, Takada, et al. (2004). Nanodrop was measured to the extracted RNA and the readings were between400-900 ng,The first run , it has been used the annealing temperature 55^{o}C which showed acceptable resulte. However, gradient PCR to the cDNAs was performed and gel electrophoresis for several annealling temperature (52.9-58 ^{o}C)to determine the exact annealling temperature, the results showed that the most appropraite annealing temperature is (54.4^{o}C) . To confirm that the melting curve had a single peak, as seen in figure (1), targeted real-time PCR was further employed. The ASD and HC groups were compared using the CT values from the obtained qPCR data.

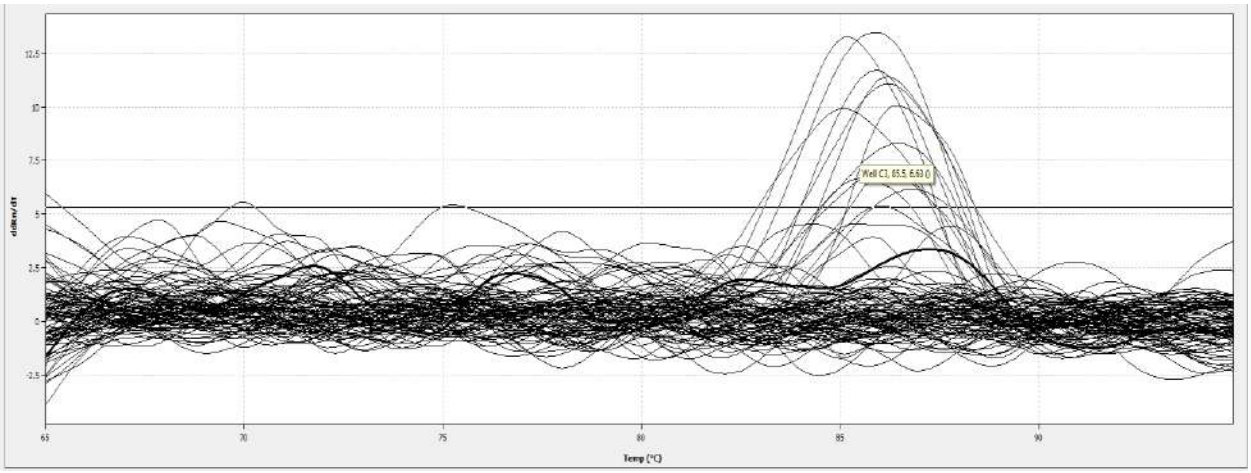

Figure-1: melting curve of *Prevotella* spp.

When 45 samples (27 control and 18 ASD)were run in RT-PCR to detect *Prevotella* spp. using SYPER green , the result showed Amplification curves of *Prevotella* spp. as shown in Figure(2).

