Supplementation of Lactobacillus Probiotic Strains Supports Gut-Brain-Axis and Defends Autistic Deficits Occurred by Valproic Acid-Induced Prenatal Model of Autism

K. Sunand[1,2,*], G. Krishna Mohan[1], Vasudha Bakshi[3]

K. Sunand[1,2,*], G. Krishna Mohan[1], Vasudha Bakshi[3]

[1]Centre for Pharmaceutical Sciences, Institute of Science & Technology, JNTU Hyderabad, INDIA.
[2]Department of Pharmacology, Anurag University, Hyderabad, INDIA.
[3]School of Pharmacy, Anurag University, Hyderabad, INDIA.

Correspondence

K. Sunand

Centre for Pharmaceutical Sciences, Institute of Science & Technology, JNTU Hyderabad; Department of Pharmacology, Anurag University, Hyderabad, INDIA.

Phone no: +91-9000077723;

E-mail: sunandpharmacy@cvsr.ac.in

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ABSTRACT

Background: Gut microbiota can interact with the brain by bidirectional communication through Gut-Brain-Axis. Gut microbiota colonization is essential for the establishment of symbiotic relation between gut and brain. A healthy gut can properly directs the brain for its functions. Autistic people are deficient in gut microbiota, a condition known as Dysbiosis. Gastro-Intestinal symptoms are comorbid conditions in autism. Re-colonization with daily supplementation of microbiota is needed in autism. **Methods:** Autism induced by Valproic acid (VPA) at a dose of 400 mg/kg, i.p. on an embryonic day (ED) 12 to the pregnant rats. Born rats exhibited many autistic features, for the treatment we selected specific Lactobacilli strains such as *L. Plantarum*, *L. Casei*, *L. Acidophilus*, *L. Bulgaricus,* with a dosage of not less than (NLT) 1 Billion Colony-forming units (CFU) /ml given orally every day for 42 days. **Results:** The results showed that Lactobacillus strains significant ameliorated the behavioral anomalies such as T-Maze, Memory, Social interaction studies as compared to the autistic group. Furthermore, Lactobacillus supplementation helped to shift the hypersertonomia (27.33±2.33 *vs* 8.167±0.72), increasesd BDNF (59.00±2.08 *vs* 48.17±0.60) increased IL-6 (46.00±1.52 *vs* 32.00±1.73) and TNF-α levels (145.0±3.21 *vs* 98.67 ± 2.028) to baseline. Histopathology examination of the cerebellum revealed that apoptosis and degeneration were reversed with lactobacillus treatment. **Conclusion:** This study proved, daily supplementation of Lactobacillus strains has reversed autistic deficits and improved immune functions might because of gut and brain symbiotic relationship.

Key words: VPA; Autism; Dysbiosis; Lactobacillus strains; Gut-Brain-Axis.

INTRODUCTION

Autism is a chronic neurodevelopmental disorder manifested by complex neurobehavioral conditions.[1] Such as deficient in communication, interaction with people, repetitive movements, behave alone, deficient in motor functions, poor eye contact, etc.[2] Autism occurs 4-5 times more often in boys than girls.[3] Possible causes of autism are genetic, environmental factors, maternal toxicity and nutritional deficiency.[4]

Gut and brain have bidirectional communication through the enteric nervous system (ENS) and vagus nerves. Such interactions can be mediated by the metabolites produced by gut bacteria called short chain fatty acids (SCFA).[5,6] The human gut is inhabited by more than a trillion (approx. 1kg) of gut bacteria (Probiotics) in the right colon.[7] Gut bacteria have symbiotic co-evolution with the host gut, in return, it improves gut health and functions by (immune support, reduces inflammatory mediators (IL-6, TNF-alpha, etc.), reduces harmful bacteria, production of nutrients and their absorption.[8,9]

Gastrointestinal (GI) symptoms such as abdominal pain, constipation, diarrhea, are common comorbidities with autism.[10] Evidences related that GI symptoms are because of dysbiosis of the gut.[11] At present, there are no effective therapies for autism. A risk-free and effective treatments are necessary for autism. Modulation of the gut microbiota is a potential therapy in people with autism. Prebiotics and probiotics and fecal microbiota transplantation are the various therapeutic opportunities.[7,11]

Probiotics, such as the lactic acid-producing bacteria belonging to Lactobacilli, Bifidobacteria and Saccharomycetes, are beneficial to the host when we provided in adequate quantities.[12,13] Many studies have shown that probiotics can prevent and treat a variety of diseases, such as obesity, depression, colorectal cancer and Crohn's disease, in animal models and humans.[14,15]

Valproic acid (VPA) is an antiepileptic drug used to treat simple or complex seizures.[16] Usage of VPA in pregnant women with epilepsy lead risk effects in the fetus such as spina bifida, cleft palate, limb defects and cardiac malformations.[17] VPA is a potent teratogen, exposure of VPA during the prenatal or postnatal period can serve as triggering factors of oxidative stress which disrupt the neuron growth and development.[18] With the VPA induced autism model is a valuable tool to investigate the neurobiology, autistic behavior and to screen the several novel therapeutic agents.[19]

MATERIALS AND METHODS

Drugs and chemicals

Unique Biotech Ltd.: Probiotic strains *L. Plantarum*- UBLP-40 (MTCC 5380), *L. Casei*- UBLC-42 (MTCC 5381), *L. Acidophilus*- UBLA-34 (MTCC 5401), *L. Bulgaricus*- Lactobacillus bulgaricus UBLB-38; Natures Velvet Lifecare: Inulin (Prebiotic); Sun Pharmaceuticals: Sodium Valproate; Rhetoric India: Rat ELISA Kits (5HT, TNF-alpha, IL-6, BDNF).

Experimental animals

Pregnant rats at the gestation age procured from the National Institute of Nutrition Hyd. Pregnant animals were maintained on standard laboratory pellet chow diet, provided water ad libitum and were kept under standard conditions at 23-25 ^{0}c, 35 to 60% humidity, and 12hr light /dark cycle. The experimental protocol was duly approved by Institutional Animal Ethics Committee (IAEC) and care of the animals was carried out as per the guidelines of CPCSEA (Protocol. No: I/IAEC/ AGI/019/2018 WR ♀).

Experimental design

On an embryonic day (ED) 12 administered VPA at a dosage 400 mg/kg, i.p.[20]. The health condition and weight of pregnant rats were monitored regularly until delivery. When the pups were born the day was recorded as postnatal day (PND) 0. On PND 8th day, rat pups are divided into 7 groups, each group with eight pups (n=8) namely,

Group I: Vehicle treated group (Inulin 3mg, p.o daily)

Group II : Autistic Group (VPA 400 mg/kg, i.p)

Group III : VPA+*L. Plantarum* (NLT 1Billion CFU/ml, p.o)

Group IV : VPA+*L. Casei* (NLT 1Billion CFU/ml, p.o)

Group V : VPA+*L. Acidophilus* (NLT 1Billion CFU/ml, p.o)

Group VI : VPA+*L. Bulgaricus* (NLT 1Billion CFU/ml, p.o)

Group VII : VPA+Multilactobacillus strains (NLT 1Billion CFU/ml, p.o).

