

## **New evidences on the altered gut microbiota in autism spectrum disorders**

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## **Abstract**

### **Background**

Autism Spectrum Disorders (ASDs) are neurodevelopmental conditions characterized by social and behavioural impairments. In addition to neurological symptoms, ASDs subjects frequently suffer from gastrointestinal abnormalities, thus implying a role of the gut microbiota in ASDs gastrointestinal pathophysiology.

### **Results**

Here we characterized the bacterial and fungal gut microbiota in a cohort of autistic individuals demonstrating the presence of an altered microbial community structure. A fraction of 90% of the autistic subjects were classified as severe ASDs. We observed a reduced relative abundance of *Bacteroidetes* in the ASDs group with *Collinsella*, *Corynebacterium*, *Dorea* and *Lactobacillus* being the bacterial taxa characterizing the gut microbiota of the ASDs cohort. On the contrary, the relative abundance of the bacterial taxa *Alistipes*, *Bilophila*, *Dialister*, *Parabacteroides* and *Veillonella* were significantly reduced in ASDs subjects. Furthermore constipation has been associated with different bacterial patterns in autistic and neurotypical subjects, with constipated autistic individuals characterized by high levels of bacterial taxa belonging to *Escherichia/Shigella* and *Clostridium cluster XVIII*. We also observed that the fungal genus *Candida* was more than two times more abundant, in terms of relative abundance, in autistic than neurotypical subjects.

### **Conclusions**

The finding that, besides the bacterial gut microbiota, also the gut mycobiota contributes to the alteration of the intestinal microbial community structure in autism opens the possibility for new potential intervention strategies aimed at the relief of gastrointestinal symptoms in ASDs.

**Keywords:** Autism spectrum disorders, gut microbiota, mycobiota, constipation, metataxonomy

## Introduction

The term “autism spectrum disorders” (ASDs) refer to a group of neurodevelopmental disorders with an early life stage onset characterized by alterations in social interactions and communication and by restricted and repetitive behaviour [1]. It is now well accepted the contribution of both genetic and environmental factors in the aetiology of ASDs [2, 3]. Among the non-neurological symptoms associated with ASDs, several studies indicate gastrointestinal (GI) symptoms as common comorbidities [4-7]. Alterations in the composition of the gut microbiota have been implicated in a wide variety of human diseases, including ASDs [8]. Since the gut microbiota makes critical contributions to metabolism, maintenance of immune homeostasis and may control central nervous system (CNS) activities through neural, endocrine and immune pathways [9] it has been hypothesized the active role of the gut microbiota in ASDs pathophysiology. There is more than a subtle link between the gut microbiota and the CNS, through the so-called “microbiome-gut-brain axis”. Indeed it has been demonstrated a direct interaction between the gut microbiota and enteric neurons [10, 11], its role in the regulation of the HPA axis [12] and the production of many chemicals important in brain functioning (*e.g.* serotonin, dopamine, kynurenine,  $\gamma$ -aminobutyric acid, SCFAs, *p*-cresol) [13, 14]. A dysbiotic microbial community could lead to systemic inflammation due to hyper-activation of T-helper 1 and T-helper 17 cell responses [15] affecting also the reactivity of peripheral immune cells to the CNS [16] and the integrity of blood-brain barrier [17] which is known to be altered in ASDs [18]. Several evidences suggested an early immune activation with chronic inflammation and cytokine dysregulation in ASDs [19, 20] and it has been shown that systemic inflammation induced by LPS provokes behavioural changes and impairs the blood-brain barrier in animal models [17, 21]. Furthermore, fungal infections that may originate from alterations in commensal bacteria population [22], could shift the indoleamine 2,3-dioxygenase’s activity [23, 24] reducing the levels of kynurenine [25], a neuroprotective agent. Despite several reports disclosed an aberrant gut microbiota in ASDs, consensus across studies has not yet been established [8]. Here we characterized the bacterial gut microbiota and the less studied gut mycobiota of subjects affected by autism through amplicon-based metataxonomics analysis of the V3-V5 region of the prokaryotic 16S rDNA and of the ITS1 region of the fungal rDNA in order to better understand the microbial community structure associated with ASDs and its impact on GI abnormalities.

## Results

### Autistic subjects harbour an altered bacterial gut microbiota

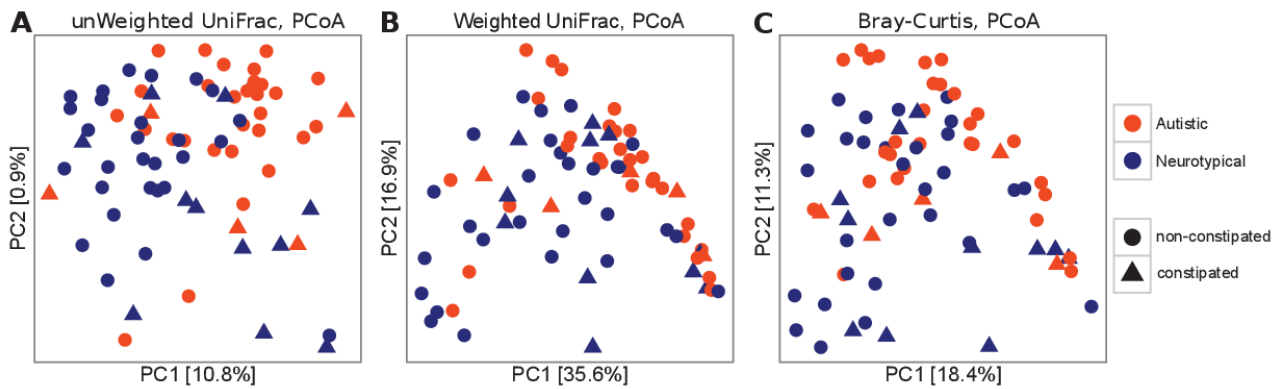
For the characterization of the gut microbiota associated with autism we recruited 40 autistic subjects (36 out of 40 autistic subjects were classified as severe ASDs, CARS value >37) and 40 neurotypical controls (Table 1, Supplementary Table 1).

**Table 1:** Characteristics of study participants

	<b>Autistic</b>	<b>Neurotypical</b>
<b>Subjects (n)</b>	40	40
<b>Age (1st - 3rd quartile)</b>	10 (5 - 17)	7 (3.6 - 12)
<b>Gender (n)</b>		
female	22.5% (9)	30% (12)
male	77.5% (31)	70% (28)
<b>Constipation (n)</b>		
constipated	12.5% (5)	27.5% (11)
non-constipated	72.5% (29)	72.5% (29)
NA	15% (6)	0% (0)
<b>Calprotectin (1st - 3rd quartile)</b>	36.9 (17.6 - 76.0) $\mu\text{g/g}$	40.9 (17 - 74.7) $\mu\text{g/g}$
constipated	39.1 (22.9 - 70.0) $\mu\text{g/g}$	27.9 (20.3 - 97.6) $\mu\text{g/g}$
non-constipated	35.9 (15.0 - 57.8) $\mu\text{g/g}$	50.5 (15.0 - 73.8) $\mu\text{g/g}$
<b>CARS (1st - 3rd quartile)</b>	47 (40 - 50.5)	NA
constipated	50 (36 - 52.0)	NA
non-constipated	48 (42 - 50.0)	NA
<b>ESR (1st - 3rd quartile)</b>	7.5 (3.25 - 17.7) mm/h	NA
constipated	22.0 (12.0 - 25.0) mm/h	NA
non-constipated	7.0 (2.7 - 11.2) mm/h	NA
<b>Serum IgA (1st - 3rd quartile)</b>	131.0 (70.0 - 172.2) mg/ml	NA
constipated	97.0 (82.0 - 153.0) mg/ml	NA
non-constipated	133.0 (67.0 - 181.0) mg/ml	NA

