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Intestinal Dysbiosis and Yeast Isolation in Stool of Subjects with Autism Spectrum Disorders

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Abstract High frequency of gastrointestinal yeast presence in ASD subjects was shown through a simple cultural approach (*Candida spp*. in 57.5 % of ASDs and no controls); the identification of aggressive form (pseudo-hyphae presenting) of *Candida spp*. at light microscope means that adhesion to intestinal mucosa is facilitated. Dysbiosis appears sustained by lowered *Lactobacillus spp*. and decreased number of *Clostridium spp*. Absence of *C. difficilis* and its toxins in both ASDs and controls is also shown. Low-mild gut inflammation and augmented intestinal permeability were demonstrated together with the presence of GI symptoms. Significant linear correlation was found between disease severity (CARs score) and calprotectin and *Clostridium spp*. presence. Also GI symptoms, such as constipation and alternating bowel, did correlate (multivariate analyses) with the increased permeability to lactulose. The present data provide rationale basis to a possible specific therapeutic intervention in restoring gut homeostasis in ASDs.

Keywords Autism · Gastrointestinal dysbiosis · Microbiota · Candida · Intestinal permeability

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Introduction

Autism spectrum disorders (ASDs) are complex neurodevelopmental conditions characterized by cognitive defects, social interaction skills impairments and communication, language and behavioral problems [2]. ASDs are thought to arise from the interaction of genetic and environmental factors [10, 32, 33, 41, 52]. Their pathogenesis is still unclear; however, several biochemical events were described as associated with ASDs [3, 4, 11, 13, 30, 47, 58, 61], as well as many gastrointestinal (GI) dysfunctions [7, 12]. A strong correlation between GI symptoms and autism severity [1], the alterations of intestinal permeability (IP) [17], the presence of inflammation [28, 38, 42, 57, 60],

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intestinal dysbiosis and microbiota changes [1, 14, 16, 21, 23, 25, 48, 61] were described. Dysbiosis could be related to yeast infection and leaky gut [9, 34]. Susceptibility to various infections in ASDs subjects is currently reported together with immune deregulation [6, 37].

Even if nothing is known on which molecular pathway is mainly responsible to trigger the GI tract in autism, it is clear that autistic children suffering of intestinal dysfunction shows increased irritability, tantrums, aggressive behavior, and sleep disturbances [7]. In addition, other common symptoms are diarrhea, constipation, vomiting/reflux, abdominal pain/discomfort, gaseousness, and foul-smelling stools [7, 12, 44]. It was proposed that alterations of the intestinal microbiota could contribute to the development of ASDs [14]. The human microbiota can be looked at "as the interface between genes and individual history of environmental exposures, thus providing new insights into the neurodevelopment and the behavioral phenotypes" [31]. The gut microbiota can modulate brain function, forming a crucial link in the bidirectional interactions between the intestine and the nervous system [19]. Various recent studies demonstrated that autistic children with gastrointestinal symptoms show major fecal microbiota alterations [1, 23, 61]. The presence of *C. perfrigens* was also reported frequently in ASD with the meaning of a repeated antibiotic therapy that disturbs the balance of physiological microflora and may change its composition, producing colonization by pathogenic bacteria, including those producing toxins [24, 25, 43, 59]. Chronic constipation was the only variable significantly associated with total urinary p-cresol concentration in the absence of *Clostridium spp*. in the gut flora of 53 Italian ASD [27].

Although several studies have been conducted on the bacterial microbiota identification in ASDs, to our knowledge few studies were focalized on fungal/yeast infections in ASDs [1, 21] until a recent retrospective study [34]; several clinicians report the presence of *Candida*-related symptoms in autistic children [35] and various hypotheses on the biochemical cascade following such an infection have been formulated [9, 36, 46, 54].

It was our main interest to examine the fecal fungal presence and its degree of development, as well as the concomitant dysbiosis, in autistic children in order to provide rationale basis to a possible specific therapeutic intervention in restoring gut microflora in ASDs [49]. To achieve our aim, we used a culture-based approach to evaluate yeast and bacterial composition, correlating the data to GI symptoms, intestinal permeability, inflammation parameters and degree of ASD disease (based on CARS score), in a group of ASD subjects compared to normal developing children.

Materials and Methods

Subjects

In this study, we recruited 80 children. From each subject, fresh stool samples were collected to evaluate inflammation (calprotectin), presence of leukocytes, aerobic and anaerobic bacteria and fungi. To each subject, a lactulose/mannitol test was administered to evaluate the intestinal permeability.

Forty-seven subjects with ASDs (40 boys and 7 girls with a mean age of 6.0 ± 2.8 years) were recruited among the patients admitted to the Child and Adolescent Neuropsychiatry Unit at the Second University of Naples and to the Department of Pediatrics of the University of Naples "Federico II", Italy between June 2011 and June 2014. Inclusion criteria were: diagnosis of ASD according to the Diagnostic and Statistical Manual of Mental Disorders, Fifth edition, Text Revision (DSM-V-TR) [2]. The compliant enrolled children were administered the Autism Diagnostic Interview-Revised version, the Childhood Autism Rating Scales, and the Autism Diagnostic Observation Schedule-Generic to verify the diagnosis of autism [39, 40, 55]; the total CARs score was used to evaluate the global degree of ASD disease; values ≤ 38 were considered low-mild degree and values >38 high degree of disease. A carefully detailed GI anamnesis was obtained for each subject, by means of the Questionnaire on Pediatric Gastrointestinal Symptoms based on Rome III Criteria, Italian version [8] to assess presence of GI symptoms and including each subjects in one of the following categories: constipation, diarrhea, alternating bowel (constipation/diarrhea), reflux, no symptoms. Among them 12 (25.5 %) were on a gluten-/casein-free diet (GFCF) since at least 1 year from recruitment, as reported by parents. Exclusion criteria were: autisms secondary to genetic syndromes; Rett syndrome; childhood disintegrative disorder; epilepsy; neurological syndromes; BMI $<$ 25th or $>$85th percentile; concomitant condition of known celiac disease (CD); and diabetes

mellitus type 1 (DMT1), concomitant condition of major diseases of the intestinal tract such as inflammatory bowel disease or hepatic disorders, as well as known and serologically proven food intolerances.