Multilactobacillus strains are made with a equal proportions of *L. Plantarum + L. Casei + L. Acidophilus + L. Rhamnosus + L. Bulgaricus* strains. The treatment duration for this study was PND 08-50 with daily supplementation of probiotic strains NLT 1 Billion CFU/ml, p.o with the guidelines of administration and storage details provided by Unique Biotech Ltd.

Rats were subjected to behavioral testing to assess Negative geotaxis, Eye-opening, Swimming performance, T-maze, Morris water maze, and Social interaction studies were performed on various postnatal days up to PND 50. All behavioral studies we performed at the Neuroscience lab, School of Pharmacy, Anurag University through a video tracking system provided by VJ Instruments.

BEHAVIORAL STUDIES

Negative geotaxis

Negative geotropism was tested on postnatal days 7 –10 by placing the mouse face down along a 45° incline in a temperature-controlled environment. Latency to turn 180° such that the head was facing upward along the incline was recorded with a maximum of 30 sec for each trial.[19]

Eye opening

Eye-opening was observed daily every day after birth PND 12-16. Eye openings were recorded and rated as follows: 0-Both eyes are closed,

1- one eye-opening, 2-Both eyes are opened.[19]

Swimming performance

An aquarium filled with water (28–29°C) was used for swimming tests on PNDs 22, 24 and 26. Each animal was put at the center of the aquarium and was observed for 5–10s. The swimming performance was evaluated according to the position of nose and head (angle) on the surface of the water. The angle of swimming was rated as follows: 0—head and nose below the surface; 1—nose below the surface; 2—nose and top of head at or above the surface but ears still below the surface; 3—the same as two except that water line was at mid-ear level; and 4—the same as three except that water line was at the bottom of ears. Thereafter, the test pups were dried and returned to the home cages. Swimming is a measure of motor development and the integration of a coordinated series of reflex responses.[19]

T-maze test

The T-maze is a spontaneous alteration test, which was performed on PND 29-31, it is used to evaluate the repetitive/restricted behavior. Five sessions were performed for each rat. For each session, the first choice of the rat in the free choice arms was evaluated, i.e., whether the rat first entered the left or right arms. The parameter analyzed was the percentage of alterations between the left and right arms, which was always assessed in relation to the arm visited in the previous session. This model is based on the natural proclivity of rats to alternate between the visited goal-arms in each trail over a series of successive trails. Thus, a higher percentage of alteration between the arms was considered normal rat behavior, whereas fewer alterations indicated cognitive inflexibility and repeated behavior. For statistical analysis, these data into scores: 0= no alterations (repeatedly visiting the same arm for all five sessions), 1=one alterations, 2= two alterations, 3= three alterations, and 4= four alterations.[21]

Morris water maze

Memory was evaluated by Morris water maze, autistic individuals have deficits in memory, and this test was performed on PND 48-50. The aquarium is made up of 4 Quadrants indicated by different colours for visual clues, it was filled with normal water and covered with milk, and a removable hidden platform was placed at one quadrant. Each animal was placed and the escape latency period was recorded, repeat the same for next day PND 49. On PND 50 platform was removed and identify escape latency period.[19]

Social interaction

Social Interaction was performed on PND 36-40, prior to the experimentation animals are separated and housed individually overnight before the experiment. Two animals from the same group, but different litters were placed into a circular cage provided by ambient light and temperature for 20 min. frequency of the following parameters is assessed: Allogroming, Anogenital Inspections, Pinning's, Play Behavior, Social exploration.[17,21]

Stereotype/ repetitive behavior

The exploratory behavior of the rat was evaluated by the open-field habituation task method. A rat was placed in a 40 cm×50 cm×60 cm open field whose brown linoleum floor was divided into 12 equal squares by white lines and left to explore it freely for 5 minutes on PND 46-48. The number of line crossings and head dipping was counted.[17]

BIOCHEMICAL PARAMETERS

Rat brains were isolated and washed with ice-cold 0.1M phosphate buffer pH-7.4 to remove the blood. Homogenize the brain with 0.1M

phosphate buffer saline solution. Then homogenate and the resultant supernatant were used for further biochemical estimations, such as AchE, 5-HT, BDNF, and antioxidants.

Estimation of acetylcholinesterase (AchE) activity

The AchE activity was measured in brain tissue by the reaction of thiocholine with dithiobisnitrobenzoate ions. The rate of formation of thiocholine from acetylcholine iodide in the presence of brain cholinesterase was measured using a spectrophotometer (Shimadzu 1800) at a wavelength of 412 nm.[22,23]

Estimation of BDNF, 5-HT, IL-6 and TNF- α

The biochemical parameters was measured in the blood and brain sample by ELISA method. To the pre-coated micro ELISA plate samples and standards were added. Then addition of further substrates to conjugate and getting of specific color. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The OD value is proportional to the concentration of Rat BDNF, 5-HT, IL-6 and TNF- α. Calculate the concentration of Rat BDNF, 5-HT, IL-6 and TNF- α in the samples by comparing the OD of the samples to the standard curve.[24-27]

HISTOPATHOLOGY

On PND 50 rats was sacrificed, isolated brains were placed immediately in 10% neutral formalin solution. Processed and embedded in paraffin. Sagittal sections of the cerebellum (5μm thick) were stained with hematoxylin and eosin (H&E) and analyzed using a light microscope for changes in the cerebellum.

STATISTICAL ANALYSIS

All data are presented as Mean ±S.E.M. The significance of difference among the groups was assessed using a one-way analysis of variance (ANOVA) followed by Kolmogorov–Smirnov test, Bonferroni's Multiple Comparison Test using GraphPad Prism 5 software and ($p < 0.05$) was considered significant.

RESULTS

Behavioral parameters

Effect of Lactobacillus strains on negative geotaxis

There was a significant increase in time taken to re-orient along the inclined plane seen in autistic group on PND 9-21, when compared to vehicle group ($p < 0.001$). Treatment with Lactobacillus strains (*L. Plantarum, L. Casei, L. Acidophilus, L. Bulgaricus*) finally decreased the time taken to re-orient ($p < 0.001$) with the autistic group, the results are shown in Tables 1 & 2.

Effect of Lactobacillus strains on Eye-opening

Delayed eye-opening was seen in the autistic group on PND 12-16 compared with the vehicle group ($p < 0.001$). Treatment with Lactobacillus strains (*L. Plantarum, L. Casei, L. Acidophilus, L. Bulgaricus*) has shown improvement in eye-openings ($p < 0.001$) the results are shown in Figures 1-3.

Effect of Lactobacillus strains on swimming performance

There was decreased swimming performance in the autistic group compared with the vehicle group ($p < 0.001$). Treatment with Lactobacillus strains (*L. Plantarum, L. Casei, L. Acidophilus, L. Bulgaricus*) showed improvement in swimming performance ($p < 0.001$) with the autistic group on PND 22, 24 and 26, the results are shown in Figures 4-6.

Effect of Lactobacillus strains on T-maze

There was a low alteration score reported in the autistic group by the T-maze test on PND 29-31 when compared with the vehicle group ($p < 0.001$). Treatment with Lactobacillus strains (*L. Plantarum, L. Casei, L. Acidophilus, L. Bulgaricus*) showed that alteration score was significantly improved when compared with an autistic group ($p < 0.05$) the results are shown in Figures 7-9.

