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MINI - REVIEW

A Mini-Review on the Correlation Between the Autistic Pathology and the Microbiome

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Abstract
Considering the latest increased awareness on the multifactorialy of the autistic pathology, as well as the possible implications of some gastrointestinal and metabolic deficiencies associated with, in the present mini-review we are describing the existing correlations between the autistic pathology and the microbiome, and also the aforementioned gastrointenstinal and metabolic associated deficiencies.

Key words*:* autism, microbiome, metabolic, gastrointestinal.

Introduction

The designation of "Autism Spectrum Disorders" (ASDs) refers to a group of ubiquitous multifactorial neurodevelopmental disorders with an early onset stage of occurrence and characterized by a growth and function impairment of the central nervous system (lack of communication and interhuman relationships, restrictive and repetitive behaviour) [1]. Compared to the 4th edition of the *Diagnostic and Statistical Manual of Mental Disorders* (DSM-IV), where a clear differentiation criterion for all ASD subtypes (autistic, Asperger's and Rett's Syndrome, Pervasive Developmental Disorder Not Otherwise Specified and Childhood Disintegrative Disorder) does not exist due to the relatively limited accuracy of DSM-IV, almost identical symptomatology and because of poor predictability of later outcome, all these impediments have been exceeded with the advent in 2013 of DSM-5. Now a single dimensional diagnostic is generally accepted: ASD. Pervasive Developmental Disorder (PDD) could be classified as a conceptual diagnostic, used to describe a patient who manifests at least two symptoms of restrained stake/cyclic conduct and three in the domain of social communication. On the other hand, Social Communication Disorder (SCD)

"counterbalances the thalers" for children under the age of 3 who do not fit in ASD's criteria [2, 3].

The etiopathogenesis of ASD is mostly unknown, on the strength of multiple causes, courses and significant range in severity of symptoms, including anxiety and gastrointestinal deficiencies [4, 5]. A gut disorder analogue to Crohn's disease is reported from time to time in autistic children, being associated to a series of non-psychiatric comorbidities symptoms such as constipation, dysentery and transition episodes constipation/dysentery [6, 7]. In recent decades, there has been an accelerated increase in the number of cases, predisposition of appearance being significantly higher in boys than that in girls [8], the information on how synapses connect and organise still remaining an obscure domain.

While genetics is responsible for about 50% of ASD cases, for example *de novo* mutations [9, 10, 11], common variations [12], or an interplay of common and rare variants [13], leads inevitably to ASD thanks to heredity who plays the main role in this context. Another factor can be represented by an exposure to various cytotoxic and genotoxic agents like lead [14], air pollution [15], pesticides [16], or antibiotic excess [17] during intrauterine life.

The life that we all know today would not be possible without microbes, each individual hosting numerous communities (archaea, bacteria, fungi and viruses) at a specific niche exercising systemic effects on host biology [18]. Co-evolving with the microbiome, in the last two decades, all the attention was focused on this symbiosis, more and more studies highlighting a close relationship between gut microflora and the brain. It goes by the popular dictum: "repair your gut, repair your brain". This collectivity fulfils essential functions for our health, including conferring protection against pathogen overgrowth, homeostasis of the intestinal mucosal barrier, wholesome, xenobiotic and drug metabolism and immunological [19] by producing short-chain fatty acids (SCFAs), aromatic amino acid derivatives (AAA), bile acids and choline with role in maintaining the interactions between its host and the neurohormonal axes [20]. Deregulation of gut flora leads to a variety of human diseases, among them, ASD, influence exerted on the far-off organs, as well as on those in the immediate vicinity taking place through various pathways [21].

Autism vs microbiome

After the completion of the Human Genome Project (HGP) in 2003, a new one has emerged in 2008 known as Human Microbiome Project (HMP). With 1.5 kilogram of biomass production, 10 times more cells compared to human ($\sim 10^{14}$) and 150 times more genes than our own DNA, alongside our "tenants" we form a super-organism [22, 23, 24]. Between 500 and 1.000 species harbour human body [25], divided in three enterotypes: *Bacteroides*, *Prevotella* and *Ruminococcus*,

each category with an impact potentially beneficial, harmful or neutral [26, 27]. Due to its interface (250-400 m^2) [28], the gastrointestinal tract houses trillions of symbiotic microbes [29, 30, 31].

