Effect of Following an "Exclusion Diet" to Modulate the Gut Microbiota of Children with Autism Spectrum Disorder

Original Article

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ABSTRACT

Background: Emerging evidence suggests a link between gut microbiota dysbiosis and the pathogenesis of autism spectrum disorder (ASD). The consumption of food additives, processed foods, trans fats, and high-sugar foods has been linked to gut dysbiosis, potentially affecting the gut-brain axis.

Aim: This study aimed to investigate whether the dietary exclusion of these foods would modulate the dysbiotic gut microbiome of children with ASD and ameliorate ASD symptoms.

Methods: Twenty Egyptian children with ASD underwent a 3-month dietary intervention that eliminated processed foods, additives, and preservatives. Stool samples were collected pre- and post-intervention for gut microbiome analysis using real-time PCR to quantify the dominant gut microbiome at the phylum, genus, and species levels. Clinical symptoms, including ASD severity, sensory impairment, and gastrointestinal symptoms, were assessed using the Autism Treatment Evaluation Checklist (ATEC), the Short Sensory Profile (SSP), and the 6-item Gastrointestinal Severity Index (6-GSI), respectively.

Results: Following 3 months of adopting the exclusion diet, children with ASD significantly improved ATEC, the total SSP, and 6-GSI scores. The sociability and health/physical/behavior subscales of the ATEC showed a significant decline in scores. Notably, the number of cases with definite sensory impairment decreased from 14 to 7 cases, and the number of ASD children with severe GSI scores decreased from 10 to 3 cases. Although the Prevotella/Bacteroides ratio increased threefold for the gut microbiome profile, the difference was insignificant. Overall, there were no significant differences in the gut microbiome profile after three months of following the exclusion diet.

Key Words: Autism spectrum disorder, dysbiosis, exclusion diet, gut microbiome, real-time PCR.

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INTRODUCTION

Autism spectrum disorder (ASD) is a heterogeneous group of neurodevelopmental disorders defined by three core impairments: deficits in communication, disruptions in reciprocal social interaction, and the presence of restricted, repetitive, and stereotyped patterns of behavior or interests[1]. ASD pathogenesis and etiology have been the subject of several hypotheses up to this point, but it is believed to stem from a combination of genetic and environmental factors[2].

The gut microbiota refers to the diverse community of microorganisms inhabiting the human gastrointestinal tract, while the gut microbiome encompasses the collective genome of these microbes[3]. It is hypothesized that the gut microbiota represent a key element of human physiology, influencing brain development and behavior via the neuroendocrine, neuroimmune, and autonomic nervous systems[4]. Alteration in the gut microbiota in individuals with ASD could contribute to immune dysregulation such as microglial activation and T regulatory cell deficits[5].

Recently, evidence supporting microbial dysbiosis in ASD has increased[6]. Gastrointestinal disorders are common comorbidities of ASD that may exacerbate ASD symptomatology[7].

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Modulating the dysbiotic gut microbiota through diet, probiotic, prebiotic, antibiotic, antifungal supplementations, and fecal microbiota transplantation have been discussed in several studies however, evidence on these interventions is limited[8-11].

Since the mid-twentieth century, a notable change in human diets has been the increased consumption of food additives, including artificial sweeteners, preservatives, food colorants, emulsifiers, stabilizers, and thickeners. Recent studies have demonstrated that the excessive consumption of various kinds of food additives, high-refined sugar foods, and trans-fatty acids plays a role in altering the gut microbiota and may have adverse effects on the health of ASD cases [12, 13]. However, not enough supporting evidence is available to support the exclusion of food additives as a validated therapeutic intervention for individuals with ASD [12].

Exclusion diets, particularly gluten-free and casein-free diets, have been explored as potential interventions for alleviating clinical symptoms in individuals with autism spectrum disorder (ASD). The proposed mechanisms by which these diets may affect improvement include modulation of the gut-brain axis, reduction of inflammation, and correction of metabolic disruptions[13-16]. The implementation of certain dietary interventions can pose challenges for children and their families, including concerns about flexibility, resource availability, children's food preferences, and adherence to the regimen[17]. That is why an exclusion diet which attempts to limit processed food, foods containing additives, and preservative may improve compliance and give rise to better outcomes, especially in resource-limited settings [18]. Therefore, the aim of this study was to determine if a simple exclusion diet may improve and modulate the dysbiotic gut microbiota of ASD cases, and whether this modulation, if it happens, would be associated with clinical improvement in ASD symptoms.

SUBJECTS AND METHODS

In this interventional study, a total of 20 children with ASD who presented at the Autism Clinic of Alexandria University Children's Hospital in Alexandria, Egypt were consecutively enrolled from March 2023 through December 2023. Thirty-five cross-matching unrelated typically developing (TD) children of cross matching age and sex were also included. The age of autistic and control children ranged between 3 to 12 years.

All autistic children were diagnosed as fulfilling the criteria for the Diagnostic and Statistical Manual of Mental Disorders Fifth Edition (DSM-5) by a pediatric neuropsychiatrist [19]. Additional assessments were done using the Childhood Autism Rating Scale -2 (CARS-2), Gastrointestinal Severity Index (GSI), Short Sensory Profile (SSP), and Autism Treatment Evaluation Checklist (ATEC) [20]. Children with ASD having known syndromes, immune deficiencies, or hepatic impairment were excluded from the study.

CARS-2 is a well-established, validated 15-item behavioural rating scale designed for diagnosing and quantitatively assessing the severity of ASD [21, 22].

The ATEC is a form that should be filled out by parents, teachers, or anybody else who regularly observes the child's behavior. It was developed by the Autism Research Institute and has been effectively used in multiple studies on ASD to assess treatment outcomes and track progress over time [23]. The ATEC consists of four subscales: Scale I, speech/language/communication (14 items; scores range from 0 to 28); Scale II, sociability (20 items; scores range from 0 to 40); scale III, sensory/cognitive awareness (18 items; scores range from 0 to 36); and scale IV, health/physical behavior (25 items; scores range from 0 to 75). A total score, ranging from 0 to 180, is calculated based on the results of these subscales. The weight assigned to each score is determined by the response and related subscale. Higher subscale and overall scores signify greater impairment in the child, and vice versa[21].

The reduction rate of ATEC score before (S1) and after (S2) the exclusion diet was used as the efficacy index (N), $N = (S1 - S2)/S1 \times 100\%$. Markedly effective: $NM \geq 50\%$, Effective: NE: 20%–50%, Ineffective: $NI < 20\%$. Total effective rate (NT) = (NM + NE) / total cases × 100% [24].

Sensory impairment was evaluated using the SSP, which includes 38 questions across 7 subsets of sensory processing functions (taste, tactile, smell, movement, visual, auditory sensitivity, and under-responsiveness/sensation seeking). Responses on the form range from 1 ("always") to 5 ("never"), based on the child's behaviors, with a maximum possible score of 190 [25, 26].

A modified short form of the GI Severity Index (6-GSI questionnaire) was used to measure gastrointestinal (GI) symptoms [27]. It consisted of six items: constipation, diarrhea, stool consistency, stool smell, flatulence, and abdominal pain. Each item was scored as 0, 1, or 2 based on its weekly frequency; a score of 0 indicated the absence of the symptom, while scores of 1 and 2 indicated the presence of the symptom with varying severity. A total score of three or less was classified as a low score, while a score greater than three was classified as a high score.

Approval of the Ethics Committee of the Faculty of Medicine, Alexandria University, was obtained, IRB number 00012098. After explaining the purpose and benefits of the study, written consent was obtained from the parents or guardians of all participants.

Dietary Intake Assessment:

To monitor short-term nutrient intake, parents were asked to complete a dietary food record for their child

during the 7 days preceding stool sample collection. The 7-day dietary recall record was utilized to assess each child's dietary patterns and served as a baseline for subsequent dietary interventions.

Sample collection, Preservation and Transport

Two stool samples were collected from each ASD case, one of them at the beginning of the study and the other one after 3 months of implementing an exclusion diet plan. Regarding the control group, only one stool sample was taken. Samples were stored at -20°C immediately after defecation at home and then transported frozen to the Microbiology laboratory at the Faculty of Medicine, Alexandria University, where they were stored at -80°C for further processing.

Gut Microbiome Analysis DNA Extraction

DNA was extracted from 180 mg stool samples using the QIAamp DNA Stool Extraction Mini Kit (Qiagen, Germany). The resulting DNA extracts were stored at

-80°C until subjected to PCR analysis.

SYBR Green Real-Time PCR

Primers:

Oligonucleotide primers targeting the 16S rDNA gene sequences of Bacteroides, Prevotella, Ruminococcus, Firmicutes, Bacteroidetes, Lactobacilli, Bifidobacteria, Akkermansia muciniphilia, Faecalibacterium prausnitzii, Bacteroides fragilis, Clostridium difficile, Desulfovibrio and Sutterella were used. Primers were also used to amplify a conserved 16S rDNA sequence present in all bacteria, universal primer, the amplification of which served as the denominator against which the amplification of the other bacteria was compared. All the primers [Invitrogen, USA] were described from previously published studies [28-31].

Detection and Quantitation

Amplification was carried out using a real-time PCR cycler (Rotor Gene Q, Qiagen, Germany) with the SensiFAST™ SYBR No-ROX PCR kit (Bioline Co., UK). Briefly, 4 pmol of forward and reverse primers were used in 20 µl reactions containing 2 µl of the DNA extract. The PCR amplification protocol included an initial denaturation step 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 conducted to assess the specificity of the amplified products. Relative quantification was automatically calculated by the Rotor-Gene software and expressed as a relative fold difference.

Intervention Protocol:

Each child's caregiver was instructed to exclude from their kids diet processed foods, additives, and preservatives. Also, junk food, soda drinks, canned juices and replace it with homemade fresh juices with fibers.

Compliance of the studied participants:

Each child's caregiver received a printed copy of the compliance sheet for the full duration of intervention in days including the weekends [90 days]. Also in this sheet, the researcher put a column for the parent to write down anything noticed on the child while being compliant to the recommended exclusion diet. The researcher followed the child's caregiver throughout the full duration of intervention to assess the compliance and document any complaints or complications. The compliance sheets were collected at the end of each child intervention for statistical analysis and then we calculated the percentage of days that the child followed the exclusion diet compared to the days that they did not follow the diet in relation to the whole length of the study.

Statistical Analysis of the Data

Data were input into the computer and analyzed using the IBM SPSS software package, version 20.0 (Armonk, NY: IBM Corp). Qualitative data were presented as frequencies and percentages. The Shapiro-Wilk test was employed to assess the normality of the distribution. Quantitative data were described using the range (minimum and maximum), mean, standard deviation, median, and interquartile range (IQR). The significance of the results was determined at the 5% level.

The tests were used: Student t-test (For normally distributed quantitative variables, to compare between two studied groups), F-test (ANOVA) (For normally distributed quantitative variables, to compare between more than two groups), Paired t-test (For normally distributed quantitative variables, to compare between two periods), Mann Whitney test (For abnormally distributed quantitative variables, to compare between two studied groups), Wilcoxon signed ranks test (For abnormally distributed quantitative variables, to compare between two groups) and Kruskal Wallis test (For abnormally distributed quantitative variables, to compare between more than two studied groups).

Shannon diversity index was used to calculate the diversity index and the dysbiosis or dissimilarity index between cases and control by applying the Bray-Curtis similarity index equation [32, 33].

RESULTS

Demographic and clinical data of the studied participants:

The present study was carried out on 20 ASD children and 35 typically developing (TD) control children with matched age and sex. Among the 20 children with ASD, 11 (55%) were male and 9 (45%) were female, resulting in a male-to-female ratio of 1.2:1. Their mean age was 8.30 ± 2.49 with a range of 4 to 12 years. Regarding their residency, 13 (65%) were recruited from urban areas while only 7 (35%) were from rural areas.

Based on the Childhood Autism Rating Scale (CARS), 15 (75%) of the 20 children with ASD had mild to moderate ASD, while 5 (25%) had severe ASD, with a mean score of 33 ± 3.02. The mean ATEC score was 79.63 ± 24.88 with a range of 41 to 118 (Table 1a). There was a statistically significant positive correlation between CARS score and total ATEC score (r=0.573, p=0.008) and speech/language/ communication (r=0.637, p=0.003), sociability (r=0.640, p=0.002) and health/physical/behavior subscales (r= 0.515, p=0.02).

Out of the 20 children with ASD, 14 (70%) showed definite sensory impairment, 5 (25%) showed probable impairment, and 1 (5%) demonstrated typical performance based on the SSP total score. The mean SSP score was 129.9 ± 17.21. The most common definite impairment was in responsiveness and seeking sensation (90%), followed by auditory filtering (60%), with the least common being in movement (15%) (Table 2).

Concerning gastrointestinal symptoms, 17 (85%) of the 20 children with ASD had at least one symptom. The mean 6-GSI score was 3.60 ± 2.62; 10 (50%) had a severe score (>3), while 7 (35%) had a moderate score (≤3), and 3(15%) had no symptoms (0) (Table 3). Flatulence was the most common GI symptom (60%), followed by abnormal stool smell (50%), constipation (45%), abdominal pain (31%), abnormal stool consistency (35%) and diarrhea (10%) (Table 3).

Clinical data of the studied participants after 3 months exclusion diet:

The mean of compliance with the exclusion diet was 65.8 ± 27.2%. Out of the 20 ASD children, 17 (85%) were ≥50% compliant with the diet, and 3 children showed <50% compliance. The mean of compliance of ASD children with mild to moderate and severe autism was 62% and 76% respectively. All severe and 12 (80%) of mild to moderate ASD cases showed ≥50% compliance. Also, the mean of compliance of ASD children with definite and probable sensory impairment and typical performance was 68%, 60% and 62% respectively.

The total ATEC score was significantly decreased in ASD children after the exclusion diet (79.6 vs 52.9, p<0.001). This significant decrease was in speech/ language/ communication, sociability, and health/physical/ behaviour subscales (Table 1a).

