# Probiotic ameliorating effects of altered GABA/glutamate signaling in a rodent model of autism

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## Abstract

# Background:

Autism spectrum disorders (ASDs) comprise a heterogeneous group of pathological conditions, mainly of genetic origin, characterized by stereotyped behavior, marked impairment in verbal and nonverbal communication, social skills, and cognition. Excitatory/inhibitory (E/I) imbalances have been recorded as an etiological mechanism of ASD. Furthermore, GABA, the main inhibitory neurotransmitter in adult life is known to be much lower in both patients and rodent models of ASD.

# Methods:

In the present study, forty young male western Albino rats, 3–4 weeks in age, weighing about 60–70 g were used. The animals were randomly assigned into six experimental groups; each of them included eight rats. Group (I) serves as control orally given phosphate-buffered saline. Groups (II, III) serve as a rodent model of ASD, orally administered a neurotoxic dose of PPA. The rats in the three therapeutic groups (IV, V, and IV) received the same doses of PPA followed by 0.2 g/kg body weight of pure *Bifidobacterium infantis, the probiotic* mixture of (ProtexinR), and pure *Lactobacillus bulgaricus* respectively for three weeks. Selected variables related to oxidative stress, glutamate excitotoxicity, and gut bacteria were measured in the six studied groups.

# Results:

Both pure or mixed *Lactobacillus* and *Bifidobacterium* were effective in ameliorating glutamate excitotoxicity as an autistic feature developed in the PPA-induced rodent model. Their therapeutic effects were mostly through, the correction of oxidative stress, restoring the depleted GABA neurotransmitter, and up-regulating the gene expression of GABA receptors. Pure *Bifidobacterium* was the most effective followed by the mixture of probiotics, and finally *lactobacillus.*

# Conclusion:

*Bifidobacteria and lactobacilli* could be used either independently or in combination as psycho-biotics to ameliorate oxidative stress and glutamate excitotoxicity as two confirmed etiological mechanisms through the gut-brain axis.

## Introduction

One of the etiologies of neurodevelopmental disorders has been known to be imbalanced neurotransmitter signaling. Levels of associated proteins, such as receptors and transporters of the neurotransmitters are usually involved in the clinical presentation of many neurological disorders among which is autism spectrum disorders (ASD). For example, γ-Aminobutyric acid (GABA), Glutamate (Glu) Serotonin (5-HT), and Dopamine (DA) are related to deficits in autism, a type of ASD. (Quaak I., 2013) Glutamatergic / GABAergic imbalance can be found in ASD and anxiety disorders with elevated glutamatergic neurotransmission as an excitatory neurotransmitter, concomitant with lower GABA as inhibitory neurotransmission. (Essa et al., 2012; El-Ansary, 2020). Glutamate increase is responsible for excitotoxicity that leads to neuronal injury, cell death, and dysfunction of surviving neurons; however, delayed disruption of excitatory glutamate circuits leads to deficits in cognitive and motor function, and in experience-dependent plasticity. GABA modulates excitatory pathways in the brain and, following injury, the loss of GABA-producing cells disrupts the balance of excitation and inhibition leading to further cell injury and apoptosis (Guerriero, 2015).

The brain-gut-microbiota axis is a bidirectional communication system enabling the crosstalk between the brain and the gut microbiota. (Dinan, T., & Cryan, J., 2017). It is composed of the central nervous system (CNS, represented by the brain), the enteric nervous system, and the digestive system. (Kim, Y., & Shin, C., 2018). This confirmed that microbiota could mediate direct effects on the GABAergic system by regulating a key synthesis and release of neurotransmitters from bacteria. It has been reported that Lactobacillus and Bifidobacterium species can produce GABA. These microbially synthesized neurotransmitters can cross the mucosal layer of the intestines indirectly affect brain functions, acting on the enteric nervous system and modulating the activation of the immune system. Repeatedly altered gut microbiota has been observed in autistic individuals (Abujamel et al., 2022; Mazzoli, & Pessione, 2016). Manipulating the gut–microbiota with probiotics and prebiotics could be used as a novel approach for treating neurodevelopmental disorders through the gut-brain axis (Nonglak Boonchooduang, 2020).

This information initiates our interest to test the effect of pure *Lactobacillus*, pure *Bifidobacterium*, and a mixture of probiotics in ameliorating selected variables related to oxidative stress, GABA/glutamate signaling, and gut microbiota. This could help to suggest the use of probiotics as an early intervention strategy in ASD.

