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ADMINISTRATION OF *LACTOBACILLUS HELVETICUS* NS8 IMPROVES BEHAVIORAL, COGNITIVE, AND BIOCHEMICAL ABERRATIONS CAUSED BY CHRONIC RESTRAINT STRESS

S. LIANG,[a,b] T. WANG,[a] X. HU,[a] J. LUO,[c] W. LI,[a,b] X. WU,[a,b] Y. DUAN[a] AND F. JIN[a]*

[a] *Key Laboratory of Mental Health, Institute of Psychology, Chinese Academy of Sciences, Beijing, China*

[b] *University of Chinese Academy of Sciences, Beijing, China*

[c] *Teacher Education College, Sichuan Normal University, Chengdu, China*

Abstract—Increasing numbers of studies have suggested that the gut microbiota is involved in the pathophysiology of stress-related disorders. Chronic stress can cause behavioral, cognitive, biochemical, and gut microbiota aberrations. Gut bacteria can communicate with the host through the microbiota–gut–brain axis (which mainly includes the immune, neuroendocrine, and neural pathways) to influence brain and behavior. It is hypothesized that administration of probiotics can improve chronic-stress-induced depression. In order to examine this hypothesis, the chronic restraint stress depression model was established in this study. Adult specific pathogen free (SPF) Sprague–Dawley rats were subjected to 21 days of restraint stress followed by behavioral testing (including the sucrose preference test (SPT), elevated-plus maze test, open-field test (OFT), object recognition test (ORT), and object placement test (OPT)) and biochemical analysis. Supplemental *Lactobacillus helveticus* NS8 was provided every day during stress until the end of experiment, and selective serotonin reuptake inhibitor (SSRI) citalopram (CIT) served as a positive control. Results showed that *L. helveticus* NS8 improved chronic restraint stress-induced behavioral (anxiety and depression) and cognitive dysfunction, showing an effect similar to and better than that of CIT. *L. helveticus* NS8 also resulted in lower plasma corticosterone (CORT) and adrenocorticotropic hormone (ACTH) levels, higher plasma interleukin-10 (IL-10) levels, restored hippocampal serotonin (5-HT) and norepinephrine (NE) levels, and more hippocampal brain-derived neurotrophic factor (BDNF) mRNA expression than in chronic stress rats. Taken together, these results indicate an anti-depressant effect of *L. helveticus* NS8 in rats subjected to chronic restraint stress depression and that this effect could be due to the microbiota–gut–brain axis. They also suggest the therapeutic potential of *L. helveticus* NS8 in stress-related and possibly other kinds of depression.

Key words: *Lactobacillus helveticus*, chronic restraint stress, depression, microbiota–gut–brain axis, BDNF, serotonin.

INTRODUCTION

Stress is unavoidable. Chronic uncontrollable stress is especially detrimental. Stressful life events can impair digestion, immune responses, endocrine function, brain function, behavior, and cognition. It is possible that they may also induce a variety of diseases such as functional gastrointestinal disorders and mental disorders, including major depression, anxiety, post-traumatic stress disorder, and drug addiction (Kessler, 1997; Wichers and Maes, 2002; Hammen, 2005; Alexander et al., 2007; Cleck and Blendy, 2008; Lupien et al., 2009; Marin et al., 2011; Moloney et al., 2014). And the chronic stress model is very common in animal studies (maternal separation, chronic unpredictable mild stress, chronic restraint stress, etc.) to mimic the etiology and pathophysiology of human depressive disorder (Glavin et al., 1994; McArthur and Borsini, 2006; Abelaira et al., 2013). However, less is known about the effects of stress on intestinal microbiota. Chronic stress (including chronic restraint stress, maternal separation, social disruption, dietary and environmental stress) can disrupt the microbiota integrity, reduce the microbiota diversity and richness (Tannock and Savage, 1974; Bailey and Coe, 1999; O'Mahony et al., 2009; Bailey et al., 2010, 2011). Different bacteria respond differently under stress. Levels of beneficial bacteria, such as genus Lactobacillus (*LB*), have been found to decrease quickly after stress, the populations of neutral and harmful bacteria like *Citrobacter rodentium* and genus Clostridium increased significantly (Tannock and Savage, 1974; Holdeman et al., 1976 March; Bailey and Coe, 1999; Bailey et al., 2010; Bangsgaard Bendtsen et al., 2012; Park et al., 2013; Bailey, 2014; Galley et al., 2014).

Until recently, scientists have started to realize the great importance of gut microbiota, as evidenced by the booming study of the human microbiome (the human

*Corresponding author.

