Can psychobiotics administration influence behavioral responses and physiological stress in healthy rats?

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Abstract

Background: There is a well-documented cross-talk between the gut and brain. Evidence is accumulating to suggest beneficial effects of psychobiotics [prebiotics, probiotics or synbiotics] on psychological distress in disease states. However, their role in healthy status remains relatively unclear. Objectives: The present study was aimed to clarify if psychobiotics could influence behavioral responses and physiological stress in healthy rats.

Methods: In the present experiment, 28 male Wistar rats were divided into four groups (healthy rats treated by Lactobacillus plantarum (L.plantarum), inulin, and their combination (synbiotic), as well as control group). Then, psychobiotics were administered to the intervention groups for 8 weeks. Behavioral tests (Morris water maze, Elevated plus maze, and forced swimming test) were performed at endpoint. Then, serum and brain levels of superoxide dismutase, malondialdehyde, glutathione peroxidase, total antioxidant capacity, brain-derived neurotrophic factor (BDNF), and serotonin were measured.

Results: Our findings indicated that unlike inulin, the administration of L.plantarum and synbiotic could ameliorate depression and anxiety-like behavior and cognitive performance (P<0.05). Serum and brain oxidative stress markers were significantly improved by synbiotic consumption. The intake of L.plantarum led to decreased oxidative stress in the hippocampus and amygdala (P<0.05). A significant increase in the hippocampal serotonin and BDNF concentration was also observed after both synbiotic and L.plantarum intake (P<0.05). In addition, there was a strong correlation of serum and brain markers with behavioral performance (P< 0.05).

Conclusion: Our study suggests that psychobiotics therapy may have favorable effects on the amelioration of some psychological disorders.
Keywords: Depression; Cognition; Oxidative stress; Probiotic; Prebiotic; Gut microbiome.

Introduction

In the past decade, numerous studies have revealed the bidirectional relationship between the gut microbiome and brain. As previously shown, gut disorders and diseases can affect the central nervous system (CNS), especially behavioral response such as cognition, anxiety, and depression-like behaviors. Therefore, the gut microbiota can be a new interesting target to change behavioral performance and brain functions. Probiotics and prebiotics are the two nutritional supplements with multiple effects in health and disease. However, there is some controversy about the synergistic effect of probiotics and prebiotics.

Psychobiotics are probiotics, prebiotics or synbiotics which have beneficial psychological effects. Lactobacillus plantarum (L.plantarum) and inulin are potentially beneficial as they affect the gut microbiome, as well as brain functions. Both inulin and L.plantarum could show promising evidence via their direct and indirect actions on mood disorders and behavioral performance.

It was reported that the intake of probiotics or prebiotics can improve depression, anxiety, and memory in some diseases. Unfortunately, findings are mostly reported in disease states in animals and human study. However, psychobiotics may have preventive and/or even ameliorative effects on cognitive and behavioral functions.

Oxidative stress is reported to underlie the pathogenesis of neuropsychological disorders. Given the widespread roles of reactive oxygen species (ROS) in neurological disorders, there has been a long effort to develop antioxidant treatments. Recent studies have shown that the use of probiotic or prebiotic could increase the activity of antioxidant enzymes.
Brain-derived neurotrophic factor (BDNF) is one of the major neurotrophins of the CNS with important roles in survival and maintenance of neurons, as well as their growth and differentiation.\textsuperscript{22} Serotonin, as an important neurotransmitter, plays a crucial role in cognitive and behavioral mechanisms.\textsuperscript{23} Though BDNF and serotonin are two apparently separate signaling systems in several brain functions, they have synergistic effects.\textsuperscript{24} Some evidence indicate that the use of some probiotics and prebiotics could improve BDNF and serotonin levels in different brain regions.\textsuperscript{9,10,25} The hippocampus, amygdala, and prefrontal cortex (PFC) are the most important regions of the brain in the creation and regulation of the behavioral process. Moreover, these regions have important interactions with each other in the regulation of neurological mechanisms.\textsuperscript{26,27} In our previous works on diabetic rats, the favorable effects of inulin and \textit{L. plantarum} and their synbiotic in the regulation of the CNS and gut function were reported.\textsuperscript{8,9,28} Therefore, we hypothesized whether the use of psychobiotics can have beneficial psychological effects in healthy rats. Overall, the aim of the present study was to evaluate the complementary effect of these supplements on brain as well as serum important parameters. In addition, three important behavioral performances and their correlations with parameters of different brain tissues were investigated to achieve new evidence.