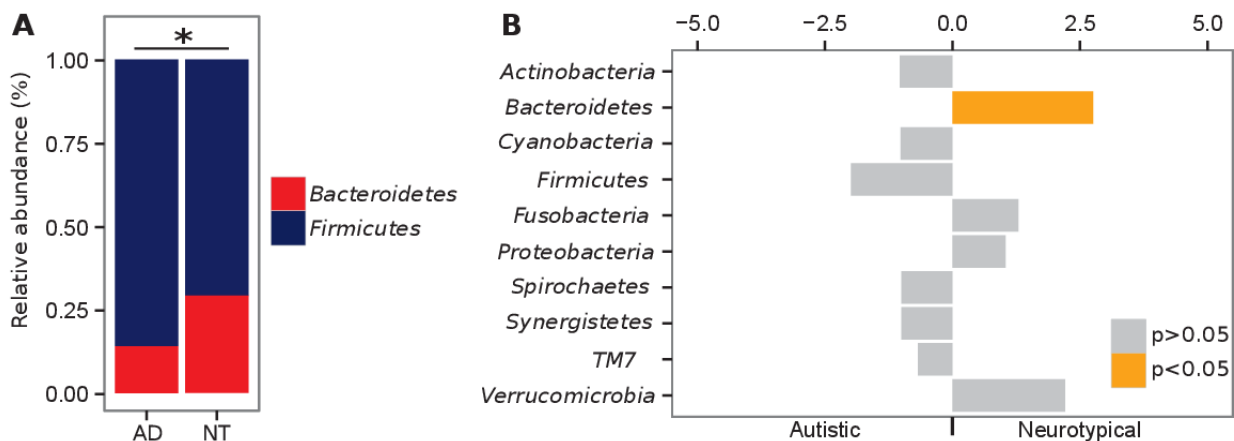
Data expressed as medians with interquartile ranges when applicable. AD, autistic subjects; NT, neurotypical subjects; NA, not applicable; CARS, childhood autism rating scale; ESR, erythrocyte sedimentation rate.

Analysis of *alpha*-diversity revealed no significant differences between autistic and neurotypical subjects (hereinafter termed AD and NT respectively). However the analysis of the *beta*-diversity calculated on the unweighted, weighted UniFrac distances and the Bray-Curtis dissimilarity, revealed that the bacterial microbiota of AD clusters apart from that of NT ( $p < 0.05$ , PERMANOVA; Figure 1, Supplementary Table 2). Since we enrolled subjects suffering from constipation among NT and AD subjects, the impact of constipation on the *beta*-diversity of the two groups of study was also analysed. We observed that constipation has a significant effect on the microbial community structure within NT subjects ( $p < 0.05$ , PERMANOVA), as expected [26, 27], but not within AD subjects (Supplementary Table 2).



**Figure 1:** PCoA of bacterial *beta*-diversity based on **A)** the unweighted and **B)** weighted UniFrac distances and **C)** Bray-Curtis dissimilarity. Autistic and neurotypical subjects are coloured in orange and blue, respectively. The constipation status of the subjects is indicated according to different shapes, circles for non-constipated and triangles for constipated individuals.

Furthermore we observed that the severity of the autistic phenotype, as measured by CARS scores, does not affect the bacterial community structure among AD individuals ( $p > 0.05$ , PERMANOVA; Supplementary Table 3). Phylum level analysis showed a clear alteration of the bacterial gut community in AD characterized by a higher *Firmicutes/Bacteroidetes* ratio ( $p < 0.005$ , Wilcoxon rank-sum test; Figure 2A) in AD than NT due to a significant reduction of the relative abundance of *Bacteroidetes* (9.2% AD, 19.4% NT) (FDR-corrected  $p < 0.05$ , Welch *t*-test; Figure 2B).



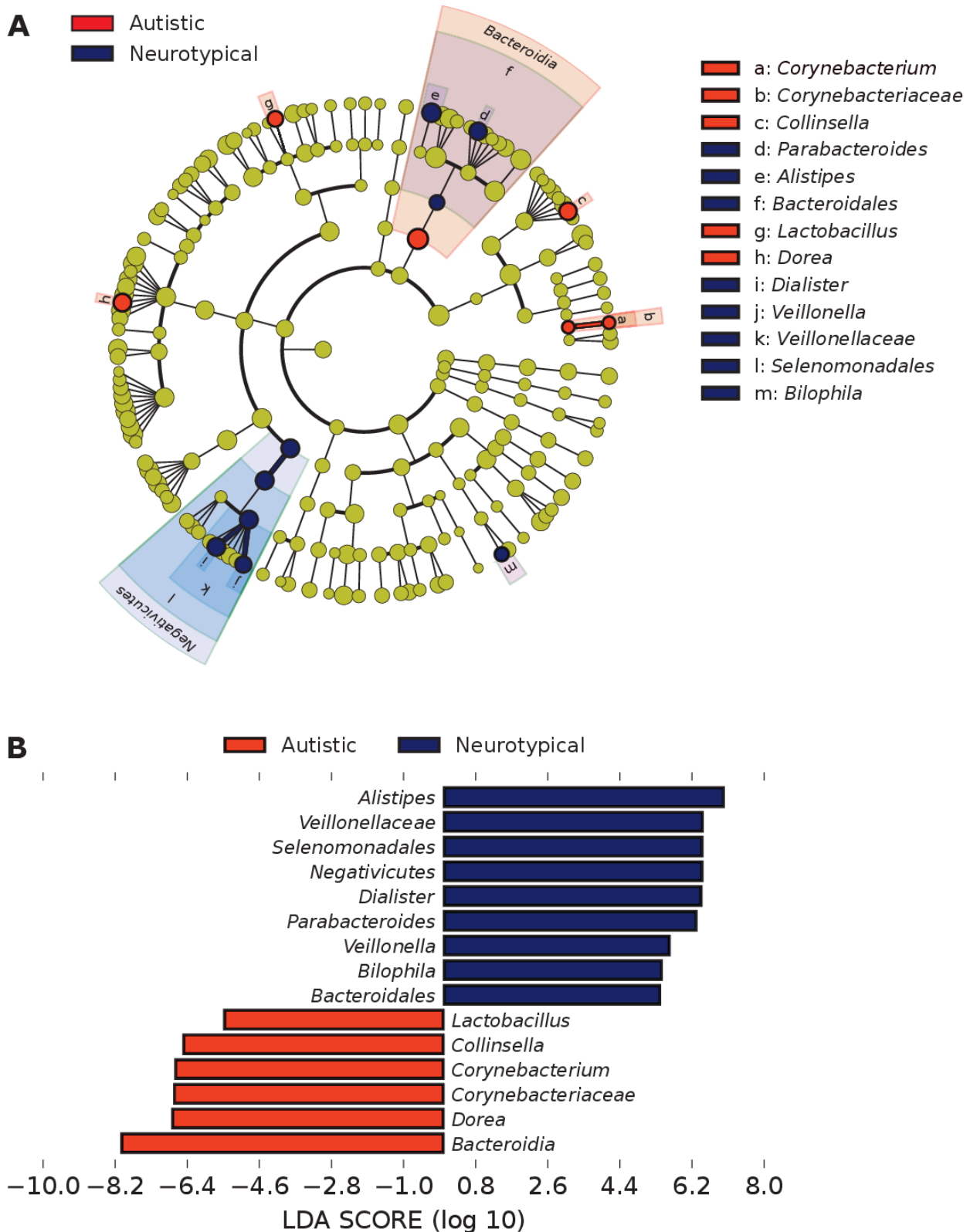
**Figure 2:** **A)** Mean relative abundances (%) of *Firmicutes* and *Bacteroidetes* in autistic (AD) and neurotypical (NT) subjects; \*  $p < 0.005$ , Wilcoxon sum-rank test on the *Firmicutes/Bacteroidetes* ratio. **B)** Welch's *t*-test statistics of the relative abundances of bacterial phyla in autistic and neurotypical subjects. Orange bars indicate significant FDR-corrected  $p$ -values adjusted for multiple comparison controlling the family-wise Type I error rate.