Abbreviations: 5-HT, serotonin; ACTH, adrenocorticotropic hormone; BDNF, brain-derived neurotrophic factor; CIT, citalopram; CON, control group; CORT, corticosterone group; CRS, chronic restraint stress group; DA, dopamine; ELISA, enzyme-linked immunosorbent assay; EPM, elevated plus-maze; HPA, hypothalamic–pituitary–adrenal; IDO, indoleamine-2,3-dioxygenase; IL-10, interleukin-10; INF-γ, interferon-gama; *LB*, lactobacillus; NE, norepinephrine; OFT, open-field test; OPT, object placement test; ORT, object recognition test; PFC, prefrontal cortex; qPCR, quantitative real-time polymerase chain reaction; SPF, specific pathogen free; SPT, sucrose preference test; SSRI, selective serotonin reuptake inhibitor; TDO, tryptophan-2,3-dioxygenase; TNF-α, tumor necrosis factor-alpha.

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second genome-metagenome) (Human Microbiome Project, 2012a,b; Nicholson et al., 2012; Yatsunenko et al., 2012). The human intestinal tract is a set of microbe-friendly organs where approximately 10^{14} of various microbes live. Collectively, they have more than 200 times as many genes as humans. They are indispensable to health and disease (Lyte, 2010; Burcelin et al., 2013). Without gut microbiota, the host would present dysfunction of the digestive system, immune system, neuroendocrine system, nervous system, behavior, and cognition (Diamond et al., 2011; Al-Asmakh et al., 2012; Manco, 2012; Clarke et al., 2013; Davari et al., 2013; Di Mauro et al., 2013; Crumeyrolle-Arias et al., 2014; Desbonnet et al., 2014). In other words, gut microbiota are essential to system development and the maturation of function (Clemente et al., 2012; Morgan et al., 2013). The normal balance of gut microbiota is also of great importance to the health of the host (Eloe-Fadrosh and Rasko, 2013). Many diseases, including cardiovascular, metabolic, autoimmune, neurodevelopment, and even psychiatric disorders, have been shown to be correlated with gut microbiota dysbiosis (Wang et al., 2011; Bekkering et al., 2013; Moloney et al., 2014; Wang and Kasper, 2014; Borre et al., 2014b).

Most researchers regard microbiota–gut–brain axis as the bidirectional communication pathway between brain and gut bacteria (Cryan and O'Mahony, 2011; Grenham et al., 2011; Borre et al., 2014a). The main pathways of microbiota–gut–brain axis are nerve routes (including vagus nerve, neurotransmitters, and neurogenesis), endocrine routes, and immune routes (Desbonnet et al., 2008b; Ochoa-Reparaz et al., 2010a; Bravo et al., 2011; Sudo, 2014; Ogbonnaya et al., 2015). Interestingly, these routes are also involved in the pathophysiology of major depression. The brain neurogenesis (especially in the hippocampus) and the brain-derived neurotrophic factor (BDNF) levels are reduced in depression patients (Marije aan het Rot et al., 2009). The monoamine neurotransmitters deficiency is an important cause of depression and a most common antidepressant treatment target (Hamon and Blier, 2013). The hypothalamic–pituitary–adrenal (HPA) system is disturbed and stress hormones are hyper-secreted in patients with severe depression (Barden, 2004). Inflammation is another character of depression, the proinflammatory cytokines levels (including IL-6, tumor necrosis factor-alpha (TNF-α) and IFN-γ) are usually enhanced while the anti-inflammatory cytokines (like interleukin-10 (IL-10)) are usually reduced (Schiepers et al., 2005; Rook and Lowry, 2008). Through the microbiota–gut–brain axis, the brain can influence gut bacteria by modulating gut physiological status, and the composition and variation of intestinal microbiota can change the central nervous system and behavior (Rhee et al., 2009; Foster, 2013; Montiel-Castro et al., 2013). Pathogenic bacteria like *C. rodentium* and *Campylobacter jejuni* infection increased anxiety-like behavior and caused memory dysfunction (Lyte et al., 2006; Goehler et al., 2008; Gareau et al., 2011). Probiotics like some *LB* species and some Bifidobacterium species administration caused improvement in the host (Desbonnet et al., 2008b, 2010; Bercik et al., 2010, 2011; Bravo et al., 2011; Arseneault-Breard et al., 2012; Davari et al., 2013; Savignac et al., 2014, 2015). Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO and WHO, 2001).

