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In-depth Understanding of Gastrointestinal (GI) Problems in Autism Spectrum Disorder (ASD): Nature, Expression, and Possible Therapeutic Approaches

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1. Abstract

Autism Spectrum Disorder (ASD) is a relatively frequent disorder with a high longitudinal diagnostic stability (Prosperi 2010), characterized by a significant individual, familial, and societal burden (Horlin et al. 2014). Gastrointestinal (GI) problems are more frequent in children with ASD than in typically developing (TD) peers (Prosperi 2016) and are associated with low functioning and high psychiatric symptoms (Prosperi et al. 2017).

This dissertation offers a possible analysis and interpretation of GI symptoms through a psychiatric perspective based on various clinical and biochemical investigations.

The first part is an overview with an introductory critical analysis of the literature and a description of the findings emerging from the specific research I have dealt with, all investigating GI problems in children with ASD. Studies concerning the microbiota-gut-brain axis with their therapeutical implications, the results of a survey concerning eating habits and the findings of a prevalence study on celiac disease are summarized.

The second part concerns a randomized controlled study on the role of probiotics on clinical and biochemical parameters funded by the Italian Ministry of Health concerning a sample of preschoolers with ASD (Santocchi et al. 2016). The results were presented by chapters, partitioning the sample into subgroups based on the data available for each experimental question.

As shown below, children with ASD and GI symptoms exhibit their disturbances with different behaviors than TD children, have a particular intestinal microbiota and fecal metabolome than children with ASD without GI symptoms, and the nature of their disturb is more likely functional than organic. Promising results emerge from a clinical trial with probiotics on GI symptoms and behavioral features for children with ASD.

Future experimental trials considering TD control samples will provide a baseline and significantly empower the robustness of some of these promising findings.

2. Introduction: state of the art of GI problems in ASD

Autism Spectrum Disorders (ASD) are neurodevelopmental disorders characterized by persistent social communication difficulties as well as restricted interests, repetitive activities and sensory abnormalities (APA 2013). The clinical presentation of ASD may vary widely in terms of severity of core features as well as psychiatric and medical comorbidities (Kohane et al. 2012, Matson 2013). Among the latter, food selectivity (FS) and gastrointestinal (GI) symptoms have been reported more frequently than in typically developing (TD) peers.

2.1. Dietary patterns and eating phenotypes at increased risk of GI problems over time

Atypical eating habits and feeding problems have often been described in association with GI symptoms in children with ASD (Field et al. 2003, Kerwin et al. 2005, Gorrindo et al. 2012, Maenner et al. 2012, McElhanon et al. 2014, Vissoker et al. 2015).

A large prospective 10 years cohort study showed a significantly increased frequency of maternally reported alterations in stooling patterns and feeding behaviors as early as 6 to 18 months of age in children who were later diagnosed with ASD compared with children with typical development and developmental delay (Bresnahan et al. 2015).

In a recent paper examining a large sample of children and adolescents with ASD compared to a control group (Babinska et al. 2020), the authors found a significant correlation between the severity of GI symptoms with FS and mealtime problems in the ASD group. Similarly, Leader and colleagues (Leader et al. 2020) found higher rates of GI symptoms in those who presented with rapid eating. The authors highlighted the importance of assessing comorbid conditions that form part of the eating phenotype, including GI symptoms while evaluating a child with ASD for feeding problems.

In literature, a growing body of research has examined dietary patterns of children with ASD, revealing in most cases a high intake of energy-dense food (e.g., sugar-sweetened beverages and snacks) (Ahearn, Castine, Nault & Green, 2001; Diolordi, del Balzo, Bernabei, Vitiello, & Donini, 2014; Emond et al., 2010; Evans et al., 2012; Malhi, Venkatesh, Bharti & Singhi, 2017; Schreck &

Williams, 2006; Schreck, Williams, & Smith, 2004) and low fruit and vegetable consumption compared to TD peers (Coulthard and Blissett 2009, Evans et al. 2012). By contrast, a minority of authors, as Herndon et al. (Herndon et al. 2009), identified significantly higher consumption of fruit in children with ASD aged 4-8 years than in TD peers. Emond et al. (Emond et al. 2010) reported that ASD toddlers consumed not only fewer vegetables, salads, and fresh fruit but also fewer sweets and fizzy drinks than TD peers. This dietary atypicality could have an impact on intestinal function.

These findings could also justify the frequently reported inadequate supply of essential nutrients (e.g., vitamins, minerals) and overconsumption of energy-dense foods in children with ASD. Food variety is a predictor of the nutritional status of children with ASD (Zimmer et al. 2012). Critical dietary behaviors, including food dislike, behavioral rigidity, reduced interest in mealtime, physical discomfort and FS, may influence eating habits and thus affect the nutritional status of individuals with ASD (Sharp et al. 2013, Brown et al. 2016), with insufficient intake of nutritional and essential foods (e.g., vitamins and minerals) (Bandini et al. 2010, Zimmer et al. 2012).

Specific nutritional deficiencies and excessive weight gain could result, which may lead to increased obesity rates (Peretti et al. 2019). In particular, it is estimated that 30.4% of children with ASD are affected by overweight or obesity, versus 23.6% of children without ASD, according to the National Survey of Children's Health in the United States (Curtin et al. 2010). The prevalence of overweight and obesity in children with ASD is greater than the overall prevalence of overweight and obesity among 5 to 17 years old children in Italy (i.e., 27.0%) (Kahathuduwa et al. 2019).

There is a growing consensus regarding the necessity to monitor nutritional status in individuals with ASD, beginning at birth (Ranjan and Nasser 2015). The United States Preventive Services Task Force suggests providers screen children for obesity starting from the age of six and offer intensive behavioral interventions to support changes in weight status (Whitlock et al. 2005, Barton 2010, Whitlock et al. 2010). “Expert Recommendations” for weight management in primary care for children with ASD advocate prevention of obesity much earlier, beginning at two years of age (Curtin et al. 2020).

In general, nutrition and eating disorders are much more frequent (5 times more) in children with ASD (46-89%) than in TD peers (Ledford and Gast 2006, Sharp et al. 2013), both isolated and in association with GI problems.

Among these, FS is frequently described in subjects with ASD, with a prevalence ranging from 18% to 89% (Schreck and Williams 2006, Bandini et al. 2010, Mari-Bauset et al. 2014, Suarez et al. 2014, Postorino et al. 2015, Prosperi et al. 2021). Before the age of two, children who are later diagnosed with ASD are more selective in their eating patterns than other toddlers (Emond et al. 2010), and atypical mealtime behaviors and food choices often persist into adolescence (Hyman et al. 2020).

In a case-control study that specifically identified the features of FS (Bandini et al. 2010), the food refusal behavior and a limited food repertoire were significantly higher in ASD compared to TD peers, while this was not the case for the domain “high-frequency single food intake”.

Therefore, the FS is not a unique concept but refers to a wide range of situations and behaviors. According to Mazzone (Mazzone 2018), these behaviors include a restricted diet to specific foods, a decreased variety in the food choices, the refusal of food, the "picking eating" behavior, a limited caloric intake, a preference for one type of food, the presence of behavioral problems at the time of the meal and rituals and obsessions inherent food.

Several studies have suggested that sensory sensitivity may lead children with ASD to restrict their food choices sticking to their preferred, tolerable, and manageable textures (Bennetto et al. 2012, Johnson 2014, Mari-Bauset et al. 2014, Suarez et al. 2014, Postorino et al. 2015, Peretti et al. 2019). Food texture and taste are thought to drive food choices and have an impact on food acceptance of children with ASD (Schreck et al. 2004, Schreck and Williams 2006, Coulthard and Blissett 2009, Hubbard et al. 2014, Postorino et al. 2015), who frequently show many foods dislikes, repetitive food choices, and resistance to new taste experiences (Sharp et al. 2013, Curtin et al. 2015, Stafford et al. 2017, Bandini et al. 2019).

Moreover, frequently undiagnosed organic or functional GI disorders and allergies in this population might play a role in food choices and food refusal (Vissoker et al. 2015, Calderoni et al. 2016, Prosperi

et al. 2017). In fact, in a certain percentage of subjects with ASD, FS could result from an underlying GI disorder (Ibrahim et al. 2009, Nikolov et al. 2009, Whitehouse et al. 2011).

A work on TD preschool children (Tharner et al. 2015) found that constipation and subsequent abdominal pain are involved in the development of FS. It has been suggested that the presence of a low-fiber diet in subjects with FS (Cooke et al. 2003, Dovey et al. 2008), may lead to the development of constipation, creating a vicious circle (Vissoker et al. 2015).

Beyond the atypical sensory profile, other factors seem to be involved in FS in subjects with ASD, as behavioral rigidity (Schreck and Williams 2006, Zimmer et al. 2012) and behavioral problems (Bennetto et al. 2012, Prosperi et al. 2017).

Behavioral problems seem to be directly related to the intensity of FS, which causes, consequently, high levels of stress within the family (Curtin et al. 2015). To confirm this, it has been shown that the parents of children with ASD and FS report more significant stress and emotional and behavioral problems than parents of children with ASD and without FS, regardless of the severity of the autistic symptoms (Postorino et al. 2015).

Some authors (Sharp et al. 2018) have shown that most subjects with ASD and severe FS are more exposed to nutritional complications without alterations of anthropometric measures. A case-control study (Malhi et al. 2017) identified a significantly higher incidence of problematic mealtime behaviors and eating disorders in children with ASD than TD children, despite energy intake, carbohydrates, lipids and BMI not significantly differed between groups. In general, the importance of monitoring FS in children and adolescents with ASD emerges due to the possible long-life persistence of an inadequate food repertoire (Bandini et al. 2017). These authors, through a follow-up evaluation about 6 years after the first assessment, showed that 44% of subjects with ASD still had a high level of FS associated with weight gain, even if the food refusal behavior was decreased significantly (from 47% to 31%), partly because of a lower variability of the foods offered by the parent.

I conducted a study on the dietary choices and their impact on weight status and relationship to FS in a group of Italian preschoolers with ASD compared to TD peers (Raspini et al. 2021). I evaluated the dietary patterns and their associations with body mass index (BMI) in 65 children with ASD and 82 TD peers, ages 1.3-6.4 years. I found that children with ASD have different dietary habits than TD children, with higher consumption of energy-dense foods and lower amounts of foods source of fibers. These dietary patterns could place them at increased risk of developing overweight, obesity, GI problems and micronutrient deficiencies later in life. Children with ASD and FS showed significantly lower annual intakes of vegetable proteins and fiber (considered essential nutrients for a healthy diet) than children with ASD without FS.

2.2. Functional rather than organic GI symptoms

As for FS and eating disorders in general, it has been shown that the incidence of GI symptoms in autism is higher than in the TD population (Erickson et al. 2005, Kohane et al. 2012, Mayer et al. 2014, Bresnahan et al. 2015, Klukowski et al. 2015) with a prevalence ranging between 9 and 91% (Fombonne and Chakrabarti 2001, Black et al. 2002, Horvath and Perman 2002, Molloy and Manning-Courtney 2003, Parracho et al. 2005, Valicenti-McDermott et al. 2008, Ibrahim et al. 2009, Nikolov et al. 2009, Smith et al. 2009, Buie et al. 2010, Mouridsen et al. 2010, Adams et al. 2011, Wang et al. 2011, Coury et al. 2012, Parmeggiani 2014, Babinska et al. 2020).

The first work that reported a high prevalence of GI alterations in individuals with ASD was conducted by Goodwin and colleagues in 1971 (Goodwin et al. 1971). Subsequently, especially in the last decade, studies on this topic have increased exponentially, reporting in some cases a positive correlation between autism severity and GI symptoms (Adams et al. 2011, Wang et al. 2011, Gorrindo et al. 2012, Chaidez et al. 2014, Tomova et al. 2015, Babinska et al. 2020). In contrast, others did not confirm it (Molloy and Manning-Courtney 2003, Chandler et al. 2013, Prosperi et al. 2017).

However, not all studies published to date agree on an increased prevalence.

This heterogeneity could be due to different causes: the different methodology (e.g., the type of the analyzed data and the time considered in the survey), the measures used to evaluate GI symptoms, the different criteria to define the GI problem or the GI symptoms considered to estimate the prevalence, the type of study (e.g., case-control studies vs. epidemiological investigations vs. retrospective studies) and the different characteristics of the study population (e.g., age, gender, criteria to define ASD and associated features).

In particular, a possible cause of conflicting results could be ascribed to the clinical features of the enrolled ASD subjects, since some GI symptoms could be difficult to be expressed by children with ASD and concurrent severe communication impairment (Carr and Owen-Deschryver 2007, Buie et al. 2010). It should be noted that a lower prevalence of GI symptoms was found in studies involving a limited number of GI symptoms more strictly defined, in which medical records were examined or the time considered was limited (Black et al. 2002, Molloy and Manning-Courtney 2003, Nikolov et al. 2009).

For example, in a study that examined pathologies such as ulcerative colitis, celiac disease and malabsorption rather than non-specific and functional GI symptoms in a sample of 545 children (449 with typical development and 96 with autism; mean age between 49.4 and 51 months), no significant differences were identified in the incidence of GI disorders between children with ASD and healthy controls (Black et al. 2002).

Conversely, the prevalence of GI disorders in ASD is higher in research that included functional and idiopathic GI symptoms, examined data from parents' reports and considered more extended periods (Horvath and Perman 2002, Parracho et al. 2005, Valicenti-McDermott et al. 2006).

A meta-analysis (McElhanon et al. 2014) was conducted on 15 studies comparing GI symptoms in ASD subjects (from birth to 18 years of age) versus TD peers, children with developmental delay and siblings. The authors found a higher prevalence of GI symptoms in children with ASD than control groups with an odds ratio (OR) of 4.42. Analysis also indicated higher rates of diarrhea (OR, 3.63), constipation (OR, 3.86), and abdominal pain (OR, 2.45).

That meta-analysis included a pioneering study published in “Pediatrics” (Ibrahim et al. 2009) on 124 children with ASD in which each child was compared to two matched control subjects. Although there were no significant differences in the incidence of GI symptoms between healthy subjects and subjects with ASD, the latter had more frequent constipation and feeding issues/food selectivity. The authors hypothesized a neurobehavioral rather than a primary organic gastrointestinal etiology.

The GI symptoms most frequently reported in children with ASD were bowel disorders (constipation, diarrhea, abdominal bloating, flatulence), chronic abdominal pain, reflux and vomiting (Horvath and Perman 2002, White 2003, Levy et al. 2007, Wang et al. 2011, Coury et al. 2012, Ferguson et al. 2019). A multicenter study of over 14,000 individuals with ASD under the age of 35 showed a higher frequency of irritable bowel syndrome (IBS) and other GI disorders in subjects with ASD compared to the general population (11.7% vs. 4.5%) (Kohane et al. 2012). Moreover, a bowel disorder was one of the most frequent complications and belonging to the three co-morbidities, along with sleep disorders and epilepsy, that tended to persist over time in the examined autistic population.

The mechanisms by which GI disorders arise are not yet fully understood. Some research has hypothesized that the cause of a higher prevalence of GI symptoms in subjects with ASD could be at least partially linked to the presence of an abnormal intestinal microbiota (Li et al. 2017, Li et al. 2019, Margolis et al. 2019).

It was also hypothesized an altered stress response (Ferguson et al. 2019), connecting the evidence of an altered stress response in some individuals with ASD (Lopata et al. 2008) and the existing link between lower GI symptoms, sensory over-responsivity, and anxiety (Mazurek et al. 2013) as well as altered psychophysiological (Ferguson et al. 2017) and endocrine (Ferguson et al. 2016) responses to stress-inducing stimuli. These findings might indicate the activation of the sympathetic nervous system and the hypothalamic-pituitary axis linked with GI disorders in ASD.

Moreover, it has been suggested that absent or delayed bowel training resulting from the alterations of sensory processing and motor problems may lead to altered GI motility and defecation physiology in ASD (Wasilewska and Klukowski 2015). Therefore, children with ASD and concomitant GI

disorders could be considered a specific subgroup, constituting a new overlap syndrome, with an immediate implication for treatment (Wasilewska and Klukowski 2015).

Several investigations have tried to determine whether GI problems could contribute to behavioural problems in ASD. Different studies have reported greater severity of problematic behaviours as irritability (Bresnahan et al. 2015), anxiety and affective disorders (Valicenti-McDermott et al. 2006, Maffini V 2008 , Fulceri et al. 2016), dysregulation and externalizing problems (Horvath and Perman 2002), rigid/compulsive behaviours (Peters et al. 2014), increased sensory sensitivity (Mazurek et al. 2013) and sleep problems (Horvath and Perman 2002, Maenner et al. 2012) in children with ASD and concurrent GI symptoms. The diagnosis of GI disorders in subjects with ASD could be very challenging due to the specific patterns of behavioral expression in these children. Thus clinicians recommend using less invasive methods of investigation before hospitalization (Buie et al. 2010). Abdominal pain, constipation or diarrhea are unpleasant and can cause impairment in attention and concentration, self-damaging acts, aggressive problem behaviors and agitation, especially in children who are not able to communicate their discomfort (Horvath and Perman 2002, Buie et al. 2010, Adams et al. 2011, Kral et al. 2013, McElhanon et al. 2014, Wasilewska and Klukowski 2015, Fulceri et al. 2016, Marler et al. 2017, Ferguson et al. 2019, Kurokawa et al. 2021). According to this view, the consensus report for evaluation, diagnosis and treatment of GI disorders in individuals with ASD (Buie et al. 2010) recommends investigating all behaviors (verbal or motor) that might indicate the presence of a GI problem, especially in those ASD individuals who are unable to communicate their discomfort effectively. For example, symptoms and signs such as sobbing ‘for no reason at all’, delayed echolalia that includes reference to pain or stomach, facial grimacing, unusual posturing, unexplained increase in repetitive behaviors, self-injuring behaviors, aggression, sleep disturbances, increased irritability and oppositional behaviors are all cited as behaviors that may be markers of abdominal pain.

In keeping with these data, two studies (Nikolov et al. 2009, Chaidez et al. 2014) that used the ‘Aberrant Behavior Checklist’(Aman et al. 1985) to evaluate the severity of a range of problem

behaviours found significantly more maladaptive symptoms (especially irritability and social withdrawal) in children with ASD and GI symptoms compared to ASD children without GI symptoms.

It has been hypothesized that behavior may have different relationships with GI symptoms at different ages, consisting in a different presentation of externalizing and internalizing symptoms associated with GI problems between young children and older children with ASD (Ferguson et al. 2019).

I investigated the prevalence and type of GI and FS symptoms in a large group of preschoolers with ASD and their possible links with core ASD features and emotional/behavioural problems (Prosperi et al. 2017) through the use of the Child Behavior Checklist (CBCL) 1.5-5 questionnaire (Achenbach and Rescorla, 2000). As many as 64/158 (40.5%) of children with ASD had at least one severe GI symptom or FS. Preschoolers with and without GI symptoms and with and without FS were significantly different on several emotional/behavioural problems and restrictive/repetitive behaviours. In contrast, they did not differ significantly on performance IQ and autistic severity. The GI plus FS group presented Sleep Problems, Self-injurious Behaviors and Anxiety Problems. Results indicated the need for early identification of GI disturbances and FS to design tailored interventions for these symptoms frequently associated with challenging behaviours in ASD.

To investigate the nature of GI problems, I considered a frequent cause of organic GI problems in pediatric populations, Celiac Disease (CD). I examined the medical records of all children and adolescents between five to eleven years old with a diagnosis of ASD evaluated between January 2014 and December 2018 at the ASD Unit of the Stella Maris Scientific Institute. Considering the prevalence of CD in a pediatric population from the same period and age, a higher prevalence in ASD was excluded (Prosperi et al. 2021). Therefore, the usefulness of offering a population screening for CD in ASD is not supported through my research. On the other hand, we must also consider that the clinical burden of unrecognized CD could be particularly important in ASD patients.

2.3. Alterations of the Microbiota-Gut-Brain axis in ASD

In recent years, research has focused on the role of bidirectional communication between the intestine and the brain (the so-called "gut-brain axis") in the etiopathogenesis of various stress-related psychopathological disturbs and neuropsychiatric conditions, including ASD, providing an essential contribution to understanding them and proposing new therapeutic perspectives (Dinan and Cryan 2020, Prosperi et al. 2021, Prosperi et al. 2022).

It has been hypothesized that the presence of alterations in the gut microbiota, a complex community of microorganisms living in the intestine and including anaerobic bacteria and viruses, protozoa, archaea, and fungi, could cause secondary effects at the level of the Central Nervous System (CNS) (Collins et al. 2012).

In fact, the gut microbiota can act on the brain functions through three main mechanisms: (i) the production of neurotransmitters (NTs), short-chain fatty acids (SCFA), essential amino acids, and their metabolites from the diet; (ii) activation of the immune system, which, in turn, can increase the levels of circulating lipopolysaccharide and some bacterial peptides, thus modifying intestinal permeability; (iii) the modulation of the vagus nerve activity through its afferents, the enteric nervous system and the neuroendocrine pathways such as the hypothalamus-pituitary-adrenal axis (Galland 2014).

It is known that gut bacteria can produce numerous metabolites that can act on the CNS (some of them are toxic, such as D-lactic acid (Thurn et al. 1985) and ammonium (Qureshi et al. 2014)) and can synthesize hormones and NTs. For example, *Lactobacillus spp.* produces Acetylcholine and Gamma-Amino Butyric Acid (GABA), *Bifidobacterium spp.* produces GABA, *Escherichia spp.* produces Norepinephrine, Serotonin, and Dopamine, *Streptococcus spp.* and *Enterococcus spp.* produce Serotonin, *Bacillus spp.* produces Norepinephrine and Dopamine (Cryan and Dinan 2012). Typically, none of these compounds passes the blood-brain barrier; it is instead NTs precursors that can modify NTs synthesis in the CNS and compounds able to compete with NTs precursors for transporters across the blood-brain barrier. However, it has been shown that in the ASD brain, there

is an altered expression of genes associated with blood-brain barrier integrity (Banks 2008, Fiorentino et al. 2016), which could explain this direct action of intestinal NTs on the CNS.

In turn, these organisms are sensitive to hormones and NTs, which impact their growth and virulence. In addition, intestinal bacteria produce in abundance short-chain fatty acids (acetate, propionate, butyrate) through the fermentation of non-digestible carbohydrates in the colon. A high level of propionate (Wang et al. 2011) and, more generally, of short-chain fatty acids (Wang et al. 2012) was found in the feces of autistic subjects, and interestingly it has been shown that intraventricular injection of propionate in a mouse model caused autistic-like behaviors in mice (MacFabe et al. 2007). In humans, subjects suffering from propionic acidemia, a genetic disorder characterized by an accumulation of propionate, have neurodevelopmental alterations with very high percentages (21%) of ASD (Cotrina et al. 2020).

An increased level of urinary dimethylamine and lower levels of hippurate and 4-cresol sulfate in children with autism may be associated with the growth of certain bacteria in the GI tract (Bacteroidetes and/or Clostridia) (Yap et al. 2010). High levels of paracresol in young ASD children could be the result of increased colonization by Clostridium spp and Pseudomonas stutzeri (Altieri et al. 2011) and this organic compound has been shown to be neuroactive even in animal models (Pascucci et al. 2020, Bermudez-Martin et al. 2021). A higher level of urinary 3- (3-hydroxyphenyl) -3-hydroxy propionic acid has been described in children with autism compared to healthy controls, possibly due to different species of Clostridia (Shaw 2010).

The brain, in turn, modulates intestinal peristalsis and sensory and secretory functions via the vagus nerve (Aziz and Thompson 1998). The vagus nerve, the supporting axis of the parasympathetic branch of the autonomic nervous system, connects the intestine to the nucleus of the solitary tract at the brain stem level and is the primary afferent pathway from the abdomen to the brain. From the spinal cord, the postganglionic sympathetic efferences project to the intestine and enter synapses with the myenteric plexus, inhibiting GI function.

Neurological and neurodevelopmental disorders like multiple sclerosis, Alzheimer's, Parkinson, ASD and attention-deficit and/or hyperactivity disorder (ADHD) have been associated with an imbalance in the gut microbiome composition (Cryan and Dinan 2012).

Regarding pediatric age, most of the studies have focused on patients with autism. Still, recently there has also been an increased interest in the involvement of the gut microbiome in the pathogenesis of ADHD and the therapeutic potential of these microorganisms in pediatric patients with this disorder (Checa-Ros et al. 2021). Relevant evidence is also emerging in a gut microbiota signature in ADHD during adulthood and across the lifespan (Richarte et al. 2021), with significant consequences for identifying therapeutical approaches.

Considering autism, the possible role of the intestinal microbiota in the etiopathogenesis of ASD has been conceptualized starting from different evidence.

First, significant dysbiosis and a change in the stability, diversity, composition, and/or metabolism of the intestinal microbiota, altered intestinal permeability (providing evidence for the so-called "leaky gut hypothesis") , and evidence of intestinal and systemic inflammation have been shown in children with ASD compared to TD peers (Bezawada et al. 2020). In pioneering research (Sandler et al. 2000), oral administration of minimal doses of Vancomycin was associated with significant improvements in children's behavior with regressive ASD. However, treatment had time-limited beneficial effects that ceased when therapy stopped; on the other hand, antibiotic treatment was not justifiable for prolonged periods. Therefore, it has been hypothesized that oral antibiotic treatment with Vancomycin could temporarily improve chronic dysbiosis (Sandler et al. 2000), indirectly reducing the increased intestinal permeability and indirectly acting on behavioral symptoms typical of ASD.

Moreover, as previously highlighted, studies on subjects with ASD from distinct geographical areas converge to identify a higher prevalence of GI symptoms in ASD than TD.

Secondarily, as in studies on animal models of ASD, the microbiota is essential for developing social relationships. By re-establishing a condition of eubiosis in the intestinal microbiota during a specific developmental time window in germ-free mice or maternal immune activation mouse model of ASD,

it is possible not only to correct the defects of permeability and intestinal dysbiosis but also to act on ASD symptoms by reducing the production and absorption of toxins in the intestine (Hsiao et al. 2013, Wang et al. 2019).

2.4. Promising therapeutic approaches for ASD considering GI and behavioral symptoms

Intriguingly, different authors have hypothesized that GI disorders and alterations in the gut microbiota could contribute to the expression of the autistic phenotype or exacerbate the severity of symptoms in subjects genetically predisposed to ASD (Finegold et al. 2010, Adams et al. 2011, de Theije et al. 2011, Mayer et al. 2014, Aldinger et al. 2015). Already gut mobilization studies of chronically constipated ASD children have shown encouraging results on core and behavioral associated symptoms of ASD (Turriziani et al. 2022)

Considering these hypotheses, treatments acting at the gut microbiota level, such as prebiotics, probiotics and fecal microbiota transplantation (FMT), promise a reduction in GI symptoms and autistic symptoms in individuals with ASD, as already partially shown by other researches (Ristori et al. 2019).

Probiotics are non-pathogenic living microorganisms considered beneficial to human health when administered in adequate quantities as a dietary supplement. They have recently been defined as "psychobiotics" (Dinan et al. 2013) because they are considered a therapeutic tool able to influence brain development and behavior through their activity in restoring the healthy balance of the intestinal microbiota, producing and/or modulating the levels of NTs. Commonly used probiotics are *Lactobacillus*, *Bifidobacterium*, *Saccharomyces Cerevisiae*, and some *Escherichia Coli* and *Bacillus* species.

Prebiotics are non-digestible substances naturally contained in some foods (such as resistant starch, non-starch polysaccharides, oligosaccharides, galacto-oligosaccharides and xylo-oligosaccharides) which selectively stimulate the growth of probiotics such as *Lactobacilli* in the intestine and *Bifidobacteria* (Saulnier et al. 2013). Promising results derive from the studies on prebiotics

(Petschow et al. 2013), although their administration in children with ASD is still in the initial experimentation phase.

