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Study of the Gut Enterotypes in Egyptian Children with Autism Spectrum Disorder

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Authors' contributions

This work was carried out in collaboration between all authors. Author SMA designed the study and wrote the protocol. Authors AMEH and MAM wrote the first draft of the manuscript. Author HA managed the selection and clinical assessment of the study participants. Authors SMAA and YSR carried out the practical work and managed the literature searches. Authors MH and AEI performed the statistical analysis. All authors read and approved the final manuscript.

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ABSTRACT

Background: Gut microbiota distribute into three enterotypes named the *Bacteroides*, *Prevotella* and *Ruminococcus*. While each person's microbial "fingerprint" is unique, there are specific patterns seen in those that are healthy and those that have specific illnesses.
Aims: The aim of the present study is to identify the enterotypes that are likely related to ASD as well as their possible role in the severity of the disease and gastrointestinal symptoms
Subjects & Methods: The study included 41 ASD patients, 45 of their neurotypical siblings and 45

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unrelated controls. Autism severity was assessed using Childhood autism rating scale (CARS). Gastrointestinal symptoms were assessed by a modified six-item Gastrointestinal Severity Index (6-GSI) questionnaire. Quantitative real-time SYBR green PCR was done for the identification and quantitation of the dominant enterotypes.

Results: Enterotype 1 (*Bacteriodes*) was the most prevalent enterotype accounting for 76.7%, 75.6% and 57.8% in patients with autism, their neurotypical siblings and unrelated controls respectively. Enterotype 3 (*Ruminococcus* dominant), it was detected nearly equal in the three groups. Enterotype 2 (*Prevotella* dominant), it was the least enterotype encountered in ASD group (4.9%) compared to 8.9% and 22.2% in the siblings and the unrelated control respectively. About 75.6% of ASD patients shared the same enterotype with their siblings. There was no significant difference between the three enterotypes as regards the CARS or 6-GSI.

Conclusion: There was no significant difference in the distribution of enterotypes in all study groups. Therefore, collapsing the whole microbiome variations into dominant enterotypes was not appropriate to identify disease association or to be used as a disease biomarker. Further studies of individual bacterial species may be more accurate to determine if there are any possible correlations between the gut microbiome and the gastrointestinal dysfunction in ASD patients.

Keywords: Autism; gut microbiome; gut enterotypes; SYBR green real-time PCR.

1. INTRODUCTION

Autism spectrum disorders (ASDs) are considered a heterogenous set of neurobehavioral diseases, characterized by alterations in social interactions and communication and by restricted and repetitive behavior [1].

In addition to the core symptoms, children with ASD often have comorbid medical conditions. [2] Among the non-neurological symptoms associated with ASDs, several studies indicate gastrointestinal (GI) disorders are a common comorbidity [3,4].

Although researchers on ASDs primarily focused on genetic associations, recent evidence has suggested that ASDs are etiologically heterogeneous. It is believed that both genetic and environmental factors, gut microbiota and dietary factors, influence the onset and development of ASD [5].

Our knowledge of the gut microbiome has excessively expanded over the last few years. The gut is inhabited with 10^{13}-10^{14} micro-organisms, a figure thought to be 10 times that of the number of human cells in our bodies and 150 times as many genomes [6].

Analysis of species abundance across different individuals suggested that the human microbiome which is composed of well-balanced host-microbial symbiotic groups of many species and genera can be grouped into three clusters, which were designated as 'enterotypes'. Each of the three enterotypes is identifiable by the variation in the levels of one of three genera: *Bacteroides* (Enterotype 1), *Prevotella* (Enterotype 2) and *Ruminococcus* (Enterotype 3) that are associated with long-term diet patterns [7].

Enterotype 1 is most common enterotype in countries consuming Western-type diet. The Western diet is characterized by high intake of salt, saturated fat, protein, sugar and is associated with gut inflammatory cell abundance. Enterotype 2 is highly prevalent in non-Westerners who consume a plant-rich diet. The association with a plant-rich diet has suggested that *Prevotella* is a beneficial microbe. Enterotype 3 is restricted to areas like rural Africa depending mainly on vegetables [8,9].