LB species are one of the most important types of probiotics (Ljungh and Wadström, 2006). In the same time, genus *LB* is widely distributed on inner and outer surfaces of the human body and throughout the entire digestive tract (Human Microbiome Project, 2012b; Russo et al., 2014). It is an essential member of gut microbiota and plays great role in host health (Evaldson et al., 1982; Ljungh and Wadström, 2006; Clemente et al., 2012; Vyas and Ranganathan, 2012; Human Microbiome Project, 2012b; Eloe-Fadrosh and Rasko, 2013). For this reason, scientists have paid a considerable amount of attention to the therapeutic effect that probiotics have on many kinds of diseases, such as irritable bowel syndrome, inflammatory bowel diseases, diabetes, chronic fatigue syndrome, hepatic encephalopathy, and autism (O'Mahony et al., 2005; Malaguarnera et al., 2007; Barouei et al., 2009; Kaur et al., 2009; Rao et al., 2009; Hörmannsperger and Haller, 2010; Critchfield et al., 2011; Davari et al., 2013; Quigley and Shanahan, 2014). During these studies, a few studies have shown that ingestion of some *LB* strains could not only rescue disorders but also attenuate emotional behavior and impairment of cognition (Sullivan et al., 2009; Bravo et al., 2011; Arseneault-Breard et al., 2012; Davari et al., 2013). NS8 is a subspecies of *Lactobacillus helveticus* strain isolated and identified by our own laboratory. It has been proven to attenuate anxiety and to improve cognition in hyperammonemia rats (Luo et al., 2014), making similar behavioral and cognition improving effects like other *L. helveticus* strains (Messaoudi et al., 2011; Ohland et al., 2013; Ohsawa et al., 2015).

Considering the modulation effects of some probiotics in mood and cognition (Cryan and Dinan, 2012; Collins and Bercik, 2013; Ohland et al., 2013; Tillisch et al., 2013; Distrutti et al., 2014; Rook et al., 2014; Borre et al., 2014a), it was here hypothesized that ingestion of some *LB* species could decrease anxiety-like and depressive-like behavior and promote cognition in depression. In order to assess these effects, the effects of *L. helveticus* NS8 administration during stress were analyzed in rats with citalopram (CIT) as a positive control. Anxiety-like behavior was detected by open-field testing and an elevated plus maze (EPM). Depressive-like behavior and cognitive function were assessed by modified sucrose preference test (SPT), object recognition test (ORT), and object placement test (OPT). To determine how *L. helveticus* NS8 influences mood and cognition, plasma stress hormones, plasma cytokines, and brain monoamine neurotransmitters were also measured using enzyme-linked immunosorbent assay (ELISA) kits. Brain BDNF mRNA expression was measured using real-time PCR.

EXPERIMENTAL PROCEDURES

Animals

Adult male specific-pathogen-free (SPF) Sprague–Dawley rats (weighing 220–240 g) were purchased from Vital River Laboratories. Rats were individually housed

during the experiment under standard laboratory conditions (12/12 h light/dark cycle, lights on at 07:00 h; 22–24 °C, 40–60% humidity). After two weeks of accommodation, rats were randomly divided into four groups: control group (CON, $n = 8$), chronic restraint stress group (CRS, $n = 8$), *Lactobacillus* group (LAC, $n = 8$, given supplemental *L. helveticus* NS8), and citalopram group (CIT, $n = 8$, given CIT hydrobromide).

In the CRS experiment (Fig. 1), rats (24/32) were restrained in polypropylene cylinders (6 cm inner diameter, with air vents at the nasal end of the cylinder and length adjusted for each rat) 6 h/d for three weeks. Body weight was measured every two days during the stress paradigm. The entire experimental protocol was approved by the Institutional Animal Care and Use Committee of the Institute of Psychology of the Chinese Academy of Sciences.

NS Lactobacillus and CIT administration

The *L. helveticus* NS8 strain was isolated from natural fermented dairy from Mongolia grasslands in the present laboratory. It was inoculated into MRS media and incubated three times at 37 °C for 18 h each. Then the *L. helveticus* NS8 strain was extracted by centrifugation at 3000 rpm for 5 min and washed twice with PBS buffer. The strain was resuspended in drinking water at a concentration of 10^9 CFU/ml. The drinking water was changed every day.

Using the serotonin (5-HT) reuptake inhibitor CIT as positive control was very common in depression-related researches (Desbonnet et al., 2010; Malkesman et al., 2012). In the present study, the method of drug administration was similar to Desbonnet 2010 (Desbonnet et al., 2010). CIT hydrobromide (manufactured by H. Lundbeck A/S. Copenhagen-DK and repackaged by Xian-Janssen Pharmaceutical Ltd., Xi'an, Shaanxi Province, China) was administered at a dose of 30 mg/kg body weight in the drinking water of each rat. Water intake was monitored 1 week prior to drug dosing and throughout the experiment. Every two days, the quantity of CIT dissolved in the drinking water was adjusted according to fluid intake and body weight in order to maintain a 30 mg/kg dosage throughout the experimental period.