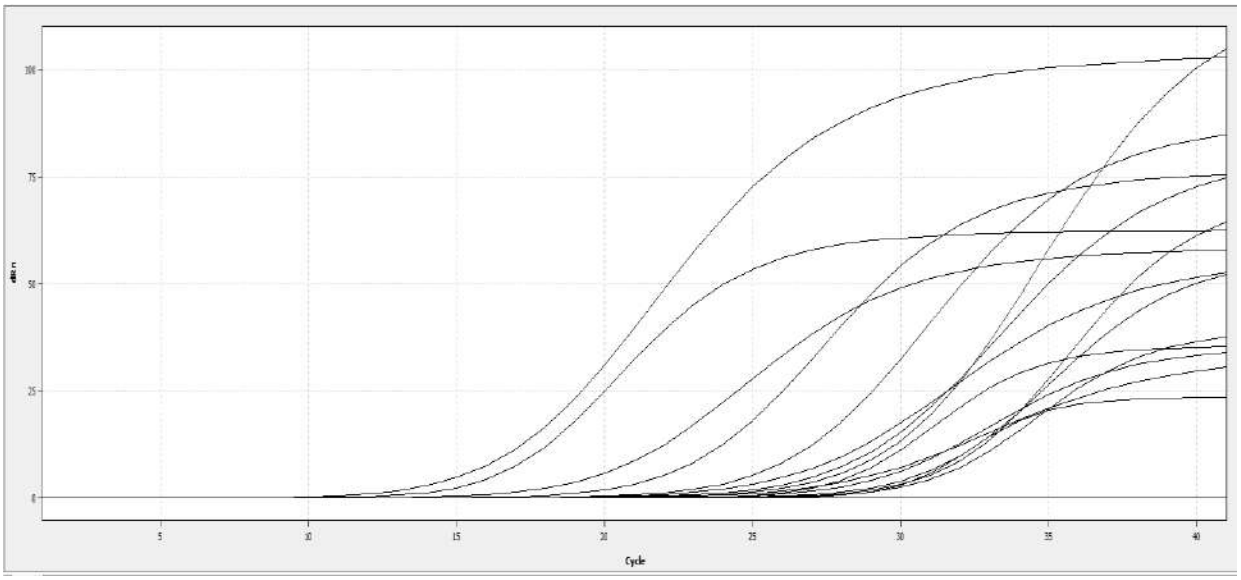

Figure 2: Amplification curves of *Prevotella* spp. using SYBR Green chemistry

Molecular study based on CT value, the frequency of *Prevotella* spp. in the current study found that twenty-two of forty-five sample (n=45) were *Prevotella* positive (40.74%) as showed in (Table-5). Out of 17 children with autism, 10 of them have *Prevotella*. Among the 27 healthy children, 12 tested positive for *Prevotella*, the present study analyses showed that participants with ASD had a lower abundance of *Prevotella* than HC group as shown in (figure-3). Although there was a difference in number of *Prevotella* spp. between the ASD children and the HC group, it was lower in the autistic than the control group, however the statically analysis revealed no significant differences in the prevalence of *Prevotella* of the two groups ($p > 0.05$).

(Table-1): *Prevotella* spp. CT values for ASD and HC groups

Sample NO.	Control	CT	Sample NO.	Autism	CT
1	C	NO	4	A	NO
2	C	NO	8	A	13.99
3	C	12.03	19	A	32.44
5	C	11.49	31	A	NO
6	C	NO	32	A	29.44
7	C	21.59	33	A	NO
9	C	NO	34	A	NO
10	C	15.32	35	A	NO
11	C	8.94	36	A	16.83
12	C	NO	37	A	26.64
13	C	NO	38	A	NO
14	C	21.6	39	A	NO
15	C	NO	40	A	32.22
16	C	NO	41	A	NO
17	C	31.47	42	A	27.59
18	C	NO	43	A	29.92
20	C	NO	44	A	29.03
21	C	NO	45	A	32.96
22	C	NO			
23	C	NO			
24	C	NO			
25	C	31.46			
26	C	NO			
27	C	28.32			
28	C	17.75			
29	C	32.22			
30	C	23.62			

NO: there is no CT, C: control group, A: autistic group

It is vital to note that CT values in quantitative PCR (qPCR) describe the cycle at which the fluorescence signal crosses a threshold, which is important to understand when analyzing the provided CT values for *Prevotella* spp. in individuals with Autism (A) and Healthy Controls (C). Higher concentrations of the target DNA in the sample are often indicated by lower CT values.