FMT or fecal bacteriotherapy is a non-drug medical treatment in which fecal material from a donor is treated in the laboratory and placed orally in the recipient as capsules, through endoscopic procedures (colonoscopy, orogastric tube) or with enema. It has become a popular treatment for refractory *Clostridium difficile* infection, obesity, chronic inflammatory bowel diseases and has been hypothesized as a therapeutic strategy for autism in recent years (Choi and Cho 2016). There is a significant impact on the microbiota up to 24 weeks, as emerging from a study concerning the duration of a single transplant (Grehan et al. 2010). Few clinical studies have evaluated the impact of FMT, like Microbiota transfer therapy (MTT), on autistic symptoms in individuals with ASD (Kang et al. 2017, Kang et al. 2019). Relevant side effects due to preventive treatment with antibiotics to favor the engraftment of the donor's microbiota in the recipient (Kang et al. 2017), and other adverse reactions, including hyperactivity, aggressive behaviors, fever, and major changes in blood chemistry (Li et al. 2021) were described considering FMT applications in ASD subjects.

The majority of studies published to date examining the gut-brain axis as one of the potential focus in the treatment of subjects with ASD deal with the use of probiotics (Blades 2000, Sandler et al. 2000, Parracho 2010, Kaluzna-Czaplinska and Blaszczyk 2012, West DO 2013, Grossi et al. 2016, Kobliner et al. 2018, Arnold et al. 2019, Liu et al. 2019, Niu et al. 2019, Santocchi et al. 2020, Mensi et al. 2021). The other ones treat the use of prebiotics (Liu et al. 2017, Grimaldi et al. 2018, Guo et al. 2018, Inoue et al. 2019) and mixed probiotics-prebiotics therapy (Shaaban et al. 2018, Sanctuary et al. 2019, Wang et al. 2020) in ASD, and only two (Kang et al. 2017, Kang et al. 2019) are about MTT, a modified FMT protocol.

Studies were conducted in seven different countries: England (n = 3), America (n = 7), Poland (n =1), Italy, (n = 3), China (n = 5), Egypt (n = 1), Japan (n = 1). Eight out of 21 studies are randomized controlled trials, possibly reducing certain sources of bias typical of studies measuring efficacies of an intervention. The subjects examined are predominantly males (569 males and 86 females with

ASD, in two studies (West DO 2013, Niu et al. 2019) sex of participants are missing). All are children and adolescents between 1 and 17 years old with ASD described in studies published mainly in the last twenty years. The sample sizes are relatively small, globally ranging from 11 to 85 subjects with a maximum of 131 subjects, and in three cases the studies are case reports (Blades 2000, Grossi et al. 2016, Kobliner et al. 2018); in more than half of the studies, the reasons for the dropout/refusal to participate and any side effects of the treatment are clearly reported (Sandler et al. 2000, Parracho 2010, Kang et al. 2017, Grimaldi et al. 2018, Shaaban et al. 2018, Arnold et al. 2019, Liu et al. 2019, Niu et al. 2019, Sanctuary et al. 2019, Santocchi et al. 2020, Wang et al. 2020, Mensi et al. 2021).

Most studies (15 out of 21) (Sandler et al. 2000, Parracho 2010, Kaluzna-Czaplinska and Blaszczyk 2012, West et al. 2013, Kang et al. 2017, Grimaldi et al. 2018, Shaaban et al. 2018, Arnold et al. 2019, Inoue et al. 2019, Kang et al. 2019, Niu et al. 2019, Sanctuary et al. 2019, Santocchi et al. 2020, Wang et al. 2020, Mensi et al. 2021) reported GI symptoms as medical comorbidity associated with ASD. No study discusses dietary habits or food intakes of enrolled subjects before and after the intervention (micronutrients and macronutrients in (Grimaldi et al. 2018) and partially in (Blades 2000)) and interference/interaction with possible concomitant drug treatment, even if in two studies these data are collected (Santocchi et al. 2020, Mensi et al. 2021). In another study, medications were excluded for at least one month before sampling (Inoue et al. 2019). Moreover, effective compliance to treatment through the examination of the fecal samples after the intervention is lacking. In a minority of cases (Parracho 2010, Grimaldi et al. 2018, Shaaban et al. 2018, Arnold et al. 2019, Sanctuary et al. 2019, Santocchi et al. 2020), the compliance to treatment is verified through other ways (e.g., measured by packet counts of returned probiotic and placebo containers).

The type of intervention varies across all of the trials, with wide variability in the strains and different formulations used (single-strain probiotic therapy (Parracho 2010, Kaluzna-Czaplinska and Blaszczyk 2012, Kobliner et al. 2018, Liu et al. 2019, Sanctuary et al. 2019), blended probiotic formulations (Sandler et al. 2000, West et al. 2013, Grossi et al. 2016, Shaaban et al. 2018, Arnold et al. 2019, Niu et al. 2019, Santocchi et al. 2020, Wang et al. 2020) or both (Mensi et al. 2021)) in

studies examining probiotics. These interventions are primarily *Bifidobacterium* and *Lactobacillus* genus based. A minority of studies tested *Streptococcus* species; the most tested strains are *Bifidobacterium longum* and *infantis* (n = 4 and 5, respectively) and *Lactobacillus acidophilus* (n =7). The therapy is mainly taken by mouth, in capsule or packet to be taken daily (once, two or three times/day), with doses ranging from 0.5 to 90 x 10^9 CFU of bacteria. Treatment duration varied between 21 days and 6 months, with only four studies reporting post-intervention follow-up outcomes (Sandler et al. 2000, Grossi et al. 2016, Grimaldi et al. 2018, Kang et al. 2019).

The changes recorded in GI symptoms and ASD severity in the subjects examined are mainly obtained through tools administered to parents rather than a direct examination of the clinicians.

The most frequently used assessment instruments for ASD-related behavior are the ATEC (Magiati et al. 2011) (n = 5) and ABC (Davenport 2011) (n = 7). In a minority of studies, specific instruments to directly assess the symptoms of autism and clinical severity are used (e.g., CARS, ADOS, ADI, CGI). For the assessment of GI symptoms, the most used tool is the 6-GSI (Adams et al. 2011) (n = 3), a modified version of the GSI that has been designed for the ASD population. Other tools used are PedsQL GI Module Scales (n = 1), questionnaire on pediatric gastrointestinal symptoms–Rome III (Rasquin et al. 2006) (n = 1), GI History survey (Hansen et al. 2008)(n = 1), and GI symptom rating scale (Svedlund et al. 1988) (n = 1; GSRS). Additionally, eight studies collect data on GI function with either unspecified questionnaires or qualitative GI diaries.

Only three out of 21 studies (14.29%) include a healthy control group, with one for probiotic intervention (Niu et al. 2019), one for prebiotic treatment (Guo et al. 2018) and one for both probiotic plus prebiotic therapy (Shaaban et al. 2018). Similarly, only six out of the 21 studies (28.57%) include a placebo group in the study design.

2.4.1 Clinical studies on probiotics

By exploring the possible applications of probiotic therapy, some studies have identified a beneficial effect of specific probiotics on some of the behavioral characteristics specific or associated with ASD

in case reports (Blades 2000, Grossi et al. 2016, Kobliner et al. 2018), small populations (Sandler et al. 2000, Parracho 2010, Kaluzna-Czaplinska and Blaszczyk 2012, West DO 2013, Shaaban et al. 2018, Arnold et al. 2019, Liu et al. 2019, Niu et al. 2019, Sanctuary et al. 2019, Wang et al. 2020), or larger samples (Mensi et al. 2021). Moreover, an improvement in GI symptoms such as constipation, stool consistency, flatulence, and abdominal pain was found (West DO 2013, Grossi et al. 2016, Shaaban et al. 2018, Arnold et al. 2019, Niu et al. 2019, Sanctuary et al. 2019, Wang et al. 2020).

In the first published double-blind placebo-controlled study (Parracho 2010), the effects of supplementation with *L. plantarum WCSF1* over three weeks (crossover study with a total duration of 12 weeks) were examined in a sample of children with ASD. In addition to a modulation of the intestinal microbiota, an improvement in stool consistency and scores obtained on a scale for evaluating behavioral and emotional problems in subjects with intellectual disabilities and global developmental delay were observed after administering the probiotic. However, a high drop-out rate affected the study's statistical power (17 out of 62 subjects completed the study).

In a cohort study (Kaluzna-Czaplinska and Blaszczyk 2012) where oral supplementation of a strain of *L. acidophilus* was administered for two months to a group of 22 autistic children, there was an improvement in the ability of concentration and carrying out orders. From baseline, no difference in reacting to other people's emotions or using eye contact was present. While intriguing, the small sample and open-label design of the study limit the relevance of the results. It's also of relevance that the total duration of the trial is double than the intervention conventionally selected in clinical trials using probiotics on ASD (Cheng et al. 2019).

In another uncontrolled clinical study (West DO 2013), positive effects were recorded due to administering a food supplement consisting of a mixture of five probiotic strains formulated with the immunomodulator Del-Immune V® (*L. rhamnosus V* lysate) in a population of 33 children with autism and concomitant GI symptoms. In addition to reporting an improvement in GI symptoms, a significant improvement in autism severity measured through the ATEC was reported after only three weeks of treatment. Similar results emerge from a subsequent uncontrolled study on 30 children with

ASD receiving three months of treatment with a probiotic mixture (strains of the species *L. acidophilus*, *L. rhamnosus*, and *B. longum*) and dried carrot (Shaaban et al. 2018). The authors found significant changes in each subdomain and total ATEC score with simultaneous improvement of the 6-Gastrointestinal Severity Index. These studies were characterized by inadequate sample size (30 and 33 ASD subjects, respectively) and open-label design with the consequent risk of overestimating the effects of probiotic therapy.

More limited results regarding probiotics in ASD emerge in a placebo-controlled study of 71 subjects with ASD (Liu et al. 2019): the authors highlighted how supplementation with *L. plantarum PS128* administered for 4 weeks resulted in a reduction in anxiety, hyperactivity, and opposition/challenge behaviors measured through CBCL and SNAP IV, although without changes in ASD symptoms. Also, the stratified analysis by age for all outcome measures adopted by the authors allowed to identify better effects on younger children than older children, underscoring the importance of early interventions. It is also to note that almost all the scores evaluating the existing impairments decreased in the placebo group at week four, suggesting that the placebo effect and confounding factors may affect the results. The importance of a placebo-controlled study design for this type of study is therefore further emphasized.

In Niu et al. (Niu et al. 2019), the authors compared a group treated with a combined intervention with probiotics (a probiotic formulation of three *Lactobacillus* and three *Bifidobacteria* strains) and behavioral therapy with a group treated with behavioral therapy only. They started from an initial sample of 114 ASD subjects in which the fecal microbiota was profiled. Still, the final subgroups were smaller, comparing 37 subjects treated with probiotics and Applied Behavioral Analysis (ABA) therapy (divided into GI and NGI subjects) with 28 subjects treated with ABA intervention only. Despite the encouraging results in the group treated with probiotics both in ASD symptoms and GI problems, there are different concerns of bias primarily related to the study design (unblinded study) where caregivers evaluated the gain, and the duration was somewhat limited (one month).

Similar results were found in a randomized, double-blind placebo-controlled study (Wang et al. 2020) using a formulation of four different probiotic strains (two of both *Lactobacillus* and *Bifidobacteria*) mixed with a prebiotic (fructooligosaccharide). However, the sample was relatively small, and the duration was not specified. Moreover, whether the effects are due only to the probiotic therapy or prebiotics/prebiotic substrate mixture cannot be determined: a probiotic-only treatment group, which could be useful in parsing out specific treatment effects, is missing.

Recently, an open-label trial on a large sample of children with ASD testing differences between *L. plantarum* ("*L. plantarum* group") and other probiotics ("OP group") in the treatment of GI and psychiatric symptoms was published (Mensi et al. 2021). The authors found that the positive effects were more evident in younger children, and the patients taking *Lactobacillus plantarum PS128* had more significant improvements and fewer side effects than the OP group.

Although such a large sample differentiates it from all other published works on this subject, several possible sources of bias limit the relevance of the results as the unblinded study design, the unbalanced number of subjects between groups, the heterogeneous treatment in the group testing other probiotics different from *L. plantarum*, and the lack of the microbiota analysis before and after treatment. As in other studies, the lack of information about microbiota changes during the treatment could limit the possible resulting correlations among the brain, clinical improvement, and specific microbiota composition in ASD.

Other studies concerning the administration of probiotics in subjects with ASD did not analyze the effects on the behavior of the enrolled subjects (Adams et al. 2011, Tomova et al. 2015) or did not reveal any improvement related to treatment with supplements (Arnold et al. 2019, Sanctuary et al. 2019).

In the randomized, double-blind trial by Sanctuary et al. (Sanctuary et al. 2019), the treatment included 5 weeks of probiotic (*B. infantis*) and prebiotic (bovine colostrum prebiotic oligosaccharides) supplementation, followed by a two-week wash-out period and finally 5 weeks of supplementation only with prebiotic. Supplementation with the combined treatment did not seem to

significantly improve irritability and stereotyped behaviors, a result that instead emerged from the treatment with the prebiotic alone. Limitations include the small sample with a very high dropout rate (8/20 completed the study) and the lack of a probiotic-only treatment group, making it very similar to the study by Wang et al.(Wang et al. 2020).

In the placebo-controlled pilot study by Arnold et al. (Arnold et al. 2019) about the use of a probiotic containing eight different bacterial species (mainly *Lactobacilli* and *Bifidobacteria*) in a sample of 13 children with ASD aged 3 to 12 years, no significant differences emerged in the increase in the quality of life or the reduction of anxiety symptoms. Still, significant improvements in GI discomfort were seen during probiotic treatment compared to the placebo treatment period. It's to highlight the small sample with a high dropout rate (10/13 completed the study) and the use of a new anxiety scale not necessarily sensitive to changes intended to be registered in the study.

2.4.2 Clinical studies on prebiotics

Seven studies have been published to date that used different types of prebiotic compounds, including carrot powder (Shaaban et al. 2018), partially hydrolyzed guar gum (Inoue et al. 2019), vitamin A (Liu et al. 2017, Guo et al. 2018), galacto- (Grimaldi et al. 2018, Sanctuary et al. 2019) and fructo-oligosaccharides (Wang et al. 2020). Some authors have examined their effects when administered alone (Liu et al. 2017, Grimaldi et al. 2018, Guo et al. 2018, Inoue et al. 2019) or associated with probiotics (Shaaban et al. 2018, Sanctuary et al. 2019, Wang et al. 2020). Therefore, in the latter studies, it is not possible to determine whether the effects/benefits are due to the specific prebiotic or its function as a substrate for some probiotic strains.

In the paper by Inoue et al. (Inoue et al. 2019), a significant decrease in microbial alpha-diversity and some cytokines and chemokines (IL-1, IL-6, and TNF-α) were highlighted in a small sample of constipated ASD children after administering a prebiotic diet based on guar gum and β-endoglucanase produced by an Aspergillus strain. Prebiotic supplementation also increased the frequency of bowel movements with a consequent higher frequency of defecations per week. The authors hypothesized

that the improvements of the gut dysbiosis and constipation symptoms could, in turn, helped attenuate the level of serum cytokines and behavioral irritability. However, the lack of control group and blinded trial, and the missing information about diets pre- and post-intervention, relativize the relevance of the results.

Grimaldi and colleagues (Grimaldi et al. 2018) found a significant increase in the *Lachnospiraceae* family and significant changes in the fecal and urinary metabolites and antisocial behavior of 30 children with ASD after a prebiotic intervention with supplementation for six weeks with Bimuno galacto-oligosaccharides (B-GOS®: 80% galacto-oligosaccharides). Despite the study's strength, which also considers the participants' dietary habits assessed by 4-days food diaries as macronutrients and micronutrients intakes, there was a high dropout rate (63% completed the study). This could further limit the power of the results on an already relatively small sample (41 enrolled subjects).

Two pilot studies of the same research group (Liu et al. 2017, Guo et al. 2018) tested vitamin A supplementation in a sample of children with ASD, showing in one case (Liu et al. 2017) a significant increase in the *Bacteroidetes*/*Firmicutes* ratio without changes in autism severity and behavioral problems, while in the other one (Guo et al. 2018) a reduction in the severity of autism and in serum levels of 5-hydroxytryptamine (which correlated positively with autistic symptoms). The somewhat conflicting results on the severity of autism and the lack of a placebo-controlled study design in both types of research limit these findings strengthen.

In conclusion, in terms of emotional-behavioral symptoms and symptoms related to ASD, some authors have found an improvement after the administration of prebiotics (Grimaldi et al. 2018, Guo et al. 2018, Inoue et al. 2019, Sanctuary et al. 2019). In contrast, others have not shown a change pre-post treatment (Liu et al. 2017) in ASD subjects. The variability in the choice of prebiotics, the simultaneous administration with probiotic strains and the few studies published to date do not allow to draw definitive conclusions about their benefits in subjects with ASD.

2.4.3 Clinical studies on Fecal Microbiota Transplantation

The first open-label study on MTT evaluated the impact of this technique on a sample of 18 autistic children aged 7 to 16 years with moderate-to-severe GI symptoms (Kang et al. 2017). An approximately 80% reduction in GI symptoms (significant improvement in constipation, diarrhea, abdominal pain, digestive problems) and symptoms related to autism were identified. The improvement persisted after eight weeks since the end of the treatment. The protocol included preliminary therapy with antibiotics for two weeks, intestinal washing, and maintenance treatment with antacid drugs. Laboratory investigations revealed partial engraftment of the donor's microbiota with consequent benefits at the level of the intestinal microenvironment (increase in *Bifidobacteria*, *Prevotella*, *Desulfivibrio*). In one case, there was an adverse dermatological reaction to Vancomycin, and in 12 subjects, an increase in hyperactivity and aggression up to three days after the end of the treatment. Despite the relevance of side effects and the complex implant procedure, the authors suggested the superiority of MTT over probiotic therapy due to the greater probability of engraftment as well as the presence of richer bacterial populations.

The authors then carried out a check on the same group of patients two years after the previous study (Kang et al. 2019), finding maintenance over time of both GI and autistic symptoms improvements and persistence of the increase of *Bifidobacteria* and *Prevotella* in the microbiota.

Although these results are promising, it should be emphasized that this procedure is still in an experimental stage and has relevant side effects due to preventive treatment with antibiotics to favor the engraftment of the donor's microbiota in the recipient.

For an overview of the studies concerning the use of prebiotics, probiotics, and FMT in subjects with ASD published up to July 2021, see Table 2.4.3.1

Table 2.4.3.1 Studies concerning the use of prebiotics, probiotics, and fecal microbiota transplantation in subjects with ASD published until July 2021

REFERENCE	COUNTRY	POPULATION	INTERVENTION		DOSE	STUDY DESIGN	MAIN RESULTS	LIMITS
Blades et al. (2000)	England	A 6-year-old boy with ASD	PRO	Not reported	Not reported	Case report	Improvement in school records and attitude against taking a variety of food during the supplementation with PRO	Case report Microbiota not analyzed The PRO type and dose are not reported
Sandler et al. (2000)	America	11 ASD (regressive onset) 10 ♂ 1 ♀ Age 3.5-7 yrs	PRO	Vancomycin + PRO (Lact acidophilus, Lact bulgaricus, Bifid bifidum)	Vancomycin (500mg/d) 3/day x 8 wks, PRO (40 X 10^9 CFU/mL) x 4 wks	Open-label trial	Short-term improvement in ASD symptoms (CARS) during Vancomycin tr	Reduced compliance during PRO tr No control group (microbiota compared with microbiota of adults) Small sample, unblinded study
Parracho et al. (2010)	England	62 ASD 59 ♂ 3 ♀ age 4-16 yrs	PRO	Lact plantarum WCFS1	4.5 x 10^{10} CFU/cp, 1 cp/day 3 wks per arm (PRO-wash out-PLA-wash out)	Randomized double blind placebo-controlled trial, cross-over	More aggressive and antisocial behaviors, anxiety problems and communication difficulties in the PLA group Improvement of the anti-social behaviors, anxiety, and communication problems No major differences in GI symptoms ↑ Lact/Enterococci and ↓ Clostridium coccoides found in the stools of ASD children as compared with PLA	Very high dropout rates (17/62 completed the study, 9 PRO and 8 PLA) No TD control group
Kaluzna-Czaplinska e Blaszczyk (2012)	Poland	22 ASD 20 ♂ 2 ♀ age 4-10 yrs Severe GI symptoms	PRO	Lact acidophilus (Rosell-11 species)	5 x 10^9 CFU/g 2/day x 2 mths	Open-label trial with self-control study	Improvement in ability of concentration and carrying out orders; no difference in reacting to other people's emotions or using eye contact	High risk of selection bias Unblinded study Microbiota not analyzed No TD control group No PLA group
West et al. (2013)	America	33 ASD ♂ ♀ missing age 3-16 yrs	PRO	DelPRO (Lact acidophilus, casei, delbrueckii+Bifid longum, bifidum + 8 mg Lact rhamnosus V lysate)	1 x 10^8 billion CFU 3 tms/day x 21 days	Open-label trial	88% subjects ↓ ATEC total score, 48% ↓ diarrhea, 52% ↓ constipation	Risk of selection bias 25/33 reported ATEC scores, 21/33 returned stool frequency diaries Unblinded study Microbiota not analyzed No TD control group No PLA group
Grossi et al. (2016)	Italy	A 12-year-old boy with ASD and severe intellectual disability	PRO	VSL#3: Bifid (breve, longum, infantis) + Lact (acidophilus, plantarum, paracasei, bulgaricus, delbrueckii) + Strept (thermophilus, salivarius) freeze-dried	9x10^{10} CFU/day x 4 wks	Case Report	↓ severity of abdominal symptoms and improvement in ASD symptoms (↓ADOS)	Case report Microbiota not analyzed

REFERENCE	COUNTRY	POPULATION	INTERVENTION		DOSE	STUDY DESIGN	MAIN RESULTS	LIMITS
Kang et al. (2017)	America	18 ASD with GI symptoms (moderate/severe) 16 ♂2 ♀ age 7-16 yrs	MTT	SHGM orally or rectally	Initial dose $2.5x10^{12}$ cells/day and maintenance dose $2.5x10^{9}$ cells/day for 7 or 8 weeks (+vancomycin + MoviPrep + Prilosec) Duration: 4 mths and 2 wks	Open-label trial	↓ 80% reduction of GI symptoms at the end of tr lasting 8 wks after tr. Behavioural symptoms of ASD significantly improved and continued improving 8 wks after tr. ↑ diversity and abundance of Bifid, Prevotella and Desulfovibrio, increased after MTT, lasting for 8 wks	ASD symptoms changes not reported Small sample, unblinded study No TD control group No PLA group
Liu et al. (2017)	China	64 ASD 55 ♂9 ♀ age 1-8 yrs	PRE	20 study participants (17 ♂3 ♀) with plasma retinol deficiency (<1.05 μmol/l) treated with VA	200000 UI once x 6 mths	Single-blind, non-randomized, interventional pilot study	Significant ↑Bacteroidetes/Firmicutes and ↓ Bifid; no change in the ASD severity or behavioral problems	No PLA group No TD control group
Grimaldi et al. (2018)	England	41 ASD 31 ♂ 10 ♀ age 4-11 yrs	PRE	Bimuno galacto-oligosaccharide (B-GOS®: 80% galacto-oligosaccharides)	1,8 gr in powder (unknown frequency) x 6 mths. At the end of intervention, patients were followed-up for 2 additional weeks.	Randomized double blind placebo-controlled trial	Improvements in anti-social behaviour After tr: ↑ Lachnospiraceae, significant changes in faecal and urinary metabolites	High dropout rates (26/41 completed the study) No TD control group
Guo et al. (2018)	China	33 ASD (28 ♂ 5 ♀) age 5.14 ± 1.33 yrs 32 TD; age 5.18 ± 0.87 yrs	PRE	VA in the 33 ASD	Single administration 200000 UI	Open-label, interventional pilot study	6 mths after administration: reduction of ASD severity and 5-hydroxytryptamine (positively correlated with ASD symptoms)	Unblinded study Microbiota not analyzed No PLA group
Kobliner et al. (2018)	America	A 16-year-old boy with ASD, OCD and SIB	PRO	Saccharomyces boulardii, 3 x 10^{9} CFU/cp	6 cp/day in the first week, then 2 cp four-times-a-day and increasing up to 12 cp/BID (= 72 x 10^{9} CFU daily) x 3 mths. After, he began weaning down by 4 cp/wks to a dose of 3 cp/BID. Activated charcoal added when ↑ OCD and SIB symptoms	Case report	↓ frequency and duration of SIB symptoms	Case report Microbiota not analyzed
Shaaban et al. (2018)	Egypt	30 ASD 19 ♂ 11 ♀ age 5-9 yrs 30 HC children (relatives) age 5-9 yrs	PRE + PRO	Lact acidophilus + Lact rhamnosus +Bifid longum and dried carrot	1 gr = $100x10^{6}$ CFU for each species 5 gr/day x 3 mths	Open-label, prospective study	↑ fecal levels of Bifid and Lact, significant improvements in ASD severity (↓ ATEC) and GI symptoms (6-GSI)	Unblinded study No TD control group

REFERENCE	COUNTRY	POPULATION	INTERVENTION		DOSE	STUDY DESIGN	MAIN RESULTS	LIMITS
Arnold et al. (2019)	America	13 ASD-GI-anxiety 6 ♂ 4 ♀ 6 ASD with PRO 4 ASD with PLA age 2-11 yrs	PRO	VISBIOME: 4 Lact strains (casei, plantarum, acidophilus, delbrueckii subsp Bulgaricus)+3 Bifid strains (longum, infantis, breve)+ 1 Strept thermophilus strain and starch	$9x10^5$ bacteria in half packet Half packet /2 tms day in the first 4 wks 1 packet/ 2 tms day if no effects are observed at 4 wks and 15 wks Duration: 4 mths and 3 wks	Randomized double blind placebo-controlled trial, crossover	PRO: ↑ Lact Improvement of GI symptoms and anxiety compared to baseline, but without statistical significance	High dropout rates (10/13 completed the study) Small sample No TD control group
Inoue et al. (2019)	Japan	13 ASD 12 ♂ 1 ♀ age 4-9 yrs	PRE	Partially hydrolyzed guar gum (Taiyo Kagaku Co.Ltd., Mie, Japan) β-endogalactomannase produced by a strain of Asp. Niger	6 gr/day Duration: 2-15 mths (median =2)	Open-label, interventional study	Significant ↓ irritability after supplementation with partially hydrolyzed guar gum	Unblinded study No TD control group No PLA group Small sample
Kang et al. (2019)	America	18 ASD with GI symptoms (moderate/severe) 16 ♂ 2 ♀ age 7-17 yrs	MTT	SHGM orally or rectally	Initial dose $2.5x10^{12}$ cells/day and maintenance dose $2.5x10^9$ cells/day for 7 or 8 weeks (+vancomycin + MoviPrep + Prilosec) Duration: 2-year follow-up	Open-label trial	Changes in gut microbiota lasted for 2 yrs, including significant ↑ in bacterial diversity and relative abundance of Bifid	ASD symptoms changes not reported Small sample, unblinded study No TD control group No PLA group
Liu et al. (2019)	China	39 ASD with PRO 41 ASD with PLA 80 ♂ 0 ♀ age 7-15 yrs	PRO	Lact plantarum PS128, 3×10^{10} CFU cp	1 cp/day X 1 mth	Randomized double blind placebo-controlled trial	↓ anxiety, hyperactivity and opposition/defiance behaviors; no change in the ASD symptoms	High dropout rates (9 out 80 subjects) Microbiota not analyzed No TD control group
Niu et al. (2019)	China	114 ASD (22 GI with PRO+ABA; 15 NGI with PRO+ABA; 28 ABA) ♂ ♀ missing 40 TD age 3-8 yrs	PRO	3 Lact strains (bulgaricus, acidophilus, casei) + 3 Bifid strains (infantis, longum, bifidum)	6 g/day (36 billion CFU in total) + ABA training Duration: 1 mth	Open-label, two-arm, randomized trial	PRO+ABA vs only ABA: ↓ Total and subdomain ATEC scores; ↓ GI in 86.4% of 22 ASD GI with PRO+ABA	Small sample, unblinded study
Sanctuary et al. (2019)	America	(20 ASD initially screened) 8 ASD with GI symptoms 7♂ 1 ♀ age 2-11 yrs	PRE + PRO	Bifidobacterium infantis in combination with a bovine colostrum product (BCP) as a source of oligosaccharides	PRO 20 billion CFU/day, BCP 5.1-10.8 gr/day 4 ASD with PRO+BCP 4 ASD with BCP 5 wks + 2 wks wash out + 5 wks	Randomized double blind trial, crossover	Combined tr: some participants ↓ frequency of GI symptoms (++pain, diarrhea, stool consistency) and some atypical behaviors (++irritability, stereotypies, hypo / hyperactivity) ↓ IL-13 and TNFα production in some participants	High dropout rates (8/20 completed the study) Lack of a control group with PLA and a PRO-only group No TD control group

