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Study of the gut Microbiome Profile in Children with Autism Spectrum Disorder: a Single Tertiary Hospital Experience

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Abstract

The role of gut microbiome was recently raised in the pathogenesis of neurodevelopmental disorders including autism spectrum disorder (ASD). The aim of this study was to elucidate changes in gut microbiome in Egyptian autistic children and its possible correlation with the severity of autism and gastrointestinal (GI) symptoms. The gut bacterial microbiome of 41 ASD children, 45 siblings, and 45 healthy controls were analyzed using quantitative SYBR Green real-time PCR technique targeting 16S rRNA of selected bacteria. The gut microbiome of ASD children and their siblings contained a higher relative abundance of *Bacteroides* as well as *Ruminococcus* than controls. *Prevotella/Bacteroides* (P/B) ratio and *Firmicutes/Bacteroidetes* (F/B) were significantly lower in both ASD cases and their siblings. The only difference between the autistic cases and their siblings was the significantly higher level of *Bifidobacterium* in siblings, which appears to offer them a protective role. There was no correlation between the altered gut microbiome and the severity of autism or GI symptoms. The current study showed an evidence of changes in the gut microbiome of autistic children compared to the unrelated control. However, the microbiome profile of siblings was more like that of autistic children than that of unrelated controls indicating that gut microbiota is affected by dietary habits, living conditions together with host genetic factors.

Keywords Gut microbiome · Autism spectrum disorders · Dysbiosis · Real time PCR

Introduction

Autism spectrum disorders (ASDs) refer to a group of neurobehavioral diseases, influenced by both genetic and environmental factors (Kim and Leventhal 2015). The significantly predominance of gastrointestinal (GI) symptoms in ASD has motivated researchers to explore the potential role of gut microbiota in ASD (Holingue et al. 2018). Also, findings of previous studies suggest that gut microbiota modification may contribute to improvement of ASD symptoms and that gut dysbiosis, or disruption of the balanced composition of gut bacteria, could represent the boundary between environmental and genetic risk factors that convey in ASD (Tomova et al. 2015; Kang et al. 2013).

The human gut microbiota is a complex population of 10^{14} microbes including bacteria, fungi, and others, whose genomes (microbiome) contain at least 100 times as many genes as the human genome (Kuczynski et al. 2012). The gut microbiota contributes to the normal metabolism and immune homeostasis and can also control the central nervous system (CNS) activities through neural, immune, and endocrine pathways (Sampson and Mazmanian 2015).

Gut bacteria have implicated in the pathogenesis of neurodevelopmental disorders through the concept of "microbiota-gut-brain axis" (Ghaisas et al. 2016). Host microbiome produces metabolites such as short-chain fatty acids (SCFA) that interferes with both normal GI and CNS functions evidenced by alteration of neurotransmitters and gap junction coupling (MacFabe 2012).

The development of culture-independent molecular techniques allowed identification and quantitation of different gut bacteria. The commonly used target for bacterial detection and enumeration is 16S rRNA gene (Matsuki et al. 2002).

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In view of these evidences, together with poor conductance of researches on gut microbiota in ASD patients in Egypt, in addition to the absence of definite medical test or biological marker for diagnosis of ASD, the present study was designed to study the predominant gut microbiome in autistic children in an attempt to identify gut bacteria which are likely related to ASD and to correlate these bacteria with the severity of autism and/or GIT manifestations.

Subjects and Methods

Subjects

Forty-one children (28 males, 13 females) diagnosed with ASD, who presented to the Autism Clinic of Alexandria University Children's Hospital, were enrolled in the study. Children aged from 2 to 18 years old were included in the study. Two control groups were also included: a non-autistic sibling group (45; 22 males and 23 females) and an unrelated healthy group in good mental and physical health (45; 28 males and 17 females). The sibling group was included to examine any impact of host genetics, environment, and/or lifestyle on the results. Children with diabetes mellitus, inflammatory bowel disease, or hepatic disorders, as well as known immunodeficiency or food intolerances were excluded from the study. None of the study subjects was taking probiotics.

Clinical Procedures

ASD was diagnosed according to DMS-V criteria, and the severity of autism was assessed using CARS (childhood autism rating scale) (Rellini et al. 2004). A written questionnaire was used to collect clinical data regarding GIT habits, dietary characteristics of cases, and controls. In addition, gastrointestinal symptoms of ASD cases were assessed with a modified six-item GI Severity Index (6-GSI) questionnaire. The score includes the number of symptoms and their severity (Adams et al. 2011).

Sample Collection, Preservation, and Transport

Stool samples were collected from cases and controls, kept at − 20 °C upon defecation at home, and within the same day delivered frozen to Alexandria University Main Microbiology laboratory, where aliquots of each sample were stored at − 80 °C for further processing.

DNA Extraction

DNA was extracted from 150-mg stool samples using ISOLATE Fecal DNA Kit (Bioline, UK) according to the manufacturers' instructions. DNA extracts were stored at − 80 °C until PCR testing. Two μL of DNA extract was used in the PCR reaction.

