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RESEARCH PAPER

Profiling of aerobic gut bacterial community isolated from faecal of autism spectrum disorder patients

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A B S T R A C T:

Intestinal microbes impact health by regulating gut homeostasis and influencing host immunity, whereas gut dysbiosis is linked to several diseases, including autism, a complex neurodevelopmental illness with multiple aetiology. We aimed to compare the bacterial populations in the gut of autistic children and their healthy siblings to better understand gut bacteria's impact on ASD. Faeces and blood samples were collected from 20 ASD individuals and 20 healthy individuals. The variations in microbial communities between the study and control groups were analysed and compared utilizing culture-dependent methods, supported by a molecular identification approach targeting the 16S RNA gene. In addition, lipopolysaccharide (LPS) level was measured in the participant's serum using enzyme-linked immunosorbent assay (ELISA). Data analysis revealed a significant difference between the two study groups in regards to gender, weight, and the usage of medication during pregnancy with a P value of 0.01, *0.05*, and 0.01, respectively. Furthermore, we observed non-significant differences in serum LPS levels between ASD children and the control group (P = 0.46). Regarding bacterial composition, 10.42%, 8.1% and 6% of the patients' faeces included *S. aureus*, *Citrobacter* spp (*Citrobacter* spp. 6.25% and *Citrobacter freundii* 2.08%) and *Klebsiella* spp (*K. pneumonia* 4.17% and *K. oxytoca* 2.08%), respectively, compared to 0% in the control group. On the other hand, the control group included *Escherichia fergusonii* (2.08%), *Shigella flexneri* (2.08%), and *Pseudomonas aeruginosa* (2.08%), which were absent in the faeces of ASD individuals. There were clear variations in the gut bacteria of the study groups. Overall, the results of this study may serve as the foundation for a more extensive metagenomics analysis of the microbiota in ASD people.

KEY WORDS: Gut bacteria, Autism spectrum disorder, Gastrointestinal symptoms, Lipopolysaccharide.
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1.INTRODUCTION:

Autism spectrum disorder (ASD) is a set of neurodevelopmental disorders marked by persistent impairments in social interactions and communication, limited interests, reciprocal social engagement, and repetitive behaviours. The incidence of ASD in children under the age of eight is estimated to be 14.6 per 1000, with a male-to-female ratio of 3:1(Sabit *et al.*, 2021). Digestive issues in particular, such as diarrhoea, constipation, and discomfort in the abdomen (Madra *et al.*, 2021), sleep disturbances (Chen *et al.*, 2021), and epilepsy (X. Liu *et al.*, 2022) are typically seen in both children and adults with ASD. In much recent research, prenatal, natal, and postnatal environmental risk factors can account for 60 to 65% of autism cases. Pregnancy-related risk factors include maternal infection, maternal physical health, pregnant women's health, folate and iron deficiencies, and medication usage. Foetal problems, umbilical cord issues, hypoxia (lack of oxygen), caesarean birth, aberrant foetal presentation, and abnormal gestational age are some of the risk factors associated with pregnancy (preterm or post-term). Among the postnatal risk factors include breastfeeding, air pollution, antibiotic use, and dietary variables (Alharthi *et al.*, 2022). Recently, there has been increased attention on the function that the gut microbiome

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plays in autism. It is well established in the literature that the human gut microbiota plays an important role in both health and disease, therefore any change in the composition may disrupt the interaction between the brain and the gastrointestinal microflora (Mehra *et al.,* 2022). Most people with ASD have gut dysbiosis, indicating a direct connection between certain disruptions in the gut bacterial population and the etiopathogenesis and severity of autism (Piras *et al.,* 2022). The bacterial population of the gut constitutes mainly 4 major phyla which are: *Bacteroides* (such as the *Bacteroides* and *Prevotella genera*), *Firmicutes* (such as *Lactobacillus*, *Clostridium* and *Ruminococcus*), *Proteobacteria* (such as the *Enterobacter* species) and *Actinobacteria* (e.g., *Bifidobacterium*) (Zafar and Habib, 2021). Numerous studies have demonstrated that the gut microbiota influences brain function via bacterial metabolites, neuroendocrine signalling, and neuroimmune signalling (J. Liu *et al.,* 2022). More recently, multiple lines of research indicate that microbial-based treatment approaches, such as faecal microbiota transplantation (FMT), antibiotics, and probiotics, have emerged as innovative therapeutic options to alleviate core and associated ASD symptoms (Johnson *et al.*, 2020, Davies *et al.*, 2021). In this study, we planned to compare the bacterial populations in the gut of patients with ASD and their families as controls in order to better understand the impact of gut bacteria on ASD. Such data helps develop cutting-edge therapy approaches to alter gut microbial communities in ASD patients.