Both *LB* and CIT were administrated until the termination of the experiment.

Behavioral testing

SPT. The day after the stress period, all subjects underwent behavioral testing. A modified version of SPT, as described by Huynh et al. was used on day 21 after the end of restraint stress (Huynh et al., 2011). The baseline SP contained a 5-day protocol. On the first 2 days, rats were given two bottles of tap water in the home cage. On the third and fourth days, one of the bottles of water was replaced by 1% sucrose solution. On the fifth day, rats were deprived of food and water for 6 h and then allowed one bottle of tap water and one bottle of 1% sucrose solution to drink freely. The consumption of tap water and 1% sucrose solution was recorded over the following 1 h. The positions of these bottles were counterbalanced across rats and switched during the test. The second SPT replicated the fifth day protocol. Sucrose preference was tested for 1 h after the end of restraint stress. Sucrose preference is expressed as the relative amount of 1% sucrose consumption over the total water consumption (the sum of 1% sucrose consumption and tap water consumption).

EPM. On day 22, anxiety-related behavior was assessed using EPM. The plus maze consists of two opposite open arms (50 * 10 cm) and two opposite closed arms (50 * 10 * 40 cm) connected by a 10-cm square center, elevated 70 cm above the floor and located in an appropriate observation room. The test was performed under dim light conditions. Rats were placed in the central area with their heads toward the open arms. Rats were allowed to move freely for 5 min. Their behavior was recorded by a CCD camera on an IBM computer with ANYMAZE software. The number of entries to open and closed arms and the time spent in each arm were recorded automatically. After each rat was tested, the EPM was cleaned with a 10% ethanol solution in order to avoid interference in subsequent tests from the animal's odors or residues.

Open-field test (OFT). On day 23, rats were introduced to an open-field apparatus (50 * 50 * 50 cm) to measure anxiety-like behavior. Their behavior was monitored for 5 min using a CCD camera. The images were captured on an IBM computer with Image ANYMAZE software. The distance traveled and the time spent in central area (12.5 * 12.5 cm) were both calculated automatically. The apparatus was cleaned after each trial as in the EPM.

ORT and OPT. Memory was assessed using an ORT on day 24 and OPT on day 25. All trials were conducted in the open field apparatus. And the specific procedures referred published researches (Beck and Luine, 2002; Bowman et al., 2006). Each trial contained a sample trial (T1) and a recognition trial (T2). The T1 was the same across all tests but T2 differed between ORT and OPT.

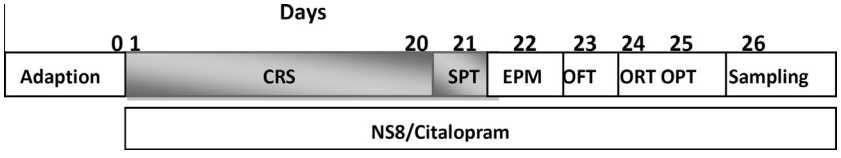

Fig. 1. Brief schema for chronic restraint stress treatment and *Lactobacillus* and citalopram intervention. SPT: sucrose preference test; OFT: open-field test; EPM: elevated plus-maze test; ORT: object recognition test; OPT: object placement test.

The interval between T1 and T2 was 3 h in both tests. In T1, two identical objects were placed at one end of the open field and amount of time spent exploring the two objects was recorded for 3 min. In T2 of ORT, one of the objects was replaced with a different object and the time spent exploring the old one (familiar object) and the new (novel) object was recorded for 3 min. The relative amount of time spent exploring the novel object over the total exploration time during T2 served as an index of object recognition. During T2 of OPT, one of these two objects was placed in a new location. Then the time spent exploring both objects, either in the new location or in the old location, was recorded for 3 min. The relative amount of time spent exploring the novel location over the total exploration time during T2 served as an index of placement recognition. Exploration was defined as when the rat sniffed at, whisked at, or looked at the object from no more than 2 cm away. The objects used for trials were toy blocks of different shapes. The novel object was counterbalanced across treatment. The field and all objects were thoroughly cleaned with a 10% ethanol solution both between T1 and T2 for individual animals and between separate trials for each animal.

Animal termination and tissue dissection

On day 26, rats were quickly killed by decapitation, and trunk blood samples were collected into pre-trilled EDTA-coated tubes. Tubes were then centrifuged at $3000\times g$ at 4 °C for 10 min. The plasma was aspirated and stored at −80 °C until further analysis. Whole brains were rapidly removed and placed on ice-cold plates. Then the prefrontal cortex (PFC) and hippocampus, were quickly dissected, frozen in liquid nitrogen and stored in −80 °C for further analysis.