Thirty-three healthy children (24 boys and 9 girls with a mean age of 7.3 ± 3.1 years) were recruited as matched controls among families of doctors, nurses, students of the Child and Adolescent Neuropsychiatry Unit and Gastroenterology at the Second University of Naples, Italy. None of these healthy children claimed any recent GI symptoms and were not affected by any major GI disease. The exclusion criteria were a concomitant condition of known CD and DMT1, a concomitant condition of major diseases of the intestinal tract such as inflammatory bowel disease or hepatic disorders, as well as known and serologically proven food intolerances. One child (MA, male, 4 years) was excluded during the study because of the suspect of celiac disease.

Informed consent was obtained from all children's parents or legally authorized representatives and identifying information was removed from each sample. The Ethics Committee of the Second University of Naples approved the study, and it was carried out in accordance with the Helsinki Declaration of 1975.

Stool Samples Examination and Culture

In this study, for each fecal sample: (a) morphological examination, (b) microscopic examination by means of Gram and May-Grünwald Giemsa staining, (c) search for toxins A/B of *C. difficilis*, (d) bacterial/yeast culture and (e) identification of bacteria and yeast colonies were performed by VITEK 2 microbial identification system.

(a) Morphological examination: the macroscopic examination was conducted detecting quantity, color, size, shape and consistency of the fecal samples; the possible presence of blood, mucus, pus, food residues or parasites was checked.

(b) Microscopic Examination of Feces was performed with both Gram staining, to verify the presence of both *Gram positive* and *Gram negative* bacteria and yeasts by means of optical microscope with 100× oil immersion objective, and May Grünwald-Giemsa staining, to distinguish between polymorphonuclear leukocytes, monocytes and lymphocytes, by means of optical microscope with 100× oil immersion objective.

(c) Most pathogenic strains of *C. difficilis* produce two toxins, Toxin A (enterotoxin) and Toxin B (cytotoxin), which are the main virulence factors for the organism. Toxin A is mildly cytopathic but induces large fluid shifts and mucosal inflammation. Toxin B is intensely cytopathic but its role in the disease process is not clearly understood. Variant strains which are Toxin A-negative, Toxin B positive are known to exist, are fully pathogenic, and capable of producing the full spectrum of disease. In this study, the Remel Xpect *Clostridium difficilis* toxin A/B test (Remel Europe, Ltd) was used; it is a rapid in vitro immunochromatographic test for the direct, qualitative detection of *Clostridium difficilis* Toxin A and/or B in human fecal specimens.

(d) Feces were weighed (analytical balance 6110 Balance—International PBI). A known quantity of the fecal sample (100 mg) was suspended in 1 ml of sterile physiological solution, homogenized by vortexing and left at room temperature for a few minutes. 10 µL of this mixture was inoculated in each culture medium. In particular, for the detection of aerobic microorganisms, the samples were inoculated into:

- Columbia CNA + 5 % sheep blood (*Staphylococcus spp./Streptococcus spp.* selective medium);
- MacConkey Agar No. 3 (APHA) (culture medium for the selective isolation and enumeration of *coliform bacteria* from clinical specimens);
- Sabouraud Dextrose Agar + Chloramphenicol + Gentamicin (culture medium for the selective isolation of fungi in samples containing mixed microbial flora).

The incubation was carried out aerobically at a temperature of 36 ± 2 °C for a period of 24 h, in the case of Columbia CNA agar and MacConkey, while it was conducted at a temperature of 29 ± 2 °C

for up to 5 days in the case of Sabouraud dextrose agar, given that in our experience yeast take more time to grow on this specific agar.

For the detection of anaerobic microorganisms, the samples were inoculated into:

- Chocolate (Vitox) (general culture medium for research of fastidious microorganisms), and the incubation was carried out in anaerobiosis, by placing the plates in special plastic envelopes to which is added a generator of CO_2. The pouches were incubated in a stove thermostat at 36 ± 2 °C for 48–72 h.

For the research of *Clostridium spp.*, the samples were subjected to a thermal shock conducted by placing the conical falcon tubes with stool samples in a thermostatic water bath, at a temperature equal to 100 °C. Then, each sample was immersed into dry ice and left there for 10–15 min, so as to quickly reduce the temperature. The thermal shock aims to destroy all bacteria in the vegetative form and promote sporulation of any spore-forming bacteria present. Finally, the feces subjected to thermal shock were again vortexed and then inoculated in a quantity equal to 10 µL in the following culture media:

- Chocolate (Vitox), (general culture medium for the detection of fastidious microorganisms);
- Brazier's *Clostridium difficilis* selective medium modified, (Medium for Isolation of *Clostridium difficilis*);

and incubation was carried out under anaerobic conditions, with the plates in the appropriate plastic envelopes to which was added a generator of CO2. The pouches were incubated in a stove thermostat at 36 ± 1 °C for 48–72 h.

After the relative periods of incubation, the direct count of any colonies grown on all kinds of culture medium (expressed as CFU/mg feces) was performed. All cultures were considered positives if bacteria and yeasts counts were $\geq 10^3$. The colonies grown on culture media were stained with Gram stain and observed by optical microscope. Moreover, each bacterial and yeast colony isolated by the primary inoculum was transferred into a fresh culture medium so that, after a further period of incubation, a pure colony and a sufficient number of bacteria or yeast were available for identification.