While each person's microbial "fingerprint" is unique, there are specific patterns seen in those that are healthy and those that have specific illnesses [7]. Therefore, this study was designed to identify the gut enterotypes of ASD patients, in an attempt to identify the enterotype that is likely related to ASD and could in turn be used to guide diagnostics and treatment options. As a control, two groups were enrolled; the first group is their neurotypical siblings sharing the same environment and some of genetic factors. The other group is cross matching healthy neurotypical children of similar age and gender with nearly similar environmental conditions.

2. SUBJECTS AND METHODS

2.1 Subjects

Forty-one autistic children, who presented to the Autism Clinic of Alexandria University Children's

Hospital, were enrolled in our study. These children were diagnosed with ASDs according to DSM-5 (Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition) [1] and CARS was used to assess severity of autism [10,11].

Forty-five siblings, as well as 45 age and sex, matched unrelated neurotypical control groups were also included. The enrolled subjects did not use any type of antibiotic or probiotic medications at least within one month prior to sample collection. The protocol was approved by the Ethics Committee of the Alexandria University Faculty of Medicine.

After obtaining an informed consent from the parents, a detailed history was taken from each case. In addition, gastrointestinal symptoms were assessed with a modified six-item GI Severity Index (6-GSI) questionnaire. Specifically, it includes only six items (constipation, diarrhoea, stool consistency, stool smell, flatulence, and abdominal pain). The absence of a symptom is 0, its presence ranges from 1 to 2 depending on the severity of the symptom, thus the score includes the number of symptoms and their severity [12].

2.2 Specimen Collection, Preservation and Transport

Stool specimens were collected from cases and controls, kept in the freezer upon defecation at home, and within the same day delivered to our laboratory frozen, where aliquots of each specimen were stored at −80°C until DNA extraction in the same week.

2.2.1 DNA Extraction

DNA was extracted from 150 mg stool samples using ISOLATE Fecal DNA Kit (Bioline, UK) according to the manufacturers' instructions. In brief, fecal samples were added directly to a bashing beads lysis tube and they were rapidly lysed by bead beating in a vortex, without the use of organic denaturants or proteinases. The DNA was then bound, isolated and purified using spin columns. The resulting DNA extracts were stored at -80°C until PCR assessment.

2.3 Quantitative SYBR Green Real-Time PCR Primers

Oligonucleotide primers targeted at the 16S rRNA gene (rDNA) sequences of *Bacteroides, Prevotella* and *Ruminocooccus* are shown in Table 1. Primers were also used to amplify a conserved 16S rDNA sequence present in all bacteria (universal primer set, recognizing domain bacteria), the amplification of which served as the denominator against which the amplification of the other bacteria was compared. All of the primer sequences were derived from the previously published studies. Primers were commercially obtained (Metabion International AG. Germany).

2.4 Detection and Quantitation of Amplified PCR Products

Amplification was performed in a light cycler (Rotor Gene Q, Qiagen, Germany) using a SensiFAST TM SYBR No-ROX PCR kit (Bioline Co. UK). In short, forward and reverse primers (4 pmol each) were used in 20 µl reactions containing 2 µl of the DNA extract.

PCR amplification was performed with initial denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. Melting curve analysis was performed from 40 to 95°C with a plate-reading step after every 1°C and held at a particular temperature for 10 seconds to check the specificity of the product formed.

Table 1. Primers used in this study

Bacteria	Primer Name	Primer Sequence (5'-3')	Product bp
Total bacteria [12]	*UnivF*	TCCTACGGGAGGCAGCAGT	500
	UnivR	GGACTACCAGGGTATCTATCCTGTT	
Bacteroides [13]	B3F	CGATGGATAGGGGTTCTGAGAGGA	238
	B3R	GCTGGCACGGAGTTAGCCGA	
Prevotella [13]	*PrevF*	CACCAAGGCGACGATCA	*286*
	PrevR	GGATAACGCCYGGACCT	
Ruminococcus [14]	*Rflbr730F*	GGCGGCYTRCTGGGCTTT	*157*
	Clep866mR	CCAGGTGGATWACTTATTGTGTTAA	

Quantitation of specific bacterial DNA was not expressed as absolute number but expressed relative to total (universal) bacteria DNA present in a stool sample by the RQ software (Qiagen) [15,16].