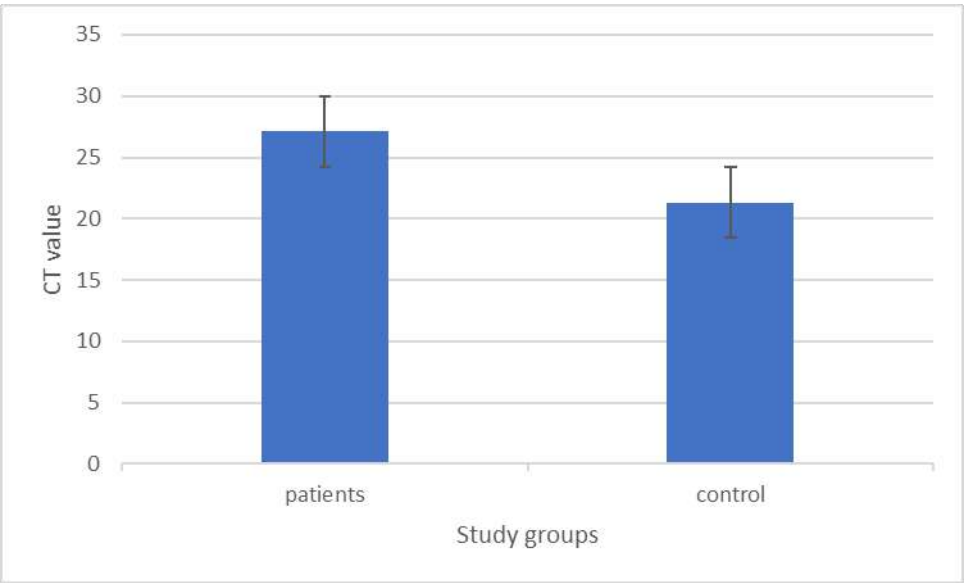

Figure-3: *Prevotella* spp. prevalence in ASD and HC group

Discussion:

Since gut microbes play a critical role in GI health maintenance, mounting evidence of GI issues occurring more frequently in children with autism suggests a connection between autism and gut microbiota. While the exact causal relationship between the various interrelated pathophysiological factors (such as diet, GI symptoms, gut microbiome profile, and autistic symptoms) is still unknown, it is critical to identify changes in the systemic microbiota and the specific microorganisms that can be targeted for the diagnosis and treatment of GI issues related to autism as well as potentially other autistic symptoms. To start achieving this long-term objective, a number of significant distinctions have been identified. First, the gut microbiota of autistic children is typically less varied. Second, there is a notable decrease in the abundance of some genera, most notably *Prevotella*, in children with autism. Finally, there are worldwide alterations in the gut microbial flora linked to autism. This goal was investigated in the culture study.

The number of samples in the current study was limited (n=45), is due to many reasons since it was challenging to meet our initial recruiting goal of 50 participants in each study group since the overall stool and questionnaire return rate from this study was lower than anticipated; we had budgeted for high return rate. This low return rate could have been influenced by the type of sample that was collected—feces. It was indicated in earlier research that "the taboo associated with the "dirtiness" of human faeces may be a key reason why some people lack the motivation to comply when it came to patients' failure to return stool samples to the researchers(Lecky et al., 2017). The other reason is cultural, parents are avoiding give anything related to their kid, in addition most private sector ASD centers established by investors didn’t want any disturbance for their projects could be raised from taking ASD children samples in spite of the task facilitating from Basrah university was demonstrated to them. The other reason was the current study condition to choose the ASD children who haven’t antibiotic for at least 3 months.

