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Title:

Role of *Lacticaseibacillus rhamnosus* SL42 in modulating wistar rat autistic behavior.

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DEDICATION

We dedicate this work to:

Our parents

Dear Parents, after God, it is thanks to you that we have finally achieved success after years of hard effort. Thank you for your help and support. You've always been an idol of complete commitment and dedication.

Alhousseini Issaka Maman Bachir

Saleh Mahmoud Warak Ibrahim

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ABSTRACT

Several studies have shown a link between brain development and autism (ASD) neurobehavior. Exposure to specific gut microbiota induces behavioral changes in animals as well as humans. Current literature indicates that the gut microbiota may affect several mechanisms related to autism. The purpose of this study was to evaluate the role of a probiotic strain from breast milk on ASD Wistar rat neurobehavior at juvenile age. A rat autistic-like model was produced by intraperitoneal injection of sodium propionic acid (PPA) to 3-week-old rats from day -4 to day 0. The rats were given the probiotic strain of *Lacticaseibacillus rhamnosus* SL42 (10^9 CFU/mL) daily from day -4 to day 14 and compared to control and PPA-induced rats without treatment. Behavioral tests (grip test, social behaviors, open field, and Elevated plus-maze) were performed on days 7 and/or 14. At the end of the experience, brain tissues were subjected to histological analysis. Significant positive effects of the SL42 probiotic strain have been observed on social interaction, and anxiety parameters. In addition, probiotic treatment corrects the PPA-induced neuron number increased in the prefrontal cortex and improves prefrontal-related behavioral functions. These data suggest that probiotic strain SL42 supplementation could be a potential therapeutic method to correct ASD-like symptoms in toddlers.

Keywords: Autism spectrum disorder, Probiotic, Wistar Rat, Propionic acid, neurobehavior.

RÉSUMÉ

Plusieurs études ont montré un lien entre le développement du cerveau et le comportement neurologique de l'autisme (TSA). L'exposition à un microbiote intestinal spécifique induit des changements comportementaux chez l'animal et chez l'homme. La littérature actuelle indique que le microbiote intestinal peut affecter plusieurs mécanismes liés à l'autisme. L'objectif de cette étude était d'évaluer le rôle d'une souche probiotique provenant du lait maternel sur le comportement neurologique des rats Wistar atteints de TSA à l'âge juvénile. Un modèle autistique a été produit par injection intrapéritonéale d'acide propionique sodique (PPA) à des rats âgés de 3 semaines, du jour -4 au jour 0. Les rats ont reçu la souche probiotique Lacticaseibacillus rhamnosus SL42 (10^9 CFU/ml) quotidiennement du jour -4 au jour 14 et ont été comparés au contrôle et aux rats induits par le PPA sans traitement. Des tests comportementaux (test d'agrippement, comportements sociaux, champ ouvert et labyrinthe en croix surélevé) ont été réalisés aux jours 7 et/ou 14. À la fin de l'expérience, les tissus cérébraux ont été soumis à une analyse histologique. Des effets positifs significatifs de la souche probiotique SL42 ont été observés sur les paramètres d'interaction sociale et d'anxiété. En outre, le traitement probiotique corrige l'augmentation du nombre de neurones induite par le PPA dans le cortex préfrontal et améliore les fonctions comportementales liées au préfrontal. Ces données suggèrent que la supplémentation de la souche probiotique SL42 pourrait être une méthode thérapeutique potentielle pour corriger les symptômes de type TSA chez les jeunes enfants.

Mots-clés : Trouble du spectre autistique, probiotique, rat Wistar, acide propionique, comportement neurologique.

ملخص

وقد أظهرت العديد من الدراسات وجود صلة بين نمو الدماغ والسلوك العصبي للتوحد (ASD). يؤدي التعرض لميكروبات أمعاء معينة إلى تغيرات سلوكية عند الحيوانات وكذلك البشر. تشير المقالات العلمية الحالية إلى أن ميكروبيوتا الأمعاء قد تؤثر على العديد من الآليات المتعلقة بالتوحد. كان الهدف من هذه الدراسة هو تقييم دور سلالة البروبيوتيك من حليب الأم على السلوك العصبي لجرذان ويستار التي تعاني من اضطراب طيف التوحد في سن الطفولة المبكرة . تم إنتاج نموذج شبيه بالتوحد لدى الجرذان عن طريق الحقن داخل الصفاق بحمض بروبيونيك الصوديوم (PPA) للجرذان البالغة من العمر 3 أسابيع من اليوم -4 إلى اليوم 0. أُعطيت الجرذان سلالة البروبيوتيك من Lacticaseibacillus rhamnosus SL42 (10^9 وحدة CFU/مل) يوميًا من اليوم -4 إلى اليوم 14، وتم مقارنتها بالجرذان السليمة والتي تم حقنها بـحمض بروبيونيك الصوديوم دون علاج. أُجريت الاختبارات السلوكية (اختبار القبضة والسلوكيات الاجتماعية والمجال المفتوح والمتاهة العالية) في اليوم 7 و/أو 14. وفي نهاية التجربة، خضعت أنسجة المخ للتحليل النسيجي. لوحظت تأثيرات إيجابية كبيرة لسلالة البروبيوتيك SL42 على التفاعل الاجتماعي ومعايير القلق. وبالإضافة إلى ذلك، يصحح العلاج بالبروبيوتيك زيادة عدد الخلايا العصبية الناجمة عن حمض بروبيونيك الصوديوم (PPA) في قشرة الفص الجبهي ويحسن الوظائف السلوكية المتعلقة بالفص الجبهي. تشير هذه البيانات إلى أن مكملات سلالة البروبيوتيك SL42 يمكن أن تكون طريقة علاجية محتملة لتصحيح الأعراض الشبيهة بالتوحد لدى الأطفال الصغار.

الكلمات المفتاحية: اضطراب طيف التوحد، البروبيوتيك، جرذ ويستار، حمض البروبيونيك، السلوك العصبي.

ACRONYMES ET ABBREVIATIONS

DSM-V: Diagnostic and Statistical Manual of Mental Disorders, fifth edition

ASD: Autism spectrum disorders

ADHD: Attention deficit hyperactivity disorder

ICD-10: International Classification of Diseases, 10th revision

ICD-11: International Classification of Diseases, 11th revision

ID: Intellectual disability

NDDs: Neurodevelopmental disorders

NDD: Neurodevelopmental disorder

MIA: Maternal immune activation

SCFAs: Short-chain fatty acids

BBB: Blood-brain barrier

IL: Interleukin

TNF-α: Tumor necrosis factor alpha

β: beta

α: alpha

TH: T helper

PDD: Pervasive developmental disorders

***B. longum* :** Bifidobacterium longum

***IBA-1*:** Ionized calcium-binding adapter molecule 1

IBD: Inflammatory bowel disease

FAO: Food and Agriculture Organization

WHO: World Health Organization

LB: *Lactobacillus*

GI : Gastrointestinal

MGB-axis : Microbiota-gut-brain axis

TJ: Tight junction

Trp: Tryptophan

EC: Enterochromaffin

QA: Quinolinic acid

NMDA: N-methyl-D-aspartate

CNS: Central nervous system

ENS: Enteric nervous system

SGLT1: Sodium glucose cotransporter1

GLUT2: Glucose transporter 2

LMBAFS: Laboratory of Beneficial Microorganisms, Functional Foods and Health

SL: *Lacticaseibacillus rhamnosus*

SI: Sociability index

SPI: Social preference index

5-HT: 5-Hydroxytryptamine(serotonin)

BDNF: brain derived neurotrophic factor

IFN-γ: Interferon gamma

PBS: phosphate buffered saline solution

PPA: propionic acid

VPA: valproic acid

HE: Histological examination

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Introduction

Autism spectrum disorders represent psychological conditions characterized by repetitive stereotypical behaviors and pervasive abnormalities in social interaction and communication. In Algeria, the number of autistic children **(Fadel *et al*., 2022)** is between 400000 and 500000 cases with a male prevalence (three to four boys for every girl). To date, rare statistical literatures have been published on ASD research.

Several neuroanatomical abnormalities have been reported to be associated with ASD. Studies have highlighted that amygdala, hippocampus, cerebellum, and prefrontal regions are structurally distinct in people with ASD **(Amaral *et al*., 2008).**

The involvement of the prefrontal lobe in the neurobiology of ASD has been documented in the literature. The prefrontal lobe has a central role in executive functions and emotion recognition and communication. These processes are both compromised in ASD. Brain imaging studies of patients with ASD have shown overgrowth in the prefrontal cortex with an increase in the number of neurons and metabolic activity **(Courchesne *et al*., 2011).**

Clinical studies showed that gastrointestinal symptoms often occur in ASD children, and their severity is related to the degree of behavioral disorders. The pathogenesis of ASD is not clear, but it has proved to be related to the gut microbiota **(Toh and Allen-Vercoe, 2015).** It is well-established that the gut tract contains trillions of bacteria that regulate the production of various signaling molecules in a host. The neurotransmitter is an endogenous metabolite that acts as a chemical messenger to carry, amplify and regulate signals between neurons and other cells. These substances play an important role in the normal life activities of the body and maybe the bridge or messenger between the gut microbiota and neurodevelopment.

Due to technology or ethics limitation, many ASD related studies cannot directly apply to humans. Animal models can be very convenient to study behaviors and mechanisms. Propionic acid (PPA) injection triggers ASD-like behaviors and neuroinflammatory reactions in animals. Like other mammals, the brains and bodies of neonatal rats develop in concert with motor skills and behaviors during the first three weeks of life. During that time, the injection of PPA acts like a neurotoxic agent, and rats exhibit abnormal behavioral patterns, such as abnormal social interactions and anxiety-like behaviour **(Al-Ghamdi *et al*., 2014).**

Therefore, we tend to determine the potential protective effect of co-administration of probiotics on autistic-like behaviors by analyzing the alteration of number of neurons and neurobehavioral activity in the prefrontal cortex of PPA-induced Wistar rat at juvenile age of three weeks.