REFERENCE	COUNTRY	POPULATION	INTERVENTION		DOSE	STUDY DESIGN	MAIN RESULTS	LIMITS
Santocchi et al. (2020)	Italy	85 ASD (30 GI and 55 NGI) 71♂ 14 ♀ Average age 4.2 yrs	PRO	De Simone formulation-Vivomixx®(1 Strept strain + 3 Bifid strains+ 4 Lact strains)	2 packets/day (900 billions of bacteria) in the first mth and 1 bust/day (450 billions of bacteria) for the next 5 months	Randomized double blind placebo-controlled trial	NGI PRO vs NGI PLA groups: ↓ ADOS GI PRO vs GI PLA groups: ↑ improvements in some GI symptoms, adaptive functioning and sensory profiles	High dropout rates (>GI group), 63/85 completed the study No information about microbiota No TD control group
Wang et al. (2020)	China	26 ASD (16 ASD with PRE+PRO; 10 ASD with PLA) 24♂ 2 ♀ age 3-9 yrs	PRE + PRO	4 PRO strains (Bifid infantis and lactis, Lact rhamnosus and paracasei) + fructooligosaccharide (FOS)	10^{10}CFU/pack/day 1, 2 or 3.6 mths	Randomized double blind placebo-controlled trial	↓ Total and subdomain ATEC scores compared to baseline ↓ Total 6-GSI score	Lack of a PRO-only group No TD control group
Mensi et al. (2021)	Italy	131 ASD 112 ♂ 19 ♀ Average age 86.1 ± 41.1 mths	PRO	Lact plantarum (105 ASD), OP (26 ASD)	Lact plantarum group: 3 x 10^{10} CFU if weight <30 kg, 6 x 10^{10} CFU if weight >30 kg OP group: prescribed PRO based on age, weight, and specific product Duration: 6 mths	Open-label trial	↑ level of shared attention (54 ASD), ↓ stereotyped movements (43 ASD), ↑ communication skills (32 ASD) and ↑ personal autonomies (23 ASD) Higher improvements in Lact plantarum group No different improvements between GI and NGI subjects	Unblinded study Unbalanced number of subjects between Lact plantarum and OP groups Heterogeneous tr in OP group Microbiota not analyzed No TD control group No PLA group

Abbreviations (alphabetic order):
↑ increase, ↓ decrease, 6-GSI: six-Gastrointestinal Severity Index, ABA: applied behavior analysis, ADI: Autism Diagnostic Interview, ASD: Autism Spectrum Disorder, Asp: Aspergillus, ATEC: Autism Treatment Evaluation Checklist, BID: two-times-a-day, Bifid: Bifidobacterium, CARS: Childhood Autism Rating Scale, CFU: colonies forming units, CGI: Clinical Global Impression, cp: capsule, FMT: fecal microbiota transplantation, GI: gastrointestinal, gr: grams, HC: healthy controls, Lact: Lactobacillus, mths: months, MTT: microbiota transfer therapy, NGI: not gastrointestinal, OCD obsessive compulsive disorder, OP other probiotics, PLA: placebo, PRE: prebiotics, PRO: probiotics, SHGM: Standardized Human Gut Microbiota, SIB self-injurious behavior, Strept: Streptococcus, TD: typically developing children, tms: times, TNFα: Tumor Necrosis Factor α, tr: treatment, VA: vitamin A, wks: weeks, yrs: years

3. A focus on GI problems in a large group of preschoolers with ASD

I was involved in a randomized controlled trial on the role of probiotics on clinical, biochemical, and neurophysiological parameters funded by the Italian Ministry of Health examining a group of Italian preschoolers with ASD (Santocchi et al. 2016). I actively participated in all the study phases: in the clinical evaluation of the patients, data collection, elaboration, and analysis of the results.

The study was a six-month double-blind randomized parallel, factorial, efficacy-controlled trial with probiotics, four parallel arms, and an allocation ratio of 1:1. The study protocol was approved by the Pediatric Ethic Committee of Tuscany Region in July 2014 (Approval Number: 126/2014) and registered with Clinicaltrials.gov (NCT02708901).

Participants were enrolled among all the patients assessed at a tertiary care university hospital (IRCCS Stella Maris Foundation - Pisa, Italy), between November 2015 and February 2018 and screened for eligibility. After recruitment, children were followed up from February 2016 to September 2018.

Inclusion criteria were:

- age-range: 18-72 months;
- ASD diagnosis according to Diagnostic and Statistical Manual of Mental Disorders-5th Edition (Association 2013) (DSM-5) performed by a senior child psychiatrist with specific expertise in clinical evaluation of ASD.

Exclusion criteria were:

- neurological syndromes or focal neurological signs;
- history of birth asphyxia, severe premature birth or perinatal injuries;
- epilepsy;
- significant sensory impairment (e.g., blindness, deafness);
- diagnosis of not functional GI disorder or Coeliac Disease;
- special diets already underway (i.e., gluten-free diet, casein-free diet, high-protein diet, ketogenic diet);
- known brain anomalies.

At the baseline assessment (T0) the children underwent a global clinical and neuropsychiatric evaluation with demographics (age, sex, parental education and employment, family and residential information), medical history, physical examination with anthropometric measurements (weight, height, head circumference), linguistic evaluation, psychometric evaluation (Griffiths 2006), ADOS 2 (Lord et al. 2012), ADI-R (Lord 1994), CARS (Schopler et al. 1980), Vineland Adaptive Behavior Scales-Second Edition – VABS-II – interview with parents for the evaluation of adaptive functioning (Sparrow et al. 2005), questionnaires filled in by parents comprising Sensory Profile – SP (Dunn 1999), Repetitive Behavior Scale – RBS-R (Lam and Aman 2007), Child Behavior Check List – CBCL 1.5-5 (Achenbach 2000), Parent Stress Index – PSI (Abidin 1995), GI Severity Index – GSI (Schneider et al. 2006). The ADOS-2 (Luyster et al. 2009, Lord et al. 2012) is a semi-structured assessment of communication, social interactions, play, imagination, and stereotyped or repetitive behaviors used as the golden standard tool for diagnosing ASD (Lord et al. 2000). It includes five modules (Toddler, 1, 2, 3, 4), each tailored to children's language level and age. At the baseline assessment we used Toddler Module for 3 children, Module 1 for 71 children, and Module 2 for 11 children. The overall score is obtained from two separate domains, Social Affect and Restricted, Repetitive Behaviors, and from the sum of both. Raw scores were converted into calibrated scores through the Calibrated Severity Score (ADOS-CSS) (Gotham et al. 2009, Hus and Lord 2014, Esler et al. 2015), created to standardize and compare ADOS-2 raw scores across different modules and ages.

Blood and fecal samples were also collected from each subject in order to perform biochemical evaluation. Specifically, blood samples were collected to measure levels of leptin, resistin and inflammatory markers: TNF- α, IL-6, CCL-2 or MCP-1 and Plasminogen Activator Inhibitor-1 (PAI-1); fecal samples were collected for the analysis of fecal calprotectin levels, microbiota and metabolome.

Blood samples were collected by venipuncture in the morning after overnight fasting, rapidly separated by centrifugation for 15 min at 4°C, and plasma samples were stored frozen at −80 °C until

assay. The cytokines were measured directly in the plasma through specific immunometric tests (MILLIPLEX MAP, human-magnetic bead panel, Millipore Corporation, Billerica, MA, USA) using an integrated multi-analyte detection platform (high-throughput technology Magpix system, Luminex xMAP technology, Austin, TX, USA).

Each sample was analyzed in duplicate. In each one, a sample was analyzed as quality control. Inter-assay variability was evaluated using two samples at different concentrations and was <10%.

Fecal samples were collected at home within two days before T0 and then stored frozen at −80 °C until assay.

Neurophysiological patterns were assessed through QEEG measures. The resting brain activity was measured by means of a 128-channel digital EEG (GES300- EGI).

Information about pharmacological treatments and food supplements in the previous 3 months were collected: parents reported an acute or episodic administration of antibiotics (28.2%), probiotics (8.2%), NSAIDs or paracetamol (14.1%), steroids (8.2%), other drugs without effects on GI symptoms (36.5%), and a chronic administration of osmotic laxatives (12.9%). Even though it is not entirely unusual that even preschoolers, if severely disturbed, already be prescribed psychoactive drugs, none of the enrolled subjects used psychotropic medications.

At the end of T0, the subjects were divided into two groups based on the presence or absence of GI symptoms (GI and NGI groups, respectively) through the GSI (Schneider et al. 2006), a composite score designed on a Likert scale to assess signs and symptoms of GI distress reported by parents in the previous two weeks (constipation, diarrhea, average stool consistency, stool smell, flatulence, abdominal pain, unexplained daytime irritability, nighttime awakening, abdominal tenderness). We adopted a GSI cut-off of 4, with at least 3 score points from the first six items of the scale, selected by Adams et al. (Adams et al. 2011) as more specifically related to GI symptoms and named the 6-GSI.

Children belonging to GI and NGI groups were randomly assigned 1:1 to supplementation with probiotics or with placebo for 6 months, according to a computer generating randomization sequence

previously determined which was made in blocks with random sequences of independent block both in the GI and in the NGI groups.

We obtained four subgroups:

- Group 1: GI+probiotic;
- Group 2: GI+placebo;
- Group 3: NGI+probiotic;
- Group 4: NGI+placebo;

Follow-up assessments at 6 months (T2) after randomization included assessing outcome measures, adverse events, concomitant treatments, and reasons for dropout.

The probiotic supplement was De Simone Formulation (DSF), a patented mixture already approved for children (marketed as Vivomixx® in EU, Visbiome® in the USA). Each packet contained 450 billion of eight probiotic strains: *Streptococcus thermophilus*, *Bifidobacterium breve*, *Bifidobacterium longum, Bifidobacterium infantis*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, Lactobacillus *para-casei*, Lactobacillus *delbrueckii subsp. bulgaricus*. This study protocol required the oral administration of DSF, dissolved directly in the mouth or in a cold, not carbonated liquid at the posology of 2 packets/day in the first month of treatment and 1 packet/day in the following 5 months. The treatment was administered to children at home by the parent(s) or the child's legal guardian. The placebo packaging and organoleptic characteristics were identical to the probiotic ones and contained 4.4 g of maltose and silicon dioxide.

The parents/caregivers filled out a weekly food diary in which they reported any suspension in the administration of the experimental treatment and any concomitant drug or food supplement. The suspension of any other intervention effective and recommended by current guidelines in ASD was not required; information on the total number of hours of rehabilitative treatment performed during the study was collected.

The study scheme and the primary and secondary outcome measures were summarized in Table 3.1.

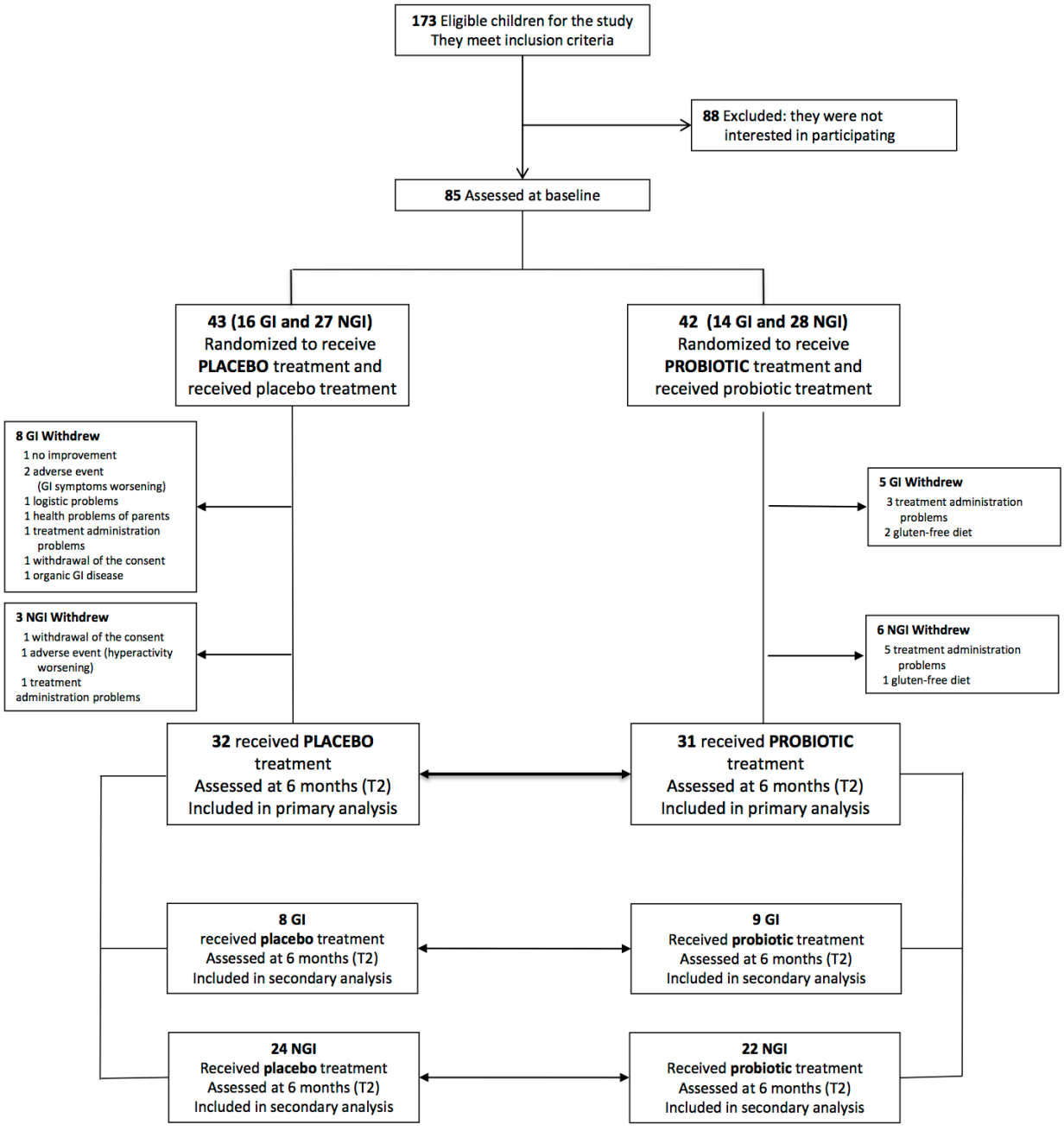
Table 3.1 Scheme of the study

TIMEPOINT of visit/assessment	Baseline	15 days (call interview)	3 months	6 months- end of the study
Enrollment				
Information and informed consent	✓			
Randomization-allocation	✓			
Interventions				
Probiotic Intervention	→	→	→	→
Placebo Intervention	→	→	→	→
Assessment				
Physical examination including anthropometric measures and abdominal tenderness	✓		✓	✓
Drug compliance and adverse event assessment		✓	✓	✓
Weekly food diaries			✓	✓
ADI-R	✓			
Outcome measures				
Primary outcome measure → ADOS 2	✓			✓
CARS	✓			✓
RBS-R	✓		✓	✓
Sensory Profile	✓		✓	✓
SCQ	✓		✓	✓
GI Severity Index	✓		✓	✓
Secondary outcome measures → CBCL 1.5-5	✓		✓	✓
PSI	✓		✓	✓
VABS-II	✓			✓
Mc Arthur-CDI	✓		✓	✓
GMDS-ER	✓			✓
Blood sample: LPS, Leptin, Resistin, TNF-α, IL-6, PAI-1	✓			✓
Urinary sample: Phtalates	✓		✓	✓
Fecal sample: Calprotectin	✓		✓	✓
High-density EEG registration: power, asymmetry index and coherence in the different frequency bands	✓			✓

Legend: *ADI-R* Autism Diagnostic Interview-Revised, *ADOS-2* Autism Diagnostic Observation Schedule- Second Edition, *CARS* Childhood Autism Rating Scale, *RBS-R* Repetitive Behavior Scale-Revised, *SCQ* Social Communication Questionnaire, *GI Severity Index* Gastro-Intestinal Severity Index, *CBCL 1.5-5* Child Behavior Checklist 1.5-5, *PSI* Parenting Stress Index, *VABS-II* Vineland Adaptive Behavior Scale-Second Edition, *Mc Arthur-CDI* MacArthur-Bates Communicative Development Inventories, *GMDS-ER* Griffiths Mental Development Scale-Extended Revised, *LPS* Lipopolysaccharide, *TNF–α* Tumor Necrosis Factor-α, *IL-6* Interleukin-6, *PAI–1* Plasminogen Activator Inhibitor-1, *EEG* Electroencephalography

One hundred seventy-three children were eligible based on the inclusion and exclusion criteria, of which 88 refused to be enrolled in the study (most of them due to the distance from the hospital or to family organizational difficulties). A total of 85 participants, 55 belonging to the NGI group and 30 belonging to the GI group, were randomized to probiotic supplementation or placebo (42 and 43 respectively) (see Figure 3.1). The study team decided to stop recruitment in February 2018, overtaking the planned sample size (76) and considering the time limit for the study completion (November 2018).

Figure 3.1 Consort flow diagram



Abbreviations: GI Gastrointestinal; NGI Non-Gastrointestinal

Baseline demographic and clinical characteristics did not significantly differ between treatment groups (Table 3.2). Of the 85 participants, all Italian, 71 (83.5%) were males and 14 (16.5%) females. The mean age at the recruitment was 4.15 years (SD: 1.08; range 2.18-6.11 years).

Table 3.2 Baseline characteristics of the sample

Characteristics	Groups (n, %) Placebo T0 (43, 51)	Probiotics T0 (42, 49)	*p*
Age, mean (SD), y	4.13 (1.00)	4.16 (1.17)	ns
Boys, No. (%)	37 (86.0)	34 (80.9)	ns
ADOS CSS [a], No (%)	43	42	
Score, mean (SD)			
Total	7.2 (2.1)	7.0 (1.4)	ns
Social Affect	6.5 (2.2)	6.3 (1.7)	ns
Restricted and repetitive behavior	8.4 (1.3)	8.1 (1.5)	ns
ADI-R [b], No (%)	36	36	
Score, mean (SD)			
Reciprocal social interaction	17.8 (4.8)	18.9 (4.8)	ns
Language and communication	11.7 (2.3)	12.3 (3.5)	ns
Repetitive behaviors and interests	5.3 (1.7)	5.5 (1.7)	ns
Age of onset	4.1 (0.8)	4.1 (0.7)	ns
SCQ [c], No (%)	43	40	
Total score, mean (SD)	15.8 (5.2)	14.2 (6.6)	ns
RBS-R [d], No (%)	43	41	
Total score, mean (SD)	21.1 (14.9)	18.5 (12.6)	ns
DQ [e], standardized test, No (%)	43	42	
Mean (SD)			
General Quotient, mean (SD)	60.5 (19.1) 25 out of 33	64.6 (16.4) 29 out of 34	ns
Developmental ret. (DQ<70), No. (%)	17 (58.6) 29 out of 43	19 (57.5) 33 out of 41	ns
VABS II [f], No (%)	43	42	
Composite Score, mean (SD)	55.4 (17.7)	63.6 (21.0)	ns
Linguistic Level [g], No (%)	43	42	
0. No words or < 5 words	26 (60.4)	20 (47.6)	ns
1. At least 5 words	10 (23.2)	11 (26.1)	ns
2. Phrases at least 3 words	6 (13.9)	9 (21.4)	ns
3. Fluent language	1 (2.3)	2 (4.7)	ns
CBCL [h], No (%)	43	41	
Score, mean (SD)			
Total Problems	62.9 (10.8)	61.5 (9.9)	ns
PSI [i], No (%)	40	37	
Score, mean (SD)			
Total Stress	76.7 (23.1)	69.8 (29.3)	ns
GI Severity Index [j], No (%)	43	42	
Score, mean (SD)			
Total 6-GSI	1.8 (1.6)	2.3 (2.2)	ns
Total GSI	3.5 (2.4)	3.8 (3.0)	ns
Child care [k], No (%)	43	42	
Total			
≥4 h/d care	14 (32.5)	14 (33.3)	ns
<4 h/d care	28 (65.1)	27 (64.2)	ns
None of the above	1 (2.3)	1 (2.3)	ns

Abbreviations: ADI-R Autism Diagnostic Interview–Revised; ADOS Autism Diagnostic Observation Schedule; CBCL 1.5-5 Child Behavior Checklist 1.5-5; CSS Calibrated Severity Score; DQ Developmental Quotient; GI gastrointestinal; GSI Gastrointestinal Severity Index; h/d hours/day; No. Number; NGI Non-Gastrointestinal; PSI Parental Stress Index; RBS-R Repetitive Behaviors Scale-Revised; SCQ Social Communication Questionnaire; SD Standard

Deviation; VABS-II Vineland Adaptive Behavior Scales-II; y years.
[a] Higher scores indicate greater severity (range of possible scores for Total, Social Affect and Restricted and Repetitive Behavior is 1-10).
[b] Higher scores indicate greater severity (ranges of possible scores: reciprocal social interaction, 0-30; language and communication, 0-26; repetitive behaviors and interests, 0-12; early onset, 0-5).
[c] Higher scores indicate greater severity (range 0-39) with a threshold of 15 compatible for a relevant impairment of social communication (some studies consider 9 in children younger than four years old).
[d] Higher scores indicate greater severity of repetitive behaviors (range 0-114).
[e] Higher scores indicate greater cognitive ability. Scores around 100 indicate normal intelligence; scores below 70 indicate a developmental delay.
[f] Higher scores indicate greater adaptive competences. Scores around 100 indicate normal adaptive capacities; scores below 70 indicate a delay with respect to age.
[g] The "Overall Level of Non-Echoed Spoken Language" item (A1 score) of the ADOS-2 was used to differentiate non-verbal (those with absent language or less than 5 words) from verbal children
[h] Higher scores indicate greater severity; a score of 63 and above is generally considered clinically significant.
[i] Higher scores indicate greater severity of parental stress index caused both by characteristics of the child and by negative experiences about the parenting role (Total Stress).
[j] Higher scores indicate greater severity of gastrointestinal symptoms; Total 6-GSI has a range of 0 to 12, Total GSI has a range of 0 to 17.
[k] We considered the daily hours of rehabilitation treatment plus the daily hours of school support teacher.

Sixty-three children completed the trial (placebo: 32, 74.4%; probiotic: 31, 73.8%) with a drop-out rate of 25.9% (22 children: 9 NGI and 13 GI) (see in Figure 3.1 reasons for discontinuation).

There were no significant differences (p=0.94) in the total number of hours of concomitant rehabilitative treatment over the six-month intervention period in those allocated to placebo (144±86 hours) compared with those assigned to probiotic supplementation (142±114 hours).

Baseline characteristics of the 22 children who dropped out at T2 were not significantly different from those of the 63 children who were followed up and included in outcome analysis, except for the GI/NGI ratio, Total GSI 9-items, Total GSI 6-items and RRB ADOS-CSS scores, which were significantly higher in children who dropped out (Table 3.3).

Table 3.3 Baseline characteristics of those followed up at 6 months versus those who dropped out

	Followed up at 6 months		Dropped out at 6 months		*p*
Characteristics	63		22		
Age, mean (SD), y	63	4.19 (1.1)	22	4.02 (1.04)	ns
Sex (male), No. (%)	63	51 (81)	22	20 (91)	ns
ADOS CSS [a], No. (%)	63		22		
Score, mean (SD)					
Total		6.9 (1.7)		7.7 (2.1)	ns
Social Affect		6.3 (2.0)		6.8 (2.0)	ns
Restricted and repetitive behavior		8.1 (1.4)		8.9 (1.4)	**.028**
ADI-R [b], No (%)	63		9		
Score, mean (SD)					
Reciprocal social interaction		18.2 (5.0)		19.8 (3.4)	ns
Language and communication		11.8 (2.9)		13.8 (3.8)	ns
Repetitive behaviors and interests		5.5 (1.8)		5.1 (1.8)	ns
Early onset		4.2 (0.8)		4.0 (0.9)	ns
SCQ [c], No (%)	62		21		
Total score, mean (SD)		14.5 (6.3)		16.7 (4.6)	ns
RBS-R [d], No (%)	63		21		
Total score, mean (SD)		20.3 (14.4)		18.4 (12.3)	ns
DQ [e], standardized test, No (%)	63		22		
Mean (SD)					
General Quotient, mean (SD)		63.0 (18.7) 49 out of 62		60.9 (15.9) 13 out of 22	ns
Developmental ret. (DQ<70), No. (%)		30 (61) 49 out of 62		8 (61) 13 out of 22	ns
VABS II [f], No (%)	63		22		
Score, mean (SD)					
Composite Score		60.4 (19.7)		57.0 (20.1)	ns
Linguistic Level [g], No (%)	63		22		
0. No words or < 5 words		31 (49)		15 (68)	
1. At least 5 words		16 (25)		5 (22)	ns
2. Phrases at least 3 words		13 (20)		2 (9)	
3. Fluent language		3 (4)		0	
CBCL [h], No (%)	63		21		
Score, mean (SD)					
Total Problems	63	61.9 (10.4)	21	63.3 (10.4)	ns
PSI [i], No (%)	63		16		
Score, mean (SD)					
Total Stress	60	72.3 (27.3)	14	78.1 (22.0)	ns
GI Severity Index [j], No (%)	63		22		
Score, mean (SD)					
Total 6-GSI		1.7 (1.8)		3.2 (2.1)	**.003**
Total GSI		3.3 (2.6)		5.0 (2.9)	**.012**
NGI subjects, No (%)		46 (73)		9 (40)	**.007**
GI subjects, No (%)		17 (26)		13 (59)	

Abbreviations: ADI-R Autism Diagnostic Interview–Revised; ADOS Autism Diagnostic Observation Schedule; CBCL 1.5-5 Child Behavior Checklist 1.5-5; CSS Calibrated Severity Score; DQ Developmental Quotient; GI gastrointestinal; GSI Gastrointestinal Severity Index; No. Number; NGI Non-Gastrointestinal; PSI Parental Stress Index; RBS-R Repetitive Behaviors Scale-Revised; SCQ Social Communication Questionnaire; SD Standard Deviation; VABS-II Vineland Adaptive Behavior Scales-II; y years.

[a] Higher scores indicate greater severity (range of possible scores for Total, Social Affect and Restricted and Repetitive Behavior is 1-10).

