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Research Article

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Molecular characterization of probiotic in autistic children stool

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ABSTRACT

The aim of this study is to compare between the count of Bifidobacterium spp. and Lactobacillus spp. as probiotic in stool of both autistic and non-autistic children. Bifidobacterium spp. and Lactobacillus spp. were assessed in stool samples of 40 autistic children and 20 healthy typical children of similar ages which consider as control group. The study indicated that the counts of both Bifidobacterium spp. and Lactobacillus spp. were lower in the stool of autistic children than that of their control group. It was significant in the case of Lactobacillus spp. with (p value 0.05) and highly significant in the case of Bifidobacterium spp. with (p value 0.000) and the count of bacteria wasn't affected by sex (male or female).

Key words: Probiotics, Autism, *Bifidobacterium spp., Lactobacillus spp.*

INTRODUCTION

Autism Spectrum Disorder (ASD) is a complex neurodevelopmental condition represents social abnormalities, communication impairments and stereotyped and repetitive behaviors. It has begun to be studied after sharp rising in the last decade [1].

The increased attention of autism was induced firstly, by the growing rates of ASD and secondary, on the common gastrointestinal (GI) manifestations in these people, where up to 90% of ASD children suffer from GI disorders e.g. gastroesophageal reflux, constipation, diarrhea, abdominal pain, vomiting and nutrition issues [2].

Recent studies have correlated gut dysfunction with ASD and suggested a possible role of the gastrointestinal (GI) microflora in severity of symptoms in autistic children [3]. Many autistic children experience severe GI problems including abdominal pain, constipation, diarrhea, and bloating. These symptoms may be due to the disruption of the indigenous gut flora promoting the overgrowth of potentially pathogenic microorganism [3].

There is considerable evidence that GI disorders are linked to intestinal dysbiosis. Gut microbiota plays a significant role in modulating human metabolism and in the development of the immune system. The cellular and biochemical pathways of gut-brain interaction provide a basis for the influences of normal gut microbiota on development neurochemistry, gene expression, and functioning of the brain [4].

Probiotic bacteria are essential for healthy gastrointestinal function. The action of probiotics on intestinal flora results in vital benefits, including protection against pathogens, development of the immune system [5] and positive effects on colonic health and host nutrition [6].

Probiotics probably function by altering the composition of the colonizing microbiota and by direct interaction with the host through immune signaling mechanisms [7].

Probiotics are capable of stabilizing the mucosal barrier by increasing g mucin expression, reducing bacterial overgrowth, stimulating mucosal immunity (secretory IgA), and synthesizing antioxidant substances [8]**.** The aim of this study was to find out whether there are differences between the count of *Bifidobacterium spp*. and *Lactobacillus spp.* as probiotic in stool of both autistic and non-autistic children.

EXPERIMENTAL SECTION

Subjects

In this study 40 autistic children and 20 non-autistic children as control were investigated. The age of control and autistic children was from 2 to 5 years old. Children with autism were recruited from the autism clinic in National Research Center. All subjects were medication-free.

Assessing the proportion of *Lactobacillus spp.* and *Bifidobacterium spp.*, which present in the stool of autistic and healthy children.

Fecal specimens: Stool specimens were collected by parents, kept in sterilized caps at +4°c and delivered to PCR laboratory within 4 hours.

Preparation of samples

A 10 g sample was taken aseptically and homogenized in 90 ml of sterilized Ringer solution (Merck, Germany) to be prepared for DNA extraction [8]**.**

DNA extraction from samples

Processed product samples, 1:10 diluted in Ringer solution, were centrifuged at 13,000 rpm for 10 min after which the supernatant was removed and the remaining pellet was subjected to DNA extraction [9]**.**

DNA extraction via modified heat shock/boiled-cell Method

1 ml sterile distilled water was added to the pellet, vortexed and subjected to heating temperature of 100°Cfor 20 min. The suspension was then cooled immediately to -20°C for 20 min and centrifuged at 13,000 rpm for 3 min before the supernatant was kept in freezer (0 - 5°C) [10]**.**

Primers

Two different genus-specific primer sets were used in this study, (g-Bifid-F/g-Bifid-R) for *Bifidobacterium* [11] and (Lacto- 16S-F / Lacto-16S-R) for *Lactobacillus* [12].