Table 1: Effect of Lactobacillus strains on Negative geotaxis.

Negative geotaxis				
Groups	PND 9	PND 11	PND 13	PND 15
Group I (Inulin 3mg, p.o daily)	1.09 ± 0.25	1.50 ± 0.50	3.50 ± 0.50	2.00 ± 1.00
Group II (Autistic group)	10.0 ± 0.81###	11.5 ± 0.50###	12.5 ± 0.50###	10.57 ± 0.39###
Group III (L. Plantarum)	7.25 ± 0.86***	7.16 ± 0.28***	6.50 ± 0.50***	7.16 ± 0.288***
Group IV (L. Casei)	7.25 ± 0.28***	7.00 ± 0.0***	7.33 ± 0.15***	6.167 ± 0.28***
Group V (L. Acidophilus)	7.92 ± 0.15*	7.50 ± 0.50***	6.76 ± 0.25***	6.033 ± 0.45***
Group VI (L. Bulgaricus)	7.67 ± 0.53**	8.16 ± 0.28***	8.33 ± 0.28***	7.23 ± 0.25***
Group VII (Multilactobacillus strains)	6.83 ± 0.28***	6.83 ± 0.28***	6.56 ± 0.92***	6.00 ± 0.50***

Negative geotaxis in rats with autism. Data expressed as mean ± SEM, n=6 for each group. ###$p < 0.001$ compared to the vehicle. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with Autistic group. (Kolmogorov–Smirnov test, Bonferroni's Multiple Comparison Test).

Table 2: Effect of Lactobacillus strains on Negative geotaxis.

Negative geotaxis			
Groups	PND 17	PND 19	PND 21
Group I (Inulin 3mg, p.o daily)	2.16 ± 0.288	2.66 ± 0.57	1.66 ± 0.76
Group II (Autistic group)	10.85 ± 0.78###	11.17 ± 1.25###	10.67 ± 1.04###
Group III (L. Plantarum)	6.00 ± 0.50***	5.16 ± 0.28***	4.33 ± 0.28***
Group IV (L. Casei)	6.33 ± 0.28***	5.83 ± 0.28***	4.40 ± 0.173***
Group V (L. Acidophilus)	5.66 ± 0.76***	6.16 ± 0.28***	5.53 ± 0.503***
Group VI (L. Bulgaricus)	7.16 ± 0.28***	6.33 ± 0.28***	5.33 ± 0.288***
Group VII (Multilactobacillus strains)	4.90 ± 0.65***	4.66 ± 0.577***	4.16 ± 0.28***

Negative geotaxis in rats with autism. Data expressed as mean ± SEM, n=6 for each group. ###$p < 0.001$ compared to the vehicle. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with Autistic group. (Kolmogorov–Smirnov test, Bonferroni's Multiple Comparison Test).
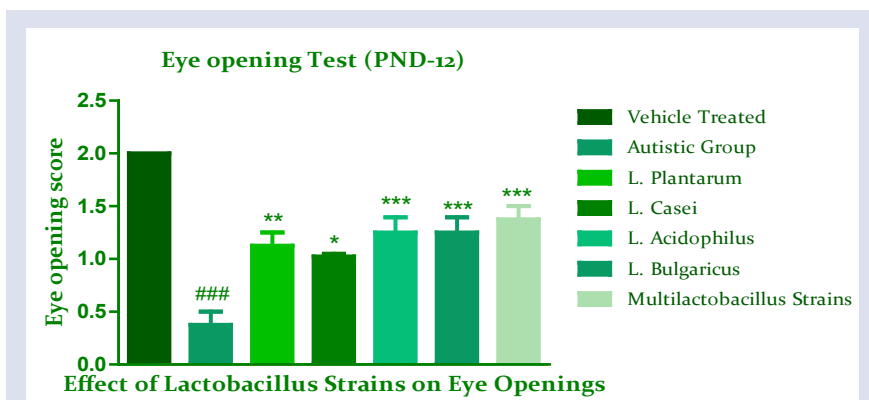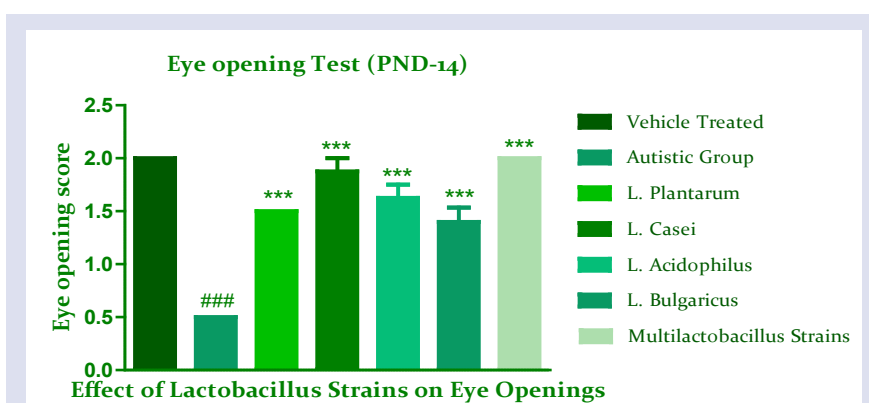
Figure 1: Effect of Lactobacillus strains on Eye openings PND 12

Eye opening test in with autism. Data expressed as mean±SEM, n=6 for each group. ###p<0.001 compared to the vehicle. *p<0.05, **p<0.01, ***p<0.001 compared with Autistic group. (Kolmogorov–Smirnov test, Bonferroni's Multiple Comparison Test)..

Figure 2: Effect of Lactobacillus strains on Eye openings PND 14.

Eye opening test in with autism. Data expressed as mean±SEM, n=6 for each group. ###p<0.001 compared to the vehicle. *p<0.05, **p<0.01, ***p<0.001 compared with Autistic group. (Kolmogorov–Smirnov test, Bonferroni's Multiple Comparison Test).
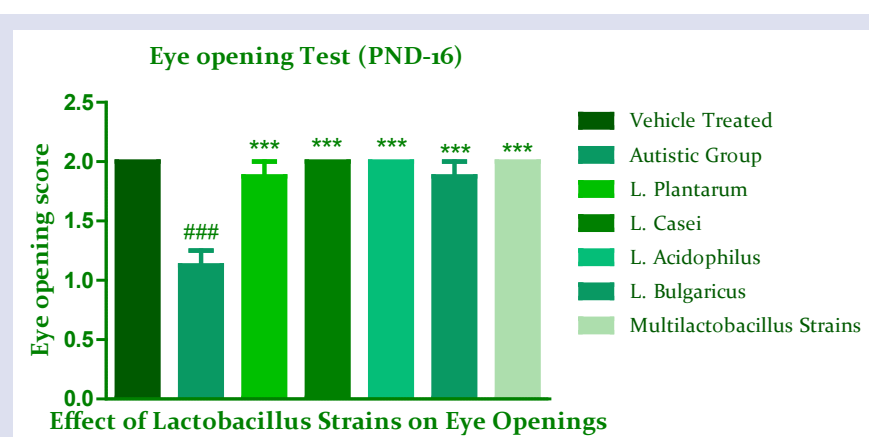

Figure 3: Effect of Lactobacillus strains on Eye openings PND 16.

