Gut health of children with autism spectrum disorder

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Abstract. Autism spectrum disorder (ASD) is a complex neurodevelopmental disorder, frequently manifested by gastro-intestinal symptoms. The investigation of alterations in the gut microbiome and its role in the gut-brain axis communication is a promising field that may aid in the understanding of ASD symptoms. The present study thus focused on a preliminary investigation of the diversity, presence and drug-resistance of particular bacterial species in the gut microbiota of ASD and neurotypical children. The study was performed in a case-control manner and the relevant information of the participants was collected using dedicated questionnaires. Stool samples were collected from urban residents and the differences in microbial communities between the study and control groups were investigated and compared using culture-dependent methods. Since phages are considered as alternatives to antibiotics, as well as a promising tool for the re-instatement of disturbed microbiotas, antibiotic resistance and phage susceptibility profiles of the isolated intestinal pathogens were defined simultaneously. As a result, antibiotic resistant and phage susceptible β-haemolytic *Escherichia coli*, but no *Clostridioides difficile* were detected in the collected sample. The differences in intestinal health and gut micro-biota between the study groups were minor. On the whole, the findings of the present study may provide the basis for an extended metagenomics study of microbiota in the ASD population.

Introduction

Autism spectrum disorder (ASD) is a broad range of neuro-developmental disorders that is generally manifested during early childhood. ASD can be presented by a lack of social interaction and communication skills, repetitive and stereo-typed behaviour, restricted interests and activities. ASD is complex and may be associated with other comorbidities (1).

It is estimated that one to two percent of the general popu-lation is affected by ASD (2), and the condition is four-fold more common among males than females (3). The recorded increase in the prevalence of ASD over the past 50 years may be attributed to changes in the case definitions, increasing awareness, the availability of specialised centres, earlier detection, improved reporting and diagnostic substitution. Therefore, the true increase cannot yet be estimated (4,5). However, demographic and geographical variables, as well as the availability of resources are considered to influence the prevalence of ASD (6).

ASD has a multifactorial aetiology, which is not yet fully understood. Epigenetic interactions between genetic and environmental factors are considered as key elements involved in the onset of this disorder (7). Notably, in recent research, gastrointestinal (GI) symptoms have been shown to have a high incidence and association with the severity of ASD (8). Research into the functions and alterations in the microbiota-gut-brain axis has become a promising field which may aid in the understanding of ASD (9).

The gut-brain axis is a bidirectional communication pathway of the intestines and central nervous system, whereby the microbiota-immune axis functions as a central mediator in their communication (10). It has been shown that alterations in the microbiome-gut-brain axis may trigger neuroinflamma-tion, myelination, microglia maturation and the modulation

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of complex behaviours, such as anxiety (11). For example, according to previous studies, increased gut permeability, or 'leaky gut syndrome' plays a crucial role in modification of the normal function of the gut-brain axis (12). With the impaired intestinal luminal barrier, bacterial metabolites, such as short-chain fatty acids, increasingly pass the membrane, enter the bloodstream and reach the brain. They can alter the levels of the cytokine expression, triggering the immune response and the production of neurotransmitters, such as dopamine and serotonin (12,13). In the cases of ASD, it has been demonstrated that the concentration of Zonulin, the protein that modulates gut permeability, is higher in children with ASD compared to healthy controls; furthermore, the severity of the behavioural symptoms has been found to be associated with an increase in Zonulin levels (14). Therefore, alterations in the gut microbiota may have a tangible effect on the function of the gut-brain axis in individuals with ASD.

The human gut microbiota is a dynamic and complex system. The development and fluctuations of this ecosystem can be affected by an individual's genetics, birth and infant feeding, diet, medication intake, the environment and geography, comorbidities, ageing, lifecycle and stress exposure (15). On the other hand, the gut microbiota itself is considered to influence the individual's metabolism, nutritional preferences, the physiology of the GI and immune systems, as well as the neurophysiological and behavioural functions of the body (16). Considering the unique homeostasis and composition of the 'normal' microbiota in each individual, the state of dysbiosis is generally difficult to define, Petersen and Round (17). The most common approach used to investigate dysbiosis in a cohort is to perform a case-controlled study.

According to a recent review article, a number of studies have reported an association between gut permeability and an increase or deprivation of several intestinal microbial genera and ASD symptoms (8). However, different literature sources refer to various findings. For example, a group of scientists (18) found lower levels of *Enterococcus* and *Bifidobacterium* and higher levels of *Clostridium, Bacteroides, Porphyromonas, Prevotella* and *Enterobacteria* in the composition of the gut microbiome of children with ASD. A recent review article summarised the results of nine studies including 254 patients with ASD (19). That study, based on a meta-analysis, revealed significant differences in abundance of *Akkermansia, Bifidobacterium, Bacteroides, Escherichia coli* (*E. coli*), and *Lactobacillus* in the total detected faecal microbiota of children with ASD compared to the controls (19). Other researchers refer to certain intestinal bacteria that may be involved in the pathogenesis of ASD, including members of the *Clostridium* genus (20). It has been shown that faecal samples from children with ASD have higher levels of *Clostridium bolteae, Clostridium histolyticum* or *Clostridium perfringens* (21-23). Some species of *Clostridium* can produce neurotoxins and may exert systemic effects that can hypothetically influence the behavioural patterns of individuals with ASD. Moreover, in a previous study, following treatment with vancomycin, the reduction of *Clostridium* yields was found to be associated with short-term behavioural improvements in children with regressive-onset autism (24). Another study, based on a mouse model, suggested a potential direct link between *Clostridioides* (*C.*) *difficile* (previously known as

Clostridium difficile) infection, gut and serum p-cresol levels, dopamine-β-hydroxylase activity and alterations in dopaminergic activity in the brain, that have direct implications in the pathogenesis of ASD (25).