PCR reaction for samples

PCR reaction for DNA extracted from samples, was carried out in a total volume of 25 µl with a reaction mixture containing 2.5 µl of 10 x PCR buffer, 1.5 µl of 25 mM MgCl2, 0.5 µl of 10 mMdNTP, 1.65 µl of 15 µM forward and reverse primers of g-Bifid-F / g-Bifid-R and Lacto-16S-F / Lacto-16S R, 0.125 µl of 5uµl-1 Taq DNA polymerase, 3 µl of genomic DNA (~10 ng) and 14.075 µl of sterile distilled water. All the reaction mixtures were obtained from Promega Corporation, Madison, USA [13]**.**

The reaction mixture in micro-centrifuge tube was amplified in a thermocycler PCR system (PTC-110TM Model, MJ Research, Inc., USA). For *Bifidobacterium spp.*, the initial denaturation was performed at 95°C for 3 min and the target DNA was amplified in 40 cycles. Each cycle consisted of denaturation (95°C, 30 s), annealing (57°C, 30 s) and extension (73°C, 60 s). The final extension step was performed at 73°C for 5 min and the holding temperature was 10°C. For *Lactobacillus spp*. for the annealing temperature which was performed at 61°C for 30 s. It is noteworthy to mention that negative control, master mix devoid of genomic DNA and positive control, a positive sample taken from previous experiments, were used simultaneously in duplicates.

Gel electrophoresis

The amplified PCR products were checked for the expected size on 1.5% (w/v) agarose gel (LE analytical grade, Promega, Madison, USA). Ten µl of each PCR amplified product and 3 µl of 6 x Loading Dye were loaded into agarose gel and run in 1 x TBE buffer (0.089 M Tris-HCl, 0.089 M Boric acid, 0.002 M EDTA, pH 8.3) (Promega, USA). A ready-to-use VC 100 bp Plus DNA Ladder - molecular weight standard (Vivantis, Italy) was used along with positive control, negative control and PCR amplified products. The PCR products were separated by an electrophoresis system at a constant voltage of 80 V for 50 min. Then, the gel was stained in ethidium bromide (Sigma, USA) staining (0.5 µgml-1) for 5 min and followed by washing with distilled water for about 30 min. Finally, the gel was visualized under UV transilluminator (Vilber Lourmat, Cedex, France) and the photos were taken using gel documentation system (Bio Rad Gel Doc 2000 Model Imaging System) [14]**.**

Standard curve for real-time PCR

In order to quantify *Lactobacillus spp.* and *Bifidobacterium spp*. in positive samples by PCR sequencing, serially diluted DNA of standard *Lactobacillus brevis* ATCC 14869 and the standard *Bifidobacterium longum* strain JCM 1260 were used to generate a standard curve for the mathematical conversion of Ct values into bacterial cell number. Ct value is the cycle number where the reaction fluorescence exceeds background fluorescence. Two different standard curves for *Bifidobacterium spp.* and *Lactobacillus spp*. were generated. Stock plasmid DNA was prepared for these two different bacteria. For the preparation of plasmid DNA, the purified PCR product of interest was prepared first. Ligation of PCR product of interest into pGEM®-T Easy Vector (Promega, Madison, USA) which consisted of a mixture of 2 x rapid ligation buffer, pGEMTR Easy Vector, purified PCR product, T4 DNA ligase and sterile distilled water. E. coli competent cells of strain JM 109 were used to carry pGEM®-T Easy Vector (Promega, Madison, USA) that had been ligated with the target sequence from each bacterial genus. The screening of plasmid insert was done, where the white colony indicated that the PCR product gene of interest was successfully cloned and transformed. The white colony was inoculated into Luria broth supplemented with ampicillin. The plasmid DNA of interest was then extracted from the cell according to the manufacturer's instruction using Wizard® Plus SV Minipreps (Promega, Madison, USA). The initial concentration of the plasmid DNA of interest was 10.6 ug/ml. The plasmid with the correct insert was then 10-folds serially diluted into six dilutions. Since the molecular weight of the plasmid DNA is known, the concentration of these dilutions were then transformed into the log copy number which was plotted against the threshold cycle (Ct) to generate the standard curve used for the absolute quantification of real-time PCR [13]**.**

Quantitative real-time PCR

After the conventional PCR, the genomic DNA obtained from the tested samples was used for the real-time PCR amplification. Genomic DNA of standard *Bifidobacterium* and *Lactobacillus* strains were included in the real-time PCR assay as a positive control while PCR mixture solution devoid of genomic DNA was used as negative control. The serially diluted bacterial standard and samples were simultaneously assayed in real-time amplification [13]**.**