**Materials and Methods**

**Animals and diet**

Twenty-eight healthy male Wistar rats (240±20g) at 6±2 weeks of age were purchased from Tabriz University of Medical Sciences (TBZMED) Laboratory Animal Center (Tabriz, Iran).
TBZMED Animal Experimentation Ethics Committee approved the whole experiments (ethical code: IR.AJAUMS.REC.1399.042) in accordance with the guidelines of the Principles of Laboratory Animal Care (NIH Publication, revised 1986). This study was carried out in compliance with the ARRIVE guidelines. The study animals were housed in polycarbonate cages and kept under standard laboratory conditions (21-24°C, 40-60% humidity, and 12:12 h light/dark cycle) and allowed free access to food and water. Initially, the rats were maintained under standard laboratory chow diet for 7 days to conform to the new condition. Then, they were randomly allocated to 4 groups (7 rats per group), as below: HSh, healthy sham; HLI, healthy rats supplemented with inulin and the *L. plantarum*; HI, healthy rats supplemented with inulin; HL, healthy rats supplemented with the *L. plantarum*.

**Preparation of the supplements**

Preparation of the supplements and the intervention method has been described, elsewhere. Briefly, *L. plantarum* ATCC 8014 was attained from TBZMED Biotechnology Research Center (Tabriz, Iran). Ten mL of the *L. plantarum* was inoculated into MRS (Man-Rogosa-Sharpe) broth and incubated in an aerobic condition 48 h at 37°C in phosphate buffered saline (PBS). Fresh bacterial suspensions were made at a concentration of 10^7 colony-forming units (CFU)/ml. Each rat was given a gastric gavage every 24 h. Based on 5% of the daily food weight, the inulin content in the rat diet was calculated. The administered inulin was dissolved in drinking water.

**Behavioral tests**
Behavioral tests were performed in a standard room. For compatibility with room conditions, the animals were maintained in the room for 2h in advance of the experiment.

**Elevated plus maze**

To evaluate anxiety-like behavior, *elevated plus maze* (EPM) test was performed. In brief, the apparatus of a black wooden Y-maze comprised a central platform with two open and two enclosed arms. Using the video-computerized tracking system, the rats were observed for 5 minutes, just after placing them on the central platform (facing the open-arm). The time passed in the open or closed arms represented the amount of anxiety in the rat. Also, the total distance was measured as an index for the activity of locomotor.

**Forced swimming test**

The forced swimming test (FST) was applied for the assay of depression-like behavior. The rats were introduced into a transparent plexiglass cylinder, filled with water at 23–25°C up to a height of 50 cm from the base. The duration of each struggling and immobility was recorded from the onset to the endpoint for five minutes. Immobility, described as the absence of all motions with the exception of movements required to keep head of the rats above the water, was calculated by subtracting the scrambling duration from the total test time.

**Morris Water Maze**
Morris water maze (MWM) test, designed for estimation of spatial learning and memory, consists of a 150 cm diameter circular pool with 70 cm high walls constructed from black PVC. This pool which contains 25°C water is filled to a depth of 40 cm. The trial started by randomly placing the rat in water facing the wall of the pool at one of the four starting points. During the learning phase, the animals were trained to locate on the platform in 60 seconds and allowed to stay there for 15 seconds in four consecutive days (the first day with the visible platform and the rest with the hidden ones). Finally, the latency to reach the platform was recorded.