# Material And Methods

### Animals

Forty young male western Albino rats, 3-4 weeks in age, weighing about 60-70 g, obtained from the experimental surgery and animal laboratory, were enrolled in the present study. The animals were randomly assigned into six experimental groups; each of them included eight rats. The animals in the control group (I) were orally given phosphate-buffered saline. The animals in the PPA-treated groups (II, III) were orally administered a neurotoxic dose of PPA (250 mg/kg body weight/day) for three days. Group II was killed after three days while group III stay alive to be killed with other groups (El-Ansary et al., 2012). The rats in the three therapeutic groups (IV, V, VI) received the same doses of PPA followed by 0.2 g/kg body weight of probiotic (ProtexinR), healthy bacteria *Bifidobacterium infantis*, and healthy bacteria *Lactobacillus bulgaricus* respectively for three weeks. ProtexinR (Somerset, UK) is a mixture of some healthy bacteria like *Bifidobacterium infantis, Bifidobacterium breve, Lactobacillus acidophilus, Lactobacillus bulgaricus*, *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Streptococcus thermophiles* with the concentration of 1 billion CFU per gram.

The rats were placed at 22 ± 1 °C with ad libitum access to water and standard chaw, and quantitative stool cultures were carried out. The experiment protocol was accordance with the ethical standards of the ethics committee responsible for animal experimentation at King Saud University, Riyadh, and was approved according to the Helsinki Declaration of 1975, as revised in 2008 (http://www.wma.net/en/20activities/10ethics/10helsinki/). (IRB NO.: KSU-SE-19-131).

### Preparation of brain tissue homogenates

By the end of the feeding trials, deeply anesthetized (by using Ketamine/Xylazine + D.W (91, respectively 9 mg/kgbw, I.P.) rats were decapitated. The brain tissues were taken from the rats in the six groups and dissected into small pieces, homogenized in bidistilled water (1:10, w/v), and stored at -30 °C until further use.

### Biochemical analyses

The quantitative determination of all tests was measured according to the manufacturer's instructions using ELISA kits from MyBioSource (San Diego, USA). Gamma-Aminobutyric Acid Receptor Subunit Alpha-1(GABRA1) (Cat No: MBS9342109), GABA (Cat No. MBS269152), glutamate (Cat No. MBS 269969), glutamine (Cat No: MBS755884) and Glutathione S-Transferase (Cat No: MBS564158). The parameters were measured in brain tissue homogenate from all the experimental animal groups, using the enzyme-linked immunosorbent assay (ELISA) technique. The applied assays were based on the method of competitive binding enzyme immunoassay technique. Descriptions of kits-assays are described below.

### Measurement of Gamma-Aminobutyric Acid Receptor Subunit Alpha-1 (GABRA1) Concentration:

This assay is based on a Quantitative Sandwich ELISA kit. from MyBioSource, USA (Cat No: MBS9342109). In the kit's plate 50µl of, Standard was added to each Standard well, 50µl sample was added to each sample well, and to each Blank/Control well Sample Diluent 50µl was added. This kit, based on the competitive enzyme immunoassay technique, uses HRP-conjugate reagent added to each well followed by incubation for 60 minutes at 37°C after being covered with a Closure Plate Membrane then 4 the plate was washed four times until excess conjugate and unbound standard, or sample was removed from the plate. Next Chromogen Solution A 50µl and Chromogen Solution B 50µl were added to each well successively to be incubated for 15 minutes at 37°C away from light to. 50µl Stop Solution was added to each well. The color in the wells was changed from blue to yellow. Finally, the Optical Density (O.D.) at 450 nm was measured by using an ELISA reader within 15 minutes after adding Stop Solution.

### Measurement of Glutamate (Glu) Concentration:

Glutamate was analyzed using an ELISA diagnostic kit from MyBioSource (San Diego, CA, USA) (Cat No. MBS269969). The principle of the kit was a Double Antibody Sandwich based on characteristics of the tested antigen with more than two valances that can identify coated antibody and detection antibody at the same time. First, the extraction samples and the standards were added together with the diluent in the extraction plate. Next derivatization with sodium hydroxide, the equalizing reagent, and D reagent in a reaction plate, covered, and shaking for 2 h, followed by addition of the Q Buffer into all wells. In the last step, the extracted samples and standard

compete with glutamate antiserum for a specific number of binding sites on the glutamate microtiter strips, a mechanism lasting for 15–20 h at 2–8°C. Free antigen and free antigen-antiserum complexes are then removed by three times wash. After that, the anti-rabbit IgG-peroxidase conjugate is added, incubated for 30 min on a shaker, and aspirated by washing.

Tetramethyl benzidine (TMB), as the enzyme-substrate, was then added to detect the antibody bound to the solid phase. The absorbance of the solution in the wells is read with the use of a microplate reader at 450 nm, and the concentration of unknown titers was calculated using the standard curve.

### Measurement of Gamma-Aminobutyric Acid (GABA)

Gamma-aminobutyric acid was analyzed using an ELISA kit from MyBioSource (San Diego, CA, USA) (Cat No. MBS269152). The kit has a microtiter plate that is pre-coated with an antibody specific to GABA. During the assay, the GABA in the standard or sample competes with a fixed quantity of biotin-labeled GABA for sites on the pre-coated monoclonal antibody. After washing the excess conjugate and unbound standard or sample from the plate, avidin conjugated to horseradish per- oxidase (HRP) was added. TMB as an enzyme-substrate was used to detect the antibody bound to the solid phase, the developed color was read at 450 nm, and the concentration of GABA was measured using the standard curve.