Chapter I: Autism Spectrum Disorders

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I.1. General information on autism

According to the DSM-V (Diagnostic and Statistical Manual of Mental Disorders, fifth edition), neurodevelopmental disorders are "a group of conditions that begin during the developmental period. They typically manifest early in development, often before the child even enters primary school. They are characterized by developmental deficits that lead to impaired personal, social, academic or professional functioning." Neurodevelopmental disorders include intellectual disabilities, communication disorders, autism spectrum disorders (ASD), attention deficit hyperactivity disorder (ADHD), learning disabilities, motor disorders, and a range of other specified and unspecified neurodevelopmental disorders **(American Psychiatric Association, 2013).**

I.1.1. Definition

For the first time, **Leo Kanner (1943; cited by Wing & Potter, 2002)** introduced the term autism as a diagnostic label to define a specific syndrome seen in young children, which manifests itself as early onset, characteristic symptomatology, and disturbed social and emotional relationships. Since then, autism has been recognized as an autism spectrum disorder (ASD), which is classified as a developmental disorder as defined in the American Psychiatric Association's DSM-5 (Diagnostic and Statistical Manual of Mental Disorders, 5th edition) and the World Health Organization's ICD-10 (International Classification of Diseases, 10th revision). Autism is characterized by significant impairment in social communication and atypical, repetitive, and/or restrictive behaviors or interests. The American Academy of Pediatrics recommends screening all infants and young children for early signs of autism at 18 months of age and again at 24 months of age. Over the years, diagnostic criteria have changed and now include a much broader spectrum **(Wing & Potter, 2002).**

These criteria, as defined by **the American Psychiatric Association (2013),** also include early onset of symptoms before the age of three and persistence throughout life.

However, diagnosis is often delayed due to the heterogeneity of symptoms, resulting from the multiple genetic and environmental origins of the disorder **(Christensen *et al.,* 2013; Masini *et al.,* 2020).** Differences in presentation between boys and girls, especially in terms of behavior in difficult situations, are notable according to ICD-11. Men tend to express their frustration aggressively, while women show emotional changes by isolating themselves. In addition,

women diagnosed with ASD associated with intellectual disability outnumber men, although they exhibit fewer restricted and repetitive behaviors **(Mandy *et al.,* 2012).**

ASD symptoms persist throughout life, negatively impacting psychosocial functioning in adulthood and later life, even without intellectual disability. Most people with ASD fail to live independently or engage in paid employment, and communication problems and social isolation can also have significant health consequences for older adults with ASD **(Howlin *et al.,* 2013).** Communication problems and social isolation can also have a significant impact on the health of older adults with ASD.

I.1.2. Diagnostic criteria

According to the **DSM-V, (2013)**, a child must have at least two of the four types of repetitive and stereotyped behaviors, as well as persistent deficits in all three sub-symptoms of communication and social interaction disorders.

I.1.2.1. Deficiencies

Persistent deficits in communication and social interaction could be observed in a variety of settings. These can be manifested by the following, either in the background or during the current period (examples are illustrative and not exhaustive):

a. Social or emotional reciprocity deficits including abnormalities in social approach and inability to normal two-way conversation, difficulties in sharing interests, emotions, and affects, and an inability to begin or respond to social interactions.

b. Deficits in nonverbal communication behaviors used in social interactions, such as faulty integrations between verbal and nonverbal communication, abnormalities in eye contact and body language, deficits in understanding and use of gestures, or a complete absence of facial expression and nonverbal communication.

c. Deficits in developing, maintaining, and understanding relationships, such as difficulties adjusting behavior to different social contexts, difficulties in sharing imaginative play, or making friends.

I.1.2.2. Behaviors

The behaviors, interests or activities are restricted and repetitive, as evidenced by at least two of the following, either in the current period or in the history (examples are illustrative and

not exhaustive): have stereotyped or repetitive movements, object use, or language (e.g., simple motor stereotypies, toy alignment, or rotation of toys, objects, echolalia, idiosyncrasy).

b. Intolerance of change, unwavering adherence to ritualized verbal or nonverbal routines or behaviors (e.g., extreme distress caused by minor changes, difficulty managing transitions, rigid ways of thinking, ritualized greetings, need to continue on the same path or eat the same foods every day or both).

c. Extremely limited and permanent interests, unusual both in intensity and purpose (such as attachment to or concern about unusual objects, excessively limited or persevering interests).

d. Hyperresponsiveness or hyporesponsiveness to sensory stimuli or unusual interest for sensory aspects of the environment, for example: apparent indifference to pain or temperature.

ASD is linked to many disorders and diseases. They can be neurodevelopmental, mental, or behavioral, such as ID or language impairment, but they can also be related to known medical or genetic conditions, such as Rett syndrome, or environmental factors such as exposure to pollutants or medications. Better patient management is possible by taking into account the severity of ASD and associated disorders **(Table 1).**

Table 1 : Severity Levels Associated with Symptoms of Social Communication and Restricted, Repetitive Behaviors (Mughal et al., 2022)

Severity Level	Required level of support	Social communication	Restricted, repetitive behaviors
Level 1 (Mild)	Requiring support	Difficulty initiating conversations and social interactions decreased interest in social interactions Unsuccessful or atypical responses to social overtures of others	Inflexibility of behavior causes significant interference with functioning in at least one area of activity
Level 2 (Moderate)	Requiring substantial support	Marked deficits in both verbal and nonverbal social communication skills limited initiation of social interactions Reduced or abnormal responses to social overtures from others	Inflexibility of behavior and difficulty coping with change cause significant interference with functioning in various areas of activity
Level 3 (Severe)	Requiring very substantial support	Severe deficits in both verbal and nonverbal social communication skills very limited initiation of social interactions Minimal response to social overtures from others	Inflexibility of behavior and extreme difficulty coping with change cause significant interference with functioning in all areas of activity

I.2. Etiology

The etiology of ASD is very complex and involves genetic, epigenetic and environmental factors (pre, peri or postnatal), taking place during development, *in utero* and during childhood. New advances in genomics and epigenomics seem to open up a new avenue for understanding the etiology of ASD.

I.2.1 Genetic factors

Heredity for ASD is very important as it is estimated to be between 50 and 90% **(Hallmayer *et al.,* 2011; Tick *et al.,* 2016).** A child's likelihood of being diagnosed with ASD varies depending on the percentage of genetic makeup they share with their parent, sibling, with ASD **(Constantino *et al.,* 2010; Risch *et al.,* 2014; Sandin *et al.,* 2014),** with an 18.7% risk of recurrence between non-twin siblings **(Ozonoff *et al.,* 2011).** For monozygotic and dizygotic twins, the probability of having the same diagnosis of ASD is 60% and 10%, respectively **(Bailey *et al.,* 1995).** Nevertheless, an effect of gender is possible on this prevalence. Indeed, a quantitative study related to sex-specific differences showed that autistic symptoms were greater in female twins than in male twins **(Robinson *et al.,* 2013).**

I.2.2. Environmental factors

It is clear that genetic factors are not the only ones responsible for the increase in ASD prevalence, as they account for only 10-25% of ASD etiology **(Bourgeron, 2015; Huguet *et al.,* 2013).** Environmental factors are therefore thought to be mainly involved in the onset of ASD. Several environmental risk factors have been clinically identified, the majority of which occur during gestation. Maternal autoimmunity confers a significantly increased risk of neurodevelopmental disorders (NDDs) in offspring, including ASD **(Chen, 2016).** Maternal obesity, diabetes, and immune-mediated diseases such as asthma also significantly increase the risk of NDD, as do exposure to toxic substances, pesticides, and air pollution. These disorders and exposures have in common immune activation and increased inflammation **(Han, 2021).** Notably, inflammation during gestation has been identified as an important risk factor for ASD and other neurological disorders, although a recent population study in Sweden identified only an association, not a cause-and-effect relationship, between maternal infection and ASD **(Atladottir, 2010; Brown, 2014; Brynge, 2022).** In support of these findings, animal models of maternal immune activation (MIA) with various immune initiators revealed ASD-related behaviors and provided evidence of innate immune activation in MIA's offspring, suggesting that early exposure to maternal inflammation may inappropriately prime the fetal immune

response and may lead to future dysfunction of this branch of the immune system **(Meyer, 2014; Patterson, 2011; Careaga *et al.*, 2017).**

I.2.3. Epigenetic risks

The presence of diseases in the mother is a significant risk factor. A meta-analysis showed that the risk of developing ASD in children with diabetic mothers is 62%, 28% in children with underweight mothers, and 36% in children with obese mothers **(Wan *et al.*, 2018).**

Several direct and indirect mechanisms act on the link between gut microbiota and microglia **(figure 1).** Under physiological conditions, bacterial metabolites such as short-chain fatty acids (SCFAs) can act directly through the vagus nerve or indirectly through modulation of the peripheral immune system.