**Preparation of serum and tissue samples**

The rats were anesthetized with pentobarbital sodium (65 mg/ kg body weight, Sigma, intraperitoneal injection). Then, five mL serum was obtained through the cardiac puncture by the use of a centrifuged run at 3000 rpm at 4°C for 20 minutes; the sera were kept frozen at -80 °C for subsequent analysis. After sacrificing the anesthetized rats, the brain was instantly removed from the cranium and the three desired regions (the hippocampus, amygdala, and PFC) were dissected out on an ice-cold plate and stored at -80°C. Brain slices were homogenized and the supernatant was separated after a 20-minute centrifugation at 6,000 rpm at 4°C. The Bradford method was used to make it compatible with the data.29

**Biochemical assays**

Superoxide dismutase (SOD), total antioxidant capacity (TAC), malondialdehyde (MDA), and glutathione peroxidase (GPx) were assayed both in the brain and whole blood of the rats to determine antioxidant status as well as oxidative stress markers which methods have been
described in our previous paper, in detail. Briefly, superoxide anion radicals were generated by the xanthine and xanthine oxidase system. The SOD activity was assessed by the degree of this reaction inhibition (by 505 nm on a spectrophotometer). MDA activity was measured through the analysis of the reaction of MDA with thiobarbituric acid (TBA), which forms an MDA-TBA adduct absorbed strongly at 535 nm. GPx activity was measured by the Paglia and Valentine method, using cumene hydroperoxide as a substrate. Nicotinamide adenine dinucleotide phosphate (NADPH) disappearance was monitored by a spectrophotometer at 340 nm. The TAC assay relies on the ability of antioxidants in the sample scavenge ABTS radical [2, 2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)], produced by peroxidase and H₂O₂ (making blue green color). Suppression of this color was measured by spectrophotometer at 600 nm. The levels of serotonin and BDNF in the serum and supernatants of all three regions were determined, according to the recommended guidelines of manufacturer's, using enzyme-linked immunosorbent assay (ELISA) kits.

**Statistical analysis**

All data were presented as means± standard error of the mean (SEM). One-way analysis of variance (ANOVA) was used for statistical analysis; when ANOVA showed a significant difference, the post hoc Tukey’s test was performed to demonstrate the differences. Data were analyzed using SPSS software (version 18). P-values below 0.05 were of statistical significance.

**Results**

**Effects of inulin and L.plantarum on oxidative stress markers**

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Treatment with synbiotic led to increased levels of SOD in the serum, amygdala, hippocampus, and PFC, compared to Sham group ($P$ for all $<0.05$). Synbiotic supplementation also led to MDA reduction in the serum, amygdala, as well as hippocampus in synbiotic group ($P<0.05$); however, this change was not significant in the PFC (Figure. 1). In addition, hippocampus levels of GPx ($P<0.05$) and TAC ($P<0.05$) increased significantly in synbiotic group, compared to Sham group. Probiotic supplementation only reduced the MDA levels in the hippocampus ($P<0.05$). However, it had no significant effect on other oxidative stress parameters (TAC, SOD, and GPx) either in the serum or the amygdala and PFC. Eight-week administration of inulin did not significantly affect serum and brain oxidative status of the healthy rats (Figure. 1).

**Effects of inulin and *L.plantarum* on serotonin and BDNF levels**

Compared to Sham group, synbiotic administration increased levels of BDNF ($P<0.05$) and serotonin ($P<0.05$) in the hippocampus. Moreover, it elevated BDNF concentration both in the PFC ($P<0.05$) and serotonin in the amygdala ($P<0.05$). Treatment with *L.plantarum* brought about increased levels of serotonin ($P<0.05$) and BDNF ($P<0.05$) only in the hippocampus, but did not show significant effects on the serum, PFC, and amygdala (Figure. 2). Supplementation with inulin did not produce significant changes in either serum or brain BDNF and serotonin levels. The *post hoc* analysis also revealed no significant differences among synbiotic, probiotic, and prebiotic groups in terms of serotonin and BDNF concentration (Figure. 2).