### Measurement of Glutamine Concentration:

For the measurement of glutamine quantity in rats' brain tissue, an ELISA kit from MyBioSource (San Diego, CA, USA) (Cat No: MBS755884) was employed. This kit, based on the competitive enzyme immunoassay technique, uses a GLN-HRP conjugate and a monoclonal anti-glutamine. On the pre-coated plate, the assay sample and the buffer are incubated for 1 h together with GLN-HRP conjugate, then decanted, and then five times washed. Then, the wells are incubated with TMB as a HRP enzyme substrate. The intensity of the yellow color that appeared after adding the stop reagent was spectrophotometrically measured at 450 nm in a microplate reader.

### Measurement of Lipid Peroxidation Concentration:

The sensitivity of measuring Thiobarbituric Acid Reactive Substances (TBARS) has made this assay the method of choice for screening and monitoring lipid peroxidation major indicator of oxidative stress. The assay has provided important information regarding free radical activity in disease states and is used for measurement of antioxidant activity, it remains the most widely employed assay used to determine lipid peroxidation.

Firstly, labeled each disposable glass test tube with sample identification. 100 µl of the sample was added. Then in each tube was added 100 µl of SDS Solution. 2.5 ml of TBA/Buffer Reagent was forcefully down the side of each group and hence, the tubes were covered with glass marble and incubated at 95 °C for 60 min then removed from incubation and cooled to room temperature in an ice bath for 10 min. samples were centrifuged at 3000 rpm for 15 min. Finally removed supernatant from samples for analysis and read the absorbance at 532 nm.

### Gene Expression

Total RNA was purified from the rat brain tissue using the RNAeasy® Lipid Tissue Mini Kit (Qiagen, Germany)(Cat No. 74104) . Purified total RNA from each sample was reverse transcribed by random hexamers of the High-capacity cDNA Reverse Transcription kit (Applied Biosystems, USA) for the preparation of complementary DNA (cDNA). The expression of GABAergic was estimated by quantitative RT-PCR (Light Cycler 480 II/96, Roche Applied Science, Switzerland) using iTaq™ Universal SYBR® Green Super mix kit, that was prepared according to the manufacturer's protocol Gene Expression Assay (Assay ID Rn00691548_m1, Applied Biosystems, USA). Gene expression of glyceraldehyde-3-phosphate dehydro- genase (GAPDH) was used as a reference gene (assay ID Rn01775763g1, Applied Biosystems, USA). And specific primers were added to the reaction mix at a final concentration of 10 pM.

### Microbial analysis:

### Fecal collection and preparation for microbial analysis

In the present study, the fecal samples of the rats from all groups were collected in sterile tubes and kept at −20 °C until further use. The microbial analysis involved the culturing of microorganisms on different media and under different incubation conditions for their preliminary identification and enumeration indicating the alteration of the gut microbiota in response to the treatment being tested.

Fecal suspensions of each treated group correspondingly were prepared by dissolving 1:10 w/v in sterile phosphate-buffered saline (PBS, 0.1M) (Zhang et al., 2014).

All samples were homogenized using a sonicator for 5 s followed by centrifugation at 5000 rpm for 10 min at −4 °C. Ten-fold serial dilutions of the fecal suspensions were then performed. One milliliter of the supernatant from the original dilution (dilution 0) was added to 9 ml sterile PBS in a tube (dilution 1). The process was repeated until dilution 4 was created, and 0.1 ml of each of the prepared dilutions were loaded and spread on the surface of different culture media. The culture media used included nutrients agar were incubated aerobically at 37 °C for 18–24 h.

### Bacterial enumeration and identification

Before incubation, the bacterial count from the different media was recorded as the colony count per plate. Data were compared between the rats' groups in the study. Preliminary bacterial identification was performed by morphological observation on the different media used. Further identification was made microscopically using the gram staining technique, where single colonies from the various culture media were selected, heated to form a smear,

subjected to a Gram staining procedure, and then observed under the microscope using an oil immersion lens.

### Statistical analyses

The results of the present study were expressed as the means ± S.D. All statistical comparisons between the control group and the PA and probiotic-treated rats groups were performed using SPSS version 16.0. One-way analysis of variance (ANOVA) tests with Dunnett's test for multiple comparisons was performed.