2.5 Statistical Analysis

Data entry and analysis were carried out using the statistical package for social sciences SPSS ver.18 PASW, SPSS ver.18, Chicago. Quantitative variables were presented as a mean and standard deviation. For qualitative variables, frequency and percentage of total were used. Comparisons between groups were carried out using Chi-Square, Fisher' s Exact, and Monte Carlo tests for qualitative variables and Kruskal Wallis test for quantitative ones. Correlations were carried out using Pearson correlation coefficient. All results were interpreted at 5% level of significance.

3. RESULTS AND DISCUSSION

3.1 Results

The characteristics of the study groups are shown in table 2. Out of the 41 ASD patients, 28 were males and 13 were females with a male to female ratio of 2.2:1. Their mean age was 5.55 ± 1.9, and their age ranged from 2.5 to 12 years. According to CARS, 35 (85.4%) out of 41 ASD patients had mild to moderate ASD, while 6 (14.6%) patients had severe ASD. The CARS mean was 31.54±5.15 for all cases. Concerning the GI manifestations, out of 41 ASD cases, 35 (85.4%) had at least one GI symptom at the time of examination, while 5 (12.2%) have no symptoms. The mean 6-GSI score was 3.37± 2.12. Abnormal stool smell was the most common GI symptom and it was reported in 30 cases (73.2%), followed by flatulence in 20 (48.8%), constipation in 17 (41.5%), abdominal pain in 12 (29.3%), abnormal stool consistency in 10 (24.4%) and diarrhea in 4 (9.8%).

There was no statistically significant correlation between CARS and 6-GSI CARS (r=-0.131, p=0.415).

Regarding the neurotypical sibling's control, out of 45, 22 (48.9%) were males and 23 (51.1%) were females with a male to female ratio of 1:1. The mean age was 4.27±3.22 and their age ranged from 0.5 to 12 years. Out of 45 unrelated neurotypical controls, 28 were males and 17 were females with male to female ratio of 1.6:1. The mean age was 5.36±2.6 and their age ranged from 2 to 12 years. None of the siblings or unrelated controls had GI symptoms at the time of examination.

Table 2. Characteristics of the study groups

	ASD cases (n=41)	Siblings controls (n=45)	Unrelated controls (n=45)
Age (years)			
Range	2.5-12	0.5 – 12	2.0 – 12
Mean ± SD	5.55 ± 1.9	4.27 ± 3.2	5.36 ± 2.6
Gender			
Male	28 (68.3%)	22 (48.9%)	28 (62.2%)
Female	13 (31.7%)	23 (51.1%)	17 (37.8%)
CARS			
Mean	31.5	NA	NA
Moderate	35 (85.4%)	NA	NA
Severe	6 (14.6%)	NA	NA
GI Symptoms			
Present	35 (85.4%)	0 (0%)	0 (0%)
Absent	6 (14.6%)	45 (100%)	45 (100%)
6-GSI mean	3.37± 2.12	0 (0%)	0 (0%)
Diarrhea	4 (9.8%)	0 (0%)	0 (0%)
Constipation	17 (41.5%)	0 (0%)	0 (0%)
Malodorous stool	30 (73.2%)	0 (0%)	0 (0%)
Flatulence	20 (48.8%)	0 (0%)	0 (0%)
Abdominal pain	12 (29.3%)	0 (0%)	0 (0%)
Abnormal stool consistency	10 (24.4%)	0 (0%)	0 (0%)