[b] Higher scores indicate greater severity (ranges of possible scores: reciprocal social interaction, 0-30; language and communication, 0-26; repetitive behaviors and interests, 0-12; early onset, 0-5).

[c] Higher scores indicate greater severity (range 0-39) with a threshold of 15 compatible for a relevant impairment of social communication (some studies consider 9 in children younger than four years old).

[d] Higher scores indicate greater severity of repetitive behaviors (range 0-114).

[e] Higher scores indicate greater cognitive ability. Scores around 100 indicate normal intelligence; scores below 70 indicate a developmental delay.
[f] Higher scores indicate greater adaptive competences. Scores around 100 indicate normal adaptive capacities; scores below 70 indicate a delay with respect to age.
[g] The "Overall Level of Non-Echoed Spoken Language" item (A1 score) of the ADOS-2 was used to differentiate non-verbal (those with absent language or less than 5 words) from verbal children
[h] Higher scores indicate greater severity; a score of 63 and above is generally considered clinically significant.
[i] Higher scores indicate greater severity of parental stress index caused both by characteristics of the child and by negative experiences about the parenting role (Total Stress).
[j] Higher scores indicate greater severity of gastrointestinal symptoms; Total 6-GSI has a range of 0 to 12, Total GSI has a range of 0 to 17.

3.1. Chapter 1. How do children with ASD show GI problems?

Children with ASD may express GI symptoms in atypical ways, especially when a concomitant communication impairment is present (Horvath and Perman 2002, Buie et al. 2010, Adams et al. 2011, Kral et al. 2013, McElhanon et al. 2014, Wasilewska and Klukowski 2015, Fulceri et al. 2016, Marler et al. 2017, Ferguson et al. 2019, Kurokawa et al. 2021).

Therefore, the main aims of the study were:

1. To investigate the correlation between GI symptoms and the presence and the type of the Associated Behaviors (AB) reported in the Consensus report (Buie et al. 2010) in preschoolers with ASD;
2. To evaluate possible differences in the expression of AB between verbal and non-verbal ASD subjects with GI problems.

3.1.1. Materials and Methods

Instruments

The tools used in this study were: the GSI (Schneider et al. 2006), the Associated Behaviors Questionnaire (ABQ), the ADOS-2 (Luyster et al. 2009, Lord et al. 2012), the RBS-R (Bodfish et al. 2000), the GMDS-ER (Griffiths 1996, Griffiths 2006), the VABS-II (Sparrow et al. 2005), the CBCL 1.5-5 (Achenbach and Rescorla 2000, Frigerio et al. 2006).

The ABQ is a questionnaire completed by parents about the behaviors reported by Buie and colleagues (Buie et al. 2010) as probably associated with pain or abdominal discomfort in ASD subjects. It includes 22 items, rated on a three-point Likert scale (absent behavior, not very present/infrequent behavior, very pronounced/very frequent behavior), which can be grouped into

three clusters (Buie et al. 2010): Verbal Behavior (VB), Motor Behaviors (MB) and Changes in overall state (C) (see Table 3.1.1).

The Performance subscale of GMDS-ER was used to measure the non-verbal skills of each child; 65 out of 85 children were evaluable with this test.

The A1 score of the ADOS-2 ("Total level of spoken language non-echolalic") was used to differentiate non-verbal (those with absent language or less than 5 words) from verbal children.

Table 3.1.1: Behaviors assessed by ABQ as possible markers of abdominal discomfort in individuals with ASD

Verbal Behaviors (VB)	**Motor Behaviors (MB)**	**Changes in overall state (C)**
Frequent clearing of throat	Facial grimacing	Sleep disturbances (difficulty getting to sleep, difficulty staying asleep)
Swallowing and/or tics	Gritting teeth	Increased irritability (exaggerated responses to stimuli)
Screaming	Wincing	Non-compliance with demands that typically elicit an appropriate response
Sobbing for no reason at all	Constant eating/drinking/swallowing	
Sighing and/or whining	Mouthing behaviors as chewing on clothes, pica	
Moaning and/or groaning	Application of pressure to abdomen	
Delayed echolalia that includes reference to pain or stomach and direct verbalizations about it	Tapping behaviors	
	Any unusual posturing	
	Agitation as jumping up and down	
	Unexplained increase in repetitive behaviors	
	Self-injurious behaviors	
	Aggressive behaviors: onset of or increase	

Statistical analysis

The Analysis of Variance (ANOVA) was used to compare males *vs* females for all variables, GI *vs* NGI subjects and verbal *vs* non-verbal subjects for the scores in the ABQ.

Correlations and regression analysis were computed to study the relationship between the scores on GI Severity Index and the scores reported in the ABQ. The multivariate regression was used to investigate the impact of the clinical variables on the differences between the GI and the NGI groups.

All statistical analyses were performed using the software Statistical Package for Social Sciences (SPSS), version 17.0 for Windows (SPSS Statistics for Windows Released 2008), with p=0.05 as the significance level.

Procedure

The correlations of the GI symptoms with the ABQ Total Score, with the three summary clusters (VB, MB and C) and with each single item of the ABQ were examined considering only the first 6 items of GSI (i.e. the 6-GI score) to avoid a possible bias due to the overlapping of survey areas between the other GSI items and ABQ items.

GI *vs* NGI subjects and verbal *vs* non-verbal subjects were compared, respectively, for their mean scores at the ABQ considering the ABQ Total Score, the three summary clusters (VB, MB and C) and each single item of the ABQ.

The correlation of the 6-GI score with the three summary clusters and with the single items of the ABQ was examined in verbal *vs* non-verbal subjects.

The scores on ADOS-2, VABS-II, RBS-R, CBCL and GMDS-ER have been used to better characterize our sample and they have been compared between the GI and the NGI groups.

3.1.2. Results

There were no significant differences in clinical variables between the GI and the NGI groups, except for the Global Score of the RBS-R, the Internalizing and Externalizing problem scores of the CBCL

(all significantly higher in the GI group than in the NGI group), and of the Communication and Daily Living adaptive scores of the VABS (significantly higher in the NGI group than in the GI group) (see Table 3.1.2).

Table 3.1.2: Statistical comparisons of clinical variables between GI and NGI groups.

Clinical variables	**GI**	**NGI**	***p*** **(NGI vs GI)**
Age (years) mean ± SD	4.06 (1.05)	4.19 (1.10)	NS
Males n (%)	26 (36.6%)	45 (63.4%)	NS
Females n (%)	4 (28.6%)	10 (71.4%)	NS
ADOS-2			
CSS Social Affect (mean ± SD)	6.77 (1.91)	6.29 (2.05)	NS
CSS Restricted Repetitive Behaviors (mean ± SD)	8.43 (1.48)	8.20 (1.45)	NS
CSS Total (mean ± SD)	7.43 (2.01)	6.95 (1.68)	NS
GMDS-ER (65 out of 85 children were evaluable with this test)			
Locomotor (mean ± SD)	70.92 (16.19)	72.88 (20.03)	NS
Personal-Social (mean ± SD)	48.96 (17.48)	57.55 (20.72)	NS
Hearing and Speech (mean ± SD)	42.87 (20.20)	47.07 (23.32)	NS
Eye-Hand Coordination (mean ± SD)	62.32 (20.07)	67.72 (24.34)	NS
Performance (mean ± SD)	67.80 (21.62)	72.26 (24.36)	NS
VABS-II			
Communication (mean ± SD)	45.47 (15.22)	54.46 (18.80)	0.0274
Daily Living (mean ± SD)	61.13 (14.29)	69.07 (17.51)	0.0365
Socialization (mean ± SD)	60.93 (13.59)	65.18 (15.83)	NS
Motor Skills (mean ± SD)	69.83 (16.34)	74.71 (17.69)	NS
Composite Score (mean ± SD)	54.40 (18.51)	62.29 (20.03)	NS
CBCL 1.5-5			
Internalizing Problems (mean ± SD)	67.48 (7.80)	62.06 (9.04)	0.0065
Externalizing Problems (mean ± SD)	59.07 (7.55)	55.82 (9.61)	NS
Total Problems (mean ± SD)	65.35 (10.02)	60.62 (10.30)	0.0469
RBS-R			
Total Score (mean ± SD)	20.07 (13.27)	19.76 (14.29)	NS
Total Endorsed Score (mean ± SD)	13.03 (6.78)	12.62 (7.57)	NS
Global Score (mean ± SD)	60.24 (20.77)	38.12 (27.06)	0.0016

Abbreviations: SD standard deviation; ADOS-2 Autism Diagnostic Observation Schedule-2; CSS Calibrated Severity Score; GMDS-ER Griffiths Mental Development Scales-Extended Revised; VABS-II Vineland Adaptive Behavior Scales-II; CBCL 1.5-5 Child Behavior Checklist 1.5-5; RBS-R Repetitive Behaviors Scale Revised.

Therefore, we examined the correlations of the 6-GI scores with the ABQ Total Scores in the whole sample (R=0.422, B=1.394, p=<0.001). In the multiple regression model, the correlation between the 6-GI scores and the ABQ Total Scores persisted even after correction for the variables that significantly differed between the GI and the NGI group (p = 0.0026).

- As far as the single AB is concerned, a positive correlation between the 6-GI scores and VB total score (p=0.009), and with "frequent clearing of throat, swallowing and/or tics" (p=0.043), "screaming" (p=0.048), "sighing and/or whining" (p=0.039), "moaning and/or groaning" (p=0.003), and "direct verbalization about pain or stomach" (p=0.015) scores was detected.
- In addition, a positive correlation between the 6-GI scores and MB total score (p=0.015), "facial grimacing" (p=0.010), "constant eating/drinking/swallowing" (p=0.002), "application of pressure to abdomen" (p=0.032), and "aggressive behaviors" (p=0.032) scores was observed.
- Moreover, a positive correlation between the 6-GI scores and C total score (p=0.041) was found.

The statistically significant correlations of the 6-GI scores with the three summary clusters (VB, MB and C) and with the singles items of the ABQ are reported in Table 3.1.3.

Table 3.1.3 Correlations between the 6-GI and the ABQ scores. Only statistically significant ($p<0.05$) scores are reported.

ABQ clusters and items	**Correlations ABQ/6-GI scores**
WHOLE SAMPLE	
VB (overall score)	R=0.281 B=0.294 p=0.009
Frequent clearing of throat, swallowing and/or tics	R=0.258 B=0.075 p=0.043
Screaming	R=0.252 B=0.110 p=0.048
Sighing and/or whining	R=0.263 B=0.089 p=0.039
Moaning and/or groaning	R=0.375 B=0.128 p=0.003
Direct verbalization about pain or stomach	R=0.309 B=0.084 p=0.015
MB (overall score)	R=0.264 B=0.526 p=0.015
Facial grimacing	R=0.325 B=0.100 p=0.010
Constant eating/drinking/swallowing	R=0.387 B=0.126 p=0.002
Application of pressure to abdomen	R=0.273 B=0.091 p=0.032
Aggressive behaviors	R=0.273 B=0.091 p=0.032
C (overall score)	R=0.222 B=0.181 p=0.041
VERBAL CHILDREN	
VB (overall score)	-
Frequent clearing of throat, swallowing and/or tics	R=0.411 B=0.119 p=0.022
Direct verbalization about pain or stomach	R=0.423 B=0.135 p=0.018
MB (overall score)	-
Constant eating/drinking/swallowing	R=0.474 B=0.184 p=0.007
C (overall score)	-
Sleep disturbances	R=0.437 B=0.207 p=0.014
NON-VERBAL CHILDREN	
VB (overall score)	R=0.561 B=0.619 p=0.001
Screaming	R=0.492 B=0.186 p=0.005
Sighing and/or whining	R=0.387 B=0.119 p=0.031
Moaning and/or groaning	R=0.540 B=0.196 p=0.002
C (overall score)	R=0.414 B=0.302 p=0.021
Sleep disturbances	R=0.362 B=0.153 p=0.045

Abbreviations: ABQ Associated Behaviors Questionnaire; GI gastrointestinal; VB verbal behaviors; MB motor behaviors; C changes in overall state; R regression; B beta regression coefficient

Thirty-nine participants (46%) were verbal and 46 (54%) were non-verbal. Among the 39 verbal subjects, 10 children (26%) were in the GI group, whereas among the 46 non-verbal subjects, 20 children (44%) were in the GI group. No statistically significant differences were found between verbal and non-verbal groups as far as the prevalence of GI subjects (p=0.086), the 6GI scores and the Total GSI scores (Table 3.1.4).

We found significantly higher scores for the GI group on the "ABQ Total Score", and in all the three ABQ cluster scores (Table 3.1.4). Furthermore, statistically significantly higher mean scores were detected in the GI group than in the NGI group in the following items: "sighing and/or whining", "moaning and/or groaning", "facial grimacing", "constant eating/drinking/swallowing", "application pressure to abdomen", "tapping behaviors", and "sleep disturbances".

Table 3.1.4: Significant differences in ABQ scores between ASD children with and without GI symptoms, and between verbal and non-verbal subjects.

	GI children n = 30	NGI children n = 55	p ≤ 0.01* ≤ 0.001**	Verbal children n = 39	Non-verbal children n = 46	p ≤ 0.01* ≤ 0.001**
6-GI Scores	4.43±1.28	0.82±0.80	<0.001**	1.74±1.74	2.39±2.16	NS
Total GSI Scores	6.73±1.70	2.04±1.53	<0.001**	3.10±2.38	4.20±2.97	NS
Associated Behaviors Total Score	14.21±5.75	9.33±6.03	0.001**			
VB (overall score)	3.67±2.29	2.55±1.87	0.017			
MB (overall score)	6.93±4.31	4.87±3.63	0.022			
C (overall score)	2.67±1.60	1.91±1.59	0.039			
Frequent clearing of throat, swallowing and/or tics				0.16±0.44	0.53±0.63	0.003
Sighing and/or whining	0.89±0.63	0.53±0.60	0.012			
Moaning and/or groaning	0.78±0.70	0.34±0.52	0.002*	0.34±0.48	0.62±0.70	0.044
Facial grimacing	0.78±0.58	0.31±0.47	<0.001**			
Constant eating/drinking/swallowing	0.80±0.82	0.22±0.50	<0.001**			
Mouthing behaviors as chewing on clothes, pica				0.29±0.56	0.64±0.75	0.022
Application of pressure to abdomen	0.61±0.79	0.25±0.44	0.010*			
Tapping behaviors	0.15±0.36	0.02±0.13	0.021			
Sleep disturbances	1.21±0.69	0.64±0.78	0.001**			

Abbreviations: GI Gastrointestinal; NGI Non-Gastrointestinal; GSI Gastrointestinal Severity Index; VB verbal behaviors; MB motor behaviors; C changes in overall state.

Three items of the ABQ were statistically significantly higher in the non-verbal group than in the verbal group: “frequent clearing of throat, swallowing and/or tics”, “moaning and/or groaning”, “mouthing behaviors”.

Then, we examined the correlations between the 6-GI symptoms and the ABQ Total Score in the verbal group (R=0.310, B=1.246, p=0.090) and in the non-verbal group (R=0.481, B=1.634. p=0.006).

In the verbal group, the 6-GI Score was positively correlated with “frequent clearing of throat, swallowing and/or tics” (p=0.022), “direct verbalization about pain or stomach” (p=0.018), “constant eating/drinking/swallowing” (p=0.007), and “sleep disturbances” (p=0.014).

The statistically significant correlations between the 6-GI score and the 3 summary clusters (VB, MB and C) and with each AB of the verbal subjects are reported in Table 3.1.3.
In the non-verbal group, the 6-GI score was positively correlated with VB total score (p=0.001), “screaming” (p=0.005), “sighing and/or whining” (p=0.031), “moaning and/or groaning” (p=0.002), C total score (p=0.021), and with “sleep disturbances” (p=0.045).
The statistically significant correlations between the 6-GI score and the three summary clusters (VB, MB and C) as well as with each AB of the non-verbal subjects are reported in Table 3.1.3.
Finally, considering the possible influence of having more internalizing or more externalizing problems on the subject's expression of abdominal discomfort, the correlations between the CBCL scores (Internalizing, Externalizing, Total Problems) and the 6-GI scores were analyzed. The Internalizing Problems and the Total Problems were significantly correlated with the 6-GI score ($p = 0.006$ $R = 0.30$, and $p = 0.036$ $R = 0.23$, respectively). We did not find a significant correlation between the 6-GI scores and the Externalizing Problems.

3.1.3. Discussion

Over the past years, the evaluation and the understanding of GI symptoms in ASD individuals are considerably improved, and crucially the importance of considering behavioral symptoms as a possible expression of GI distress is repeatedly emerging (Gorrindo et al. 2012, Maenner et al. 2012, Peters et al. 2014, Marler et al. 2017, Holingue et al. 2018, Margolis et al. 2018, Ferguson et al. 2019, Kurokawa et al. 2021).
This is the first study that seeks to investigate the possible association between all the AB and GI symptoms, with the aim of contributing to a better comprehension of the way by which ASD subjects signal the presence of a GI problem.
Results pointed out to significantly higher scores in ABQ Total scores as well as in the scores of the three clusters proposed by Buie and colleagues (Buie et al. 2010) in subjects with significant GI

symptoms than in subjects without, with some AB significantly associated with the presence of an underlying GI problem. The abovementioned association was maintained independently from clinical variables -involving the adaptive functioning and emotional-behavioural psychopathology- that distinguish GI from NGI subjects. These findings could provide a new interpretation for some events that are usually considered part of autistic symptomatology (Mannion 2014, Holingue et al. 2018). The sudden appearance of self- and other-directed aggression, an increase in motor activity or in repetitive behaviors and a worsening of global adaptive behaviors in ASD subjects could suggest clinicians starting or increasing a psychopharmacological treatment that instead could be prevented through a correct diagnosis and appropriate treatment of the underlying GI problem. Accordingly, the contribution of GI symptoms to the onset of sudden irritability or aggressive behavior in non-verbal ASD children has already been reported (Horvath et al. 1999, Maenner et al. 2012). In addition, the findings showing that ASD subjects with GI problems have worse clinical functioning than ASD subjects without GI problems, independently from the severity of autistic symptoms are confirmed (Prosperi et al. 2017). Of note, the presence of more substantial internalizing or externalizing symptoms may influence the subject's expression of abdominal discomfort.

Behaviors like sighing and/or whining, moaning and/or groaning, facial grimacing and sleep disturbances are easily associable to the discomfort of an underlying GI problem. Other manifestations like constant eating/drinking/swallowing, application pressure to abdomen and tapping behaviors are more difficult to predict in these subjects. Since the action of constantly eating small amounts of food or repetitively swallowing with a feeling of obstruction (dysphagia) are key symptoms of gastro-esophageal reflux (GER), a link between “constant eating/drinking/swallowing” behavior and GER can be hypothesized. This result could be particularly relevant, since GER is a problem difficult to identify in the early stage, which often leads to medical complications, and usually requires invasive diagnostic techniques (Rybak et al. 2017). “Holding the abdomen” has already been referred as constipation in children and adolescents with ASD (Furuta et al. 2012), and it could be the only visible sign of a health issue with a high rate of hospitalization (Sparks et al.

2018). It has also been associated with GERD, intestinal inflammation, malabsorption and maldigestion (Buie et al. 2010, Margolis et al. 2018). “Tapping behaviors” have been only recently considered as a possible expression of GI problems (Buie et al. 2010), since they were frequently interpreted as a behavior specific to autism (McDougle et al. 1995). In all these cases, the modalities of onset (e.g., sudden, or gradual) and other anamnestic information are needed for symptom characterization and proper treatment options.

Previously, it has been suggested that the presence of these AB is not useful per se for the identification of GI problems, since they are very common also in children without GI problems (Maenner et al. 2012). Buie and colleagues (Buie et al. 2010) pointed out that the motor behaviors are not specific to GI problems but may be indicative of pain or discomfort also arising in other parts of the body. A positive correlation between the 6-GI score and all the three ABQ clusters was found, showing for the first time specificity of these behaviors. In particular, some specific behaviors seem to represent a direct expression of a GI problem in ASD subjects: vocal behaviors like “frequent clearing of throat, swallowing and/or tics”, “screaming”, “sighing and/or whining”, “moaning and/or groaning”, “direct verbalization about pain or stomach”, and motor behaviors like “facial grimacing”, “constant eating/drinking/swallowing”, “application of pressure to abdomen” and “aggressive behaviors”, as well as “changes in overall states”.

As far as the comparison between verbal and non-verbal children, the two groups showed several significant differences when the 6-GI Score was considered. “Mouthing behaviors” seemed to differentiate verbal from non-verbal subjects. “Frequent clearing of throat, swallowing and/or tics”, “direct verbalization about pain or stomach” and “constant eating/drinking/swallowing” are symptoms that characterized the verbal group with higher 6-GI scores, whereas “screaming”, “sighing and/or whining” and “moaning and/or groaning” were more representative for the non-verbal group with higher 6-GI scores. Notably, “sleep disturbances” were present in both groups, in agreement with previous literature (Williams 2010, Maenner et al. 2012, Mannion et al. 2013). Therefore, non-verbal subjects with a more relevant GI symptomatology show more frequently vocal behavior and

changes in the global state than verbal subjects with a GI disturb. Interestingly, there were no significant differences in the prevalence and severity of GI symptoms between verbal and non-verbal subjects. In a recent review, the authors concluded that it was not possible to examine the association between GI symptoms and verbal skills in subjects with ASD, since the majority of studies did not distinguish the prevalence of GI symptoms on the basis of expressive language level (e. g. verbal vs non-verbal subjects) (Holingue et al. 2018). By evaluating the inverse relationship, previous investigations on relatively small samples detected both lower verbal abilities (Gorrindo et al. 2012) and no differences of verbal skills (Williams 2012, Chandler et al. 2013) in ASD children with GI problems compared to ASD children without GI problems. This result supports the view that non-verbal ASD children differ from verbal ASD patients only in the modalities of GI discomfort expression, but not in the prevalence or global intensity of GI symptoms. We can hypothesize that non-verbal children activate a minimally verbal behavior to report a physical discomfort (AB as "screaming", "sighing and/or whining" and "moaning and/or groaning"), while verbal subjects more frequently verbalize their pain (AB as "direct verbalization about pain or stomach"). Importantly, the relatively low average age and the high proportion of subjects not evaluable at the psychometric test (i.e. more functionally compromised) could partly explain the significantly higher presence of behaviors like "frequent clearing of throat, swallowing and/or tics" in verbal subjects, as well as the low prevalence of "delayed echolalia that includes reference to pain or stomach" in both groups and in the total sample. Crucially, this result indicates that verbal subjects with autism use direct verbalizations, rather than echolalia, to signal discomfort.

It is important to be aware of the atypical presentation of GI symptoms (Leader 2015). Usually, the questionnaires that investigate the GI symptoms in ASD children are based almost exclusively on the symptoms reported by the parents rather than on the visible signs (Valicenti-McDermott et al. 2008, Nikolov et al. 2009, Williams 2012, Chandler et al. 2013, Mannion et al. 2013, Mazurek et al. 2013). The presence of significant GI symptoms has been evaluated through the administration of parent-report questionnaire and this could represent a limitation, particularly in younger subjects or in

children with more severe autistic symptoms (Holingue et al. 2018). However, a high concordance between parental reports and gastroenterologist evaluations has been reported (Gorrindo et al. 2012, Margolis et al. 2018).

In the future, the sensitivity and the specificity of these results could be enhanced by providing a complete gastroenterological evaluation in ASD subjects with higher GI total scores or at least in the positive cases (Margolis et al. 2018). Conversely, this protocol could be recommended in hospitals with limited economic resources.

The GSI does not evaluate the presence of some frequently reported GI symptoms, such as GER and vomiting and the cut-off we used was based on the examination of the previous literature but is not yet validated. Furthermore, a potential bias derived from the expected numerical disparity between GI and NGI subjects may affect the interpretation of results. Finally, these results are limited to functional GI disorders: therefore, additional research on the associated behaviors in children with organic GI problems is needed.

In summary, these results encourage researchers to adapt the current tools used to investigate GI symptomatology in ASD subjects, through the addition of items that are also based on objective measures. It is important to enhance clinicians' awareness of the high co-occurrence of GI symptoms in ASD subjects, so that targeted investigations can be undertaken (Mannion 2014).

In addition, a future comparison between these results and those obtained from a group of age and sex-matched typical controls could be useful to better understand the features of the AB related to the GI symptomatology, independently from an ASD diagnosis.

Our understanding of GI problems in ASD in relation to AB has come a long way, but further studies and more systematic research are warranted.

These results were published in BMC Pediatrics (Prosperi et al. 2019).

3.2. Chapter 2. Are children with ASD and GI problems "inflamed"?

Recently, the contribution of immune dysregulation has been described as a common feature of ASD, and alterations in circulating cytokine levels have been repeatedly reported (Mead and Ashwood 2015, Saghazadeh et al. 2019).

Although a systematic review about proinflammatory markers in more than 3900 children and/or adolescents with neuropsychiatric disorders including ASD (Mitchell and Goldstein 2014) found preliminary evidence for the role of inflammation and pro-inflammatory state in these conditions, until now conflicting and irreproducible findings have been detected in various studies.

Some authors have proposed interleukin (IL)-6, tumor necrosis factor-alpha (TNF)-α, and macrophage chemoattractant protein-1 (CCL2) as potentially involved in brain inflammation at least in a subgroup of subjects with ASD (Theoharides et al. 2012).

A recent meta-analysis of 25 studies revealed a higher concentration of pro-inflammatory cytokines interferon (IFN)-γ, IL-1β, IL-6, and TNF-α in children with ASD compared with controls (Saghazadeh et al. 2019). Increased levels of IL-6 and IL-8 were found to be predictive biomarkers for ASD risk in a study analyzing circulating cytokine patterns from neonatal blood (Heuer et al. 2019). High levels of IL-6 in the brain could determine alterations of synapse formation, dendritic spine development, and neuronal circuit balance (Wei et al. 2012), while in plasma they have been associated with increased stereotypical behaviors and with regressive forms of ASD (Ashwood et al. 2011). Conversely, TNF-α has a critical role in regulating synaptic strength and plasticity (Steinmetz and Turrigiano 2010), and his levels have been positively correlated with ASD severity (Inga Jacome et al. 2016). High CCL2 levels could be instead considered as a signal of microglia/astroglia activation (Vargas et al. 2005), and have been associated with higher aberrant behavior scores and more impaired adaptive functioning (Ashwood et al. 2011).

Similarly, GI problems that frequently occur in ASD subjects seem to be caused by inappropriate immune activation and pro-inflammatory processes of the digestive tract (Rose et al. 2018). It has

been shown that the level of stress-responsive cytokines, like IL-6 and TNF-α, are increased both in ASD subjects (Ashwood et al. 2011) and in the general population in association to GI symptoms (von Kanel et al. 2006, Lyte et al. 2011), pointing to a link between peripheral inflammation and neuroinflammation. Particularly, high levels of TNF-α can influence the intestinal epithelial barrier, possibly contributing to GI problems (Gorrindo et al. 2012) and intestinal permeability, and also to ASD onset as recently suggested by the "leaky gut" hypothesis (Coury et al. 2012). The myeloid dendritic cells, which produce among others TNF- α and IL-6, have been associated with increased GI symptoms in ASD as well as increased amygdala volume and regressive autism (Breece et al. 2013). More recently, other authors (Ferguson et al. 2016, Rose et al. 2018) did not confirm an association between the symptoms of the lower GI tract and levels of TNF-α or IL-6, however their levels were correlated with irritability, socialization, and intelligence in ASD subjects.