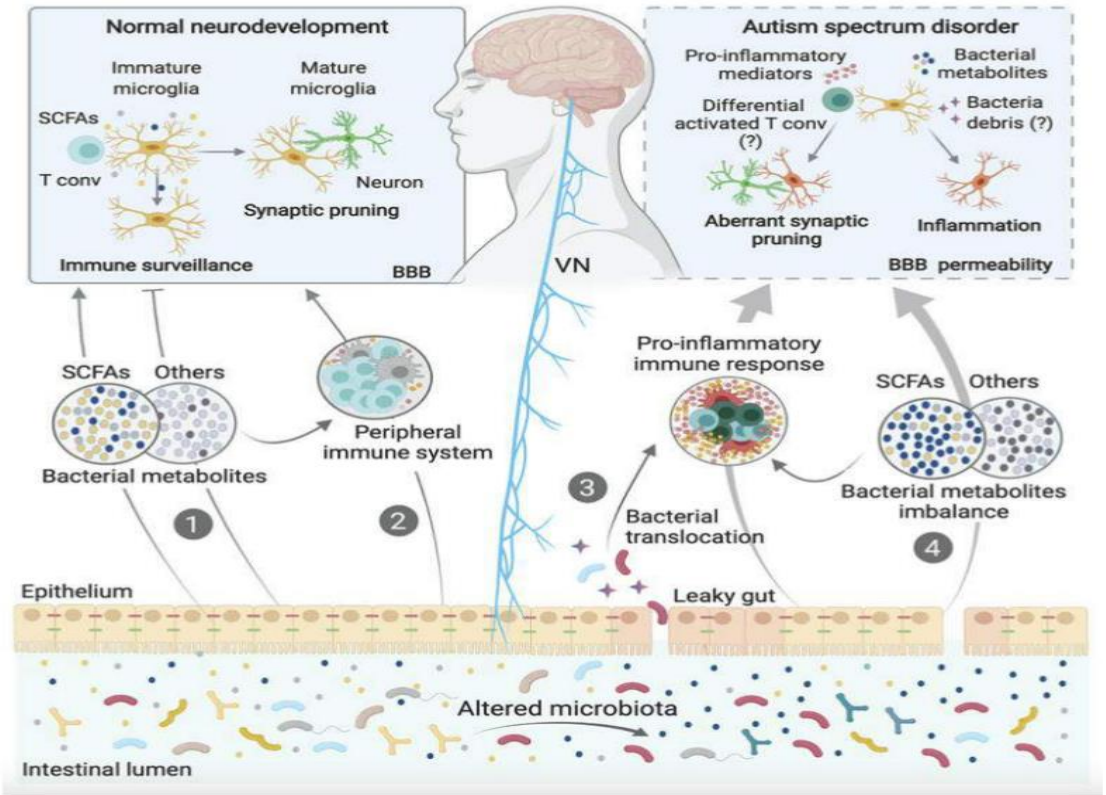

Figure 1: Interactions between gut microbiota and microglia in ASD **(Davoli-Ferreira *et al.*, 2021).**

In ASD, dysbiosis induces greater intestinal permeability **(figure 2)** and thus the translocation of bacteria, and an imbalance of SCFAs and other bacterial metabolites (**figure explanation** 3-4). This results in a proinflammatory response from the peripheral nervous system, and thus produces pro-inflammatory molecules and cytokines, which can directly activate the vagus nerve and cross the blood-brain barrier (BBB). This pro-inflammatory response has consequences for microglial functions, including monitoring, synaptic pruning, and inflammation, which contributes to autistic symptoms according to **Davoli-Ferreira *et al.* (2021)**.

In addition, an association between obesity and maternal diabetes, whether pre-gestational or gestational, increases the prevalence rate of ASD associated with ID by more than 3% **(Li *et al.*, 2016).** Other diseases, such as autoimmune diseases, viral and bacterial infections, are associated with the risk of developing ASD **(Jiang *et al.*, 2016; Zerbo *et al.*, 2015).** Infections will cause an activation of the maternal immune system and an increase in pro-inflammatory molecules, cytokines and chemokines, which have the ability to cross the blood-brain barrier (BBB) but also to modify the characteristics of this barrier **(Zawadzka *et al.*, 2021).**

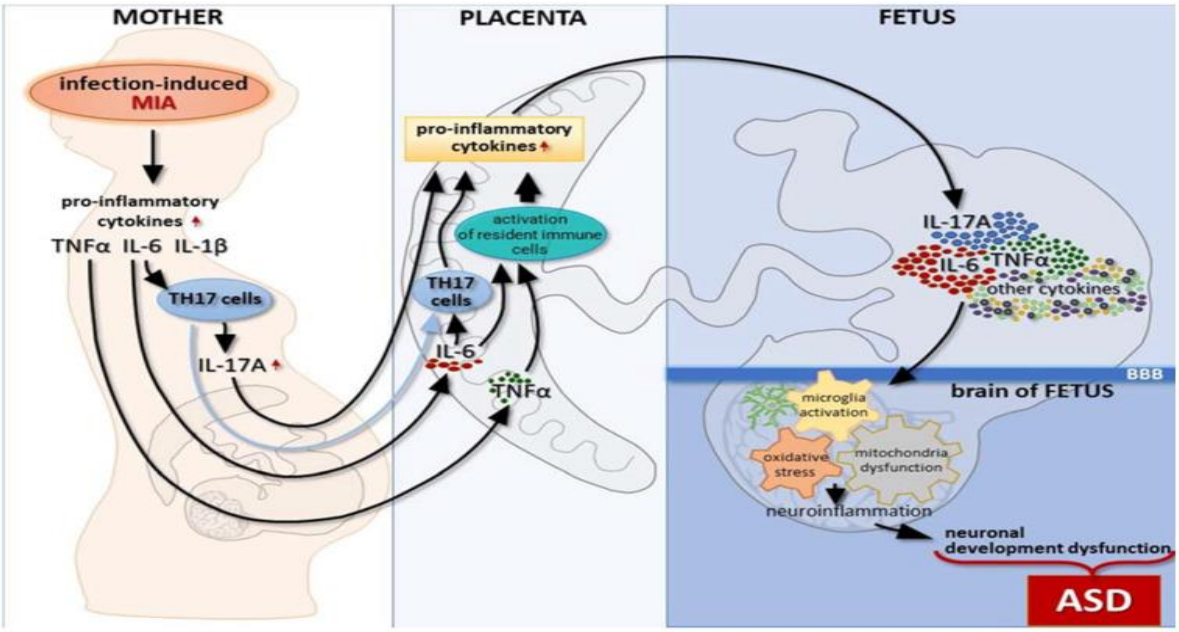

Figure 2: Immunological modification of the placenta and fetal brain **(Zawadzka *et al.*, 2021).**

In response to systemic inflammation during pregnancy leading to dysfunction of neural development in offspring. Infection during pregnancy activates the mother's immune system, which releases pro-inflammatory cytokines, such as IL-6, Il-1β, TNF-α and others. The high level of IL-6 leads to the activation of maternal TH17 cells. As a result, IL-17A is released and, together with IL-6 and TNF-α, can reach the placenta, where they additionally activate resident immune cells, resulting in increased production of pro-inflammatory cytokines, including IL-6. In addition, activated maternal TH17 cells also transmigrate across the placenta and enhance cytokine production, which affects placental function and causes damage. This allows cytokines to pass to the developing fetus and enhance the production of fetus-derived cytokines. Then, proinflammatory cytokines cross the BBB and trigger a cascade of neuroinflammation **(Zawadzka *et al.*, 2021).**

I.3. Therapeutic strategy

Currently, there is no drug treatment that can cure autism spectrum disorder (ASD). However, taking certain treatments can improve behaviors that interfere with socialization.

In order to prevent depression, anxiety or problematic behaviors related to PDD, sleep disorders, drug treatment can be combined with non-drug educational and therapeutic interventions. The Gut Microbiota may be a novel therapeutic target in this illness, with potential for treatment using certain antibiotics, microbiota transplantation, and diet modulation with prebiotics and probiotics **(Dinan and Cryan, 2017).**

The fact remains that the medications used to treat many mental disorders may not be effective or even tolerated by patients. Alternate therapies using prebiotics or probiotics may be of benefit. Nonetheless, further investigation is needed, particularly regarding the composition of the microbiota and how diet and prebiotics or probiotics influence the microbiota. This new field may bring possibilities of new and inexpensive treatments **(Lankelma et al., 2015).**

After probiotic supplementation, the brain activity of ASD children (showing an improvement in excitatory/inhibitory imbalance) suggested that probiotics can promote a change in brain activity in ASD children toward that of controls. Moreover, probiotic administration was found to promote a shift in brain connections toward a more typical pattern with respect to coherence and asymmetry. Importantly, probiotics could significantly improve the brain function of animals with ASD. For example, immunohistochemical analysis of brain tissues showed that *B. longum* CCFM1077 could ameliorate microglia activities in the

cerebellum of autistic rats, as evidenced by the decreased *IBA-1* protein expression **(Kong et al., 2022).**

Chapter II:

The Gut Microbiota: Role and Implication in Autism Spectrum Disorders

Chapter II: The Gut Microbiota: Role and Implication in Autism Spectrum Disorders.

II.1. General concept

The gut microbiota or gut flora refers to all the microorganisms that make up the digestive system. It is estimated that there are up to 10^{18} microorganisms weighing up to 2 kg in the human digestive tract, including bacteria (10^{14}), which is 10 times more bacterial cells than the number of human cells, yeasts, fungi and viruses **(M *et al.*, 2018).** The gut microbiota occupies a prominent place among the microbiota of the human body, extending over 400 m^2 of the intestinal wall, making it an entire organ. By creating a symbiotic bond, bacteria play a critical role in maintaining immune and metabolic balance, as well as an essential protective system against external pathogens.

II.1.1. Dysbiosis in Autism Spectrum Disorder

A high-fat maternal diet during pregnancy alters the microbiota in newborns and may be associated with ASD in men **(Connolly *et al.*, 2016).** Breastfeeding is associated with a lower risk of ASD if continued for 6 months, while formula-fed infants have a greater representation of *Clostridium difficile* in the gut **(Schultz *et al.*, 2016).** Antibiotic treatments, even if undertaken for a short period of time, can induce long-lasting alterations in the gut microbiota, both in humans and in animal models**. Yassour *et al.* (2016)** demonstrated that children treated with antibiotics during the first three years of life have different compositions of the gut microbiome, while **Korpela *et al.* (2016)** showed that a sustained alteration of the gut microbiota in children after macrolide antibiotic treatment may be associated with obesity and asthma. Gut dysbiosis is reported in several conditions, such as immunological abnormalities, Crohn's disease, obesity, inflammatory bowel disease (IBD), and abnormal behaviors in children (including those with ASD).

There is evidence that autistic people have problems with maldigestion and malabsorption. A recent study analyzed the duodenal mucosal microbiome of autistic subjects and found no difference in the oral microbiome diversity compared to the healthy control group, as well as no difference in disaccharidase activities **(Kushak *et al.*, 2017).** The oral microbiota has recently been studied, but no difference was found in the richness and diversity of the

microbiota in saliva samples from individuals with Alzheimer's disease and healthy children. However, minor but statistically lower bacterial diversity was observed in toddlers with ASD compared to controls **(Qiao *et al.,* 2018).** In particular, a low prevalence of *Prevotella*, a commensal microorganism involved in saccharide metabolism and vitamin biosynthesis, was detected in saliva and dental samples from people with ASD. As autistic patients are thought to have impaired carbohydrate digestion **(Williams *et al.,* 2012),** restoration of relative *Prevotella* deficiency may have therapeutic potential for ASD symptoms.