CARS: Childhood Autism Rating Scale.
6-GSI: modified six-item GI Severity Index

Enterotype 1 (*Bacteroides*) was the most prevalent enterotype encountered in all study groups. Enterotype 1 was detected in 32 (78%) ASD cases, 34 (75.6%) siblings and 26 (57.8%) unrelated controls. On the other hand, 10 (22.2%) unrelated controls had enterotype 2 in comparison to only two (4.9%) ASD cases and four (8.9%) siblings. For Enterotype 3, it was detected in seven (17.1%) ASD cases, seven (15.6%) siblings and nine (20%) unrelated controls. Regarding the distribution of different enterotypes within each of the study groups; Enterotype 1 was significantly higher than Enterotype 2 or 3 in ASD group (p value=<0.001). Similarly, Enterotype 1 was statistically significantly higher than the 2 other enterotypes (2 & 3) in both siblings and unrelated healthy control group. (p value=<0.05). Concerning the distribution of different enterotypes in the three study groups, Enterotype 1 was significantly higher in ASD group when compared to the unrelated healthy controls (p value= 0.049), while, Enterotypes 2 & 3 were significantly lower in ASD group than in the unrelated healthy controls (p=0.049). There was no statistically significant difference in the distribution of Enterotypes between the ASD group and their siblings group (p=0.858) or between the siblings and the unrelated healthy control group (p=0.143) (Table 3).

Table 4 shows the relation between enterotypes of ASD patients and different variables like; age, gender, ASD severity and GI symptoms. There was no statistically significant difference between the three enterotypes and the different clinical variables. Similarly, no statistically significant difference was observed when comparing the enterotypes with different variables (age, gender) in both neurotypical siblings and the unrelated control (Data not shown, p=>0.05).

According to CARS, out of 41 ASD cases, 35 (85.4%) had mild to moderate autism, while only six cases (14.6%) had severe autism and this showed a statistically significant difference (p=<0.001). Comparison between children with mild to moderate ASD and those with severe ASD regarding their age, gender, CARS score and GI symptoms revealed no statistically significant difference between the two ASD severity groups and the different variables (Data are not shown).

When studying the enterotypes similarity among the ASD cases and their siblings, they were divided into: enterotype similar group, 31 cases (75.6%) and enterotype non-similar group, 10 cases (24.39%). By comparing the two groups it was found that enterotype 1 was the most prevalent (87.1%) in the enterotype similar group. On the other hand, 50% of the non-similar group had enterotype 1 while 50% had enterotype 3. This difference was statistically significant (p=0.006).

3.2 DISCUSSION

In recent years, the gut microbiota has emerged as a topic of great interest in medical research. Recent investigations have shown altered gut microbiota composition in patients with neurodevelopmental disorders [17,18].

Given the crucial role of gut microorganisms in maintaining GI health and increasing evidence of more frequent occurrence of GI problems in autistic children, this strongly implies a link between autism and gut microbiota [19].

According to the results of the current study, out of the 41 ASD cases, 35 (85.4%) had at least one gastrointestinal symptom at the time of

Table 3. The distribution of the three Enterotypes in the three study groups

Enterotypes	ASD Cases [A] (n=41)	Siblings Controls[B] (n=45)	Unrelated Controls [C] (n=45)
Enterotype 1	32 (78%)[a*]	34 (75.6%)[d*]	26 (57.8%)[g*]
Enterotype 2	2 (4.9%)[b]	4 (8.9%)[e]	10 (22.2%)[h]
Enterotype 3	7 (17.1%)[c]	7(15.6%)[f]	9 (20%)[i]
Statistical Test	Monte-Carlo X^2= 7.698		
P value	0.103		

**Statistically significant at p ≤0.05*
P value: A vs B; <0.858, A vs C; <0.049, B vs C=0.143.*
a vs b; <0.001, a vs c; <0.001*, b vs c =0.096.*
d vs e; <0.001, d vs f; <0.001*, e vs f =0.366.*
g vs h=0.008, g vs i=0.004*, h vs i =0.819.*

Table 4. Relation of demographic and clinical characteristics of ASD cases and different Enterotypes