Enzyme-linked immunosorbent assay (ELISA) analysis

Plasma cytokines and stress hormones were common biological indexes in chronic restraint stress model (Naert et al., 2011; Voorhees et al., 2013). In the present experiment, levels of plasma cytokines (including IL-10, interferon-gama (INF-γ), TNF-α) and stress hormones corticosterone (CORT) and adrenocorticotropic hormone (ACTH) were all measured using ELISA kits (Cusabio Biotech Co., Ltd., Wuhan, China), according to the manufacturer's instructions.

The PFC and hippocampus were two crucial parts for cognition and mood regulation (Marije aan het Rot et al., 2009; Duman and Duman, 2015). And the monoamine transmitters' content and BDNF mRNA expression in these regions were often detected in animal behavior studies (Desbonnet et al., 2010; Naert et al., 2011; O'Mahony et al., 2011; Chiba et al., 2012). In the present experiment, the right PFC and right hippocampus samples were homogenized in phosphate-buffered saline (0.1 mol L^{-1}) on ice. The homogenate was centrifuged (3500 rpm for 10, at 4 °C). The supernatants were aspirated and stored at −80 °C until further analysis. Monoamine neurotransmitters including 5-HT, norepinephrine (NE), and dopamine (DA) in the supernatants were also detected using ELISA kits (Cusabio Biotech Co., Ltd., Wuhan, China), according to the manufacturer's instructions.

Quantitative real-time polymerase chain reaction (qPCR)

The total RNA in the left PFC and left hippocampus was isolated using TRNzol reagent according to the manufacturer's instructions (Tiangen Biotech Co. Ltd., Beijing, China). Then the RNA samples were converted to double-stranded cDNA using a TIANScript Reverse Transcription Kit (Tiangen Biotech Co. Ltd., Beijing, China). The cDNA samples collected were used in subsequently qPCR for measurement of mRNA expressions of GADPH (housekeeping gene, forward 5′–3′: ATGACTCTACCCACGGCAAG; reverse 5′–3′: TACTCAGCACCAGCATCACC), and (BDNF, forward 5′–3′: AAGCCGAACTTCTCACATGATGA; reverse 5′–3′: TGCAACCGAAGTATGAAATAACCATAG). The qPCR reaction was performed in an Applied Biosystems7300 system using SYBR® Premix Ex Taq™ (Takara Bio, Japan). Using the 7300 SDS software, the relative quantification of each sample was analyzed and each mean $2^{-\triangle CT}$ was calculated later. The BDNF mRNA expression is presented as percentage related to GADPH.

Statistical analysis

All data were presented as mean ± SEM. The body weight data were analyzed by a repeated measures analysis of variance (ANOVA). Other data were analyzed by a one-way ANOVA. And the gene expression data were logarithmic transformed before ANOVA because of the heterogeneity of variance. Tukey HSD testing was used for the post hoc test. A correlation analysis was performed between behavioral results and biological outcomes using Pearson's correlation coefficient (r_p). Differences were considered statistically significant when $P < 0.05$.

RESULTS

Body weight

The body weight was measured every two days from day 0 to day 21. Repeated measures ANOVA found a significant effect of time ($F(11,328) = 400.237$, $P < 0.001$), time * treatment ($F(33,308) = 15.975$, $P < 0.001$), and group ($F(3,28) = 3.769$, $P = 0.02$). Then one-way ANOVA and post hoc test found chronic restraint stress retarded the body weight growing from day 8 compared to CON, while *LB* and CIT treatment could not promote body weight gain, see Table 1.

SPT

The SPT was conducted on day 21 after the end of restraint stress. ANOVA indicated significant differences between four groups with respect to sucrose consumption ($F(3,28) = 9.68$, $P < 0.001$) (Fig. 2). Post hoc analyses revealed that the CRS group consumed

Table 1. Chronic restraint stress retarded the body weight growth

Day	CON	CRS	CRS/LAC	CRS/CIT
0	339.38 ± 6.28	344.25 ± 10.05	342.5 ± 6.83	340.88 ± 6.10
2	349 ± 7.64	338.25 ± 10.57	335.13 ± 6.65	327.75 ± 6.92
4	357.63 ± 7.18	340.13 ± 10.92	337.88 ± 6.45	331.13 ± 7.04
6	368 ± 7.61	343.13 ± 11.41	339.88 ± 5.49	335.25 ± 6.62
8	375.88 ± 7.65	344.38 ± 10.72*	342.88 ± 5.69	337.88 ± 6.31
10	387.88 ± 7.91	351.13 ± 11.71*	349 ± 6.34	346.88 ± 6.74
12	393 ± 8.31	354.88 ± 12.08*	354.5 ± 6.76	352.38 ± 6.62
14	397.13 ± 8.73	357.63 ± 11.58*	356.88 ± 7.26	359.13 ± 6.74
16	405.88 ± 9.21	364.63 ± 11.98*	362 ± 7.28	364.63 ± 6.92
18	413.63 ± 9.27	365.25 ± 11.8**	366.13 ± 7.4	367.25 ± 6.43
20	417.5 ± 8.79	366.25 ± 11.78**	368.63 ± 6.77	372.63 ± 7.49
25	431.13 ± 10.57	389.88 ± 13.73*	394.88 ± 8.90	390.88 ± 7.32