**Effects of inulin and *L.plantarum* on anxiety and depression**
Compared with Sham group, synbiotic and probiotic groups had more OAT (open arms duration/total time×100) \((P<0.05)\) and less CAT (close arms duration/total time×100) \((P<0.05)\), an indicator of anxiety improvement (Figure. 3). Intervention groups (probiotic, prebiotic, and synbiotic) showed no significant differences in distance moved in the central area of the maze, as compared to Sham group. Also, measurement of the total motion to assess locomotor activity showed no significant difference among the synbiotic, probiotic, and prebiotic groups, compared to Sham group. One-way repeated measures ANOVA revealed that probiotic and synbiotic treatment led to a significant reduction of immobilization time \((P<0.05)\) in FST, in comparison with Sham group (Figure. 3).

**Effects of inulin and *L.plantarum* on learning and memory**

The intervention groups displayed the same performance in locating the hidden platform in the first to the third day of learning phase, compared to Sham group \((P<0.05)\). But on the fourth day of learning phase, the escape latency decreased in probiotic \((P<0.05)\) and synbiotic \((P<0.05)\) groups (Figure. 4). In the spatial probe test performed on day five, the swimming time allotted within the target quadrant by the synbiotic group was increased, compared to Sham group \((P<0.05)\). Whilst, the probiotic and prebiotic group did not have a significant difference in the time elapsed within the target quadrant, as compared with Sham group. Furthermore, Post hoc analysis confirmed no statistical variation among the performance of the intervention groups in the MWM (Figure. 4).

**Discussion**
There is growing evidence that the administration of psychobiotics may have protective and ameliorative effects on behavioral disorders.\textsuperscript{34} The present study was conducted to provide a new insight into the effects of psychobiotics on psychological behaviors in three major brain regions that play important roles in the regulation of behaviors. It was also aimed to examine blood and brain parameters to compare the initial production of oxidative stress markers, serotonin, and BDNF under normal conditions and observe their changes after supplementation. According to our findings, the administration of the synbiotic could alleviate depression and anxiety-like behavior and enhance learning and memory. Accordingly, there were strong correlations of serum and brain parameters with behavioral responses (Figure. 5).

The synbiotic supplementation resulted in the ameliorated oxidative status in the serum, hippocampus, and amygdala. Furthermore, the intake of the \textit{L. plantarum} could significantly improve markers of oxidative stress in the hippocampus (Figure. 1). D'souza \textit{et al.}\textsuperscript{21} showed that probiotic supplements are effective antioxidants and may be beneficial for combating the adverse effects of ROS through decreased inflammation and increased antioxidant enzymes such as GPx and SOD. Huang \textit{et al.}\textsuperscript{38} also reported the increased activity of SOD, GPx, and catalase by the intake of \textit{L. plantarum} K68 (10\textsuperscript{9} CFU/mL), leading to improved hyperglycemia, IR, and hyperlipidemia in rats with insulin resistance. Moreover, our results demonstrated that there is a positive correlation between oxidative markers of the serum and brain regions and anxiety and cognition performance (Figure. 5). In our previous works, we reported that the consumption of synbiotic, inulin, and \textit{L. plantarum} could ameliorate antioxidant levels in the serum, hypothalamus, and amygdala of diabetic rats. Antioxidant enzymes would be considered a potential target for the prevention of memory decline. Liu \textit{et al.}\textsuperscript{37} found that catalase and SOD keep cognitive functions
safe against harms. Oxidative stress can be obtained from various sources, usually observed in normal conditions. As mentioned earlier, oxidative stress can damage the CNS function and behavioral process. Recently, oxidative stress indices, known as risk factors for some diseases, appear to be prospective biomarkers for early prediction of diseases in healthy people. Also, one of the mechanisms to improve the antioxidant capacity in the brain could probably be via increased serotonin concentration; however, its clear mechanism is yet to be known. Hence, boosting the antioxidant system could protect against brain damage as well as prevent behavioral disorders. Psychobiotics could prevent nerve oxidative damage via increasing antioxidative enzyme levels.