C. difficile is an emerging nosocomial pathogen, common in cases of antibiotic-associated diarrhoea and pseudomembranous colitis. However, *C. difficile* is naturally resistant to ampicillin, amoxicillin, cephalosporins, clindamycin and fluoroquinolones. The limited number of antibiotics that are active against *C. difficile*, such as metronidazole, vancomycin and fidaxomicin become increasingly less effective, due to the accumulation of resistance in the *C. difficile* population (26). Due to various reasons, patients with ASD often undergo intensive antibiotic treatment courses that can lead to acute *C. difficile* infections (27). *C. difficile* can produce two large toxins: ToxinA (308 kDa) and ToxinB (270 kDa), which are glucosyltransferases and can inactivate Rho, Rac and Cdc42 (small GTPases) within target cells. This leads to the ability of the toxin to disrupt the tight junctions of epithelial barriers and increase gut permeability (28). Therefore, the prevalence of *C. difficile* in the intestinal biome of individuals with ASD has become a main topic of research.

The first epidemiological study of autism in Georgia was performed between the years 2007-2009. That analysis was based on tests and questionnaires. According to the collected data, one out of 110 children suffered from ASD. This frequency was approximating the statistical average of Europe at that time (29). In another study in 2017 (30), the levels of the bacterial metabolite, *p*-cresol, were measured in the urine of children with ASD, epilepsy and healthy controls, and were found to be elevated in the both study groups compared to the controls. However, the diversity or presence of particular bacterial species in the gut microbiota of Georgian children with ASD has not been investigated to date, at least to the best of our knowledge.

The present study aimed to accomplish a preliminary bacteriological investigation of the gut microbiota and its association with the intestinal health of children with ASD. In particular, the present study focused on the prevalence of A/B toxins producing *C. difficile* and the abundance of *Bifidobacteria, Lactobacteria, E. coli* and *Enterococcus*, as well as antibiotic resistant pathogens in the stool samples collected from an urban population with ASD and neurotypical children from Tbilisi, Georgia. Since phages are considered as alternatives to antibiotics, as well as a promising tool for the re-instatement of disturbed microbiotas, antibiotic resistance and phage susceptibility profiles of the isolated intestinal pathogens were defined simultaneously. The present study was performed in a case-control manner.

Patients and methods

Study design. The study included two cohorts, one composed of 30 children diagnosed with ASD and a control group of 27 neurotypical children, all aged between 2 to 17 years and permanently residing in Tbilisi, Georgia. The enrolment criteria for the patient group included the following: A previous diagnosis of with ASD in accordance to DSM-5 (https://psychiatry.org/Psychiatrists/Practice/DSM), the permanent residence of the family in the Tbilisi urban area

and an age not >17 years; applicants with neurotypical siblings in the same age groups were prioritized. The present study was approved by the Scientific Research Ethics Committee of the Ilia State University, Tbilisi, Georgia (issued on October, 2019). Parental oral consents were obtained prior to the initiation of the study.

The participants of the ASD cohort were assessed via the autistic diagnostic observation schedule (ADOS) (https://www.pearsonclinical.com.au/products/view/502). Dedicated questionnaires were designed and information concerning birth type, infant feeding, diet, GI symptoms, antibiotic treatment history and other medically relevant data were collected from the parents of the children in the ASD and the control group.

Samples collection and bacteriological analysis. Fresh faecal samples were collected in sterile containers and immediately transferred to the Eliava Bacteriophage Analytical-Diagnostic Canter (BADC), Tbilisi, Georgia. A total of 57 faecal samples were collected for the study.

RIDASCREEN *C. difficile* Toxin A/B (C0801, R-biopharm AG) enzyme immunoassay was used for the qualitative determination of the *C. difficile* toxins A and B in the fresh stool specimens. The test was performed according to the manufacturer's instructions.