Genus level analysis showed that the top ten most abundant genera in both AD and NT subjects were *Bifidobacterium*, *Bacteroides*, *Faecalibacterium*, *Unknown Lachnospiraceae*, *Blautia*, *Ruminococcus*, *Clostridium XI*, *Streptococcus*, *Gemmiger* and *Lachnospiraceae incertae sedis* (Supplementary Fig. 1, Supplementary Table 4). Interestingly, the genus *Prevotella* was only barely

represented in AD with respect to NT (0.05% AD, 1.5% NT), in agreement with a previous study on the gut microbiota in ASD children [28] although this difference of relative abundance was not supported by the statistical analysis. We further analysed the bacterial community structure associated with AD and NT by using LEfSe, an algorithm for high-dimensional biomarker discovery which uses linear discriminant analysis (LDA) to estimate the effect size of each taxa which are differentially represented in cases and controls [29]. LEfSe analysis revealed a significant increase of the relative abundance of different bacterial taxa in AD than in NT among which *Collinsella*, *Corynebacterium*, *Dorea* and *Lactobacillus* and a significant reduction of the taxa *Alistipes*, *Bilophila*, *Dialister*, *Parabacteroides* and *Veillonella* in AD than in NT ( $p < 0.01$ , Wilcoxon rank-sum test;  $LDA > 2.0$ ; Figure 3).

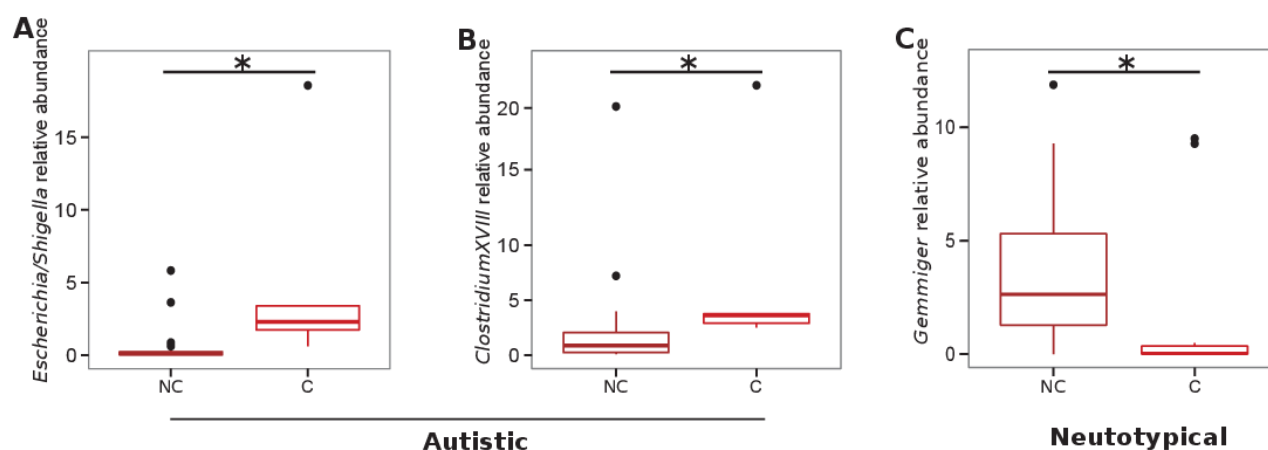
### **Constipation select different bacterial taxa in autistic subjects and neurotypical healthy controls**

Autistic subjects frequently suffer of GI comorbidities [4-7] and constipation is a GI symptom often reported in these subjects, known to alter the physiology of the human GI tract and the gut microbiota itself [27, 30, 31]. Correlation analysis of the bacterial relative abundances between constipated and non-constipated subjects, both autistic and neurotypical, revealed that among the most abundant bacterial genera (with relative abundance  $> 0.5\%$  and detectable in at least the 70% of the investigated subjects) the taxa *Gemmiger* and *Ruminococcus* anti-correlates with the constipation status (Spearman's correlation  $r = -0.39$  and  $-0.36$ , respectively; FDR-corrected  $p < 0.05$ ; Supplementary Table 5) while *Escherichia/Shigella* and *Clostridium cluster XVIII* positively correlates with this GI symptom (Spearman's correlation  $r = 0.31$  and  $0.38$ , respectively; FDR-corrected  $p < 0.05$ ; Supplementary Table 5). We further compared the relative abundance of these taxa among constipated and non-constipated subjects within and between groups. We observed that *Escherichia/Shigella* and *Clostridium cluster XVIII* were significantly more abundant in constipated AD compared to the non-constipated ones (FDR-corrected  $p < 0.05$ , Wilcoxon rank sum test; Figure 4A and 4B) while no differences have been detected between constipated and non-constipated NT for these taxa. On the other hand, the genus *Gemmiger* was significantly less abundant in constipated compared to non-constipated NT (FDR-corrected  $p < 0.05$ , Wilcoxon rank sum test; Figure 4C). Remarkably, no significant differences have been observed in the levels of faecal calprotectin between AD and NT as well as between constipated and non-constipated subjects in both groups (Table 1 and Supplementary Table 1). Furthermore we analysed the levels of other two biomarkers of inflammations *i.e.* serum IgA and ESR in the autistic subjects and we did not observe significant differences among constipated and non-constipated AD (Table 1 and Supplementary Table 1).



**Figure 3:** **A)** Cladograms generated by LEfSe indicating differences in the bacterial taxa between autistic (AD) and neurotypical (NT) subjects. Nodes in orange indicate taxa that were enriched in AD compared to those in NT, while nodes in blue indicate taxa that were enriched in NT compared to those in AD. **B)** LDA scores for the bacterial taxa differentially abundant between autistic (AD) and neurotypical (NT) subjects. Positive and negative LDA scores indicate the bacterial taxa enriched in NT and AD subjects, respectively. Only the taxa having a  $p < 0.01$  (Wilcoxon rank-sum test) and  $LDA > 2.0$  are shown in the figure legend

Therefore, while constipation resulted in a significant increase of *Escherichia/Shigella* and *Clostridium cluster XVIII* no differences have been observed in the levels of inflammation between constipated and non-constipated autistic subjects suggesting that constipation and the related alterations of the gut microbiota in autistic subjects as well as in neurotypical individuals is not associated with an increase of intestinal inflammation. It should be noted that the number of enrolled constipated subjects was quite low and therefore these analyses could be underpowered.