(e) VITEK 2 microbial identification system (bioMérieux) was used for identification of all bacteria and yeast grown on culture media. Specific VITEK cards were used for identification: GN ID Card for *Gram negative* bacterial identification, GP ID Card for *Gram positive* bacterial identification, YST ID Card for yeast identification, NH ID Card for *Neisseria*, *Haemophilus* and other fastidious *Gram negative* bacteria identification, ANC ID Card for *anaerobic bacteria* and *coliform bacteria* identification.

Calprotectin Determination

Fecal calprotectin (FC) was detected by means of enzyme-linked immunosorbent assay (ELISA) (Calprest, Eurospital, Italy). Briefly, this method is based on the use of a polyclonal antibody against calprotectin in an ELISA system, with the addition of a final colored product. Normal values were estimated to be <100 mg/g stool (adult and children) on the basis of previous reports [5, 51] and our own laboratory experience [17].

Intestinal Permeability

Intestinal permeability (IP) was assessed with the lactulose/mannitol (LA/MA) test. The test was administered once to all recruited children. The LA/MA test is considered a valuable and noninvasive test for monitoring barrier function of the small intestine. The procedure is based on the simultaneous oral administration of two sugar probes of different molecular sizes and absorption routes and the assessment of the concentration of each molecule in the urine. The LA/MA test was administered as previously described [17]. Briefly, an oral isosmolar load of the 2 probes—5 g of Lactulose (LA) and 2 g of mannitol (MA)—is orally administered to fasting subjects and urine samples are collected for the following 5 h. The LA/MA detection in the urine samples was performed by high-performance anion exchange chromatography with pulsed amperometric detection, as previously described [29]. Intestinal permeability is expressed as the ratio of the recovered percentage of lactulose versus mannitol (LA/MA). The cut-off value for the normal range was set at LA/MA <0.030 [29].

Statistical Analysis

Statistical analysis of quantitative and qualitative data, including descriptive statistics, was performed. Continuous data are expressed as mean ± standard deviation (SD), unless otherwise specified. Frequency analysis was performed with Chi-square test or Fisher's exact test, and in multiple comparisons, the Cochran's Q test, as needed. The intergroup differences were assessed by one-way analysis of variance test for parametric variables. The correlation analysis was performed using univariate and multivariate analysis. Particularly, Student's t test was used to evaluate whether the Pearson's linear correlation coefficient R was significantly different to zero. Data were analyzed with MATLAB statistical toolbox version 2008 (MathWorks, Natick, MA, USA) for 32-bit Windows. All tests with p value <0.05 were considered significant.

Results

Main results obtained are: (a) presence of *Candida spp*. in 27 out of 47 (57.5 %) ASDs as compared to controls (none) (Fig. 1); (b) absence of *Clostridium difficilis* detected by culture and toxins production in both ASDs and controls (Fig. 2); (c) *Lactobacillus spp*. detection in 13 ASDs only (27.7 %) as compared to controls (68.8 %); (d) higher amounts of recovered Lactulose (IP test) in 38.8 % of ASDs as compared to 18.8 % of controls (Fig. 3); (e) significant correlation

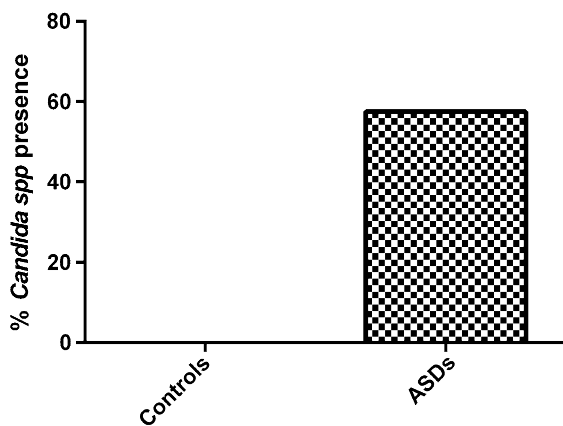

Fig. 1 *Candida spp*. was isolated from 57.5 % of ASDs (27 among 47 investigated subjects) samples and no controls. The % is shown

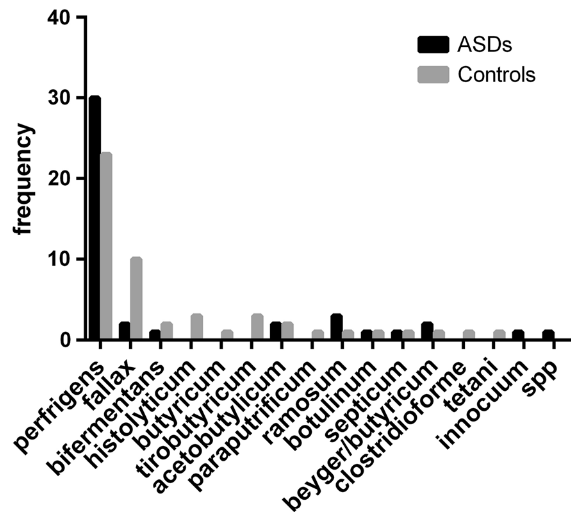

Fig. 2 *Clostridium spp*. was isolated from the stools of 35 ASDs (74.5 %) and 30 controls (93.8 %). *C.perfrigens* was the most frequently found; ASD samples were less rich in the number of *Clostridium* species with respect to controls (10 vs 16 species)

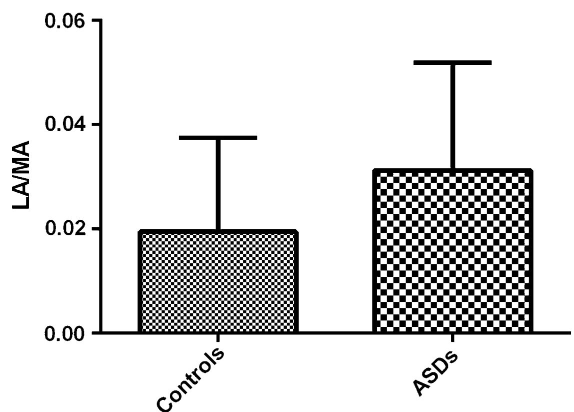

Fig. 3 Intestinal permeability evaluation was carried out by means of the lactulose/mannitol test; LA/MA mean values (mean + SD) are reported, showing a higher trend of values in ASDs versus controls. The % LA recovery resulted significantly higher in ASDs versus controls ($p < 0.05$)

of *Clostridium spp*. presence and calprotectin values with CARS in multivariate analysis (Table 5); (f) of GI symptoms in 70.2 % ASDs and no controls, with a mild correlation by multivariate analyses of constipation and alternating bowel versus increased permeability to Lactulose. In Table 1, the summary of clinical and laboratory parameters is reported.