SYBR Green Real-Time PCR

The real-time PCR protocol was performed as previously described by Tomova et al. (2015). Specific PCR primers were used to target selected phyla, genera, or species constituting the gut microbiota *(Bacteroides, Prevotella*, *Ruminococci, Bacteroidetes, Firmicutes, Bifidobacterium, Lactobacillus, Clostridium difficile, Desulfovibrio, Sutterella)*. In addition to a broad-range primer targeting conserved 16SrRNA sequence of total bacteria, the amplification of which served as the denominator against which the amplification of other bacteria was estimated. Primers (Metabion International AG, Germany) used in the study were previously described (Tomova et al. 2015; Bergstrom et al. 2012; Williams et al. 2012; Ramirez-Farias et al. 2009; Penders et al. 2005; Rinttila et al. 2004; Nadkarni et al. 2002) and are listed in Table 1.

Amplification was performed in real-time PCR cycler, the Rotor-Gene Q (QIAGEN, Germany) using a SensiFAST™ SYBR® No-ROX PCR kit (Bioline Co., UK). It was performed in 20 μL reaction volume containing 5 pmol/μL of each primer. The reaction consisted of initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. Melting curve analysis was performed to check the specificity of the amplified products. Quantitation of specific bacterial DNA was expressed as relative quantitation (the cycle threshold (Ct) at which DNA for a specific target was detected relative to the cycle threshold (Ct) at which universal bacterial DNA was detected). This relative quantification is calculated automatically by the Rotor-Gene software and expressed as relative fold difference.

Statistical Analysis of the Data

Data entry and analysis were carried out using the Statistical Package for Social Sciences version 20 (SPPS PASW Statistics, Chicago) (Kirkpatrick and Feeney 2013).

Quantitative variables were presented in the form of range, mean, median, and standard deviation. On the other hand, studied qualitative variables were presented as frequency and percentage from total. Comparisons between the different study groups were carried out using Chi-Square, Fisher's Exact, and Monte Carlo tests for qualitative variable and Mann-Whitney and Kruskal-Wallis tests for quantitative ones. For assessing correlations between different quantitative variables, Spearman's correlation coefficient was calculated. All results were interpreted at 5% level of significance where the difference between the study groups is considered significant if P is ≤ 0.05. To evaluate the degree of variation of the microbial community structure within a sample, we measured the alpha diversity by employing the Shannon diversity index (Shannon 1948), and to evaluate the degree of similarity

Table 1 Primers used in the present study

Primer Sequence (5′–3′)	Target	Amplicon size (bp)
TCCTACGGGAGGCAGCAGT GGACTACCAGGGTATCTATCCTGTT	Total bacteria	~466 bp
GGARCATGTGGTTTATTCGATGAT AGCTGACGACAACCATGCAG	*Bacteroidetes (*Phylum*)*	~126 bp
GGAGYATGTGGTTTAATTCGAAGCA AGCTGACGACAACCATGCAC	*Firmicutes (*Phylum*)*	~126 bp
CGATGGATAGGGGTTCTGAGAGGA GCTGGCACGGAGTTAGCCGA	*Bacteroides*	~238 bp
CACCAAGGCGACGATCA GGATAACGCCYGGACCT	*Prevotella*	~283 bp
GGCGGCYTRCTGGGCTTT CCAGGTGGATWACTTATTGTGTTAA	*Cluster IV Ruminococcus*	~157 bp
TCGCGTC(C/T)GGTGTGAAAG CCACATCCAGC(A/G)TCCAC	*Bifidobacterium spp.*	~243 bp
AGCAGTAGGGAATCTTCCA CACCGCTACACATGGAG	*Lactobacillus spp.*	~341 bp
TTGAGCGATTTACTT CGGTAAAGA TGTACTGGCTCACCTTTGATATTCA	*Clostridium difficile*	~500 bp
ATACCCTGGTAGTCCACGCT CCACATACTCCACCGCTTGT	*Desulfovibrio spp.*	~160 bp
CGCGAAAAACCTTACCTAGCC GACGTGTGAGGCCCTAGCC	*Sutterella spp.*	~260 bp

between ASD cases and siblings or controls, we employed the Bray-Curtis similarity index (Bray and Curtis 1957).

Results

In the present study, the 41 ASD patients had male to female ratio of 2.2:1. Their mean age was 5.55 ± 1.9 ranging from 2.5 to 12 years. According to CARS, 35 (85.4%) had mild to moderate ASD (CARS < 36, ranging from 30–< 36), while six (14.6%) patients had severe ASD (≥ 36, ranging from 36 to 45) with a mean score of 34.54 ± 5.15. Regarding the GI manifestations, 35 (85.4%) ASD patients had at least one symptom at the time of examination, while only six (14.6%) patients had no symptoms. The mean 6-GSI score was 3.37 ± 2.12. Abnormal stool smell was the most common GI symptom (30 cases; 73.2%), followed by flatulence (20; 48.8%), constipation (17; 41.5%), abdominal pain (12; 29.3%), abnormal stool consistency (10; 24.4%), and diarrhea (4; 9.8%). None of the siblings or healthy controls had GI symptoms at the time of examination; therefore, 6-GSI score was not assessed. There was no statistically significant correlation between CARS and 6-GSI ($r = -0.131$, $p = 0.415$) in ASD cases (Table 2).