In contrast with our study, Davari et al. demonstrated that daily consumption of a mixture of probiotics (L.acidophilus, B.lactis, and L.fermentum) for 56 days could not significantly ameliorate oxidative stress markers in healthy rats. There are some differences between their studies and ours. In their study, the number of probiotics (CFU/mL) for intervention was not mentioned and each rat was kept in a separate cage (one animal per cage). While in our study, every four rats were housed in a cage. Bacterial species also differed in the two studies which can be concluded that the species and strain of probiotics could have their own unique effects on oxidative status.

In the present work, we demonstrated a significant increase in the hippocampal serotonin and BDNF concentration after synbiotic and L.plantarum intake. The administration of synbiotic could increase PFC BDNF and amygdala serotonin concentrations, as well (Figure. 2). Burokas et al. reported that the administration of galacto-oligosaccharides (GOS and) fructo-oligosaccharides (FOS) combination (GOS+FOS) in chronic stress could increase the expression of gamma-aminobutyric acid B1 (GABA_B1), GABA_B2, and BDNF in the hippocampus of mice. In addition,
after FOS+GOS intake, elevation of serotonin level in the PFC was observed. BDNF and serotonin are extensively distributed in the CNS and affect various brain functions such as survival, maintenance, growth, differentiation of neurons, and consequently, physiological behaviors.\textsuperscript{22,23} Supplementations have probably increased serotonin and BDNF levels through oxidative pathway. Our previous work was indicative of a strong inverse correlation between the elevated MDA and reduced amygdala BDNF and serotonin levels in the diabetic rats.\textsuperscript{9} In the study of Salim \textit{et al.}\textsuperscript{40}, oxidative stress reduced the amygdala and hippocampal BDNF concentration. Moreover, Shankaran \textit{et al.}\textsuperscript{41} indicated that induced oxidative stress can result in depleted brain serotonin level in the striatum and hippocampus which can be improved by vitamin E and vitamin C intake. Consistent with our finding, in a study conducted by Toldy \textit{et al.}\textsuperscript{43}, nettle consumption as an antioxidant could not increase BDNF and nerve growth factor (NGF), despite reducing oxidative stress in both cerebellum and frontal lobes. Therefore, other mechanisms may also be involved with changes in the levels of nerve parameters. In a study,\textsuperscript{10} FOS+GOS consumption could ameliorate behavioral responses, as well as GABA\textsubscript{B2}, BDNF, and serotonin levels via regulated HPA (The hypothalamic-pituitary-adrenal) activity (i.e. plasma and hypothalamic corticosterone), improved dysbiosis, and increased short-chain fatty acids (SCFAs) concentrations. Additionally, the combination of the two prebiotics was more effective than their separate use. In our study, inulin was not probably able to significantly alter the gut microbial composition. On the other hand, it seems that the prebiotic intake may be more helpful in pathologic conditions.\textsuperscript{8,9} Our results on behavior responses indicated that the synbiotic and \textit{L.plantarum} consumption had significant effects on learning, memory, depression, and anxiety in the healthy rats (Figure. 3, 4). Also, a strong correlation of behavioral tests was found with oxidative markers, serotonin and
BDNF concentration in different brain regions (Figure. 5). The beneficial effects of psychobiotics on behavioral disorders have been reported in several diseases, as well.\textsuperscript{9,44,45} However, studies on healthy individuals or animals are confined. Allen \textit{et al.}\textsuperscript{16} demonstrated that the administration of \textit{B. longum} 1714 (10\textsuperscript{9} CFU/mL) resulted in the modulation of stress and amelioration of memory performance in addition to cortisol reduction in healthy volunteers. In another study, Jeong \textit{et al.}\textsuperscript{46} indicated that compared to the control group, older rats treated with \textit{L. plantarum} C29 (2\times10\textsuperscript{9} CFU/mL) for 8 weeks had a significant increase in the expression of cAMP response element binding protein (CREB) genes and hippocampal BDNF, as well as improvement in cognitive behavior via inhibiting NF-κB signaling pathway. Our findings also indicated a positive correlation of memory and learning with PFC and hippocampal BDNF (Figure.5). In addition, the improvement of anxiety like-behavior was consistent with increasing levels of serotonin in the amygdala and hippocampus (Figure.5). It was previously shown that the levels of these parameters decrease in anxiety and depression-like behaviors and other disorders that lead to cognitive impairment.\textsuperscript{24} It was reported that any change in the levels of neurotrophins in the hippocampus and amygdala of mice was strongly correlated with anxiety.\textsuperscript{39} In another research by Takada \textit{et al.}\textsuperscript{47}, it was demonstrated that the administration of \textit{L. casei} could regulate stress in both healthy subjects and rats. They showed that \textit{L. casei} intake declined levels of corticotropin-releasing factor (CRF) and cortisol in the hypothalamus of rats which probably affected the vagus nerve signaling to the brain and decreased activity of the HPA axis. Furthermore, Bravo \textit{et al.}\textsuperscript{48} indicated that \textit{L. rhamnosus} (10\textsuperscript{9} CFU/mL) consumption resulted in reduced anxiety (EPM) and depression (FST) as well as decreased levels of mRNA expression.
of GABA\textsubscript{B1b} in the hippocampus, amygdala, and locus coeruleus, compared with healthy control mice.