Eye opening test in with autism. Data expressed as mean±SEM, n=6 for each group. ###p<0.001 compared to the vehicle. *p<0.05, **p<0.01, ***p<0.001 compared with Autistic group. (Kolmogorov–Smirnov test, Bonferroni's Multiple Comparison Test).

Effect of Lactobacillus strains on Morris water maze

There was poor cognition seen in the autistic group with the identification of hidden platform on PND 48-50 when compared with the vehicle group (p<0.001). Treatment with Lactobacillus strains (*L. Plantarum, L. Casei, L. Acidophilus, L. Bulgaricus*) showed that latency

to identify the hidden platform was significantly increased than autistic subjects (p<0.001) the results are shown in Table 3.

Effect of Lactobacillus strains on Social interaction

In the autistic group, there was a lower level of social interaction seen when compared to the vehicle group (p<0.001) on PND 36-
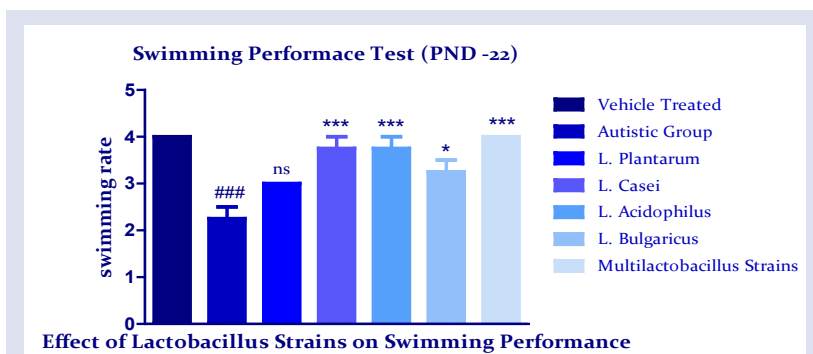
Figure 4: Effect of Lactobacillus strains on swimming performance PND 22.

Swimming performance in rats with autism. Data expressed as mean±SEM, n=6 for each group. ###p<0.001 compared to the vehicle. *p<0.05, **p<0.01, ***p<0.001 compared with Autistic group. (Kolmogorov–Smirnov test, Bonferroni's Multiple Comparison Test).
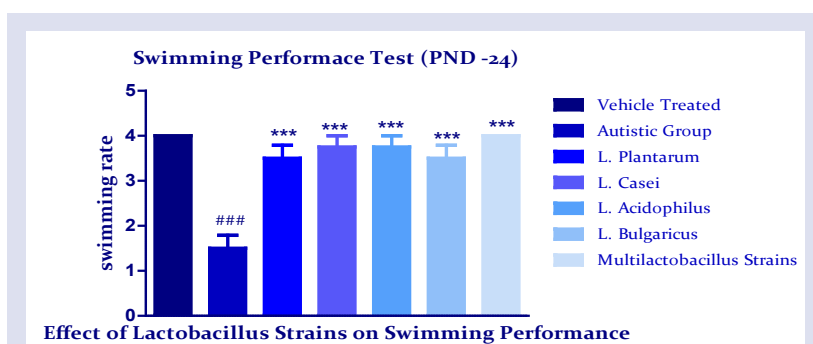

Figure 5: Effect of Lactobacillus strains on swimming performance PND 24.

Swimming performance in rats with autism. Data expressed as mean±SEM, n=6 for each group. ###*p*<0.001 compared to the vehicle. *p*<0.05, ***p*<0.01, ****p*<0.001 compared with Autistic group. (Kolmogorov–Smirnov test, Bonferroni's Multiple Comparison Test).
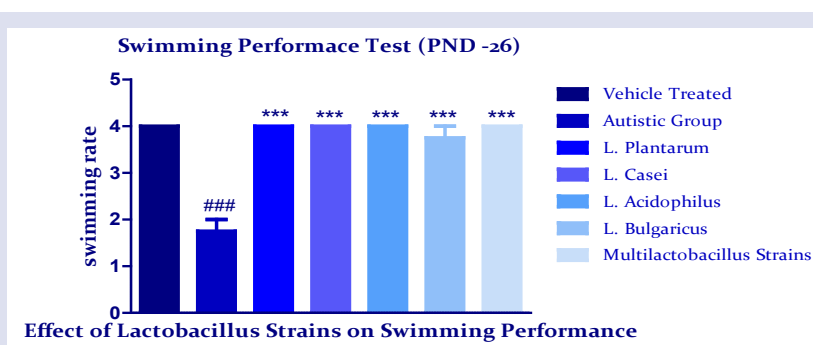

Figure 6: Effect of Lactobacillus strains on swimming performance PND 26.

Swimming performance in rats with autism. Data expressed as mean±SEM, n=6 for each group. ###*p*<0.001 compared to the vehicle. *p*<0.05, ***p*<0.01, ****p*<0.001 compared with Autistic group. (Kolmogorov–Smirnov test, Bonferroni's Multiple Comparison Test).

40. Treatment with Lactobacillus strains (*L. Plantarum, L. Casei, L. Acidophilus, L. Bulgaricus*) shown improvement in all social interaction activities (*p*<0.001) when compared with the autistic group. The results are shown in Table 4.

Effect of Lactobacillus strains on Repetitive/ Stereotype Behavior

There was a decrease in exploration and nature of behavior in autistic rats when compared with the vehicle group (*p*<0.001) on PND 46-48. Treatment with Lactobacillus strains (*L. Plantarum, L. Casei, L. Acidophilus, L. Bulgaricus*) improved their stereotype behavior

(*p*<0.001) when compared with the autistic group. The results are shown in Table 5.

Biochemical parameters

Estimation of IL-6 & TNF-α

Prenatal induction of autism resulted in significant increase in IL-6 & TNF-α levels (*p*<0.001) when compared with the vehicle group. Lactobacillus strains treatment significantly attenuated the increased IL-6 & TNF-α levels (*p*<0.001) when compared with the autistic group. The results are shown in Table 6.
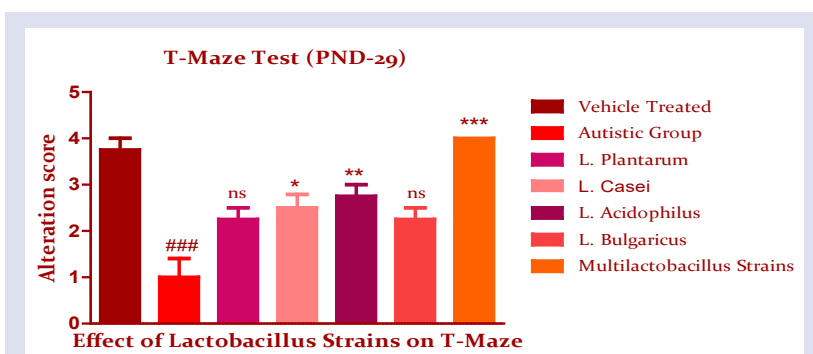
Figure 7: Effect of Lactobacillus strains on T-Maze PND 29.