Concerning the percentage of cases showed effective reduction rate in the total ATEC score in ASD children, it was markedly effective reduction (≥50%) in 6 cases (30%), effective (20%–50%) in 8 (40%) and ineffective (<20%) in 6 cases (30%). The exclusion diet plan was associated with a marked decrease in the score of sociability and health/ physical/behaviour subscales in 12 (60%) and 13 (65%) of ASD children, respectively (Table 1b). However, there was no statistically significant correlation between the percent of compliance with that of the reduction rate in ATEC Scale and subscales in ASD group (Table 1c).

Regarding Regarding SSP score, there was a statistically significant increase in the Total SSP Score after the exclusion diet (130 vs 146, p <0.001), i.e. decrease of the sensory impairment in ASD cases. Also, after diet only 7 cases showed definite

sensory impairment versus 14 cases before diet (p=0.012). There was a significant increase in movement sensitivity (p *value*: 0.002), low energy (p=0.007), under responsive/ seek sensation (p=0.023), and taste/smell sensitivity (p=0.037) sub scores (Table 2).

Concerning the GI symptoms, the GSI was significantly decreased after 3 months diet plan (mean GSI: 3.6 vs 1.6, p= 0.001). There was a statistically significant decrease in flatulence (p=0.002), stool smell (p= 0.012), and abdominal pain (p =0.035) sub scores (Table 3). After 3 months following the exclusion diet the number of ASD children with severe GSI score (≥4) decreased from 10 (50%) to 3 (15%).

Gut Microbiome Analysis Results:

Table (4) shows the relative abundance of studied gut microbiome and ratios in ASD children before and 3 months after following the exclusion diet and the typical developing (TD) control group.

As shown in Table (4), dysbiotic gut profile was evidenced by the significantly higher levels of Bacteroidetes (p <0.001), Bacteroides (p <0.001) and Sutterella (p = 0.012) as well as the lower abundance of Desulfovibrio (p=0.037) and lower Firmicutes/Bacteroidetes ratio (F/B)

(p<0.001) and Prevotella/Bacteroides (P/B) (p= 0.005) ratios of ASD children compared to TD children.

After diet modification, none of the studied bacteria or the ratios or indices were statistically different from

their values for the ASD children before diet modification. Although the P/B increased three folds (0.11 vs 0.31), yet the difference was not statistically significant (p= 0.709).

When comparing microbiome of ASD children after their diet modification to that of TD children, it was noted that the was no change in Bacteroidetes, Bacteroides as well as F/B and P/B ratios. However, the relative abundance of Ruminococcus became significantly higher and B. fragilis became significantly lower in ASD group after diet modification compared to TD group (p= 0.011 and 0.001, respectively). It was also noted that, after diet modification, the relative abundance of Desulfovibrio have increased (p=0.037) while Sutterulla has decreased (p=0.012). The dissimilarity index and the diversity index were neither significantly affected by diet modification (Table 4).

In the present study, there was no statistically significant difference in the gut microbiome of ASD children with mild to moderate and severe ASD (Supplementary table S1). However, in severe ASD cases specifically, the relative abundance of B. fragilis showed significant increase after diet modification (5.20E-4 vs 3.83E-2, p= 0.043) (Supplementary table S2).

While comparing the relative abundance of each studied bacteria with the sensory performance subgroups before the implementation of exclusion diet, no statistically significant difference was found in the gut microbiome between children with ASD who had definite or probable sensory impairment and those with typical sensory performance. After the implementation of the exclusion diet, the relative abundance of B. fragilis was significantly increased after diet in ASD cases with probable and typical performance (2.60E-3 vs 1.52E-2, p=0.046). In ASD children with definite sensory impairment the P/B increased 5 times after diet (0.05 vs 0.27); however, the difference did not reach statistical significance (p =0.363) (Supplementary table S2).

Concerning the mean 6-GSI score before the implementation of the exclusion diet, No statistically significant difference was found in the gut microbiome profile among ASD children with no symptoms, mild-moderate GSI, and severe GSI. Also, after the implementation of the exclusion diet, the gut microbiome showed no statistically significant difference between ASD

children with GSI scores less than 4 and those with scores of 4 or greater. Although the P/B ratio in ASD children with GSI ≥4 increased 2 times after diet (0.05 vs 0.12); however, the difference remained statistically insignificant (p=0.386) (Supplementary table S3).

Table (1a): Comparison between ATEC parameters before and after the exclusion diet

	Before (n = 20)	After (n = 20)	p
Speech/language/communication			
Min. – Max.	0.0 – 28.0	2.0 – 26.0	0.044*
Mean ± SD.	19.40 ± 6.82	16.85 ± 7.94	
Sociability			
Min. – Max.	5.0 – 30.0	0.0 – 20.0	<0.001*
Mean ± SD.	18.05 ± 7.22	7.60 ± 6.05	
Sensory/cognitive awareness			
Min. – Max.	6.0 – 30.0	1.0 – 26.0	0.266
Mean ± SD.	17.70 ± 6.43	15.75 ± 7.98	
Health/physical/behavior			
Min. – Max.	11.0 50.0 –	2.0 – 44.0	<0.001*
Mean ± SD.	25.55 ± 13.32	12.70 ± 12.32	
Total ATEC			
Min. – Max.	41.0 118.0 –	11.0 – 106.0	<0.001*
Mean ± SD.	79.63 ± 24.88	52.90 ± 28.08	

p: *p value* for comparing between before and after diet modification.
*: Statistically significant at $p \leq 0.05$

Table S1a: Comparison between mild to moderate and severe ASD cases.

Bacteria	CARS		*p*
	Mild to Moderate (*n* = 15)	Severe (*n* = 5)	
Firmicutes			
Min. – Max.	1.32E-1 – 6.39E-1	1.20E-1 – 3.52E-1	0.197
Mean ± SD.	3.73E-1 ± 1.62E-1	2.84E-1 ± 9.43E-2	
Median	4.23E-1	3.27E-1	
IQR	2.44E-1 – 4.60E-1	2.93E-1 – 3.30E-1	
Bacteroidetes			
Min. – Max.	3.19E-1 – 7.98E-1	2.28E-1 – 6.49E-1	0.800
Mean ± SD.	5.26E-1 ± 1.22E-1	5.04E-1 ± 1.84E-1	
Median	5.23E-1	6.08E-1	
IQR	4.81E-1 – 5.84E-1	4.03E-1 – 6.34E-1	
Prevotella			
Min. – Max.	1.47E-5 – 5.48E-1	2.89E-3 – 4.78E-1	1.000
Mean ± SD.	1.25E-1 ± 1.88E-1	1.26E-1 ± 2.05E-1	
Median	2.84E-2	8.87E-3	
IQR	1.89E-3 – 1.80E-1	4.51E-3 – 1.36E-1	
Bacteroides			
Min. – Max.	4.59E-2 – 5.66E-1	6.23E-2 – 3.30E-1	0.197
Mean ± SD.	3.46E-1 ± 1.75E-1	2.30E-1 ± 1.12E-1	
Median	3.78E-1	2.67E-1	
IQR	2.13E-1 – 4.93E-1	1.74E-1 – 3.18E-1	
Ruminococcus			
Min. – Max.	5.80E-5 – 1.28E-1	7.26E-3 – 1.03E-1	0.306
Mean ± SD.	3.63E-2 ± 3.86E-2	5.84E-2 ± 4.32E-2	
Median	2.51E-2	6.78E-2	
IQR	4.14E-3 – 4.58E-2	1.99E-2 – 9.42E-2	
Lactobacilli			
Min. – Max.	1.68E-5 – 4.17E-2	1.30E-3 – 1.55E-2	0.866
Mean ± SD.	7.01E-3 ± 1.10E-2	5.71E-3 ± 5.59E-3	
Median	4.17E-3	3.81E-3	
IQR	7.13E-4 – 5.96E-3	3.66E-3 – 4.28E-3	
Bifidobacteria			
Min. – Max.	2.07E-3 – 1.09E-1	1.05E-3 – 1.91E-1	0.933
Mean ± SD.	3.76E-2 ± 4.13E-2	7.46E-2 ± 8.35E-2	
Median	1.06E-2	5.09E-2	
IQR	5.83E-3 – 6.45E-2	1.27E-3 – 1.29E-1	

IQR: Inter quartile range; SD: Standard deviation; *: Statistically significant at $p \leq 0.05$

Table 1b: Percentage of effective reduction in ATEC Score after exclusion diet.

% of Reduction	NI (<20%)	NE (20%–50%)	NM (≥50%)	NT (≥20%)
Speech/language/ communication	14 (68.4%)	2 (10.5%)	4 (21.1%)	6 (31.6%)
Sociability	4 (20.0%)	4 (20.0%)	12 (60.0%)	16 (80.0%)
Sensory/cognitive awareness	12 (60.0%)	5 (25.0%)	3 (15.0%)	8 (40.0%)
Health/physical/ behavior	4 (20.0%)	3 (15.0%)	13 (65.0%)	16 (80.0%)
Total ATEC	6 (30.0%)	8 (40.0%)	6 (30.0%)	14 (70.0%)

NI: Ineffective; NE: Effective; NM: Markedly effective; NT: Total effective reduction rate

Table (S1b): Comparison between mild to moderate and severe ASD cases "continue".

Bacteria	CARS		*p*
	Mild to Moderate (*n* = 15)	Severe (*n* = 5)	
Akkermansia muciniphila			
Min. – Max.	4.99E-6 – 3.65E-1	2.60E-5 – 1.03E-1	
Mean ± SD.	2.84E-2 ± 9.36E-2	3.47E-2 ± 4.87E-2	0.933
Median	2.88E-4	3.55E-4	
IQR	1.52E-4 – 4.55E-3	8.06E-5 – 7.01E-2	
Faecalibacterium prausnitzii			
Min. – Max.	2.50E-3 – 2.60E-1	2.73E-2 – 1.70E-1	
Mean ± SD.	9.28E-2 ± 6.47E-2	9.47E-2 ± 5.86E-2	0.933
Median	7.23E-2	1.09E-1	
IQR	5.60E-2 – 1.07E-1	4.44E-2 – 1.23E-1	
Clostridium difficile			
Min. – Max.	0.0E+0 – 0.0E+0	0.0E+0 – 0.0E+0	
Mean ± SD.	0.0E+0 ± 0.0E+0	0.0E+0 ± 0.0E+0	1.000
Median	0.0E+0	0.0E+0	
IQR	0.0E+0 – 0.0E+0	0.0E+0 – 0.0E+0	
Desulfovibrio			
Min. – Max.	4.92E-6 – 3.03E-1	7.08E-2 – 4.12E-1	
Mean ± SD.	1.12E-1 ± 7.47E-2	2.09E-1 ± 1.29E-1	0.081
Median	9.99E-2	1.80E-1	
IQR	6.59E-2 – 1.33E-1	1.42E-1 – 2.38E-1	
Sutterulla			
Min. – Max.	3.73E-3 – 6.44E-2	4.36E-3 – 1.16E-1	
Mean ± SD.	2.47E-2 ± 1.72E-2	3.35E-2 ± 4.72E-2	0.735
Median	2.50E-2	9.12E-3	
IQR	8.14E-3 – 3.50E-2	7.66E-3 – 3.06E-2	
Bacteroides fragilis			
Min. – Max.	3.19E-5 – 3.56E-1	1.20E-5 – 4.08E-2	
Mean ± SD.	6.66E-2 ± 1.20E-1	9.81E-3 ± 1.76E-2	0.266
Median	5.59E-3	5.20E-4	
IQR	6.66E-4 – 4.46E-2	5.45E-5 – 7.65E-3	

IQR: Inter quartile range SD: Standard deviation *: Statistically significant at $p \leq 0.05$

Table 1c: Correlation between percentages of compliance and the reduction in ATEC parameters.

Correlation between percentage of compliance and the percentage of reduction in ATEC	r_S	*p*
Speech/language/communication	0.074	0.764
Sociability	0.389	0.090
Sensory/cognitive awareness	0.356	0.123
Health/physical/behavior	0.316	0.175
Total ATEC	0.316	0.174

r_S: Spearman coefficient

Table (S1c): Comparison between mild to moderate and severe ASD cases "continue"

	CARS		*p*
	Mild to Moderate (*n* = 15)	Severe (*n* = 5)	
P/B			
Min. – Max.	0.00003 – 5.86	0.01 – 1.79	
Mean ± SD.	1.05 ± 1.87	0.48 ± 0.75	0.800
Median (IQR)	0.09 (0.0 – 1.05)	0.14 (0.03 – 0.41)	
F/B			
Min. – Max.	0.17 – 1.18	0.19 – 1.43	
Mean ± SD.	0.76 ± 0.36	0.69 ± 0.46	0.553
Median (IQR)	0.81 (0.45 – 1.10)	0.58 (0.51 – 0.73)	
Diversity index			
Min. – Max.	1.41 – 1.99	1.25 – 2.05	
Mean ± SD.	1.70 ± 0.16	1.79 ± 0.33	0.390
Median (IQR)	1.70 (1.60 – 1.80)	1.93 (1.73 – 2.01)	
DSI			
Min. – Max.	27.0 – 70.0	27.0 – 58.60	
Mean ± SD.	48.33 ± 11.09	44.12 ± 12.07	0.480
Median (IQR)	47.0 (43.0 – 55.50)	47.0 (38.0 – 50.0)	

IQR: Inter quartile range; SD: Standard deviation

Table (2): Comparison between SSP parameters Before and After exclusion Diet

	Before		After		*p*
	No.	%	No.	%	
Tactile					
Definite	7	35.0	6	30.0	
Probable	4	20.0	5	25.0	0.796
Typical	9	45.0	9	45.0	
Min. – Max.	17.0 – 35.0		20.0 – 35.0		0.530
Mean ± SD.	28.20 ± 5.19		29.0 ± 4.51		
Taste/smell					
Definite	5	25.0	2	10.0	
Probable	3	15.0	2	10.0	0.144
Typical	12	60.0	16	80.0	
Min. – Max.	4.0 – 20.0		10.0 – 20.0		0.037*
Mean ± SD.	14.50 ± 4.84		17.30 ± 3.64		
Movement					
Definite	3	15.0	1	5.0	
Probable	10	50.0	3	15.0	0.001*
Typical	7	35.0	16	80.0	
Min. – Max.	3.0 – 15.0		9.0 – 15.0		0.002*
Mean ± SD.	11.80 ± 2.95		13.95 ± 1.79		
Under responsive					
Definite	18	90.0	15	75.0	
Probable	2	10.0	3	15.0	0.132
Typical	0	0.0	2	10.0	
Min. – Max.	8.0 – 25.0		9.0 – 28.0		0.023*
Mean ± SD.	16.20 ± 4.96		19.35 ± 5.61		

Auditory					
Definite	12	60.0	10	50.0	
Probable	4	20.0	3	15.0	0.336
Typical	4	20.0	7	35.0	
Min. – Max.	8.0 – 28.0		16.0 – 30.0		0.129
Mean ± SD.	18.65 ± 4.72		20.70 ± 4.45		
Low Energy					
Definite	9	45.0	2	10.0	
Probable	4	20.0	3	15.0	0.007*
Typical	7	35.0	15	75.0	
Min. – Max.	5.0 – 30.0		14.0 – 30.0		0.006*
Mean ± SD.	22.85 ± 6.90		27.45 ± 4.22		
Visual					
Definite	4	20.0	8	40.0	0.655
Probable	8	40.0	2	10.0	
Typical	8	40.0	10	50.0	
Min. – Max.	5.0 – 25.0		8.0 – 25.0		0.704
Mean ± SD.	17.65 ± 4.85		18.30 ± 5.21		
Total SSP					
Definite	14	70.0	7	35.0	
Probable	5	25.0	6	30.0	0.012*
Typical	1	5.0	7	35.0	
Min. – Max.	88.0 – 159.0		114.0 – 172.0		0.001*
Mean ± SD.	129.9 ± 17.21		146.1 ± 16.43		

p: *p value* for comparing between before and after diet modification
*: Statistically significant at $p \leq 0.05$.