**Table 1**: Mean ±S.D. of all the measured variables in the brain homogenates of the six studied groups:

| | Control | PPA | PPA+ | PPA+BIF | PPA+LAC | PPA+MIX |
|---|---|---|---|---|---|---|
| **Glutamine** | 136.4 ± 16.95 | 58.41 ± 14.36$^{a**}$ | 77.76 ± 14.39$^{a**}$ | 113.3 ± 16.67$^{bc*}$ | 58.74 ± 10.18$^{d*}$ | 72.11 ± 18.05$^{d**}$ |
| **Glutamate** | 3.883 ± 0.6326 | 1.061 ± 0.2552 | 1.931 ± 0.4517 | 2.773± 0.8548 | 1.214 ± 0.1813 | 2.033 ± 0.7631 |
| **GABA** | 283.5 ± 59.00 | 143.0 ± 24.02$^{a**}$ | 152.3 ± 34.06$^{a*}$ | 347.8 ± 80.01$^{bc*}$ | 142.0 ± 29.95$^{d*}$ | 278.7 ± 67.55$^{b*}$ |
| **$GABAR_A$** | 9.912 ± 1.587 | 1.752 ± 0.8360$^{a**}$ | 3.096 ± 0.9080$^{a**}$ | 6.479 ± 2.550$^{bd*}$ | 1.324 ± 0.7396$^{a*}$ | 4.493 ± 2.594$^{a*}$ |
| **Lipid peroxides** | 10.17 ± 3.528 | 43.61 ± 5.181$^{a**}$ | 51.24 ± 6.843$^{a**}$ | 33.30 ± 6.274$^{ac**}$ | 40.68 ± 6.111$^{a**}$ | 47.09 ± 10.41$^{a**}$ |
| **GSH** | 81.62 ± 19.59 | 26.21 ± 15.94$^{a*}$ | 41.16 ± 24.21 | 69.70 ± 45.15 | 27.39 ± 11.51 | 90.67 ± 27.27$^{bce*}$ |
| **GST** | 18.55 ± 3.219 | 5.690± 3.775 | 9.010 ± 3.237 | 17.83 ± 6.741 | 6.135 ± 2.628 | 18.38 ± 5.644 |

(a) Control vs all groups, (b) PPA vs all groups, (c) PPA+ vs therapeutics group, (d) PPA+BIF vs

PPA+LAC and PPA+BIF vs PPA+MIX, (e) PPA+LAC vs PPA+MIX.

(*). The mean difference is significant at P≤ 0.001 level,

**Table 2**: Mean ±S.D. of all the measured variables Ratios of (I) GABA\GABARA, (II) GABA\Glutamate, (III) Glutamine\Glutamate) in the brain homogenates of the six studied groups:

| | Control | PPA | PPA+ | PPA+BIF | PPA+LAC | PPA+MIX |
|---|---|---|---|---|---|---|
| **Glutamine\glutamate** | 25.00 ± 9.945 | 16.18 ± 7.730 | 28.72 ± 13.32 | 30.90 ± 9.489 | 25.38 ± 11.18 | 39.86 ± 12.84$^{b*}$ |
| **GABA\Glutamate** | 83.27 ± 26.58 | 47.92 ± 19.39 | 64.26 ± 67.62 | 81.82 ± 28.73 | 61.63 ± 40.50 | 83.06 ± 44.28 |
| **GABA\ $GABAR_A$** | 25.32 ± 7.092 | 11.32 ± 5.397$^{a*}$ | 12.64 ± 10.00$^{a*}$ | 13.05 ± 8.337$^{a*}$ | 11.21 ± 3.899$^{a*}$ | 10.59 ± 5.425$^{a*}$ |

(a) Control vs all groups, (b) PPA vs all groups,

(*). The mean difference is significant at P≤ 0.001 level.

**Table 3:** Mean ± S.D of **$GABAR_A$, $GABAR_B$, and $GABAR_G$ selected subunits gene** expression in brain homogenates of male western albino young rats, in all groups:

| | Control | PPA | PPA+ | PPA+BIF | PPA+LAC | PPA+MIX |
|---|---|---|---|---|---|---|
| **$GABAR_A1$** | 1 ± 0.409 | 0.0716 ± 0.0089* | 1.878 ± 0.086* | 2.493 ±0.291** | 2.049 ± 0.0789* | 0.0371 ± 0.00073** |
| **$GABAR_A2$** | 1 ± 0.162 | 0.0116 ± 0.007** | 0.924± 0.056401* | 1.574 ± 0.336* | 0.310 ± 0.044* | 0.0259 ± 0.0026** |
| **$GABAR_A3$** | 1 ± 0.027 | 0.00036± 5.18046E-05** | 0.648 ± 0.024** | 1.132 ± 0.119** | 0.256± 0.0213** | 0.00059 ± 0.00015** |
| **$GABAR_A5$** | 1 ± 0.108 | 0.0070 ± 0.0011** | 0.564 ± 0.030** | 0.683 ± 0.038** | 0.142± 0.004** | 0.019 ± 0.002** |
| **$GABAR_B2$** | 1 ± 0.0195 | 0.066 ± 0.0137* | 1.076 ± 0.135* | 1.608 ± 0.269* | 0.677 ± 0.662 | 0.038± 0.00196** |
| **$GABAR_B3$** | 1 ± 0.158 | 0.00025 ± 0.00011* | 1.089 ± 0.0064* | 2.099 ± 0.215** | 0.561± 0.087* | 6.81E-05 ± 8.92E-05* |
| **$GABAR_G2$** | 1± 0.199 | 0.0410± 0.00322* | 0.611 ± 0.028* | 1.181 ± 0.1609* | 0.2875 ± 0.0266* | 0.01715± 0.0017* |

(*). The mean difference is significant at P≤ 0.001 level,

(**) The mean difference is significant at P≤ 0.0001 level.