The body weight changes of four groups during the experiment are shown in the above table. All values are expressed as mean ± SEM. Groups: $N = 8$/group.*$P < 0.05$,**$P < 0.01$ compared to the control.

less sucrose solution than the control group ($P = 0.001$), LAC group ($P = 0.001$) and CIT group ($P = 0.001$).

EPM

Chronic restraint stress influenced anxiety behavior in EPM on day22 (Fig. 3), for time spent in open arms $F(3,28) = 5.789$, $P = 0.003$, for open arm entries $F(3,28) = 3.920$, $P = 0.019$ and for closed arm entries $F(3,28) = 7.276$, $P = 0.001$. The CRS group rats entered the open arms fewer times ($P = 0.019$) than control rats did. Rats given *L. helveticus* NS8 stayed longer in open arms than CRS rats ($P = 0.017$). And rats administrated with CIT entries more times in closed arms than other groups.

OFT

In the OFT on day 23, chronic restraint stress also affected rats' performance in center area (time in center area $F(3,28) = 3.089$, $P = 0.043$; distance traveled in the center area $F(3,28) = 7.82$, $P = 0.001$). As shown in Fig. 4D, C, the CRS group stayed less time in the center area ($P = 0.03$) and traveled less distance in center area ($P = 0.001$) than the control group.

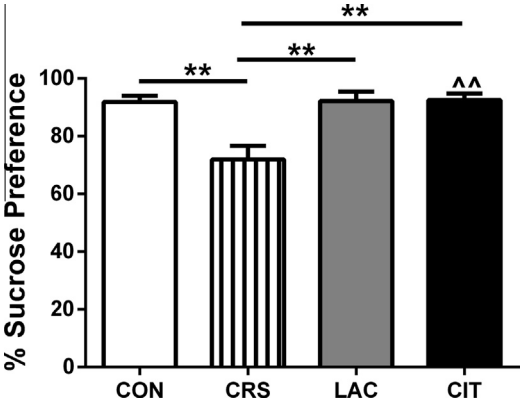

Fig. 2. *Lactobacillus helveticus* NS8 supplementation increased sucrose preferences in SPT as citalopram intervention did. Depression-related sucrose preferences were presented in the above figure. All values are expressed as mean ± SEM. $N = 8$/group. **$P < 0.01$.

Ingestion of *L. helveticus* NS8 was associated with more distance traveled in the center area ($P = 0.002$) than in the CRS group. Administration of CIT did not show any influence on behavior in the OFT.

ORT and OPT

Fig. 5A, B shows performance of rats in ORT. In ORT on day 24, the four groups presented different exploration patterns ($F(3,27) = 4.802$, $P = 0.008$). Chronic restraint stress decreased the percentage of time exploring new object compared to the control ($P = 0.039$). *L. helveticus* NS8 was associated with more object exploration than in the CRS groups ($P = 0.025$). CIT treatment showed no effect in ORT.

Fig. 5C, D shows the performance of rats in OPTs on day 25. There were significant differences between the four groups in placement recognition ($F(3,27) = 6.191$, $P = 0.002$). Post hoc tests showed that the CRS group spent less time exploring the object in novel location than control rats ($P = 0.024$), while *LB* and CIT groups both showed more novel location exploration time than the CRS group ($P = 0.002$ and $P = 0.048$, respectively).

Plasma CORT and ACTH levels

To elucidate the molecular basis of these behavioral changes, we first measured the stress hormones in plasma (Fig. 6). The four groups showed significant differences in both CORT ($F(3,28) = 18.905$, $P < 0.001$) and ACTH ($F(3,28) = 9.559$, $P < 0.001$) levels. The CRS rats showed higher CORT ($P = 0.011$) and ACTH ($P = 0.02$) levels than control rats. Administration of *L. helveticus* NS8 was associated with lower CORT levels and ACTH levels than in the CRS group ($P < 0.001$ and $P = 0.019$, respectively). Administration of CIT showed no effect on either CORT or ACTH levels.