Routine bacteriological examination of the same stool samples was performed according to the Eliava BADC Standard Operational Procedures for the investigation of dysbiosis. The enumeration of *Bifidobacteria*, *Lactobacteria*, *E. coli* and *Enterococcus* spp., and the detection of facultative and aerobic enteric pathogens, such as haemolytic *E. coli*, *Salmonella* spp., *Shigella* spp., *Klebsiella* spp., *Citrobacter* spp., *Morganella morganii*, as well as *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and coccoid species, including *Staphylococcus* spp. and *Streptococcus* spp. was performed as described below. A total of 1 g of faecal sample was added to 9 ml sterile saline solution and mixed to obtain a suspension. Further 10-fold serial dilutions were performed. A total of 0.1 ml from the dilutions: 10^{-1}, 10^{-3} and 10^{-5} were plated or inoculated in enrichment (brain hart infusion (BHI), 5% sheep blood agar), selective (brilliant green bile broth, MRS, casein yeast mannitol salt, phenylethanol, Endo, Sabouraud and triple sugar iron agars) or differential (MacConkey, SS, cetrimide, bile esculin azide and Herella agar) medias. All media listed above were produced by Eliava Media Production Ltd. In particular, BHI agar (Eliava Media Production Ltd.) plates were used for determining the total bacterial counts, 5% sheep blood agar for the detection of α- and β-haemolysis, mannitol salt agar for the selection of *Staphylococcus* spp., phenylethanol agar for *Streptococcus* spp., brilliant green bile broth, Endo, triple sugar iron agar and MacConkey agar for *Enterobacteriaceae* (*E. coli*, *Klebsiella*, etc.), SS agar for the detection of *Salmonella* and *Shigella* species, cetrimide agar for *Pseudomonas aeruginosa* and Herellea agar for *Acinetobacter baumannii;* bile esculin azide agar was used to enumerate *Enterococcus spp.* and MRS medium for *Lactobacillus* spp., Sabouraud agar for yeasts, and casein yeast soft agar (0.7%) with supplement for the cultivation of *Bifidobacteriacea*e. The plates and tubes were incubated in optimal growth conditions of the target species, with anaerobic environment of <0.1% of oxygen, >15% of CO_2 where needed.

The combination of microscopy and biochemical tests were implemented for further identification of the isolates. The strains exhibiting α- or β-hemolysis and isolates identified as pathogens underwent antibiotic and phage susceptibility testing. Antibiotic susceptibility was determined using the Kirby-Bauer disk method (31) and interpreted according to the Clinical and Laboratory Standards Institute criteria (32). Phage susceptibility testing was conducted using the spot test assay (33) using five commercially available polyvalent phage preparations: PYO, INTESTI, FERSIS, SES and ENKO bacteriophages (Eliava Biopreparations, Tbilisi, Georgia) including the components against *Staphylococcus* spp, *Streptococcus* spp., *Enterococcus* spp., *E. coli*, *Pseudomonas aeruginosa* and *Proteus* spp.

TheiIsolation of presumptive *C. difficile* was performed at the in-house laboratory of the Eliava Institute of Bacteriophages, Microbiology and Virology (Tbilisi, Georgia). Loop-fulls of the same stool sample dilutions 10^{-1}, 10^{-3}, 10^{-5} were cultured on *C. difficile* selective agar (CCFA; Biolife Italiana), differential chrome-agar (CHROMagar, DRG International, Inc.) and a blood agar base with 5% of sheep blood (Eliava Media Production Ltd.). The plates were incubated in an anaerobic jar with a gas-pack and anaerobic indicator pad (GasPak, Becton, Dickinson and Company (BD) BBL) at 37˚C for 48 h. *C. difficile* ATCC 43255 and ATCC 43593 (American Type Culture Collection) were cultured along with the samples as a positive control for growth conditions. The selected colonies were Gram-stained (Gram staining kit, Carl Roth GmbH + Co. KG) and examined for catalase activity by applying 3% hydrogen peroxide solution (Imedi) to the smear of bacterial colony.

Phenotypically *C. difficile* isolates were identified as follows: Gram-positive and catalase-negative bacilli with endospores, displaying γ-haemolytic grey colonies with umbonate edges on 5% sheep blood agar, fluorescence under ultraviolet light on chrome-agar and circular, raised, opaque grey colonies, 4 to 6 mm in diameter on CCFA. Furthermore, presumptive *C. difficile* isolates were grown on a chrome-agar medium for genome extraction. DNA was isolated using the UltraClean Microbial DNA Isolation kit (15800-250, MO BIO Laboratories, Inc.) according to the manufacturer's instructions. End-Point PCR was performed using the C. difficile Detection kit (TM37100, Norgen Biotek Corp.) as per the manufacturer's instructions. The gel electrophoresis of 20 μl PCR products was conducted at 150 V for 30 min on a 1.4% (w/v) agarose gel, with 10 μl ethidium bromide. The results were visualised using a transilluminator (Bio-Rad Laboratories, Inc.). An overview of the study design is presented in Fig. 1.