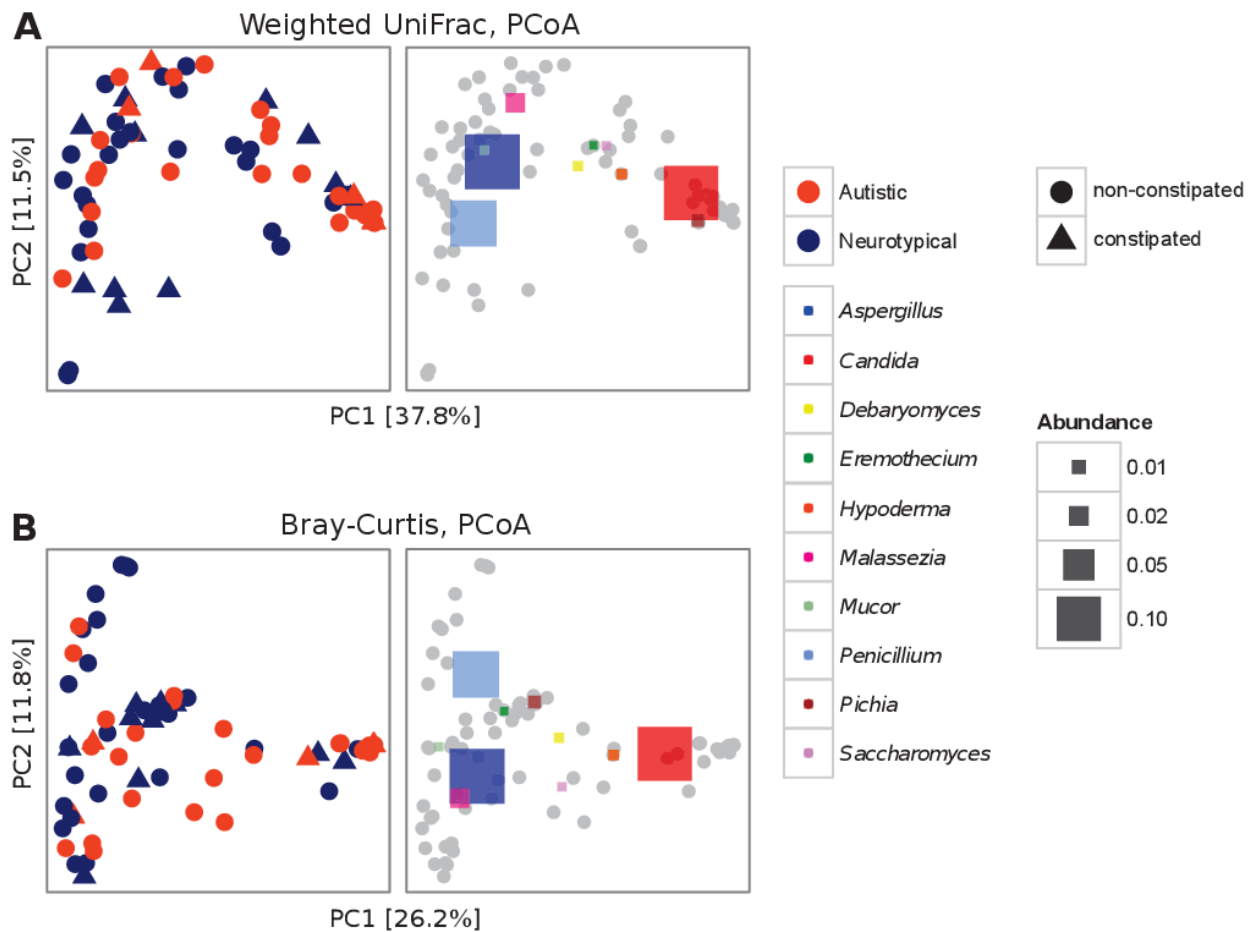


**Figure 4:** Box plot representation of the relative abundances of bacterial genera correlating with the constipation status of the subjects enrolled in this study. Comparisons between **A, B**) constipated (C) and non-constipated (NC) autistic subjects and **C**) constipated (C) and non-constipated (NC) neurotypical subjects; \* FDR-corrected  $p < 0.05$ , Wilcoxon sum-rank test.

### Autistic subjects harbour an altered gut mycobiota

We then investigated the gut mycobiota of our study cohort through amplicon-based sequencing of fungal ITS1 region. High quality fungal sequences were detected respectively in 35 out of 40 autistic subjects and 38 out of 40 NT. As occurred for the bacterial gut microbiota we did not observe significant differences in fungal *alpha*-diversity between AD and NT. The analysis of *beta*-diversity revealed that the gut mycobiota of AD was different compared to NT as calculated by PCoA and PERMANOVA on the weighted UniFrac distance and Bray-Curtis dissimilarity ( $p < 0.05$ , Figure 5). As for the bacterial *beta*-diversity, constipation showed a significant effect within NT subjects ( $p = 0.046$ , PERMANOVA on Bray-Curtis dissimilarities) but not within AD subjects (Supplementary Table 6). Furthermore the severity of the autistic phenotype does not affect the gut mycobiota community structure among AD individuals ( $p > 0.05$ , PERMANOVA; Supplementary Table 3). An in-depth analysis of the gut mycobiota lead to the identification of 50 fungal taxa fully classified to the genus level and 30 only partially classified. Genus level analysis showed *Aspergillus* (24.2% AD; 28% NT), *Candida* (37.7% AD; 14.1% NT), *Penicillium* (13.2% AD; 23.5% NT) and *Malassezia* (3.05% AD; 3.3% NT) as the most abundant and widely distributed genera in our study cohort in terms of relative abundance (Supplementary Fig. 2, Supplementary Table 7).





**Figure 5:** PCoAs of fungal *beta*-diversity based on **A**) weighted UniFrac distance and **B**) Bray-Curtis dissimilarity. The right panel of the graphs **A** and **B** shows the same PCoA coordinates with the most abundant OTUs superimposed as coloured squares, with the size being proportional to the mean relative abundance of the taxon across all samples (grey dots). Autistic and neurotypical subjects are coloured in orange and blue, respectively. The constipation status of the subjects is indicated according to different shapes, circles for non-constipated and triangles for constipated individuals.

Although the relative abundance of the genus *Candida* was more than twice as much in AD than NT we did not identify significant differences among the two study groups (Welch *t*-test, FDR-corrected *p*-value=0.09, raw *p*-value=0.006; Supplementary Fig. 3) probably due to a larger dispersion of values in AD when compared to NT ( $p < 0.001$ ; Levene's test). However the superimposition of the most abundant genera over the PCoA plots revealed that high levels of *Candida* abundance was associated with a group of subjects mainly affected by autism (Figure 5) suggesting that *Candida* could play a role in the altered microbial community associated with the autistic subjects. Correlation analyses among the most abundant fungi and bacteria (with relative abundance  $> 0.5\%$  and detectable in at least the 70% of the investigated subjects) revealed no significant correlations among autistic subjects while a significant positive correlation between the genera *Aspergillus* and *Bifidobacterium* was found within NT individuals (Spearman's  $r = 0.6$ , FDR-corrected  $p = 0.004$ ) (Supplementary Table 8).