Even though there is a tendency to believe that each individual possesses the same gut flora, this assumption is not entirely true, various genetic studies revealing an inter-individual variation, this maybe having to do with the layout and co-incidental expansion of the habitat in an already existing niche formed by genetics, time and nourishment [32, 33]. For example, in the case of twins, they have an almost identical microbiome compared to that of their brethren, finally forming similarities that are not encountered in unrelated persons [34, 35]. Although the gut of an unborn baby is theoretically sterile in the mother's womb, the development of the microbiota starts immediately after the child passes into the birth channel, where a large amount of microbial communities from faecal, vaginal and skin shapes the offspring's microbiota [36]. In the last decade, caesarean sections (CS) have increased dramatically [37], neonates presenting less numbers of *Bifidobacteria* species primarily [38, 39], a woman lately confined without knowing that she is exposing her newborn to a series of epidemiological illnesses like anaphylactic reactions, adiposity, asthma and autoimmune diseases [40]. It is well documented that breastfeeding plays an important role in the subsequent development of the baby. Apart from that, pro- and prebiotics offer an alternative to manipulate the early colonization of the gut [41].

As mentioned above, gut flora fulfils essential functions for the metabolism, concomitantly with the maintenance of immune homeostasis and control of the central nervous system (CNS) through immune, endocrine and neural pathways [42]. In the gastrointestinal tract (GI), cells from the central, peripheral and enteric nervous system form a dense network, in association with hypothalamic-pituitary-adrenal (HPA) axis giving birth to the so-called gut-brain axis (GBA) [21, 43]. An interdependence between the gut flora and enteric neurons has been demonstrated [44, 45], as well as its regulating role upon HPA axis [46], and the production of important chemicals involved in brain's optimal functioning [47, 48].

Deregulations that occur at the level of this "micro-world" could lead to a petulant activity of T-helper 1 and 17 cells [49], affecting the reactivity of the peripheral immune cells response to the "main core" [50], and followed finally by a disturbance along the integrity of blood-brain barrier (BBB). Studies on the importance of BBB revealed that it provides protection against bacterial lipopolysaccharide and other toxins [51, 52].

The results of the studies published over the years support the concept of bidirectional gut-brain interactions and *vice versa*, some of them regarding a

better understanding of the influence exerted by gut flora on phenotypic attributes being summarized in Table 1.

Table 1. Influence exerted by GBA deregulation on phenotypic attributes

Murine model	Attribute/ Category	Procedure	Main observations	Note
Wistar rats	Anxiety-like behavior/ Behavioral	Daily administration of a probiotic formulation containing *Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175	Anxiety-like behavior was significantly reduced in rats after two weeks of administration	[53]
BALB/c mice	Anxiety-like behavior/ Behavioral	Daily administration of *Bifidobacterium longum* 1714 *Bifidobacterium breve* 1205, Escilatopram or vehicle	After six weeks of treatment, both Bifidobacteria species and Escitalopram reduced anxiety	[54]
BALB/c mice	Depression-like behavior/ Behavioral	Administration of *Lactobacillus rhamnosus* (JB-1)	Treatment with *Lactobacillus rhamnosus* (JB-1) reduced depression-like behavior	[55]
Sprague-Dawley rats	Depression-like behavior/ Behavioral	Administration of *Bifidobacterium infantis*	Treatment with *Bifidobacterium infantis* reduced depression-like course	[56]
Germ-free and BALB/c mice	17-deoxy-cortisol/ Hormonal	Comparative study	Increased hypothalamic corticosterone in germ-free mice	[57]
Germ-free adult mice	Blood-Brain Barrier/ Neurochemical	Exposure to a pathogen-free gut microbiota	A decrease of blood-brain barrier permeability concomitant with an regulation of tight junction protein expression	[58]
Germ-free mice	Peripheral serotonin/ Neurochemical	Exposure to spore-forming bacteria from mouse and human	An increased production of serotonin	[59]

Germ-free C57/BL6 mice	Colony-stimulating factor 3/ Neurochemical	Granulocytosis, Neutrophil homeostasis and host resistance importance against prolonged anbitiotic exposure	A decreased colony-stimulating factor 3 levels is associated with host susceptibility to *Escherichia coli* K1 and *Klebsiella pneumoniae* sepsis	[60]
Germ-free mice	Noradrenaline, dopamine and serotonin/ Neurochemical	Exposure to gut microbiota during early life	An increased dopamine, noradrenaline and serotonin levels	[61]
Germ-free and CC Swiss Webster mice	Serotonin and serotonin receptor/ Neurochemical	Comparative study	An evelation of oxitriptan and 5-hydroxyindoleacetic acid in germ-free mice compared to the control	[62]

In spite of numerous pre- and clinical studies, its mechanism, or rather, the mechanisms of action remain largely unknown. It is certain that ASD can be considered the result of a combination of exogenous and genetic factors. Some of them will be discussed below.