T-maze test in rats with autism. Data expressed as mean±SEM, n=6 for each group. ###p<0.001 compared to the vehicle. *p<0.05, **p<0.01, ***p<0.001 compared with Autistic group. (Kolmogorov–Smirnov test, Bonferroni's Multiple Comparison Test).

Figure 8: Effect of Lactobacillus strains on T-Maze PND 30.

T-maze test in rats with autism. Data expressed as mean±SEM, n=6 for each group. ###p<0.001 compared to the vehicle. *p<0.05, **p<0.01, ***p<0.001 compared with Autistic group. (Kolmogorov–Smirnov test, Bonferroni's Multiple Comparison Test).

Figure 9: Effect of Lactobacillus strains on T-Maze PND 31.

T-maze test in rats with autism. Data expressed as mean±SEM, n=6 for each group. ###p<0.001 compared to the vehicle. *p<0.05, **p<0.01, ***p<0.001 compared with Autistic group. (Kolmogorov–Smirnov test, Bonferroni's Multiple Comparison Test).
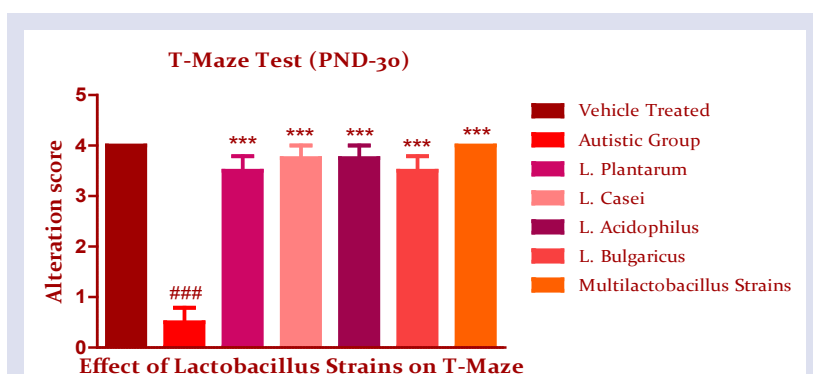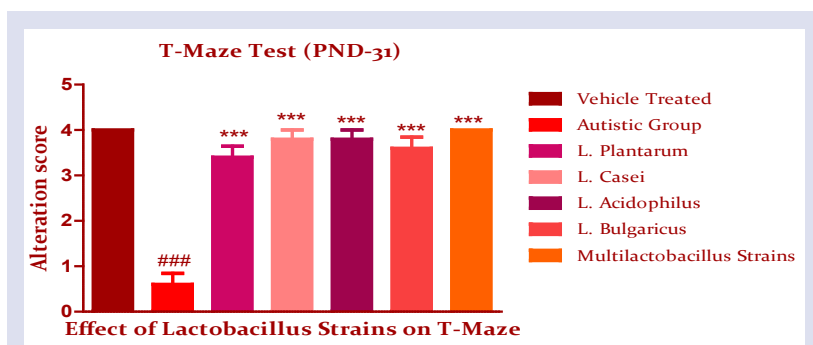
Table 3: Effect of Lactobacillus strains on Memory test (Morris water maze).

Morris water maze			
Groups	PND 48	PND 49	PND 50
Group I (Inulin 3mg, p.o daily)	50.25 ± 3.750	40.00 ± 2.88	14.00 ± 0.57
Group II (Autistic group)	137.0 ± 6.69###	146.7 ± 17.64###	122.7 ± 7.21###
Group III (L. Plantarum)	79.25 ± 2.49***	56.67 ± 4.41***	26.67 ± 3.33***
Group IV (L. Casei)	80.50 ± 2.10***	48.00 ± 1.52***	25.00 ± 3.33***
Group V (L. Acidophilus)	72.00 ± 2.12***	31.67 ± 1.66***	16.67 ± 4.41***
Group VI (L. Bulgaricus)	81.75 ± 1.65***	55.00 ± 2.88***	26.67 ± 1.66***
Group VII (Multilactobacillus strains)	61.50 ± 2.53***	23.33 ± 4.41***	9.66 ± 1.202***

Memory test (Morris water maze) in rats with autism. Data expressed as mean ± SEM, n=6 for each group. ###*p*<0.001 compared to the vehicle. *p*<0.05, **p*<0.01, ***p*<0.001 compared with Autistic group. (Kolmogorov–Smirnov test, Bonferroni's Multiple Comparison Test).

Table 4: Effect of Lactobacillus strains on Social interaction.

	Social interaction				
Groups	**Allogrooming**	**Anogenital inspection**	**Pinnings**	**Play behavior**	**Social exploration**
Group I (Inulin 3mg, p.o daily)	13.52 ± 0.33	27.50 ± 1.04	20.75 ± 1.25	8.66 ± 0.33	21.50 ± 0.64
Group II (Autistic group)	2.25 ± 0.85###	9.25 ± 1.109###	8.250 ± 1.65###	1.00 ± 0.57###	8.75 ± 0.85###
Group III (L. Plantarum)	6.00 ± 0.40**	16.00 ± 0.40***	14.00 ± 0.91**	4.00 ± 0.57*	12.50 ± 0.28*
Group IV (L. Casei)	11.25 ± 0.47***	17.50 ± 0.28***	15.75 ± 0.47***	4.66 ± 0.66**	14.75 ± 0.28***
Group V (L. Acidophilus)	12.00 ± 0.40***	22.00 ± 0.91***	16.50 ± 1.04***	5.00 ± 0.57**	12.75 ± 0.62*
Group VI (L. Bulgaricus)	7.750 ± 0.47***	14.00 ± 0.40**	14.00 ± 0.40**	4.33 ± 0.33**	13.50 ± 1.04**
Group VII (Multilactobacillus strains)	12.50 ± 0.64	24.00 ± 0.48***	17.25 ± 0.47***	8.00 ± 0.57***	17.50 ± 0.64***

Social interaction in rats with autism. Data expressed as mean ± SEM, n=6 for each group. ###*p*<0.001 compared to the vehicle. *p<0.05, **p<0.01, ***p<0.001 compared with Autistic group. (Kolmogorov–Smirnov test, Bonferroni's Multiple Comparison Test).

Table 5: Effect of Lactobacillus strains on repetitive/stereotypic-like behaviors.

	Repetitive/stereotypic-like behaviors		
Groups	**Rearing's**	**Grooming**	**Hole pocking**
Group I (Inulin 3mg, p.o daily)	24.50 ± 0.64	15.00 ± 1.08	20.00 ± 1.08
Group II (Autistic group)	7.25 ± 0.47###	6.75 ± 0.62###	9.75 ± 0.85###
Group III (L. Plantarum)	12.75 ± 0.62***	10.25 ± 0.47*	15.75 ± 0.47***
Group IV (L. Casei)	14.50 ± 0.28***	10.75 ± 0.47**	15.25 ± 1.03***
Group V (L. Acidophilus)	16.50 ± 0.28***	12.50 ± 0.50***	16.50 ± 0.64***
Group VI (L. Bulgaricus)	13.50 ± 1.04***	10.50 ± 0.47*	13.50 ± 0.28*
Group VII (Multilactobacillus strains)	19.25 ± 0.85***	13.50 ± 0.64***	18.50 ± 0.64***

Repetitive/stereotypic-like behaviors in rats with autism. Data expressed as mean ± SEM, n=6 for each group. ###*p*<0.001 compared to the vehicle. *p<0.05, **p<0.01, ***p<0.001 compared with Autistic group. (Kolmogorov–Smirnov test, Bonferroni's Multiple Comparison Test).