Statistical analysis. The exposure status was measured using standardised questionnaires and biological samples. The measure of association between control and study group was expressed as odds ratios (ORs). Post-hoc statistical evaluation of odds data was performed using the GIGAcalculator (www.gigacalculator.com), in a 2-by-2 table, with two-sided P-value and confidence interval at the level of 95%. Data were visualised using Datawrapper software (www.datawrapper.de). Recall bias was excluded due to non-ASD symptom-specific questionnaires.
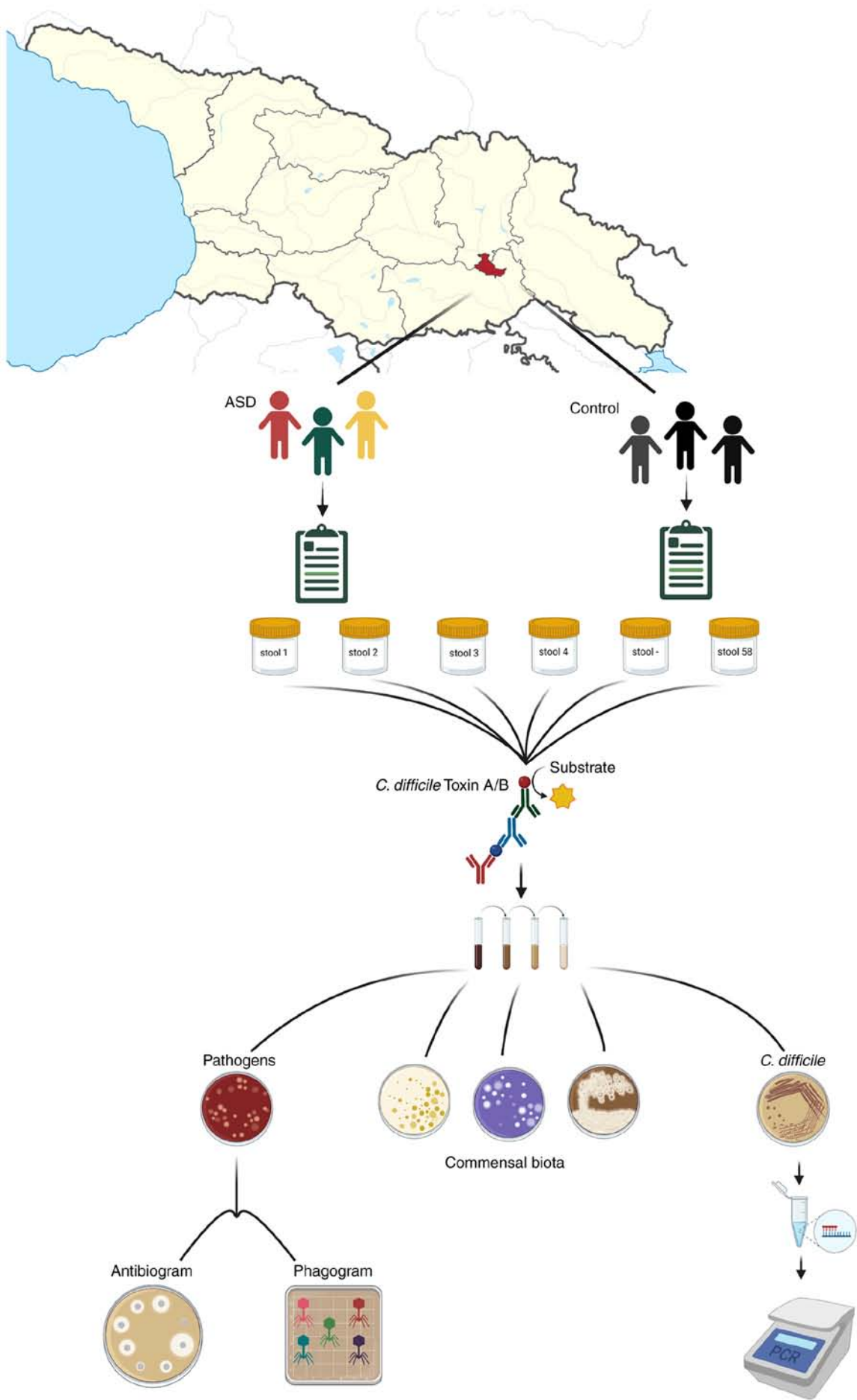
Figure 1. Graphical representation of the study design flow. The image was created using BioRender.com (www.biorender.com).

Table I. Age, sex and distribution of gastrointestinal symptoms between the study groups.

Group	Average age (years)	Sex	Exhibiting GI symptoms	Variety of GI symptoms	% Individuals	No. of individuals
ASD	10.8±3.2	30/30 Male	70%	Constipation	33.3	10
		0/30 Female		Diarrhoea	23.3	7
				Eructation	26.7	8
				Flatulence	40	12
				Heartburn	10	3
				Exhibiting >1 symptom	30	9
Control	5.8±2.7	19/27 Male	37%	Constipation	14.8	4
		8/27 Female		Diarrhoea	3.7	1
				Eructation	11.1	3
				Flatulence	25.9	7
				Heartburn	0	0
				Exhibiting >1 symptom	14.8	4

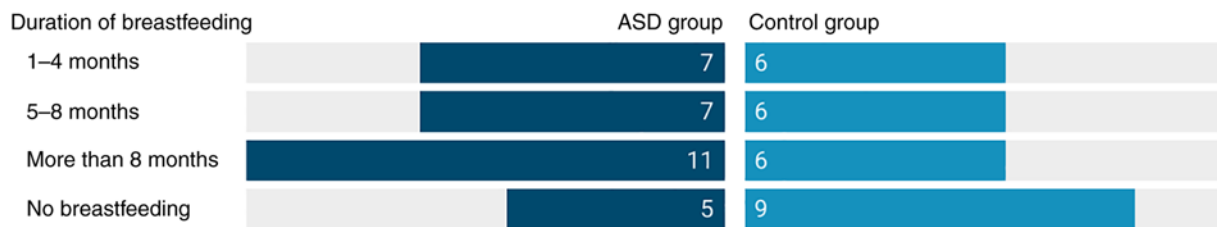ASD, autism spectrum disorder; GI, gastrointestinal.