Table (S2a): Comparison between mild to moderate and severe ASD cases before and after diet

Bacteria	Mild to Moderate CARS (n = 15)		Severe CARS (n = 5)	
	Before Diet	After Diet	Before Diet	After Diet
Firmicutes				
Min. – Max.	1.32E-1 – 6.39E-1	5.30E-2 – 7.91E-1	1.20E-1 – 3.52E-1	2.40E-1 – 6.18E-1
Mean ± SD.	3.73E-1 ± 1.62E-1	3.56E-1 ± 2.29E-1	2.84E-1 ± 9.43E-2	4.50E-1 ± 1.65E-1
Median	4.23E-1	3.22E-1	3.27E-1	5.34E-1
IQR	2.44E-1 – 4.60E-1	1.74E-1 – 5.10E-1	2.93E-1 – 3.30E-1	3.10E-1 – 5.49E-1
Z (p)	Z = 0.114 (p = 0.910)		Z = 1.483 (p = 0.138)	
Bacteroidetes				
Min. – Max.	3.19E-1 – 7.98E-1	2.87E-1 – 8.45E-1	2.28E-1 – 6.49E-1	1.71E-1 – 8.43E-1
Mean ± SD.	5.26E-1 ± 1.22E-1	5.08E-1 ± 1.49E-1	5.04E-1 ± 1.84E-1	5.74E-1 ± 2.69E-1
Median	5.23E-1	5.23E-1	6.08E-1	6.47E-1
IQR	4.81E-1 – 5.84E-1	4.13E-1 – 5.59E-1	4.03E-1 – 6.34E-1	4.50E-1 – 7.57E-1
Z (p)	Z = 0.511 (p = 0.609)		Z = 0.405 (p = 0.686)	
Prevotella				
Min. – Max.	1.47E-5 – 5.48E-1	7.56E-5 – 5.39E-1	2.89E-3 – 4.78E-1	1.53E-2 – 1.87E-1
Mean ± SD.	1.25E-1 ± 1.88E-1	1.59E-1 ± 1.75E-1	1.26E-1 ± 2.05E-1	1.09E-1 ± 7.52E-2
Median	2.84E-2	1.00E-1	8.87E-3	1.28E-1
IQR	1.89E-3 – 1.80E-1	2.53E-3 – 2.81E-1	4.51E-3 – 1.36E-1	4.72E-2 – 1.69E-1
Z (p)	Z = 0.852 (p = 0.394)		Z = 0.405 (p = 0.686)	
Bacteroides				
Min. – Max.	4.59E-2 – 5.66E-1	1.02E-1 – 5.97E-1	6.23E-2 – 3.30E-1	1.11E-1 – 5.65E-1
Mean ± SD.	3.46E-1 ± 1.75E-1	3.83E-1 ± 1.57E-1	2.30E-1 ± 1.12E-1	4.06E-1 ± 1.83E-1
Median	3.78E-1	4.07E-1	2.67E-1	4.02E-1
IQR	2.13E-1 – 4.93E-1	3.39E-1 – 4.76E-1	1.74E-1 – 3.18E-1	3.99E-1 – 5.53E-1
Z (p)	Z = 0.682 (p = 0.496)		Z = 1.483 (p = 0.138)	
Ruminococcus				
Min. – Max.	5.80E-5 – 1.28E-1	1.26E-3 – 1.05E-1	7.26E-3 – 1.03E-1	1.46E-2 – 1.71E-1
Mean ± SD.	3.63E-2 ± 3.86E-2	4.33E-2 ± 3.11E-2	5.84E-2 ± 4.32E-2	6.15E-2 ± 6.41E-2
Median	2.51E-2	4.32E-2	6.78E-2	4.87E-2
IQR	4.14E-3 – 4.58E-2	1.70E-2 – 6.14E-2	1.99E-2 – 9.42E-2	1.61E-2 – 5.72E-2
Z (p)	Z = 0.511 (p = 0.609)		Z = 0.135 (p = 0.893)	
Lactobacilli				
Min. – Max.	1.68E-5 – 4.17E-2	2.97E-6 – 7.22E-2	1.30E-3 – 1.55E-2	1.45E-4 – 8.02E-2
Mean ± SD.	7.01E-3 ± 1.10E-2	1.05E-2 ± 1.90E-2	5.71E-3 ± 5.59E-3	1.70E-2 ± 3.54E-2
Median	4.17E-3	1.76E-3	3.81E-3	1.24E-3
IQR	7.13E-4 – 5.96E-3	1.14E-3 – 1.09E-2	3.66E-3 – 4.28E-3	8.80E-4 – 2.35E-3
Z (p)	Z = 0.000 (p = 1.000)		Z = 0.674 (p = 0.500)	
Bifidobacteria				
Min. – Max.	2.07E-3 – 1.09E-1	7.06E-4 – 4.72E-1	1.05E-3 – 1.91E-1	7.82E-3 – 2.88E-1
Mean ± SD.	3.76E-2 ± 4.13E-2	5.74E-2 ± 1.21E-1	7.46E-2 ± 8.35E-2	7.24E-2 ± 1.21E-1
Median	1.06E-2	1.58E-2	5.09E-2	2.11E-2
IQR	5.83E-3 – 6.45E-2	8.79E-3 – 3.39E-2	1.27E-3 – 1.29E-1	1.04E-2 – 3.48E-2
Z (p)	Z = 0.170 (p = 0.865)		Z = 0.135 (p = 0.893)	

IQR: Inter quartile range; SD: Standard deviation; Z: Wilcoxon signed ranks test
p: *p value* for comparing between before diet and after diet; *: Statistically significant at $p \leq 0.05$

Table (S2b): Comparison between mild to moderate and severe ASD cases before and after diet "continue"

Bacteria	Mild to Moderate CARS (≤35) (*n* = 15)		Severe CARS (>35) (*n* = 5)	
	Before Diet	After Diet	Before Diet	After Diet
Akkermansia muciniphila				
Min. – Max.	4.99E-6 – 3.65E-1	2.02E-6 – 3.20E-2	2.60E-5 – 1.03E-1	1.34E-4 – 8.80E-2
Mean ± SD.	2.84E-2 ± 9.36E-2	5.80E-3 ± 1.01E-2	3.47E-2 ± 4.87E-2	3.16E-2 ± 3.84E-2
Median	2.88E-4	4.86E-4	3.55E-4	1.56E-2
IQR	1.52E-4 – 4.55E-3	1.01E-4 – 5.22E-3	8.06E-5 – 7.01E-2	3.24E-4 – 5.40E-2
Z (p)	Z = 0.454 (*p* = 0.650)		Z = 0.405 (*p* = 0.686)	
Faecalibacterium prausnitzii				
Min. – Max.	2.50E-3 – 2.60E-1	1.10E-2 – 3.92E-1	2.73E-2 – 1.70E-1	1.20E-1 – 4.05E-1
Mean ± SD.	9.28E-2 ± 6.47E-2	1.24E-1 ± 1.06E-1	9.47E-2 ± 5.86E-2	1.93E-1 ± 1.20E-1
Median	7.23E-2	1.02E-1	1.09E-1	1.36E-1
IQR	5.60E-2 – 1.07E-1	5.39E-2 – 1.42E-1	4.44E-2 – 1.23E-1	1.31E-1 – 1.72E-1
Z (p)	Z = 0.909 (*p* = 0.363)		Z = 1.753 (*p* = 0.080)	
Clostridium difficile				
Min. – Max.	0.0E+0 – 0.0E+0	0.00E+0 – 1.44E-5	0.0E+0 – 0.0E+0	0.00E+0 – 9.58E-7
Mean ± SD.	0.0E+0 ± 0.0E+0	1.08E-6 ± 3.71E-6	0.0E+0 ± 0.0E+0	1.92E-7 ± 4.28E-7
Median	0.0E+0	0.00E+0	0.0E+0	0.00E+0
IQR	0.0E+0 – 0.0E+0	0.00E+0 – 0.00E+0	0.0E+0 – 0.0E+0	0.00E+0 – 0.00E+0
Z (p)	Z = 1.342 (*p* = 0.180)		Z = 1.000 (*p* = 0.317)	
Desulfovibrio				
Min. – Max.	4.92E-6 – 3.03E-1	1.36E-2 – 2.99E-1	7.08E-2 – 4.12E-1	1.71E-1 – 3.47E-1
Mean ± SD.	1.12E-1 ± 7.47E-2	1.19E-1 ± 9.10E-2	2.09E-1 ± 1.29E-1	2.60E-1 ± 8.46E-2
Median	9.99E-2	8.88E-2	1.80E-1	2.62E-1
IQR	6.59E-2 – 1.33E-1	5.76E-2 – 1.85E-1	1.42E-1 – 2.38E-1	1.78E-1 – 3.40E-1
Z (p)	Z = 0.114 (*p* = 0.910)		Z = 0.674 (*p* = 0.500)	
Sutterulla				
Min. – Max.	3.73E-3 – 6.44E-2	2.62E-4 – 8.13E-2	4.36E-3 – 1.16E-1	2.08E-3 – 4.92E-2
Mean ± SD.	2.47E-2 ± 1.72E-2	3.20E-2 ± 3.22E-2	3.35E-2 ± 4.72E-2	1.77E-2 ± 1.85E-2
Median	2.50E-2	1.44E-2	9.12E-3	1.04E-2
IQR	8.14E-3 – 3.50E-2	6.26E-3 – 6.76E-2	7.66E-3 – 3.06E-2	9.17E-3 – 1.75E-2
Z (p)	Z = 0.284 (*p* = 0.776)		Z = 0.944 (*p* = 0.345)	
Bacteroides fragilis				
Min. – Max.	3.19E-5 – 3.56E-1	7.97E-6 – 6.10E-1	1.20E-5 – 4.08E-2	1.90E-3 – 1.34E-1
Mean ± SD.	6.66E-2 ± 1.20E-1	6.03E-2 ± 1.54E-1	9.81E-3 ± 1.76E-2	4.97E-2 ± 5.32E-2
Median	5.59E-3	1.36E-2	5.20E-4	3.83E-2
IQR	6.66E-4 – 4.46E-2	6.79E-3 – 2.86E-2	5.45E-5 – 7.65E-3	9.86E-3 – 6.45E-2
Z (p)	Z = 0.341 (*p* = 0.733)		Z = 2.023* (*p* = 0.043*)	

IQR: Inter quartile range; SD: Standard deviation; Z: Wilcoxon signed ranks test
p: *p value* for comparing between before diet and after diet
*: Statistically significant at $p \leq 0.05$

Table (S2c): Comparison between mild to moderate and severe ASD cases before and after diet "continue"

Bacteria	Mild to Moderate CARS (≤35) (*n* = 15)		Severe CARS (>35) (*n* = 5)	
	Before Diet	After Diet	Before Diet	After Diet
P/B				
Min. – Max.	0.00003 – 5.86	0.0002 – 3.74	0.01 – 1.79	0.03 – 0.47
Mean ± SD.	1.05 ± 1.87	0.68 ± 1.07	0.48 ± 0.75	0.31 ± 0.17
Median (IQR)	0.09 (0.0 – 1.05)	0.22 (0.01- 0.73)	0.14 (0.03 – 0.41)	0.32 (0.31 0.43 –)
Z (p)	Z = 0.511 (*p* = 0.609)		Z = 0.135 (*p* = 0.893)	
F/B				
Min. – Max.	0.17 – 1.18	0.11 – 1.73	0.19 – 1.43	0.28 – 3.61
Mean ± SD.	0.76 ± 0.36	0.74 ± 0.55	0.69 ± 0.46	1.27 ± 1.36
Median (IQR)	0.81 (0.45 – 1.10)	0.54 (0.39 -1.12)	0.58 (0.51 – 0.73)	0.83 (0.41- 1.22)
Z (p)	Z = 0.227 (*p* = 0.820)		Z = 0.405 (*p* = 0.686)	
Diversity index				
Min. – Max.	1.41 – 1.99	1.02 – 2.07	1.25 – 2.05	1.62 – 2.19
Mean ± SD.	1.70 ± 0.16	1.72 ± 0.23	1.79 ± 0.33	1.85 ± 0.24
Median (IQR)	1.70 (1.60 – 1.80)	1.79 (1.64 1.84 –)	1.93 (1.73 – 2.01)	1.72 (1.71 -1.99)
t (p)	t = 0.360, (*p* = 0.725)		t = 0.271, (*p* = 0.800)	
DSI				
Min. – Max.	27.0 – 70.0	40.0 – 69.0	27.0 – 58.60	25.0 – 59.0
Mean ± SD.	48.33 ± 11.09	53.59 ± 9.86	44.12 ± 12.07	46.36 ± 12.86
Median (IQR)	47.0 (43.0 – 55.50)	55.0 (44.94 -59.50)	47.0 (38.0 – 50.0)	50.0 (45.80- 52.0)
t (p)	t = 1.895, (*p* = 0.079)		t = 0.226, (*p* = 0.832)	