Besides, a particular type of cytokines called adipokines seems to be implicated in the pathogenesis of inflammatory CNS disorders and ASD (Rodrigues et al. 2014) despite the findings obtained so far are mostly controversial. Adipokines, or adipocytokines, are active proteins secreted by white adipose tissue with functions similar to hormones in inter-organ communication (Pan and Kastin 2007) and their dysregulation has been implicated in obesity, type 2 diabetes, cardiovascular disease and recently, in peripheral tissue insulin resistance and inflammation (Kwon and Pessin 2013). Leptin, adiponectin and resistin are the only three molecules that belong exclusively to the class of adipokines and they have been studied in a limited number of researches concerning autism. Increased levels of leptin, decreased levels of resistin and a negative correlation between the levels of adiponectin and the severity of social impairment were found in the plasma of ASD subjects vs. controls (Rodrigues et al. 2014). Previously, Blardi et al. (Blardi et al. 2009, Blardi et al. 2010) found higher levels of leptin in patients with Rett syndrome in comparison with healthy female subjects, as reported by Ashwood et al. (Ashwood et al. 2008) in patients with autism compared to TD controls. Leptin dysregulation has been proposed as a mechanism of psychopathology associated with mental health

disorders (Valleau and Sullivan 2014), and elevated circulating leptin was consistently found in childhood neurodevelopmental disorders, including ASD (Ashwood et al. 2008).

Resistin has been implicated in the pathogenesis of several inflammatory CNS disorders (Bastard et al. 2006) and its levels are related to immune changes in autistic subjects: it has been shown that proinflammatory cytokines may increase the expression of messenger-RNA resistin (Kaser et al. 2003) with a positive correlation between increasing resistin levels and inflammatory serum cytokines (Nehus et al. 2012). A recent case-control study (Ghaffari et al. 2016) found that resistin levels were increased in ASD subjects compared to healthy controls. To date, no studies have investigated differences in adipokines' levels in ASD subjects with or without GI symptoms.

Distally regulated by some cytokines (i.e., IL-6, IL-1, and TNF-α), the plasminogen activator inhibitor-1 (PAI-1) has been hypothesized to directly influence brain functions causing a neuronal dis-connectivity due to abnormal neuronal migration. PAI-1 may regulate microglial migration and phagocytosis in an autocrine or paracrine manner playing an important role in the regulation of brain microglial activities in health and disease (Jeon et al. 2012). Moreover, his locus in human maps very close to or within a region in chromosome 7 linked to autism. No association was found between the presence of ASD and a particular polymorphism of the PAI-1 gene promoter that affects the PAI-1 plasma levels (Persico et al. 2001).

Therefore, the aims of this pilot study were:

1- To investigate the plasmatic levels of several proinflammatory molecules (TNF-α, IL-6, CCL2, leptin, resistin, and PAI-1) in preschoolers with ASD;
2- To explore the correlation between their plasmatic levels and behavioral profiles in preschoolers with ASD to detect possible specific subgroups within the ASD heterogeneity.

3.2.1. Materials and Methods

Instruments

The tools used in this study were: ADOS-2 (Lord C 2012), GMDS-ER (Griffiths 2006), VABS-II (Sparrow SS 2005.), CBCL 1.5-5 (Achenbach 2000), RBS-R (Bodfish 1999), SCQ (Rutter M 2003). The "*Overall Level* of *Non*-Echoed *Spoken Language*" item (A1 score) of the ADOS-2 was used to differentiate non-verbal (those with absent language or less than 5 words) from verbal children: 39 participants (46%) were verbal and 46 (54%) were non-verbal.

The demographic and clinical characteristics of all the participants and in no-verbal vs. verbal groups are reported in Table 3.2.1.

Table 3.2.1. Clinical characteristics of the total sample and in non-verbal vs. verbal group.

	Total Sample (*n* = 85; 100%)	**Non-Verbal (*n* = 46; 54%)**	**Verbal (*n* = 39; 46%)**	***p***	***p*, Age-adjusted**
AGE (years) mean ± SD	4.14 ± 1.08 (range 2.18–6.11)	3.74 ± 0.96	4.62 ± 1.02	<0.0001	-
MALES	71 (83.5%)	38 (44.7%)	33 (38.8%)	NS	-
FEMALES	14 (16.5%)	8 (9.4%)	6 (7.1%)		-
Weight (Kg)	17.70 ± 3.09	17.06 ± 3.1	18.56 ± 2.89	0.026	NS
BMI (Kg/m^2)	15.95 ± 1.66 (range 12.75–21.43)	16.07 ± 1.74	15.82 ± 1.54	NS	NS
Head Circumference (cm)	51.21 ± 1.69 (range 55–46)	51.31 ± 1.83	51.09 ± 1.54	NS	NS
ADOS-2 CSS Score (mean ± SD)					
Social Affect	6.43 ± 2.05	7.06 ± 1.73	5.74 ± 2.09	0.002	n.a.*
Restricted and Repetitive Behaviors	8.23 ± 1.46	8.56 ± 1.36	7.95 ± 1.50	NS	n.a.*
Total	7.05 ± 1.85	7.72 ± 1.50	6.41 ± 1.90	0.0007	n.a.*
GMDS-ER (mean ± SD)					
Performance Quotients	70.75 ± 23.33	61.47 ± 19.42	78.75 ± 23.73	0.0018	n.a.*
VABS-II (mean ± SD) Quotients					
Communication	50.86 ± 17.79	40.76 ± 10.24	63.69 ± 17.43	<0.0001	n.a.*
Daily Living	66.56 ± 17.07	60.46 ± 13.14	73.13 ± 18.16	0.0002	n.a.*
Socialization	63.55 ± 15.02	57.35 ± 10.36	71.15 ± 16.53	<0.0001	n.a.*
Motor Skills	71.88 ± 17.85	70.89 ± 17.64	75.46 ± 16.75	NS	n.a.*
Composite Score	59.40 ± 19.53	52.96 ± 17.52	67.23 ± 19.61	0.0007	n.a.*
CBCL 1.5-5 T-score (mean ± SD)					
Internalizing Problems	63.85 ± 9.06	64.98 ± 8.30	62.72 ± 9.64	NS	NS
Externalizing Problems	57.10 ± 9.09	56.71 ± 8.68	57.20 ± 9.55	NS	NS
Total Problems	62.28 ± 10.51	62.73 ± 10.68	61.69 ± 10.24	NS	NS
Sleep Problems	58.21 ± 9.11	59.62 ± 10.45	56.44 ± 6.83	NS	NS
Attention Problems	64.15 ± 8.21	64.66 ± 8.47	63.56 ± 7.98	NS	NS
Aggressive Behavior	56.58 ± 7.13	56.27 ± 5.93	56.95 ± 8.38	NS	NS
Attention Deficit/Hyperactivity Problems	59.31 ± 7.70	59.58 ± 7.51	59.00 ± 8.00	NS	NS
RBS-R (mean ± SD)					
Total Score	19.87 ± 13.87	17.67 ± 10.25	22.41 ± 16.91	NS	NS
Total Endorsed Score	12.76 ± 7.27	11.91 ± 5.88	13.74 ± 8.58	NS	NS
Low Index	9.44 ± 6.07	9.33 ± 5.67	9.56 ± 5.59	NS	NS
High Index	10.25 ± 9.91	8.09 ± 7.11	12.79 ± 12.04	0.028	0.028
SCQ (mean ± SD)					
Total Score	14.98 ± 5.90	16.72 ± 5.28	13.18 ± 6.16	0.006	NS

Abbreviations (alphabetic order): ADOS-2 Autism Diagnostic Observation Schedule-2; BMI Body Mass Index; CBCL 1.5-5 Child Behavior Checklist 1.5-5; CSS Calibrated Severity Score; GMDS-ER Griffiths Mental Development Scales-Extended Revised; n.a. not applicable; NS not significant; RBS-R Repetitive Behaviors Scale Revised; SCQ Social Communication Questionnaire; SD standard deviation; VABS-II Vineland Adaptive Behavior Scales-II.

Again, the modified version of the GSI (Schneider et al. 2006) was used to split the subjects into two groups (GI vs. NGI).

To detect possible specific subgroups within the ASD heterogeneity, all preschoolers were divided into regressive or non-regressive (early-onset -EO-ASD-) autism based on the presence/absence of a history of loss of competences such as language or social competences (Hansen et al. 2008); children belonging to regressive group were further divided in those with regression plus a previous developmental delay (Reg + DD) and those without a previous developmental delay (Reg − DD). According to Kern et al. (Kern et al. 2014), "regression plus developmental delay" was defined as a significant lag in the appearance of normal developmental milestones with a later loss of previously acquired skills.

Statistical Analysis

Descriptive statistics were computed for selected demographic variables across diagnostic groups. Contingency tables were used to perform the frequency analysis. Since the molecule's values were not normally distributed, we used log-transformed values with parametric statistic tests and non-parametric tests to compare GI vs. NGI subjects (Mann-Whitney test) and to compare EO ASD vs. Reg-DD vs. Reg + DD (Kruskall-Wallis test) for all the selected molecules.

Correlation and regression analysis were computed to study the relationship between the molecules and the identified clinical parameters. Findings with p value <0.05 were considered significant. StatView software (version 5.0.1; SAS Institute, Abacus Concept Inc., Berkeley, CA, USA) was used for data analyses.

To discriminate different subgroups of ASD children based on biomarker levels, we performed Principal Component Analysis (PCA) using as correlated variables: sex, BMI, age, and cytokine levels (TNF-α, IL6, CCL2, leptin, resistin and PAI 1). After log transformation and auto scaling (e.g., mean-centered and divided by standard deviation of each variable) PCA was performed using MetaboAnalystR 1.0.3 (Xia Lab, McGill University, Montreal, Canada). We checked quality control

of samples using PCA that allowed us to label the 85 samples as outlier so it was excluded from downstream analysis.

3.2.2. Results

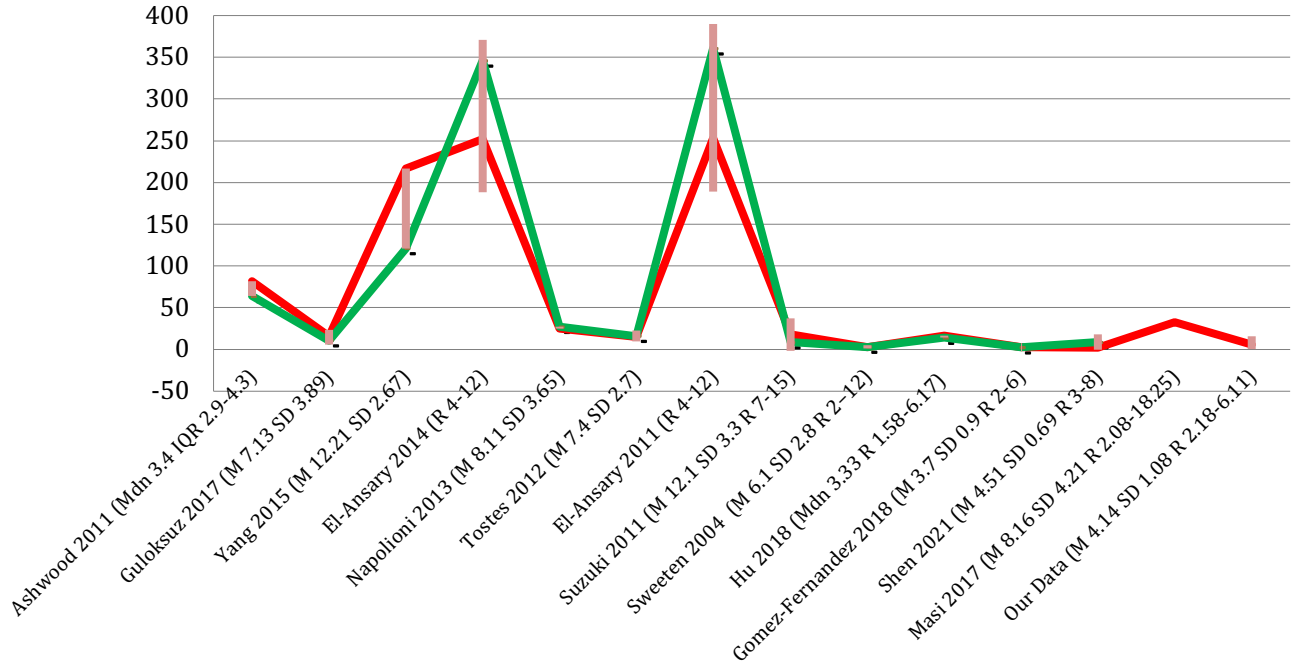
Thirty children (35%) were in the GI group and 55 (65%) in the NGI group. Among the 30 GI subjects, 20 children (67%) were in the non-verbal group, whereas among the 55 NGI, 26 children (47%) were in the non-verbal group. No statistically significant differences were found in the prevalence of GI subjects between verbal and non-verbal groups ($p = 0.086$). As concerns sex distribution, no differences were found in the prevalence of females in GI versus NGI groups neither verbal versus non-verbal groups ($p = 0.560$ and $p = 0.804$, respectively).

As concerns clinical variables, there were no significant differences between the GI and the NGI groups, with the exception of the Global Score of the RBS-R (60.24 ± 20.77 vs. 38.12 ± 27.06; $p = 0.0016$), the Internalizing and Total problem scores of the CBCL (all significantly higher in the GI group than in the NGI group: 67.48 ± 7.80 vs. 62.06 ± 9.04, $p = 0.0065$ and 65.35 ± 10.02 vs. 60.62 ± 10.30, $p = 0.0469$, respectively), and of the Communication and Daily Living adaptive scores of the VABS (significantly higher in the NGI group than in the GI group: 45.47 ± 15.22 vs. 54.46 ± 18.80 $p = 0.0274$ and 61.13 ± 14.29 vs. 69.07 ± 17.51 $p = 0.0365$, respectively).

As concerns proinflammatory cytokines, the mean level of TNF-α was 6.12 (SD 2.40) pg/mL, IL-6 was 5.99 (SD 16.17) pg/mL and CCL2 was 127.22 (SD 58.81).

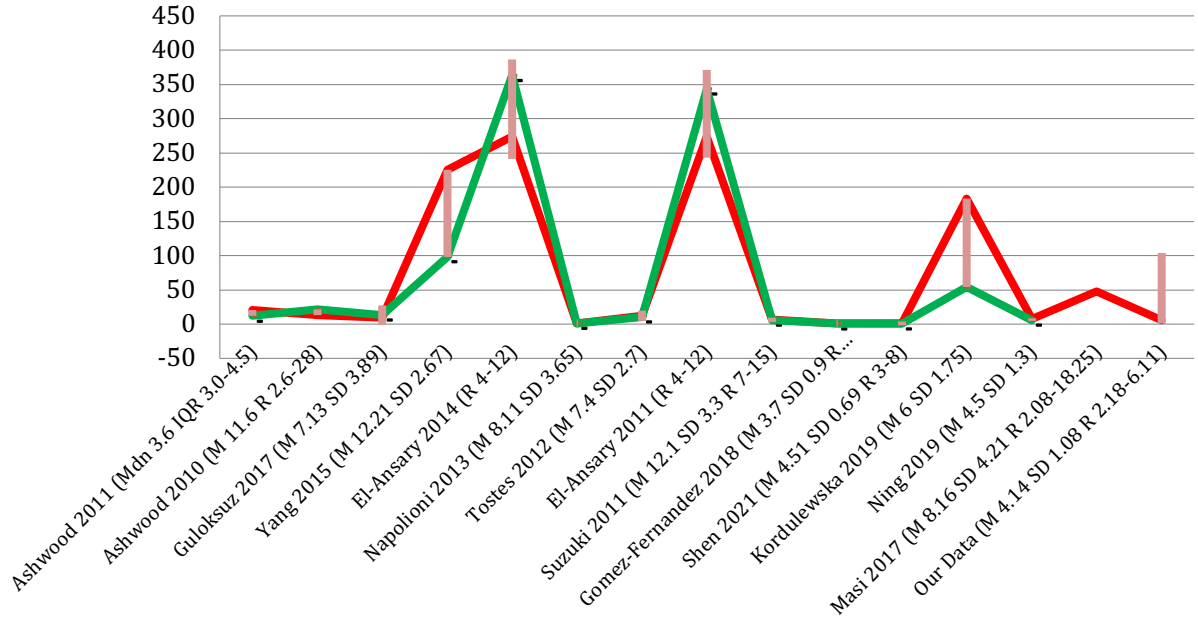
In order to compare the mean values of the plasmatic cytokines with those already published in similar researches, we examined the literature and we used, when available, the reported mean basal values of TNF-α, IL-6, and MCP-1 in studies about children with ASD with similar age, isolated or compared to TD children as shown in Figure 3.2.1a, 3.2.1b and 3.2.1c, respectively.

Figure 3.2.1a the mean values of TNF-α in the ASD (in dark red) and TD (in green) populations with the minimum and maximum values (in light red) in the studies published to date. The age of the examined ASD population is shown in brackets.



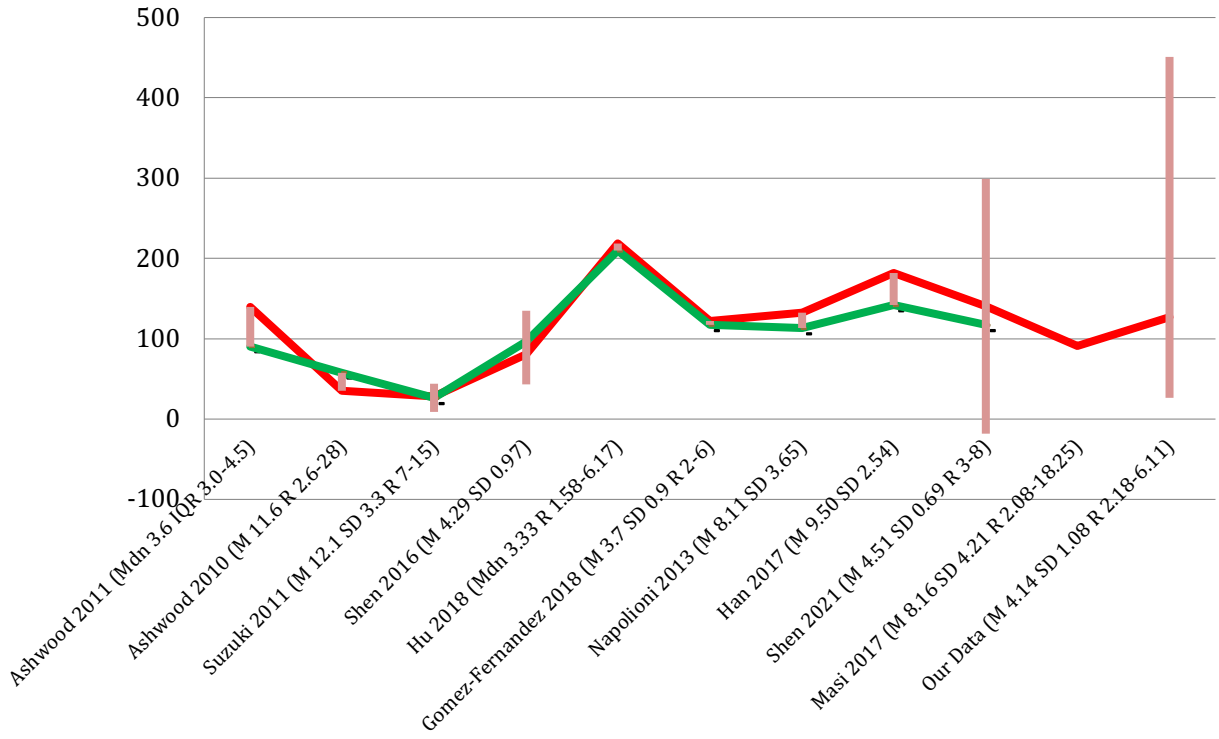
Abbreviations: M mean; Mdn Median; IQR Interquartile Range SD Standard Deviations R range

Figure 3.2.1b the mean values of IL-6 in the ASD (in dark red) and TD (in green) populations with the minimum and maximum values (in light red) in the studies published to date. The age of the examined ASD population is shown in brackets.



Abbreviations: M mean; Mdn Median; IQR Interquartile Range SD Standard Deviations R range

Figure 3.2.1c the mean values of MCP-1 in the ASD (in dark red) and TD (in green) populations with the minimum and maximum values (in light red) in the studies published to date. The age of the examined ASD population is shown in brackets.



Abbreviations: M mean; Mdn Median; IQR Interquartile Range SD Standard Deviations R range

As shown in Table 3.2.2, we did not find significant differences in the levels of plasmatic cytokines between GI and NGI group except for resistin levels ($p = 0.032$). No difference in plasma biomarker levels was found between non-verbal and verbal groups.

Regarding the onset of autism, the mean values of cytokines were not statistically significantly different between EO-ASD and regressive subgroups. Nevertheless, comparing cytokines levels in the EO-ASD subgroup with the two types of regressive preschoolers (with and without DD), resistin and PAI-1 levels were statistically significantly higher in the Reg + DD group than in the other two groups, the EO-ASD and the Reg-DD ones ($p < 0.01$ for all).

Table 3.2.2 Comparisons between the cytokine levels in GI vs. NGI groups, in EO ASD (a) vs. Reg-DD (b) vs. Reg+DD (c) subgroups and No-Verbal vs. Verbal groups. The mean levels of each cytokine in the total sample are also reported.

					a	b	c				
	Total Sample	**NGI 55 Subjects**	**GI 30 Subjects**	***p*** **(NGI vs. GI)**	**EO ASD**	**Reg – DD**	**Reg + DD**	**ANOVA *p* Value**	**NO VERBAL 46 Subjects**	**VERBAL 39 Subjects**	***p*** **(No-V vs. V)**
N (%)	85 (100)	55 (64.7%)	30 (35.3%)		57 (67.0)	14 (16.5)	14 (16.5)				
TNF-α, m (SD) pg/mL range 0.74–16.09	6.12 (2.40)	5.84 (2.01)	6.63 (2.95)	ns	6.09 (3.16)	6.76 (3.16)	5.56 (2.56)	ns	6.52 (2.57)	5.63 (2.50)	ns
IL-6, m (SD) pg/mL range 0.80–104.00	5.99 (16.17)	4.70 (13.83)	8.34 (19.80)	ns	5.74 (14.47)	10.82 (27.24)	2.18 (0.90)	ns	4.67 (6.96)	7.54 (22.69)	ns
CCL2, m (SD) pg/mL range 26.36–451.00	127.22 (58.81)	131.61 (66.86)	119.16 (39.90)	ns	126.85 (56.03)	135.10 (53.15)	120.84 (76.74)	ns	125.38 (53.73)	129.39 (64.95)	ns
Leptin, m (SD) pg/mL range 0.03–4.83	1.14 (0.89)	1.19 (0.96)	1.06 (0.76)	ns	1.26 (1.01)	0.96 (0.50)	0.88 (0.55)	ns	1.01 (0.80)	1.30 (0.97)	ns
Resistin, m (SD) ng/mL range 8.1–96.8	22.89 (13.63)	24.50 (14.37)	19.82 (11.74)	0.032	20.97 (10.45)	18.30 (9.68)	35.14 (20.75)	0.0003 (c > a) 0.0007 (c > b)	23.65 (15.78)	21.96 (10.60)	ns
PAI-1, m (SD) ng/mL range 5.5–91.2	26.04 (18.96)	27.52 (20.27)	23.24 (16.13)	ns	23.28 (12.74)	22.46 (14.04)	40.67 (33.68)	0.0018 (c > a) 0.0090 (c > b)	28.66 (22.86)	22.88 (12.32)	ns

Abbreviations (in alphabetic order): ASD: Autism Spectrum Disorder; CCL2: Macrophage Chemoattractant Protein-1; EO ASD: early onset of ASD without a history of loss of competences; GI: gastrointestinal; IL-6: interleukin-6; ns: not significant; PAI 1: Plasminogen Activator Inhibitor-1; Reg – DD: regression without a previous developmental delay; Reg + DD: regression with a previous developmental delay; SD: standard deviation; TNF-α: Tumor Necrosis Factor-alpha.

Finally, after the correlation analysis between each molecule and all the clinical parameters, CCL2 levels negatively correlated with CBCL1.5-5 Internalizing and Total problems ($p = 0.0003$, $R = 0.383$ and $p = 0.013$, $R = -0.272$, respectively) and with RBS-R total scores ($p = 0.05$, $R = 0.21$), and positively correlated with VABS-II Motor Skills ($p = 0.019$, $R = 0.25$). TNF-α and PAI-1 levels negatively correlated with age ($p = 0.0005$, $R = -0.37$ and $p = 0.024$, $R = -0.25$, respectively); Leptin levels positively correlated with Body Mass Index ($p = 0.002$, $R = 0.34$) and negatively correlated with CBCL1.5-5 Internalizing problems ($p = 0.0086$, $R = -0.29$).

PCA analysis showed that the variability within the components explains the subdivision in clusters (NGI vs. GI and EO-ASD vs. Reg − DD vs. Reg + DD) with a low percentage (PC1 = 21.3% and PC2 = 19.0%), indicating that the two and three groups respectively are not partially separated but overlapped (Figure 3.2.2).

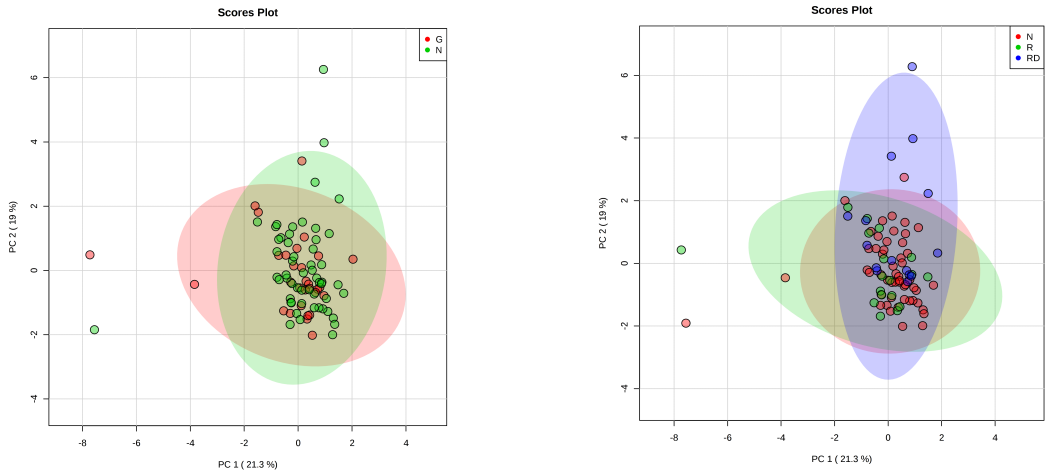


Figure 3.2.2 In the left plot, the Principal Component Analysis in gastrointestinal (red) and non-gastrointestinal subjects (green) is presented; in the right plot the PCA based on the ASD onset is presented: subjects with early-onset in red, regression without a previous developmental delay in green, regression plus a previous developmental delay in blue.

3.2.3. Discussion

Our study fits within the complexity and the heterogeneity of studies that examine inflammation and immunity dysfunctions in ASD subjects, moving the field forward into the investigation of biological biomarkers to discriminate possible endophenotypes. The narrow age range considered, the detailed

clinical characterization with specific and gold-standard tools for ASD evaluation, and an enough large sample represent the strengths of the study.

First, we found that the single and the mean values of our cytokines were lower than those expected in subjects with systemic inflammation (Monastero and Pentyala 2017, Zhu et al. 2017, Naqvi et al. 2019). These findings are in agreement with a part of the literature on this topic in which there is an absence of any atypical profile in the expression of relevant plasma cytokines both within ASD subjects and in comparison with TD children (Gomez-Fernandez et al. 2018). Regarding plasmatic cytokines, it should be highlighted that in literature the reference values and in particular those relating to the pediatric age, to date, are not definitively characterized. Despite our attempt to define specific subgroups based on cytokines levels and anthropometric measures using PCA, in our sample different endophenotypes were not identified. These results exclude the possibility that bringing all cases together in a single ASD group could have hidden significant results in one specific subgroup of preschoolers, as previously hypothesized (Careaga et al. 2017, Guloksuz et al. 2017). Consequently, our findings do not support the use of anti-inflammatory therapies in ASD children, not even in a specific subgroup of ASD subjects as previously suggested (Careaga et al. 2010).