Out of the 45 siblings, 22 (48.9%) were males and 23 (51.1%) were females, with mean age of 4.31 ± 3.23 (0.5–12 years). Out of 45 controls, 28 (62.2%) were males and 17 (37.8%) were females. Their mean age was 5.36 ± 2.6 (2–12 years) (Table 2).

Microbiota Analysis

Phylum Level Analysis

Bacterial phylum analysis showed no significant difference regarding the relative abundance of *Bacteroidetes* and *Firmicutes* in the three study groups ($p = 0.456$, $p = 0.233$, respectively) (Table 3). However, a clear difference was observed when comparison was made between the three study groups regarding *Firmicutes/Bacteroidetes* (F/B) ratio. F/B ratio was significantly lower in both ASD cases (2.05) and their siblings (1.54) when compared to healthy controls (4.61) ($p = 0.028$, $p = 0.002$, respectively), while no significant difference was found between ASD cases and their siblings regarding F/B ratio ($p = 0.389$) (Fig. 1).

Genus Level Analysis

Bacteroides' as well as *Ruminococcus*' relative abundance was significantly higher in ASD cases (1.35E-01, 2.55E-02, respectively) as well as in their siblings (1.38E-01, 3.27E-02, respectively) than in healthy controls (3.64E-02, 1.07E-02, respectively) (p value = < 0.001, $p = 0.003$, respectively). Pairwise comparison confirmed the statistical significant difference between both ASD cases and healthy controls (p = < 0.001, $p = 0.002$, respectively), as well as between siblings and healthy controls (p = < 0.001, $p = 0.004$) in the relative abundance of *Bacteroides* and *Ruminococcus*, respectively.

Comparing the relative abundance of *Prevotella* between the three study groups revealed non-statistically significant difference in their relative abundance means ($p = 0.095$);

Table 2 Demographic and clinical characteristics of the study subjects

Clinical data	ASD cases ($n = 41$)		Siblings ($n = 45$)		Healthy controls ($n = 45$)	
Sex						
Male	28	68.3	22	48.9	28	62.2
Female	13	31.7	23	51.1	17	37.8
Age						
Min.–max.	2.5–12		0.5–12		2–12	
Mean ± SD.	5.55 ± 1.9		4.31 ± 3.23		5.36 ± 2.61	
CARS						
Mild to moderate (< 36)	35	85.4				
Severe (≥ 36)	6	14.6	ND	ND	ND	ND
Score (min.–max.)	30–45					
Score (mean ± SD)	34.5 ± 5.15					
GI symptoms						
Present	35	85.4				
Malodorous stool	30	73.2	0	–	0	–
Flatulence	20	48.8	0	–	0	–
Constipation	17	41.5	0	–	0	–
Abdominal pain	12	29.3	0	–	0	–
Abnormal stool consistency	10	24.4	0	–	0	–
Diarrhea	4	9.8	0	–	0	–
6-GSI						
Score (min.–max.)	0–9		ND	ND	ND	ND
Score (mean ± SD)	3.37 ± 2.12					

CARS childhood autism rating scale, *6-GSI* modified six-item gastrointestinal Severity Index, *GI* gastrointestinal, *ND* not done

however, pairwise comparison showed a statistically significant difference in bacterial relative abundance means between the ASD cases (4.28E-03) and healthy controls (1.23E-02) ($p = 0.043$) (Table 3).

To further study the abundance of *Prevotella* in ASD patients, *Prevotella* to *Bacteroides* ratio (P/B ratio) was calculated and compared between the three study groups. P/B ratio is a measure of overall gut microbiota balance as it is considered a proxy for gut enterotypes. P/B ratio was significantly lower in both ASD cases (3.75E-02) and their siblings (7.20E-02) when compared to healthy controls (4.35E-01) ($p = < 0.001$, $p = 0.001$, respectively) (Fig. 2).

No statistically significant difference in relative abundance of *Bacteroides*, *Ruminococcus*, *Prevotella*, or P/B ratio was observed between ASD cases and their siblings ($p = 0.762$, $p = 0.959$, $p = 0.093$, $p = 0.167$, respectively) (Fig. 2).

Species Level Analysis

Two types of beneficial bacteria were investigated, *Bifidobacterium* spp. and *Lactobacillus* spp. Comparison between the three study groups revealed that *Bifidobacterium* spp. relative abundance was significantly higher in the siblings group (1.21E-01) than in both ASD cases (9.59E-02) and healthy controls (5.38E-02) ($p = 0.05$). Pairwise comparison confirmed a statistical significant difference only between the group of siblings and the healthy controls. ($p = 0.017$), while the relative abundance of *Lactobacillus* spp. was lower in ASD cases (3.17E-03) compared to siblings (3.43E-03) as well as to healthy controls (3.63E-03), but this difference was not statistically significant ($p = 0.754$) (Table 3).