IQR: Inter quartile range; SD: Standard deviation; Z: Wilcoxon signed ranks test
t: Paired t-test
p: *p value* for comparing between before diet and after diet
*: Statistically significant at $p \leq 0.05$

Table (3): Comparison between GI severity index parameters Before and After exclusion Diet

GSI	Before Diet		After Diet		*p*
	No.	%	No.	%	
Constipation					
0	11	55.0	12	60.0	
1	3	15.0	6	30.0	0.225
2	6	30.0	2	10.0	
Diarrhea					
0	18	90.0	19	95.0	
1	0	0.0	1	5.0	0.180
2	2	10.0	0	0.0	
Stool Consistency					
0	13	65.0	16	80.0	
1	6	30.0	4	20.0	0.102
2	1	5.0	0	0.0	
Stool Smell					
0	10	50.0	15	75.0	
1	4	20.0	4	20.0	0.012*
2	6	30.0	1	5.0	
Flatulence					
0	8	40.0	12	60.0	
1	4	20.0	7	35.0	0.002*
2	8	40.0	1	5.0	
Abdominal Pain					
0	12	60.0	18	90.0	
1	7	35.0	2	10.0	0.035*
2	1	5.0	0	0.0	
GI severity index					
No symptoms (0)	3	15.0	6	30.0	
Moderate (1 – 3)	7	35.0	11	55.0	0.008*
Severe (≥4)	10	50.0	3	15.0	
Min. – Max.	0.0 10.0 –		0.0 5.0 –		0.001*
Mean ± SD.	3.60 ± 2.62		1.60 ± 1.54		

GSI: GI severity index
*: Statistically significant at $p \leq 0.05$

Table (S3a): Comparison between the three subgroups of Total SSP score of ASD cases before diet.

Bacteria	Total SSP			U	*p*
	Definite difference (38 – 141) (*n* = 14)	Probable difference (142 – 154) (*n* = 5)	Typical performance (155 – 190) (*n*=1[#])		
Firmicutes					
Min. – Max.	1.20E-1 – 6.39E-1	3.25E-1 – 5.41E-1			
Mean ± SD.	3.35E-1 ± 1.57E-1	4.38E-1 ± 7.73E-2	1.32E-1[#]	22.00	0.257
Median	3.29E-1	4.50E-1			
IQR	1.66E-1 – 4.52E-1	4.23E-1 – 4.51E-1			
Bacteroidetes					
Min. – Max.	2.28E-1 – 6.49E-1	3.44E-1 – 6.96E-1			
Mean ± SD.	5.11E-1 ± 1.21E-1	4.92E-1 ± 1.33E-1	7.98E-1[#]	29.00	0.622
Median	5.31E-1	4.84E-1			
IQR	4.78E-1 – 5.90E-1	4.12E-1 – 5.23E-1			
Prevotella					
Min. – Max.	1.47E-5 – 4.78E-1	9.99E-3 – 4.97E-1			
Mean ± SD.	7.53E-2 ± 1.43E-1	1.81E-1 ± 2.03E-1	5.48E-1[#]	15.00	0.070
Median	6.69E-3	9.17E-2			
IQR	6.33E-4 – 5.73E-2	3.72E-2 – 2.69E-1			
Bacteroides					
Min. – Max.	6.23E-2 – 5.26E-1	4.59E-2 – 5.66E-1			
Mean ± SD.	3.31E-1 ± 1.57E-1	2.80E-1 ± 2.25E-1	3.00E-1[#]	29.00	0.622
Median	3.29E-1	1.86E-1			
IQR	2.39E-1 – 4.75E-1	1.34E-1 – 4.68E-1			
Ruminococcus					
Min. – Max.	1.70E-3 – 1.28E-1	5.80E-5 – 7.69E-2			
Mean ± SD.	4.83E-2 ± 4.33E-2	2.75E-2 ± 3.11E-2	2.29E-2[#]	24.00	0.343
Median	3.44E-2	1.85E-2			
IQR	7.26E-3 – 9.42E-2	4.92E-3 – 3.69E-2			
Lactobacilli					
Min. – Max.	1.68E-5 – 1.55E-2	4.06E-4 – 4.17E-2			
Mean ± SD.	3.98E-3 ± 3.82E-3	1.55E-2 ± 1.67E-2	3.22E-4[#]	15.00	0.070
Median	3.74E-3	8.29E-3			
IQR	1.30E-3 – 4.60E-3	5.34E-3 – 2.19E-2			
Bifidobacteria					
Min. – Max.	1.05E-3 – 1.91E-1	3.14E-3 – 5.70E-2			
Mean ± SD.	5.70E-2 ± 6.12E-2	2.74E-2 ± 2.69E-2	2.07E-3[#]	31.00	0.754
Median	3.32E-2	1.06E-2			
IQR	4.74E-3 – 1.07E-1	9.76E-3 – 5.64E-2			

IQR: Inter quartile range; SD: Standard deviation; U: Mann Whitney test
p: *p value* for comparing between the three subgroups of Total SSP score

Table (S3b): Comparison between the three subgroups of Total SSP score of ASD cases before diet "continue"

Bacteria	Total SSP			U	*p*
	Definite difference (38 – 141) (*n* = 14)	Probable difference (142 – 154) (*n* = 5)	Typical performance (155 – 190) (*n*=1[#])		
Akkermansia muciniphila					
Min. – Max.	2.60E-5 – 3.65E-1	4.99E-6 – 1.20E-3	2.88E-4[#]	19.00	0.156
Mean ± SD.	4.27E-2 ± 9.79E-2	3.46E-4 ± 4.85E-4			
Median	1.12E-3	1.86E-4			
IQR	8.16E-5 – 3.26E-2	1.18E-4 – 2.20E-4			
Faecalibacterium prausnitzii					
Min. – Max.	2.73E-2 – 2.60E-1	2.50E-3 – 1.88E-1			
Mean ± SD.	1.01E-1 ± 6.17E-2	8.20E-2 ± 6.86E-2	4.19E-2[#]	30.00	0.687
Median	7.75E-2	7.98E-2			
IQR	6.09E-2 – 1.23E-1	4.80E-2 – 9.17E-2			
Clostridium difficile					
Min. – Max.	0.0E+0 – 0.0E+0	0.0E+0 – 0.0E+0			
Mean ± SD.	0.0E+0 ± 0.0E+0	0.0E+0 ± 0.0E+0	0.0E+0[#]	35.00	1.000
Median	0.0E+0	0.0E+00			
IQR	0.0E+0 – 0.0E+0	0.0E+0 – 0.0E+0			
Desulfovibrio					
Min. – Max.	4.92E-6 – 4.12E-1	4.59E-2 – 1.29E-1			
Mean ± SD.	1.56E-1 ± 1.09E-1	9.84E-2 ± 3.24E-2	4.94E-2[#]	23.00	0.298
Median	1.40E-1	9.99E-2			
IQR	7.08E-2 – 2.02E-1	9.61E-2 – 1.21E-1			
Sutterulla					
Min. – Max.	4.36E-3 – 1.16E-1	3.73E-3 – 3.30E-2			
Mean ± SD.	3.19E-2 ± 2.98E-2	1.51E-2 ± 1.31E-2	1.56E-2[#]	20.00	0.186
Median	2.91E-2	8.26E-3			
IQR	8.01E-3 – 3.96E-2	5.70E-3 – 2.50E-2			
Bacteroides fragilis					
Min. – Max.	1.20E-5 – 3.56E-1	3.19E-5 – 1.31E-2			
Mean ± SD.	7.31E-2 ± 1.22E-1	4.78E-3 ± 5.32E-3	5.01E-5[#]	23.00	0.298
Median	1.30E-2	4.96E-3			
IQR	5.20E-4 – 4.49E-2	2.32E-4 – 5.59E-3			

IQR: Inter quartile range; SD: Standard deviation; U: Mann Whitney test
p: *p value* for comparing between the three subgroups of Total SSP score

Table (S3c): Comparison between the three subgroups of Total SSP score of ASD cases before diet "continue"

	Total SSP			Test of Sig.	*p*
	Definite difference (38 – 141) (*n* = 14)	Probable difference (142 – 154) (*n* = 5)	Typical performance (155 – 190) (*n*=1[#])		
P/B					
Min. – Max.	0.00003 – 4.54	0.02 – 5.86			
Mean ± SD.	0.52 ± 1.25	1.80 ± 2.52	1.83[#]	U= 22.00	0.087
Median (IQR)	0.05 (0.001 – 0.24)	0.28 (0.16 – 2.67)			
F/B					
Min. – Max.	0.19 – 1.43	0.65 – 1.12			
Mean ± SD.	0.71 ± 0.39	0.92 ± 0.20	0.17[#]	U= 23.00	0.298
Median (IQR)	0.66 (0.30 – 1.11)	0.94 (0.81 – 1.09)			
Diversity index					
Min. – Max.	1.25 – 2.05	1.41 – 1.82			
Mean ± SD.	1.77 ± 0.22	1.66 ± 0.15	1.48[#]	t= 1.035	0.315
Median (IQR)	1.78 (1.61 – 1.93)	1.68 (1.67 – 1.70)			
DSI					
Min. – Max.	27.0 – 65.0	36.0 – 47.0			
Mean ± SD.	47.69 ± 11.12	41.60 ± 4.62	70.0[#]	t= 1.170	0.258
Median (IQR)	49.0 (44.0 – 56.0)	42.0 (38.0 – 45.0)			

IQR: Inter quartile range; SD: Standard deviation
t: Student t-test; U: Mann Whitney test
p: *p value* for comparing between the three subgroups of Total SSP score

Table (4): Comparison between the gut microbiome before and after exclusion diet

Bacteria	ASD		TD	*p value*
	Before	After		
Firmicutes				
Min. – Max.	1.20E-1 – 6.39E-1	5.30E-2 – 7.91E-1	5.72E-2 – 8.95E-1	p_1=0.478, p_2=0.720, p_3=0.713
Median	3.41E-1	3.26E-1	3.73E-1	
IQR	2.30E-1 – 4.52E-1	2.32E-1 – 5.42E-1	2.50E-1 – 5.90E-1	
Bacteroidetes				
Min. – Max.	2.28E-1 – 7.98E-1	1.71E-1 – 8.45E-1	2.28E-2 – 8.74E-1	p_1=0.940, p_2<0.001*, p_3<0.001*
Median	5.24E-1	5.34E-1	1.18E-1	
IQR	4.45E-1 – 5.99E-1	4.13E-1 – 6.22E-1	6.68E-2 – 4.10E-1	
Prevotella				
Min. – Max.	1.47E-5 – 5.48E-1	7.56E-5 – 5.39E-1	1.20E-3 – 3.87E-1	p_1=0.411, p_2=0.958, p_3=0.231
Median	2.74E-2	1.14E-1	2.12E-2	
IQR	3.02E-3 – 2.03E-1	5.67E-3 – 2.49E-1	7.21E-3 – 1.07E-1	
Bacteroides				
Min. – Max.	4.59E-2 – 5.66E-1	1.02E-1 – 5.97E-1	6.83E-4 – 4.34E-1	p_1=0.167, p_2<0.001*, p_3<0.001*
Median	3.23E-1	4.05E-1	3.64E-2	
IQR	1.80E-1 – 4.72E-1	3.39E-1 – 5.19E-1	1.36E-2 – 1.20E-1	
Ruminococcus				
Min. – Max.	5.80E-5 – 1.28E-1	1.26E-3 – 1.71E-1	7.08E-5 – 2.03E-1	p_1=0.654, p_2=0.162, p_3=0.011*
Median	2.79E-2	4.60E-2	1.09E-2	
IQR	6.09E-3 – 7.24E-2	1.64E-2 – 6.17E-2	4.99E-3 – 3.83E-2	