2. Materials and Methods

2.1 Ethical approval

The study protocol was approved by the Ethical Committee of Koya University and the sample collection was approved by Erbil's general directorate of health.

2.2 Study subjects

Twenty samples (17 males and 3 females) were diagnosed with ASD and stated by the diagnostic and statistical manual of mental disorders, who presented to the Lanwe Centre for autistic children in Koya City, and Gashbin Centre in Erbil city, aged between 4-16 years old were enrolled in this study. Furthermore, twenty samples (10 males, 10 females) were collected from their neurotypical siblings as a healthy control group, aged between 5-18 years old. All the samples were collected from November 2021 to April 2022.

2.3 Sample collection

Fresh faecal samples were collected in a sterile container by the patient's parents at home. All the faecal samples were delivered in the ice box to the Science and Health Research Centre (SHRC) within the same day and kept in a refrigerator at (4°C) until analysis.

Furthermore, 3 ml of blood samples were collected from all participants using the standard venepuncture method. The serum was separated by centrifuging at 12000 rpm (Thermo Scientific, Germany) for 20 minutes and kept in a deep freezer (-80°C) until analysis.

2.4 Bacterial identification

2.4.1 Bacterial culture

A loop full of the stool sample was suspended in (1ml) of nutrient broth and homogenised well using a vortex (Scientific Industries, USA). Then 100 µl of the stool suspension was transferred onto the following culture media; Nutrient Agar, Mannitol salt agar, MacConkey agar and Eosin methylene blue agar. The plates were incubated in aerobic conditions at 37°C for 24 hours. In each plate set, single distinct colonies with various characteristics and morphologies were processed to a pure culture procedure before being preserved at -80°C in 25% glycerol for subsequent analysis.

2.4.2 Microscopic examination

Gram stain was used to determine the bacterial shape, and arrangement present in each sample using a commercially available gram stain kit (CDH, cat.no, 010721, India).

2.4.3 Biochemical tests

Diagnosis of the pure bacterial isolates was performed using the following biochemical tests; Triple Sugar Iron Agar (TSI), Urease, Simmons citrate utilization, Indole, Methyl red (MR), Voges Proskauer (VP), Motility, Oxidase, and catalase test.

2.5 Lipopolysaccharides evaluation

Human Lipopolysaccharides (LPS) concentration was determined in the serum of all the study participants using a commercially available Enzyme-Linked Immunosorbent Assay (ELISA) (BT LAB, cat no. E1791Hu, China) according to the manufacturer's instructions.

2.6 DNA extraction from bacteria

Genomic DNA was extracted from 1.5 ml bacterial culture in nutrient broth, using a commercially available genomic DNA extraction kit (FAVORGEN, cat no. FABGK100, Taiwan) according to the manufacturer's instructions. DNA

purity and concentration were measured by Nanodrop spectrophotometry (NanoVue Plus, UK). The extracted DNA was stored at (-20°C) until PCR testing.

2.7 Polymerase chain reaction (PCR)

A universal broad-ranged set of primers were used to target the conserved 16S rRNA gene sequence in the DNA samples isolated from bacterial isolates. The primers were P1F (5'-TGAAGAGTTTGATCATGGCTCAG-3'), and P1R (5'-TTCCCCTACGGTTACCTTGT-3'). The PCR reaction was performed in a thermal cycler (Biorad, USA). The reaction was run in a final volume of 20 µl mixture, containing 10 µl of 2x Taq master mix (ADDBIO, Sweden), 1 µl P1F, 1 µl P1R, 2 µl of 1ng/ µl DNA template, and 6 µl PCR water. For bacterial DNA, conventional PCR was started with an initial denaturation of 95°C for 5min and 27 cycles of 95°C for 30 secs, followed by annealing at 58°C for 30 sec and extension at 72°C for 1 min, followed by a final extension at 72°C for 5min. Negative control was used in each PCR protocol without a DNA template. The PCR products were electrophoresed in 1% TBE (Tris-Borate-EDTA) agarose gel ran at 80V for 1 hour and then visualized using a Gel documentation system (Syngene, UK). The expected size of the PCR products should be about 1500 bp (Hama-Ali and Hasan, 2023).