Figure 2. Duration of infant breastfeeding. Data were collected from the mothers of participants in the ASD and control groups. ASD, autism spectrum disorder.

Results

General group data. The ASD group was formed by all males, including one pair of siblings. The age range in this group varied from 4 to 17 years, with a mean age of 10.8±3.2 years. The control group included six pairs of neurotypical siblings and 5 children were siblings of the ASD group participants. Sex distribution was as follows: 19 (70.4%) males and eight (29.6%) females. The mean age was 5.8±2.7 years, ranging from 2 to 15 years (Table I). All participants from the both groups originated and resided in the same geographical area (Tbilisi, Georgia).

Nutrition and comorbidities. The average maternal age was the same for both groups, with 30.3±6.5 for the ASD and 30.3±4.3 for the control group. In the ASD group, 18 children (60%) were delivered vaginally and 25 (83.3%) were breastfed, eight participants (26.6%) had a restricted diet, 19 (63.3%) were fed without restrictions and 3 children (10%) had mild aversions. Another 3 children (10%) had endocrine comorbidities, 14 (46.6%) demonstrated other types of comorbidities and 10 (33.3%) had a reported history of various allergic reactions. In the control group, 11 children (40.7%) were delivered

naturally, 18 (66.6%) were breastfed; no dietary restrictions were reported. Only two cases of comorbidities were reported, and 7 participants (25.9%) reported allergies. The detailed information related to infant feeding for both groups is presented in Fig. 2.

GI symptoms. At the time of the examination, in the ASD group, 70% of the participants had at least one GI symptom (constipation, diarrhoea, eructation, flatulence or heartburn). The most frequent symptom was constipation (33.3%, 10 participants), followed by diarrhoea (23.3%, 7 participants) and the least frequent was heartburn affecting (only 10%, 3 participants). It should be noted that 30% (9 participants) exhibited more than one GI symptom simultaneously. In the control group, only 37% of the participants noted at least one GI symptom, with most and least frequent being flatulence (25.9%, 7 children) and diarrhoea (3.7%, 1 child), respectively. The details regarding age, sex and GI symptoms are summarised in Table I.

History of antibiotic use. According to the data collected through the questionnaires, in the ASD group, 93% of the participants underwent antibiotic therapy between the ages
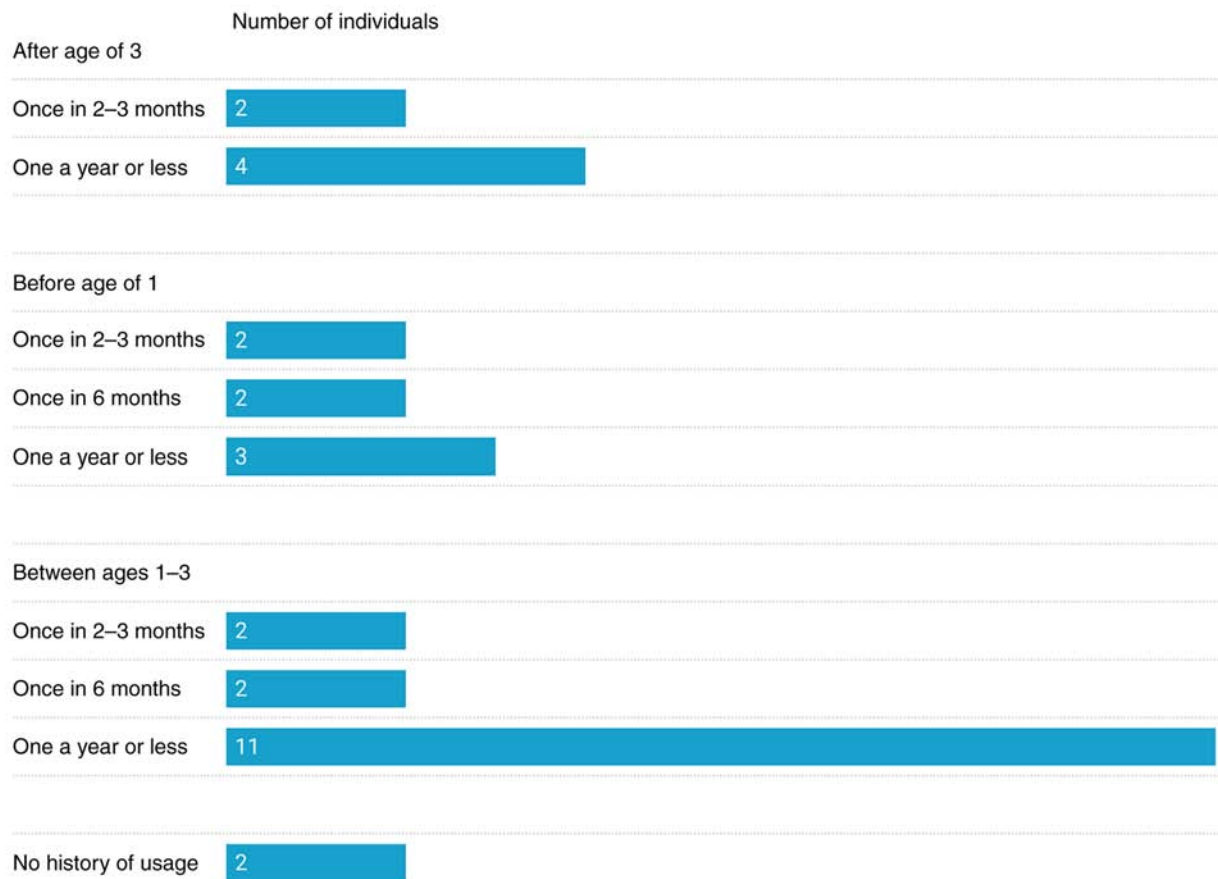
Figure 3. Details regarding antibiotic usage in the autism spectrum disorder group. Age and frequency of antibiotic intake as reported by the parents of the participants.