The PCR program consisted of an initial denaturation step, amplification step (40 cycles) and a melting-curve determination step. The condition for the amplification was as same as the conventional PCR. Following amplification, melting temperature analysis of PCR products was performed to determine the specificity of the PCR. The melting curves were obtained by slow heating at 0.2°Cs-1 increments from 60 - 99°C, with continuous fluorescence collection. Analysis of PCR amplification and melting curves were done by Rotor- Gene Real-Time Data Acquisition and Analysis Software version 1.7 (Corbett Research, Australia). Measurement of the SYBR Green fluorescence was performed at the end of each amplification step and continuously during the melt-curve analysis [15]**.** A melting curve would be generated at the end of amplification for monitoring specificity of PCR reaction

Statistical analysis

Data were analyzed using one-way ANOVA and t –test to evaluate the differences between groups. All calculations were performed with XL Statistics 5.0 and Microsoft Excel 2007. Data are presented as means ± SD. For data correlation was used Pearson correlation coefficients [16]. P values less than 0.05 were considered significant.

RESULTS AND DISCUSSION

We enrolled in this study 40 autistic children recruited from the autistic child clinic of the National Research Centre (from December 2015 to February 2016). Also, twenty normal healthy typically developing children of matched age and sex served as control.

In autistic group: Females represented 35.0 % of the patients and males represented 65.0 % of the patients.

In control group: Females represented 50.0 % of the children and males represented 50.0 % of the children, as shown in table (1).

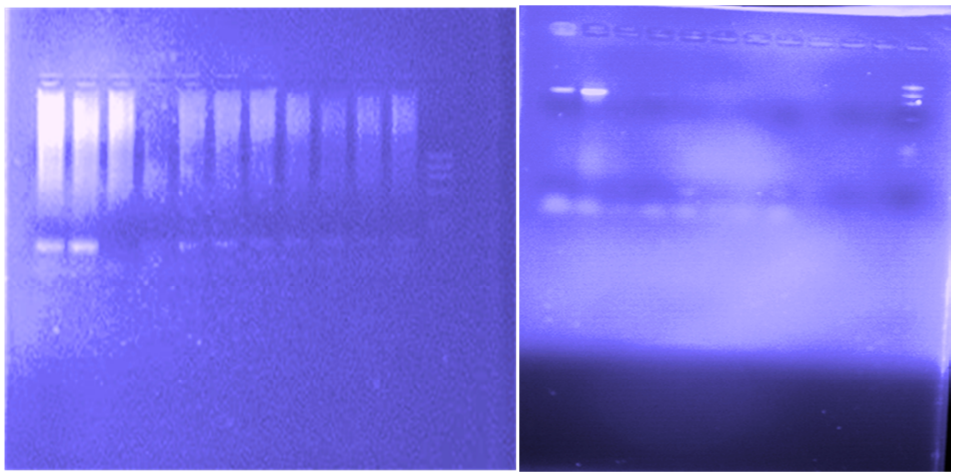
Table 1 Descriptive data of the children

		Control	Autistic
Sex	**Female**	10(50.0%)	14 (35.0%)
	Male	10(50.0%)	26 (65.0%)
Total		20 (100%)	40 (100%)

Table (2) shows that at the start of the study the mean weight of autistic children (26.91 ± 5.32) was higher than that of control group (25.35 ± 4.61) and the mean BMI of autistic children was significantly higher than that of control group with (*P value 0.009*).

Table 2 Baseline anthropometric measures of both groups

Variable	Autistic	Control	P-value
Weight (kg)	26.91 ± 5.32	25.35 ± 4.61	0.291
Height (cm)	112.48 ± 8.27	112.38 ± 7.80	0.964
BMI (kg/m^2)	17.04 ± 1.36	16.08 ± 1.00	0.009



(a) **(b)**

Fig.1. Gel electrophoresis images of genomic DNA extracted from (a) *Lactobacillus spp*. and (b) *Bifidobacterium spp*

Figure 1 show that the DNAs extracted from (*Lactobacillus ssp*. and *Bifidobacterium spp*.) respectively were observed for degradation by agarose gel electrophoresis. It was observed that all DNAs produced bands at the uppermost part of the gel.