2.8 DNA sequencing

The 16S rRNA gene amplicons from each bacterial DNA were sent out for sequencing by the Sanger method at Macrogen, South Korea, Biotech Corporation, Korea. A universal primer named P2F (5'-GTAATACGGAGGGTGCAAGC-3') was used to ensure the sequencing of the V4 region of the 16S rRNA gene. The trimmed DNA sequences were uploaded to the EzBiocloud database to obtain sequence per cent similarities and possible identities with their closely related species (Yoon *et al.,* 2017).

2.9 Statistical analysis

All statistical analyses were carried out with Statistical Package for Social Science (SPSS), version 26 (SPSS, Inc., Chicago, IL, USA). Chi-squared test was used to compare the categorical data, and the T-test was used to compare the mean of the continuous data. Logistic regression analysis was used to assess the risk factors. All *P* values of $\geq$ 0.05 were considered statistically significant.

3. Results

3.1 General characteristics of ASD and neurotypical healthy siblings

In the present study, a total of 40 samples were collected from 20 ASD children and 20 healthy neurotypical siblings as healthy control. The 20 ASD children had male to female ratio of 17:3 (85% male and 15% female). Their average age was 8.2±3.1, ranging from 4 to 16 years old and BMI= 19.9±6.3. Regarding gastrointestinal manifestation, all the patients had at least one symptom at the time of examination. Diarrhoea (7 cases; 35%) and constipation (7; 35%) were the most common symptoms, followed by indigestion (5; 25%), and nausea (2; 10%).

Out of the 20 healthy siblings, male to female ratio was 10:10 (50% male and 50% female). Their average age was 9.6±3.3, ranging from 5 to 18 years old and BMI= 18.2±2.1. None of the siblings had any gastrointestinal symptoms at the time of examination.

Chi-squared analysis showed no significant difference regarding age, height, weight, diet, taking antibiotics, prescription medication, medical history, birth method of delivery and breastfeeding duration between the two study groups. On the other hand, gender, BMI, and medication during pregnancy had significant differences between both groups (Table 1).

The general characteristics that showed a significant difference in the chi-squared comparison were further analysed using logistic regression analysis by calculating the OR and CI of 95%. The data analysis showed that male children are three times more likely to be autistic than female children (OR=3, *p*=0.12, CI lower=0.5 and CI upper=15.1). In addition, mothers who used medication during pregnancy were eight times more likely to give birth to an autistic child (OR=8, p=0.8, CI lower=0.7 and CI upper 10.8). Finally, the autistic children were one time more likely to be overweight (BMI>20) (OR=1, P=0.3, CI lower= 0.9 and CI upper=1.2).