On the other hand, three dysbiotic bacteria, *C. difficile, Desulfovibrio* spp., *and Sutterella* spp., were analyzed. *C. difficile* showed very low relative abundance (0.00E+00) among the three study groups with no statistical significant difference between the three groups ($p = 0.07$). Similarly, although the relative abundance of *Desulfovibrio* spp. was higher in ASD cases (2.81E-01) than in siblings (2.36E-01) and healthy controls (2.15E-01), this difference was statistically insignificant ($p = 0.801$). Also, *Sutterella* spp. relative abundance was statistically non-significantly higher in ASD children (1.41E-02) and their siblings (1.45E-02) in comparison to healthy controls (1.03E-02), ($p = 0.49$) (Table 3).

Table 3 Comparison of the bacterial relative abundances and ratios means in the study groups

Bacteria	ASD cases[A] ($n = 41$)	Siblings[B] ($n = 45$)	Healthy controls[C] ($n = 45$)	Test of significance	p value
Bacteroidetes	1.31E-01 (7.26E-02–2.64E-01)	1.52E-01 (6.41E-02–3.57E-01)	1.01E-01 (4.05E-02–2.63E-01)	$^{KW}\chi^2 = 1.572$	0.456
Firmicutes	4.95E-01 (9.56E-05–6.46E-01)	3.30E-01 (5.93E-05–6.03E-01)	4.11E-01 (2.56E-01–6.04E-01)	$^{KW}\chi^2 = 2.911$	0.233
F/B ratio	2.05E + 00 (8.88E-04–6.44E + 00)	1.54E + 00 (4.82E-04–4.39E + 00)	4.61E + 00 (1.09E + 00–1.02E + 01)	$^{KW}\chi^2 = 10.257^*$	*0.006**
Bacteroides	1.35E-01 (6.92E-02–3.84E-01)	1.38E-01 (5.46E-02–3.69E-01)	3.64E-02* (1.22E-02–1.58E-01)	$^{KW}\chi^2 = 17.492^*$	*< 0.001**
Prevotella	4.28E-03 (1.16E-03–2.39E-02)	9.44E-03 (2.61E-03–3.33E-02)	1.23E-02 (2.72E-03- 3.53E-02)	$^{KW}\chi^2 = 4.713$	0.095
P/B ratio	3.75E-02 (7.61E-03–1.90E-01)	7.20E-02 (1.29E-02–3.21E-01)	4.35E-01* (1.39E-03–1.06E-01)	$^{KW}\chi^2 = 22.517^*$	*< 0.001**
Cluster IV Ruminococcus	2.55E-02 (1.42E-02–7.98E-02)	3.27E-02 (8.28E-03–1.10E-01)	1.07E-02* (3.38E-03–3.07E-02)	$^{KW}\chi^2 = 11.795^*$	*0.003**
Bifidobacterium spp.	9.59E-02 (2.59E-02–2.62E-01)	1.21E-01# (4.89E-02–2.60E-01)	5.38E-02 (1.62E-02–1.99E-01)	$^{KW}\chi^2 = 5.926^*$	*0.05**
Lactobacillus spp.	3.17E-03 (3.30E-04–3.17E-02)	3.43E-03 (1.27E-03–4.64E-02)	3.63E-03 (7.51E-04–4.43E-02)	$^{KW}\chi^2 = 0.565$	0.754
C. difficile	0.00E+00 (0.00E+00–2.92E-06)	0.00E+00 (0.00E+00–2.89E-06)	0.00E + 00 (0.00E + 00–0.00E + 00)	$^{KW}\chi^2 = 5.307$	0.07
Desulfovibrio spp.	2.81E-01 (1.48E-01–4.46E-01)	2.36E-01 (1.73E-01–4.32E-01)	2.15E-01 (1.26E-01–4.98E-01)	$^{KW}\chi^2 = 0.444$	0.801
Sutterella spp.	1.41E-02 (3.14E-03–3.28E-02)	1.45E-02 (3.65E-03–4.76E-02)	1.03E-02 (2.70E-03–2.85E-02)	$^{KW}\chi^2 = 1.427$	0.49

Median (interquartile range) of relative abundance of the various bacteria are shown

*Statistically significant at $p \leq 0.05$

$^{KW}\chi^2$ Calculated value for Kruskal Wallis test

Bacteriodes: A vs B 0.762, B vs C = <0.001*, A vs C = <0.001* (pairwise comparison; Calculated by Mann-Whitney)

Prevotella: A vs B 0.093, B vs C = 0.64, A vs C = 0.043* (Calculated by Mann-Whitney)

Ruminococcus: A vs B 0.959, B vs C = 0.004*, A vs C = 0.002* (Calculated by Mann-Whitney)