**Table 6: Effect of Lactobacillus strains on proinflammatory cytokines levels.**

	Proinflammatory cytokines	
Groups	**IL-6 (pg/ml)**	**TNF- α (pg/ml)**
Group I (Inulin 3mg, p.o daily)	25.67 ± 1.764	89 ± 3.64
Group II (Autistic group)	46.00 ± 1.528###	145.0 ± 3.215###
Group III (L. Plantarum)	29.00 ± 0.577***	107.3 ± 3.383***
Group IV (L. Casei)	30.17 ± 0.6009***	111.7 ± 2.028***
Group V (L. Acidophilus)	33.50 ± 0.2889***	124.7 ± 0.88**
Group VI (L. Bulgaricus)	36.67 ± 1.202**	115.3 ± 3.283***
Group VII (Multilactobacillus strains)	32.00 ± 1.732***	98.67 ± 2.028***

Proinflammatory cytokines levels in rats with autism. Data expressed as mean ± SEM, n=6 for each group. ###*p*<0.001 compared to the vehicle. *p<0.05, **p<0.01, ***p<0.001 compared with Autistic group. (Kolmogorov–Smirnov test, Bonferroni's Multiple Comparison Test).

**Table 7: Effect of Lactobacillus strains on BDNF, 5-HT, AchE.**

	BDNF, 5-HT, AchE		
Groups	**BDNF (pg/ml)**	**5-HT (ng/ml)**	**AchE (µM/min/mg tissue)**
Group I (Inulin 3mg, p.o daily)	45.00 ± 1.55	5.267 ± 0.318	1.30 ± 0.15
Group II (Autistic group)	59.00 ± 2.082###	27.33 ± 2.33###	7.15 ± 0.38###
Group III (L. Plantarum)	49.33 ± 0.88***	16.00 ± 1.00***	2.254 ± 0.25***
Group IV (L. Casei)	46.17 ± 0.44***	9.33 ± 0.881***	3.25 ± 0.47***
Group V (L. Acidophilus)	50.00 ± 0.577**	15.67 ± 0.88***	3.69 ± 0.34***
Group VI (L. Bulgaricus)	49.00 ± 1.525***	12.67 ± 0.667***	5.20 ± 0.44**
Group VII (Multilactobacillus strains)	48.17 ± 0.60***	8.167 ± 0.72***	1.33 ± 0.144***

BDNF, 5-HT, AchE levels in rats with autism. Data expressed as mean ± SEM, n=6 for each group. ###*p*<0.001 compared to the vehicle. *p<0.05, **p<0.01, ***p<0.001 compared with Autistic group. (Kolmogorov–Smirnov test, Bonferroni's Multiple Comparison Test).
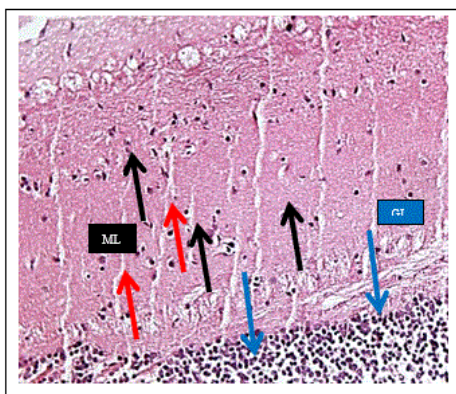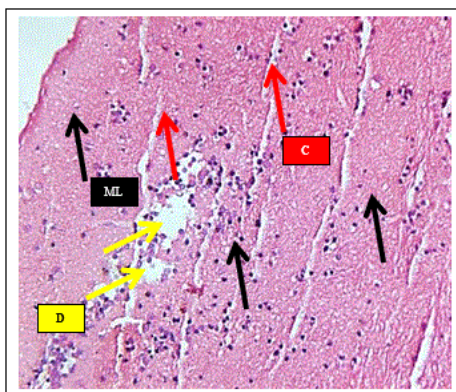
a) **Vehicle treated group**

- Black arrow: Normal Molecular layer of cerebellum
- Red arrow: Capillary
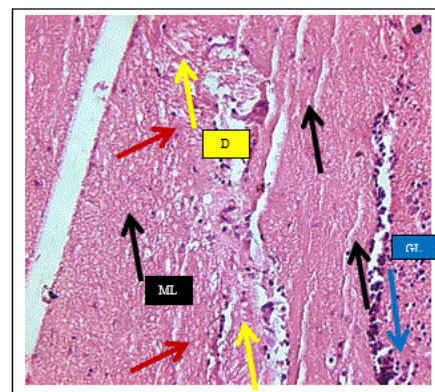- Blue arrow: Granular layer of cerebellum
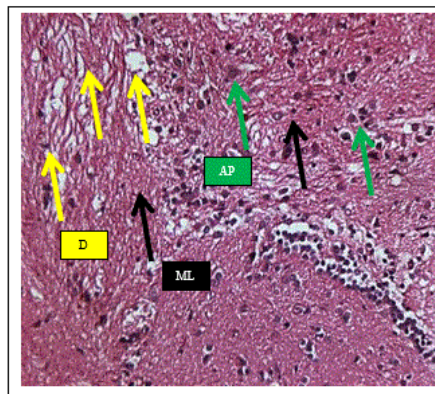
b) **Autistic group**

Black arrow: Molecular layer not appeared normal
Green arrow: Apoptosis of many neurons
Yellow arrow: Damaged neurons

c) **L. Plantarum group**

- Black arrow: Normal Molecular layer of cerebellum
- Red arrow: Capillary
- Yellow arrow: Mild vacuolar degeneration of neurons

d) **L. Casei group**

Black arrow: Molecular layer appeared normal
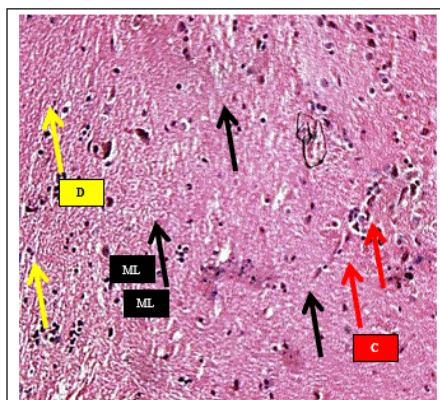Brown arrow: Recovering of neurons
Yellow arrow: Damaged neurons

e) **L. Acidophilus group**

- Black arrow: Normal Molecular layer of cerebellum
- Red arrow: Capillary
- Yellow arrow: Mild vacuolar degeneration of neurons