Table 1 Summary of the *clinical and laboratory parameters* evaluated in the two groups of children (ASDs = autistic and controls = healthy)

Parameters	ASDs		Controls	
	Mean ± SD	Range	Mean ± SD	Range
A				
LA%	0.55 ± 0.606	0.06–2.90	0.249 ± 0.280	0.04–1.20
MA%	19.97 ± 13.73	2.20–63.60	16.22 ± 9.26	0.93–32.70
LA/MA	0.044 ± 0.076	0.005–0.45	0.019 ± 0.018	0.002–0.079
Calprotectin	40.29 ± 66.53	0–407.50	38.77 ± 40.32	14–193.10
Candida spp	$4.34 \times 10^4 \pm 2.92 \times 10^5$	$0–2 \times 10^6$	0.00 ± 0.00	–
CARS	38.01 ± 6.26	27.50–52.00	–	–

	Present %	Absent %	Abnormal %	Present %	Absent %	Abnormal %
B						
Lactobacilli	27.66 (13/47)	72.34 (34/47)	–	68.75 (22/32)	31.25 (10/32)	–
Anaerobic spp.	61.70 (29/47)	38.30 (18/47)	–	53.13 (17/32)	46.87 (15/32)	–
Clostridium spp.	74.47 (35/47)	25.53 (12/47)	–	93.75 (30/32)	6.25 (2/32)	–
Candida spp.	57.45 (27/47)	42.55 (20/47)	–	0.00 (0/32)	100.00 (32/32)	–
Presence of leukocytes	14.89 (7/47)	85.11 (40/47)	–	0.00 (0/32)	100.00 (32/32)	–
GI symptoms	70.21 (33/47)	29.79 (14/47)	–	–	–	–
Constipation	34.04 (16/47)	–				
Diarrhea	6.38 (3/47)	–				
Alternating bowel	27.65 (13/47)	–				
Reflux	2.13 (1/47)	–				
Calprotectin	–	–	8.51 (4/47)	–	–	6.25 (2/32)
LA/MA	–	–	38.30 (18/47)	–	–	18.75 (6/32)

Section A: quantifiable parameters are reported (mean, SD, range). Section B: presence, absence and abnormal values frequency is reported

Morphological examination of the stool samples revealed normal consistency of 68 % ASDs; the remaining being more or less loose. Among controls 82 % had normal consistency. No blood, mucus, pus, food residues or parasites were detected, showing no significant differences among the investigated groups. Microscopic examination showed the presence of yeast cells and granulocytes in most of the autistic stool samples as compared to none of the controls.

Candida spp. was detected in the stools of 27 ASDs (57.5 %) and in none of the controls (0 %) (Fig. 1; Table 4, $p = 8.67 \times 10^{-6}$). It is important to underline that the first identification is obtained through light microscope observation (Fig. 4) followed by culture under special described conditions (see "Materials and Methods" section). In ASDs, *C. albicans* was most frequently identified, but other species were also found

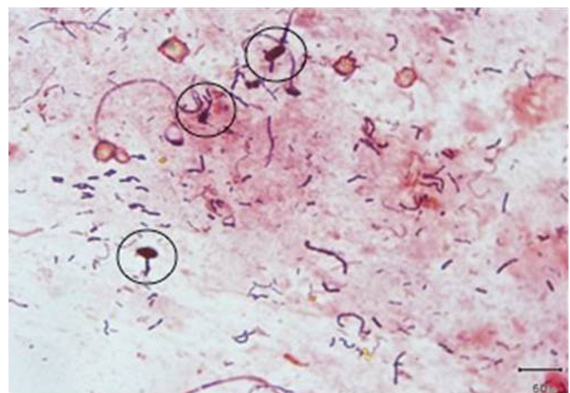

Fig. 4 *Candida spp*. was identified at light microscopy by examination of fresh stool samples, before culture approach confirming its presence. As shown in this example, *Candida* was often found in the aggressive form (*circles* pseudo-hyphae presenting) that facilitates adhesion to intestinal mucosa [50]

Table 2 *Candida spp*. was detected in the stools of 27 ASDs among the 47 investigated

Candida spp.	% of cases (ASDs)
C. albicans	59.26
C. glabrata	7.41
C. tropicalis	7.41
C. lusitaniae	7.41
C. parapsilosis	11.11
C. krusei	7.41
Total cases	27

C.albicans was the most frequently isolated (16 times/27 total cases). Other species were also found as shown

(Table 2). In four subjects, counts were $>10^3$ CFU/mg; the other ranging 20–110 CFU/mg. Among the 27 ASDs in whom *Candida spp*. was detected, three only were on GFCF with counts ranging 110–13 $\times$ 10^3 CFU/mg. Its presence/amounts did not correlate with any other investigated parameter even taking into consideration the GFCF diet regimen ($p > 0.05$, McNemar's exact test).