Lactobacilli				
Min. – Max.	1.68E-5 – 4.17E-2	2.97E-6 – 8.02E-2	1.60E-4 – 5.43E-1	p_1=0.709, p_2=0.310, p_3=0.167
Median	3.99E-3	1.64E-3	4.53E-3	
IQR	1.16E-3 – 5.96E03	9.03E-4 – 1.09E-2	7.51E-4 – 3.65E-2	
Bifidobacteria				
Min. – Max.	1.05E-3 – 1.91E-1	7.06E-4 – 4.72E-1	2.91E-4 – 4.06E-1	p_1=0.765, p_2=0.259, p_3=0.286
Median	1.31E-2	1.60E-2	3.73E-2	
IQR	3.94E-3 – 8.48E-2	9.11E-3 – 3.86E-2	9.92E-3 – 1.65E-1	
Akkermancia muciniphila				
Min. – Max.	4.99E-6 – 3.65E-1	2.02E-6 – 8.80E-2	1.23E-5 – 1.74E-1	p_1=0.940, p_2=0.773, p_3=0.903
Median	3.22E-4	6.03E-4	7.64E-4	
IQR	9.98E-5 – 1.18E-2	1.61E-4 – 1.71E-2	1.81E-4 – 6.70E-3	
Faecalibacterium prausnitzii				
Min. – Max.	2.50E-3 – 2.60E-1	1.10E-2 – 4.05E-1	4.86E-3 – 4.50E-1	p_1=0.079, p_2=1.000, p_3=0.298
Median	7.61E-2	1.21E-1	7.94E-2	
IQR	4.96E-2 – 1.23E-1	7.04E-2 – 1.58E-1	3.29E-2 – 1.98E-1	
Clostridium difficile				
Min. – Max.	0.0E+00 – 0.0E+00	0.0E+00 – 1.44E-5	0.0E+00 – 5.17E-5	p_1=0.109, p_2=0.052, p_3=0.881
Median	0.0E+00	0.0E+00	0.0E+00	
IQR	0.0E+00 – 0.0E+00	0.0E+00 – 0.0E+00	0.0E+00	
Desulfovibrio				
Min. – Max.	4.92E-6 – 4.12E-1	1.36E-2 – 3.47E-1	2.11E-2 – 5.83E-1	p_1=0.601, p_2=0.037*, p_3=0.107
Median	1.16E-1	1.36E-1	1.71E-1	
IQR	7.08E-2 – 1.84E-1	7.05E-2 – 2.40E-1	1.07E-1 – 3.63E-1	
Sutterulla				
Min. – Max.	3.73E-3 – 1.16E-1	2.62E-4 – 8.13E-2	6.51E-4 – 6.01E-2	p_1=0.709, p_2=0.012*, p_3=0.093
Median	2.25E-2	1.24E-2	5.90E-3	
IQR	7.84E-3 – 3.50E-2	6.26E-003 – 5.82E-2	2.93E-3 – 2.37E-2	
Bacteroides fragilis				
Min. – Max.	1.20E-5 – 3.56E-1	7.97E-6 – 6.10E-1	1.97E-6 – 2.17E-1	p_1=0.232, p_2=0.146, p_3=0.001*
Median	5.28E-3	1.69E-2	1.29E-3	
IQR	1.73E-4 – 4.26E-2	6.79E-3 – 4.54E-2	5.32E-5 – 6.75E-3	
P/B				
Min. – Max.	0.00003 5.86 –	0.0002 3.74 –	0.05 – 5.22	Zp1=0.709 Up2=0.005* Up3=0.012*
Median	0.11	0.31	0.67	
IQR	0.01 1.10 –	0.01 0.49 –	0.33 – 1.94	
F/B				
Min. – Max.	0.17 1.43 –	0.11 3.61 –	0.12 – 18.03	$^{Z}p_1$=0.940 $^{U}p_2$<0.001* $^{U}p_3$=0.001*
Median	0.75	0.57	2.75	
IQR	0.40 1.10 –	0.39 1.26 –	0.96 – 5.68	
Diversity index				$^{t0}p_1$=0.647, $^{t}p_2$=0.229, $^{t}p_3$=0.113
Min. – Max.	1.25 – 2.05	1.02 – 2.19	1.22 – 2.04	
Mean ± SD.	1.72 ± 0.21	1.75 ± 0.23	1.65 ± 0.24	
Dissimilarity index				
Min. – Max.	27.0 70.0–	25.0 69.0 –		$^{t0}p_1$=0.160
Mean ± SD.	47.28 11.18 ±	51.78 10.81 ±		

IQR: Inter quartile range

p_1: *p value* for comparing ASD children Before and after diet modification.

p_2: *p value* for comparing TD controls and ASD children before diet modification.

p_3: *p value* for comparing between TD controls and ASD children after diet modification *: Statistically significant at $p \leq 0.05$

Table (S4a): Comparison between the three subgroups of Total SSP score of ASD cases before & after diet

Bacteria	Definite difference (38 – 141) (*n* = 14)		Probable / Typical (>141) (*n* = 6)	
	Before Diet	After Diet	Before Diet	After Diet
Firmicutes				
Min. – Max.	1.20E-1 – 6.39E-1	5.30E-2 – 7.91E-1	1.32E-1 – 5.41E-1	2.64E-1 – 7.07E-1
Mean ± SD.	3.35E-1 ± 1.57E-1	3.57E-1 ± 2.34E-1	3.87E-1 ± 1.43E-1	4.34E-1 ± 1.64E-1
Median	3.29E-1	3.11E-1	4.37E-1	3.93E-1
IQR	1.66E-1 – 4.52E-1	1.24E-1 – 5.49E-1	3.25E-1 – 4.51E-1	3.22E-1 – 5.22E-1
Z (p)	Z = 0.471 (*p* = 0.638)		Z = 0.734 (*p* = 0.463)	
Bacteroidetes				
Min. – Max.	2.28E-1 – 6.49E-1	1.71E-1 – 8.43E-1	3.44E-1 – 7.98E-1	4.06E-1 – 8.45E-1
Mean ± SD.	5.11E-1 ± 1.21E-1	5.07E-1 ± 1.82E-1	5.43E-1 ± 1.73E-1	5.66E-1 ± 1.85E-1
Median	5.31E-1	5.34E-1	5.04E-1	4.97E-1
IQR	4.78E-1 – 5.90E-1	3.42E-1 – 5.96E-1	4.12E-1 – 6.96E-1	4.20E-1 – 7.34E-1
Z (p)	Z = 0.157 (*p* = 0.875)		Z = 0.105 (*p* = 0.917)	
Prevotella				
Min. – Max.	1.47E-5 – 4.78E-1	7.56E-5 – 3.81E-1	9.99E-3 – 5.48E-1	2.51E-3 – 5.39E-1
Mean ± SD.	7.53E-2 ± 1.43E-1	1.04E-1 ± 1.17E-1	2.42E-1 ± 2.36E-1	2.46E-1 ± 1.99E-1
Median	6.69E-3	7.36E-2	1.80E-1	2.69E-1
IQR	6.33E-4 – 5.73E-2	2.55E-3 – 1.69E-1	3.72E-2 – 4.97E-1	4.96E-2 – 3.50E-1
Z (p)	Z = 1.538 (*p* = 0.124)		Z = 0.105 (*p* = 0.917)	
Bacteroides				
Min. – Max.	6.23E-2 – 5.26E-1	1.02E-1 – 5.65E-1	4.59E-2 – 5.66E-1	3.44E-1 – 5.97E-1
Mean ± SD.	3.31E-1 ± 1.57E-1	3.55E-1 ± 1.68E-1	2.83E-1 ± 2.01E-1	4.69E-1 ± 1.12E-1
Median	3.29E-1	4.01E-1	2.43E-1	4.54E-1
IQR	2.39E-1 – 4.75E-1	1.50E-1 – 4.51E-1	1.34E-1 – 4.68E-1	3.68E-1 – 5.95E-1
Z (p)	Z = 0.471 (*p* = 0.638)		Z = 1.572 (*p* = 0.116)	
Ruminococcus				
Min. – Max.	1.70E-3 – 1.28E-1	1.26E-3 – 1.71E-1	5.80E-5 – 7.69E-2	2.59E-2 – 8.14E-2
Mean ± SD.	4.83E-2 ± 4.33E-2	4.64E-2 ± 4.72E-2	2.67E-2 ± 2.79E-2	5.11E-2 ± 2.08E-2
Median	3.44E-2	3.30E-2	2.07E-2	4.95E-2
IQR	7.26E-3 – 9.42E-2	1.46E-2 – 5.72E-2	4.92E-3 – 3.69E-2	3.40E-2 – 6.62E-2
Z (p)	Z = 0.220 (*p* = 0.826)		Z = 1.153 (*p* = 0.249)	
Lactobacilli				
Min. – Max.	1.68E-5 – 1.55E-2	2.97E-6 – 8.02E-2	3.22E-4 – 4.17E-2	1.76E-3 – 7.22E-2
Mean ± SD.	3.98E-3 ± 3.82E-3	9.34E-3 ± 2.17E-2	1.30E-2 ± 1.61E-2	1.85E-2 ± 2.73E-2
Median	3.74E-3	1.30E-3	6.82E-3	6.61E-3
IQR	1.30E-3 – 4.60E-3	1.45E-4 – 2.35E-3	4.06E-4 – 2.19E-2	2.13E-3 – 2.18E-2
Z (p)	Z = 0.910 (*p* = 0.363)		Z = 0.734 (*p* = 0.463)	
Bifidobacteria				
Min. – Max.	1.05E-3 – 1.91E-1	7.06E-4 – 4.72E-1	2.07E-3 – 5.70E-2	3.66E-3 – 1.57E-1
Mean ± SD.	5.70E-2 ± 6.12E-2	6.68E-2 ± 1.38E-1	2.32E-2 ± 2.62E-2	4.81E-2 ± 5.68E-2
Median	3.32E-2	1.51E-2	1.02E-2	2.93E-2
IQR	4.74E-3 – 1.07E-1	7.82E-3 – 2.53E-2	3.14E-3 – 5.64E-2	1.35E-2 – 5.57E-2
Z (p)	Z = 0.408 (*p* = 0.683)		Z = 1.363 (*p* = 0.173)	

IQR: Inter quartile range; SD: Standard deviation; Z: Wilcoxon signed ranks test
p: *p value* for comparing between before diet and after diet; *: Statistically significant at $p \leq 0.05$

Table (S4b): Comparison between the three subgroups of Total SSP score of ASD cases before & after diet "continue"

Bacteria	Definite difference (38 – 141) (*n* = 14)		Probable / Typical (>141) (*n* = 6)	
	Before Diet	After Diet	Before Diet	After Diet
Akkermansia muciniphila				
Min. – Max.	2.60E-5 – 3.65E-1	2.02E-6 – 8.80E-2	4.99E-6 – 1.20E-3	2.89E-6 – 1.86E-2
Mean ± SD.	4.27E-2 ± 9.79E-2	1.57E-2 ± 2.63E-2	3.36E-4 ± 4.34E-4	4.12E-3 ± 7.19E-3
Median	1.12E-3	2.71E-4	2.03E-4	1.19E-3
IQR	8.16E-5 – 3.26E-2	1.34E-4 – 2.25E-2	1.18E-4 – 2.88E-4	4.86E-4 – 3.28E-3
Z (p)	Z = 0.785 (*p* = 0.433)		Z = 1.572 (*p* = 0.116)	
Faecalibacterium prausnitzii				
Min. – Max.	2.73E-2 – 2.60E-1	1.10E-2 – 4.05E-1	2.50E-3 – 1.88E-1	5.13E-2 – 1.73E-1
Mean ± SD.	1.01E-1 ± 6.17E-2	1.54E-1 ± 1.28E-1	7.53E-2 ± 6.35E-2	1.11E-1 ± 4.39E-2
Median	7.75E-2	1.26E-1	6.39E-2	1.08E-1
IQR	6.09E-2 – 1.23E-1	5.64E-2 – 1.72E-1	4.19E-2 – 9.17E-2	8.43E-2 – 1.44E-1
Z (p)	Z = 1.413 (*p* = 0.158)		Z = 1.153 (*p* = 0.249)	
Clostridium difficile				
Min. – Max.	0.00E+0 – 0.00E+0	0.00E+0 – 1.82E-6	0.00E+0 – 0.00E+0	0.00E+0 – 1.44E-5
Mean ± SD.	0.00E+0 ± 0.00E+0	1.98E-7 ± 5.32E-7	0.00E+0 ± 0.00E+0	2.40E-6 ± 5.88E-6
Median	0.00E+0	0.00E+0	0.00E+0	0.00E+0
IQR	0.00E+0 – 0.00E+0	0.00E+0 – 0.00E+0	0.00E+0 – 0.00E+0	0.00E+0 – 0.00E+0
Z (p)	Z = 1.342 (*p* = 0.180)		Z = 1.000 (*p* = 0.317)	
Desulfovibrio				
Min. – Max.	4.92E-6 – 4.12E-1	1.36E-2 – 3.47E-1	4.59E-2 – 1.29E-1	7.80E-2 – 2.50E-1
Mean ± SD.	1.56E-1 ± 1.09E-1	1.64E-1 ± 1.22E-1	9.02E-2 ± 3.52E-2	1.30E-1 ± 6.43E-2
Median	1.40E-1	1.75E-1	9.80E-2	1.08E-1
IQR	7.08E-2 – 2.02E-1	5.21E-2 – 2.62E-1	4.94E-2 – 1.21E-1	8.88E-2 – 1.47E-1
Z (p)	Z = 0.157 (*p* = 0.875)		Z = 1.572 (*p* = 0.116)	
Sutterulla				
Min. – Max.	4.36E-3 – 1.16E-1	1.31E-3 – 8.13E-2	3.73E-3 – 3.30E-2	2.62E-4 – 7.94E-2
Mean ± SD.	3.19E-2 ± 2.98E-2	2.78E-2 ± 2.85E-2	1.52E-2 ± 1.17E-2	2.99E-2 ± 3.47E-2
Median	2.91E-2	1.24E-2	1.19E-2	1.44E-2
IQR	8.01E-3 – 3.96E-2	9.17E-3 – 4.92E-2	5.70E-3 – 2.50E-2	3.68E-3 – 6.71E-2
Z (p)	Z = 0.847 (*p* = 0.397)		Z = 0.314 (*p* = 0.753)	
Bacteroides fragilis				
Min. – Max.	1.20E-5 – 3.56E-1	7.97E-6 – 6.10E-1	3.19E-5 – 1.31E-2	2.61E-3 – 9.06E-2
Mean ± SD.	7.31E-2 ± 1.22E-1	6.96E-2 ± 1.59E-1	3.99E-3 ± 5.13E-3	2.98E-2 ± 3.51E-2
Median	1.30E-2	1.75E-2	2.60E-3	1.52E-2
IQR	5.20E-4 – 4.49E-2	8.27E-3 – 3.83E-2	5.01E-5 – 5.59E-3	2.65E-3 – 5.25E-2
Z (p)	Z = 0.345 (*p* = 0.730)		Z = 1.992 (*p* = 0.046)	

IQR: Inter quartile range; SD: Standard deviation; Z: Wilcoxon signed ranks test
p: *p value* for comparing between before diet and after diet; *: Statistically significant at $p \leq 0.05$