Plasma cytokines

The plasma pro-inflammatory cytokines IFN-γ and TNF-α and anti-inflammatory cytokine IL-10 were also detected to evaluate the inflammatory situation (Fig. 7). A one-way ANOVA displayed significant differences across

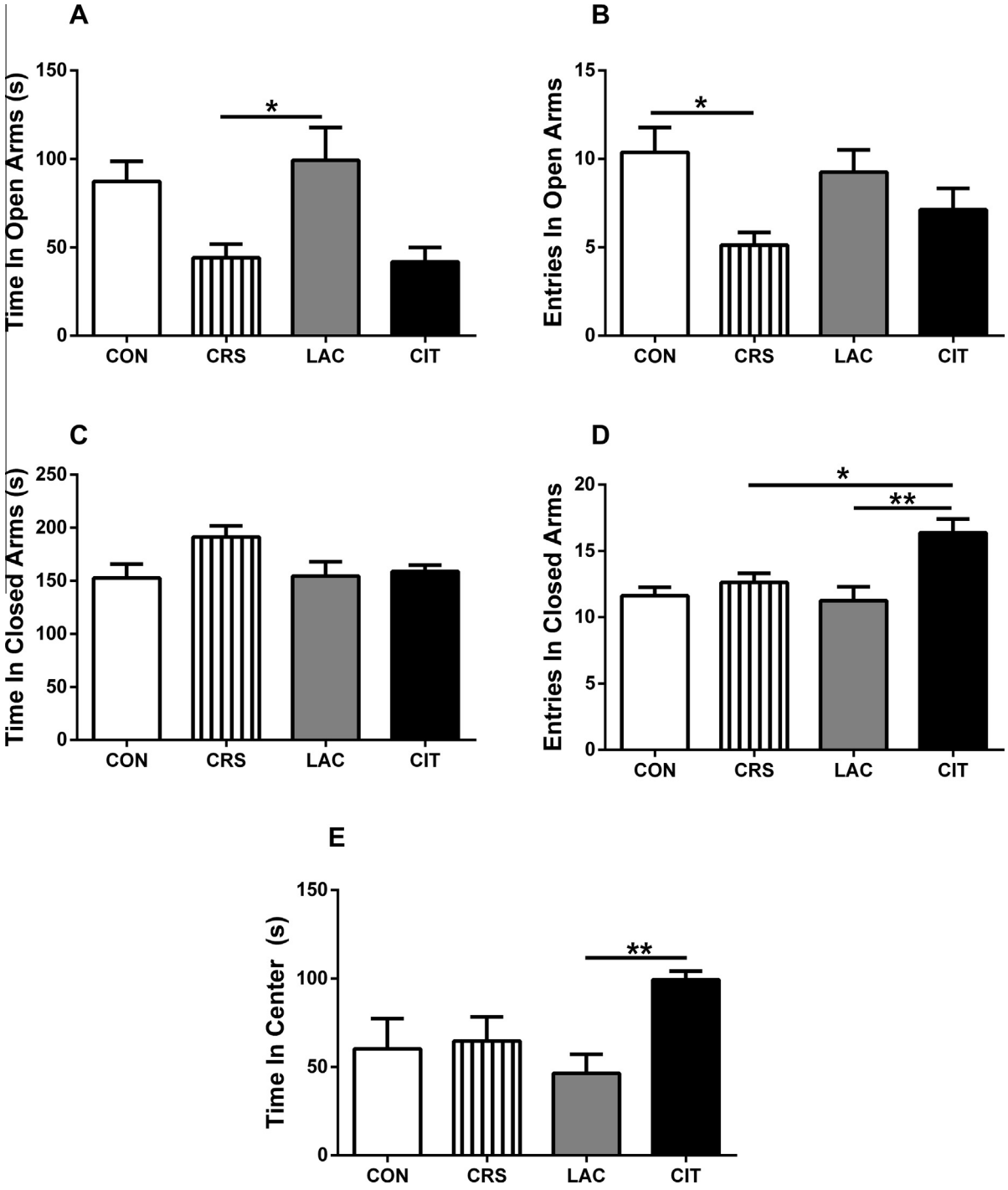

Fig. 3. *Lactobacillus helveticus* NS8 supplementation increased time spent in open arms in EPM while citalopram intervention did not. The anxiety-related behaviors in the elevated plus maze are shown in the above figure. Panel A, panel C and panel E show the time spent in open arms, closed arms and center areas individually. Panel B and panel D show the entries in open arms and closed arms respectively. All values are expressed as mean ± SEM. $N = 8$/group. $^{*}P < 0.05$, $^{**}P < 0.01$.

groups in IL-10 content ($F(3,28) = 19.263$, $P < 0.001$), IFN-γ content ($F(3,28) = 11.703$, $P < 0.001$) and TNF-α content ($F(3,28) = 13.152$, $P < 0.001$). As shown in Fig. 7, chronic restraint stress increased IFN-γ ($P = 0.003$) levels and TNF-α ($P = 0.05$) levels, decreased IL-10 levels ($P = 0.002$) compared to the control group. Although *L. helveticus* NS8 supplementation did not change the IFN-γ and TNF-α levels, it was associated with higher IL-10 levels ($P < 0.001$) than in the CRS group. Although CIT intervention did not influence IL-10 levels it did reduce IFN-γ ($P = 0.004$) and TNF-α ($P < 0.001$) levels.