Clostridium spp. was detected in the stools of 35 ASDs (74.5 %) and 30 controls (93.8 %) (Fig. 2; Table 4, $p = 0.0143$). The most frequent species found was *C.perfrigens*, and other species were often associated with it (Fig. 2). *C.perfringens* counts were $>10^3$ CFU/mg more often in ASDs (64.9 %) than in controls (19.4 %). *C.difficilis* was detected in none of the investigated samples, as well as its related Toxins A and B. *Clostridium spp*. presence shows a significant mild correlation with CARS scores at multivariate analysis (Table 5).

The search for other anaerobic microorganisms resulted in the detection of various species (Table 3) in the stools of 29 ASDs (61.7 %) and 17 controls (53.1 %); more anaerobic species were present in the stool samples from controls than ASDs. *Enterobacteriacee* were found in all but one stool samples of ASDs, while they were undetectable in six controls (18.2 %).The microscopic examination of feces with Gram staining showed the presence of *Gram + cocci* in all but two ASDs samples and in all but one control.

Presence of *Lactobacillus spp*. was different among groups: in 13 ASDs (27.7 %) and in 22 controls (68.8 %) ($p = 7.28\times10^{-4}$). By means of microscopic examination, yeast cells (40.4 %) and leukocytes (14.9 %) were also shown in ASDs stool samples (Fig. 4). Control samples were all negative for leukocytes (Table 4, $p = 0.0108$) and yeast cells/hyphae presence.

Calprotectin values resulted >100 mg/g stool in 4 ASDs (8.5 %; range 100–408 mg/g) and 2 controls (6.3 %; range 100–193 mg/g), and the difference was not statistically significant ($p > 0.05$). Calprotectin values well correlate with CARs score in multivariate analysis (Table 5) with a $p = 0.0044$ ($R = 0.622$).

Intestinal permeability resulted altered in 38.3 % of ASDs with respect to 18.8 % of controls (Fig. 3). The recovery of Lactulose during the permeability test was in fact higher in ASDs than controls ($p = 0.0312$; Table 4). Among ASDs with altered IP (i.e. LA/MA > 0.030), 36.4 % were on GFCF and 45.5 % on

Table 3 Various species of anaerobic microorganisms were found in stool from ASDs and controls

Anaerobic spp.	% of cases (ASDs)	% of cases (controls)
Peptococcus	13.64	0
Bacteroides	31.82	11.11
Bacillus	25.00	25.94
Fusobacterium	27.27	18.52
Eubacterium limosum	2.27	11.11
Bifidobacterium	0	18.52
Lepto. buccalis	0	3.70
Mobiluncus	0	3.70
Gemella morbillorum	0	3.70
Perv. intermedia	0	3.70
Total cases	44	27

Their absolute frequence is shown. Microbial species, more than 1/subjects, were found in 29 ASDs and 17 controls

Table 4 Results of statistical comparison of the various investigated parameters among the two groups

Test characteristics	Hypothesis	Test type	p value
LA (A vs N)	$\boldsymbol{\mu_1 = 0.550 > \mu_2 = 0.249}$	**ANOVA**	**0.0312**
MA (A vs N)	$\mu_1 = 19.97 > \mu_2 = 16.22$	ANOVA	0.267
LA/MA (A vs N)	$\mu_1 = 0.044 > \mu_2 = 0.019$	ANOVA	0.136
Calprotectin (A vs N)	$\mu_1 = 40.29 > \mu_2 = 38.77$	ANOVA	0.914
Calprotectin (A vs N)	8.51 %(4/47) > 6.25 %(2/32)	χ^2 with Yates correction	0.0476
***Candida spp*. (A vs N)**	**57.45 %(27/47) > 0 %(0/32)**	**χ^2 with Yates correction**	**8.67×10^{-6}**
***Lactobacillus* (A vs N)**	**27.66 %(13/47) < 68.75 %(22/32)**	**χ^2 with Yates correction**	**7.28×10^{-4}**
***Clostridium spp*. (A vs N)**	**74.47 %(35/47) < 93.75 %(30/32)**	**Fisher exact test**	**0.0143**
Leukocytes (A vs N)	**14.89 % (7/47) > 0 % (0/32)**	**Fisher exact test**	**0.0108**
LA/MA (A vs N)	38.30 % (18/47) > 18.75 % (6/32)	Fisher exact test	0.053

A = ASDs and N = controls. The sole statistically significant comparisons are in bold. Presence of *Candida spp*. and reduced presence of *Lactobacillus* in ASD versus controls are strongly significant. In ASDs, higher mean values of recovered lactulose (LA) account for the difference in intestinal permeability values (LA/MA)

regular diet. At multivariate analysis, a correlation was found between increased Lactulose absorption and two GI symptoms: constipation and alternating bowel (Table 6).

Significant gastrointestinal symptoms were present in 33 ASDs (70.2 %) as from the administration of the Questionnaire on Pediatric Functional Gastrointestinal Symptoms; constipation accounted for 50 %, alternating bowel for 40 %; diarrhea was present in two cases and reflux in one case.

To determine the ASD disease severity, total CARs score was evaluated; mean ± SD = 38.0 ± 6.3, with a range 27.5–52.0. CARS scores strongly correlated with calprotectin values and mildly with *Clostridium spp* presence in multivariate analysis (Table 5).

Statistical comparison of all data is reported in Table 4. Statistically significant differences among the two investigated groups (ASDs vs Controls), as already described in details, were found in: Lactulose excretion during LA/MA test; *Candida spp* presence; *Lactobacillus spp* reduced presence; *Clostridium spp* reduced presence and variety; and fecal leukocytes presence. The correlation analyses conducted among most relevant parameters are shown in Table 5. Specific correlation analysis of GI symptoms and other parameters is shown in Tables 6 and 7. Particularly, among all considered symptoms (Table 6), we observed a mild significant correlation in multivariate analysis between LA and constipation (p value = 0.0086) and alternating bowel (p value = 0.0167).