Table (S4c): Comparison between the three subgroups of Total SSP score of ASD cases before & after diet "continue"

	Definite difference (38 – 141) (*n* = 14)		Probable / Typical (>141) (*n* = 6)	
	Before Diet	After Diet	Before Diet	After Diet
P/B				
Min. – Max.	0.00003 – 4.54	0.0002 – 3.74	0.02 – 5.86	0.01 – 1.57
Mean ± SD.	0.52 ± 1.25	0.59 ± 1.07	1.80 ± 2.25	0.59 ± 0.59
Median (IQR)	0.05 (0.001 – 0.24)	0.27 (0.01 – 0.47)	1.05 (0.16 – 2.67)	0.45 (0.10 – 0.95)
Z (p)	Z = 0.910 (*p* = 0.363)		Z = 1.572 (*p* = 0.116)	
F/B				
Min. – Max.	0.19 – 1.43	0.11 – 3.61	0.17 – 1.12	0.44 – 1.68
Mean ± SD.	0.71 ± 0.39	0.88 ± 0.93	0.80 ± 0.36	0.86 ± 0.51
Median (IQR)	0.66 (0.30 – 1.11)	0.50 (0.28 – 1.22)	0.88 (0.65 – 1.09)	0.65 (0.47 – 1.29)
Z (p)	Z = 0.031 (*p* = 0.975)		Z = 0.105 (*p* = 0.917)	
Diversity index				
Min. – Max.	1.25 – 2.05	1.02 – 2.19	1.41 – 1.82	1.63 – 2.07
Mean ± SD.	1.77 ± 0.22	1.72 ± 0.26	1.63 ± 0.15	1.82 ± 0.15
Median (IQR)	1.78 (1.61 – 1.93)	1.72 (1.62 – 1.84)	1.68 (1.48 – 1.70)	1.82 (1.72 – 1.87)
t (p)	t = 0.502, (*p* = 0.624)		t = 4.607, (*p* = 0.006*)	
DSI				
Min. – Max.	27.0 – 65.0	25.0 – 69.0	36.0 – 70.0	40.0 – 55.0
Mean ± SD.	47.69 ± 11.12	52.91 ± 12.21	46.33 ± 12.31	49.17 ± 6.68
Median (IQR)	49.0 (44.0 – 56.0)	54.50 (45.80 – 61.0)	43.50 (38.0 – 47.0)	50.50 (44.0 – 55.0)
t (p)	t = 1.275, (*p* = 0.225)		t = 0.671, (*p* = 0.532)	

IQR: Inter quartile range; SD: Standard deviation; Z: Wilcoxon signed ranks test
t: Paired t-test; *p*: *p value* for comparing between before diet and after diet
*: Statistically significant at $p \leq 0.05$

Table (S5a): Comparison between the three subgroups of GI severity index in ASD cases before diet.

Bacteria	GI severity index			H	*p*
	No symptoms (0) (*n* = 3)	Mild-Moderate (1 – 3) (*n* = 7)	Severe (≥4) (*n* = 10)		
Firmicutes					
Min. – Max.	1.32E-1 – 5.41E-1	2.93E-1 – 6.39E-1	1.20E-1 – 5.51E-1	1.059	0.589
Mean ± SD.	2.71E-1 ± 2.34E-1	4.05E-1 ± 1.20E-1	3.36E-1 ± 1.48E-1		
Median	1.41E-1	3.52E-1	3.53E-1		
IQR	1.37E-1 – 3.41E-1	3.26E-1 – 4.51E-1	1.66E-1 – 4.52E-1		
Bacteroidetes					
Min. – Max.	4.84E-1 – 7.98E-1	4.03E-1 – 6.96E-1	2.28E-1 – 6.34E-1	1.862	0.394
Mean ± SD.	5.97E-1 ± 1.74E-1	5.55E-1 ± 1.13E-1	4.73E-1 ± 1.33E-1		
Median	5.10E-1	5.78E-1	5.13E-1		
IQR	4.97E-1 – 6.54E-1	4.74E-1 – 6.29E-1	3.44E-1 – 5.89E-1		
Prevotella					
Min. – Max.	3.72E-2 – 5.48E-1	4.50E-4 – 4.78E-1	1.47E-5 – 4.97E-1	4.227	0.121
Mean ± SD.	2.98E-1 ± 2.56E-1	1.40E-1 ± 1.78E-1	6.33E-2 ± 1.53E-1		
Median	3.08E-1	9.17E-2	9.43E-3		
IQR	1.73E-1 – 4.28E-1	3.02E-3 – 2.03E-1	6.33E-4 – 2.84E-2		
Bacteroides					
Min. – Max.	6.79E-2 – 3.00E-1	4.59E-2 – 5.66E-1	6.23E-2 – 5.26E-1	2.759	0.252
Mean ± SD.	1.67E-1 ± 1.20E-1	3.56E-1 ± 1.75E-1	3.35E-1 ± 1.63E-1		
Median	1.34E-1	3.30E-1	3.53E-1		
IQR	1.01E-1 – 2.17E-1	2.93E-1 – 4.82E-1	1.86E-1 – 4.75E-1		
Ruminococcus					
Min. – Max.	2.76E-3 – 7.69E-2	5.80E-5 – 1.03E-1	1.70E-3 – 1.28E-1	0.588	0.745
Mean ± SD.	3.42E-2 ± 3.83E-2	5.05E-2 ± 4.07E-2	3.80E-2 ± 4.28E-2		
Median	2.29E-2	3.69E-2	2.46E-2		
IQR	1.28E-2 – 4.99E-2	2.25E-2 –8.44E-2	4.92E-3 – 5.34E-2		
Lactobacilli					
Min. – Max.	3.22E-4 – 6.57E-3	1.68E-5 – 1.55E-2	2.86E-4 – 4.17E-2	0.872	0.647
Mean ± SD.	2.43E-3 ± 3.58E-3	5.23E-3 ± 5.29E-3	8.98E-3 ± 1.30E-2		
Median	4.06E-4	3.81E-3	4.23E-3		
IQR	3.64E-4 – 3.49E-3	1.84E-3 – 6.82E-3	3.10E-3 – 5.04E-3		
Bifidobacteria					
Min. – Max.	2.07E-3 – 9.76E-3	1.05E-3 – 1.29E-1	1.27E-3 – 1.91E-1	3.225	0.199
Mean ± SD.	5.52E-3 ± 3.90E-3	3.85E-2 ± 4.86E-2	6.51E-2 ± 6.13E-2		
Median	4.74E-3	1.06E-2	5.67E-2		
IQR	3.41E-3 – 7.25E-3	3.12E-3 – 6.15E-2	8.99E-3 –1.07E-1		

IQR: Inter quartile range; SD: Standard deviation; H: H for Kruskal Wallis test
p: *p value* for comparing between the three subgroups of GI severity index

Table (S5b): Comparison between the three subgroups of GI severity index in ASD cases before diet "continue"

Bacteria	GI severity index			H	*p*
	Normal (0) (*n* = 3)	Moderate (1 – 3) (*n* = 7)	Severe (≥4) (*n* = 10)		
Akkermansia muciniphila					
Min. – Max.	8.16E-5 – 2.88E-4	4.99E-6 – 3.65E-1	5.25E-5 – 7.01E-2		
Mean ± SD.	1.97E-4 ± 1.05E-4	6.80E-2 ± 1.36E-1	1.23E-2 ± 2.29E-2	0.837	0.658
Median	2.20E-4	3.55E-4	7.91E-4		
IQR	1.51E-4 – 2.54E-4	1.06E-4 – 5.51E-2	1.18E-4 – 1.63E-2		
Faecalibacterium prausnitzii					
Min. – Max.	4.19E-2 – 1.88E-1	2.50E-3 – 1.23E-1	2.73E-2 – 2.60E-1		
Mean ± SD.	9.37E-2 ± 8.18E-2	7.34E-2 ± 4.12E-2	1.07E-1 ± 7.01E-2	0.575	0.750
Median	5.11E-2	8.26E-2	7.61E-2		
IQR	4.65E-2 – 1.20E-1	5.27E-2 –1.00E-1	6.79E-2 – 1.52E-1		
Clostridium difficile					
Min. – Max.	0.0E+0 – 0.0E+0	0.0E+0 – 0.0E+0	0.0E+0 – 0.0E+0		
Mean ± SD.	0.0E+0 ± 0.0E+0	0.0E+0 ± 0.0E+0	0.0E+0 ± 0.0E+0	0.000	1.000
Median	0.0E+0	0.0E+0	0.0E+0		
IQR	0.0E+0 – 0.0E+0	0.0E+0 – 0.0E+0	0.0E+0 – 0.0E+0		
Desulfovibrio					
Min. – Max.	4.94E-2 – 9.61E-2	4.59E-2 – 4.12E-1	4.92E-6 – 3.03E-1		
Mean ± SD.	7.21E-2 ± 2.34E-2	1.81E-1 ± 1.18E-1	1.25E-1 ± 8.60E-2	3.711	0.156
Median	7.07E-2	1.42E-1	1.10E-1		
IQR	6.01E-2 – 8.34E-2	1.20E-1 – 2.13E-1	7.08E-2 – 1.80E-1		
Sutterulla					
Min. – Max.	8.26E-3 – 3.70E-2	3.73E-3 – 1.16E-1	4.36E-3 – 6.44E-2		
Mean ± SD.	2.03E-2 ± 1.49E-2	3.79E-2 ± 3.70E-2	2.12E-2 ± 1.96E-2	1.503	0.472
Median	1.56E-2	3.29E-2	1.40E-2		
IQR	1.19E-2 – 2.63E-2	1.99E-2 – 3.63E-2	7.32E-3 – 2.75E-2		
Bacteroides fragilis					
Min. – Max.	5.01E-5 – 5.59E-3	3.19E-5 – 1.97E-1	1.20E-5 – 3.56E-1		
Mean ± SD.	2.25E-3 ± 2.94E-3	4.21E-2 ± 7.08E-2	7.46E-2 ± 1.38E-1	1.012	0.603
Median	1.10E-3	7.65E-3	7.32E-3		
IQR	5.75E-4 – 3.35E-3	2.51E-3 – 4.26E-2	2.32E-4 – 4.49E-2		

IQR: Inter quartile range; SD: Standard deviation; H: H for Kruskal Wallis test
p: *p value* for comparing between the three subgroups of GI severity index

Table (S5c): Comparison between the three subgroups of GI severity index in ASD cases before diet "continue"

	GI severity index			Test of Sig.	*p*
	Normal (0) (*n* = 3)	Moderate (1 – 3) (*n* = 7)	Severe (≥4) (*n* = 10)		
P/B					
Min. – Max.	0.28 – 4.54	0.001 – 5.86	0.00003 – 2.67		
Mean ± SD.	2.21 ± 2.16	1.18 ± 2.16	0.33 ± 0.83	H= 4.692	0.096
Median (IQR)	1.83 (1.05 – 3.18)	0.16 (0.01 – 1.10)	0.05 (0.00 – 0.14)		
F/B					
Min. – Max.	0.17 – 1.12	0.51 – 1.11	0.19 – 1.43		
Mean ± SD.	0.52 ± 0.52	0.75 ± 0.25	0.80 ± 0.42	H= 1.558	0.459
Median (IQR)	0.28 (0.22 – 0.70)	0.65 (0.59 – 0.91)	0.85 (0.30 – 1.15)		
Diversity index					
Min. – Max.	1.48 – 1.68	1.41 – 2.05	1.25 – 2.01		
Mean ± SD.	1.59 ± 0.10	1.76 ± 0.24	1.74 ± 0.21	F= 0.768	0.479
Median (IQR)	1.61 (1.55 – 1.65)	1.73 (1.62 – 1.96)	1.78 (1.70 – 1.82)		
DSI					
Min. – Max.	38.0 – 70.0	38.0 – 55.0	27.0 – 65.0		
Mean ± SD.	54.67 ± 16.04	46.29 ± 5.47	45.76 ± 12.85	F= 0.756	0.485
Median (IQR)	56.0 (47.0 – 63.0)	47.0 (43.5 – 48.5)	47.0 (36.0 – 57.0)		

IQR: Inter quartile range; SD: Standard deviation
F: F for One way ANOVA test; H: H for Kruskal Wallis test
p: *p value* for comparing between the three subgroups of GI severity index

Table (S6a): Comparison between the three subgroups of GI severity index in ASD cases before and after diet