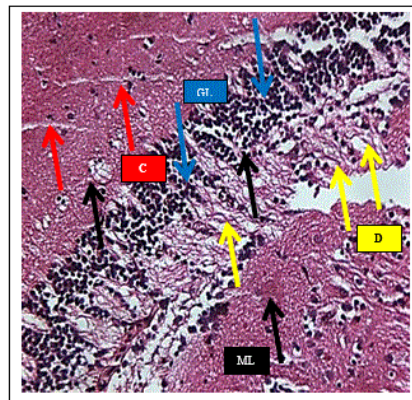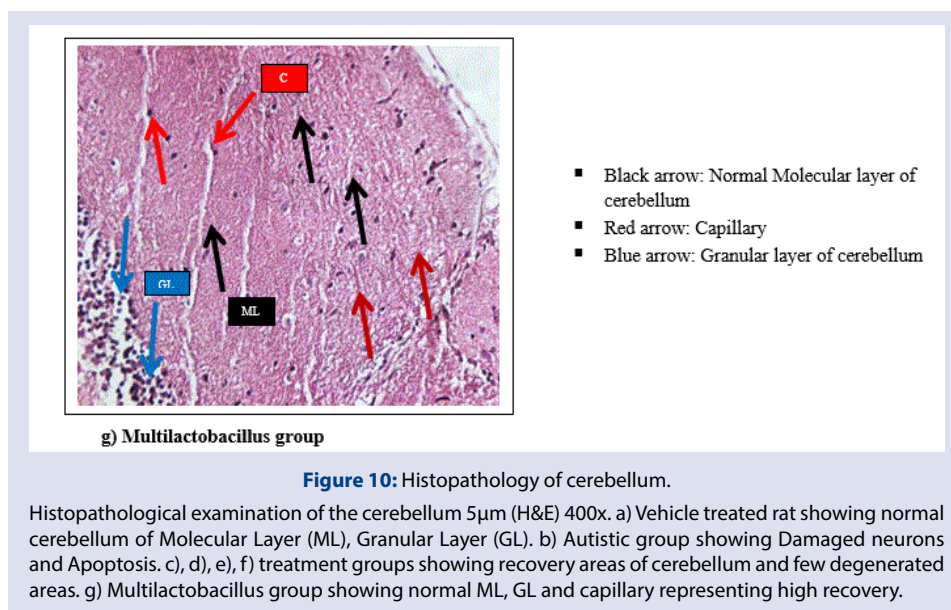f) **L. Bulgaricus group**

Black arrow: Molecular layer appeared normal
Brown arrow: Recovering of neurons
Yellow arrow: Damaged neurons

- Black arrow: Normal Molecular layer of cerebellum
- Red arrow: Capillary
- Blue arrow: Granular layer of cerebellum

g) Multilactobacillus group

Figure 10: Histopathology of cerebellum.

Histopathological examination of the cerebellum 5μm (H&E) 400x. a) Vehicle treated rat showing normal cerebellum of Molecular Layer (ML), Granular Layer (GL). b) Autistic group showing Damaged neurons and Apoptosis. c), d), e), f) treatment groups showing recovery areas of cerebellum and few degenerated areas. g) Multilactobacillus group showing normal ML, GL and capillary representing high recovery.

Estimation of BDNF

Significantly elevated levels of BDNF were seen in autistic group ($p<0.001$) when compared with the vehicle group. Lactobacillus strains treatment decreased the BDNF levels ($p<0.001$) when compared with the autistic group. The results are shown in Table 7.

Estimation of 5HT

Autistic rats have shown hypersertonomia when compared with the vehicle group ($p<0.001$). Lactobacillus strains treatment significantly decreased serotonin levels activity ($p<0.001$) when compared with the autistic group. The results are shown in Table 7.

Effect of Lactobacillus strains on AChE levels

In the autistic group increased acetylcholinesterase activity seen when compared with the vehicle group ($p<0.001$). Lactobacillus strains treatment significantly attenuated the increased enzyme activity ($p<0.001$) when compared with the autistic group. The results are shown in Table 7.

DISCUSSION

According to the results of this study, prenatal model of autism resulted in delayed delivery, low birth weight of pups and behavioral anomalies. The lactobacillus strains treatment ameliorated the autistic like behaviors. The offspring who exposed to VPA in the uterus showed impairment in social interaction, memory and other behavioral tests.

Negative geotaxis is an assessment parameter to motor performance of cerebellum. In autistic group increase time to reorient on the inclined plane.[19,28,29]. Reorientation time was decreased by lactobacilli. There was a delay in Eye-openings in autistic rats[19] on PND 12-16, which were become normal in the lactobacillus treatment.

Swimming performance was evaluated to measure motor development. Lower swimming activity is seen in autistic group[19,30]. Swimming rate was improved in lactobacillus treatment from PND 24 onwards. Hippocampus essential in spatial memory and learning behavior. In the T-maze test lack of spontaneous alterations is observed in the autistic group[1]. With the lactobacillus treatment it re-established the spontaneous alteration.

Morris water maze test an important tool to assess the cognitive ability. Autistic group increased latency to swim and identification of hidden platform is seen[19]. With lactobacillus treatment improved navigation skills and memory performance contributing its impact on Gut-Brain communication.

One of the important confirmation test for autism is social interaction. This test is used to assess the different patterns of behavior, communication and play behavior. With the results social interaction studies in rats were similar to autistics. In our study we identified impairments of play behavior, pinning, social explorations and repetitive behavior become normal with the autistic subjects.[1,19,30].

Cytokines including TNF-α, IFN-γ, IL-1β, and IL-6, 12 have been reported to be elevated in the blood of autistic subjects [31]. In the CNS IL-6 can trigger inflammation and demyelination[32]. Our study evaluated the levels of TNF-α in serum, IL-6 in the cerebellum. In autistic animals IL-6 and TNF-α levels were increased with the lactobacillus treatment IL-6 and TNF-α activity become normal by their immune supportive mechanisms[33,34].

BDNF is a neurotrophic factor (NF) that supports the growth, survival of developing and mature neurons of cholinergic, dopaminergic, and serotonergic neurons[35]. NF is more active in the hippocampus, cortex, and forebrain involved in learning, memory and higher thinking. In our study increased BDNF levels seen in serum of autistic subjects[36,37]. Treatment with lactobacillus strains BDNF activity normal with that of vehicle group.

Hyperseretonomia is seen in 25-50% autistic cases, hyperserotonemia is considered to be one of the most-well replicated findings in neurobiology.[38] 5-HT or Serotonin is originated in the gut has a pivotal role in Gut-Brain communication. Some biological factors that cause the platelet hyperserotonemia may play a role in the early development of the autistic brain.[39,40] in our study we examined hyperseretonomia [41, 42] in autistic group treatment with lactobacillus strains attenuated the hyperseretonomia condition, improved social behavior and communication by attributing to its effect on balancing 5-HT release from gut.

Acetylcholine is involved in the learning and memory, acetylcholine (ACh) in the synaptic cleft by hydrolysed by acetylcholinesterase (AChE).[43] A good level of acetylcholine has a regulating role in attention, cognition, social interactions and stereotypical behaviors.[44-47] in results due to excessive oxidative stress by VPA up-regulation of acetylcholinesterase seen in the autistic group.[48] Treatment with

lactobacillus strains significantly decreased AchE levels by attenuating the enzyme activity.

Histopathology results of cerebellum reveals damage and apoptosis of molecular layer, granular layer seen in the autistic group.[28] Treatment with lactobacillus strains reveals mild degeneration, few necrotic cells reported in *L. Plantarum* and *L. Bulagricus*. Cerebellum become normal is seen *L. Acidophilus* and *L. Casei* and Multilactobacillus strain treatment groups. The recovery of cerebellum providing an important link connecting probiotics role in Gut and Brain communication, immune and brain health support.