**Table4:** Estimation change of microorganisms in all groups. MCA, MacConkey agar; NA, Nutrient agar; MHA Mellur Henton agar; Blood agar. [(-): no growth, (+) Weak growth, (++): Medium growth, (+++): Strong growth.]:

| Isolated Organisms | Media and incubation conditions | Control | PPA+ | PPA + BIF | PPA + LAC | PPA + MIX |
|---|---|---|---|---|---|---|
| Enterobacteriaceae (Gram-negative rod, lactose fermenters) | MCA /Aerobic 37˚C/24hr | + | - | ++ | +++ | ++ |
| *Staphylococcus* and/or Bacilli (Gram-positive cocci/ rod or Gram-negative rod) | N.A / Aerobic 37˚C/24hr | - | - | +++ | + | ++ |
| Moraxella spp<br>Gram-negative | MHA / Aerobic 37˚C/24hr | ++ | + | ++ | ++ | ++ |
| Gram-pe/Gram-negative rod and positive cocci | Blood /Aerobic 37˚C/24hr | - | ++ | ++ | - | + |

(-): No growth; (+): Weak growth; (++) Moderate growth; (+++) High growth

## Discussion

Oxidative stress is one of the chief penalties of glutamate-induced neurotoxicity. However, it is not possible to define a unidirectional cause/effect relationship between the two signaling as etiological mechanisms of ASD, since oxidative stress and excessive intracellular ROS can also induce excitotoxicity by stimulating extracellular glutamate release (Pellegrini-Giampietro et al., 1990) and releasing calcium from mitochondria into the cytosol (Richter and Kass, 1991). From another viewpoint, it has already been revealed that astrocytic glutamine synthetase is especially vulnerable to ROS-induced inactivation, which compromises the whole glutamate-glutamine cycle and contributes to an increase in extracellular glutamate levels and resulting in excitotoxicity (Schor, 1988). Additionally, the presence of ROS has been shown to decrease glutamate transporter activity, impairing synaptic clearance of glutamate further contributing to the increase in extracellular glutamate concentration (Anderson and Swanson, 2000). Collectively this could support the remarkable increase of lipid peroxides concomitant with the decrease of glutathione, GST, glutamine, and glutamine/glutamate ratios in PPA-treated groups as a

rodent model of ASD (Table 1 and Figs. 1(I-V)- Table 2 and Figs. 2 (III). This is in good agreement with numerous previous works which demonstrate alteration in multiple biomarkers related to oxidative stress and glutamate excitotoxicity in PPA-induced rodent model of autism (El-Ansary et al., 2012, 2016, Aldbass et al., 2013). Lower glutamine could lead to a much higher toxic effect of glutamate as an excitatory neurotransmitter, while lower GABA and GABA receptors could be easily related to the E/I imbalance repeatedly reported in autistic patients and rodents models (Al- Gadani et al., 2009; El-Ansary et al., 2017). Reversal of GABAergic/ glutamatergic imbalance was recently reported as a medical hypothesis to treat ASD (El-Ansary, 2020). Among the integrated strategy to reverse E/I imbalance was the use of probiotics as a source of GABA and inducer of GABA receptor gene expression.

Moreover, Table 1 and Figs. 1(I-V)-Table 2 and Figs. 2 (III) demonstrate the significant improvement of lipid peroxides, glutathione, GST, glutamine, and glutamine/glutamate ratios in probiotics-treated rats. This is in good agreement with the previous work by Duranti et al (2020) in which they reported a slight decrease in glutamate concentration after the growth of *B. dentium*. While glutamate levels were somewhat unchanged in their study which is in good agreement with the current study, they observe increased concentrations of GABA and glutamine in *B. dentium* conditioned fully defined bacterial media, called ZMB1, for 18 h.

GABAergic neurons produce GABA from glutamate using glutamic acid decarboxylase (GAD) and this synthesized GABA is packaged into synaptic vesicles at synaptic terminals through vesicular GABA transporters (VGATs). Synaptically released GABA binds to both presynaptic and postsynaptic GABA receptors (GABAA and GABAB) and suppresses the excitation as a neurotoxic mechanism repeatedly reported in ASD (El-Ansary, 2016 &2020).