P/B ratio: A vs B = 0.167, B vs C = 0.001*, A vs C < 0.001* (Calculated by Mann-Whitney)

F/B ratio: A vs B = 0.389, B vs C = 0.002*, A vs C = 0.028* (Calculated by Mann-Whitney)

Bifidobacterium: A vs B = 0.069, B vs C = 0.017*, A vs C = 0.46 (Calculated by Mann-Whitney)

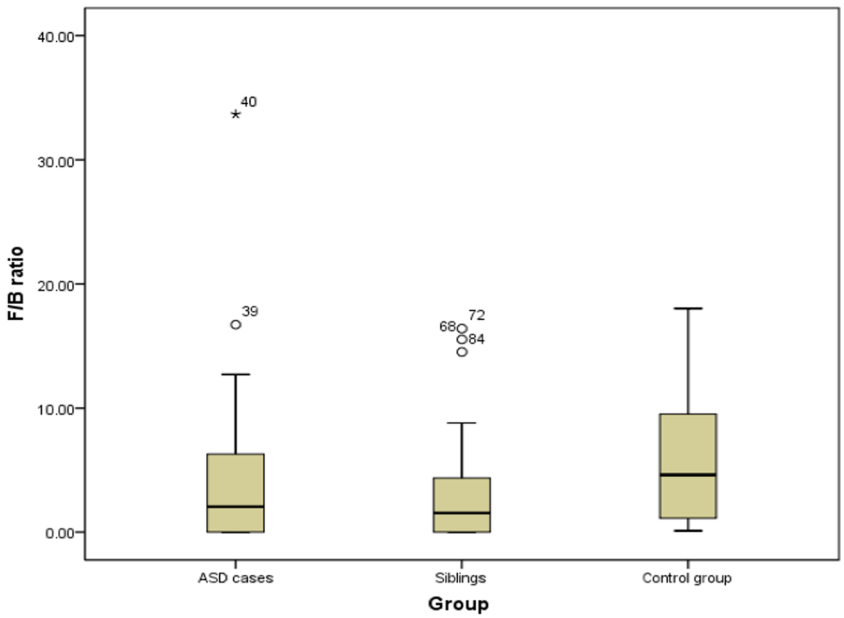

Fig. 1 The relative abundance of F/B ratio in the studied groups, the thick line in the middle of the box represents the median, the box represents the interquartile range (from 25th to 75th percentiles), and the whiskers represents the minimum and maximum

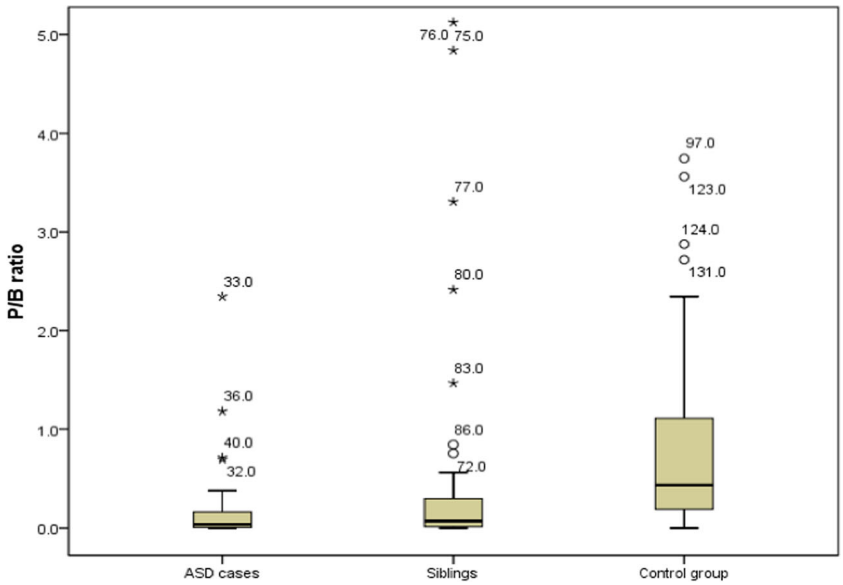

Fig. 2 The relative abundance of P/B ratio in the studied groups, the thick line in the middle of the box represents the median, the box represents the interquartile range (from 25th to 75th percentiles), and the whiskers represents the minimum and maximum

Correlation Between the Gut Microbiota and Autism Severity

Comparison between children with mild-moderate ASD (35 cases) and those with severe ASD (six cases) regarding their age, gender, CARS score, GI symptoms, and GSI score revealed no statistically significant difference between the two ASD severity groups except for weight, which was significantly lower in severe cases ($p = 0.013$) (Data not shown).

When comparing the two severity groups regarding the bacterial relative abundance, children with severe ASD had significantly higher relative abundance of *Firmicutes* (6.43E-01) than mild-moderate ASD cases (4.58E-01), ($p = 0.027$). However, for the other bacteria, the differences were not statistically significant ($p = > 0.05$). Similarly, none of the F/B or P/B ratios showed a statistically significant difference between the two severity groups (Table 4).