Discussion

Gut microbiota contributes to nearly every aspect of the host's growth and development, not confined to the gastrointestinal tract; a large number of diseases and dysfunctions have been tentatively associated with an imbalance in the composition, numbers, or habitat of the gut microbiota [56]. Besides the more or less obvious implications in the GI tract (inflammation, malignancies, etc.), alterations of intestinal microbiota have been described/hypothesized in other body districts so far from the gut as the brain [15, 19].

Different components of GI microbiota contribute to many aspects of the normal host physiology such as breakdown of dietary constituents, development of mucosal immunity and modulation of the gastrointestinal development. Gut microbiota also provides its host with a physical barrier to incoming pathogens by competitive exclusion, consumption of nutrient sources, and production of antimicrobial substances [56].

In healthy individuals, the host immune system maintains homeostasis with the resident microbiota and mycobiota [50], and the resident eukaryotes are mutualistic or commensal important components. Disturbance of the network of competitive commensal bacteria can lead to increased susceptibility to *Candida spp.* infections, and mucosal diseases are strongly correlated to filamentous forms like invasive hyphae and pseudo-hyphae [50].

Table 5 Univariate and multivariate linear correlation analyses among some of the investigated parameters in ASDs group

Parameters	Univariate analysis R (p value)	Multivariate analysis Multiple linear correlation coefficient = 0.489
(LA/MA)/*Candida spp.*	−0.050 (0.783)	$R_{partial} = 0.021$; p value = 0.932
(LA/MA)/CARS	−0.117 (0.538)	$R_{partial} = -0.292$; p value = 0.226
(LA/MA)/Calprotectin	−0.010 (0.593)	$R_{partial} = 0.153$; p value = 0.531
(LA/MA)/*Lactobacilli*	−0.149 (0.408)	$R_{partial} = 0.161$; p value = 0.511
(LA/MA)/Anaerobic	−0.001 (0.996)	$R_{partial} = -0.066$; p value = 0.789
(LA/MA)/*Clostridium*	0.201 (0.261)	$R_{partial} = 0.358$; p value = 0.133
(LA/MA)/Leukocyte	−0.063 (0.728)	$R_{partial} = 0.115$; p value = 0.639
(LA/MA)/Sex	0.116 (0.522)	$R_{partial} = 0.252$; p value = 0.297
(LA/MA)/Symptom	0.103 (0.567)	$R_{partial} = -0.352$; p value = 0.139
		Multiple linear correlation coefficient = 0.461
LA/*Candida spp.*	−0.189 (0.293)	$R_{partial} = -0.173$; p value = 0.479
LA/CARS	−0.0151 (0.937)	$R_{partial} = -0.014$; p value = 0.956
LA/Calprotectin	0.062 (0.742)	$R_{partial} = 0.019$; p value = 0.939
LA/*Lactobacilli*	−0.266 (0.135)	$R_{partial} = 0.058$; p value = 0.814
LA/*Anaerobi*	0.044 (0.807)	$R_{partial} = 0.035$; p value = 0.886
LA/*Clostridium*	0.281 (0.113)	$R_{partial} = 0.038$; p value = 0.879
LA/Leukocyte	−0.163 (0.364)	$R_{partial} = 0.224$; p value = 0.357
LA/Sex	0.231 (0.197)	$R_{partial} = 0.176$; p value = 0.470
LA/Symptom	0.239 (0.180)	$R_{partial} = -0.096$; p value = 0.695
		Multiple linear correlation coefficient = 0.728
CARS/*Candida spp.*	−0.134 (0.385)	$R_{partial} = 0.146$; p value = 0.551
CARS/LA	−0.0151 (0.937)	$R_{partial} = 0.014$; p value = 0.956
CARS/Calprotectin	0.278 (0.075)	**$R_{partial} = 0.622$; p value = 0.0044**
CARS/(LA/MA)	−0.117 (0.538)	$R_{partial} = -0.292$; p value = 0.226
CARS/*Lactobacilli*	−0.082 (0.598)	$R_{partial} = 0.156$; p value = 0.524
CARS/*Anaerobi*	−0.129 (0.403)	$R_{partial} = -0.193$; p value = 0.429
CARS/*Clostridium*	0.065 (0.673)	**$R_{partial} = 0.493$; p value = 0.0322**
CARS/Leukocyte	0.051 (0.742)	$R_{partial} = 0.071$; p value = 0.774
CARS/Sex	0.008 (0.958)	$R_{partial} = -0.229$; p value = 0.347
CARS/Symptom	−0.0004 (0.998)	$R_{partial} = -0.412$; p value = 0.080
		Multiple correlation coefficient = 0.368
Candida spp./(LA/MA)	−0.050 (0.783)	$R_{partial} = 0.021$; p value = 0.932
Candida spp./Calprotectin	−0.0193 (0.90)	$R_{partial} = -0.158$; p value = 0.519
Candida spp./LA	−0.189 (0.293)	$R_{partial} = -0.173$; p value = 0.479
Candida spp./CARS	−0.134 (0.385)	$R_{partial} = 0.146$; p value = 0.551
Candida spp./Lactobacilli	0.263 (0.063)	$R_{partial} = 0.090$; p value = 0.713
Candida spp./Anaerobi	−0.0433 (0.772)	$R_{partial} = 0.0017$; p value = 0.994
Candida spp./Clostridium	−0.225 (0.128)	$R_{partial} = -0.159$; p value = 0.515
Candida spp./Leukocyte	0.132 (0.376)	$R_{partial} = 0.240$; p value = 0.323
Candida spp./Sex	−0.218 (0.141)	$R_{partial} = 0.122$; p value = 0.619

Table 5 continued

Parameters	Univariate analysis R (p value)	Multivariate analysis Multiple linear correlation coefficient = 0.489
Candida spp./Symptom	−0.240 (0.121)	$R_{partial} = 0.068$; p value = 0.783