Second, we did not observe significant differences in the levels of circulating cytokines between GI and NGI ASD children, except for resistin. Notably, there is too scant relevant research on this topic in ASD subjects (Rodrigues et al. 2014, Ghaffari et al. 2016) to draw valid and accurate conclusions. Thus, the role of adipokines needs further studies, in particular, in correlation with GI symptomatology in ASD considering also the influence of fat mass in plasmatic levels of adipokines. These findings suggest that the frequently reported GI symptoms in ASD children seem to be independent from an inflammatory condition, confirming a not yet clarified meaning of these symptoms (Kang et al. 2014). Previously, only a modest relationship between GI symptoms and TNF-α levels was detected (Ashwood et al. 2011, Ferguson et al. 2016), in one case (Ferguson et al. 2016) in significantly older subjects (school-aged children and adolescents) than ours. Specifically, when Ferguson et al. (Ferguson et al. 2016) considered only inferior GI symptoms (as we did) they did not

identify any statistically significant correlations, in line with the findings that TNF-α levels are independent from the presence of GI symptoms (Jyonouchi et al. 2005, Rose et al. 2018). Some authors (Furlano et al. 2001, Torrente et al. 2002, Ashwood et al. 2004, Ashwood and Wakefield 2006) have measured the presence of cytokine-producing cells directly in the bowel of subjects with ASD, and found a local high level of these cells in patients with GI symptoms, supporting a local role of the inflammatory cytokines in altering intestinal epithelial barrier and thus in contributing to GI symptoms. Besides, we confirm our previous findings showing that ASD subjects with GI problems have worse clinical functioning than ASD subjects without GI problems, independently from the severity of autistic symptoms (Prosperi et al. 2017).

We did not find any significant correlations between the basal levels of TNF-α and IL-6 and the autistic features of the total sample, similarly to some investigations (Guloksuz et al. 2017, Masi et al. 2017) and in contrast to others (Enstrom et al. 2010, Ashwood et al. 2011, Ferguson et al. 2016, Xie et al. 2017). Moreover, we found a positive, though weak, correlation between CCL2 and better functioning of children, evaluated with the CBCL1.5-5, RBS-R and VABS-II, in contrast with studies reporting a significant correlation between higher CCL2 plasmatic levels and more severe impairment of the autistic condition (Ashwood et al. 2011, Careaga et al. 2017). Further studies are necessary to disentangle the controversial findings on the possible role of some cytokines as sensible markers of the impairment in ASD children.

Third, we found that the group with regression plus developmental delay prior to the onset of ASD (16.5% of the sample) was significantly different from the rest of the sample as far as the higher plasmatic levels of resistin and PAI-1. We could suggest that Reg + DD children represent a specific subgroup with a definite biological profile and a specific clinical feature. However, using the PCA method, we did not identify the Reg + DD group as a particular cluster of patients, making the individuation of a specific endophenotype unlikely in this sample. Future studies are needed to retest the robustness of these findings before we can consider them as reliable.

In addition, we did not identify any significant correlation between the levels of cytokines and the presence or absence of regression of skills prior to the onset of autism. This result is in accordance with the majority of similar investigations, but in contrast with others where an association, although weak, between regressive autism and TNF-α (Gladysz et al. 2018), or lower plasma leptin levels (Ashwood et al. 2008) was found. Previous studies detected higher basal plasmatic levels of IL-1β (Ashwood et al. 2011, Napolioni et al. 2013), IL-5 (Napolioni et al. 2013), IL-17 (Napolioni et al. 2013) and higher levels of neural cell adhesion molecule (NCAM) (Gomez-Fernandez et al. 2018)—a molecule playing a role in cell–cell adhesion, neurite outgrowth, synaptic plasticity, learning and memory—in subjects with a regression of skills prior to the onset of autism. More broadly, ASD subjects with regression have been repeatedly identified as different in pathophysiological findings from ASD subjects without regression both in terms of neuroanatomy (Nordahl et al. 2011), and EEG patterns (Valvo et al. 2016). However, there is an urgent need to study the clinical regression in ASD, since a clear understanding of the definition, prevalence, etiopathogenesis, age of onset, and outcome profiles of this complex phenomenon is far from being concluded (Boterberg et al. 2019, Zhang et al. 2019).

We must consider this study as a pilot investigation with several limitations. Compared to other authors who have shown an inflammatory profile conducting proteomic studies in plasma (Abraham et al. 2019) or have measured pro-inflammatory cytokines panels in ASD subjects (Rose et al. 2018), we focused our analysis on six cytokines, limiting the possible range of our results. The changes in the expression of cytokines due to subjects' age (Hartel et al. 2005) have already been described, and we cannot exclude that our results on inflammatory markers could be age-specific; in addition, we have to consider that sex, sleep-wake cycle and the percentage of fat mass, which could increase that variability (Masi et al. 2015, Mantovani et al. 2016) representing possible interfering factors, have not been assessed in this study. Moreover, the low number of females within our sample of preschoolers with ASD did not allow us to accurately investigate possible sex differences in pro-inflammatory cytokine profiles.

In conclusion, despite the above-mentioned limitations and the existing controversies within the studies about the role of cytokines in ASD and the extreme variability of their findings, our study finds no evidence of the presence of inflammatory condition in ASD subjects, except for resistin. Our findings do not support the use of anti-inflammatory therapies in ASD children, and paves the way for the search of alternative hypotheses for the etiology of GI symptoms in subjects with ASD. Although our findings showed a specific plasmatic cytokine profile in ASD children with a history of a regressive way of onset within a previous developmental delay, the specific endophenotype for these subjects has not been identified.

These findings were published in Brain Sciences (Prosperi et al. 2019).

3.3. Chapter 3. Do children with ASD and GI problems have a particular intestinal microbiota and fecal metabolome?

Some microbiota perturbations have been consistently found in ASD subjects, suggesting the possible role of the gut microbiota as a contributing factor in the etiopathogenesis of ASD (Coretti et al. 2018). Several studies indicated that decreases of *Bifidobacterium* spp. and increases of *Bacteroides* spp. in stool samples characterize the microbiota of ASD individuals (Ding et al. 2017). An increase in *Prevotella* spp., belonging to the same phylum *Bacteroidetes*, has also been frequently detected. Moreover, variations of different clostridial clusters, *Sutterella* and *Akkermansia* genera, gave contradictory results across studies. Some bacterial species have been found almost exclusively in the intestinal microbiota of autistic patients, such as *Alkaliflexus* (Finegold et al. 2010) and *Sutterella* (Williams et al. 2012); other bacterial types were found only in healthy subjects, such as *Weissella* (Finegold et al. 2010). Interestingly, a recent theory concerning the pathogenesis of autism gives a specific etiopathogenetic role to bacteria such as *Clostridia*, *Desulfovibrio* and *Bifidobacterium* (Heberling et al. 2013).

The advent of techniques for the bulk measurement of genome, transcriptome, proteome, and metabolome has offered means to study microbial community's overall traits. In this framework, the

metabolome is considered the most convenient representation because minimal perturbations of the microbial profile may strongly impact molecules' concentration through cascade phenomena and pleiotropic effects (Laghi et al. 2014). For this reason, fecal metabolome has often been considered as a link between microbiota and host, especially concerning GI symptoms (Ursell et al. 2014, Tursi et al. 2016). Moreover, the concentration of some molecules in feces has been found to differentiate ASD subjects and their typical counterparts. For instance, Kang and colleagues (Kang et al. 2018) found that isopropanol and p-cresol concentrations were significantly higher in the feces of children with ASD than in controls, while gamma-aminobutyric acid showed an opposite trend. High concentrations of glutamate (De Angelis et al. 2013) and short-chain fatty acids (Wang et al. 2012) have also been detected in ASD children.

Interestingly, the totality of the studies focusing on the fecal metabolome features in ASD has investigated the differences between subjects with and without this disorder while ignoring potential correlations between metabolome and ASD severity.

The present work aimed to identify possible correlations between water-soluble fecal metabolome, fecal microbiota, calprotectin levels, and ASD severity in a group of ASD preschoolers with or without GI symptoms.

3.3.1. Materials and Methods

Subjects

A total of 80 ASD preschoolers were considered for this part of the study. The clinical data and the fecal samples were collected at baseline.

Procedure and clinical assessment

The participants were divided into three subgroups characterized by low, moderate, and high ADOS-CSS scores, according to validated cut-offs for these three categories (low severity 1–4; moderate severity 5–7; high severity 8–10).

Characteristics of the recruited subjects in the whole sample and in the three severity subgroups are reported in Table 3.3.1.

Table 3.3.1 Characteristics of the subjects in the whole sample and in the three severity subgroups.

	Whole Sample	Low-ADOS	Moderate-ADOS	High-ADOS
n	80	6	42	32
NGI/GI	52/28	4/2	29/13	19/13
Females/Males	14/66	2/4	9/33	3/29
Age (years)	4.14 ± 1.01	3.60 ± 1.01	4.29 ± 1.18	4.05 ± 0.97
BMI (Kg/m^2)	16.00 ± 1.66	15.21 ± 1.61	15.81 ± 1.61	16.40 ± 1.69

Abbreviations: NGI, Children without gastrointestinal symptoms; GI, Children with gastrointestinal symptoms; BMI, Body Mass Index.

Metabolomics Analysis by ^{1}H-NMR: fecal metabolome was characterized and quantified by ^{1}H-NMR, an analytical platform that grants a high reproducibility and requires minimal sample preparation.

Microbiota Analysis: DNA was extracted from fecal samples stored at −80 °C by QIAamp PowerFecal DNA Kit (Qiagen, Hilden, Germany). According to the manufacturer's instructions, each sample was homogenized in a 2-mL bead beating tube containing garnet beads. DNA was eluted with Solution C6 and stored at −20 °C for further real-time qPCR analysis. The quantification of total bacteria was performed using a universal primer set specific for 16S rDNA of domain bacteria and conditions, as reported elsewhere (Nadkarni et al. 2002).

Calprotectin Analysis: According to the manufacturer's instructions (BÜHLMANN fCAL® ELISA, Buhlmann, Switzerland), fecal calprotectin levels were determined by means of a commercially available Enzyme-Linked Immunosorbent Assay (ELISA).

Statistical analysis

Statistical analysis was conducted in an R computational language (Team 2019), while the artwork was refined by GIMP (version 2.10, www.gimp.org (accessed on 23 September 2021)). Comparisons were performed by *t*-test or two-way ANOVA with interaction on data checked for normality and normalized when needed according to Box and Cox (Box and Cox 1964). Correlations were investigated according to Spearman. A *p*-value of 0.05 was accepted as a limit for significance.

Trends underlying groups of molecules were highlighted by robust principal component analysis (rPCA) models (Hubert et al. 2005), by employing the PcaHubert algorithm implemented in the rrcov package. In the first stage, the algorithm detects outlying samples according to their distance from the others along and orthogonally to the PCA plane. In the second stage, the optimal number of principal components (PCs) are determined. A score plot and a correlation plot summarize the main features of a rPCA model. The former represents the samples in the PC space, therefore evidencing the overall structure of the data. The latter report the correlations between the concentration of each variable and the model's components, thus showing which molecule mostly determines the structure of the data.

Metabolic pathways over-representation analysis was performed by Fisher exact test, by employing Reactome (https://reactome.org (accessed on 23 September 2021)) as a pathways' database.

3.3.2. Results

3.3.2.1. Fecal Metabolome

Links with gastrointestinal disease. Fecal metabolome analysis allowed the identification and quantification of 59 molecules. To evaluate the metabolome's features related to GI symptoms, we applied a t-test on each molecule's concentration and found that twelve of them significantly differed between children with and without GI symptoms (GI and NGI children, respectively; Table 3.3.2.1a).

Low vs. high autism severity autism diagnostic observation schedule (ADOS) scores. To evaluate the metabolome's features potentially related to autism independently from the presence of GI

symptoms, we focused on children classified as low-ADOS and high-ADOS, and we applied a two-way ANOVA (GI symptoms—ADOS) on each molecule. A preliminary investigation by Spearman correlation allowed us to observe that the specific samples considering ADOS symptoms were independent of GI symptomatology, measured as a GI score ($p = 0.60$, $r = 0.06$). We found twelve molecules that were significantly affected by ADOS (Table 3.3.2.1b).

Table 3.3.2.1 Concentration (mmol/L; median (IQR)) in feces of the molecules significantly different between children (a) without (NGI) and with gastrointestinal symptoms (GI); (b) with high and low Autism Diagnostic Observation Schedule 2 (ADOS 2) scores. Legend: ↑higher; ↓lower.

1a.

	NGI	GI	*p*-value	GI vs. NGI
Acetate	6.32×10^{-2} (4.06×10^{-2})	7.58×10^{-2} (4.99×10^{-2})	0.042	↑
Alanine	6.12×10^{-3} (2.10×10^{-3})	4.80×10^{-3} (1.62×10^{-3})	0.012	↓
Ethanol	4.62×10^{-4} (8.20×10^{-4})	5.78×10^{-4} (9.06×10^{-4})	0.048	↓
Formate	1.20×10^{-4} (5.59×10^{-5})	1.42×10^{-4} (9.45×10^{-5})	0.006	↑
Isoleucine	1.68×10^{-3} (8.23×10^{-4})	1.43×10^{-3} (5.31×10^{-4})	0.039	↓
Leucine	4.34×10^{-3} (2.31×10^{-3})	3.61×10^{-3} (1.16×10^{-3})	0.034	↓
Methionine	9.03×10^{-4} (3.75×10^{-4})	7.87×10^{-4} (3.02×10^{-4})	0.014	↓
Orotate	5.89×10^{-5} (4.18×10^{-5})	7.70×10^{-5} (6.42×10^{-5})	0.015	↑
Phenylalanine	1.34×10^{-3} (6.20×10^{-4})	1.19×10^{-3} (4.83×10^{-4})	0.037	↓
Propionate	1.82×10^{-2} (1.20×10^{-2})	2.23×10^{-2} (1.30×10^{-2})	0.035	↑
Tyrosine	2.76×10^{-3} (1.16×10^{-3})	2.47×10^{-3} (1.02×10^{-3})	0.048	↓
Uridine	4.55×10^{-5} (3.82×10^{-5})	6.51×10^{-5} (5.33×10^{-5})	0.003	↑

1b.

	Low-ADOS	High-ADOS	*p*-value Low vs. High-ADOS
1,3-Dihydroxyacetone	8.08×10^{-5} (6.53×10^{-5})	1.67×10^{-4} (1.65×10^{-4})	0.037
Acetate	4.17×10^{-2} (1.14×10^{-2})	7.87×10^{-2} (3.72×10^{-2})	0.011
Aspartate	2.16×10^{-3} (4.78×10^{-4})	1.18×10^{-3} (6.75×10^{-4})	1.78×10^{-4}
Ethanol	1.97×10^{-4} (2.91×10^{-4})	1.02×10^{-3} (1.10×10^{-3})	0.007
Fucose	7.40×10^{-5} (3.47×10^{-5})	1.37×10^{-4} (8.47×10^{-5})	0.021
Isoleucine	2.15×10^{-3} (4.07×10^{-4})	1.34×10^{-3} (7.11×10^{-4})	0.006
Leucine	5.40×10^{-3} (9.46×10^{-4})	3.39×10^{-3} (2.04×10^{-3})	0.007
Methionine	1.19×10^{-3} (2.78×10^{-4})	8.04×10^{-4} (3.35×10^{-4})	0.004
N-Methylhydantoin	1.89×10^{-5} (8.73×10^{-6})	3.93×10^{-5} (4.35×10^{-5})	0.026
Orotate	3.12×10^{-5} (1.55×10^{-5})	6.34×10^{-5} (6.89×10^{-5})	0.011
Phenylalanine	1.57×10^{-3} (2.81×10^{-4})	1.16×10^{-3} (6.09×10^{-4})	0.005
Tyrosine	3.24×10^{-3} (6.49×10^{-4})	2.42×10^{-3} (1.13×10^{-3})	0.011

To observe the overall distribution of the samples from low-ADOS and high-ADOS children in the space constituted by this reduced group of metabolites, a rPCA model calculation distinguishing GI and NGI children was performed (Figure 3.3.2.1A). Moreover, a Boxplot representation summarizing the position of these groups along with PC 1 was done (Figure 3.3.2.1B), and a correlation plot

reporting the correlation between each substance's importance over PC 1 and its concentration (Figure 3.3.2.1C).

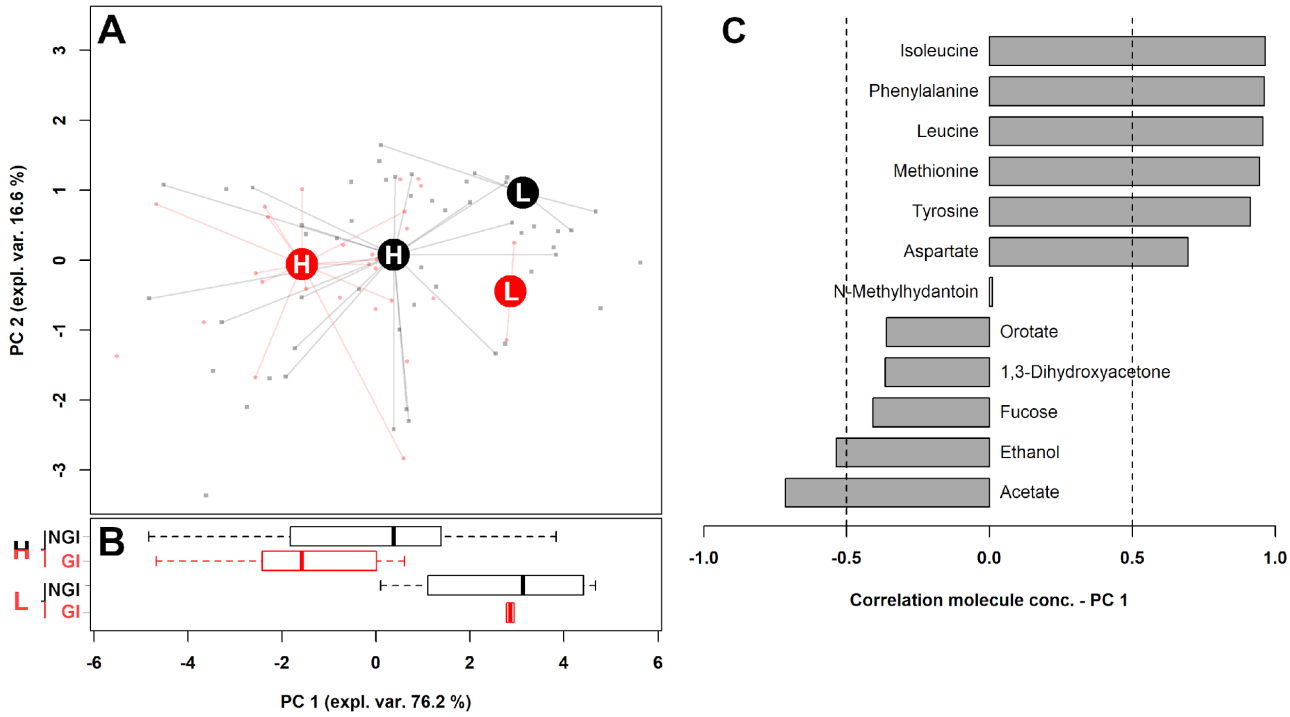


Figure 3.3.2.1 rPCA model calculated on fecal samples metabolomics of molecules reported in Table 3.3.2.1b distinguishing children within low-ADOS (L) and high-ADOS (H) groups. (A) The score plot reports the samples joined with lines to their median values, represented with big filled circles. Children without (NGI) and with gastrointestinal symptoms (GI)are evidenced with black or red colors, respectively. (B) Boxplot summarizing the position of the groups along with PC 1. (C) Correlation plot reporting the correlation between each substance's importance over PC 1 and its concentration. Gray bars highlight significant correlations ($p < 0.05$).

Three principal components (PCs) were included (described in detail in Table 3.3.2.S1), the first of which accounted for 76.2% of the samples' variance overall represented by the model.

Along with this PC, the samples appeared as distributed according to ADOS, with low-ADOS and high-ADOS children appearing at positive and negative PC 1 scores, respectively. To check the authenticity of the differences highlighted between high and low ADOS subjects, patients with intermediate ADOS were employed as a test set, by excluding them from any calculation and by predicting them through their projection over the PCA space based on the other subjects.

Moderate autism severity. The significantly different molecules between low-ADOS and high-ADOS children were also quantified in the feces of moderate-ADOS children (Table 3.3.2.2), which were then projected over the rPCA space calculated before (Figure 3.3.2.2).

Both moderate-ADOS + NGI and moderate-ADOS + GI children appeared as characterized by PC 1 scores intermediate to the corresponding low-ADOS and high-ADOS. In addition, the presence of GI symptoms separated NGI and GI children by 17.39%, a value that is intermediate between the values of 6.34% and 41.31% observed above.

Table 3.3.2.2 Concentration (mmol/L; median (IQR)) in the samples from moderate-ADOS children of the molecules significantly different between samples from high-ADOS and low-ADOS children.

	NGI	GI	*p* value
1,3-Dihydroxyacetone	1.50×10^{-4} (1.51×10^{-4})	2.59×10^{-4} (1.90×10^{-4})	NS
Acetate	6.52×10^{-2} (3.96×10^{-2})	5.49×10^{-2} (2.03×10^{-2})	NS
Aspartate	1.26×10^{-3} (6.79×10^{-4})	1.22×10^{-3} (6.84×10^{-4})	NS
Ethanol	3.59×10^{-4} (3.45×10^{-4})	5.42×10^{-4} (9.63×10^{-4})	NS
Fucose	1.01×10^{-4} (7.41×10^{-5})	1.15×10^{-4} (4.43×10^{-5})	NS
Isoleucine	1.68×10^{-3} (9.22×10^{-4})	1.55×10^{-3} (3.83×10^{-4})	NS
Leucine	4.40×10^{-3} (2.03×10^{-3})	3.94×10^{-3} (9.69×10^{-4})	NS
Methionine	9.26×10^{-4} (3.31×10^{-4})	8.07×10^{-4} (1.67×10^{-4})	NS
N-Methylhydantoin	3.15×10^{-5} (2.31×10^{-5})	3.97×10^{-5} (1.93×10^{-5})	NS
Orotate	6.04×10^{-5} (3.61×10^{-5})	7.25×10^{-5} (3.75×10^{-5})	NS
Phenylalanine	1.35×10^{-3} (7.01×10^{-4})	1.19×10^{-3} (1.97×10^{-4})	NS
Tyrosine	2.73×10^{-3} (1.23×10^{-3})	2.49×10^{-3} (4.87×10^{-4})	NS

Abbreviations: NGI, Children without gastrointestinal symptoms; GI, Children with gastrointestinal symptoms, NS, not significant

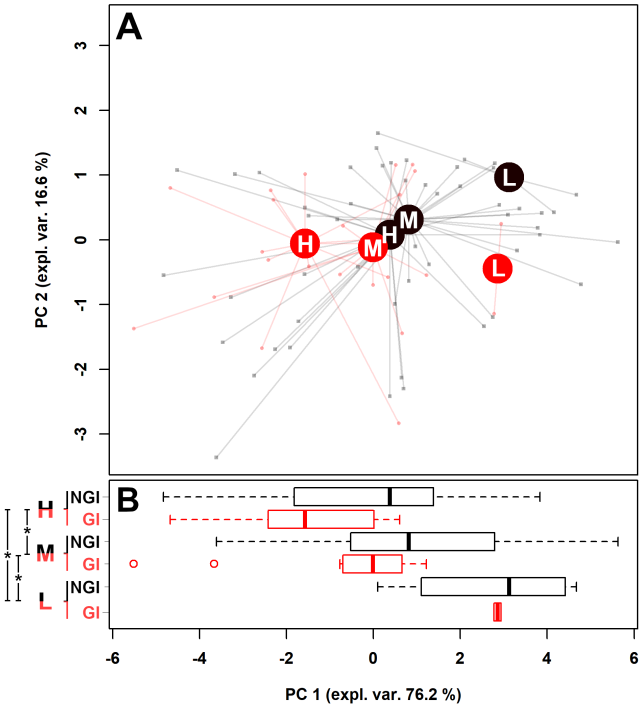


Figure 3.3.2.2 rPCA model calculated on the fecal metabolomics related with the molecules reported in Table 3.3.2.1b and adding the values relative to moderate-ADOS group (Table 3.3.2.2). (**A**) rPCA model of Figure 1 where moderate-ADOS children (M) were added within low-ADOS (L) and high-ADOS (H) groups. The score plot reports the samples joined with lines to their median values, represented with big, filled circles. Children without (NGI) and with gastrointestinal symptoms (GI) are evidenced with black or red colors, respectively. (**B**) Boxplot summarizing the position of the groups along PC 1. Asterisks denote significant ($p < 0.05$) pairwise differences. Children without (NGI) and with gastrointestinal symptoms (GI) are evidenced with black or red colors, respectively.

To obtain from the 12 molecules hints about the metabolic pathways most likely related to autism severity, we set up an overrepresentation analysis. Eight of them were found to be involved in the metabolism of proteins: 1,3-dihydroxyacetone, acetate, fucose, aspartate, isoleucine, leucine, phenylalanine, and tyrosine. Among them, the presence of five amino acids suggested that the alteration of the metabolism of proteins could be linked to their synthesis (Figure 3.3.2.3).

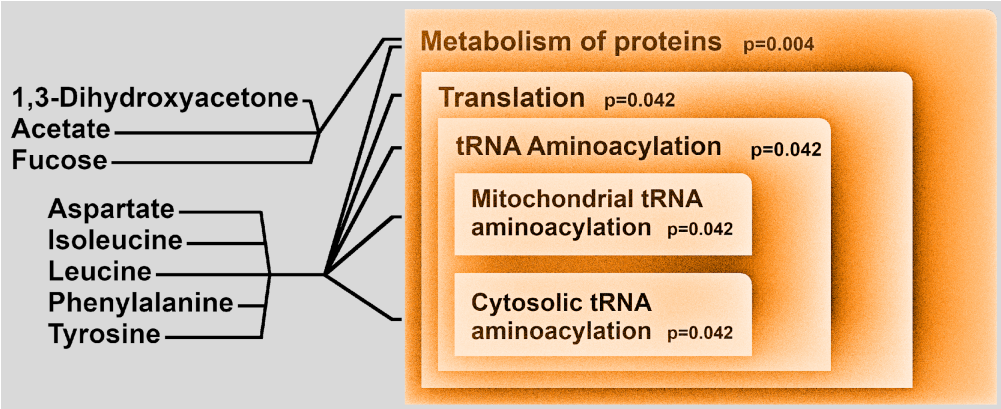


Figure 3.3.2.3. Metabolic pathways over-representation analysis based on the molecules significantly differed between low-ADOS and high-ADOS children. Orange colors are used for pathways significantly over-represented. Pathways are sub-leveled following Reactome's hierarchy.

Table 3.3.2.S1**:** Correlation coefficients describing the rotation of the PCA space reported in figure 3.3.2.1 and 3.3.2.2 with respect to the original variable's (molecules concentrations) system.