Correlation between the relative abundance of each bacterium and the severity of autism (CARS) in ASD cases was analyzed. Although relative abundances of *Bacteroidetes*, *Firmicutes*, *Prevotella*, *Ruminococcus*, *Bifidobacterium* spp., *Lactobacillus* spp., and *C. difficile* were positively correlated with CARS, while *Bacteroides*, *Desulfovibrio* spp., and *Sutterella* spp. were negatively correlated with CARS, none of these correlations was statistically significant ($p = > 0.05$). It was also observed that F/B ratio had statistically insignificant weak negative correlation with CARS ($p = 0.972$), while P/B ratio had a weak nonsignificant positive correlation ($p = 0.534$) (Table 5).

Correlation Between the Gut Microbiota and GI Severity Index (6-GSI)

Comparison of the relative abundance of each of the studied bacterial means in the ASD cases in relation to the 6-GSI score using Kruskal-Wallis test revealed no statistical significant difference between the ASD patients with high, low, or zero 6-GSI score, $p = > 0.05$ (Data not shown).

When investigating the correlation of the selected bacteria and 6-GSI using Spearman's correlation, most of the studied bacteria showed weak negative correlations with 6-GSI, but none of these correlations was statistically significant ($p = > 0.05$). Both F/B and P/B ratios were negatively correlated with GSI but still not statistically significant ($p = 0.213$ and $p = 0.063$, respectively) (Table 5).

Alpha Diversity

Shannon diversity index demonstrated a similar degree of microbial diversity in the three studied groups (mean diversity index of ASD cases = 1.46, of siblings = 1.55, and of healthy controls = 1.44). No statistical significant difference was observed between the three studied groups using ANOVA test (Table 6). Moreover, when studying Pearson correlation between the diversity index of ASD cases and their age, CARS, and 6-GSI scores, no statistical significant correlation was detected with their age ($r = 0.064$, $p = 0.690$), CARS ($r = 0.120$, $p = 0.456$), or 6-GSI ($r = 0.117$, $p = 0.467$).

Similarity Index

Bray-Curtis similarity index was performed to study the similarity index between both the ASD cases and their siblings and between ASD cases and healthy controls. The mean similarity index between cases and their siblings was 0.59, while similarity index between cases and healthy controls was 0.55. Comparing similarity index between cases/siblings and cases/healthy controls demonstrated no significant statistical difference ($t = 0.856$, $p = 0.395$) (Table 7).

Table 4 Comparison of bacterial relative abundance in mild-moderate vs severe ASD

Bacteria	Mild-moderate ($n = 35$)	Severe ($n = 6$)	Test of significance	p value
Bacteroidetes	1.30E-01 (6.79E-02–2.56E-01)	1.82E-01 (1.29E-01–8.03E-01)	Z = − 1.531	0.126
Firmicutes	4.58E-01 (5.78E-05–6.02E-01)	6.43E-01 (5.82E-01–8.06E-01)	Z = − 2.217*	*0.027**
F/B ratio	2.02E+00 (5.86E-04–6.65E+00)	3.36E+00 (9.10E-01–5.35E+00)	Z = − 0.406	0.684
Bacteroides	1.46E-01 (5.49E-02–3.82E-01)	1.24E-01 (7.44E-02–3.41E-01)	Z = 0	1.0
Prevotella	4.28E-03 (1.01E-03–2.32E-02)	3.90E-03 (1.02E-03–2.17E-01)	Z = − 0.166	0.868
P/B ratio	3.75E-02 (6.69E-03–1.64E-01)	4.61E-02 (7.54E-03–8.70E-01)	Z = − 0.369	0.712
Cluster IV Ruminococcus	2.54E-02 (1.05E-02–8.44E-02)	2.85E-02 (2.28E-02–3.86E-02)	Z = − 0.055	0.956
Bifidobacterium spp.	9.70E-02 (2.66E-02–2.96E-01)	4.81E-02 (7.85E-03–2.57E-01)	Z = − 0.922	0.356
Lactobacillus spp.	6.72E-03 (2.81E-04–3.45E-02)	2.06E-03 (5.84E-04–7.41E-02)	Z = − 0.037	0.971
C. difficile	0.00E+00 (0.00E+00–1.09E-06)	0.00E+00 (0.00E+00–6.44E-05)	Z = − 0.642	0.521
Desulfovibrio spp.	2.81E-01 (1.38E-01–4.30E-01)	2.74E-01 (1.48E-01–4.85E-01)	Z = − 0.037	0.971
Sutterella spp.	1.41E-02 (3.87E-03–3.45E-02)	1.14E-02 (2.71E-03–8.05E-02)	Z = − 0.148	0.883

Median (interquartile range) of relative abundance of the various bacteria are shown

Z: Z-score of Mann-Whitney U test

*Statistically significant at $p \leq 0.05$

Discussion

According to the results of the current study, it was observed that GIT symptoms were reported in 85.4% of ASD patients. The actual incidence of gastrointestinal disorders among ASD patients is under debate. It has been estimated to range between and 90%, depending on the study (Buie et al. 2010). GIT complications in ASD children may contribute to the severity of the disorder. However, according to the findings of the current study, there was no statistically significant correlation between CARS and 6-GSI among ASD children. This finding is consistent with the results of Kang et al. (2013) and Mazefsky et al. (2014) who did not find a significant correlation between the 6-GSI score and any of the measures of autism severity.