## Discussion

The gut microbiota is a crucial factor for the maintenance of GI tract functions and immune homeostasis. It is well known that dysbiosis of the GI tract could lead to inflammation and immune activation in several pathologies [15]. The frequent occurrence of GI symptoms in autistic subjects imply the possible involvement of the gut microbiota in ASDs gastrointestinal pathophysiology, further supported by the speculations on the increased incidence of ASDs cases due to “Western” habits (*i.e.* diet, medications and excessive overall hygiene) that can affect the composition of the gut microbiota [32]. Several studies demonstrated alterations in the bacterial gut microbiota of ASDs individuals, even if the differences reported in these studies were in some cases discordant, possibly due to variance in sampling strategies and methodologies used [8]. In addition, our recent findings showed an altered gut microbiota in Rett syndrome [33], a genetically determined neurodevelopmental disorder previously categorized in the ASDs group sharing some features of these conditions. We characterized the gut microbiota associated with autism, disclosing an altered microbial community both at bacterial and fungal level. We observed a significant increase in the *Firmicutes/Bacteroidetes* ratio in autistic subjects due to a significant reduction of *Bacteroidetes* in these individuals. Several inflammatory conditions have been related to an increase in the *Firmicutes/Bacteroidetes* ratio such as IBDs [34] and obesity [35]. Consistently with these observations, an increased *Firmicutes/Bacteroidetes* ratio has been reported also in subjects with autism [36, 37]. Furthermore we discovered that the relative abundances of the genera *Collinsella*, *Corynebacterium*, *Dorea* and *Lactobacillus* were significantly increased in the gut microbiota of autistic subjects with respect to that of the neurotypical subjects while the relative abundance of the genera *Alistipes*, *Bilophila*, *Dialister*, *Parabacteroides* and *Veillonella* were significantly reduced in these individuals. A recent study on a mouse model of ASDs demonstrated that treatments with a PSA<sup>+</sup> *Bacteroides fragilis* strain restore autism-related behavioural and GI abnormalities, also reducing the reported high levels of *Lachnospiraceae* and 4-ethylphenylsulfate, a metabolite produced by this bacterial family related to *p*-cresol, a putative metabolic marker for autism [38]. Overall, these data are consistent with our findings and remark the importance of *Bacteroidetes* in ASDs pathophysiology. Moreover *Lactobacillus* resulted to be enriched in the gut microbiota of autistic individuals while *Dialister* and *Veillonella* resulted to be depleted, in line with the results obtained in previous studies [28, 39]. Since constipation is a common gastrointestinal problem in subjects with ASDs [4-7] we compared our data between constipated and non-constipated subjects in order to evaluate the contribution of constipation in shaping the gut microbiota of autistic subjects. Indeed it has been proposed that GI symptoms may be related to ASDs [40]. The evidence that the taxa belonging to the *Clostridium cluster XVIII* and the putative pro-inflammatory

*Escherichia/Shigella* [41, 42] positively correlated with the constipation status of the subjects as well as their enrichment in constipated autistic subjects supports the hypothesis that GI problems and related alterations of the gut microbiota may contribute to ASDs gastrointestinal symptoms [40]. Because of their ability to produce exotoxins and propionate that may exacerbate autistic symptoms [43], the role of clostridia in ASDs has been extensively explored. The species belonging to the *Clostridium cluster XVIII* have been shown to produce exotoxins [44] and to promote conditions favouring inflammation [45, 46] although other studies observed their potential ability to induce homeostatic T-reg responses [47]. It is also interesting to underline the occurrence of a subclinical acute phase response in ASDs plasma, as evidenced by advanced proteomic analysis [48].

Despite the importance given to the implications of the gut microbiota in health and disease few reports have explored the relevance of the fungal component of the gut microbiota in GI (patho)physiology [49]. Furthermore, none of the published studies on ASDs' gut microbiota have assessed the fungal gut community structure associated with autism. Our dataset of autistic subjects displayed a different fungal community structure compared to neurotypical subjects. In particular the genus *Candida* was one of the most abundant taxa in the gut mycobiota of this study cohort, being two times more abundant in AD than NT. To the best of our knowledge this is the first time that alterations of the intestinal fungal microbiota are associated with ASDs. Although *Candida* is one of the most common and abundant genus of the human gut mycobiota [50, 51], its implication in phenomena of fungal dysbiosis have been reported in several GI and inflammatory conditions [52-54] as well as in Rett syndrome [33]. It is therefore possible that alteration of the intestinal fungal population driven by an expansion of *Candida* in the gut mycobiota of autistic individuals may negatively impact on GI abnormalities through cytokine dysregulation. The gut microbiota, in particular some species of *Lactobacillus*, modulates the immunological responses to *Candida* in the GI tract by providing tryptophan-derived aryl-hydrocarbon receptor ligands that stimulate the immune system, principally ILC3 cells, to produce IL-22 [55]. Together with IL-17, IL-22 avoids the excessive proliferation of *Candida* and other fungal commensals in the gut. It is therefore possible that alterations of the gut microbiota in ASDs could lead to an expansion of the *Candida* population preventing from a full restoration of the bacterial community structure. Indeed it has been observed that alterations of the bacterial gut microbiota due to prolonged antibiotic usage and the subsequent colonization with *C. albicans* prevents from a full restoration of the bacterial community structure resulting in altered abundances of *Bacteroidetes*, *Lactobacillaceae*, *Ruminococcaceae* and *Lachnospiraceae* [56].

## Conclusions

Here we observed an altered intestinal microbial community associated with autism, both at bacterial and fungal level. Furthermore such alterations of the gut microbiota did not depend by the constipation status of autistic individuals although a larger cohort would be necessary to validate these findings. Our results encourage an in-depth, extensive study on the impact of the gut microbiota in the GI physiology and neuroplastic changes in ASDs, as well as the integration of such data with immunology and metabolomics to further establish the relevance of the gut microbiota in the ASDs pathophysiology.

## Methods

### Study participants and samples handling and collection

We recruited 40 subjects with clinical diagnosis of autism (average age  $11.1 \pm 6.8$ ; sex, male:female, 31:9) and we compared them with 40 age and sex-matched neurotypical healthy subjects (average age  $9.2 \pm 7.9$ ; sex, male:female, 28:12). Autistic subjects with clinically evident inflammatory conditions were excluded. Constipation and inflammation (*i.e.* serum IgA, erythrocyte sedimentation rate and faecal calprotectin levels) were also assessed. The autistic subjects were consecutively admitted to the Child Neuropsychiatry Unit of the University Hospital of Siena, and ASDs were diagnosed according with the Diagnostic and Statistical Manual of Mental Disorders, 5th Edition [1], and evaluated using Autism Diagnostic Observation Schedule and Autism Behaviour Checklist. Childhood Autism Rating Scale scores (CARS) [57] were calculated by an experienced child neuropsychiatrist. Average CARS values were  $46.2 \pm 6.8$  (value range 32-57); a fraction of 90 % (36/40) were classified as severe ASDs (CARS value  $>37$ ), with 10 % (4/40) being moderately severe ASDs (CARS values from 30 to 36) (Supplementary Table 9). No specific comorbidities in the autistic cohort were present with the single exception of a coexisting celiac disease in two patients (5 %). Constipation has been defined according to Rome III criteria [58]. Stool samples from enrolled subjects were collected, aliquoted as it is and stored at  $-80^{\circ}\text{C}$  until analysis. All subjects of this study were under a Mediterranean-based diet and no antibiotics, probiotics or prebiotics have been taken in the 3 month prior the sample collection. None of the subjects were on anti-inflammatory or antioxidant drugs. The study was conducted after the approval by the Institutional Review Board of the Siena University Hospital (AOUS, Siena, Italy) and all written informed consents were obtained from either the parents or the legal tutors of the enrolled subjects, in compliance with national legislation and the Code of Ethical Principles for Medical Research Involving Human Subjects of the World Medical Association (Declaration of Helsinki).

### Faecal Calprotectin Assay

Calprotectin determination was performed by using a polyclonal antibody in an enzyme-linked immunosorbent assay (Calprest, Eurospital, Trieste, Italy) according to the manufacturer's instructions. Calprotectin values < 50 µg/g per stool sample were considered normal.