Table 1 and Fig. 1 (VI and VII) also demonstrate the significant increase of GABA and GABA receptors in probiotic-treated rats with *Bifidobacterium* strains being the more effective followed by the probiotic mixture, while *lactobacillus* was the least effective. This could find support in the previous work of Yunes et al (2016) in which they screened the ability of 135 human-derived *lactobacillus* and *bifidobacterium* strains to produce GABA from glutamate. They reported that the most efficient GABA-producers were *Bifidobacterium* strains which are in good agreement with the results of the current study. Their data indicate that *gad* genes as well as the ability to produce GABA are widely distributed among *lactobacilli* and *bifidobacteria* which could help the use of both bacterial strains independently or in combination as psychobiotics (Yunes et al., 2016). Another support could be found in the more recent study of Duranti et al (2020) in which Groningen rats fed on *B. adolescentis* strains- supplemented diet, stimulate the in vivo production of GABA highlighting their potential implication in gut-brain axis interactions.

Up to this, we can suggest that probiotics have demonstrated an effect in the form of mono and multi-strains, and so they could help in treating infectious diarrhea, antibiotic-associated diarrhea, and *Clostridium difficile*-associated diarrhea, which is one of the problems in ASD patients related to GI co-morbidity (Wasilewska, et al., 2015; Doenyas, 2018; Abdellatif et al., 2020).

Balanced neural circuits are especially important for proper social and emotional behavior, language processing, and higher-order cognition. Research in this area has shown that alterations in GABAergic signaling inhibition activities could result in a loss of balance in neural circuits and lead to a disproportionately high level of excitation. Importantly, evidence accumulated from previous studies indicates that disruption in GABAergic signaling transmission could be a potential underlying mechanism that leads to the development of autism (Eagleson et al., 2010; LeBlanc & Fagiolini, 2011).

Specific ratios between selected pairs of metabolite concentrations (metabolite ratios) have been introduced in the past as biomarkers in many biomedical applications (Petersen et al., 2012). Glutamate/GABA, and glutamate/ glutamine ratios were used to evaluate the biochemical and functional relationship of impaired glutamate signaling in ASD patients and both recorded much higher predictive values (Al-Otaish et al., 2018).

Table 2 and Fig. 2 (I-III) demonstrate the relative ratios of GABA/GABAAa, GABA/glutamate, and glutamine/glutamate. These three ratios ascertained the impaired glutamate signaling in response to PPA-induced neurotoxicity in the rodent model, together with the remarkable ameliorative effects of the three used probiotic interventions, most noticeably seen as much higher GABA/glutamate, and glutamine/glutamate ratios. As previously discussed, this is in good agreement with multiple clinical or experimental works on ASD (El-Ansary et al., 2012, 2016, Aldbass et al., 2013).

The high significant decrease or down regulation of all the seven studied GABA receptor subunits in PPA-treated rats could support the validity of this model and explain the recently recorded alteration in social interaction as an autistic feature in male rats treated with PPA (Abuaish et al., 2020). With lower and dysfunctional GABA receptors, imbalanced E/I signaling could be induced as an autistic feature (Table 3 and Fig. 3). It is very clear that the three used probiotics intervention strategies demonstrate significant up- regulation of the studied GABA receptor subunits gene expression with pure *Bifidobacterium* strains being the more potent followed by the probiotic

mixture, while *lactobacillus* was the least effective. This can be supported through the previous work of Bravo et al (2011) which prove the effectiveness of probiotics in the up-regulation of GABA receptor subunits and highlight the possible mechanistic vision into the potential effect of probiotics in treating anxiety-like behavior (Rudolph and Möhler, 2004; Cryan and Kaupmann, 2005).

Table 4 demonstrates the change of microorganisms between the six studied groups at the end of the study. The decrease of lactate producers in PPA-treated rats compared to controls and the ameliorative effect of the three used probiotics could be easily observed. In an attempt to support this we can consider the previous reports of Kang and colleagues (2013) which recorded a much lower growth rate of certain species of lactate fermenters in subjects with autism (Lindsay et al., 2006; Liu et al., 2019). Their decrease can partly explain the high levels of lactate observed in autistic patients (Benjamin et al., 2011). There was a noticeable increase in, *Staphylococcus*, and *Moraxella* species in probiotic-treated rats compared to the PPA-induced model. This is in good agreement with the recent work of Forsyth (2020) and Al-Dera (2021) which reported a decrease in the abundance of certain bacteria among which is *Moraxella* in autistic individuals and the PPA-rodent model of ASD respectively. Lower *Moraxella* in the PPA-rodent model was attributed to leaky gut as an accepted phenomenon relating gut microbiota to brain disorders through the gut-brain axis.

The remarkable increase of *Moraxella* in the probiotic-treated groups could find support in the previous work of van den Broek et al (2018) which recorded the inhibitory effect of lactobacilli against the respiratory tract pathogen *Moraxella catarrhalis*.

## In Conclusion

The study indicates that pure or mixed lactobacillus and *bifidobacterium* can be used to ameliorate glutamate excitotoxicity induced in a rodent model of autism, mostly through, the amelioration of oxidative stress, increasing the depleted GABA and up-regulated the gene expression of GABA receptors. Collectively, these data highlight the important role of probiotics in the bidirectional communication of the gut–brain axis and suggest that certain organisms may prove to be useful as therapeutic strategy.