II.2. Maternal microbiota during gestation and lactation

When studying the role of the microbiota in pregnancy, it is crucial to consider the stage at which the essential interaction between the host and its microbes begins. In other words, when does the developing fetus first encounter microbes? While more than 100 years ago, the hypothesis that we are born germ-free was put forward **(Tissier, 1900 cited by Ballongue et al., 1993)** and many doctors still believe it, there is now ample evidence challenging this assumption and suggesting that a bacterial presence already exists in the fetus-placental unit **(Hu *et al.,* 2013; Aagaard *et al.,* 2014).** It has also been shown that the mode of delivery, vaginal or caesarean section, has effects on the initial microbiota of the newborn, which then changes significantly depending on the diet and the overall environment of the child throughout the first two years of life, until stabilization.

A healthy pregnancy (**figure 3**) is characterized by an increase in bacterial load and profound alterations in the composition of the gut microbiota **(Collado *et al.,* 2008; Koren *et al.,* 2012).** During the first trimester of pregnancy, the gut microbial composition is similar to that of healthy non-pregnant women. However, from the first to the third trimester, the composition of the gut microbiota changes dramatically.

These changes are characterized by an increased abundance of members of the phyla *Actinobacteria* and *Proteobacteria,* as well as a reduction in individual richness **(Koren *et al.* 2012).** In addition, levels of *Faecalibacterium,* a butyrate-producing bacterium with anti-inflammatory activities, which is depleted in patients with metabolic syndrome **(Haro *et al.,* 2015),** are significantly decreased during the third trimester of pregnancy. Diversity between subjects (beta diversity) increases in the third trimester, associated with weight gain, insulin insensitivity, and higher fecal cytokine levels, reflecting inflammation **(Koren *et al.,* 2012).**

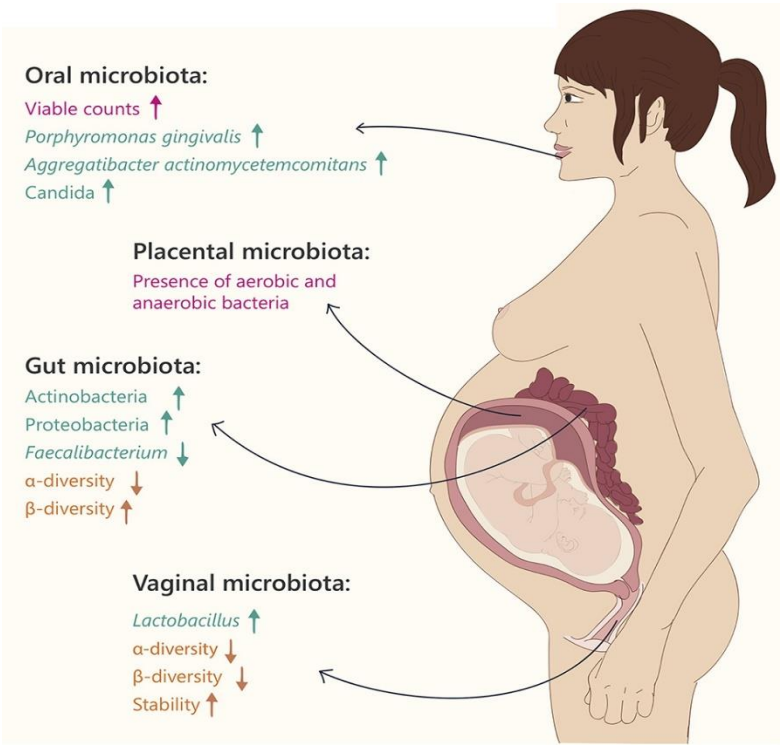

Figure 3: **Changes in the microbiome during pregnancy (Nuriel-Ohayon *et al.*, 2016).** *The text and arrows refer to general changes (pink), changes in specific taxonomy (green), and community diversity (orange).*

The mother is probably the external factor that has the greatest influence on the composition of the microbiota that colonizes the digestive tract during the first days of life in relation to postnatal intimate contact between mother and child during delivery, feeding and care, but also before birth during gestation. Studies of potential bacterial transmission across the placental barrier have shown that bacteria are detected in the umbilical cord, amniotic fluid, and fetal membranes (**Jimenez *et al.*, 2005**). In addition, meconium, the first feces resulting from the ingestion of amniotic fluid, is not sterile but has a complex microbial community (**Hu *et al.*, 2013; Collado *et al.*, 2016**).

Interestingly, many bacterial genera of the meconium microbiota, including Enterococcus and Escherichia, are commonly found in the gastrointestinal tract **(Jimenez *et al.*, 2005).** To test whether gut bacteria from the mother can be transferred to the fetus, **Jiménez *et al.* (2005)** inoculated pregnant mice with genetically labelled *Enterococcus faecium* and analyzed bacteria present in the meconium of full-term caesarean offspring. A higher abundance of bacteria was

found in the meconium of offspring born to inoculated mothers, and *E. faecium* was able to be cultured from their meconium, unlike the control group **(Jimenez *et al.*, 2008).**

The composition of the microbiota present in meconium reflects the health of the mother and influences the health of the newborn.

Similarities between the maternal and newborn microbiota are also found several days or even months after birth. Comparison of maternal fecal microbiota a few days before delivery with that of newborns during the first week of life and in the longer term (three months) shows that 11 strains of *Bifidobacterium longum* subsp. *longum* are found in the maternal feces and in those of their child at birth but also at three months (**Makino *et al.*, 2011**).

II.3. Probiotics

II.3.1 Definition

The term probiotic derives from the two Greek words (*pros*) and (*bios*) which literally means "for life" as opposed to the term antibiotic which means against life.

According to FAO and WHO, probiotics are live microorganisms (bacteria or yeasts) that, when consumed in adequate amounts, produce a beneficial effect on the health of the host beyond traditional nutritional effects**. (Gordon & Klaenhammer, 2011; Ravel *et al.*, 2011).**

They can be present or introduced in certain foods (food supplements) or in certain medicines (e.g., Lacteol® containing *Lactobacillus* LB). The most well-known probiotics are lactic acid bacteria (*Lactobacillus*, *Streptococcus* and *Lactococcus*) and Bifidobacteria, which are widely used in yogurt and other fermented dairy products **(Spear *et al.*, 2014).**

Probiotics exert their beneficial effects through a variety of mechanisms, including lowering gut pH, decreasing colonization and invasion by pathogenic organisms, and altering the host's immune response.

The main probiotic microorganisms known to date are lactic acid bacteria (lactobacilli, bifidobacteria, propionibacteria, *Escherichia coli* and enterococci) and yeasts (*Saccharomyces boulardii*) **(Ouwehand et al., 2002). Table 2** summarizes the microbial species considered probiotics.

Table 2: Microorganisms considered probiotics **(Wijegunawardhana, 2023).**

Lactobacillus	Bifidobacterium	Other lactic acid bacteria	Non-lactic acid bacteria
Lactobacillus acidophilus	*Bifidobacterium adolescentis*	*Enterococcus faecalis*	*Bacillus cereus var. toyoi*
Lactobacillus amylovorus	*Bifidobacterium animalis*	*Enterococcus faecium*	*Enterococcus faecalis*
Lactobacillus brevis	*Bifidobacterium adolescentis*	*Lactococcus lactis*	*Enterococcus faecium*
Lactobacillus casei	*Bifidobacterium bifidum*	*Leuconostoc mesenteroides*	*Escherichia coli Nissle*
Lactobacillus cellobiosus	*Bifidobacterium breve*	*Pediococcus acidolactici*	*Lactococcuslactis subsp. lactis*
Lactobacillus crispatus	*Bifidobacterium infantis*	*Streptococcus thermophilus*	*Leuoconostocmes enteroides*
Lactobacillus delbrueckii subsp. bulgaricus	*Bifidobacterium lactis*	*Sporolactobacillus inulinus*	*Propionibacterium freudenreichii*
Lactobacillus fermentum	*Bifidobacterium longum*		*Pediococcus acidilactici*

II.3.1. *Lactobacillus*

Lactobacilli are Gram-positive, catalase-negative, non-spore-forming, rod-shaped bacteria that produce lactic acid as the main end product of fermentation.