Both the Pearson's linear correlation coefficient R and the correspondence p values are reported. The sole statistically significant comparisons are in bold. By analysis, it resulted a multiple linear correlation coefficient $R = 0.728$. The correlation was only significant for *CARS* by *Calprotectin* (p value = 0.0044) and *Clostridium* (p value = 0.0322)

R = Pearson's linear correlation coefficient; (p value)

The partial correlation coefficient $R_{partial}$ is the coefficient of correlation of the variable with the dependent variable, adjusted for the effect of the other variables in the linear regression model. The dichotomic variables, such as *Sex* (M/F), *Lactobacilli* (present/absent), *Anaerobic* (present/absent), *Clostridium* (present/absent), *Leukocyte* (present/absent) and *Symptom* (present/absent), were defined with numeric values assigning 1/0 to present/absent or M/F, respectively, and defining for them cumulative distributions

Table 6 Cochran's Q test was applied to individuate significant differences among symptoms type in ASDs group

Cochran's Q test	
Patient numbers	47
Cochran's Q statistic	19.87
DF	3
p value	<0.001
Variable	**Different (p value <0.05) from variable nr**
(1) Alternating bowel	(3)
(2) Diarrhea	(4)
(3) Reflux	(1) (4)
(4) Constipation	(2) (3)

There is a range of indications that alterations of microbiota in the gut might contribute to the pathogenesis of various diseases [56]. It was hypothesized that the principal contributor to the gastrointestinal disturbances among autistic individuals is an abnormal composition of gut microbiota [19, 20]. Several groups have studied the intestinal microbiota of autistic populations and found different composition of many microbial species compared to healthy controls. These ASD-related microbial species variations mainly include *Clostridium spp.* (various strains), *Ruminococcus*, *Bacteroidetes*, *Bacteroides, Firmicutes* and *Desulfovibrio* species [1, 16, 23–25, 43, 48, 62]. Interestingly, antibiotic treatment of ASD children did not only lead to gastrointestinal improvements, but also improvements in cognitive skills [53].

Yeast infection was rarely investigated in ASDs [1, 21] until a very recent retrospective study [34]; related symptoms were reported [35] and biochemical cascades following such an infection were tentatively formulated [9, 54]. Adams et al. [1] showed no differences of yeast infection among stools from ASDs

Table 7 Univariate and multivariate linear correlation analyses of CARS and LA with symptom type in ASDs group

Parameters: symptom type	Univariate analysis R (p value)	Multivariate analysis Multiple correlation coefficient = 0.30
Constipation/CARS	−0.022 (0.893)	$R_{partial} = -0.0046$; p value = 0.787
Alternating bowel/CARS	−0.073 (0.656)	$R_{partial} = -0.0036$; p value = 0.831
Diarrhea/CARS	0.130 (0.424)	$R_{partial} = 0.0097$; p value = 0.569
Reflux/CARS	0.161 (0.322)	$R_{partial} = 0.145$; p value = 0.247
		Multiple correlation coefficient = 0.535
Constipation/LA	0.326 (0.074)	**$R_{partial} = 0.487$; p value = 0.0086**
Alternating bowel/LA	0.218 (0.239)	**$R_{partial} = -0.448$; p value = 0.0167**
Diarrhea/LA	0.118 (0.537)	$R_{partial} = 0.014$; p value = 0.941
Reflux/LA	0.234 (0.205)	$R_{partial} = -0.306$; p value = 0.113

Both the Pearson's linear correlation coefficient R and the correspondent p values are reported

R = Pearson's linear correlation coefficient; (p value)

and healthy controls, while Ekiel et al. [21] report quantitative differences. Kantarcioglu et al. [34] in a recent retrospective study investigating a large number of ASDs subjects reported the presence of *Candida spp*. in a large percentage of patients compared to controls that is in strict agreement with the present results.

Although cultural techniques can be looked at as lacking specificity and sensitivity and molecular ones have been developed and are currently used, we choose the cultural approach because: (1) we were looking for a simple clinically applicable method to evaluate yeast population in the stool of children, and (2) when we started this work, there were no molecular techniques available to detect yeasts in stool.

In the present study, we demonstrated the presence (high CFU counts) of *Candida spp*. in more than half of the investigated stool samples from ASDs patients. This and previous studies are based on cultural approaches for yeast detection given that molecular biology methods were not available until very recently [26]. In the present work, we applied a cultural approach conducted with incubation in Sabouraud Dextrose Agar + Chloramphenicol + Gentamicin from 2 up to 5 days; in our experience, yeasts take more time to grow on this selective agar. This could explain previous negative results from other Authors. Our results show that *C.albicans* is largely the most represented species, in accordance with the recent report by Kantarcioglu et al. [34]. Although morphological examination showed no significant differences among the investigated groups, microscopic examination revealed to be a very useful first step to identify samples rich of yeast cells and granulocytes; it can be looked at, therefore, as a useful and potential tool in lab practice.

Even if GI symptoms were present in 70.2 % of ASDs, their presence does not correlate with the detected counts of *Candida spp*., meaning that they are not due to its presence. *Candida spp.* is usually present in the gastrointestinal system where it colonizes mucosal surfaces in asymptomatic manner; its growth is inhibited by the competition and suppression of resident flora. Increased counts of *Candida spp.*, even in the absence of GI symptoms, could be looked at as precocious index of intestinal dysbiosis. It cannot be excluded that, in its presence, ammonia and toxins are released, absorption of carbohydrates and minerals lowered, possibly contributing to autistic behavior [9].