In the present study, phylum level analysis showed no significant difference regarding the level of *Bacteroidetes* and *Firmicutes* in the three study groups. However, some previous studies reported increased *Bacteroidetes* and decreased *Firmicutes* in ASD cases compared to the normal control (De Angelis et al. 2015; Finegold et al. 2010). It should be mentioned that the majority of species in the *Bacteroidetes*

Table 5 Correlation between the bacterial relative abundances and CARS and 6-GSI in ASD cases

Bacteria	CARS		6-GSI	
	r	p	r	p
Bacteroidetes	0.071	0.658	−0.032	0.841
Firmicutes	0.155	0.333	−0.236	0.137
F/B ratio	−0.006	0.972	−0.199	0.213
Bacteroides	−0.051	0.749	0.090	0.576
Prevotella	0.043	0.787	−0.280	0.076
P/B ratio	0.10	0.534	−0.293	0.063
Ruminococcus	0.034	0.834	−0.008	0.959
Bifidobacterium spp.	0.212	0.183	−0.124	0.440
Lactobacillus spp.	0.026	0.870	−0.054	0.735
Desulfovibrio spp.	−0.016	0.923	−0.008	0.962
C difficile	0.169	0.290	0.093	0.564
Sutterella spp.	−0.101	0.532	0.062	0.701

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

r: Spearman's correlation coefficient, p: p value

Table 6 Alpha diversity estimates of the three study groups

Alpha diversity	ASD Cases	Siblings	Healthy controls
Range	0.34–2.01	0.83–1.99	1.03–1.84
Mean	1.458	1.551	1.442
S.D.	0.301	0.231	0.227
F	2.176		
p	0.118		

S.D.:Standard deviation, *F* calculated value for ANOVA test, *p p* value

Table 7 Comparison of Bray-Curtis similarity index between ASD cases/their siblings and ASD cases/healthy controls

Bray-Curtis	ASD Cases/siblings	ASD cases/healthy controls
Mean S.D.	0.59 0.2	0.55 0.2
tp	0.856 0.395	

t calculated value for Student's *t* test, *p p* value

produce propionic acid and other short-chain fatty acids as end products of their metabolism, and a previous study has shown that when propionic acid is injected into cerebral ventricles of rats, the rats show biologic and pathologic characteristics of autism (MacFabe et al. 2007). The relative abundance of *Bacteroidetes* reported in the current study may appear to be incongruent with previously reported results, since the current study was based on fecal samples rather than using biopsies as in the previous studies. Several studies have reported differences in the composition of fecal versus mucosal microflora (Gillevet et al. 2010; Marteau et al. 2001). The loss of *Bacteroidetes* from the gut epithelium due to cell death or injury can result in increased passage of *Bacteroidetes* into the feces. Thus, a higher level of *Bacteroidetes* in feces is not an indication that *Bacteroidetes* are found at higher levels in the microbiome (Marteau et al. 2001).

In the present study, F/B ratio was significantly lower in both ASD group and their siblings when compared to healthy controls. Finegold et al. (2010) reported similar results, whereas Williams et al. (2011) observed an opposite trend in ileum and cecum biopsy samples from neurotypical children than those from autistic children. However, no significant difference in F/B ratio was observed in the study by Kang et al. (2013) On the other hand, an increased F/B ratio has been reported in subjects with autism in the study of Tomova et al. (2015)

Despite some *Bacteroides* species have some beneficial effects including supplying energy to the host by facilitating fermentation, promoting T cell-dependent immune responses, and limiting the colonization of pathogens in the GI tract, other species are pathogenic to humans (Wexler 2007). By comparing the relative abundance of *Bacteroides* in the three study groups, it was observed that ASD cases and their siblings had significantly higher *Bacteroides* than the healthy controls, and this agreed with previous studies which reported that higher numbers of the *Bacteroides* were found in ASD children, and this might be responsible for GI pathology in children with autism (Finegold et al. 2010). A previous pyrosequencing analysis of the mucoepithelial bacteria revealed significant dysbiosis with increased *Ruminococcaceae* in ASD cases (Momozawa et al. 2011). The same was reported in the current study as ASD cases and their siblings had significantly higher *Ruminococcus* compared to the healthy controls. In the present study, although the relative abundance of *Prevotella* was decreased in ASD cases, this was not statistically significant. Similar to our results, Kang et al. (2013) reported that *Prevotella* was the most significantly altered genus between autistic and neurotypical subjects and decreases dramatically in abundance in stool samples from autistic patients. It should be noted that *Prevotella* plays a key role in digesting carbohydrate-rich food. It also has essential genes for biosynthesis of vitamin B1, which was reported to lessen ASD symptoms.