### Pyrosequencing and Data analysis

Total DNA extraction from faecal samples (250mg, wet weight) was performed using the FastDNA™ SPIN Kit for Feces (MP Biomedicals, Santa Ana, CA, USA) following manufacturer's instructions. For each DNA sample, we amplified respectively the bacterial 16S rRNA genes using a primer set specific for V3-V5 hypervariable region (F357: 5'-TCCTACGGGAGGCAGCAG-3' and R937: 5'-TGTGCGGGCCCCGTCAATT-3') and the Internal Transcribed Spacer (ITS) using a primer set specific for fungal ITS1 rDNA region (18SF: 5'-GTAAAAGTCGTAACAAGGTTTC-3' and 5.8S1R: 5'-GTTCAAAGAYTCGATGATTCAC-3') [59] containing adaptors, key sequence and barcode sequences as described by the 454 Sequencing System Guidelines for Amplicon Experimental Design (Roche, Basel, Switzerland). The PCR products obtained were then purified, quantified and pooled in equimolar way in a final amplicon library. The 454 pyrosequencing was carried out on the GS FLX+ system using the XL+ chemistry following the manufacturer's recommendations (Roche, Basel, Switzerland). Raw 454 data were demultiplexed using the Roche's sff file software and submitted to the European Nucleotide Archive (ENA) with accession numbers PRJEB15418 and PRJEB15420. Sample accessions IDs and metadata are available in Supplementary Table 9. Reads were pre-processed using the MICCA pipeline (v. 0.1) (<http://www.micca.org>) [60]. operational taxonomic units (OTUs) were assigned by clustering the sequences with a threshold of 97% pairwise identity and their representative sequences were classified using the RDP classifier version 2.7 on 16S data and using the RDP classifier version 2.8 [61] against the UNITE fungal ITS database [62] on ITS1 data. Template-guided multiple sequence alignment (MSA) was performed using PyNAST [63] (v. 0.1) against the multiple alignment of the Greengenes [64] database (release 13\_05) filtered at 97% similarity for bacterial sequences and through de novo MSA using T-Coffee [65] for fungal sequences. Fungal taxonomy assignments were then manually curated using BLASTn against the GenBank's database for accuracy. High quality fungal sequences have been also manually filtered out for sequences belonging to *Agaricomycetes* (unlikely to be residents of the human gut due to their ecology [66]). The phylogenetic tree was inferred using micca-phylogeny [67]. Sampling heterogeneity was reduced by rarefaction. *Alpha* (within-sample richness) and *beta*-diversity (between-sample dissimilarity) estimates were computed using the phyloseq R package [68]. Permutational MANOVA (PERMANOVA) test was performed using the `adonis()` function in the R

package *vegan* with 999 permutations. Permutations have been constrained within age groups (corresponding to 0-2 y/o, 3-10 y/o, 11-17 y/o and >18 y/o) or gender to evaluate possible biases related to the unequal age and gender distributions among subjects using the “*strata*” argument within the *adonis()* function. Two-sided, unpaired Welch *t*-statistics were computed using the function *mt()* in the *phyloseq* library and the *p*-values were adjusted for multiple comparison controlling the family-wise Type I error rate (minP procedure) [69]. Spearman's correlation tests were computed using the *psych* R package [70]. Linear discriminant effect size analysis (LEfSe) was performed to find features (taxa) differentially represented between autistic and neurotypical subjects. LEfSe combines Kruskal-Wallis test or pairwise Wilcoxon rank-sum test with linear discriminant analysis (LDA). It ranks features by effect size, which put features that explain most of the biological difference at top. LEfSe analysis was performed under the following conditions:  $\alpha$  value for the statistical test equal to 0.01 and threshold on the logarithmic LDA score for discriminative features equal to 2.0 [29]. All statistical analyses were performed using R [71] and *p*-values were FDR-corrected [72].

### Abbreviations

AD, autistic subjects; ASDs, autism spectrum disorders; CARS, childhood autism rating scale; CNS, central nervous system; ESR, erythrocyte sedimentation rate; FDR, false discovery rate; GI, gastrointestinal; IBD, inflammatory bowel disease; ITS, Internal Transcribed Spacer; NT, neurotypical subjects; OTU, operational taxonomic unit; PCoA, principal coordinates analysis; PERMANOVA, permutational multivariate analysis of variance; SCFAs, short chain fatty acids.

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### Author contribution

FS, CDF, DC and AC conceived the study and designed the experiments. FS performed the experiments and wrote the manuscript. DR and SL analysed and collected the clinical data. FS analysed the metagenomics data. DA and CD supervised and contributed to data analysis. CDFe, JH, SL, DR and AC recruited subjects and collected specimens. CDFe, JH, SL, DR and AC provided

further clinical patient information and reagents for the study. AC, DC and CDF approved the final manuscript. All the authors critically reviewed and approved the manuscript.

### Availability of supporting data

Raw sequences are available in the European Nucleotide Archive (ENA) with accession numbers PRJEB15418 and PRJEB15420.

### Conflict of interest

Authors declare no conflict of interests.

### Ethics approval and consent to participate

The study was conducted after the approval by the Institutional Review Board of the Siena University Hospital (AOUS, Siena, Italy) and all written informed consents were obtained from either the parents or the legal tutors of the enrolled subjects, in compliance with national legislation and the Code of Ethical Principles for Medical Research Involving Human Subjects of the World Medical Association (Declaration of Helsinki).

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### References

1. Association AP: **Diagnostic and Statistical Manual of Mental Disorders (DSM-5®)**: American Psychiatric Pub; 2013.
2. Kim YS, Leventhal BL: **Genetic epidemiology and insights into interactive genetic and environmental effects in autism spectrum disorders**. *Biological psychiatry* 2015, **77**(1):66-74.
3. Colvert E, Tick B, McEwen F, Stewart C, Curran SR, Woodhouse E, Gillan N, Hallett V, Lietz S, Garnett T *et al*: **Heritability of Autism Spectrum Disorder in a UK Population-Based Twin Sample**. *JAMA psychiatry* 2015, **72**(5):415-423.
4. McElhanon BO, McCracken C, Karpen S, Sharp WG: **Gastrointestinal symptoms in autism spectrum disorder: a meta-analysis**. *Pediatrics* 2014, **133**(5):872-883.
5. Hsiao EY: **Gastrointestinal issues in autism spectrum disorder**. *Harvard review of psychiatry* 2014, **22**(2):104-111.
6. Furuta GT, Williams K, Kooros K, Kaul A, Panzer R, Coury DL, Fuchs G: **Management of constipation in children and adolescents with autism spectrum disorders**. *Pediatrics* 2012, **130** Suppl 2:S98-105.
7. Gorrindo P, Williams KC, Lee EB, Walker LS, McGrew SG, Levitt P: **Gastrointestinal dysfunction in autism: parental report, clinical evaluation, and associated factors**.