Table 1 General characteristics of ASD and neurotypical healthy siblings

Characteristics n=40	Control n=20 n (%)	ASD n=20 n (%)	*P*-value
Gender			
Male	10(50)	17(85)	**0.01**
Female	10(50)	3(15)	
Age (mean ±SD)	9.6±3.3	8.2±3.1	0.19
Height (cm) (mean ±SD)	127 ±22.9	119±16.3	0.2
Weight (Kilo) (mean ±SD)	30.8±12.8	27.8±8.57	0.38
BMI (mean ±SD)	18.2±2.1	19.9±6.3	0.27
Healthy	18(90)	13(65)	**0.05**
Overweight	2(10)	7(35)	
Diet			
Gluten and sweet free	0(0)	3(15)	0.07
No specific diet	20(100)	17(85)	
Taking probiotics	0(0)	0(0)	0
Prescription medication			
Glunidin	0(0)	1(5)	0.30
Levetractam	0(0)	1(5)	
Multivitamin	2(10)	1(5)	
Multivitamin+Omega3	0(0)	3(15)	
Vitamin D	1(5)	1(5)	
Respadol	0(0)	1(5)	
No medication	17(85)	12(60)	
Antibiotic use within 3 months			
Amoxicillin	2(10)	2(10)	0.57
Azithromycin	1(5)	1(5)	
Respadol	0(0)	2(10)	
No medication	17(85)	15(75)	
Medical history			
Multivitamin	0(0)	2(10)	0. 40
Vitamin D	1(5)	3(15)	
Respadol	0(0)	3(15)	
Respal and Omega3	1(5)	3(15)	
No	18(90)	9(45)	
Medication during pregnancy			
Yes	1(5)	7(35)	**0.01**
No	19(95)	13(65)	
Birth methods of delivery			
C- section	10(50)	10(50)	1
Natural	10(50)	10(50)	
Type of milk			
Breast	3(15)	4(20)	0.63
Commercial	6(30)	8(40)	
Both	11(55)	8(40)	
Breastfeeding duration			
1-2 years	13(65)	11(55)	0.74
3-4 years	6(30)	7(35)	
≥5 years	1(5)	2(10)	

3.2 Identification of the bacterial isolates

Microscopic examination by gram staining was applied to all the bacteria isolates. The results showed that the bacteria isolated from the stool sample of ASD children were 20% gram-positive and 80% gram-negative. Furthermore, the bacteria isolated from their neurotypical siblings were 100% gram-negative.

The bacterial isolates were further diagnosed by biochemical tests. A total of 96 different bacterial colonies were isolated from the stool culture, of which 48 (50%) of them were from ASD children. *E. coli* was the most common isolate (32; 66.67%), followed by *Staphylococcus aureus* (5; 10.42%), *E. coli* inactive (4; 8.33%), *Citrobacter* (3; 6.25%), *Klebsiella pneumonia* (2; 4.17%), *Citrobacter freundii* (1; 2.1%), and *Klebsiella oxytoca* (1; 2.1%) (Figure 1, A).

On the other hand, of the 48 (50%) bacterial isolates that were from neurotypical siblings, *E. coli* (44; 91.67%) was on the top, followed by *E. coli inactive* (1; 2.08%), *E. fergusonii* (1;2.08%), *Shigella flexneri* (1; 2.08%), and finally *Pseudomonas aeruginosa* (1; 2.08%) (Figure 1, B).

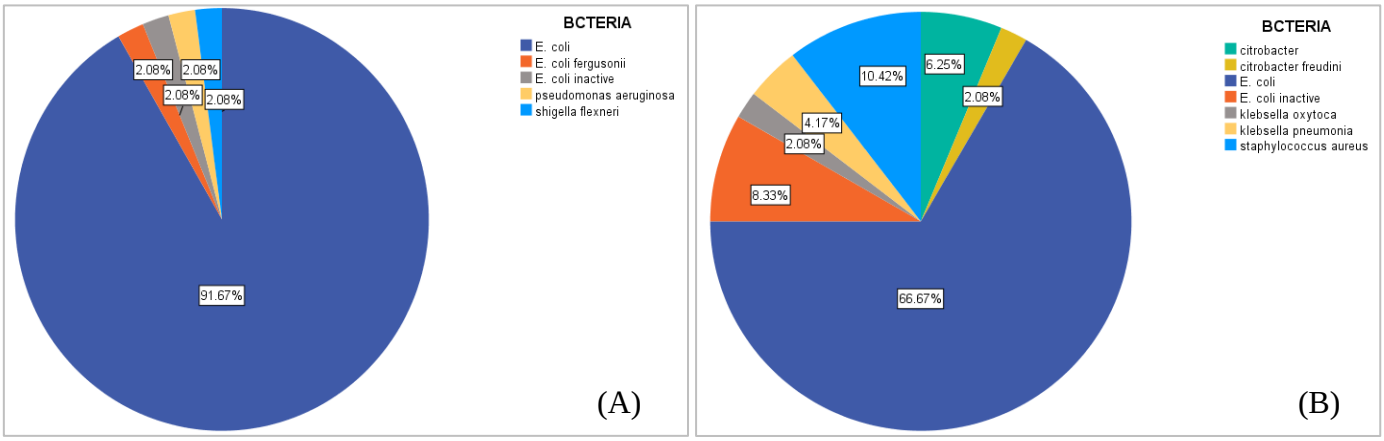

Figure 1 Pie charts showing the relative abundance of the bacterial species isolated from the stool samples of (A) ASD children and (B) healthy control group.