Table II. Classes of antibiotics used in the ASD group.

Antibiotic classes	No. of individuals	% Individuals
Penicillins	12	40.0
Cephalosporins	7	23.3
Quinolones	1	3.3
Macrolides	3	10.0
Aminoglycosides	1	3.3
Unknown	7	23.3
Never used	2	6.7

The numbers shown are based on data provided by parents of the participants. ASD, autism spectrum disorder.

of 0 to 3 years. In total, the parents of 7 participants (23.3%) reported the usage of penicillin, cephalosporin or macrolides before the age of one, and 15 participants (50%) between the ages of 1 and 3 years. The parents of 6 participants (20%) recalled the usage of penicillin, cephalosporin, quinolones or aminoglycosides after the age of three. In addition, 9 children (30%) had a history of antibiotic usage and deficiency in normal bacterial composition (as diagnosed by BADC) even though they did not have recorded GI symptoms. In total, 2 participants (7%) had no history of antibiotic usage, but exhibited constipation and deficiency in normal bacterial composition. In the control group, the parents of 20 participants (74%) reported usage of antibiotics at some point in the past. However, only one parent could recall which antibiotic was prescribed. The details of antibiotic usage in the ASD group are illustrated in Fig. 3, while the classes of used antibiotics are presented in Table II.

Bacteriological analysis. A near detection level of toxin A/B was found in only one stool sample from the ASD group. A child with the history of antibiotic therapy with penicillin in early childhood (1 to 3 years) exhibited diarrhoea, belching and flatulence. The parents of another participant with a history of frequent use of penicillin drugs and quinolones after the age of three, suffering with the interchangeable constipation and diarrhoea, bloating and flatulence, reported of a previously diagnosed *C. difficile* infection; however, at the time of the examination, toxin producing *C. difficile* was not detected. Otherwise, A/B toxin producing *C. difficile* infection was not detected or reported by any other participants from either group. The bacteriological examination of the samples and following species-specific PCR testing of the isolates did not confirm the presence of *C. difficile* in any of the provided samples.

According to the stool analysis performed by the BADC, no *Candida*-like fungal growth was detected in any of the tested samples. All participants, apart from one in the control

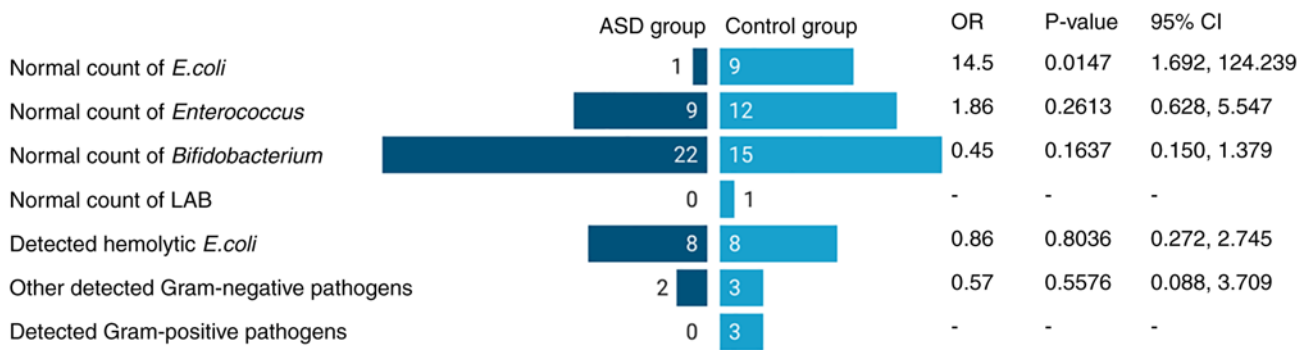|	ASD group	Control group	OR	P-value	95% CI
Normal count of *E.coli*	1	9	14.5	0.0147	1.692, 124.239
Normal count of *Enterococcus*	9	12	1.86	0.2613	0.628, 5.547
Normal count of *Bifidobacterium*	22	15	0.45	0.1637	0.150, 1.379
Normal count of LAB	0	1	-	-	-
Detected hemolytic *E.coli*	8	8	0.86	0.8036	0.272, 2.745
Other detected Gram-negative pathogens	2	3	0.57	0.5576	0.088, 3.709
Detected Gram-positive pathogens	0	3	-	-	-