ASD Cases (n=41)	Enterotype 1 (n=32)	Enterotype 2 (n=2)	Enterotype 3 (n=7)	Statistical test	P value
Age					
Mean age ± SD	5.4 ± 2.04	5.3 ± 1	6.3 ± 1.32	Kruskal Wallis	0.26
Age range	2.5-12	4.6 - 6	3.5 - 7.5	X^2=2.696	
Gender					
Male no. (%)	24 (85.7%)	1 (3.6%)	3 (10.7%)	Monte Carlo	0.259
Female no. (%)	8 (61.5%)	1 (7.7%)	4 (30.8%)	X^2=3.065	
ASD Severity					
Mean CARS ± SD	31.4 ± 5.67	33 ± 4.24	31.7 ± 2.69	Kruskal Wallis X^2=0.686	0.71
GI Symptoms					
6-GSI mean	3.5 ± 2.12	1 ± 1.41	3.3 ± 2.06	Kruskal Wallis X^2=2.909	0.233
Low 6-GSI	14 (77.8%)	2 (11.1%)	2 (11.1%)	Monte Carlo	0.212
High 6-GSI	18 (78.26%)	0	5 (21.74%)	X^2= 3.224	
Diarrhea	4 (100%)	0 (0%)	0 (0%)	Monte Carlo X^2=1.247	0.657
Constipation	12 (70.6%)	0 (0%)	5 (29.4%)	Monte Carlo X^2=4.213	0.117
Malodorous stool	24 (80%)	1(3.3%)	5 (16.7%)	Monte Carlo X^2=0.612	1
Flatulence	16 (80%)	0 (0%)	4 (20%)	Monte Carlo X^2=2.12	0.531
Abdominal pain	10 (83.3%)	0 (0%)	2 (16.7%)	Monte Carlo X^2=0.89	0.853
Abnormal stool consistency	8 (80%)	0 (0%)	2 (20%)	Monte Carlo X^2=0.718	1
No GIT symptoms	4 (66.7%)	1 (16.6%)	1 (16.6%)	Monte Carlo X^2 = 2.12	0.321

**Statistically significant at p ≤0.05*

examination. Results of the current study are consistent with a recently published meta-analysis of GI symptoms in ASD patients by Mc Elhanon et al. [4] that gathered data from published peer-reviewed journals concluded that GI symptoms are more common in children with ASD than control children, although the highly variable methodologies between the studies. This meta-analysis showed that children with ASD, in contrast to control groups, experienced significantly more general GI symptoms and the most common GI symptoms was overproduction of intestinal gases/flatulence [4].

Also, a survey conducted in USA on 412 children with autism reported that 84.1% of the autistic patients had at least one of GI symptoms, compared with 31.2% of the healthy siblings [20].

Nevertheless, the actual incidence of gastrointestinal disorders among ASD patients is under debate. In fact, it has been estimated to range between 9% and 90%, depending on the study [21,22]. These variations in prevalence of GI problems can be accounted as the result of methodological differences across studies, such as: variations in the criteria used to define a GI problem and the number of different GI symptoms considered. Another reason is the difficulty of diagnosis of gastrointestinal problems in these subjects as many -in fact- cannot express pain or discomfort through verbal and/or nonverbal channels.

In the present study, these challenges have been overcome by using the 6-GSI score, depending on specified symptoms observed by the parents or caregivers. It was applied to the assessment of the gastrointestinal symptoms and its severity. CARS was also used for the assessment of autism severity.

However, according to findings of the current study, there was no statistically significant

correlation between CARS and 6-GSI among ASD children. This finding is consistent with results of a study by Kang and colleagues [23] who conducted the study on 20 children with ASD and did not find a significant correlation between the 6-GSI score and any of the measures of autism severity [23].

Similar results were reported by Mazefsky et al. [24] who stated that there were no associations between the presence of GI problems and autism symptom severity [24].

On the other hand, Adam et al. [19], reported that gastrointestinal symptoms (assessed by the 6-GSI) were strongly and very significantly correlated with the severity of autism (assessed by the Autism Treatment Evaluation Checklist, ATEC). Children with 6-GSI scores above 3 had much higher ATEC total scores than those with 6-GSI-scores of 3 or lower. Our results may be due limited number of severe cases as they represented only 6 cases of the total 41 ASD cases [19].