- Autism research : official journal of the International Society for Autism Research* 2012, **5**(2):101-108.
8. Mayer EA, Padua D, Tillisch K: **Altered brain-gut axis in autism: comorbidity or causative mechanisms?** *BioEssays : news and reviews in molecular, cellular and developmental biology* 2014, **36**(10):933-939.
  9. Sampson TR, Mazmanian SK: **Control of brain development, function, and behavior by the microbiome.** *Cell host & microbe* 2015, **17**(5):565-576.
  10. Barajon I, Serrao G, Arnaboldi F, Opizzi E, Ripamonti G, Balsari A, Rumio C: **Toll-like receptors 3, 4, and 7 are expressed in the enteric nervous system and dorsal root ganglia.** *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society* 2009, **57**(11):1013-1023.
  11. Brun P, Giron MC, Qesari M, Porzionato A, Caputi V, Zoppellaro C, Banzato S, Grillo AR, Spagnol L, De Caro R *et al*: **Toll-like receptor 2 regulates intestinal inflammation by controlling integrity of the enteric nervous system.** *Gastroenterology* 2013, **145**(6):1323-1333.
  12. Sudo N: **Role of microbiome in regulating the HPA axis and its relevance to allergy.** *Chemical immunology and allergy* 2012, **98**:163-175.
  13. De Vadder F, Kovatcheva-Datchary P, Goncalves D, Vinera J, Zitoun C, Duchampt A, Backhed F, Mithieux G: **Microbiota-generated metabolites promote metabolic benefits via gut-brain neural circuits.** *Cell* 2014, **156**(1-2):84-96.
  14. Ray K: **Gut microbiota: microbial metabolites feed into the gut-brain-gut circuit during host metabolism.** *Nature reviews Gastroenterology & hepatology* 2014, **11**(2):76.
  15. Kamada N, Seo SU, Chen GY, Nunez G: **Role of the gut microbiota in immunity and inflammatory disease.** *Nature reviews Immunology* 2013, **13**(5):321-335.
  16. Berer K, Krishnamoorthy G: **Commensal gut flora and brain autoimmunity: a love or hate affair?** *Acta neuropathologica* 2012, **123**(5):639-651.
  17. Stolp HB, Dziegielewska KM, Ek CJ, Potter AM, Saunders NR: **Long-term changes in blood-brain barrier permeability and white matter following prolonged systemic inflammation in early development in the rat.** *The European journal of neuroscience* 2005, **22**(11):2805-2816.
  18. Onore CE, Nordahl CW, Young GS, Van de Water JA, Rogers SJ, Ashwood P: **Levels of soluble platelet endothelial cell adhesion molecule-1 and P-selectin are decreased in children with autism spectrum disorder.** *Biological psychiatry* 2012, **72**(12):1020-1025.
  19. Onore C, Careaga M, Ashwood P: **The role of immune dysfunction in the pathophysiology of autism.** *Brain, behavior, and immunity* 2012, **26**(3):383-392.
  20. Theoharides TC, Asadi S, Patel AB: **Focal brain inflammation and autism.** *Journal of neuroinflammation* 2013, **10**:46.
  21. Stolp HB, Johansson PA, Habgood MD, Dziegielewska KM, Saunders NR, Ek CJ: **Effects of neonatal systemic inflammation on blood-brain barrier permeability and behaviour in juvenile and adult rats.** *Cardiovascular psychiatry and neurology* 2011, **2011**:469046.
  22. Oever JT, Netea MG: **The bacteriome-mycobiome interaction and antifungal host defense.** *European journal of immunology* 2014, **44**(11):3182-3191.
  23. De Luca A, Montagnoli C, Zelante T, Bonifazi P, Bozza S, Moretti S, D'Angelo C, Vacca C, Boon L, Bistoni F *et al*: **Functional yet balanced reactivity to *Candida albicans* requires TRIF, MyD88, and IDO-dependent inhibition of Rorc.** *Journal of immunology* 2007, **179**(9):5999-6008.
  24. Cheng SC, van de Veerdonk F, Smeekens S, Joosten LA, van der Meer JW, Kullberg BJ, Netea MG: ***Candida albicans* dampens host defense by downregulating IL-17 production.** *Journal of immunology* 2010, **185**(4):2450-2457.



25. Bozza S, Fallarino F, Pitzurra L, Zelante T, Montagnoli C, Bellocchio S, Mosci P, Vacca C, Puccetti P, Romani L: **A crucial role for tryptophan catabolism at the host/*Candida albicans* interface.** *Journal of immunology* 2005, **174**(5):2910-2918.
26. Vandeputte D, Falony G, Vieira-Silva S, Tito RY, Joossens M, Raes J: **Stool consistency is strongly associated with gut microbiota richness and composition, enterotypes and bacterial growth rates.** *Gut* 2016, **65**(1):57-62.
27. Zhu L, Liu W, Alkhoury R, Baker RD, Bard JE, Quigley EM, Baker SS: **Structural changes in the gut microbiome of constipated patients.** *Physiological genomics* 2014, **46**(18):679-686.
28. Kang DW, Park JG, Ilhan ZE, Wallstrom G, Labaer J, Adams JB, Krajmalnik-Brown R: **Reduced incidence of *Prevotella* and other fermenters in intestinal microflora of autistic children.** *PloS one* 2013, **8**(7):e68322.
29. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, Huttenhower C: **Metagenomic biomarker discovery and explanation.** *Genome biology* 2011, **12**(6):R60.
30. Zoppi G, Cinquetti M, Luciano A, Benini A, Muner A, Bertazzoni Minelli E: **The intestinal ecosystem in chronic functional constipation.** *Acta paediatrica* 1998, **87**(8):836-841.
31. Son JS, Zheng LJ, Rowehl LM, Tian X, Zhang Y, Zhu W, Litcher-Kelly L, Gadow KD, Gathungu G, Robertson CE *et al*: **Comparison of Fecal Microbiota in Children with Autism Spectrum Disorders and Neurotypical Siblings in the Simons Simplex Collection.** *PloS one* 2015, **10**(10):e0137725.
32. Wang Y, Kasper LH: **The role of microbiome in central nervous system disorders.** *Brain, behavior, and immunity* 2014, **38**:1-12.
33. Strati F, Cavalieri D, Albanese D, De Felice C, Donati C, Hayek J, Jousson O, Leoncini S, Pindo M, Renzi D *et al*: **Altered gut microbiota in Rett syndrome.** *Microbiome* 2016, **4**(1):41.
34. Frank DN, St Amand AL, Feldman RA, Boedeker EC, Harpaz N, Pace NR: **Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases.** *Proceedings of the National Academy of Sciences of the United States of America* 2007, **104**(34):13780-13785.
35. Turnbaugh PJ, Backhed F, Fulton L, Gordon JI: **Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome.** *Cell host & microbe* 2008, **3**(4):213-223.
36. Williams BL, Hornig M, Buie T, Bauman ML, Cho Paik M, Wick I, Bennett A, Jabado O, Hirschberg DL, Lipkin WI: **Impaired carbohydrate digestion and transport and mucosal dysbiosis in the intestines of children with autism and gastrointestinal disturbances.** *PloS one* 2011, **6**(9):e24585.
37. Tomova A, Husarova V, Lakatosova S, Bakos J, Vlkova B, Babinska K, Ostatnikova D: **Gastrointestinal microbiota in children with autism in Slovakia.** *Physiology & behavior* 2015, **138**:179-187.
38. Hsiao EY, McBride SW, Hsien S, Sharon G, Hyde ER, McCue T, Codelli JA, Chow J, Reisman SE, Petrosino JF *et al*: **Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders.** *Cell* 2013, **155**(7):1451-1463.
39. Finegold SM, Dowd SE, Gontcharova V, Liu C, Henley KE, Wolcott RD, Youn E, Summanen PH, Granpeesheh D, Dixon D *et al*: **Pyrosequencing study of fecal microflora of autistic and control children.** *Anaerobe* 2010, **16**(4):444-453.
40. Adams JB, Johansen LJ, Powell LD, Quig D, Rubin RA: **Gastrointestinal flora and gastrointestinal status in children with autism--comparisons to typical children and correlation with autism severity.** *BMC gastroenterology* 2011, **11**:22.