Figure 4. Results of bacteriological analysis. Number of samples with normal commensal microbial counts and count of samples with undesirable bacteria detected in them, as well as calculated OR values, with P-values presented for each group. ASD, autism spectrum disorder; OR, odds ratio; LAB, *Lactobacillus*.
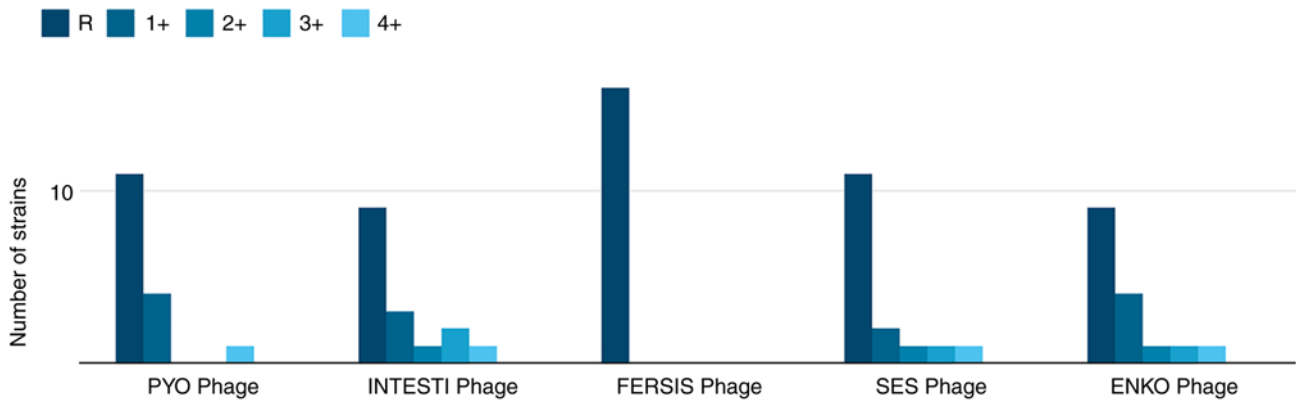

Figure 5. Phage susceptibility. The susceptibility patterns of isolated β-haemolytic *E. coli* cultures against five different phage cocktails are given. R, resistant; 1+/2+, intermediate activity; 3+/4+, sensitive.

group exhibited a deficiency in *Lactobacillus* spp. counts. In the ASD group, only 3% of the participants exhibited normal counts of commensal *E. coli*, 30% had satisfactory counts of *Enterococcus spp.*, and 73.3% had regular *Bifidobacterium* counts. β-haemolytic *E.coli* was isolated from 26.7% of samples, and drug-resistant *Klebsiella* spp. and *Pseudomonas aeruginosa* were found in two different samples; however, despite this fact, those participants had not reported any GI symptoms. In the control group, 33.3% had normal counts of the commensal *E. coli*, 44.4% had a satisfactory count of *Enterococcus* spp., and only 55.6% had regular *Bifidobacterium* counts. β-haemolytic *E. coli* was isolated from 29.6% of samples, *Klebsiella* spp. and drug-resistant *Pseudomonas aeruginosa*, *Morganella morganii* and *Staphylococcus aureus* were isolated from five different samples as well. The OR calculation for normal *Bifidobacterium* counts was 0.45 with significance level of P=0.1637, suggesting that the disruption in *Bifidobacterium* biota was more frequent in the control group than in the ASD group, although at a low level of significance. For *Enterococcus,* the analysis revealed an OR of 1.86 (P=0.2613), suggesting that the disruption in *Enterococcus* was 2-fold the odds in the ASD than in the control group, also with low significance. For the normal *E. coli* count, the OR value was 14.5 (P=0.0147), indicating that the ASD group had 14-fold the odds of a disruption in *E. coli* counts than the control. The overview of the bacteriological analysis is presented in Fig. 4.

Phage and antibiotic susceptibility. A total of 16 encountered strains of β-haemolytic *E. coli* isolated from both groups underwent antibiotic- and phage-susceptibility tests against five antibiotic classes and five commercial phage preparations. All strains isolated from the ASD group demonstrated resistance to penicillin, which was often associated with resistance to aminoglycoside and in some cases, to cephalosporin group antibiotics. All isolates obtained from the control group also revealed resistance to penicillin, often associated with aminoglycosides and cephalosporins, and rarely to the group of tetracycline antibiotics (Table SI).