Maternal risk factors associated with the microbiome

There are strong evidences suggesting that infections during pregnancy could negatively influence the normal development of the newborn by later manifesting specific ASD symptoms [63, 64, 65]. For decades it has been thought that they are germ-free, but recent studies using meconium samples revealed that the colonization begins *in utero* [66, 67]. As mentioned at the beginning of chapter II, the delivery mode could influence the infant's gut flora in terms of acquisition potentially harmful microbes such as *Salmonella*, *Campylobacter* and *Shigella* species, *Yersinia enterocolitica* or *Shiga toxin-producing Escherichia coli* [68], to the detriment of the beneficial ones like *Bifidobacterium*, *Eubacterium*, *Lactobacillus* [27]. Thus, a dysbacteriosis in the mother's microbiota may be the endpoint of response to environmental or hereditary risk factors. Consistent with this concept, epidemiological studies conducted on human and animals have shown that the mother's diet during gestation, especially fat-rich alimentation and metabolic conditions (especially obesity and diabetes) could amplify the possibility of acquiring a neurobiological disorder in the offspring [69, 70]. Stress-related HPA deficiency during pregnancy creates an imbalance, effect passed onto the neonate at birth with unwanted and long-lasting repercussions [71, 72].

Alterations of the microbiome associated with ASD

It has been noticed that ASD patients present alterations in the composition of gut flora compared to the control. Kang and his team showed a less diversity and distinct category of microbes in children with ASD, including *Coprococcus*, *Prevotella* and *Veillonellaceae* genus whose species are responsible for fermentation and carbohydrate deglutition [73]. Also, an analysis of faecal microflora from children with regressive autism revealed an elevated abundance of *Clostridium*species compared to the stools of the control [74]. It has also been seen at phylum level in children with ASD an increased ratio of *Bacteroidetes* and *Firmicutes* [75].

Immune system deregulation and association with gut microflora

Progressive evidence indicates that the immune system deregulation contributes equally next to other factors mentioned earlier in the development, respectively the pathophysiology of ASD. Such aberrations have been described in both young and old age subjects defined by pro- and inflammatory reactions on the brain and cytokine profiles in the cerebrospinal fluid and blood followed by a weakening of immune cell activity due to an increased presence of brain-specific auto-antibodies [76]. Neuroinflammatory reactions underway are presented as well as in postmortem brain specimens, existing an overexpression of microglia, in parallel with an exaggerated production of proinflammatory cyto- and chemokines including small inductible cytokine A2, tumour necrosis factor (TNF)-α, lymphocyte activating factor 1β, interleukin 6, natural killer cell stimulatory factor 2 and interferon gamma (IFNγ) [77, 78]. For example, commensal species *Bacteroides fragilis* can improve symptoms of multiple sclerosis and intestinal deficiencies through a detain of T helper 17 cells reaction and enhancing levels of human cytokine synthesis inhibitory factor by producing T regulatory cells [79, 80]. Despite the multitude of reported studies where a deterioration of the immune system has been found, the exact mechanism of action against the integrity of the microbiome in the case of patients with ASD remains an aspect that needs to be deepened.

Microbial metabolites associated with ASD

Liquid-chromatography-mass spectrometry (LC-MS) is an eloquent example of the analysis of alterations of various metabolites in patients suffering from ASD along with the improvement of these techniques. By analyzing samples of faecal and urinary from ASD children was revealed a substantial concentration of short-chain fatty acids (SCFAs) and 4-methylphenol compared to the control [81, 82]. Propionic acid (PPA), is a short-chain fatty acid produced by ASD-associated gut bacteria like *Clostridium*, *Bacteroides* and *Desulfovibrio* in the gastrointestinal tract. Studies performed on rodent highlighted that rats treated with propionic acid

displayed limited concern action, impaired liveable conduct and perception as well as an inductive inherent neuroinflammatory reply [83, 84, 85]. Another major metabolite 4-methylphenol and its derivates p-cresyl sulphate, p-cresyl glucuronate and free p-cresol levels could be considered as biomarkers in children with ASD, especially in women and males who are affected more severely [82, 86]. Butyric acid (BA) is another short-chain fatty acid produced by anaerobic bacteria that modulates transepithelial transport and participates in mitochondrial optimum's function, stimulates oxidative phosphorylation and fatty acid oxidation [87].