Bacteria	GSI <4 (n = 10)		GSI ≥4 (n = 10)	
	Before Diet	After Diet	Before Diet	After Diet
Firmicutes				
Min. – Max.	1.32E-1 – 6.39E-1	5.30E-2 – 7.07E-1	1.20E-1 – 5.51E-1	1.10E-1 – 7.91E-1
Mean ± SD.	3.65E-1 ± 1.61E-1	3.61E-1 ± 2.17E-1	3.36E-1 ± 1.48E-1	3.99E-1 ± 2.22E-1
Median	3.41E-1	3.17E-1	3.53E-1	4.14E-1
IQR	2.93E-1 – 4.51E-1	2.40E-1 – 5.22E-1	1.66E-1 – 4.52E-1	2.24E-1 – 5.49E-1
Z (p)	Z = 0.255 (p = 0.799)		Z = 0.663 (p = 0.508)	
Bacteroidetes				
Min. – Max.	4.03E-1 – 7.98E-1	1.71E-1 – 8.45E-1	2.28E-1 – 6.34E-1	2.87E-1 – 6.47E-1
Mean ± SD.	5.67E-1 ± 1.25E-1	5.54E-1 ± 2.30E-1	4.73E-1 ± 1.33E-1	4.96E-1 ± 1.16E-1
Median	5.57E-1	5.11E-1	5.13E-1	5.44E-1
IQR	4.84E-1 – 6.49E-1	4.06E-1 – 7.57E-1	3.44E-1 – 5.89E-1	4.35E-1 – 5.60E-1
Z (p)	Z = 0.459 (p = 0.646)		Z = 0.561 (p = 0.575)	
Prevotella				
Min. – Max.	4.50E-4 – 5.48E-1	1.99E-4 – 5.39E-1	1.47E-5 – 4.97E-1	7.56E-5 – 3.01E-1
Mean ± SD.	1.87E-1 ± 2.04E-1	2.01E-1 ± 1.83E-1	6.33E-2 ± 1.53E-1	9.31E-2 ± 1.07E-1
Median	1.14E-1	1.82E-1	9.43E-3	5.44E-2
IQR	3.14E-3 – 3.08E-1	4.72E-2 – 3.50E-1	6.33E-4 – 2.84E-2	2.51E-3 – 1.69E-1
Z (p)	Z = 0.255 (p = 0.799)		Z = 0.866 (p = 0.386)	
Bacteroides				
Min. – Max.	4.59E-2 – 5.66E-1	1.02E-1 – 5.97E-1	6.23E-2 – 5.26E-1	3.33E-1 – 5.95E-1
Mean ± SD.	2.99E-1 ± 1.79E-1	3.26E-1 ± 1.94E-1	3.35E-1 ± 1.63E-1	4.52E-1 ± 8.41E-2
Median	3.09E-1	3.56E-1	3.53E-1	4.33E-1
IQR	1.34E-1 – 4.53E-1	1.17E-1 – 5.00E-1	1.86E-1 – 4.75E-1	3.99E-1 – 5.38E-1
Z (p)	Z = 0.255 (p = 0.799)		Z = 1.580 (p = 0.114)	
Ruminococcus				
Min. – Max.	5.80E-5 – 1.03E-1	1.26E-3 – 8.14E-2	1.70E-3 – 1.28E-1	2.27E-3 – 1.71E-1
Mean ± SD.	4.56E-2 ± 3.86E-2	3.27E-2 ± 2.61E-2	3.80E-2 ± 4.28E-2	6.29E-2 ± 4.78E-2
Median	3.10E-2	2.16E-2	2.46E-2	5.61E-2
IQR	1.99E-2 – 7.69E-2	1.46E-2 – 4.87E-2	4.92E-3 – 5.34E-2	3.40E-2 – 7.81E-2
Z (p)	Z = 0.764 (p = 0.445)		Z = 1.478 (p = 0.139)	
Lactobacilli				
Min. – Max.	1.68E-5 – 1.55E-2	2.97E-6 – 2.72E-2	2.86E-4 – 4.17E-2	4.87E-5 – 8.02E-2
Mean ± SD.	4.39E-3 ± 4.83E-3	4.96E-3 ± 8.19E-3	8.98E-3 ± 1.30E-2	1.92E-2 ± 3.09E-2
Median	3.09E-3	1.95E-3	4.23E-3	1.42E-3
IQR	4.06E-4 – 6.57E-3	1.24E-3 – 4.92E-3	3.10E-3 – 5.04E-3	8.80E-4 – 2.18E-2
Z (p)	Z = 0.459 (p = 0.646)		Z = 0.051 (p = 0.959)	
Bifidobacteria				
Min. – Max.	1.05E-3 – 1.29E-1	7.06E-4 – 5.57E-2	1.27E-3 – 1.91E-1	7.17E-3 – 4.72E-1
Mean ± SD.	2.86E-2 ± 4.28E-2	1.43E-2 ± 1.59E-2	6.51E-2 ± 6.13E-2	1.08E-1 ± 1.56E-1
Median	7.25E-3	1.04E-2	5.67E-2	3.01E-2
IQR	3.09E-3 – 5.09E-2	3.66E-3 – 1.62E-2	8.99E-3 – 1.07E-1	1.58E-2 – 1.57E-1
Z (p)	Z = 0.357 (p = 0.721)		Z = 0.764 (p = 0.445)	

IQR: Inter quartile range; SD: Standard deviation; Z: Wilcoxon signed ranks test
p: *p value* for comparing between Before Diet and After Diet; *: Statistically significant at $p \leq 0.05$

Table (S6b): Comparison between the three subgroups of GI severity index in ASD cases before and after diet "continue"

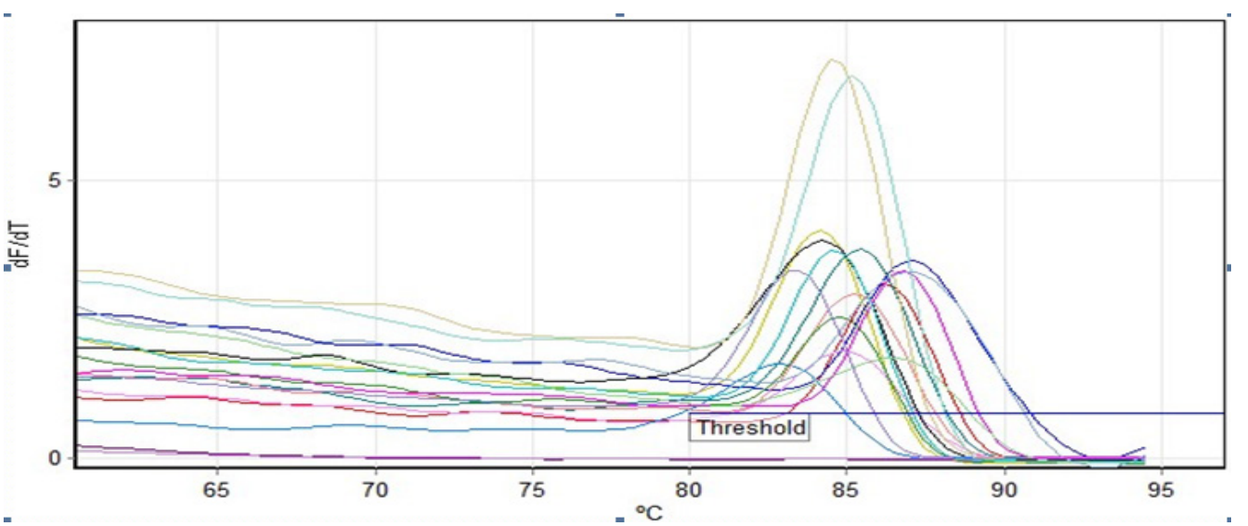
Bacteria	GSI <4 (*n* = 10)		GSI ≥4 (*n* = 10)	
	Before Diet	After Diet	Before Diet	After Diet
Akkermansia muciniphila				
Min. – Max.	4.99E-6 – 3.65E-1	1.49E-5 – 1.86E-2	5.25E-5 – 7.01E-2	2.02E-6 – 8.80E-2
Mean ± SD.	4.76E-2 ± 1.16E-1	4.75E-3 ± 6.90E-3	1.23E-2 ± 2.29E-2	1.98E-2 ± 3.03E-2
Median	2.54E-4	1.07E-3	7.91E-4	4.66E-4
IQR	8.16E-5 – 7.23E-3	2.18E-4 – 7.16E-3	1.18E-4 – 1.63E-2	1.19E-5 – 3.20E-2
Z (p)	Z = 0.051 (*p* = 0.959)		Z = 0.051 (*p* = 0.959)	
Faecalibacterium prausnitzii				
Min. – Max.	2.50E-3 – 1.88E-1	1.10E-2 – 1.36E-1	2.73E-2 – 2.60E-1	4.61E-2 – 4.05E-1
Mean ± SD.	7.95E-2 ± 5.21E-2	8.64E-2 ± 4.66E-2	1.07E-1 ± 7.01E-2	1.96E-1 ± 1.30E-1
Median	7.18E-2	9.78E-2	7.61E-2	1.58E-1
IQR	4.44E-2 – 1.09E-1	5.13E-2 – 1.22E-1	6.79E-2 – 1.52E-1	1.19E-1 – 3.17E-1
Z (p)	Z = 0.255 (*p* = 0.799)		Z = 1.682 (*p* = 0.093)	
Clostridium difficile				
Min. – Max.	0.00E+0 – 0.00E+0	0.00E+0 – 9.58E-7	0.00E+0 – 0.00E+0	0.00E+0 – 1.44E-5
Mean ± SD.	0.00E+0 ± 0.00E+0	9.58E-8 ± 3.03E-7	0.00E+0 ± 0.00E+0	1.62E-6 ± 4.53E-6
Median	0.00E+0	0.00E+0	0.00E+0	0.00E+0
IQR	0.00E+0 – 0.00E+0	0.00E+0 – 0.00E+0	0.00E+0 – 0.00E+0	0.00E+0 – 0.00E+0
Z (p)	Z = 1.000 (*p* = 0.317)		Z = 1.342 (*p* = 0.180)	
Desulfovibrio				
Min. – Max.	4.59E-2 – 4.12E-1	1.36E-2 – 2.62E-1	4.92E-6 – 3.03E-1	2.15E-2 – 3.47E-1
Mean ± SD.	1.48E-1 ± 1.11E-1	1.43E-1 ± 8.98E-2	1.25E-1 ± 8.60E-2	1.65E-1 ± 1.26E-1
Median	1.20E-1	1.48E-1	1.10E-1	1.15E-1
IQR	7.07E-2 – 1.87E-1	8.88E-2 – 2.29E-1	7.08E-2 – 1.80E-1	6.30E-2 – 2.99E-1
Z (p)	Z = 0.051 (*p* = 0.959)		Z = 0.663 (*p* = 0.508)	
Sutterulla				
Min. – Max.	3.73E-3 – 1.16E-1	2.62E-4 – 7.94E-2	4.36E-3 – 6.44E-2	1.31E-3 – 8.13E-2
Mean ± SD.	3.26E-2 ± 3.21E-2	2.42E-2 ± 2.95E-2	2.12E-2 ± 1.96E-2	3.27E-2 ± 3.06E-2
Median	3.18E-2	9.91E-3	1.40E-2	2.00E-2
IQR	9.12E-3 – 3.70E-2	3.68E-3 – 4.92E-2	7.32E-3 – 2.75E-2	9.17E-3 – 6.80E-2
Z (p)	Z = 1.070 (*p* = 0.285)		Z = 1.172 (*p* = 0.241)	
Bacteroides fragilis				
Min. – Max.	3.19E-5 – 1.97E-1	1.90E-3 – 1.34E-1	1.20E-5 – 3.56E-1	7.97E-6 – 6.10E-1
Mean ± SD.	3.02E-2 ± 6.10E-2	2.58E-2 ± 4.07E-2	7.46E-2 ± 1.38E-1	8.95E-2 ± 1.85E-1
Median	5.28E-3	1.00E-2	7.32E-3	2.86E-2
IQR	5.45E-5 – 4.08E-2	5.30E-3 – 2.02E-2	2.32E-4 – 4.49E-2	1.03E-2 – 6.45E-2
Z (p)	Z = 0.764 (*p* = 0.445)		Z = 0.968 (*p* = 0.333)	

IQR: Inter quartile range; SD: Standard deviation; Z: Wilcoxon signed ranks test
p: *p value* for comparing between Before Diet and After Diet; *: Statistically significant at $p \leq 0.05$

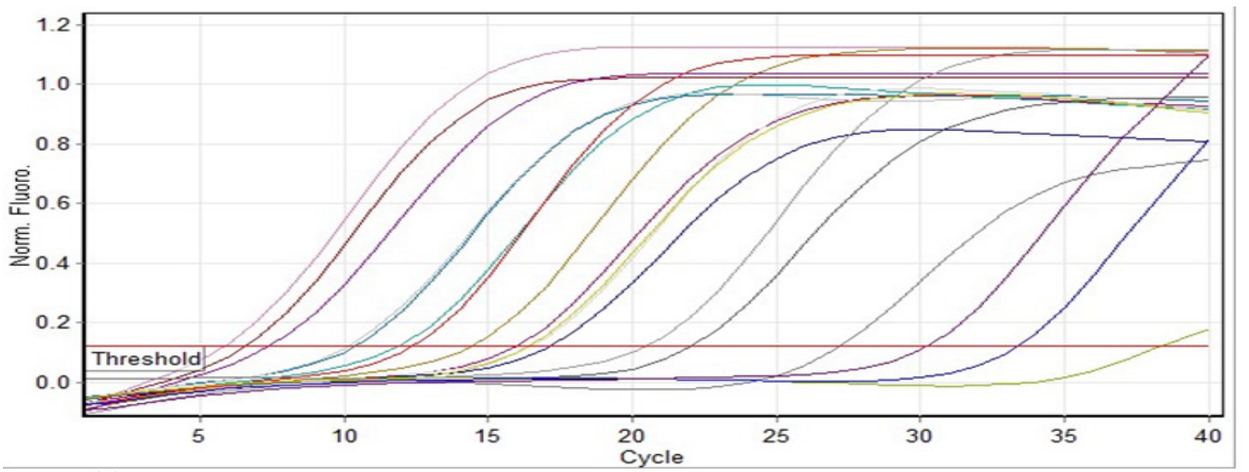
Table (S6c): Comparison between the three subgroups of GI severity index in ASD cases before and after diet "continue"

	GSI <4 (n = 10)		GSI ≥4 (n = 10)	
	Before Diet	After Diet	Before Diet	After Diet
P/B				
Min. – Max.	0.0010 – 5.86	0.001 – 3.74	0.00003 – 2.67	0.0002 – 0.51
Mean ± SD.	1.49 ± 2.10	0.97 ± 1.21	0.33 ± 0.83	0.20 ± 0.22
Median (IQR)	0.34 (0.01 – 1.83)	0.41 (0.10 – 1.57)	0.05 (0.001 – 0.14)	0.12 (0.01 – 0.47)
Z (p)	Z = 1.070 (p = 0.285)		Z = 0.866 (p = 0.386)	
F/B				
Min. – Max.	0.17 – 1.12	0.11 – 3.61	0.19 – 1.43	0.20 – 1.73
Mean ± SD.	0.68 ± 0.34	0.92 ± 1.07	0.80 ± 0.42	0.84 ± 0.50
Median (IQR)	0.62 (0.51 – 1.09)	0.49 (0.28 – 1.29)	0.85 (0.30 – 1.15)	0.80 (0.40 – 1.22)
Z (p)	Z = 0.153 (p = 0.878)		Z = 0.153 (p = 0.878)	
Diversity index				
Min. – Max.	1.41 – 2.05	1.02 – 1.87	1.25 – 2.01	1.61 – 2.19
Mean ± SD.	1.71 ± 0.22	1.66 ± 0.24	1.74 ± 0.21	1.85 ± 0.19
Median (IQR)	1.68 (1.57 – 1.93)	1.72 (1.62 – 1.81)	1.78 (1.70 – 1.82)	1.82 (1.69 – 1.99)
t (p)	t = 0.531, (p = 0.608)		t = 1.443, (p = 0.183)	
DSI				
Min. – Max.	38.0 – 70.0	25.0 – 69.0	27.0 – 65.0	40.0 – 69.0
Mean ± SD.	48.80 ± 9.67	50.20 ± 12.46	45.76 ± 12.85	53.37 ± 9.26
Median (IQR)	47.0 (42.0 – 55.0)	51.0 (44.0 – 55.0)	47.0 (36.0 – 57.0)	56.0 (45.80 – 59.0)
t (p)	t = 0.287, (p = 0.781)		t = 2.023, (p = 0.074)	

IQR: Inter quartile range; SD: Standard deviation; Z: Wilcoxon signed ranks test; t: Paired t-test
p: *p value* for comparing between Before Diet and After Diet; *: Statistically significant at $p \leq 0.05$



(A)



(B)

Fig. 1: Melting curve (A), and PCR amplification plot (B) of ASD Child case
(A) The melting curve illustrates that each primer has a distinct peak, representing its unique melting point (eg. 87°C for total bacteria primer, 83°C for Bacteroidetes primer, 89°C for Bifidobacteria primer and 83°C for Firmicutes primer, etc..).
(B) The PCR amplification plot shows a sharp, exponential increase in fluorescence at the beginning of the reaction and low cycle threshold (Ct), indicating detectable levels of target DNA.