In 2020, a taxonomic reorganization of lactic acid bacteria reclassified more than 300 species from 7 genera and 2 families into a single family, *Lactobacillaceae*, with 31 genera including Lactobacillus, *Paralactobacillus, Pediococcus, Weissella, Fructobacillus, Convivina, Oenococcus, Leuconostoc* and 23 new genera that include organisms previously classified as Lactobacillus species **(Zheng et *al*., 2020).**

II.2.1. Bacterial Composition of Breast Milk

According to **Boix-Amoros *et al.* (2016),** it is estimated that a breastfed infant who consumes about 800 ml of milk per day could ingest between 10^5 and 10^7 bacteria **(figure 3).** According to **Hunt *et al.* (2011)** and **Jost *et al.* (2013),** colostrum and human milk contain several types of bacteria, such as *Staphylococcus, Streptococcus, Propionibacterium, Bacteroides, Faecalibacterium, Roseburia, Lactobacillus and Bifidobacterium*, which are transmitted to infants and influence bacterial colonization **(figure 4).** It is possible that these bacteria in breast milk originate from the migration of gut bacteria that travel to the mammary gland **(Fernandez *et al.*, 2013).**

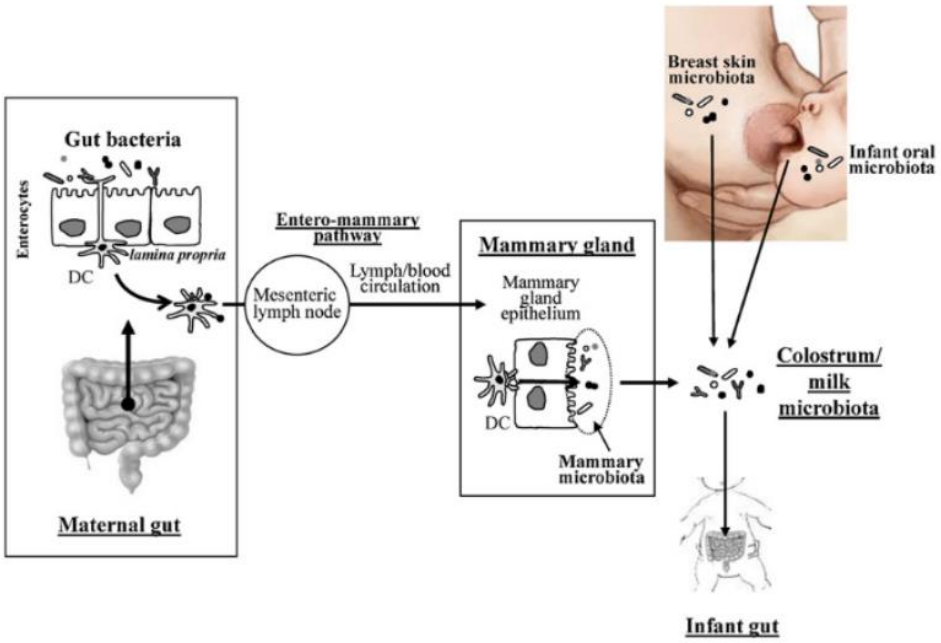

Figure 4: Transmission of the microbiome from mother to child (**Fernández *et al.*, 2013**)

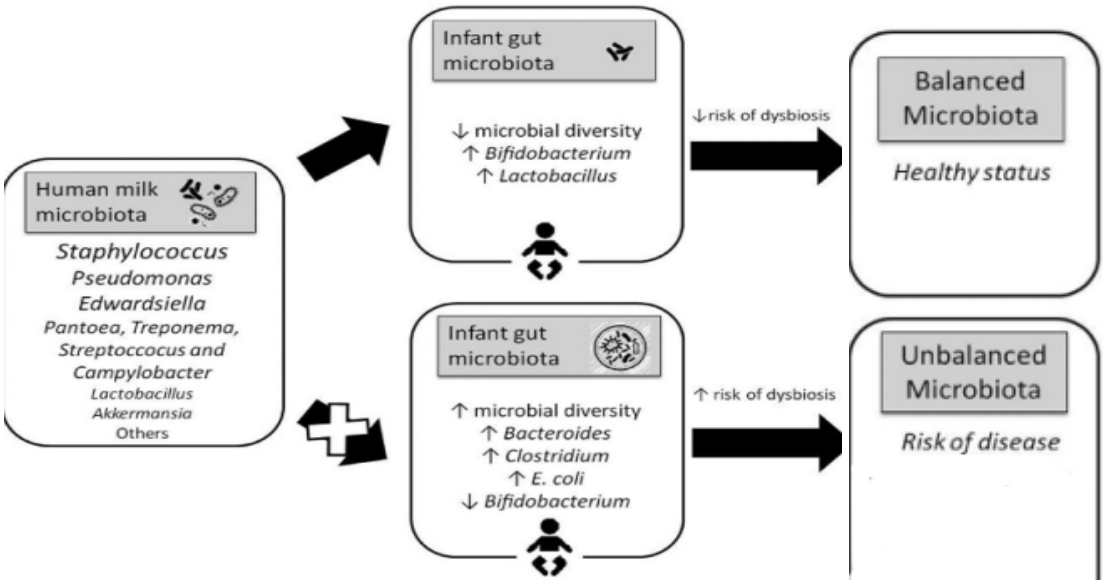

Figure 5: Composition of the microbiota of human milk and comparison between the microbiota of breastfed children **(Gomez-Gallego *et al.*, 2016).**

II.4. Involvement of the microbiota in autism spectrum disorders

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by deficits in social interaction and communication, repetitive behaviors and restricted interests, and sensory perception abnormalities. Some ASD patients have gastrointestinal (GI) disorders, dysbiosis of the gut microbiota, and altered levels of metabolites produced by this microbiota. Research supports the existence of a microbiota-gut-brain axis through which the microbiota can communicate with the brain via peripheral relays such as the vagus nerve, the GI tract or the immune system.

An emerging communication pathway is *via* microbial metabolites that can act on these peripheral relays or directly on the brain. Thus, disruptions of the microbiota-gut-brain axis could contribute to the development of ASD symptoms **(figure 5)** *via* microbial metabolites **(Hughes *et al.*, 2018)**.

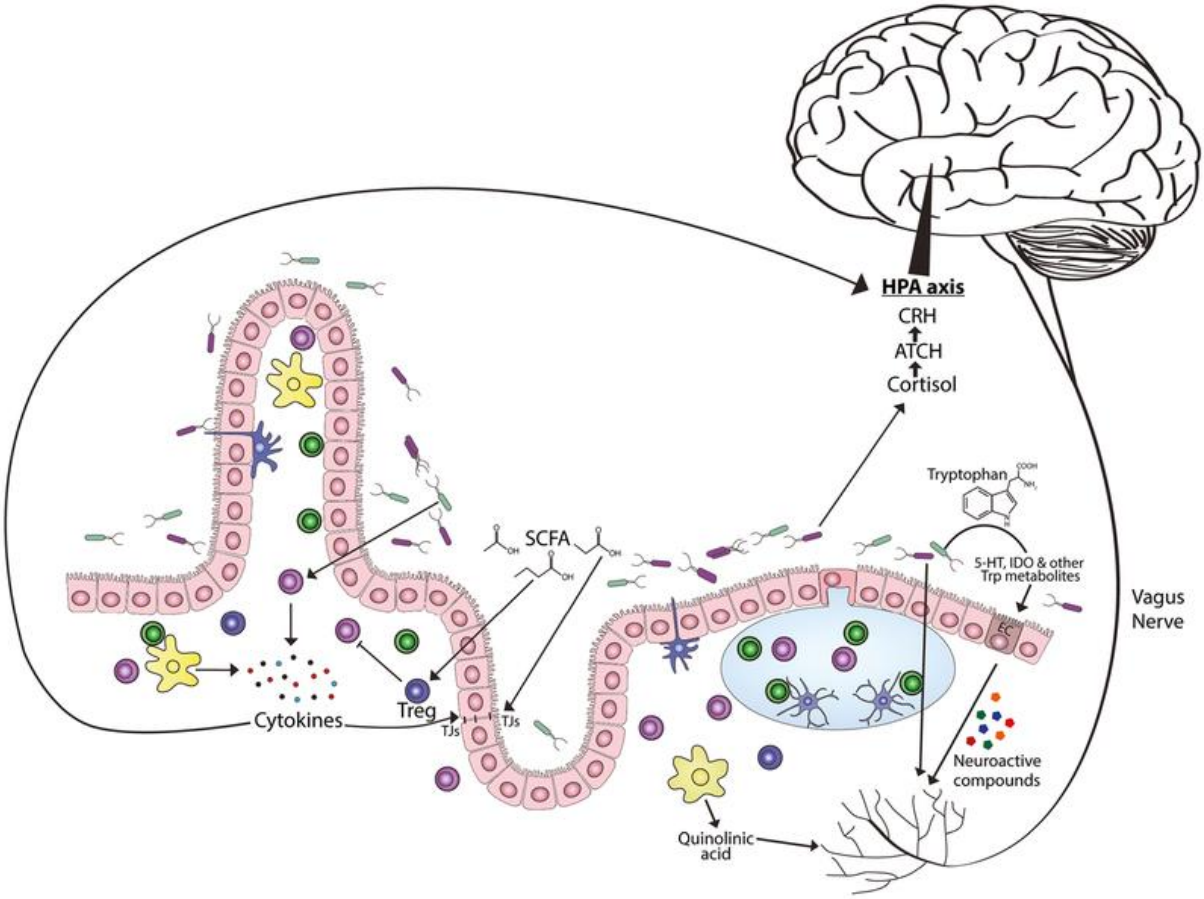

Figure 6: Mechanisms of the microbiota-gut-brain axis **(Hughes *et al.*, 2018).**

Possible mechanisms of the MGB-axis are being actively investigated and include neuroimmune pathways, neural communication through the vagus nerve, influence of metabolites produced by the microbiota, microbial-derived neurotransmitters, and the significant influence the microbiota have on tryptophan, kynurenine, and serotonin metabolism. Short-chain fatty acids (SCFA) can promote peripheral T regulatory (Treg) cell expansion as well as influence tight junction (TJ) proteins and intestinal barrier function. Microbiota regulate tryptophan (Trp) metabolites by degrading Trp to indole-derivatives or through kynurenine and serotonin pathways, such as increasing expression of tryptophan hydroxylase (Tph)1 in enterochromaffin (EC) cells. Dysbiosis can promote activation of immune cells, including

macrophages that produce quinolinic acid (QA) through an alternative kynurenine pathway, a known excitotoxic N-methyl-D-aspartate (NMDA) receptor agonist.

Activated immune cells also produce proinflammatory cytokines which can further disrupt microflora and impact intestinal barrier function. Neural communication can also occur through the vagus nerve *via* signaling from hormones and neurotransmitters release by gut endocrine cells and immune cells. Breech of the intestinal barrier would also allow direct pattern recognition sensing due to Toll-like receptor expression on afferent fibers.

The schematic diagram of the microbiota-gut-brain axis **(figure 6)** represents the bidirectional connection between the central nervous system (CNS) and the enteric nervous system (ENS) via vagus nerves, which carry neurotransmitters such as serotonin, tetanus neurotoxin, and microbial metabolites such as SCFAs produced by microbial action. Millions of immune cells in the enteric nervous system perform immune-mediated functions and maintain healthy microbial colonization .