	PC 1	PC 2	PC 3
1,3-Dihydroxyacetone	-1.07×10^{-1}	-7.82×10^{-1}	4.52×10^{-1}
Acetate	-3.02×10^{-1}	-2.82×10^{-1}	-4.51×10^{-1}
Aspartate	3.25×10^{-1}	-1.17×10^{-1}	1.45×10^{-1}
Ethanol	-1.45×10^{-1}	-1.39×10^{-1}	3.86×10^{-1}
Fucose	-5.26×10^{-2}	-6.51×10^{-2}	2.91×10^{-2}
Isoleucine	3.90×10^{-1}	-1.14×10^{-1}	-5.93×10^{-2}
Leucine	4.03×10^{-1}	-1.04×10^{-1}	-6.99×10^{-2}
Methionine	4.08×10^{-1}	-9.83×10^{-2}	-7.20×10^{-2}
N-Methylhydantoin	-3.52×10^{-2}	-4.43×10^{-2}	1.98×10^{-1}
Orotate	-5.14×10^{-2}	-4.63×10^{-1}	-6.04×10^{-1}
Phenylalanine	3.85×10^{-1}	2.84×10^{-3}	-7.58×10^{-3}
Tyrosine	3.67×10^{-1}	-1.47×10^{-1}	-4.89×10^{-2}

3.3.2.2. Fecal Microbiota and Intestinal Inflammation

The absolute abundance of total bacteria, lactobacilli, bifidobacteria, *Akkermansia muciniphila*, *Bacteroides*, *Prevotella*, and *Sutterella* are reported in Table 3.3.2.S2, while Table 3.3.2.S3 reports the relative abundance of the same bacterial groups.

Table 3.3.2.S2**:** Copy numbers of selected bacteria in the feces of children.

	Total subjects	**NGI**	**GI**	**Low ADOS**		**Moderate ADOS**		**High ADOS**	
				NGI	**GI**	**NGI**	**GI**	**NGI**	**GI**
Total bacteria	10.25 (0.41)	10.21 (0.34)	10.37 (0.31)	10.21 (0.09)	10.38 (0.23)	10.26 (0.31)	10.21 (0.35)	10.19 (0.56)	10.43 (0.21)
Lactobacilli	4.92 (1.25)	4.88 (1.14)	4.94 (1.32)	5.10 (0.51)	5.28 (0.34)	4.72 (1.11)	4.90 (1.44)	4.86 (1.22)	4.99 (1.28)
A. muciniphila	6.79 (3.98)	6.99 (3.88)	6.14 (4.16)	6.89 (2.19)	8.13 (0.07)	7.25 (2.29)	5.70 (4.10)	5.81 (4.53)	6.00 (4.34)
Bifidobacteria	8.55 (0.53)	8.56 (0.50)	8.53 (0.62)	8.36 (0.97)	8.22 (0.14)	8.55 (0.40)	8.51 (0.36)	8.57 (0.52)	8.58 (0.79)
Bacteroides	9.09 (0.67)	9.05 (0.58)	9.33 (0.57)	9.19 (0.18)	8.87 (0.48)	8.97 (0.54)	9.23 (0.61)	9.04 (0.80)	9.45 (0.55)
Prevotella	4.06 (1.17)	4.11 (1.07)	3.91 (1.55)	3.85 (1.02)	5.78 (1.92)	4.15 (2.83)	3.86 (0.64)	4.04 (0.84)	4.00 (2.40)
Sutterella	6.69 (1.39)	6.67 (1.50)	6.93 (1.21)	6.68 (0.31)	5.01 (1.50)	6.57 (1.19)	6.22 (1.21)	6.67 (2.20)	7.37 (0.47)

Values reported represent the median (IQR) of log10 concentration/g of feces

Table 3.3.2.S3**:** Relative abundance of selected bacteria in the feces of children.

	Total subjects	**NGI***	**GI***	**Low ADOS**		**Moderate ADOS**		**High ADOS**	
				NGI	**GI**	**NGI**	**GI**	**NGI**	**GI**
Lactobacilli	-5.32 (1.34)	-5.38 (1.14)	-5.20 (1.50)	-5.10 (0.34)	-5.10 (0.12)	-5.53 (1.28)	-5.25 (1.97)	-5.37 (1.01)	-5.18 (1.37)
A. muciniphila	-3.52 (4.01)	-3.49 (3.62)	-4.00 (4.33)	-3.37 (2.29)	-2.25 (0.30)	-3.00 (2.58)	-4.16 (4.04)	-3.81 (4.24)	-4.63 (4.53)
Bifidobacteria	-1.75 (0.35)	**-1.70 (0.37)**	**-1.86 (0.39)**	-1.84 (0.79)	-2.16 (0.37)	-1.70 (0.35)	-1.79 (0.32)	-1.64 (0.40)	-1.96 (0.64)
Bacteroides	-1.15 (0.49)	-1.20 (0.46)	-1.01 (0.50)	-1.02 (0.21)	-1.52 (0.26)	-1.23 (0.44)	-0.98 (0.42)	-1.08 (0.50)	-1.01 (0.48)
Prevotella	-6.12 (1.13)	-6.06 (1.14)	-6.24 (1.37)	-6.32 (1.15)	-4.61 (1.70)	-5.97 (2.85)	-6.36 (0.84)	-6.17 (0.97)	-6.23 (2.26)
Sutterella	-3.53 (1.04)	-3.53 (1.04)	-3.48 (0.97)	-3.49 (0.44)	-5.38 (1.27)	-3.52 (1.11)	-3.82 (1.14)	-3.74 (1.83)	-3.13 (0.66)

Values reported represent the median (IQR) of the difference $\log_{10}$(concentration of targeted bacteria) - $\log_{10}$(concentration of total bacteria)
*Significant differences ($p < 0.05$) between NGI and GI are reported in bold font

These microorganisms were chosen because several publications reported significant changes in their fecal levels in autistic subjects compared to healthy individuals (Ding et al. 2017). The amount of total bacteria was similar between NGI and GI subjects, while the relative abundance of bifidobacteria was significantly lower in GI than in NGI children ($p = 0.032$). Moreover, although not significant, the relative abundance of *Prevotella* and *Sutterella* was higher in GI subjects, while the relative abundance of *A. muciniphila* was lower in the same subjects.

Intestinal inflammation in GI and NGI children was evaluated by fecal calprotectin. The median concentration of fecal calprotectin was not significantly different between NGI (79.27 μg/g, with an IQR of 131.15) and GI groups (69.50 μg/g, with an IQR of 131.21). Besides, no significant difference was observed between NGI and GI patients in the amount of calprotectin even when age-based stratification of children was considered.

In adults and children over 4 years old, values of fecal calprotectin below 50 μg/mg are generally viewed as normal. Intermediate levels (in the 50 and 200 μg/mg range) are considered to indicate low-grade intestinal inflammation, while values above 200 μg/mg are viewed as associated with pathology above (Bjarnason 2017). The relative amount of *Akkermansia muciniphila* showed a negative correlation ($p = 0.041$, $r = -0.32$) with intermediate fecal calprotectin levels (50–200 μg/g). This correlation was stronger ($p = 0.0002$, $r = -0.49$) when all values of calprotectin higher than 50 μg/g were considered. On the contrary, *Prevotella* was directly correlated with levels of calprotectin higher than 200 μg/g ($p = 0.0030$, $r = 0.75$).

Correlation among microorganisms. As reported in Table 3.3.2.S4, a positive linear correlation between the relative abundance of lactobacilli and bifidobacteria was observed in the entire study group ($p = 0.0008$, $r = 0.38$).

Table 3.3.2.S4**:** Correlation among relative abundance of bacteria in the feces of children.

	Sutterella	*Prevotella*	*Bacteroides*	Bifidobacteria	*A. muciniphila*
Lactobacilli	-0.36	0.38	-	0.38	-
A. muciniphila	-	-	-0.33	-	
Bifidobacteria	-0.37	-	-0.35		
Bacteroides	0.54	-			
Prevotella	-				

Positive correlations were also observed between lactobacilli and *Prevotella* and between *Bacteroides* and *Sutterella*. Negative correlations were observed across the entire study group for bifidobacteria with *Bacteroides*, for lactobacilli with *Sutterella*, and for *Akkermansia* with *Bacteroides*. Finally, a negative correlation of bifidobacteria with *Sutterella* was consistently present in the entire study group and in NGI and GI groups observed separately.

Low vs. High ADOS. We first focused on children of the low-ADOS and high-ADOS groups. A two-way ANOVA (ADOS—GI) showed that ADOS was not significantly associated with any bacterial groups. Focusing on GI problems, *Sutterella* showed a relative abundance significantly higher in GI subjects ($p = 0.036$), while *Prevotella* showed an opposite trend ($p = 0.056$).

We searched for microorganisms that could have determined the trends of the samples highlighted by the rPCA model of Figure 3.3.2.1. We found that the relative abundance of bifidobacteria ($r = 0.38$, $p = 0.021$) and *Sutterella* ($r = -0.48$, $p = 0.0029$) significantly correlated with samples' scores along PC 1.

Extension to the moderate ADOS group. When the observation was extended to include moderate group, no links between ADOS or GI disease and microorganisms' relative abundance could be highlighted. For completeness, the absence of correlation was confirmed when correlations were calculated between microorganism's abundance and ADOS score. In contrast, as highlighted in Table 3.3.2.3, when considering all the subjects studied, the significant correlation between bifidobacteria ($r = 0.46$) and *Sutterella* ($r = -0.35$) relative abundance and PC 1 was confirmed, and one with lactobacilli could be noticed ($r = 0.27$, $p = 1.96 \times 10^{-2}$).

Table 3.3.2.3 Key correlations in all the subjects studied among microbiology, calprotectin, and metabolomics data.

		Lact.	Akk.	Bifi.	Bact.	Prev.	Sutt.	Calpr.
Molecules altered only by GI	Formate	0.32	-	-	-	0.28	-	-
	Uridine	-	-	−0.26	0.30	-	-	−0.4
	Alanine	0.27	0.45	0.33	−0.46	-	−0.28	-
	Propionate	-	-	-	-	0.35	-	-
Molecules altered by GI and ADOS	Acetate	−0.23	-	−0.34	-	-	0.22	-
	Ethanol	−0.23	−0.28	-	-	-	0.28	-
	Isoleucine	0.37	-	0.51	−0.26	-	−0.38	-
	Leucine	0.41	0.27	0.54	−0.32	-	−0.41	-
	Methionine	-	0.23	0.33	-	-	-	-
	Orotate	-	-	−0.30	-	-	-	-
	Phenylalanine	0.32	-	0.46	-	-	−0.34	-
	Tyrosine	-	-	0.31	-	-	−0.32	-
Molecules altered only by ADOS	Aspartate	-	-	-	-	-	−0.44	-
	N-Methylhydantoin	-	-	-	-	−0.26	-	-
	1,3-Dihydroxyacetone	-	-	−0.23	-	-	-	-
	Fucose	-	-	-	0.26	-	0.31	−0.44
Metabolomics PC 1		**0.27**	**-**	**0.46**	**-**	**-**	**–0.35**	-

3.3.3. Discussion

This study aims to identify whether water-soluble fecal metabolome, microbiota, and calprotectin levels correlate with SA levels in a group of ASD preschoolers. The metabolomic analysis highlighted a close relationship between the ASD severity and the fecal metabolic profile. Specifically, the 12 molecules differentiating low-ADOS and high-ADOS children can pave the way for further progress into the possible role of fecal metabolomics as a biomarker in autism.

A previous investigation showed that the urine metabolome of young autistic children correlates with their clinical profile severity (Mussap et al. 2020). In this study, the six molecules significantly higher in the low-ADOS group than in the high-ADOS group were all amino acids, whereas none of the six molecules higher in the High-ADOS group was an amino acid. So, these metabolites seem to distinguish children with severely impaired behaviors from those with lower severity of autism symptoms. De Angelis et al. (De Angelis et al. 2015) have found that proteolytic bacteria (e.g.,

Clostridium and *Bacteroides*) hydrolyzed proteins and peptides, producing consistent amounts of free amino acids (FAA) detectable in the fecal samples of ASD children. In a previous study, the same authors (De Angelis et al. 2013) reported higher levels of aspartate, an excitatory neurotransmitter acting on *N*-methyl-D-aspartate (NMDA) receptors in the feces of children with ASD, compared to TD peers. Kang and colleagues (Kang et al. 2018) also observed relatively higher concentrations in their ASD group, which may reflect its potential contribution to ASD symptoms associated with *N*-methyl-D-aspartate (NMDA) receptor dysfunction. In fact, dysfunctional ionotropic NMDA receptors have been recently linked to multiple forms of ASD and emerging evidence showed that d-aspartate and d-serine are important neuromodulators of glutamatergic transmission (Burket and Deutsch 2019). These data suggest that targeting the NMDA receptor could have promising therapeutic potential in ASDs and experimental studies have been conducted with this aim (Crespi 2019).

The lack of other studies investigating the correlations between autism severity and amino acids concentrations prevents further speculation besides the significant correlation between fecal aspartate levels and ADOS.

Among the fecal non-amino acidic molecules, which significantly vary between children with high and low ASD severity, three molecules (fucose, 1,3-dihydroxyacetone, *N*-methylhydantoin) are of particular interest since they characterized the highest severity group, both with and without GI symptoms.

As far as we know, fucose's role has never been studied in subjects with ASD to date. Considering that synaptic plasticity, neurite outgrowth and neuron morphology are regulated by fucosylation and are responsible for several cognitive processes, including learning and memory, this molecule deserves further investigation (Schneider et al. 2017). The fucose is released in the colon by commensal intestinal bacteria (from some strains of Bifidobacteria) that cleave fucose residue from the chain of glycolipid and utilize it as the carbon source (Ke et al. 2020). Therefore, since various bacterial strains metabolized fucose, the different fucose concentrations in these fecal samples could be justified by a different gut microbial composition. Moreover, it has been shown that fucose has an

anti-inflammatory role against intestinal infections (Pickard et al. 2014), modulating the interaction between gut microbiota and bile acid in animal models (Ke et al. 2020).

Concerning dihydroxyacetone, it contributes to the oxidative phosphorylation pathway in the mitochondria to generate ATP. Therefore, the alteration of dihydroxyacetone levels in ASD, and in the High-ADOS group, in particular, could be ascribed to the hypothesis that mitochondrial dysfunction is associated in a subset of subjects with ASD (Rossignol and Frye 2012). The role of the *N*-methylhydantoin remains even more enigmatic and unexplored in autism; it is a bacterial metabolite, i.e., the product of degradation of creatinine by bacteria (Shimizu et al. 1989). To the best of our knowledge, it has never been studied in autism, and further investigations are needed to clarify its role.

Moreover, these results indicate that the above-mentioned metabolites discriminate between children with severely impaired behaviors and low impaired behaviors. In fact, the mild-moderate profile is positioned in the middle, indicating a continuous trend between the severity of ASD and the fecal concentration of these molecules. Thus, to the best of our knowledge, the current study represents the first attempt to identify a fecal metabolomic cluster distinguishing different levels of autism severity.

Metabolic pathways over-representation analysis based on the molecules that significantly differentiate between low-ADOS and high-ADOS children showed the involvement of proteins' metabolism, particularly the tRNA aminoacylation, both at the mitochondrial and cytosolic level. Aminoacyl-tRNA synthetases (ARSs) are a ubiquitously expressed family of nuclear enzymes responsible for charging tRNAs with their relative amino acids, therefore fundamental for the first step in protein synthesis. The role of tRNA synthetases has been studied in neurological and neuromuscular disorders (Boczonadi et al. 2018) and changes in protein synthesis have been previously observed in mouse models of ASD/intellectual disability (Auerbach et al. 2011, Barnes et al. 2015). In fact, the de novo protein synthesis plays a pivotal role in regulating the synaptic function and plasticity; mutations in several genes involved in the regulation of protein synthesis have been

identified as risk factors for the development of ASD with associated intellectual disability (Bhakar et al. 2012).

Therefore, we could conclude that the over-representation analysis of the molecules significantly differing between low-ADOS and high-ADOS children highlighted a metabolic pathway that might potentially be involved in ASD.

Besides metabolomics analysis, the microbiota characterization that we performed indicated that microorganisms and inflammation mediate the link between metabolome and severity of autism.

The recent literature detected a different microbial composition between ASD children and TD controls (Finegold et al. 2010, Averina et al. 2020), suggesting the presence of dysbiosis in ASD children (Hughes et al. 2018). De Angelis et al. (De Angelis et al. 2013) studied children with a pervasive developmental disorder (not otherwise specified) and Autistic Disorder (AD) in comparison to TD controls, demonstrating a higher deviation of relative abundance of fecal microbiota in the AD group compared to the other two groups.

These results showed no difference in the absolute abundance of total bacteria between NGI and GI subjects. Conversely, the relative abundance of bifidobacteria was significantly lower in GI than in NGI children. Other studies investigating the gut microbial composition in ASD with or without GI symptomatology concluded that autism-related changes in both overall diversity and individual *genus* abundances were correlated with the presence of autistic symptoms, but not with their diet patterns (Kang et al. 2013). In fact, notwithstanding it is well known that children with ASD are at higher risk of GI symptoms (McElhanon et al. 2014), few studies have characterized the different microbial profiles between ASD subjects with and without GI symptoms and considered them in the statistical analyses (Wang et al. 2011, Wang et al. 2013, Strati et al. 2017, Rose et al. 2018, Liu et al. 2019). These results provide evidence of altered gut microbiota in ASD children with GI symptoms, adding to the complexity of microbial differences in autism. The significantly lower relative levels of bifidobacteria in ASD children with GI symptoms than those without GI symptoms confirm previous results of protective effects of these microbes for the gut and human health in general (Fukuda et al.

2011). However, the current findings do not allow us to establish whether these bacteria are implicated as a cause or a consequence of some GI symptoms.

In these results, the fecal calprotectin concentration was elevated and not significantly different in subjects with and without GI symptoms. The observed values were similar to those reported by Zhu et al. (Zhu et al. 2016) in TD Chinese children of similar ages. Conversely, the elevated calprotectin concentration was directly correlated with *Prevotella* and inversely correlated with *Akkermansia*, suggesting an inflammatory and protective role of these two groups of microorganisms, respectively. In this context, some species of *Akkermansia* have been specifically observed in children with ASD (De Angelis et al. 2013, Kang et al. 2013), but their pathogenic role remains to be established.

To the best of our knowledge, we are among the few authors to have tested the link between the severity of autistic symptoms and the microbiota composition (Needham et al. 2021), highlighting the association between the SA and microbiota in parallel with the metabolomics analysis. By examining the severity of the autistic symptomatology, we did not observe differences in bacterial levels between Low-ADOS and High-ADOS groups. On the other hand, searching for microorganisms that could have determined the trends of the molecules highlighted by the rPCA model identifying low- and high-ADOS groups, we found that the relative abundance of Bifidobacteria and *Sutterella* significantly correlated with samples' scores along with PC 1. This result suggests a link between microbial status and characteristics of metabolome profile in the two groups with different ASD severity. When considering all the subjects, the significant correlation between the relative abundance of bifidobacteria and *Sutterella* and PC 1 was confirmed (r = 0.46 and -0.35, respectively).

As mentioned above, bifidobacteria and lactobacilli, i.e., important components of the human gut microbiome, have health-promoting properties and contribute to the host homeostasis (Fukuda et al. 2011, Hempel et al. 2012). Crucially, *Bifidobacterium* and *Lactobacillus* levels positively correlated with amino acids and negatively correlated with the molecules characterizing the high-ADOS groups.

This last result allows us to hypothesize a link between fecal microbiota composition and water-soluble fecal metabolome as well as the higher relative abundance of *Lactobacillus* and *Bifidobacterium,* which indicates a metabolomic profile characterized by a lower ASD severity degree. Concerning *Sutterella*, other authors have previously reported high levels of these bacteria in the stools of children with ASD compared to healthy controls (Wang et al. 2013), as well as high rates of these bacteria in GI biopsies taken from ASD children with GI symptoms (Williams et al. 2012). The consequences of an increase in *Sutterella* in the fecal populations are not yet known; however, it is possible that under certain conditions, these bacteria can cause infections (Williams et al. 2012).

We acknowledge the following limitations of this study.

Firstly, we did not consider the dietary patterns of the enrolled subjects, which may have influenced their fecal microbiota, especially considering the frequent presence of food selectivity in this population. Secondly, our results are limited to examining the fecal metabolome: urinary metabolomic studies may provide more interpretable data about CNS effects than fecal metabolomic, which do not reflect absorption rates and first-passage metabolism by the liver. Moreover, fecal metabolome was characterized and quantified by ^{1}H-NMR that has indeed high reliability, but also low sensitivity as compared to mass spectrometry-based techniques. Thirdly, we did not consider other members of gut microbial communities in addition to the investigated bacterial taxa as potential contributors to the metabolite profiles. Finally, the sample size of the low-ADOS group was smaller than the ones of the other groups.

In conclusion, this study represents the first one to detect a direct correlation between degrees of autism severity, as well as the features of the water-soluble fecal metabolome, fecal microbiota, and calprotectin levels in a group of ASD preschoolers. These results pave the way for subtyping the ASD population through the identification of specific metabolomic endophenotypes with the final aim of contributing to personalized therapies, given the need for evidence for personalized biopsychosocial interventions with this population.

These findings were published in Metabolites (Laghi et al. 2021).

3.4. Chapter 4. Possible therapeutic approaches

ASD can be considered a relatively frequent disorder with a high longitudinal diagnostic stability (Pierce et al. 2019), characterized by a significant individual, familial, and societal burden (Horlin et al. 2014). To date, the available therapies are mainly of the behavioral type, and the possibility of using a supplementary treatment that acts on the gut-brain axis, easily administered, with limited side effects and low costs, might be attractive in ASD. Studies from ASD-like animal models demonstrated not only that the microbiota is essential for social development (Desbonnet et al. 2014), but also that restoring the normal components of gut microbiota with probiotics may correct the intestinal permeability defects, altered microbial composition, and ASD-related abnormalities though the reduction of gut production and absorption of toxins (Hsiao et al. 2013, de Theije et al. 2014, de Theije et al. 2014).

As shown, the clinical studies published to date that have examined integrative treatment with probiotics and/or prebiotics and FMT are few and show heterogeneous results (Mayer et al. 2014). Most authors, however, found a benefit of these therapies not only on GI disorders but also on behavioral problems and severity of autism symptoms, both in RCT and in non-randomized studies. As already highlighted (Patusco and Ziegler 2018), not all these positive results reach statistical significance, and it is unclear if the period of supplementation that each study considers is long enough to expect behavioral changes. Moreover, the placebo effect should be considered, especially when parents rated the improvement through questionnaires (Whalley and Hyland 2013, Jones et al. 2017), and a placebo group is lacking in many studies examining treatment in ASD. However, in literature, it should be noted a good truthfulness of parents about the emotional and behavioral problems of their child (Glascoe 2003).

In general, significant limitations are the relatively limited study samples, frequently characterized by considerable dropout rates, participants that are exclusively children and adolescents, and study designs that often are unblinded trials. In addition, immediate or short-term effects are examined while few studies analyze whether the benefits are maintained even at follow-up (Sandler et al. 2000,

Grossi et al. 2016, Grimaldi et al. 2018, Kang et al. 2019). Considering FMT studies, some side effects may occur during the engraftment procedure of the microbiota and can be severe, requiring a suspension of the therapy (Kang et al. 2017), with side effects over time unknown.

As emerges from the methodological quality assessment of the studies published to date, only one (Liu et al. 2019) is a RCT with a low overall risk-of-bias (see Tables 3.4.1 and 3.4.2).

Table 3.4.1 Results of methodological quality assessment of randomized controlled trials (RCT) considering probiotic, prebiotic and FMT as a treatment in children with ASD.

First author, year	Randomization process	Deviations from intended interventions	Missing outcome data	Measurement of the outcome	Selection of the reported result	Overall risk-of-bias
Parracho, 2010	Low	High	High	Low	Low	High
Grimaldi, 2018	Low	Low	High	Low	Unclear	High
Arnold, 2019	Low	Low	Low	High	Low	High
Liu, 2019	Low	Low	Low	Low	Low	Low
Sanctuary, 2019	Low	Low	High	Low	Low	High
Wang, 2020	Low	High	High	Low	High	High

Table 3.4.2 Results of methodological quality assessment of non-randomized studies considering probiotic, prebiotic and FMT as a treatment in children with ASD.

	Pre-intervention		**At intervention**	**Post-intervention**				
First author, year	Bias due to confounding	Bias in selection of participants into the study	Bias in classification of interventions	Bias due to deviations from intended interventions	Bias due to missing data	Bias in measurement of outcomes	Bias in selection of the reported result	Overall risk-of-bias
Blades, 2000								
Sandler, 2000	High	High	Low	High	High	Low	Low	High
Kaluzna-Czaplinska, 2012	Low	High	High	Low	Low	High	Low	High
West, 2013	High	High	Low	High	High	High	High	High
Grossi, 2016								
Kang, 2017 and 2019	High	High	Low	High	High	Low	High	High
Liu, 2017	Unclear	Low	Low	Low	High	Low	High	High
Guo, 2018	High	High	Low	High	Low	High	High	High
Kobliner, 2018								
Shaaban, 2018	Low	Low	Low	High	Unclear	Low	High	High
Inoue, 2019	Low	Unclear	Unclear	High	Low	Low	High	High
Niu, 2019	Low	Unclear	High	High	High	Low	High	High
Mensi, 2021	High	Low	High	High	Low	High	High	High

RCT were evaluated using the Revised Cochrane-risk of-bias tool for randomized trials (RoB 2) (Sterne et al. 2019), which includes six domains of bias. Non-randomized studies were evaluated using the "risk of bias in non-randomized studies - of Interventions" (ROBINS-I) (Sterne et al. 2016), which includes seven domains of bias divided into three stages (pre-, at, and post-intervention stage). If at least one of the domains was rated as high, the trial was considered at a high risk of bias. If all domains were judged as low, the trial was considered at low risk of bias. Otherwise, the trial was considered as having an unclear risk of bias.

It is to note that this study is characterized by a relevant dropout rate (11%) and that there are other possible sources of bias, such as lack of registration of participants' dietary intakes and examination of comorbidities associated with ASD. Moreover, the pre-post intervention changes in the microbiota and their relationship with the recorded changes in behavior are not examined. Therefore, it is not possible to establish whether biological changes in the gut mediate changes in behavior.

Considering previous results (Liu et al. 2019, Mensi et al. 2021), where the ages of participants were relevant to expect more or fewer changes, further studies are needed to understand the exact correspondence between the type of treatment and the available benefit by age group. In both studies, the authors comprehensively assessed ASD symptoms using clinical assessment tools and caregiver questionnaires, and this way of proceeding is also desirable for future studies examining this topic.

More results emerge from the studies on probiotics rather than those on prebiotics and FMT. As it emerges from literature, probiotics, beyond the type of probiotic and duration of treatment, positively modifies the fecal microbiota. It has been shown a reduction of *Clostridia,* an increase in *Lactobacilli, Enterococci* and *Bifidobacteria* and, a normalization of the *Bacteroidetes/Firmicutes* ratio, a decrease in *Candida* and a decrease in intestinal inflammation and permeability in children with ASD (Parracho 2010, Kaluzna-Czaplinska and Blaszczyk 2012, Tomova et al. 2015, Shaaban et al. 2018, Arnold et al. 2019).

The consensus about types and doses of probiotics to be administered in ASD is lacking, ranging from single strain and multi-strains probiotics. Considering RCT studies examining the effects of probiotics exclusively, the most promising seems to be *L. plantarum WCFS1* (Parracho 2010), *L. plantarum PS128* (Liu et al. 2019) and Visbiome (Arnold et al. 2019), with positive results either in GI problems, the severity of autism and psychiatric symptoms. Unfortunately, long-term benefits are unknown because in no one of these studies, postintervention follow-up outcomes are done, hypothesizing temporary effects lasting only as long as the probiotic was administered.