In total, five polyvalent commercial phage preparations with lines active against *E. coli* were used on β-haemolytic *E. coli* isolates; eight strains out of the 16 tested isolates appeared to be sensitive to at least one preparation. The overall phage susceptibility of the isolated pathogens is summarised in Fig. 5.

Discussion

It has been shown that the type of childbirth delivery, infant feeding and medication intake has a profound influence on development of an individual's microbiome (34). C-section, perinatal antibiotic intake and formula feeding are associated with microbiome perturbation and may influence infants' neurocognitive development (35). There is still a controversial view about the link between C-section and the development of ASD. A 2019 meta-analysis, involving >20 million individuals,

reported that children delivered via C-section were ~30% more likely to be diagnosed with ASD than those born vaginally (36). At the same time, other research has indicated that toddlers without breastfeeding in the first 6 months of life have higher odds of developing ASD when compared to those who were exclusively breastfed (37). The results of the present study did not confirm these observations. In particular, the percentage and duration of breastfeeding, as well as vaginal deliveries were higher in the ASD group. However, a higher frequency of early-childhood antibiotic intake and more frequent GI symptoms were reported in the ASD group.

The present study tried to link the ASD symptoms to the composition of the gut microbiota and the particular role of *C. difficile* in the associated neurological disorders. No *C. difficile* was found in the faecal samples of the participants, which is in agreement with the findings of Khalil *et al* (38), whereas no association between *C. difficile* and GI manifestation in ASD was observed.

Some differences in the commensal microbiota counts were detected between the two groups, with more frequent deficits in *Bifidobacterium* in the control group and more frequent disruptions in *Enterococcus* and *E. coli* in the ASD group. The results of the present study partially correspond to the data from literature, where the lower abundance of *Bifidobacteria*, *Enterococcus* and *E. coli* among children with ASD has been reported (19). In the present study, the slightly higher incidence of normal counts of *Bifidobacteria* in the ASD cohort may be attributed to the frequent administration of probiotics containing these bacteria. As a popular over-the-counter supplement in Georgia, probiotics are frequently advised by pharmacists and doctors for the normalisation of bowel movements. However, the questions related to probiotic use were not included in the questionnaire.

Drug-resistant pathogens were encountered in the samples from both groups, where isolates from the control group were more diverse than those in the ASD group. The results of antibiotic sensitivity tests of β-haemolytic *E. coli* demonstrated slightly different resistance patterns in the ASD and control groups. In phage susceptibility testing, 50% of β-haemolytic *E. coli* isolates were susceptible to at least one phage preparation, which is a promising result. Due to the limited number of the tested strains, it was impossible to identify significant differences in the phage sensitivity patterns between the ASD and control groups. At same time, no association between antibiotic and phage sensitivity patterns was observed.

In order to regulate GI disturbances in patients with ASD, various personalised dietary approaches, such as the use of different probiotics and prebiotics, or exclusion diets (gluten- and casein-free) and microbial transfer therapy have been suggested and implemented with varying levels of success (1). In some cases, even antibiotic therapy is prescribed to manage ASD symptoms, which can be devastating for the commensal microbiota of the individual (39). As an alternative safer treatment option, phages are gaining increasing momentum. They can be used as highly specific and natural antibacterial agents that can specifically eliminate target multi-drug resistant bacteria, or as a prebiotic agent that can help regulate dysbiosis. As phage products can be adapted for personalised use, it is possible to develop phage-based tailored preparations catering to the needs of individual patients with ASD and thus decrease the overuse of antibiotics, avoiding antibiotic associated side-effects and resistant development or offer an option for individuals with antibiotic-incompatibilities. For example, the present study, children with β-haemolytic *E. coli* and at same time, low commensal *E. coli* counts, would benefit from highly specialised, preferably strain specific phages, which only lyse β-haemolytic *E. coli* and do not attack commensal strains, thus ensuring pathogen elimination without further degradation of commensal microbiota.

Due to the unique nature of human microbiotas, the further understanding of the functions and alterations of the microbiome-gut-brain axis and its implications on the ASD aetiology would benefit from longitudinal observations with a personalized approach.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

All authors (EK, KM, NB, NG, SK, TE, VB, NS, NaC, EJ, GN, TT, MM, NiC and IA) participated in the conception and design of the study, revised, edited or reviewed the submitted versions of the manuscript. In addition, EK, KM, NB, NG, NS, NaC, EJ, GN, TT, MM contributed to sample collection, processing and microbiological analysis. SK, TE, VB, NiC and IA participated in recruitment as well as data collections from the cohorts. NiC, KM and IA contributed to funding acquisitions and EK, SK and NiC participated in data analysis, and in the writing and processing of the manuscript. NiC and IA coordinated the project. All authors have read and approved the final manuscript. NiC and SK confirmed the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was approved by the Scientific Research Ethics Committee of the Ilia State University, Tbilisi, Georgia (issued on October, 2019). Parental oral consents were obtained prior to the initiation of the study and the parents provided consent for the information of their children to be published.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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