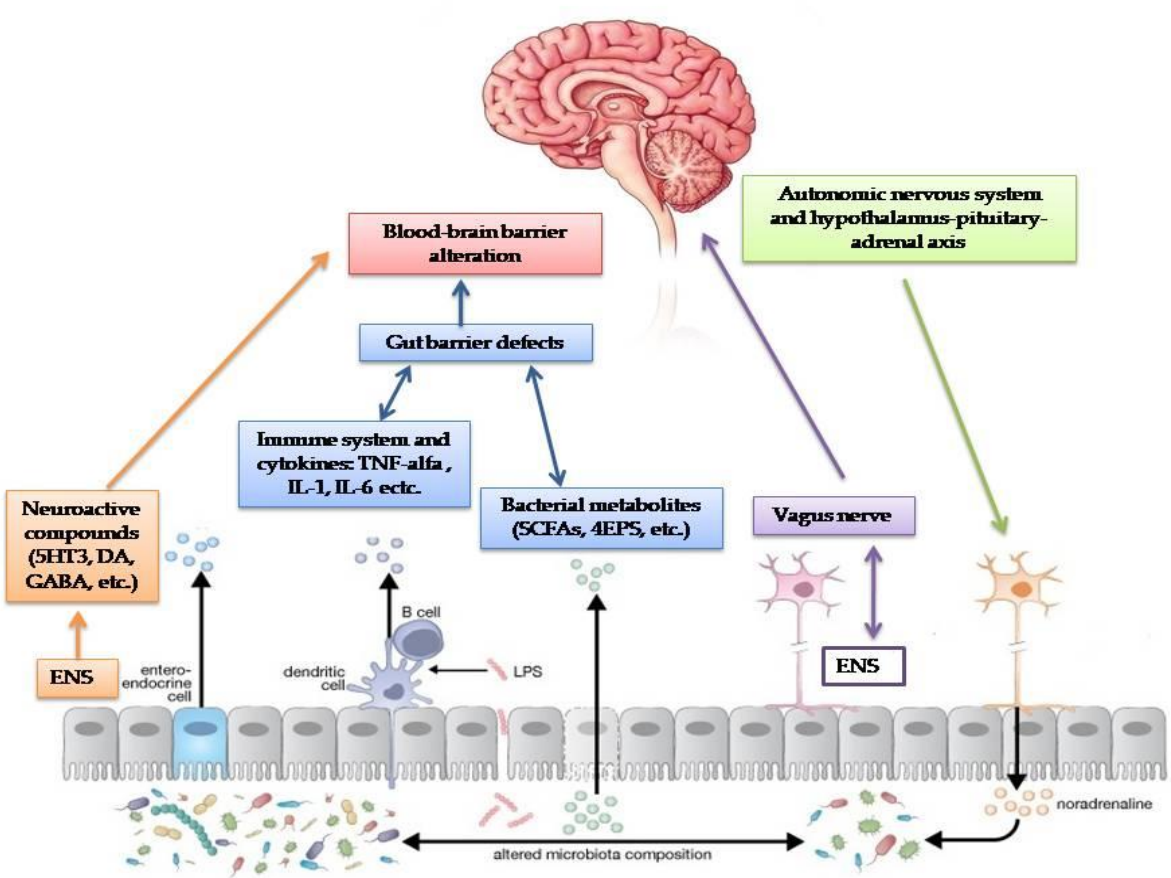

Figure 7: SCFAs and Autism spectrum disorder, It stimulates oxidative phosphorylation and fatty acid oxidation, which is critical for mitochondrial function. Because it upregulates physiological stress pathways, it may be beneficial in a variety of neurological functions such as depression and dementia **(Sarkar et al., 2016).**

Some essential stages of neurological development are influenced by the microbiota, such as neurogenesis, blood-brain barrier (BBB) formation and integrity, microglia maturation, myelination, neurotransmitter and receptor synthesis, and neurotrophin expression. It is therefore essential to ensure a bacterial balance in order to ensure normal neurological development. Thus, a disruption in gut health during crucial phases of development, especially during the postnatal and perinatal periods, can cause neurodevelopmental disorders such as autism spectrum disorder (ASD) (**Wu *et al.*, 2020**).

ASD is part of a complex set of neurodevelopmental disorders that manifest as disrupted communication and social interaction disorders, accompanied by repetitive and restrictive behaviours. Autism ranks first among ASD. The specific etiopathogenesis of ASD is unknown. The causes are many and varied, including genetic and environmental elements such as nutritional deficits, overloads or deficiencies, exposure to viruses, exposure to pesticides (glyphosate), immune system problems, embryonic neural tube closure abnormalities, etc. (**Fattorusso *et al.*, 2019**). In addition, it is frequently observed that many people with ASD also face major gastrointestinal problems such as abdominal pain, chronic diarrhea, vomiting, constipation, gastroesophageal reflux disease, intestinal infections, and increased intestinal permeability. However, it remains difficult to assess gastrointestinal symptoms because there is underdiagnosis in these patients with ASD, especially in some non-verbal children who make it difficult to recognize symptoms (**Fattorusso *et al.*, 2019**).

Several recent clinical studies have shown that these gastrointestinal symptoms are related to the severity of their deficiency. Indeed, behavioral symptoms (anxiety, aggression, self-harm) are improved after taking steps to treat gastrointestinal illness (**Bjørklund *et al.*, 2020**). It is possible that these gastrointestinal symptoms are a manifestation of the underlying inflammatory process of these ASDs.

The precise origin of these autism spectrum disorders is still unknown, which makes it possible to take into account the microbiota, due to the associated gastrointestinal disorders, as one of

the actors likely to play a role in these diseases. According to the initial hypothesis, the alteration of the gut microbiota would lead to the colonization of pathogenic bacteria that produce neurotoxins in abnormal quantities, which would contribute in part to the manifestation of ASD symptoms (**Fattorusso *et al.,* 2019**).

It stimulates oxidative phosphorylation and fatty acid oxidation, which is critical for mitochondrial function. Because it upregulates physiological stress pathways, it may be beneficial in a variety of neurological functions such as depression and dementia **(Sarkar *et al.,* 2016).**

Analyses of fecal samples from autistic children have indeed revealed significant dysbiosis. An imbalance is found in bacterial populations, especially in the Bacteroid/Firmicutes phylum, with an increase in Bacteroids and a decrease in Firmicutes. In addition, there is a decrease in the levels of the genus *Bifidobacterium* (which has anti-inflammatory properties) as well as a decrease in the genera *Prevotella*, *Coprococcus* and *Veillonellaceae* (which are responsible for carbohydrate fermentation and bleeding).

Indeed, autistic people suffer from malabsorption and maldigestion due to the reduced expression of disaccharides and SGLT1 (sodium glucose cotransporter 1) and GLUT2 (glucose transporter 2) transporters. This malabsorption of mono and disaccharides has an impact on the bacterial composition of the microbiota. Thus, there is a greater quantity of low molecular weight sugars in the large intestine, favoring intestinal fermentation bacteria to the detriment of those that degrade polysaccharides. However, there is a reduction in *Prevotella* spp. which degrades polysaccharides and synthesizes vitamin B1, thus leading to vitamin deficiency **(Socala *et al.,* 2021).**

In addition, there is a greatly increased presence (10 times more) of species of the genus *Clostridium* (**Mangiola *et al.,* 2016**). Indeed, gastrointestinal disorders in autistic children are thought to be caused in particular by an increase in *Clostridium* and *Sutterella*.

Chapter III: Materials and Methods.

Chapter III: Materials and Methods.

The present work was carried out in the Laboratory of Beneficial Microorganisms, Functional Foods and Health (LMBAFS) of Abdelhamid Ibn Badis University (Mostaganem). It consists in evaluating the role of *Lacticaseibasillus rhamnosus* SL42 on the modulation of the induced-autistic behavior in juvenile Wistar rats.

III.1. Materials

III.1.1. Origin of the probiotic strain

The probiotic strain *Lacticaseibacillus rhamnosus* SL42 **(GenBank: OQ300076.1)** was provided by the Laboratory of Beneficial Microorganisms, Functional Foods and Health (probiotsanté team, LMBAFS). The strain was isolated in 2018 from the breast milk of a healthy Algerian young mother.

MRS broth from de Man, Rogosa, and Charpe **(de Man et *al.*, 1960**), supplemented with 0.05% HCl-cystein, was used for cultivating SL42.

III.1.2. Animal model and housing conditions

15 male Wistar rats at 3 weeks' age, weighing between 80 and 100g, were used in the establishment of autism-like animal model. Rats were purchased from the Pasteur Institute (Algiers).

III.2. Methods

III.2.1. Autistic-rat model

All animal procedures were approved by the Animal Research Ethics Committee of LMBAFS laboratory, Mostaganem University (Approval number: 51/2412; Date: 22/11/2023). The experiments comply with the ARRIVE guidelines and were performed in accordance with EU Directive 2010/63/EU for animal testing. Upon their delivery, the rats were randomly housed in cage **(figure 7)** at room temperature of 23±°C, humidity of 55-60% and a 12 h light/dark cycle (lights on: 07:00) and had *ad libitum* access to food (standard rodent chow, Bouzaréa, Algiers) and water. Furthermore, the rats were divided into three groups, the first group is the control: the non- PPA induced group which underlined intraperitoneal injections of saline, administered daily during five consecutive days. The second is PPA group: the PPA-

induced group with intraperitoneal injections of sodium propionate (at a dose of 500 mg/kg; 250 mg/mL 0.26 M, PBS 0.1 M pH 7.4), administered daily **(figure 8)** during five consecutive days. In addition, 0.5 mL/day saline was administered *via* orogastric gavage during the treatment period for control and PPA groups **(figure 8)**.

The third is PPA+ SL42 group: the PPA-induced group with intraperitoneal injections of sodium propionate, administered daily during five consecutive days, and treated daily (-4 day to day 14) *via* orogastric gavage with 0.5mL of the probiotic strain *Lacticaseibacillus rhamnosus SL42:* 10^9 CFU/mL).

The successful establishment of the model was confirmed through behavioral evaluation. Then, the onset of the study was immediate for about 14 days following the scheme illustrated in (**figure 9)**.