41. Morgan XC, Tickle TL, Sokol H, Gevers D, Devaney KL, Ward DV, Reyes JA, Shah SA, LeLeiko N, Snapper SB *et al*: **Dysfunction of the intestinal microbiome in inflammatory bowel disease and treatment.** *Genome biology* 2012, **13**(9):R79.
42. Arthur JC, Gharaibeh RZ, Mühlbauer M, Perez-Chanona E, Uronis JM, McCafferty J, Fodor AA, Jobin C: **Microbial genomic analysis reveals the essential role of inflammation in bacteria-induced colorectal cancer.** *Nature communications* 2014, **5**.
43. Frye RE, Rose S, Slattery J, MacFabe DF: **Gastrointestinal dysfunction in autism spectrum disorder: the role of the mitochondria and the enteric microbiome.** *Microbial ecology in health and disease* 2015, **26**:27458.
44. Stiles BG, Pradhan K, Fleming JM, Samy RP, Barth H, Popoff MR: **Clostridium and bacillus binary enterotoxins: bad for the bowels, and eukaryotic being.** *Toxins* 2014, **6**(9):2626-2656.
45. Matsuda H, Fujiyama Y, Andoh A, Ushijima T, Kajinami T, Bamba T: **Characterization of antibody responses against rectal mucosa-associated bacterial flora in patients with ulcerative colitis.** *Journal of gastroenterology and hepatology* 2000, **15**(1):61-68.
46. Woting A, Pfeiffer N, Loh G, Klaus S, Blaut M: **Clostridium ramosum promotes high-fat diet-induced obesity in gnotobiotic mouse models.** *mBio* 2014, **5**(5):e01530-01514.
47. Atarashi K, Tanoue T, Oshima K, Suda W, Nagano Y, Nishikawa H, Fukuda S, Saito T, Narushima S, Hase K: **Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota.** *Nature* 2013, **500**(7461):232-236.
48. Cortelazzo A, De Felice C, Guerranti R, Signorini C, Leoncini S, Zollo G, Leoncini R, Timperio AM, Zolla L, Ciccoli L *et al*: **Expression and oxidative modifications of plasma proteins in autism spectrum disorders: Interplay between inflammatory response and lipid peroxidation.** *Proteomics Clinical applications* 2016.
49. Rizzetto L, De Filippo C, Cavalieri D: **Richness and diversity of mammalian fungal communities shape innate and adaptive immunity in health and disease.** *European journal of immunology* 2014, **44**(11):3166-3181.
50. Scanlan PD, Marchesi JR: **Micro-eukaryotic diversity of the human distal gut microbiota: qualitative assessment using culture-dependent and -independent analysis of faeces.** *The ISME journal* 2008, **2**(12):1183-1193.
51. Strati F, Di Paola M, Stefanini I, Albanese D, Rizzetto L, Lionetti P, Calabro A, Jousson O, Donati C, Cavalieri D *et al*: **Age and Gender Affect the Composition of Fungal Population of the Human Gastrointestinal Tract.** *Frontiers in microbiology* 2016, **7**:1227.
52. Li Q, Wang C, Tang C, He Q, Li N, Li J: **Dysbiosis of gut fungal microbiota is associated with mucosal inflammation in Crohn's disease.** *Journal of clinical gastroenterology* 2014, **48**(6):513.
53. Luan C, Xie L, Yang X, Miao H, Lv N, Zhang R, Xiao X, Hu Y, Liu Y, Wu N *et al*: **Dysbiosis of fungal microbiota in the intestinal mucosa of patients with colorectal adenomas.** *Scientific reports* 2015, **5**:7980.
54. Iliev ID, Funari VA, Taylor KD, Nguyen Q, Reyes CN, Strom SP, Brown J, Becker CA, Fleshner PR, Dubinsky M *et al*: **Interactions between commensal fungi and the C-type lectin receptor Dectin-1 influence colitis.** *Science* 2012, **336**(6086):1314-1317.
55. Zelante T, Iannitti RG, Cunha C, De Luca A, Giovannini G, Pieraccini G, Zecchi R, D'Angelo C, Massi-Benedetti C, Fallarino F *et al*: **Tryptophan catabolites from microbiota engage aryl hydrocarbon receptor and balance mucosal reactivity via interleukin-22.** *Immunity* 2013, **39**(2):372-385.
56. Erb Downward JR, Falkowski NR, Mason KL, Muraglia R, Huffnagle GB: **Modulation of post-antibiotic bacterial community reassembly and host response by Candida albicans.** *Scientific reports* 2013, **3**:2191.

57. Schopler E, Reichler RJ, DeVellis RF, Daly K: **Toward objective classification of childhood autism: Childhood Autism Rating Scale (CARS)**. *Journal of autism and developmental disorders* 1980, **10**(1):91-103.
58. Longstreth GF, Thompson WG, Chey WD, Houghton LA, Mearin F, Spiller RC: **Functional bowel disorders**. *Gastroenterology* 2006, **130**(5):1480-1491.
59. Findley K, Oh J, Yang J, Conlan S, Deming C, Meyer JA, Schoenfeld D, Nomicos E, Park M, Program NIHISCCS *et al*: **Topographic diversity of fungal and bacterial communities in human skin**. *Nature* 2013, **498**(7454):367-370.
60. Albanese D, Fontana P, De Filippo C, Cavalieri D, Donati C: **MICCA: a complete and accurate software for taxonomic profiling of metagenomic data**. *Scientific reports* 2015, **5**:9743.
61. Wang Q, Garrity GM, Tiedje JM, Cole JR: **Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy**. *Applied and environmental microbiology* 2007, **73**(16):5261-5267.
62. Koljalg U, Nilsson RH, Abarenkov K, Tedersoo L, Taylor AF, Bahram M, Bates ST, Bruns TD, Bengtsson-Palme J, Callaghan TM *et al*: **Towards a unified paradigm for sequence-based identification of fungi**. *Molecular ecology* 2013, **22**(21):5271-5277.
63. Caporaso JG, Bittinger K, Bushman FD, DeSantis TZ, Andersen GL, Knight R: **PyNAST: a flexible tool for aligning sequences to a template alignment**. *Bioinformatics* 2010, **26**(2):266-267.
64. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P, Andersen GL: **Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB**. *Applied and environmental microbiology* 2006, **72**(7):5069-5072.
65. Notredame C, Higgins DG, Heringa J: **T-Coffee: A novel method for fast and accurate multiple sequence alignment**. *Journal of molecular biology* 2000, **302**(1):205-217.
66. Hibbett DS: **A phylogenetic overview of the Agaricomycotina**. *Mycologia* 2006, **98**(6):917-925.
67. Price MN, Dehal PS, Arkin AP: **FastTree 2--approximately maximum-likelihood trees for large alignments**. *PloS one* 2010, **5**(3):e9490.
68. McMurdie PJ, Holmes S: **phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data**. *PloS one* 2013, **8**(4):e61217.
69. Westfall PH, Young SS: **Resampling-based multiple testing: Examples and methods for p-value adjustment**, vol. 279: John Wiley & Sons; 1993.
70. Revelle W: **psych: procedures for psychological, psychometric, and personality research. R package version 1.3.10**. Northwestern University, Evanston, IL. In.; 2013.
71. Team RC: **R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, 2012**. In.: ISBN 3-900051-07-0; 2014.
72. Benjamini Y, Hochberg Y: **Controlling the false discovery rate: a practical and powerful approach to multiple testing**. *Journal of the Royal Statistical Society Series B (Methodological)* 1995:289-300.