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Gastrointestinal microbiota in children with autism in Slovakia

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HIGHLIGHTS

- Fecal microbiota in autistic children in Slovakia differs from controls and siblings.
- There is a correlation of the autism severity with the severity of GI dysfunction.
- In our study *Desulfovibrio* spp. abundance is associated with the severity of autism.
- Probiotic supplementation normalizes bacterial balance in fecal microbiota.
- No joined influence of oxytocin, testosterone, DHEAS and fecal microbiota on autism.

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ABSTRACT

Development of Autism Spectrum Disorders (ASD), including autism, is based on a combination of genetic predisposition and environmental factors. Recent data propose the etiopathogenetic role of intestinal microflora in autism. The aim of this study was to elucidate changes in fecal microbiota in children with autism and determine its role in the development of often present gastrointestinal (GI) disorders and possibly other manifestations of autism in Slovakia. The fecal microflora of 10 children with autism, 9 siblings and 10 healthy children was investigated by real-time PCR. The fecal microbiota of autistic children showed a significant decrease of the *Bacteroidetes*/*Firmicutes* ratio and elevation of the amount of *Lactobacillus* spp. Our results also showed a trend in the incidence of elevated *Desulfovibrio* spp. in children with autism reaffirmed by a very strong association of the amount of *Desulfovibrio* spp. with the severity of autism in the Autism Diagnostic Interview (ADI) restricted/repetitive behavior subscale score. The participants in our study demonstrated strong positive correlation of autism severity with the severity of GI dysfunction. Probiotic diet supplementation normalized the *Bacteroidetes*/*Firmicutes* ratio, *Desulfovibrio* spp. and the amount of *Bifidobacterium* spp. in feces of autistic children. We did not find any correlation between plasma levels of oxytocin, testosterone, DHEA-S and fecal microbiota, which would suggest their combined influence on autism development. This pilot study suggests the role of gut microbiota in autism as a part of the "gut-brain" axis and it is a basis for further investigation of the combined effect of microbial, genetic, and hormonal changes for development and clinical manifestation of autism.

1. Introduction

Autism spectrum disorders (ASD) are pervasive developmental disorders, characterized by social abnormalities, communication impairments, and stereotyped and repetitive behaviors. The incidence of ASD in 2010 was 1.47% in the USA [1] and it has been increasing worldwide for last decade. The interest in autism is based firstly, on the escalating incidences of ASD and secondarily, on the common gastrointestinal (GI) manifestations in these people. Up to 90% of children with ASD suffer from GI disorders such as gastroesophageal reflux, constipation, diarrhea, abdominal pain, vomiting and nutrition issues [2–4]. A direct correlation between the severity of autism and gastrointestinal symptoms has been shown [4,5]. Understanding the pathophysiology of the GI morbidity in ASD might be important for the early identification of ASD-related pathology and for guiding the therapy of GI symptoms and perhaps ASD.

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.

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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 [6–10]. The variations in the composition of gut microbes are associated with changes in the normal functioning of the nervous system and behavior [11,12]. For the last several years an escalating number of studies show the changes of gut microbiota in patients with ASD.

Possible involvement of a microbial element in pathogenesis of autism was shown for the first time in 1998, when the hypothesis was introduced that *Clostridium tetani* neurotoxin ascends along the vagus nerve route from the intestinal tract to the CNS causing the symptoms of autism [13]. Later the positive effect of the antibiotic vancomycin on the GI symptoms in autistic patients confirmed the involvement of gut bacteria [14]. Unfortunately, the effect was temporal and could be explained by bacteria spore formation [15]. Establishment of dysbiosis in autistic children [16], and health improvement after using probiotics [17] only rapidly increased the evidence, that the composition of gut microbiota is associated with changes in the normal functioning of the nervous system and behavioral changes in ASD. A recent pyrosequencing study serves as convincing evidence of dysbiosis in autistic subjects, including not only a change of ratio of normally present microorganisms, but a significant increase of bacterial diversity [18]. The investigation of the dominant intestinal bacterial phyla in metagenomic analyses disclosed a significant change in the *Bacteroidetes*/*Firmicutes* ratio in the feces of autistic children comparing to healthy or neurotypical children with GI symptoms [18,19]. Various bacterial species have been shown to be involved in dysbiosis in children with autism, particularly they had a higher bacterial incidence of *Desulfovibrio* spp. [20] and some *Clostridium* clusters [13,16,21] than the control children population. The diversity of bacterial species was also higher in autistic compared to neurotypical children. For example, they had more *Clostridia* species [18,22]. Conversely, other species were significantly reduced in gut microbiota of autistic children, like *Akkermansia muciniphila* and *Bifidobacterium* spp. [23]. And finally some bacterial species have been shown to be present almost exclusively in autistic gut microbiota, such as *Alkaliflexus* [18] and *Sutterella* [24] or some other bacteria were present but only in healthy subjects, as *Weissella* [18].