## Declarations

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**Availability of data:**

The datasets generated analyses during the current study are available from the corresponding author on reasonable request.

**Author's contributions:**

RBK : Data acquisition

MO : Data acquisition

AA : Co-drafting of the manuscript

AMA: Experimental work

HAS: Experimental work

DAS: Microbiology data

AE: Conceptualization, and drafting the manuscript

**Ethical approval:** Ethical approval from the Institutional Review Board was obtained.

**Consent for publication:** All authors read the manuscript and agree to publish

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## Figures

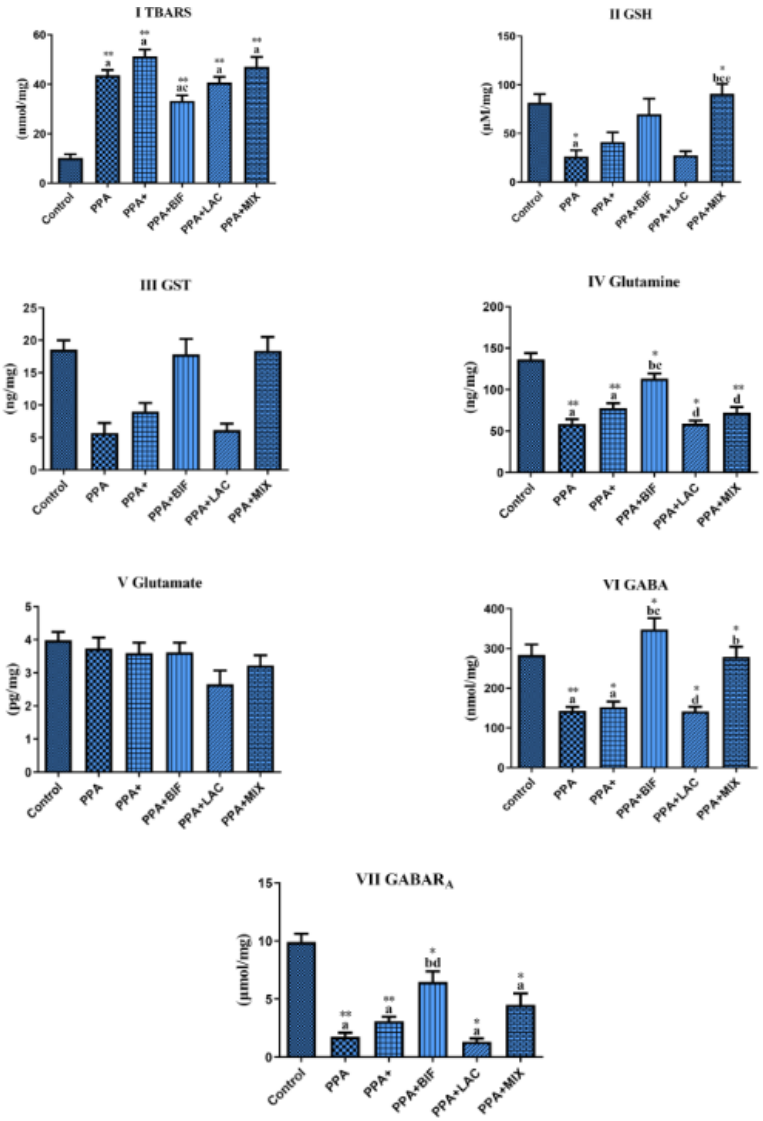



Figure 1

**Concentrations of ((I)TBARS, (II)GSH, (III)GST, (IV)Glutamine, (V)Glutamate, (VI)GABA, (VII)GABAR$_A$)** in brain homogenates of male western albino young rats, in all groups. (a) Control vs all groups, (b) PPA vs all groups, (c) PPA+ vs therapeutic groups, (d) PPA+BIF vs PPA+LAC and PPA+BIF vs PPA+MIX, (e) PPA+LAC vs PPA+MIX. (*) The mean difference is significant at P 0.001 level, (**) The mean difference is significant at P 0.0001 level.