III.2.2. Weight monitoring

The animals were weighed using an electronic scale **(figure 9)** according to a defined schedule: before and throughout induction experiments (-4^{th} day, 3^{rd} day, 7^{th} day, and 14^{th} day).

III.2.3. Behavioral assessments

ASD-like behavior was scored using the Super Maze tracking software (Shanghai Xin Ruan). The behavior assessments were conducted during the day, between 09:00 and 18:00. Each rat was placed in a clean cage with a small layer of padding (1 cm) to minimize stress and discourage digging. The rats were given 10 min getting used to the new environment before the behavioral experiment.

III.2.3.1. The Grip strength test

A simple method and the most convenient to objectively quantify the muscle strength of rats. It allows the study of neuromuscular functions by determining the peak of maximum strength developed by the rodent. The grip strength tests have been widely used alone or in combination with other tests to assess the phenotypes of animals with neuromuscular disorders and to evaluate the effects of various chemicals on motor performance **(Takechita *et al*., 2017).**

Each rat was fixed horizontally **(figure 10)**, and then pulled steadily by the root of the tail away from the T bar until its grip was broken. The peak of the grip strength was measured. Each rat was subjected to three such trials, and the average was used as the grip strength. The grip strength was measured at 3, 7, and 14 days. It is represented as the gram-force.

III.2.3.2. The sociability test

Before the test, rats were allowed to explore the apparatus for 15 min. For the sociability test (**figure 11**), a strange Wistar rat (never seen by the subjects) of the same age and sex was placed in one chamber of the device (40 × 40 × 40 cm), while an empty cage was placed in the other chamber of the device. One subject was placed in the middle chamber and the sliding doors between the chambers were opened to initiate the sociability phase.

For the sociability preference test (**figure 12**), a rat familiar to the reference rat is placed in a cage in a chamber at one end. Another, whom he does not know, is placed at the other end.

Time duration in each test was 10 min and the inner surfaces of the walls and ground of the apparatus were cleaned with 70% ethanol before and after each trial.

The parameter evaluated in the sociability phase was the "sociability index (SI)," and that in the social preference phase was the "social preference index (SPI)". SI and SPI were calculated using the following formulas:

Sociability index (SI) = Time spent with animal (s) / Time spent with empty cage (s)

Social preference index (SPI) = Time spent with strange animal (s) / Time spent with familiar animal (s).

III.2.3.3. The Open Field Test

- **Principle**

The Open Field test, originally described by **(Hall, 1934; cited by Çalışkan et al., 2024),** assesses the locomotor and exploratory activity as well as the emotional state of the animal. This test indicates locomotor activity and anxious behavior, respectively. The latter is all the more pronounced when the rat spends more time in the peripheral area. As for the central area, its exploration represents a sign of less anxiety.

- **Experimental design**

The device (**figure 14**) consists of a base surrounded by plexiglass parapets measuring 100×100 cm) and 40 cm respectively. The floor is in the form of squares of (14.28 × 14.28 cm) on each side. It has been divided into 3 zones: central zone, middle zone and peripheral zone.

The test begins after the animal is placed in the center of the device and its movements are filmed for 5 minutes. The device is cleaned with alcohol at the end of the test.

- **Variables Measured**

- Time spent in the middle part: Animal with little anxiety.

- Time spent in the peripheral part: Anxious animal.

- Distance travelled: Estimation of locomotor activity.

- Recovery time: Exploratory activity.

- The number of times in the center of the device: Animal with little anxiety.

III.2.3.4. The Elevated plus-maze Test

• Principle

The elevated plus-maze **(figure 15)** is one of the most widely used anxiety tests in rodents. It is based on the work of **(Montgomery, 1955; Chen et al., 2024)** and has been validated in rats by **(Pellow et al., 1985).** The device consists of a four-pronged elevated plus-maze with two open and two closed arms. Exploring rats with open arms shows less anxious behavior. On the other hand, the more the animal is located in the outstretched arms, the more its behavior is designated as anxious.

• Experimental design

The device consists of 2 open arms (100 x 10 cm) perpendicular to 2 closed arms (100 x 10 cm) and a neutral central area at the intersection of the 4 arms. The maze is raised 50 cm from the ground.

• Procedure

The test begins after the animal has been placed in the central area, with the head pointing towards the closed arm, which is the same for all rats. The animals are filmed for 5 minutes under white light. The maze board as well as the walls of the closed arms were cleaned with alcohol and dried after each animal has passed.

• Variables Measured

- Time spent in the central area of the device: Reduced locomotor activity.

- Number of depth estimates: Exploration ability of the rat.

- Time spent with open arms: Animal with little anxiety.

- Time spent in closed arms: Very anxious animal.

III.2.6. Sacrifice and Organ Removal

At the end of the experimental period, the animals are weighed, and subsequently euthanized *via* cervical dislocation. Simultaneously, the brain tissues and intestines of the animals were removed and weighed (**figure 18**). Immediately thereafter, the prefrontal cortex, cerebellum and ventral hippocampus were quickly dissected. Brain tissues were then fixed in formaldehyde in order to make histological sections. Other dissected tissues were stored in Eppendorf tubes at -80°C under nitrogen gas until the day of the biochemical analysis.

First, 50 mg tissue samples (cerebellum and cortex) were homogenized in 500 µL of 0.1 M phosphate buffered saline solution (PBS, pH 7.4) with an ultrasonic homogenizer for 5 min. Afterwards, the homogenized tissues were centrifuged at 10,000 rpm for 15 min, and the supernatants of the tissues were transferred to Eppendorf tubes. Organ supernatants were subjected to cytokines analysis, and performed by a foreign company (R & D Systems, Minneapolis, MN, USA).

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III.2.7. Statistical analysis

The mean ± standard error of the mean or standard deviation was used to present experiment's data for statistical analysis implementation (SPSS statistics 26, Chicago, IL, USA). Statistical significance was determined using Student's t-test, and one-way ANOVA was used for parametric tests. Differences at $P < 0.05$ were considered statistically significant.

III.2.8. Histology

The animals were slaughtered on the morning of the 15th day after a 12-hour fasting, chloroform was used as an anesthetic agent.

The intestines, and brains were then incised, weighed, rinsed with physiology water and then fixed in formol at 10% for histological study (figure...). The latter was carried out by HE staining (Hematoxyline-Eosine) by the Pathological Anatomy Laboratory of Makour Hamou Hospital, wilaya of Ain Defla. The organs taken were submitted to different successive steps according to the **Hould** method (1984).

The histological blades thus prepared were observed in the laboratory of the Kharouba Faculty of Medicine **(Mostaganem University)** using an optical microscope equipped with a digital color camera. (Optika Vision Lite 2.1). The images captured had a resolution of 600 pixels, ensuring high visual quality for the detailed description.

Chapter IV:

Results and discussion

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Chapter IV: Results and Discussion.

IV.1. The changes in rat weight during the study

The evolution of rat weights (g) before PPA-induction (day 0, D0) and after 3 days (D3), 7 days (D7), and the last day of the challenge (14th day, D14), are illustrated in **Figure 17.**

The changes in weight of the different studied groups (Control, PPA, PPA+SL42) reveal an exponential evolution from D0 to D14. The weights in PPA group differed from the other groups at the end of the study where lower values were recorded, while for the control and PPA+ SL42 groups, the increase was clear at the end of the experiment. However, no significant differences were calculated for those results ($P>0.05$).

It seems that weight gain had no connection to the chemical induction since in the study of **Qi *et al.* (2021),** rat weight losses were only observed in the fecal transplant group, compared to the control and VPA groups. In the contrary, in the work of **Bhalla and Mehan (2022),** adult Wistar rats made autistic by PPA-induction had the lowest weights compared to controls and were slightly lower compared to the group treated with 4-hydroxyisoleucine.

IV.2. Behavioral changes during the experience

During the study, PPA-induced rats expressed a high appetite, and the intake was one and a half times that of the control and the probiotic-treated groups.The autistic-rats reflected also a remarkable hypersensitivity and were most of time inactive and/or reluctant in their responses, preferring to spend day-time at the corner side of the cage as shown in **Figure 18.**

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IV.2.1. The results of the grip strength test

The rodent grip strength test was developed decades ago and is a putative measure of muscular strength. This test has been included in the functional observational battery to screen for neurobehavioral toxicity, and changes in grip strength. This test has been interpreted as evidence of motor neurotoxicity. People with autism have defects in motor function, some of which have been found in rats **(Wu *et al*., 2016).**

In the present study, we examined motor function with a grip strength test and observation of spontaneous locomotion.

The forelimbs grip strength was monitored and registered with electronic scale. Results are shown in **figure (19)**. PPA-induced group showed a decrease in muscle strength of 0.94 ± 0.42 kg compared to PPA+probiotic and control rats, respectively: 0.13±0.31 *P≤0.05), 0.142±0.5 (**P≤0.01). Similarly, **Schwingel *et al.* 2023** showed that the VPA group rats exhibited a delayed onset in limb grip test compared to the control.

In this study, we investigated whether the efficacy of early or concomitant probiotic treatment throughout autism-like animal induction could bring improvement in rat balance and behavior. Our results showed that the PPA+probiotic group exhibited better results compared to PPA-induced group, and where the decrease in rat's strength in the last group was more pronounced (*P≤0.05).

A previous study showed that PPA injection resulted in memory deficits and walking imbalance observed through various behavioral models for autistic rat **(Dubey *et al*., 1981).**

IV.2.2. The Sociability scores

The results for the social preference test are shown in **(Figure 20).** The different parameters measured made it possible to evaluate the social preference of a rat, knowing that a normal rat will tend to discover what is new to it.

We first examined animals for their social preference (SI). The PPA-induced group showed a significantly lower SI scores (1.58 ± 1.22) than that of the control (2.07 ± 1.58, *P≤0.05) group.

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Further, no significant difference was observed between the treatment group PPA+SL42 (1.84 ± 1.02, P>0.05) and the PPA-group.