The hypothesis of the pathogenesis of ASD includes multiple mechanisms, a combination of genetic predisposition and environmental factors. Although there have been advances in identifying a genetic cause in ASD, recent studies of concordant twins suggest there is a stronger environmental component than previously believed [25,26]. Our study is aimed to make a step forward to elucidating the importance of environmental factors and possibly in combination with other factors leading to developing the disease.

Recent data suggest the connection of intestinal microflora content on health status of autistic individuals, and its possible etiological role in people with autistic predisposition. One of the latest theories of autism pathogenesis includes the etiopathogenetic role of specific bacteria (*Clostridia*, *Desulfovibrio* and *Bifidobacterium*) [27]. This is the reason why in our pilot study we investigated the intestinal microflora in children with autism in Slovakia and the specificity of their dysbacteriosis, compared to their siblings and to control group of neurotypical children. Moreover, we have also investigated the changes of microflora in a group of autistic children after probiotic therapy. Although over 50 bacterial phyla have been described in the human gut microbiota, we focused our study on the two most dominant, the *Bacteroidetes* and the *Firmicutes*. Special attention was paid to the bacteria described as being involved in manifestations of ASD (*Lactobacillus*, *Bifidobacterium*, *Clostridia*, *and Desulfovibrio*). For more complex investigation in the development of autism, we also looked at these children's oxytocin plasma levels and at such neurosteroids as testosterone and DHEAS, that were implicated to play an important role in autism development [28–30] and their possible coaction with intestinal microflora.

2. Materials and methods

2.1. Subjects

We enrolled in our study 10 autistic children, their 9 non-autistic siblings, and 10 non-autistic children as a control. 9 of 10 autistic children underwent probiotic intervention. The age of autistic children was from 2 to 9 years, siblings — from 5 to 17 years and control children from 2 to 11 years old. In the group of autistic subjects were included 9 boys and one girl, in the group of siblings — 7 boys and two girls, the control group consisted of 10 boys. Children with autism were recruited from the local Autism Centre for children in Bratislava, Slovakia. All autistic children were diagnosed as meeting criteria for ICD-10 childhood autism by a clinical child psychologist in cooperation with the child psychiatrist. Additional assessment was done in our study using the Childhood Autism Rating Scale (CARS) and the Autism Diagnostic Interview (ADI) (two of the children had no additional ADI assessment). Control subjects were recruited through local pediatricians. All control subjects had no psychiatric conditions confirmed by child psychiatrists according to their examinations and parent interview. All subjects were medication–free. Written informed consent was obtained from parents of participating children. The protocol was approved by the Ethics Committee of the Comenius University Faculty of Medicine. The study conformed to the code of ethics stated in the Declaration of Helsinki.

2.2. Clinical procedures

Psychological evaluation of the children with autism was performed using CARS [31] designated for identification and differential diagnostics of children with autism [32,33], and ADI [34], a semistructured interview for parents, who respond to the questions about a patient's behavior. We used the adjusted ADI version for research with 35 items in 4 content areas: social/reciprocal interaction, communication, speech and language and restricted/repetitive behavior, rated on 3 or 4 (5)–point scale.

Questions about GI symptoms were selected on the basis of claims made about potential links with ASD. The GI condition was evaluated based on the parental questionnaires, where the absence of a symptom was 0, its presence ranged from 1 to 4 depending on the severity of the symptom, thus the score includes the number of symptoms and their severity.

2.3. Probiotic supplementation

Dietary supplementation of one capsule of “Children Dophilus” containing 3 strains of *Lactobacillus* (60%), 2 strains of *Bifidumbacteria* (25%) and one strain of *Streptococcus* (15%) was given orally three times a day for 4 months.

2.4. Fecal specimens

Stool specimens were collected by parents, kept at +4 °C and delivered to our laboratory within 4 hours, where aliquots of 200 mg of each specimen were frozen at −80 °C until DNA extraction. The other aliquots were diluted 1:2 in phosphate-buffered saline (pH 7.2) containing 1% protease inhibitor cocktail (Sigma-Aldrich, Steinheim Germany) and centrifuged at 12,000 g at 4 °C for 10 min. Supernatants were collected and kept frozen at −80 °C until TNF-α detection.

DNA extraction from the fecal samples was performed from 200 mg of stool by QIAamp DNA Stool Mini Kit, (Qiagen, Hilden, Germany), according to the manufacturer's instructions with the final elution volume 100 μl of distilled water. The DNA concentration was determined by NanoDrop 1000 Spectrophotometer, (Thermo scientific, MA, USA).