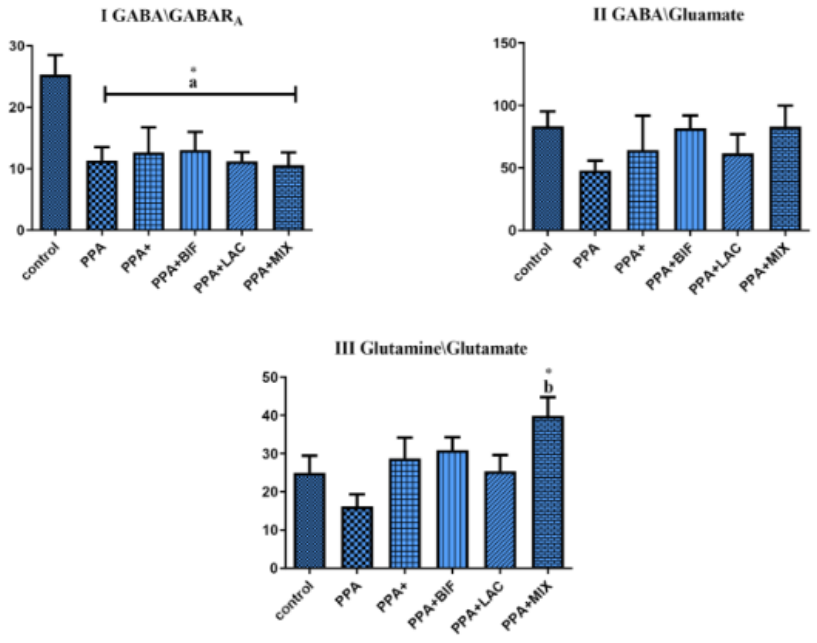
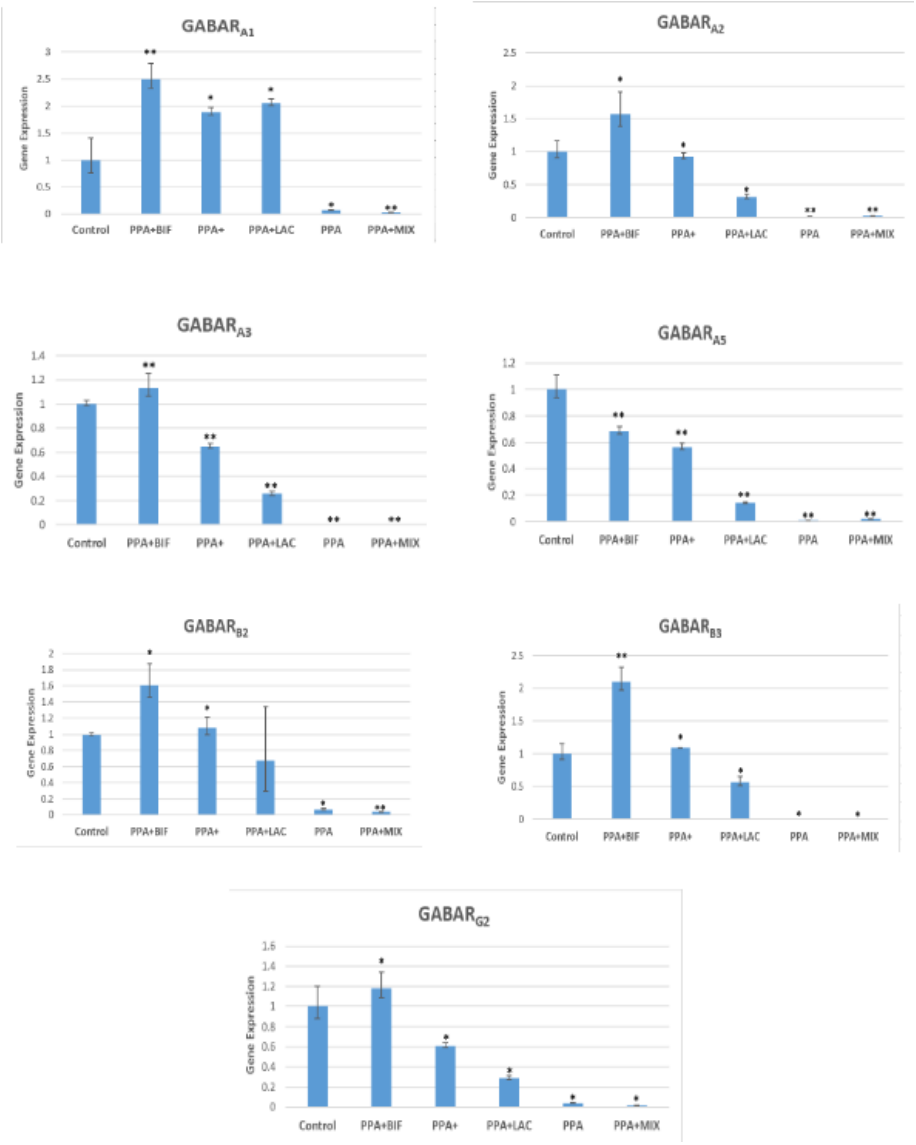



Figure 2

**Ratio of ((I)GABA\GABAR$_A$, (II)GABA\Glutamate, (III)Glutamine\Glutamate)** in brain homogenates of male western albino young rats, in all groups. (a) Control vs all groups, (b) PPA vs all groups. (*) The mean difference is significant at P 0.001 level.




## Figure 3

Effect of probiotic treatments on GABAR$_{A,}$ GABAR$_{B,}$ and GABAR$_{G}$ *selected subunits* gene expression in brain homogenates of male western albino young rats, in all groups.