Surveys of humans from around the world have revealed differences in gut microbiota composition among geographically separated populations. However, globalization has affected people's eating habits, leading many of them to consume high-fat and high-calorie foods which have adverse effects on human health [9,25].

It was observed in the present study that Enterotype 1 (*Bacteroides* dominant) was the most prevalent enterotype encountered in all study groups. Enterotype 1 was detected in 78% ASD* cases, 75.6% siblings and 57.8% unrelated controls. Also, this enterotype was significantly higher in ASD cases and their siblings than healthy neurotypical unrelated control. For Enterotype 3 (*Ruminococcus* dominant), it was detected nearly equal in the three groups. It was detected in 7 (17.1%) ASD cases, 7 (15.6%) siblings and 9 (20%) unrelated controls. Regarding Enterotype 2 (*Prevotella* dominant), it was the least enterotype encountered in ASD group (4.9%) compared to 8.9% and 22.2% in the siblings and the unrelated control respectively.

These results agree with all studies that revealed that Enterotype 1 is most common in countries consuming Western-type diet and Enterotype 3 is restricted to areas like rural Africa depending mainly on vegetables [7-9].

Regarding Enterotype 2 (*Prevotella* dominant), current result agrees with other studies reported that *Prevotella* is much decreased in neurological and autoimmune diseases associated with gut dysbiosis [8,26].

Similar to the current observation, Finegold et al. [27] also reported the depletion of *Prevotella* in autistic children in contrast to its prevalence in sibling controls, but no further discussion with statistical analysis followed this observation [27]. Moreover, a study by Son et al. [28], reported that *Prevotella* dominant enterotype was observed at lower levels in autistic samples than the neurotypical control and this is consistent with the current results [28].

By examining the association between the characteristics of the participants such as age and gender, in comparison with their enterotypes, no significant difference was evident neither in the ASD group nor the two control groups and this observation is consistent with what was reported in a previous study [23,29].

By investigating the distribution of the three gut enterotypes in the ASD cases according to the autism severity (CARS), there was no significant difference. Furthermore, there were no specific GI symptoms associated with different gut enterotype profile within the autistic group. The current results agree with observations reported in a previous study which showed that autism-related changes in the bacterial diversity and individual genus abundance were not correlated with the severity of autistic symptoms [23,27,28].

Unexpectedly in the current study, there was no statistical difference between the ASD cases and their siblings regarding their enterotypes distribution. On the other hand, both ASD cases and their siblings displayed statistically significant difference compared with the unrelated neurotypical group. Moreover, 75.6% of the ASD cases had enterotypes similar to their siblings. This observation was consistent with the results of a previous study [28].

Son et al. (2015) explained why their study which compared ASD children with neurotypical siblings, did not replicate differences reported between ASD children with unrelated controls in previous studies. Although the neurotypical siblings of ASD children had altered microbiomes compared to that of unrelated children, the studies tracking siblings of ASD children have detected vulnerabilities in some neurocognitive domains in the absence of an ASD diagnosis

suggesting that some of the neurotypical siblings may exhibit a broader subclinical ASD phenotype [28,30,31].

Another explanation is that the environmental factors such as dietary habits and the living conditions together with host genetics may play an essential role in shaping the gut enterotype of the study cases and related siblings controls [32].

4. CONCLUSIONS

Enterotype 1 is dominant in all study groups and significantly higher in ASD cases and their siblings than unrelated control. On the other hand Enterotype 2 was the least enterotype encountered in ASD group. About 75.6% of ASD cases had similar enterotypes as their siblings.

There was no significant difference in the distribution of enterotypes in all study groups. Therefore, collapsing the whole microbiome variations into dominant enterotypes was not appropriate to identify disease association or to be used as a disease biomarker. Otherwise studying the individual bacterial species and genera maybe more accurate to determine if there are any possible correlations between the gut microbiome alteration and GI dysfunction and the severity of the disease in ASD children.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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