Much focus has been given to the presence and abundance of *Clostridium* in the intestines of autistic children [1]. Finegold et al. [24] hypothesized that (1) the relapse of some autistic kids after antibiotic treatment is caused by the presence of *Clostridium* spores, (2) the incidence of autism is related to the widespread exposure to *Clostridium* spores, and (3) the increase of multiple autism cases within a single family is also related to contact with spores [24]. The presence of *C. perfrigens*, frequently reported in ASD, was linked to repeated antibiotic therapy leading to changes of microflora composition and producing colonization by pathogenic bacteria, including those producing toxins [16, 23, 43, 48, 59]. Finegold et al. [23] studied fecal samples from 13 children with late-onset autism and eight controls. They used basic anaerobic culturing techniques to count and isolate microorganisms, followed by polymerase chain reaction (PCR) targeting the 16S rDNA to identify the isolates cultivated. The number and type of *Clostridium* and *Ruminococcus* species in children with autism differed from the control children. Song et al. [59] found that *Clostridium* cluster groups had mean cell counts significantly higher than those of control children. Parracho et al. [48] used another culture-independent technique, fluorescence in situ hybridization (FISH) targeting *Clostridium* groups, and reported differences in the gut microflora of children with ASD compared to healthy children. In their study, levels of the *Clostridium histolyticum* group (toxin producers) of bacteria were higher in the ASD children compared to typical children. Similarly, we found that stool samples were rich in *Clostridium spp*. both in ASD and controls and that *C. perfrigens* was the most represented. Even if total counts are less in ASD than controls, high counts of *Clostridium spp.* ($>10^3$ CFU/mg) were present in ASD more frequently than controls and the positive correlation with CARS confirms the importance of this bacterial species for eubiosis of the gut. ASD samples were less rich in number of *Clostridium spp*. with respect to controls (10 versus 16 species); a decrease of species richness has been described in the microbiota of many pathological conditions [19, 50, 56]. Absence of Toxins A and B confirmed the fact that *C.difficilis* was undetectable in all examined sample.

In the present study, presence of *Lactobacillus spp*. was significantly (Table 4, $p = 7.28 \times 10^{-4}$) less frequent in ASDs (27.7 %) than controls (68.8 %); mainly four anaerobic species (*Peptococcus, Bacterioides, Fusobacterium* and *Bacillus*) were represented

in ASD with higher frequency than control stool samples. Lower levels of *Bifidobacter* [1, 16], higher levels of *Bacteroidetes* [16, 24], were already reported, while higher levels of *Lactobacillus* (all strains) in ASD were reported [1]. The reduced amounts and species variety of *Lactobacillus spp*. could cause a diminished control on mucosal homeostasis that appears to be confirmed by the increased Lactulose amounts with a possible derangement of tight junctions.

Various microbiota members contribute to the maintenance of intestinal epithelium barrier integrity through maintenance of cell-to-cell junctions and promotion of epithelial repair following injury. Several probiotic strains of *Lactobacillus* have been shown to contribute to the maintenance of tight junctions in intestinal epithelia, providing protective effect in the face of pathogen assault or intestinal injury [45]. Integrity of epithelial barrier has been assessed, in the present study, by administering a dual probe intestinal permeability test, the Lactulose/Mannitol (LA/MA) test. Previous data [17, 18] on impaired intestinal barrier are confirmed in the present group of autistic children, most of them reporting LA/MA values >0.030. The lack of statistical difference among the mean LA/MA values of the two groups could be due to the high variability of the samples. However, in the present study, a significant difference in Lactulose absorption between ASDs and controls was demonstrated, thus confirming the increased LA/MA values among ASDs. The augmented permeability to lactulose could be consequence of a lack/reduction of protecting effect on tight junctions by the reduced presence of *Lactobacilli* and/or of any other beneficial species not presently investigated.

Any attempted correlation of LA/MA and %LA values versus increase/decrease of bacterial/yeast species gave not significant results. Being on a GFCF did not correlate with presence/absence or increase/decrease of any microbial/yeast species while confirming a restoring effect on intestinal permeability (data not shown). The increased absorption of Lactulose correlates with GI symptoms (Table 6), in particular with constipation and alternating bowel, possibly indicating the importance of GI discomfort in aggravating ASD symptoms.

GI symptoms presence did not correlate with CARS score. The lack of correlation GI symptoms/severity of ASD disease is in contrast with what previously reported [1]. The difference could be due to the different scales used to evaluate both GI symptoms and disease degree. ASD disease degree was in fact presently evaluated on the basis of the well recognized CARS, used in the diagnosis of autism and based on behavioral observation besides referred symptoms. In the present study, the gastrointestinal symptoms were assessed as "GI disease categories", based on Rome III Criteria for functional symptoms [8]; this questionnaire, administered to the parents of all children (cases and controls), is a well recognized and validated instrument to assess GI problems in pediatric patients, and it is even more useful in children with lack of communication. The questionnaire was chosen and used following previous consensus statements [7].

Finally, low rates of gut inflammation, detected by means of fecal calprotectin, were found in the presently investigated samples, confirming previous results by us and other authors [17, 22]. In the few ASD-positive samples, with low-mild augmented calprotectin values, no correlation was found with either increase or decrease of bacterial/yeast species. A correlation was, however, present with disease degree (CARS score), thus possibly confirming that gut inflammation also could participate to gut discomfort ending in influence on behavior.

Conclusions

Increased counts of gastrointestinal *Candida albicans* presence in ASD subjects were shown through a simple and cheap diagnostic cultural approach. Future studies could be aimed to verify whether yeast elimination therapy is useful to manage ASDs GI and behavioral symptoms. Changes of microbiota composition were shown as well, in that ASD stool samples are less rich of microbial species than controls. Low-mild gut inflammation and augmented intestinal permeability were confirmed; inflammation correlated to disease severity (CARS) and intestinal permeability impairment was related to GI symptom type. Although the described differences among ASDs and controls could be due to presence of functional GI disorders vs absence of functional GI disorders, the present data provide rationale basis to a possible specific therapeutic intervention in restoring gut homeostasis in ASDs.

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