Similar trends were observed in the study of **Adıgüzel *et al*. (2022)** using VPA-autistic model. The results for the social preference index "SPI" are shown in **Figure 21**. In this test and contrary to normal rat which tend to discover what is new to it, autistic rat will not. Our results clearly showed a significant low SPI in the PPA group (1.4 ± 1.40) compared to the SL42 PPA+ group (2.15 ± 6.02, *P≤0.05) and the control group (5.00 ±10.14, **P≤0.01). According to the SI and SPI data, the rat model of autism induced by PPA in this study was effective, and caused impairment in social interaction and repetitive behavior. Our results are also consistent with different studies on mice and rats using VPA-induction **(Schneider *et al*., 2008; Kataoka *et al*., 2013).** Additionally, previous research suggests that both multi- and single-strain probiotic therapies can improve three-chamber social behavior test attributes in autism models. Two studies using the VPA-induced experimental autism models found that probiotic treatments with different *Lactobacillus* species (1×10^9 CFU/day for 4- and 6-weeks) significantly improved sociability and social preference scores **(Sunand *et al*., 2020; Kong *et al*., 2021).**

IV.2.3. The assessment of anxiety in open field test

The results of the open field test after 7 and 14 days of the experience are shown in **Figure 22**. The various parameters measured made it possible to evaluate the locomotor activity and the level of anxiety of the rats, knowing that the exposure to chronic stress leads to a reduction in the distance travelled during the open field test. The open field test is commonly used to assess anxiety in rats. Avoidance behavior in the central area has been identified as an indication of anxiety **(Kraeuter *et al*., 2019).**

Observations at day 7 **(table 3 and figure 22)** showed stark differences in the total distance travelled (*P ≤ 0.05), the average speed (*P ≤ 0.05), the number of entries to the new zone (**P ≤ 0.01), the number of exits from the new zone (**P ≤ 0.01), and the time spent in the new zone (**P ≤ 0.01) between rats from the PPA and the other groups (control and PPA+SL42 groups).

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Likewise, observations on the total distance travelled, the average speed, and the time spent in the new zone, recorded at day 14 **(table 3 and figure 23)** were of similar trend but statistically significant. No differences ($P > 0.05$) were registered between the PPA+SL42 and the PPA groups for the rest of attributes.

Limited research has been conducted on the impact of probiotic treatments on anxious behavior in autism-rodent models. At our modest knowledge, only two studies on the efficiency of probiotic therapies using *Bacteroides fragilis* for the first one, and three *Lactobacillus* strains in the second as autism models, confirm our findings **(Hsiao *et al*., 2013; Kong *et al*., 2021).** It seems that our probiotic strain SL42 could improve anxiety as an associated symptom to autism.

IV.2.3. The results of the Elevated plus-maze test

The Elevated plus-maze test, which measures spatial memory and insistence on sameness, is one of the most commonly used behavioral tests in chemical-induced autism models. A subject with high spatial memory remembers the arms it discovered during the habituation phase, so it tends to enter an arm that it has not visited frequently. Repeated visits to the same arm are associated with a lower rate of spontaneous alternation, and so stereotyped behavior **(Sarnyai *et al*., 2000).**

In our study, PPA induction negatively affected the spontaneous alternation mainly characterised by a low total distance travelled **(table 4).** However, probiotic treatment improved PPA-induced spontaneous alternation and the arms remembering deficits in the Elevated seplus-maze test **(figures 24 and 25),** clearly (**$P \leq 0.01$) after the second test at the 14^{th} day then at the 7^{th} day.

These findings suggest that administering probiotic during induction and over 14 days can successfully mitigate the core ASD behaviors induced by PPA.

Although different *Bifidobacterium* strains have been reported to significantly improve the spontaneous alternation rate in experimental Alzheimer's models **(Kobayashi *et al*., 2017),** data on the effectiveness of probiotic or prebiotic treatment on spontaneous alternation in autism are lacking. To the best of our knowledge, there is only one study of **Adıgüzel *et al*. (2022)** who evaluate the positive effects of probiotics and prebiotics on spontaneous alternations in autism.

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In a post hoc study, **Kong *et al.* (2023)** demonstrated that the concurrent supplementation of oral probiotic *Lactobacillus plantarum* PS128 and intranasal oxytocin in young children and teens with ASD may improve autonomic function indices.

Neurodevelopmental and neurodegenerative illnesses share several molecular and cellular neurobiological processes. Most occurrences of mental disease are not hereditary but emerge spontaneously due to a multitude of environmental factors such as psychological stress, depression, traumatic brain damage, diet, physical and intellectual activity **(Blaney *et al*., 2013).**

IV.3. Brains of the different groups

IV.3.1. Relative weight

Figure 26 shows the results of the relative mean brain weight (g) of rats in the different experimental groups. It showed a slight ($P > 0.05$) increase in cortical weights in the PPA+SL42 group compared to the control and the PPA groups. On the other hand, the weights of the cerebellum show almost no significant ($P > 0.05$) differences between the PPA and PPA+SL42 groups, but were slightly ($P > 0.05$) small in the control.

IV.3.2. Macroscopic view of brains

To gain a better understanding of the structural basis underlying brain damage in PPA-group and improvement by SL42 treatment, we first globally viewed the morphology of the fresh brains from each group after 14^{th} day of post-induction **(Figure 27).**

PPA+SL42 rats showed normal brain structure without edema and bleeding, and the midline was straight and not shifted. PPA-treated rat's brain exhibited shifted midline, and bleeding spots (**Figure 27b**, black and red arrows). However, PPA+SL42 group showed improved brain morphology similar to that of the control rats (**Figure 27a and c**). Thus, probiotic treatment during PPA-induction and two weeks' forwards (post-induction) protects Wistar rat brain from autism-associated damage.

IV.3.3. Histological examination of brains

Histological examination HE showed an increase in the number of cells in the prefrontal cortex in PPA rats in comparison to other groups. However, their soma size decreased in comparison to the other groups. Moreover, probiotic treatment with SL42 strain effectively reduced changes compared to PPA-treated rats and did not change the number and size of

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neurons compared to the control group. The role of the frontal lobe in the neurobiology of ASD has been extensively documented in the literature **(Gilbert *et al*., 2017).**

In general, SL42 co-administration with PPA effectively reduced the number of neurons compared to the PPA-rat. The possible underlying mechanism might occur *via* the decreased oxidative stress, which decreases neuron number increased in the prefrontal cortex and improves prefrontal-related behavioral functions. Moreover, **Margedari *et al.* (2024)** showed similar positive effect while studying vitamin C in VPA-induced rats.

The HE staining has highlighted that chemical administration using PPA causes significant histological changes in the brain, such as the loss of neurons in some layers of the prefrontal and somatosensory cortices and decreased levels of parvalbumin-positive interneurons in the parietal and occipital cortices.

Various methods have been suggested to generate ASD-like animal models, including administration of PPA or VPA. Both PPA and VPA have similar effects including inhibition of histone deacetylase, altering carnitine activity and mitochondrial metabolism. However, VPA has several side effects, including hepatic steatosis, hepatotoxicity, hemorrhagic pancreatitis, encephalopathy, and metabolic disorders such as obesity **(Bai *et al*., 2017).**

As GABA plays a critical role in the development of neural connections, changes in GABA levels due to PPA exposure in early neurodevelopmental processes may cause autism-like behavioral phenotypes by disrupting the neural balance **(Choi *et al*., 2018).**

CONCLUSION

Autism is a severe neurodevelopmental disorder characterized by impaired social interactions, communication deficits, repetitive behavior, and restricted interests. However, the exact cause of the disorder is unknown. The gut-brain axis may play a role in the etiology and manifestation of ASD, as the gut microbiome interacts with core symptoms, Gl symptoms, and behavioral difficulties.

Propionic acid (PPA), being available endogenously as an intermediary product of cellular fatty acid metabolism and end product produced by the intestinal tract, skin, and oral mucosa bacteria. Although PPA may be beneficial at optimum levels, excessive PPA appears to have several undesirable effects on health. PPA can readily cross the gut-blood, blood-brain, and placental barriers. PPA particularly gets concentrated intracellularly, producing deleterious effects on the developing brain. This becomes noteworthy in the context of ASD since PPA affects cell signaling, neurotransmitter synthesis, mitochondrial function, lipid metabolism, immune function, neuroinflammation, and gene expression, all of which have been implicated in ASD.

The fact remains that the medications used to treat many mental disorders may not be effective or even tolerated by patients. Alternate therapy using probiotics may be of benefit. Nonetheless, further investigation is needed, particularly regarding the composition of the microbiota and how diet and prebiotics or probiotics influence the microbiota. This new field may bring possibilities of new and inexpensive treatments.

This study suggests that PPA administration can cause abnormal neural cell organization, leading to autism-like neurobehaviors in rats such as reduced exploratory activity and increase isolative behavior. This study allows us to see the effect of probiotic treatments in terms of how they affect autism symptoms.

This study shows many positive results and benefits of using probiotics to treat autism in rats. Behaviorally, the rats treated with probiotics behaved normally, similar to the controls, as opposed to the autistics, who showed decreased activity, curiosity, poor muscle strength, increased stress, anxiety, and introversion. Histologically, autistic rats' brains contained a greater number of cells in the prefrontal cortex as well as smaller cells.

Probiotic treatment improved social interaction, anxiety, and repetitive behaviors. These findings highlight autism-related behavioral and molecular parameters that are enhanced by microbial modulation. Although some bacterial species are known to be effective in the pathways of the microbiome-gut-brain axis, Furthermore, the roles of molecules formed by the fermentation of various substrates by specific strains in autism-related pathways are critical for the prognosis of autism. However, further research is needed into the specific mechanisms of action and long-term effects of these interventions. The microbiome's diversity, susceptibility to a wide range of factors, and intricate connection to both physiological and neurological processes all contribute to the nuanced findings reported in this study and various studies.

In sum, we demonstrated that probiotics administration had ameliorating effects on PPA-induced autism-like behavioral, biochemical, and histopathological changes in rats. Our results suggest that probiotics administration may ultimately lead to neuroprotective and neuromodulator effects via regulating the gut microbiota.

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