Gastroenterological and Metabolic Relevance

The possibility of a link between the microbiome deregulation and the intestinal microflora thanks to frequency of gastrointestinal deficiencies in patients with ASD is a very debated issue at present. There are both pros and cons in favour of this hypothesis, researchers claiming the realization and reliability of conducting such studies on larger cohorts (thousands or tens of thousands). A significant percentage of individuals exhibit symptoms similar to irritable bowel disease (IBD) or Crohn's syndrome [88]. Among the most common signs we can cite: constipation and diarrhea in the first place, vomiting episodes, abdominal cramps, respectively upward pain, unusual odour of the stool or lack of appetite [89, 90]. Besides these, states like anxiety, depression, intentional injury or aggression have been as well observed [91]. Recently, a mega-analysis of 14.000 individuals under the age of 35 with ASD revealed a higher prevalence of IBD next to other gastrointestinal disorders compared to the control [92]. GI pathology such as intestinal mucosa damage and "leaky gut'' phenomena has been observed in a study including non-autistic first-degree relatives highlighting the role of heritability in this context [93]. One genetic risk factor associated with a subgroup of ASD patients with co-occurring comorbid GI symptoms is a variant in the promoter of the MET receptor tyrosine kinase, known to be involved in brain development in GI repair [94]. Also variants of serotonergic transporters (hSERT) are participants in autism [95, 96], and beyond its well-known implication as a brain's neurotransmitter, 5-hydroxytryptamine maintains the gut's normal activity [97, 98]. Mitochondria, very dynamic intracellular organelles containing their own genome and protein translation machinery, an eventual dysfunction being recognized as a pawn in ASD. With essential roles in generating adenosine triphosphate (ATP) and energy transduction, cell signalling, apoptosis and oxidative metabolism of eukaryotic cells, it is not surprising that mitochondrial dysfunction is associated with various diseases, including metabolic unrests, neurodegenerative disorders and tumorigenesis [99, 100]. Ketogenic diet (KD) has proven to be a powerful tool with a remarkable improvement in mitochondrial optimum activity used for many decades to treat epilepsy, and it has also been

shown to increase the functionality of those devices along with the potentiation of additional molecular targets for comorbid ASD-associated symptoms [101]. Inborn errors of metabolism such as phenylketonuria, recessive autosomal metabolic disorders, histidinemia and dihydropyrimidine dehydrogenase deficiency, deregulation of purine metabolism, adenylosuccinate lyase, an over-activity of 5'nucleotidase or a poor one of phosphoribosylpyrophosphate synthetase, aspects described in what follows bringing a dramatic melioration in symptomatology [102]. Another surprise is supported by the finding that a large number of persons with ASD have medical conditions as epilepsy, with a predominant prevalence of epilepsy-resistant treatment in children with ASD compared to those without ASD, these indications suggesting that current treatments are far from optimal. A lot of information on how metabolic disturbances in biotin, creatinine, carnitine, cholesterol activity and so on is just starting to appear [103].

Given the observation that the emergence of neurobehavioral symptoms and chronic dysentery occurred as a result of repeated antibiotic treatments in a subgroup of children with regressive ASD, a hypothesis has been postulated according to which that this outcome is constituted by the activity of a toxin produced by a species of the *Clostridium* genus. In the case of regressive autism, there is a transition period between normal or approximately normal development, followed by a cessation characterized by a progressive regression of the cognitive processes previously acquired [104]. One of the most significant features of this hypothesis is the existence of a number of up to 10 times more species belonging to *Clostridium* genus especially (*Clostridium histolyticum* - clusters I and II, *Clostridium butyricum*, *Clostridium difficile*, *Clostridium ramosum, Clostridium bolteae*) [105, 106, 107]. However many members of this heterogeneous group are non-pathogenic bacilli who contribute to the gut's homeostasis [108]. Another category of strictly anaerobic gram negative bacteria with implications in ASD is represented by *Sutterella* genus. Following biopsies taken from the intestinal tract of subjects with ASD, a significant prevalence of *Sutterella* species was observed. Based on their results, it can be concluded that *Sutterella* genus is an integrated part in such situations compared to typical intestinal dysfunctions [109]. In support to this hypothesis is the additional evidence that Wang brings. Following the analysis of faecal samples of young subjects, *Sutterella* and *Ruminococcus torques* were dominant [110]. While *Bacteroidetes* and *Prevotella* are commonly considered to be "pillars of resistance" to colon integrity, ASD manifestations could be considered the results of a disproportionality between beneficial/harmful species.