Considering the present evidence as well as our previous works,\textsuperscript{9,16,46,48} several mechanisms have been proposed for the effects of psychobiotics on behavioral responses through intestinal microbial changes. First, as a result of improved microbial composition, lipopolysaccharide (LPS) production as well as immune and inflammatory responses reduce; subsequently, leading to the alleviation of ROS production.\textsuperscript{47,49} Second, the regulation of HPA hyperactivity in most studies has a direct correlation with anxiety and depression. The most important factor involved in this pathway is cortisol, directly linked to increased neuropsychological disorders.\textsuperscript{47,50} Third, the psychobiotics affect the gut-vagus nerve-brain axis which leads to improved CNS neurotrophins (like BDNF) and neurotransmitters (like serotonin).\textsuperscript{9,51} Although valuable findings were obtained in the present study, there were some limitations. We could not assess changes of the microbial population which could be very helpful. Our results could be more comprehensive, if the microbial composition of the rats were also examined. Finally, supplementation with other probiotics and prebiotics in different doses is proposed, as varied results might be produced.

**Conclusion**

In the current study, it was demonstrated that synbiotic supplementation could improve antioxidant enzymes, brain BDNF, and serotonin concentration as well as cognitive and behavioral performance of the healthy rats after 8 weeks. Also, it was found that most of the changes occurred in the hippocampus, compared to the amygdala and PFC, following the intervention. Our finding well demonstrated a strong correlation of serum and brain levels of the parameters with behavioral
responses. These findings offer new evidence for health effects of psychobiotics as therapeutic agents for the prevention or amelioration of behavioral disorders. Further studies are warranted to clarify the mechanisms of psychobiotics on the gut-brain axis.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
MM, MSA, SH, and GA designed the study, carried out data analyses, performed the statistical analyses and prepared the first draft of the manuscript. KBV and VH conceived the study and edited the manuscript. MSA, MM, and SMM commented on study design, data analyses, inference
of the results, and critically edited the manuscript. All authors read and approved the final manuscript.

**Conflict of interest**

The authors declare that they have no competing interests.

**Consent for publication**

All authors agreed to the submission and approved the final version of the manuscript.

**Ethics approval and consent to participate**

All experiments were approved by the TBZMED Animal Experimentation Ethics Committee in accordance with the guidelines of the Principles of Laboratory Animal Care (NIH Publication, revised 1986). As laboratory animals were used in this study, consent to participate was not required.