Table (3): shows that both *Bifidobacterium spp.* and *Lactobacillus ssp.* count were lower in the stool of autistic children than that of their control group. It was significant in the case of *Lactobacillus spp.* with *(p value 0.05*) and highly significant in the case of *Bifidobacterium spp.* with (*p value 0.000)*

Table 3 The count of *Lactobacillus spp.* and *Bifidobacterium spp.* in stool of both groups using PCR

	Bifidobacterium spp.		***Lactobacillus spp.***	
	Control	**Autistic**	**Control**	**Autistic**
Mean	7.55 ±0.02	6.56 ±0.06	7.92 ±0.04	7.18 ±0.05
P value	0.000		0.047	

Table (4) shows that both *Bifidobacterium spp.* and *Lactobacillus spp.* count were lower in the stool of (male &female) autistic children than that of their control group. It was significant in the case of *Lactobacillus spp.* for both male and female and highly significant in the case of *Bifidobacterium spp.* The deficiency in the count of bacteria wasn't affected by sex (male or female).

Table 4 The count of *Lactobacillus spp.* and *Bifidobacterium spp.* in stool of males and females in autistic and healthy children using RCR

	Bifidobacterium spp.				***Lactobacillus spp.***			
	Female		**Male**		**Female**		**Male**	
	control	**Autistic**	**Control**	**Autistic**	**control**	**Autistic**	**Control**	**Autistic**
Mean	7.52±0.02	6.59±0.06	7.58±0.02	6.53± 0.06	7.94±0.03	7.14±0.04	7.90±0.01	7.22±0.05
P value	0.000		0.001		0.042		0.02	

Estimates of ASD in pediatric populations have rising sharply over the past decade [17]. We enrolled in these study forty autistic children the range from2-5 years old, with females representing 14 (35.0 %) and males 26 (65.0

%).Also, we included twenty normal healthy typically developing children of same age and sex representing the control group.

The anthropometric measurements (weight, height and BMI) were calculated for all participants and found that the autistic children were overweight than healthy children. In this study we found that the autistic patients had a highly significant mean Body Mass Index (BMI) than their matched control group with P value 0.009.

Our results are in agreement with [18] who found that the majority of the autistic children were overweight or obese than healthy children of same gender and age. Also, [19] reported that autistic children have high mean Body Mass Index (BMI) compared with healthy children of same gender and age, and the gain in weight in autistic children may be due to the higher caloric intake and they have suggested an association between weight gain and the use of antipsychotic medications such as resperidone, commonly used for the treatment of these individuals.

Sharma et al. [20] reported that autistic children were more likely to be obese than healthy children. The incidence of obesity in autistic children was 30.4% and 23.6% among non-autistic children. Shabayek [21] estimated the prevalence of obesity among autistic males and females as 15.8% and 16.1%, respectively.

Our results didn't agree with [22] who found that the Body Mass Index (BMI) of autistic children was significantly lower than that of healthy children of same gender and age. They reported that the exact reason for lowering BMI among autistic children is unknown. It might be due to the abnormal eating behavior of autistic children, which might result in reduced energy intake, another factor of lowering the Body Mass Index (BMI) of autistic children might be their hyperactivity.

Emond et al. [23] did not find any significant differences between the Body Mass Index (BMI) of autistic children and healthy children of same gender and age, despite parents reporting that the autistic children were difficult, selective and demanding eaters, who started eating solids relatively late and resisted trying new foods and that didn't agree with our results.

The stool samples for all participants were tested using quantitative real time PCR prior to the start of the study to compare the levels of beneficial bacteria (mainly *Bifidobacterium* and *Lactobacillus species*) between both the autistic group and the control group.

We found that the counts of both *Bifidobacterium spp.* and *Lactobacillus spp.* were lower in the stool of autistic children than that of their control group. It was significant in the case of *Lactobacillus spp.* with (*p value 0.047*) and highly significant in the case of *Bifidobacterium spp.* with (*p value 0.000)* and the count of bacteria wasn't affected by sex (male or female).

These differences in beneficial bacteria lead to an imbalance in the gut microbiota composition for autistic children [24].

In agreement with our study [25] observed the deficiency of the count of *Bifidobacteria* in children with autism compared with normal children. [26-27] reported that *Bifidobacterium spp.* were low in stool of autistic children.
The deficiency of beneficial intestinal microflora may lead to inflammation or immune dysfunction, food intolerance and constipation or diarrhea [28]. The cause in the deficiency of beneficial bacteria appears to partly relate to the excessive use of oral antibiotic without medical supervision which can alter gut flora. Loss of normal gut flora can result in overgrowth of pathogenic flora which may lead to constipation and several problems.

However, [29] reported that their was no significant differences in the composition of the microbiota of autistic children compared with healthy children of same gender and age. They suggest that GI dysfunction observed in the ASD population could be caused by a variety of other factors, including elevated anxiety and self-restricted diets. A study by [29] found no significant differences in the gut microbiota of autistic children as compared to their healthy children of same gender and age.

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