3.3 Serum levels of LPS

T-test analysis showed non-significant (p=0.46) differences in serum LPS levels between ASD children (226.6±249.6) and the control group (301.1±376.3). Further

3.3 DNA amplification

The extracted genomic DNA from 15 bacterial isolates (fourteen-gram negative and one gram-positive based on the biochemical tests) were amplified by polymerase chain reaction (PCR) using P1F and P1R primers to target the conserved 16SrRNA gene of bacterial isolates. The targeted DNA sequence was successfully amplified in all DNA (100%) samples. All the positive PCR products were around the 1500bp band of the DNA marker, indicating a successful PCR reaction (Figure 3). Finally, no DNA band was observed in the negative control lane.

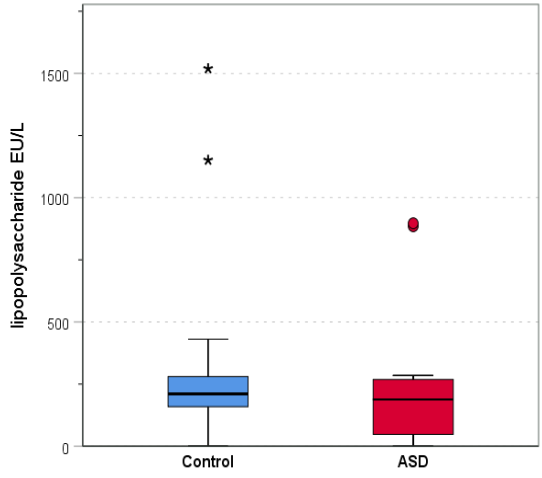

Figure 2 Box plots showing the distributions of serum Lipopolysaccharide levels between ASD and Control. The line in the middle of the box represents the mean value, and the asterisks indicate the outliers.

4. Discussion

In the present study, the results had shown that the male-to-female ratio was 17:3 (85% male and 15% female). Although the specific cause is unknown, the fact that men are diagnosed with ASD more commonly than women has led to important theories about the nature and cause of ASD, such as the excessive male brain, female protective effect, and female autism phenotypic theories (Napolitano *et al.,* 2022). Empathizing and systemizing are the two dimensions for understanding sex differences in people, according to the Extreme Male Brain Theory. Accordingly, systematisation, rather than empathy, matches the male brain better. On the other hand, the cognitive profile of the female brain is described differently. As a result, ASD can be considered an extreme case of the usual male profile (Baron-Cohen *et al.*, 2005). The idea of a "Female Protective Model/Effect" was created as a result of the continuously observed rise in male prevalence. This model assumes that the risk for ASD can be measured, that it is spread throughout the population, and that females are protected from the disorder's impacts (Werling, 2016). When exposed to risk factors, females appear to be shielded from developing autism, and their risk burden threshold is higher than that of males before their ASD manifests (for example, genetic variations or environmental exposures). Additionally, theories on the Female Autism Phenotype predict the existence of a female-specific manifestation of autistic abilities and needs, which fits improbably with existing, male-based conceptual frameworks of ASD (Lai & Baron-Cohen, 2015). The existence of the female ASD phenotype is supported by evidence that women and girls with ASD are more socially motivated and have better relationship skills than men with ASD (Bargiela *et al.,* 2016). People with ASD typically have comorbid disorders, and they are more likely to have GI symptoms such as constipation, diarrhoea, and abdominal pain, which will worsen the quality of their life (Leader *et al.,* 2022). According to our findings, every patient experienced at least one of the GI symptoms at the time of the examination. Including seven cases of diarrhoea (35%), seven cases of constipation (35%), five cases of indigestion (25%), and two cases of nausea (10%). These gastrointestinal problems have an unknown cause. Nevertheless, these seem to be related to alteration in the gut bacteria, specifically in the overgrowth of pathogenic bacteria (such as *Clostridium* spp.) and the reduction of beneficial bacteria (such as *Bifidobacterium* and *Lactobacillus*) (Jendraszak *et al.*, 2021).