In agreement with the Food and Agriculture Organization of the United Nations and in accordance with the guidelines established by the World Health

Organization, probiotics can be defined as "living microorganisms which, in adequate doses, confer a shield to the host by generally improving the state of health" [111]. Modern techniques for manipulating the gut flora by using probiotics have demonstrated an extraordinary ability to suppress pathogens in epithelium, intestines, but also in regulating immune cells activity [112]. The beneficial role of probiotics in supporting intestinal flora homeostasis in both normal and dysbacterial conditions is frequently discussed in clinical practice, with a considerable interest as indicated by expanding markets, some of which are proving to have benefits in situations of gastrointestinal deficiencies [113, 114]. For probiotics to exert their effects in order to restore the microbiome homeostasis, microbes must first be able to survive the route through intestinal tract, but also to continue their spreading and evolving in the presence of the bile [36]. The lactic acid producing bacteria (*Lactobacillus*, *Bifidobacteria, Saccharomycetes* or *Lactococcin*) is currently among the most recommended for use, preventing or even treating diseases like obesity [115], colorectal cancer [116] and Crohn's disease [117]. Unlike probiotics, prebiotics are food supplements administrated to stimulate the growth and/or activity of potentially beneficial bacteria [118]. In contrast, synbiotics are a mixture of the two biotic categories mentioned above, whose main purpose is to increase the living and colonization of those already existing in the intestinal tract [119]. Faecal Microbiota Transplantation (FMT) and Microbiota Transfer Therapy (MTT) are also tools intended for the reconstruction of the intestinal flora. Although the two techniques are quite similar, FMT involves the transfer of faecal microflora from a healthy individual to a diseased subject, while MTT is essentially a modified FMT protocol. The reliability of the two has been thoroughly analyzed, ultimately proving to be strong alternatives for treating irritable bowel syndrome and inflammatory bowel disease [120, 121], or other ASD-associated symptoms [122]. Studies on how such entities shape human microbiome in ASD are summarized in Table 2.

Table 2. Human epidemiologic studies on the ASD symptomatology

Model	Treatment	Main observations	Note
33 children with ASD	Delpro® Probiotic containing Lactobacillus acidophilus/casei/delbruecki, Bifidobacterium longum/bifidum and 8 mg of Del-Immune V® powder	88% of individuals reported a significantly decrease of Autism Treatment Evaluation Checklist (ATEC), 52% a decreased in constipation severity and 48% in diarrhea with two cycles (21 days therapy period and 21 post-treatment)	[123]
12 year old boy with ASD, severe cognitive	Administration of a mixture probiotic VSL#3 containing $9x10^{10}$ Bifidobacterium *breve*/*longum* and *infantis*,	After 4 weeks of treatment and 4 months follow-up this probiotic reduced significantly the severity of abdominal symptoms concomitant with	[124]

disability and celiac disease	$8x10^{10}$ *Lactobacillus acidophilus/plantarum/casei/bulgaricus/delbrueckii* subsp and $20x10^{10}$ *Streptococcus thermophilus/salivarius* subsp.	an improvement in Autistic core symptoms	
3 autistic and 3 non-autistic children (*in vitro* model)	Administration of a prebiotic who contains galactooligosaccharide (B-GOS) containing oligogalactosyllactose, lactose, dextrose and galactose	With 2g/daily has been increased the number of *Bifidobacterium* species in both situations alongside acetate and butyrate	[125]
10 autistic children, 9 brethren and 10 healthy	Administration of a probiotic who contains *Lactobacillus*, *Bifidobacterium* and *Streptococcus* three times per day	After 4 months of treatment, the amount of *Bacteroidetes/Firmicutes* ratio was normalized, in parallel with an increased level of *Desulfovibrio* and *Bifidobacterium* spp	[126]
22 autistic children with GI dysfunctions	Twice per day orally administration of a probiotic who contains *Lactobacillus acidophilus* (strain Rosell-11 containing $5x10^9$ CFU/g)	After 2 months of treatment the level of D-arabinitol was reduced in the urine of the subjects	[127]
11 children with regressive-onset ASD	Orally administration of a mixture probiotic containing *Lactobacillus acidophilus/bulgaricus* and *bifidum* ($40x10^9$ colony-forming units/mL) for 4 weeks and 500 mg of Vancomycin 4 times per day for 8 weeks	After 12 weeks of treatment, general condition improvement previously acquired has regressed with discontinuation of treatment. Fecal SCFAs level was higher in ASD subjects	[128]

Conclusions

Thus our mini-report presented here is confirming a strong correlation between the autistic pathology and the microbiome, as well as the aforementioned described gastrointenstinal and metabolic associated deficiencies.

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