**Abbreviations**

BDNF, Brain-derived neurotrophic factor; CAT, close arms duration/total time×100; CFU, colony-forming units; CNS, central nervous system; CREB, cAMP response element binding protein; CRF, corticotropin-releasing factor; ELISA, enzyme-linked immunosorbent assay; EPM, Elevated plus maze; FOS, fructo-oligosaccharides; FST, forced swimming test; GABAB1, gamma-aminobutyric acid B1; GOS, galacto-oligosaccharides; GPx, glutathione peroxidase; HI, healthy rats treated by inulin; HL, healthy rats treated by the *L. plantarum*; HLI, healthy rats treated...
by the *L. plantarum* and inulin; HPA, The hypothalamic–pituitary–adrenal; HSh, healthy sham group; LPS, lipopolysaccharide; MDA, malondialdehyde; MRS, Man-Rogosa-Sharpe broth; MWM, Morris water maze; NADPH, Nicotinamide adenine dinucleotide phosphate; OAT, open arms duration/total time×100; PBS, phosphate buffered saline; PFC, prefrontal cortex; ROS, reactive oxygen species; SCFAs, short-chain fatty acids; SOD, Superoxide dismutase; TAC, total antioxidant capacity; TBA, thiobarbituric acid.
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**Figure 1.** Effects of *L. plantarum* and inulin treatment on oxidative stress markers of the healthy control and intervention groups of the rats (n=7 per group). SOD, GPx, TAC, and MDA concentration in the (A-D) serum, (E-H) amygdala, (I-L) PFC, and (M-P) hippocampus. HSh, healthy sham; HLI, healthy rats treated by *L. plantarum* and inulin; HI, healthy rats treated by inulin; HL, healthy rats treated by *L. plantarum*; One-way analysis of variance, followed by post
hoc Tukey’s test, was used. All values are expressed as means ± SEM. *P<0.05 compared with the HSh.

**Figure 2.** Effects of *L. plantarum* and inulin treatment on serotonin and BDNF levels of the healthy control and intervention groups of the rats (n=7 per group). Serotonin and BDNF concentration in (A, B) serum, (C, D) amygdala, (E, F) hippocampus, and (G, H) PFC. HSh, healthy sham; HLI, healthy rats treated by *L. plantarum* and inulin; HI, healthy rats treated by
inulin; HL, healthy rats treated by *L.plantarum*. One-way analysis of variance, followed by post hoc Tukey’s test, was used. All values are expressed as means ± SEM. *P<0.05 compared with the HSh.

**Figure 3.** Effects of *L.plantarum* and inulin treatment on anxiety and depression-like behaviors of the rats (n=7 per group). Bars show time (seconds) spent in the (A) open arms and (B) OAT (open arms duration/total time×100) during a 5-min elevated plus maze test (EPM). (C) The total number of closed arm entrance was measured to assess locomotor activity. (D) Duration of immobility in the FST. HSh, healthy sham; HLI, healthy rats treated by *L.plantarum* and inulin;
HI, healthy rats treated by inulin; HL, healthy rats treated by *L. plantarum*. One-way analysis of variance, followed by post hoc Tukey’s test, was used. All values are presented as means ± SEM. * P<0.05 compared with the HSh.

**Figure 4.** Effects of *L. plantarum* and inulin treatment on learning and memory in the Morris water maze (MWM) (n=7 per group). (A) Duration of time in which animals remained in the target quadrant (B) Time elapsed by rats to locate the platform. (C) Heat map (Thermal maps are characterized based on tracking data from the position of mice in the environment. Red color indicates that the animal has spent more time in that area, while the blue one demonstrates that the
animal has spent less time in that area. If the red color is more around the rescue platform, it indicates a stronger memory and learning performance of the animal) of MWM in different treatment groups. HSh, healthy sham; HLI, healthy rats treated by *L.plantarum* and inulin; HI, healthy rats treated by inulin; HL, healthy rats treated by *L.plantarum*. One-way analysis of variance, followed by *post hoc* Tukey’s test, was used. All values are presented as Means ± SEM.

* P<0.05, compared with the HSh.
**Figure 5.** Correlations of oxidative stress and neurological parameters in serum and brain regions with behavioral tests (A-J). Correlations were computed by Pearson correlation coefficient test. Data were expressed as Means±SEM. P<0.05 was regarded as statistically significant.