CONCLUSION

In conclusion, by comparing all behavioral and biochemical results and histopathology of the cerebellum, It can be emphasized that daily supplementation with Lactobacillus strains provides a healthy gut by the enrichment of microbial diversity and re-colonization of useful bacteria that can be able to alter the brain functionality by Gut-Brain-Axis. Among we studied for four lactobacillus strains, *L. Acidophilus* and *L. Casei* given potential outcomes in recovering autism. Multilactobacillus combination is more approachable therapy, it can advances the benefit of probiotic treatment. According to our results, daily supplementation of Lactobacillus is very effective in the recovery of autism associated Dysbiosis. The present findings of this research work contributing to an important understanding on influence of gut microbiota on brain health. Further our studies will continue on selected probiotic strains of Bifidobacterium, Saccharomyces, Streptococcus and their combination with potent natural drugs.

HIGHLIGHTS

- VPA at a dose of 400mg/kg, i.p. on ED 12 to the pregnant rats has induced autism in born rats.

- Lactobacillus strains (*L. Plantarum*, *L. Casei*, *L. Acidophilus*, *L. Bulgaricus*) with good potential health benefits are selected for the study.

- Daily supplementation with Lactobacillus strains NLT 1 Billion CFU/ml ameliorated the Behavioral & Biochemical deficits.

- Lactobacillus has proven its therapeutic efficacy in autism-associated dysbiosis.
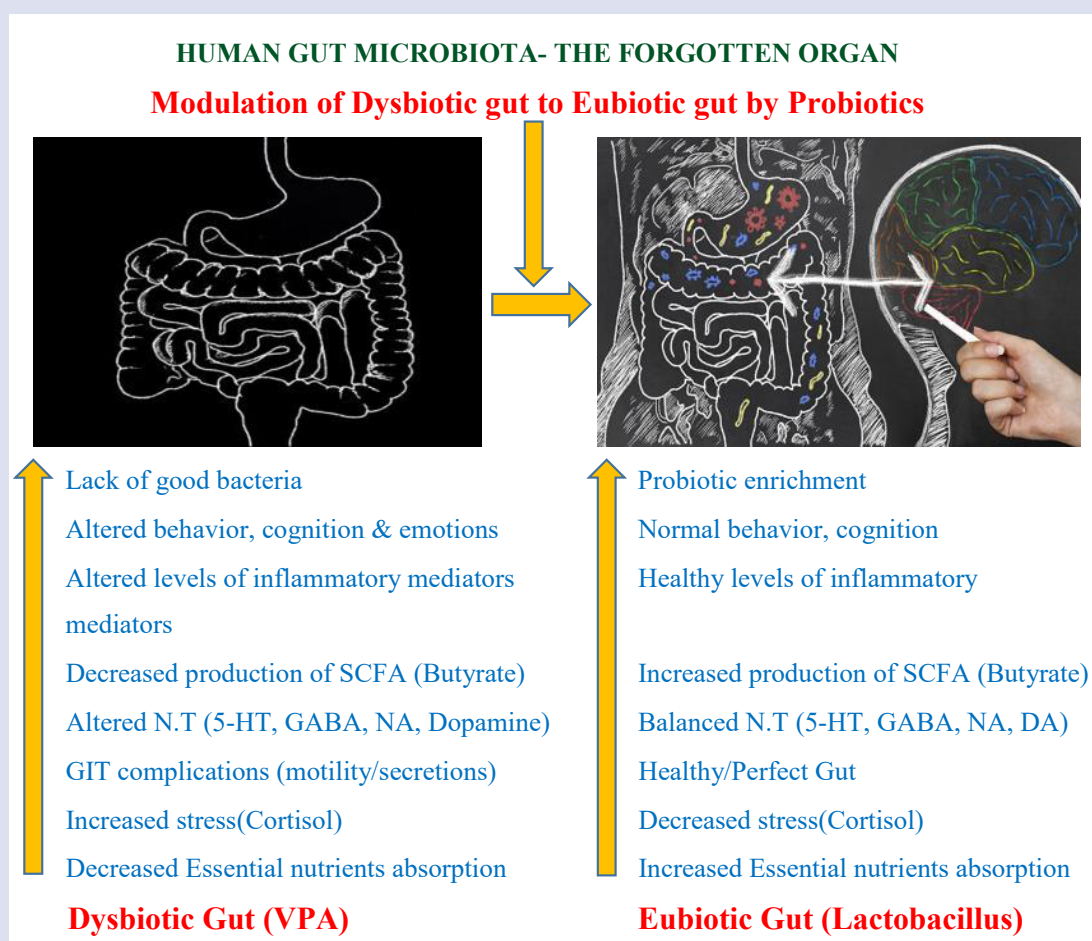
ACKNOWLEDGMENT

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GRAPHICAL ABSTRACT

HUMAN GUT MICROBIOTA- THE FORGOTTEN ORGAN

Modulation of Dysbiotic gut to Eubiotic gut by Probiotics

Lack of good bacteria

Altered behavior, cognition & emotions

Altered levels of inflammatory mediators mediators

Decreased production of SCFA (Butyrate)

Altered N.T (5-HT, GABA, NA, Dopamine)

GIT complications (motility/secretions)

Increased stress(Cortisol)

Decreased Essential nutrients absorption

Dysbiotic Gut (VPA)

Probiotic enrichment

Normal behavior, cognition

Healthy levels of inflammatory

Increased production of SCFA (Butyrate)

Balanced N.T (5-HT, GABA, NA, DA)

Healthy/Perfect Gut

Decreased stress(Cortisol)

Increased Essential nutrients absorption

Eubiotic Gut (Lactobacillus)

ABOUT AUTHORS

K. Sunand: Assistant Professor, Centre for Neuroscience, Department of Pharmacology, Anurag Group of Institutions. He is pursuing a Doctorate at JNTUH. He has expertise in animal experimentation, his main domains of research are Neuroscience (Autism, Depression, Motor disorders, Alzheimer's and Cerebral ischemia), PCOS, Probiotics. His current research work on Gut microbiota role in brain health. He is the Life member of IPS, APTI Professional bodies, and also a member of IAEC.

Dr. G.Krishna Mohan: Professor, Center for Pharmaceutical Sciences, Institute of Science & Technology (IST), JNTUH. He has 4 Patents and many publications over Elsevier, Scopus Journals. He guided many Ph. D Students. His research domains are Anti-diabetic activity of various traditionally used medicinal plants, Evaluation of Immunomodulatory activity of various traditionally used medicinal plants, Isolation of bioactive phytochemical agents, Green synthesis of nanoparticles for development of NDDS.

Dr. Vasudha Bakshi: Dean, School of Pharmacy has 18 years of Academic experience. She has more than 100 research publications & 4 Patents. She organized various National & International Conferences, Workshops, Faculty development programs. She delivered lectures in many National & International conferences, FDPs. She is a member of PCI Inspectors. She is the Life member of IPS, IPA, APTI Professional bodies. She is the Chairman/ Member Secretary IAEC.