Further, the ratio gradient between *Prevotella* and *Bacteroides* was reported to be a good measure for characterizing gut microbiota (Kang et al. 2013). According to the results of the current study, P/B ratio was significantly lower in both ASD cases and their siblings when compared to healthy controls; interestingly, the siblings displayed an intermediate P/B ratio. This finding agrees with the results reported by Kang et al. (2013)

From our previous findings, F/B and P/B ratios could be used as potential biomarkers of gut dysbiosis in ASD children especially in cases showing no significant differences regarding the abundance of the different gut microbiota.

The importance of the microbial community balance in maintaining healthy gut flora has been recognized. The relationship between autism and gut microbiota has been previously studied with either focusing on harmful bacteria or to already-known beneficial bacteria (Finegold et al. 2010). The relative abundance of *Bifidobacterium* spp. as well as of *Lactobacillus* spp. did not differ significantly among the three study groups in the present study, although some studies reported that the composition of *Lactobacillus* spp. and *Bifidobacterium* spp. differed between ASD patients and healthy children (Adams et al. 2011; Wang et al. 2011; Finegold et al. 2010). Nevertheless, other studies only suggested that alterations might be found in a minority of ASD cases (Gondalia et al. 2010). Although the relative abundance of *Bifidobacterium* spp. did not differ significantly between ASD cases and healthy controls, it was significantly higher in the siblings group, which appears to have a protective role for those siblings. A recent systematic review and meta-analysis analyzed nine studies, including 254 patients with ASD. This meta-analysis suggested an association between ASD and alteration of microbiota composition in children (Mingyu et al. 2019).

On the other hand, previous studies comparing autistic and control groups have suggested that some bacteria (*Clostridium, Sutterella, Desulfovibrio* spp.) are more related to autistic symptoms, as well as to disease severity (Wang et al. 2013; Gondalia et al. 2012). In the present study, no statistically significant difference in *C. difficile*, *Sutterella*, and *Desulfovibrio* spp. level was found between ASD patients and control groups. Thus, our study suggests that none of the potentially pathogenic species tested could be responsible for ASD symptoms in Egyptian children.

In the current work, ASD participants were divided into two mild-moderate ($n = 35$) and severe ASD ($n = 6$) severity groups. Comparing age, sex, weight, GI symptoms, and 6-GSI score showed no statistical significant differences between the

two groups except for weight, which was significantly lower in severe cases. Comparison of the two groups regarding the bacterial relative abundances revealed no statistical significant differences in all studied bacteria except for *Firmicutes,* which was higher in severe cases. This is in agreement with a previous study, which reported no significant difference in bacterial relative abundances between autism severity groups (Gondalia et al. 2012) Absence of statistical significant difference between the two groups may be due to small sample size as the severe group represented only six ASD cases.

Since most ASD cases in this study had GI problems, it was speculated that microbial differences might be associated with the presence of GI symptoms. However, the 6-GSI score was not correlated with the changes in microbiome profiles among autistic children. Similarly, Son et al. (2015) reported no significant differences regarding the microbiome composition within the ASD group when comparing those with and without GI dysfunction.

Shannon index demonstrated that our three study groups exhibited a similar degree of alpha diversity. An expected finding, as a limited number of bacterial genera and/or species, was targeted in this study. It has been previously reported that gut microbial diversity is a new biomarker of health and metabolic capacity, as high microbial gut diversity has the ability to protect the human gut from environmental stresses (Stecher et al. 2007). Finegold et al. (2010) observed a higher microbial diversity in autistic children, while Kang et al. (2013) found that autistic children tend to have a less diverse gut microbiome.

Several studies reported that the microbiota of siblings was more similar to ASD children than to the healthy controls (Wang et al. 2011; Finegold et al. 2010), and this is consistent with results of the present study. This could be explained by similar diet, living conditions, and other environmental factors having role in shaping the gut microbiome. There is also a possibility of transmission of bacteria from autistic children to siblings (Finegold et al. 2010).

Conclusions

The current study showed an evidence of changes in the gut microbiome of ASD children compared to the unrelated controls. However, the microbiome profile of siblings was more like that of autistic children than that of unrelated controls. These observations may highlight the importance of the interplay between environmental and host genetic factors in shaping the gut microbiome. This study also emphasizes the importance of identification of microbiome and specific microorganisms' changes that can be targeted for diagnosis as well as for treatment of ASD. The findings of the present study could potentially guide implementation of dietary interventional plans that impact the gut microbiota and improve ASD symptoms.

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Author Contributions All authors contributed to the study conception and design. All authors performed material preparation, data collection, and analysis. All authors wrote the draft of the manuscript, read, and approved the final manuscript.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval All procedures performed in the study involving human participants were in accordance with the ethical standards of the institutional research committee (Medical Research Ethics Committee of Alexandria Faculty of Medicine, Egypt) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Statement of Informed Consent An informed written consent was obtained from each individual's parent or guardian prior to inclusion in the study.

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