The current study had used the classic method like culture technique to identify the anaerobic bacteria, however the result was growing the aerobic bacteria like *Enterococcus faecium* , *Escherichia coli* , *Lactococcus garvieae* , *Enterococcus faecalis* , *Brevibacillus parabrevis* , *Lactobacillus salivarius* , and *Klebsiella pneumonia*, Iraqi study was carried out about this type of bacteria and the result wasn't statistically significant(Nasser et al., 2021) therefor we used the genetic method like RT-qPCR to detect the anaerobic bacteria. Three studies—De Angelis *et al*., 2013; Zou *et al*., 2020 and Ding *et al*., 2021—stated that *Prevotella* abundance in children with ASD is greater than HC, while three studies—Pulikkan *et al*., 2018; Kang *et al*., 2013; Wu *et al*., 2019 and Chen *et al*., 2021—said it was reduced level in ASD group (Korteniemi et al., 2023). In the current study and based on CT values, 40.74% of 45 samples had *Prevotella*. Ten of the seventeen autistic youngsters have *Prevotella*. Twelve of the 27 healthy kids had positive *Prevotella* tests, as indicated in (figure- 4). The prevalence of *Prevotella* did confirm the gut microbiota alteration between the two groups however differ significantly between the two groups, according to a statical analysis ($p > 0.05$) was unnoticeable. *Prevotella* was the fully recognized genera in current study where there was the greatest relative abundance difference between neurotypical and autistic patients. *Prevotella* capacity to break down a wide range of plant polysaccharides is drawing more and more attention to itself as a commensal bacteria in the human large intestine(Kang *et al*., 2013). *Prevotella* species were highly prevalent in African children whose diet is rich in grains(Filippo *et al*., 2010), suggesting that diet habits have an impact on the quantity of *Prevotella* in the human stomach in addition to the fact that the bacteria is essential for the digestion of foods high in carbohydrates, these ideas have been endorsing by Wu (2011). It was recently revealed that diets high in carbohydrates caused the gut microbiota to shift towards an enterotype rich in *Prevotella* spp. It's interesting to note that there are substantial deficits in disaccharide metabolism in autistic children, such as reduced upper GI tract lactase activity, and according to Williams *et al.* , unabsorbed mono- and disaccharides have the potential to enter the large intestine and lead to an unbalanced gut environment (Williams *et al*., 2011). In this environment, bacteria that break down polysaccharides, including *Prevotella*, may be outcompeted by mono- and disaccharide fermenters. Lipopolysaccharide (LPS) is present in the cell walls of numerous pathogenic gram-negative bacteria-*Prevotella* spp. Included-, which can lead to damage in various tissues, including the brain(Minami *et al*., 2007) .The induction of inflammation in the brain by Lipopolysaccharides (LPS) results in an augmentation of the permeability of the blood-brain barrier, thereby promoting the accumulation of elevated levels of mercury within the cerebrum. This accumulation may potentially exacerbate symptoms associated with Autism Spectrum Disorder (ASD), additionally, *Prevotella* species have vital genes for the production of vitamin B1(Arumugam *et al*., 2011), It was said to reduce the symptoms of ASD(Lonsdale *et al*., 2002). Finegold *et al*. (2010) also observed, in line with present study findings, that *Prevotella* levels were lower in autistic children than in siblings who were not affected by the condition. It has been showed in the current study a succinct analysis based on the information supplied :(NO) Denotes that, during the number of cycles observed, the amplification was unable to meet the threshold. (CT Points) Low CT values (e.g., 8.94, 11.49, 12.03): These samples had a greater *Prevotella* spp. abundance. Moderate abundance is seen in intermediate CT (e.g., 13.99, 15.32,

17.75, 21.59, 23.62, 28.32). A decreased abundance of *Prevotella* spp. is indicated by a high CT value (e.g., 29.44, 29.92, 31.46, 31.47, 32.22, 32.44, 32.96). At Comparing Groups: Autism (A) some samples had greater CT values that indicate lesser abundance; others have lower CT values that reflect higher abundance. and Control (C) Variability in CT values indicates that the number of *Prevotella* spp. varies amongst control samples. *Prevotella* spp. DNA may have been present in higher starting concentrations in samples with lower CT values. Present study demonstrates that the gut microbiota changing is real, although a statical analysis revealed no significant differences in the prevalence of *Prevotella* of the two groups ($p > 0.05$).to support the result of the current study by including by a large number of ASD samples. One of the predominant gut microorganisms that are enhanced by ruminant diet supplements of fish oil is *Prevotella*. Omega-3 fatty acids, which are important for healthy brain development, are precursors of fish oil(Simopoulos, 2011). The current study findings emphasize the value of regional studies on gut microbiota and the necessity of additional research in this field, especially with regard to children in Iraq, in order to shed light on the connection between gut microbiota alteration in children with autism,

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