Interestingly, those women who used medication during pregnancy were eight times more likely to give birth to an autistic child, the association was significant with a P value of 0.01. It's possible that prenatal antibiotic exposure altered the composition of the newborn's gut microbiota and contributed to the development of ASD (Hamad *et al.,* 2019). LPS, compounds found on the outer membrane of Gram-negative bacteria, can pass from the gut into the bloodstream when there is a leaky gut. As a result of LPS activating immune cells, pro-inflammatory cytokines are secreted more often, resulting in systemic low-grade inflammation. The increase in LPS in autistic individuals' bloodstream correlates with social interaction scores (Emanuele *et al.,* 2010) Our results revealed no significant changes between the control group and children with ASD in terms of blood LPS levels, which is in agreement with (Carissimi *et al.,* 2019). In the aspect of bacterial composition, 10.42%, 8.1% and 6% of the patients' faeces included *S. aureus*, *Citrobacter* spp (*Citrobacter* spp. 6.25% and *Citrobacter freundii* 2.08%) and *Klebsiella* spp (*K. pneumonia* 4.17% and *K. oxytoca* 2.08%), respectively, compared to 0% in the control group. On the other hand, the control group included *Escherichia fergusonii* (2.08%), *Shigella flexneri* (2.08%), and *Pseudomonas aeruginosa* (2.08%), which were absent in the faeces of ASD individuals. The aforementioned bacterial species were isolated and identified using conventional cultivating techniques, and then the results were subsequently confirmed by sequencing the 16s rRNA genes in representative bacterial isolates (Figure 3). In order to better understand the functioning and alterations of the microbiome-gut-brain axis and its implications on the development of ASD, metagenomic studies with a specialised methodology are necessary.

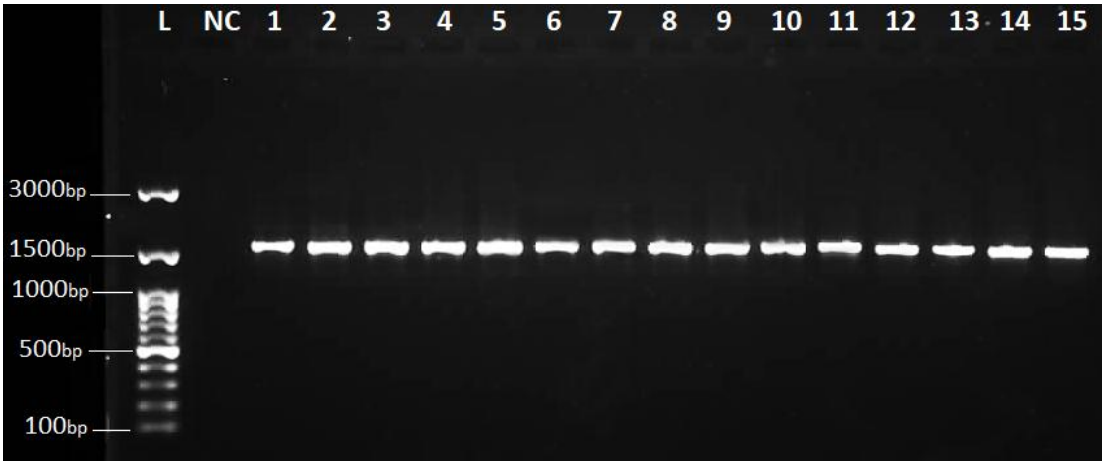

Figure 3 DNA amplification targeting conserved 16S rRNA gene of the bacterial isolates. Lane (L) contains a 100bp DNA ladder (GeneDirex, USA). Lane (NC) contains negative control, and lanes (1 to 15) contain PCR products from examined bacterial samples. Gel electrophoresis was performed on 1% agarose gel with 5µl of 3x Ethidium bromide solution (at a final concentration of 2%) and ran at 80V for 1h.

5. Conclusion

In conclusion, our results showed that children with ASD have higher pathogenic bacterial diversity in their gut, such as *Citrobacter, Klebsiella, and Staphylococcus* strains compared with healthy children. In addition, there was a significant correlation between developing ASD and BMI, gender, and medication during pregnancy.

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