Considering GI symptoms, it has been suggested that practitioners may consider probiotic therapy in children with ASD and severe GI dysfunction (e.g., constipation or diarrhea) because they may

experience some reduction in symptoms (Patusco and Ziegler 2018). Encouraging results, especially in contrasting GI symptoms, also emerge in studies analyzing prebiotics (Grimaldi et al. 2018, Inoue et al. 2019, Sanctuary et al. 2019). However, only one RCT study (Grimaldi et al. 2018) has been published to date that has examined the role of a prebiotic without confounding bias resulting from the simultaneous use of probiotics, showing the modulation of the gut microbiota composition in subjects assuming prebiotics. Bimuno galacto-oligosaccharide did not significantly affect GI symptoms but showed a significant reduction in anti-sociability scores when combined with an exclusion diet. Therefore, the role of prebiotics must be deepened whether used alone or as a substrate for certain probiotic strains.

Moreover, considering the heterogeneity of ASD and that medications are specific for only specific ASD subgroup as suggested by results of pharmacological trials in this population (Hollander and Uzunova 2017, Veenstra-VanderWeele et al. 2017), none of the studies examining probiotics and/or prebiotics select the participants based on their intestinal microbiota limiting possible positive results.

Much more needs to be done in research examining FMT in subjects with ASD. Despite the encouraging results, also considering long-term lasting after the end of the treatment (Kang et al. 2019), this area of research is in its infancy. To date, no RCT studies are available on this topic, and the preparation procedure of its applications (as MTT) that require antibiotics, antiacid drugs, and bowel cleanse could be complex and not free from concerns, mainly when applied in children and adolescents with ASD. Moreover, as already showed (Sandler et al. 2000, Vrieze et al. 2014, Freedberg et al. 2015), the effects of the preparation on the microbiota and its role in the benefits for the host beyond the microbiota transplant (Kang et al. 2017) must be considered. The sole research examining MTT in ASD subjects has shown an increase of overall bacterial diversity and relative abundances of *Bifidobacteria, Prevotella, and Desulfovibrio*, among other taxa, most of which persisted two years after the end of treatment (Kang et al. 2019).

Taken together, these findings suggested the need for randomized, placebo-controlled trials to yield more rigorous results.

The current study was a RCT (randomized control trial) evaluating the effects of supplementation with the De Simone Formulation or Vivomixx® (DSF) on ASD core symptoms, GI symptoms, plasma and fecal inflammatory biomarkers in ASD preschoolers with and without GI symptoms.

3.4.1. Materials and Methods

See introduction to section 3. for a complete description of the materials and methods.

The sample size calculation was based on the primary outcome assumption in the intervention and control groups, the severity level of ASD symptomatology, measured with the ADOS-CSS. In a previous study, the ADOS-CSS decreased in 62% of preschoolers whereas it was the same or worse in 37% of the children after 6 months of "as usual" treatment (Narzisi et al. 2015). Sample size calculations were performed using the nQuery advisor 6.2 software. Assuming a response rate of 62% in the placebo group and 90% in the probiotic group, it was calculated that 38 patients per treatment arm would be sufficient to achieve 90 % power in detecting a treatment difference based on 1-tail $\chi 2$ test at a significance level of 0.05. The main statistical analysis included all participants who had data for the primary endpoint in the group to which they had originally been randomized. Analyses were performed both on the binary outcome measure assumed for sample size calculation (rates of subjects with a decrease in ADOS-CSS vs rates of subjects whereas it was the same or worse) and on continuous outcome measure (changes in mean ADOS-CSS). Quantitative data are presented as mean ± standard deviation (SD). A comparison between different points of time-course was performed by t-student test. The difference between several independent groups was compared by two-way ANOVA. Statistical analysis was performed using Statview 5.0.1 software (SAS Institute, Inc., Cary, NC, USA). A p value <0.05 was considered statistically significant.

3.4.2. Results

3.4.2.1. Efficacy: primary outcome in the whole sample

From baseline to T2, the Total ADOS-CSS decreased in 45.2% (14/31, [95%CI, 27.7% to 62.7%]) of children treated with probiotic and in 28.1% (9/32, [95%CI, 12.5 % to 43.7%]) of children treated with placebo. This difference was not statistically significant (risk ratio=1.60; risk difference=0.17; P = 0.16). Mean Total ADOS-CSS scores decreased from 6.84 to 6.19 in the probiotic group and increased from 6.97 to 7.00 in the placebo group, with a difference that did not reach statistical significance (Mean change probiotic vs placebo -0.65 vs +0.03 [95%CI, -0.68 to +0.08]; P = 0.08) (Table 3.4.2.1).

3.4.2.2. Efficacy: secondary clinical outcomes in the whole sample

From baseline to T2, the other pre-specified clinical secondary outcomes showed no significant differences in the probiotic vs the placebo group (Table 3.4.2.1)

Table 3.4.2.1 Efficacy measures at baseline and after 6-months in the two treatment groups

	Placebo (32)			Probiotics (31)			p ANOVA Pla /Pro T0	p ANOVA Pla/Pro T0-T2
Characteristics	**T0**	**T2**	**Change T0-T2**	**T0**	**T2**	**Change T0-T2**		
Age, mean (SD), y	4.09 (0.97)	4.62 (0.98)	0.52	4.29 (1.22)	4.82 (1.23)	0.53	ns	ns
Boys, No. (%)	27 (84.4)	27 (84.4)	n. a.	24 (77.4)	24 (77.4)	n. a.	ns	ns
ADOS CSS [a]								
Total	6.97 (1.91)	7.00 (1.80)	0.03	6.84 (1.39)	6.19 (1.56)	-0.65	ns	ns
Social Affect	6.41(2.21)	6.09 (1.82)	-0.31	6.26 (1.79)	5.35 (1.56)	-0.90	ns	ns
Restricted and repetitive behavior	8.22(1.31)	8.53 (1.34)	0.31	7.94 (1.57)	8.23 (1.45)	0.29	ns	ns
SCQ [b]	16.06(5.54)	13.90 (6.19)	-2.16	12.83 (6.68)	11.97 (6.71)	-0.87	0.042	ns
RBS-R [c]	22.31 (15.47)	19.13 (12.10)	-3.18	18.32 (13.17)	14.37 (8.01)	-3.96	ns	ns
DQ [d]**, standardized test**								
General Quotient	62.29 (20.12)	61.14 (20.13)	-1.15	65.91 (18.06)	69.27 (20.09)	3.36	ns	ns
VABS II [e]								
Composite Score	57.00 (16.74)	59.72 (16.38)	2.72	63.87 (22.12)	67.39 (22.29)	3.52	ns	ns
CBCL [f]								
Total Problems	62.84 (10.97)	57.30 (9.05)	-5.54	60.94 (9.94)	57.80 (7.92)	-3.14	ns	ns
PSI [g]								
Total Stress	74.76 (24.98)	61.03 (32.58)	-13.72	70.03 (29.63)	66.62 (31.15)	-3.41	ns	ns
GI Severity Index [h]								
Total 6-GSI	1.38 (1.45)	1.29 (1.19)	-0.08	2.06 (2.14)	1.23 (1.48)	-0.83	ns	ns
Total GSI	2.91 (2.19)	2.16 (1.57)	-0.74	3.61 (2.92)	2.53 (2.19)	-1.08	ns	ns
	Pla T0-T2 (%)			**Pro T0-T2 (%)**				
Linguistic Level [i]	↓	=	↑	↓	=	↑		
	0	87.50	12.50	9.68	70.97	19.35	ns	ns

Abbreviations: ADI-R Autism Diagnostic Interview–Revised; ADOS Autism Diagnostic Observation Schedule; CBCL 1.5-5 Child Behavior Checklist 1.5-5; CSS Calibrated Severity Score; D Definite Difference; DQ Developmental Quotient; GI gastrointestinal; GSI Gastrointestinal Severity Index; IQ Intelligence Quotient; No. Number; NGI Non-Gastrointestinal; Pla: Placebo; Pro: Probiotics; P Probable Difference; PSI Parental Stress Index; RBS-R Repetitive Behaviors Scale-Revised; SCQ Social Communication Questionnaire; SD Standard Deviation; T Typical Performance; VABS-II Vineland Adaptive Behavior Scales-II; y years. ↓ is to be understood as worsened compared to the previous evaluation, **=** is to be understood as unchanged from the previous evaluation, ↑ is to be understood as improved compared to the previous evaluation. Means, and standard deviations are reported.

[a] Higher scores indicate greater severity (range of possible scores for Total, Social Affect and Restricted and Repetitive Behavior is 1-10).

[b] Higher scores indicate greater severity (range 0-39) with a threshold of 15 compatible for a relevant impairment of social communication (some studies consider 9 in children younger than four years old).

[c] Higher scores indicate greater severity of repetitive behaviors (range 0-114).

[d] Higher scores indicate greater cognitive ability. Scores around 100 indicate normal intelligence; scores below 70 indicate a developmental delay.

[e] Higher scores indicate greater adaptive competences. Scores around 100 indicate normal adaptive capacities; scores below 70 indicate a delay with respect to age.

[f] Higher scores indicate greater severity; a score of 63 and above is generally considered clinically significant.

[g] Higher scores indicate greater severity of parental stress index caused both by characteristics of the child and by negative experiences about the parenting role (Total Stress).

[h] Higher scores indicate greater severity of gastrointestinal symptoms; Total 6-GSI has a range of 0 to 12, Total GSI has a range of 0 to 17.

[i] The "Overall Level of Non-Echoed Spoken Language" item (A1 score) of the ADOS-2 was used to differentiate non-verbal (those with absent language or less than 5 words) from verbal children.

3.4.2.3. Efficacy: secondary exploratory analyses on GI and NGI parallel arms

One of the original aims of this study was to evaluate the effects of probiotics on ASD core symptoms, GI symptoms, and plasma and fecal inflammatory biomarkers in ASD children with and without GI symptoms. For this purpose the randomization was made independently in the GI and NGI groups, to obtain four parallel arms. At the end of recruitment, the sample size of each arm did not reach the target already determined for the whole sample; the GI group, already less numerous, was also affected by a bigger drop-out rate than the NGI one. Therefore, secondary exploratory analyses among subgroups were performed. The four parallel arms were well balanced for the total number of hours of rehabilitative treatments (GI placebo: 175± 91, GI Probiotic 156±68, NGI placebo 134± 84, NGI probiotic 137±129 $p>0.05$ for all the comparisons). In the NGI group we found a significant decrease both in the primary outcome measure, Total ADOS-CSS scores (which decreased from 6.72 to 5.91 in the probiotic group and increased from 6.96 to 7.17 in the placebo group; mean change probiotic vs placebo, - 0.81 vs + 0.21 [95%CI, -0.76 to +0.20]; P = 0.026), and in Social-Affect ADOS-CSS (mean change probiotic vs placebo -1.14 vs -0.04 [95%CI, -1.01 to +0.06]; P = 0.027). In the GI group, statistically significant effects were found in GI symptoms (Total GSI, Total 6-GSI, stool smell and flatulence mean scores), and in adaptive functioning (Receptive Skills, Domestic Skills and Coping Skills VABS-II subscales) for which probiotic therapy was associated with greater improvements than placebo (Table 3.4.2.2). In the GI group a significantly higher proportion of children in the probiotic group than in placebo group showed a normalization of Sensory Profile scores in the Multisensory Processing subscale (87% vs 28%, respectively, p= 0.001).

Table 3.4.2.2 Efficacy Measures with Significant Changes from Baseline to 6-Months in the Subgroups

GI subjects	T0 (baseline)	T2 (after 6 months)	**Change T0-T2**	**p ANOVA Pla/Pro T0**	**p ANOVA Pla/Pro T0-T2**
VABS II					
Receptive (v-scale)[a]					
Placebo Group	6.75 (3.10) [2 to 11]	7.12 (2.53) [3 to 10]	0.38 [-1 to 2]	ns	0.0104
Probiotics Group	4.78 (3.03) [2 to 10]	7.11 (3.14) [3 to 12]	2.33 [1 to 6]		
Domestic (v-scale) [a]					
Placebo Group	12.50 (2.27) [10 to 16]	13.37 (3.16) [9 to 19]	0.87 [-1 to 3]	ns	0.047
Probiotics Group	9.44 (5.50) [0 to 15]	12.66 (2.74) [10 to 17]	3.22 [0 to 14]		
Coping Skills (v-scale) [a]					
Placebo Group	11.25 (2.12) [9 to 16]	9.75 (4.59) [2 to 18]	-1.50 [-8 to 2]	ns	0.0115
Probiotics Group	9.11 (4.01) [0 to 14]	10.22 (2.17) [6 to 13]	1.11 [-1 to 10]		
Total 6-GSI [b]					
Placebo Group	3.50 (0.93) [3 to 5]	2.00 (1.53) [0 to 4]	-1.75 [-3 to 0]	0.009	0.0191
Probiotics Group	5.00 (1.22) [4 to 7]	1.67 (1.66) [0 to 5]	-3.33 [-6 to 0]		
Total GSI [c]					
Placebo Group	5.75 (1.03) [4 to 7]	3.43 (1.81) [1 to 6]	-2.28 [-5 to 0]	ns	0.0416
Probiotics Group	7.22 (1.99) [4 to 10]	2.89 (2.31) [0 to 7]	-4.33 [-8 to -2]		
GSI, Stool Smell [d]					
Placebo Group	0.25 (0.71) [0 to 2]	0.14 (0.38) [0 to 1]	-0.15 [-1 to 0]	<0.001	<0.001
Probiotics Group	1.88 (0.33) [1 to 2]	0.56 (0.88) [0 to 2]	-1.32 [-2 to 0]		
GSI, Flatulence [d]					
Placebo Group	0.43 (0.79) [0 to 2]	0.86 (0.90) [0 to 2]	0.43 [0 to 1]	ns	0.0187
Probiotics Group	0.56 (0.88) [0 to 2]	0.33 (0.50) [0 to 1]	-0.23 [-1 to 0]		
NGI subjects	T0 (baseline)	T2 (after 6 months)		**p ANOVA Pla/Pro T0**	**p ANOVA Pla/Pro T0-T2**
ADOS CSS Social Affect [e]					
Placebo Group	6.37 (2.30) [2 to 10]	6.33 (1.71) [3 to 9]	-0.04 [-4 to 4]	ns	0.027
Probiotics Group	6.09 (2.00) [1 to 10]	4.95 (1.56) [2 to 7]	-1.14 [-5 to 2]		
ADOS CSS Total Score [e]					
Placebo Group	6.96 (1.90) [3 to 10]	7.17 (1.79) [4 to 10]	0.21 [-4 to 4]	ns	0.026
Probiotics Group	6.73 (1.49) [3 to 10]	5.91 (1.63) [3 to 9]	-0.82 [-4 to 2]		

Abbreviations: ADOS Autism Diagnostic Observation Schedule; D Definite Difference; GSI Gastrointestinal Severity Index; No. Number; NGI Non-Gastrointestinal; Pla: Placebo; Pro: Probiotics; P Probable Difference; SD Standard Deviation; T Typical Performance; VABS-II Vineland Adaptive Behavior Scales-II. Means, standard deviations and ranges are reported.

[a] Higher scores indicate greater adaptive competences. Scores around 100 indicate normal adaptive capacities; scores below 70 indicate a delay with respect to age.

[b] Range of possible scores, 0 to 12; higher scores indicate greater severity.

[c] Range of possible scores, 0 to 17; higher scores indicate greater severity.

[d] Range of possible scores, 0 to 2; higher scores indicate greater severity.

[e] Range of possible scores, 1 to 10; higher scores indicate greater severity.

3.4.2.4. Biochemical Secondary outcomes

No statistically significant changes in plasma biomarkers and in fecal calprotectin levels were found from baseline to T2 in all the subjects who completed the study (Table 3.4.2.3).

Table 3.4.2.3 Biomarkers at Baseline and 6-Months in the Two Treatment Groups.

	Placebo T0	Placebo T2	Pla T0-T2	Probiotic T0	Probiotic T2	Pro T0-T2	*p* ANOVA Pla/Pro T0	*p* ANOVA Pla/Pro T0-T2
	mean (SD)	mean (SD)		mean (SD)	mean (SD)			
Plasmatic Biomarkers								
IL-6 pg/ml	3.61 (6.18)	3.54 (3.63)	-0.08	3.24 (4.32)	3.33 (2.63)	0.09	ns	ns
Leptin pg/ml	1.21 (1.03)	1.32 (1.09)	0.11	1.23 (0.82)	1.13 (0.96)	-0.10	ns	ns
TNF-α pg/ml	6.47 (2.79)	6.16 (2.34)	-0.32	5.45 (2.14)	5.82 (2.16)	0.37	ns	ns
PAI-1 ng/ml	28.79 (23.20)	27.31 (12.54)	-1.48	28.34 (18.15)	32.46 (23.86)	4.11	ns	ns
Fecal Biomarker								
Calprotectin µgr/gr	128.43 (171.87)	204.61 (438.11)	+76.18	138.12 (196.32)	129.50 (139.67)	-8.62	ns	ns

Abbreviations: IL-6: interleukin-6; PAI 1: Plasminogen Activator Inhibitor-1; TNF-α: Tumor Necrosis Factor-alpha; ns: not significant.

3.4.2.5. Safety

No serious Adverse Event (AE) was reported. All treatment-emergent AEs were transient and mild in severity. A total of three participants, all treated with placebo, discontinued treatment because of an AE, reporting a worsening of GI symptoms (2) and a worsening of hyperactivity (1). Two participants, both treated with probiotic, reported GI symptoms (abdominal pain and diarrhea), during the first ten days of treatment, but these symptoms were transient and both children continued the treatment and completed the trial.

3.4.3. Discussion

In this double-blind, randomized controlled six-months trial completed in 63 children with ASD, the supplementation with probiotic mixture DSF resulted in no statistically significant difference in autism severity compared with placebo. These results are not consistent with some previous findings of significant improvements in ASD symptoms in response to probiotic administration (Parracho 2010, West DO 2013, Shaaban et al. 2018). Specific strengths of the current study – i.e., the double-blind study protocol and the inclusion of reliable tools to assess outcomes- could explain the differences with those previous investigations. For example, we have used the ADOS-2 (a semi-structured direct observation of the child specifically designed for ASD and administrated by an expert clinician following appropriate training) that is considered a gold-standard method of assessment for ASD in both research and clinical practice, even if its capacity to detect changes over time may be questioned (McConachie et al. 2015, Bieleninik et al. 2017). Other studies (West DO 2013, Shaaban et al. 2018) have described significantly superior benefits of probiotics than placebo using more subjective instruments such as parent-report interviews or questionnaires.

Our study's novel and promising finding is the significant decline in ADOS CSS scores (both Total and Social-Affect scores) in the NGI group treated with probiotics instead of those obtained in the placebo group. Although deriving from a secondary analysis, this result is significant from a clinical point of view, especially in the light of the abovementioned psychometric properties of the used tool.

In fact, a mean reduction of 0.81 in Total ADOS CSS and of 1.14 in Social-Affect ADOS CSS over six months constitutes a clinically significant decrease of ASD symptoms (Gotham et al. 2009). Not all previous trials with probiotics examined their effect, considering the presence/absence of GI symptoms (Patusco and Ziegler 2018). Our result suggests that ASD children with and without GI symptoms could represent two different populations and that probiotics interventions could potentially provide different effects, likely due to distinct microbiota targets. Previous studies have already suggested that differences in the microbiome (Luna et al. 2016, Arnold et al. 2019) are independent of GI dysfunction. Luna et al. (Luna et al. 2016) argued that larger and well-designed studies are still needed to determine whether microbial composition may stratify ASD children beyond the GI symptoms. Within this framework, a positive impact of probiotics on autism severity in children without pre-existing GI symptoms supports the complexity of the microbiota-gut-brain axis, warranting further studies on this subgroup of ASD subjects.

As far as GI symptoms, our findings are partially consistent with those reported by some trials (Parracho 2010, West DO 2013, Shaaban et al. 2018), which showed significant effects of probiotic supplementation in reducing GI symptoms in children with ASD (Patusco and Ziegler 2018). Parracho et al. (Parracho 2010) reported significantly fewer “hard” and more “formed” stools in children treated with probiotic therapy compared with placebo. Shaaban et al. (Shaaban et al. 2018) found significant improvement in GI symptoms after three months of probiotic supplementation when measured through 6-GSI, particularly on constipation, stool consistency, flatulence, and abdominal pain. West et al. (West DO 2013) detected a considerable decrease in constipation and diarrhea after probiotic therapy. Our results are also in line with those reported in a recent pilot study performed in 13 ASD children, 3–12 years of age, which showed significant improvement in GI complaints in children treated with DSF compared with children treated with placebo (Arnold et al. 2019).

In the subgroup of children with GI symptoms, we found a positive effect of probiotics not only on GI symptoms but also on adaptive functioning and multisensory processing. The significant improvement in multisensory processing in children with GI symptoms could be an expression of the

interdependence between these two classes of symptoms, and their simultaneous amelioration could have led to the beneficial effect on adaptive functioning, as previously described (Thye et al. 2018). Taken together, these different results on NGI and GI groups of children suggest that the effects of probiotic supplementation in ASD children may be due to distinct mechanisms. The well-known neurobiological heterogeneity of ASD implies that each medication is likely to benefit only a subset within the spectrum of affected children, as suggested by results of pharmacological trials in this population (Hollander and Uzunova 2017, Veenstra-VanderWeele et al. 2017). The described positive effect on both GI and NGI children paves the way for identifying those ASD subjects who can respond to probiotic supplementation beyond GI symptoms and even beyond GI inflammatory status. In fact, in the current study, the supplementation with DSF compared with placebo resulted in no significant effects on the levels of plasma and fecal inflammatory biomarkers. In a previous investigation, we have reported that the values of these biomarkers were in the normal range already at baseline (Prosperi et al. 2019); thus, we do not confirm the two previous studies (Kaluzna-Czaplinska and Blaszczyk 2012, Tomova et al. 2015) reporting some positive effects of probiotics on biomarkers of inflammation, and we could hypothesize that the behavioral effect of probiotics is not mediated by a reduction in systemic or intestinal inflammation.

Indeed, the exact mechanisms by which probiotics exert potential therapeutic effects are not already completely identified. They probably go beyond the down-regulation of inflammatory cytokines and refer to other effects on gut barrier permeability, immunomodulation, and restoration of altered gut microbiota (Ng et al. 2019). This is particularly true for the high concentration multi-strain probiotics such as DSF, which has been proven to exert positive effects on the balance among different CD4 T-cell subsets and Th17 cell subsets, on the integrity of the gut epithelial barrier, on modulating intraepithelial lymphocytes density and enterocyte apoptosis (d'Ettorre et al. 2017).

Compared with previous trials of probiotics in ASD, the strengths of this study include its duration, rigorous double-blinding and simultaneous assessment of several clinical and biochemical outcome measures. Unlike in previous trials, we also controlled for additional rehabilitative treatments to

ensure that the changes we detected were closely related to the probiotic supplementation. Furthermore, the research protocol administered to the ASD patients seemed very well accepted by parents, children, and staff, with high compliance and adherence to all the procedures. Lastly, our trial confirms the data of previous studies reporting few and transient side effects during probiotic therapy (Patusco and Ziegler 2018), also adding information about the safety of probiotic supplementation in a pediatric population and over a longer period of treatment than previously reported (Firth et al. 2019).

Several limitations must be noted. Firstly, the significant dropout rates, although satisfactory considering the duration of the study, may have affected the trial's ability to detect significant differences between the two main treatment groups reliably. This seems to have affected particularly the subjects within the GI group, in which almost half of the participants dropped out. Consequently, children who dropped out were substantially comparable to those who completed the trial in all clinical variables, except for higher levels of GI symptoms. This discrepancy between the two groups could impact the study's ability to detect other possible significant differences in the whole spectrum of GI symptoms. Moreover, 173 children were eligible, and 85 participated, which is less than 50% recruitment rate, possibly introducing a bias by selecting patients that reached the end of the study in some way. Most of the families refused to participate due to the distance from the hospital or to family organizational difficulties.

A second limit is that the use of the ADOS evaluation as an outcome measure in clinical trials has been recently disputed (McConachie et al. 2015), mainly because it lacks sensitivity to detect changes in short periods.

Nevertheless, the field of trials with medication treatments in ASD is still challenged by the lack of objective outcome measures adequately sensitive and specific to change in social symptoms (Anagnostou et al. 2015). Indeed, most studies have used parent-report questionnaires, which lack adequate inter-rater reliability, test-retest reliability, and/or internal validity or are frequently affected by a high placebo effect on parental perception (Jones et al. 2017). Third, the choice of assessing GI

symptoms with GSI (a tool not yet validated and providing information based on parent input without added diary) may have affected the reliability of data we collected about GI symptoms. Nevertheless, in a recent literature review (Holingue et al. 2018), comparing different approaches to the measurement of GI symptoms (including Autism Treatment Network, Rome criteria, and GSI) in 84 studies on ASD samples, the authors found that no symptom prevalence proportions differed significantly or was associated with the type of questionnaire. Finally, this study did not provide information about microbiota and metabolomic changes during the treatment; future studies need to carry out these analyses in order to search for correlation between the brain, clinical improvement and specific microbiota composition to develop precision medicine in ASD.

In conclusion, a six-month probiotic supplementation did not result in statistically significant changes in autism symptoms in the whole sample of ASD preschoolers. Nevertheless, for the first time to our knowledge, we have observed in children without GI symptoms treated with probiotics significant modification of core ASD symptoms measured by the ADOS-CSS scores (specifically Social-Affect domain) that are unrelated to the specific intermediation of the probiotic effect on GI symptoms. As far as children with GI symptoms, the six-month supplementation with DSF showed significant effects, compared to placebo, in improving not only GI symptoms but also multisensory processing and adaptive functioning.

All these findings could pave the way for further studies on larger subgroups of ASD to improve precision medicine in ASD. These results were published in Frontiers in Psychiatry (Santocchi et al. 2020).

4. Conclusions and Future Perspectives

GI disorders arise very early in the development of an individual with ASD and represent a challenge for practitioners and caregivers, both in suspecting presence and finding efficacious interventions for their management.

Although there is a greater awareness of their presence today, the literature published on this topic is not exhaustive. In this view, this thesis offers an interpretation of GI symptoms based on the results of several clinical studies and in light of previous findings. The hypothesis that children with ASD and GI symptoms are inflamed is not supported, nor is evidenced an organic nature of the disorder, confirming a not yet clarified meaning of these symptoms.

Moreover, it remains to be clarified whether microbiota alterations are implicated in the onset of ASD or occur subsequently and how they are linked with GI symptoms. The worsening of autistic symptoms could occur either due to a GI disorder or through indirect pathways linked to the microbiota-gut-brain axis.

To date, the available therapies for ASD are mainly of the behavioral type, and the possibility of using a supplementary treatment that acts on the gut-brain axis, easily administered, with limited side effects and low costs, might be attractive in ASD. The positive impact of probiotics on core autism symptoms in a subset of ASD children, independently from the specific intermediation of the probiotic effect on GI symptoms, is promising. Considering the variability of therapies, the samples size, the duration of treatment and the tools used to evaluate the outcome, the results arising from the gut-brain axis as one of the potential focuses of treatment in children with ASD are still partial. They do not allow the establishment of a conclusive beneficial effect of probiotics and other interventions on the symptoms of autism. Furthermore, future studies that will examine conditions frequently comorbid with ASD, such as ADHD or other neurodevelopmental disorders, will have to define their contribution in the response or not to these treatments.

Considering ASD heterogeneity, it would be desirable to select alternative therapies with food supplements based on the specific characteristics of both the subjects with ASD and the host's microbiota to individualize the treatment.

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