DISCUSSION

Our results showed a significant improvement after 3 months following the exclusion diet evident by a significant decrease in the total ATEC score as well as the ATEC sub-scales, except for the sensory/cognitive awareness subscale; the speech/language/communication, sociability as well as health and physical abilities subscales were all significantly decreased. At the level of individual cases, the total ATEC score showed a markedly effective decrease in 30% of cases and effective in 40% of cases, that is totally effective in 70% of cases. As regards the subscales, the reduction of sociability and health/physical/ behavior sub scores was totally effective in 80% of cases but for the speech/language/communication subscale in only 32% of cases.

A significant decrease in ATEC score was also reported by ***Wang et al.***, after 30 to 60 days of probiotics and fructo-oligosaccharide intervention, and a significant reduction in severity of autistic symptoms was in speech/language/ communication and sociability subscales [34]. This conforms also with results of preliminary study of probiotics supplement, where the total and the four domains of ATEC

score decreased after probiotics supplement [35]. Another study assessed the effect of ketogenic versus gluten free diet in ASD children and both diet groups showed significant improvement in ATEC after 6 months [36].

Regarding sensory impairment, 70% of our participants had definite sensory impairment, 25% had probable impairment, and only one (5%) had a typical performance at the start of the present study. After 3 months, 35% of our participants showed definite sensory impairment, 30% had probable impairment, and 35% had a typical performance. There was a significant improvement in movement sensitivity, low energy/weak, under responsive/ seek sensation, and taste/smell sensitivity sub scores. The SSP total score improved by 12.5%, which was similar to ***Abele et al.***, who reported a 13% improvement following specific carbohydrate diet [16]. This also conforms with another study that reported the improvement of ASD children in hyperactivity, irritability, attention, aggression/ agitation, anxiety, cognition, sensory sensitivity, and the ability to fall asleep after following the Feingold diet, a diet without any artificial food additives [37].

Although the improvement in sensory skills was not evident in ATEC score, however, the more detailed SSP score revealed significant improvement in several sensory parameters as movement sensitivity, low energy/ weak, under-responsive/seek sensation, and taste/smell sensitivity. This highlights the importance of taking more than one scale to monitor the patient improvement.

As regards the GI manifestations, 85% of our study participants had at least one GI symptom. The mean 6-GSI score was 3.6 with 50 % had severe score. Flatulence and abnormal stool smell were the most common GI symptoms. After 3 months, there was a significant decrease in the 6-GSI score to 1.6 and only 15% showed severe score. There was a statistically significant decrease in stool smell, flatulence, and abdominal pain sub scores. Similar findings were reported by ***Berding et al.***, where a healthy diet, characterized by higher intake of vegetables, fruits, legumes, nuts, and seeds, along with lower sugar consumption, was linked to a more favorable gut microbiome and improved gastrointestinal (GI) symptom scores [38]. However, in our study, despite improvements

in GSI scores and GI symptoms, these changes were not associated with significant alterations in the gut microbiome.

Shaaban et al. reported a significant improvement in the total 6-GSI score, with notable reductions in the scores for abdominal pain, constipation, stool consistency, and flatulence following probiotic supplementation[39]. In contrast, a randomized controlled single blinded trial demonstrated no significant differences in the gastrointestinal symptoms between ASD children on gluten free diet and ASD children on gluten containing diet[40]. Differences between various studies might be

caused by various factors, including the compliance rate of patients as well as the type of diet followed and types of food categories that were either excluded or introduced.

In this study, the ASD children exhibited dysbiosis, evidenced by their lower Firmicutes/Bacteroidetes (F/B) and Prevotella/Bacteroides (P/B) ratios, and higher relative abundance of Bacteroidetes and Bacteroides compared to the TD control group. Our exclusion diet modulated slightly the gut microbiota, reflected by an increase in the Prevotella/Bacteroides (P/B) ratio, which rose by threefold, however, this change not achieve statistical significance. Despite the lack of significant changes in the relative abundances of individual bacterial taxa, the observed increase in the P/B ratio might indicate functional shifts within the microbial community, potentially influencing gut metabolic processes evident by significant decrease in flatulence, stool smell, and abdominal pain GSI sub scores.

In terms of clinical outcomes, the number of cases with definite sensory impairment decreased from 14 to 7 cases and the number of children with severe GSI score decreased from 10 to 3 following the dietary intervention. The P/B ratio increased fivefold and twofold after the diet in children with definite sensory impairment and severe GSI score respectively, yet this difference was also not statistically significant.

In addition, by comparing our cases after 3 months with TD control, there was a significant rise in abundances of B. fragilis and Ruminococcus that which might be an indirect indicator of changes in the microbiome that were diet-associated.

The role of Ruminococcus in ASD is a subject of debate and ongoing research. Some studies have suggested that certain species or strains of Ruminococcus may be more prevalent in individuals with ASD compared to neurotypical individuals. On the other hand, because of its possible influence on arginine metabolism, there is a hypothesis that links autism to decreased Ruminococcus abundance which might lead to increased blood arginine concentrations, which can produce more neurotoxic nitric oxide [41].

Bacteroides fragilis plays a protective role against intestinal inflammatory conditions through production of interleukin-10 (IL-10) released by regulatory T cells and production of polysaccharide A which is an immunomodulatory molecule [42].

A trial to evaluate the effect of exclusion diets and prebiotics on ASD children showed that children on exclusion diets (mainly gluten and casein free) reported significantly lower scores of abdominal pain and bowel movement, along with a reduced abundance of Bifidobacterium spp. but increased presence of F. prausnitzii and Bacteroides spp.[43]. Newell et al., showed that ketogenic diet increased the F/B ratio in ASD-mimicking black and tan brachyury (BTBR) mice [44], while another study found no significant difference in the total abundance of bacteria or in the number of bacterial species in the fecal sample of BTBR mice before and after ketogenic diet [45].

The observed improvements in clinical outcomes in the present study, including the reduction in 6-GSI and ATEC scores and the increase in SSP scores after three months of dietary intervention, suggest that the diet contributed to slight gut microbiota modulation but a marked clinical improvement. However, given the relatively short duration of the follow-up (three months), it is possible that more pronounced microbiota shifts or stronger associations with clinical outcomes may emerge over a longer timeframe. It could be suggested that the reason behind the disproportionate improvement in gut microbiome (which was slight) with the marked clinical improvement might be owing to other mechanisms than gut microbiome, such as reduction of the systemic inflammation following the exclusion diet, potentially leading to improvements in ASD symptoms inflammatory mediators. However, this point was not tackled in our study but is recommended to be followed up in future work.

The improvement in ASD symptoms in the present study is comparable to other studies using specific diets and probiotics [46]. Also, all severe ASD cases were compliant probably due to their eagerness and desperation for improvement. In addition, our regimen is more affordable, which is crucial given Egypt's high inflation rates and rising food prices, particularly for low-income families. An exclusion diet based on preparing food from raw ingredients, rather than processed and ready-made options, may offer a more cost-effective approach for families of children with ASD. In contrast, special diets like gluten-free, casein-free, or ketogenic diets can impose additional financial burdens, alongside the existing medical, therapeutic, and educational expenses [47].

LIMITATIONS

There are some limitations in this study such as small sample size. Follow-up of patients for a longer time might have shown more significant improvement in ASD parameters. However, due to the self- funded nature of our research, follow up was not possible beyond the 3 months period owing to its associated higher expenses. Another limitation of our study is that we utilized real-time PCR while most of the studies use sequencing analysis, therefore, our findings might not encompass the full spectrum of microbiome variations found in the stool samples.

CONCLUSION

Mounting evidence confirmed the alterations in the gut microbial composition in ASD children. We concluded that exclusion of processed foods, additives, and preservatives may serve as a potential dietary therapy in children with ASD, leading to improvement in sociability, behavior, sensory processing, and gastrointestinal symptoms comparable to other probiotics and special exclusion diets interventions studies.

LIST OF ABBREVIATIONS

ATEC: Autism Treatment Evaluation Checklist

BTBR: Black and tan brachyury (a strain of mouse model has phenotypic similarities to ASD humans)

CARS: Childhood Autism Rating Scale

DSM-5: Diagnostic and Statistical Manual of Mental Disorders Fifth Edition criteria

STATEMENTS AND DECLARATIONS

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study follows the principles of the Declaration of Helsinki. Ethical approval was obtained from the Faculty of Medicine's Ethics Committee at Alexandria University, with IRB number 00012098. Parents or guardians of all study participants provided written consent following an explanation of the study's purpose and benefits.

CONSENT FOR PUBLICATION

Not applicable.

DATA AVAILABILITY

All data will be provided by the corresponding author upon reasonable request.

COMPETING INTERESTS

The authors declare that they have no conflict of interest.

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AUTHORS' CONTRIBUTIONS

All All authors critically reviewed and approved the final manuscript for publication. SMA and WB worked together to design the study protocol. EO and ME were responsible for data collection and patient recruitment. SMA and ME performed the gut microbiome analysis and contributed to the interpretation of the data. SMA, WB, and EO handled patient management. WM assisted with diet implementation and monitored patient adherence to the diet plan. SMA, EO, and ME contributed to writing the article, reviewed it critically, and approved the final manuscript for publication.

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تأثير اتباع "حمية الإقصاء" على تعديل ميكروبيوتا الأمعاء لدى الأطفال المصابين باضطراب طيف التوحد

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المقدمة: تشير الأدلة الناشئة إلى وجود ارتباط بين اضطراب ميكروبيوتا الأمعاء وتطور مرض التوحد. وقد تم ربط استهلاك المواد المضافة إلى الطعام، والأطعمة المعالجة، والدهون المتحولة، والأطعمة عالية السكر باضطراب ميكروبيوتا الأمعاء، مما قد يؤثر على محور الأمعاء والدماغ. هدفت هذه الدراسة إلى التحقيق فيما إذا كان استبعاد هذه الأطعمة من النظام الغذائي سيؤدي إلى تعديل ميكروبيوتا الأمعاء لدى أطفال التوحد المصابة بالاضطراب الميكروبيوتا وتحسين أعراض التوحد.

خضع عشرون طفلاً مصريًا مصابًا بالتوحد لتدخل غذائي لمدة ثلاثة أشهر تم خلاله استبعاد الأطعمة المعالجة، والمواد الحافظة والإضافات. تم جمع عينات براز قبل وبعد التدخل الغذائى لتحليل ميكروبيوتا الأمعاء باستخدام تقنية تفاعل البوليمراز المتسلسل لقياس الميكروبيوم المعوى السائد في الأمعاء على مستويات الفيلوم، والجنس، والنوع. تم تقييم الأعراض السريرية بما في ذلك شدة التوحد، وضعف الحواس، والأعراض المعوية باستخدام قائمة تقييم علاج التوحد ومؤشر شدة اضطرابات الجهاز الهضمي وبروفايل الحواس القصير.

النتائج: بعد ثلاثة أشهر من اتباع النظام الغذائي الاستبعادي، أظهر الأطفال المصابون بالتوحد تحسنًا كبيرًا في درجات تقييم علاج التوحد ومؤشر شدة اضطرابات الجهاز الهضمي وبروفايل الحواس القصير. وكذلك انخفاضا كبيرا في الجزء الخاص بمقياس السلوك الاجتماعى والجزء الخاص بالمقياس الصحى والبدنى والسلوكى في قائمة تقييم علاج التوحد. ومن الجدير بالذكر أن عدد الحالات التي كانت تعاني من ضعف حسي واضح انخفض من ١٤ إلى ٧ حالات، كما انخفض عدد الأطفال المصابين باضطراب طيف التوحد مع درجة شديدة من اضطرابات الجهاز الهضمى من ١٠ الى ٣ حالات.

بالنسبة لبروفايل ميكروبيوم الأمعاء، على الرغم من زيادة نسبة البريفوتيلا الى العصوانية ثلاثة أضعاف، إلا أن الفرق لم يكن ذا دلالة إحصائية. بشكل عام، لم تُلاحظ أي اختلافات جوهرية في ملف ميكروبيوم الأمعاء بعد ثلاثة أشهر من اتباع نظام الاستبعاد الغذائي.