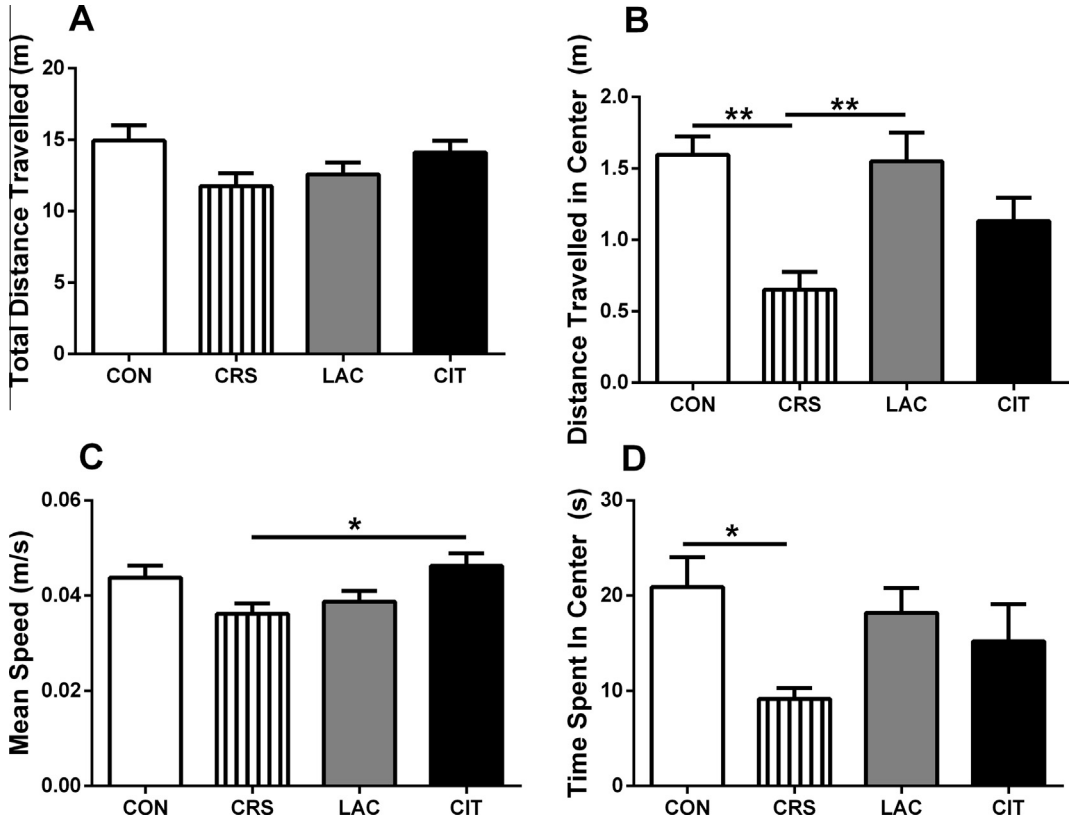

Fig. 4. *Lactobacillus helveticus* NS8 supplementation increased distance traveled in center in OFT while citalopram intervention did not. The anxiety-related behaviors in the open field test are shown in the above figure. Panel A and panel B show the distance traveled in entire area and in center respectively. Panel C shows the mean speed of rats. Panel D shows the time spent in center area. All values are expressed as mean ± SEM. $N = 8$/group. $^{*}P < 0.05$, $^{**}P < 0.01$.

BDNF

The BDNF mRNA expression in the PFC and hippocampus were measured to explain cognition-related changes (Fig. 8). A one-way ANOVA after logarithmic transformation (Log 10) showed the mRNA expression to have significant differences in the hippocampus ($F(3,8) = 16.582$, $P = 0.001$). Post hoc testing showed that chronic restraint stress rendered BDNF mRNA expression in the hippocampus ($P = 0.003$) lower than in the control group, and *L. helveticus* NS8 and CIT was associated with more BDNF mRNA expression ($P = 0.024$ and $P = 0.001$, respectively) in the hippocampus than in the CRS group.

Brain monoamine neurotransmitters

The anxiety and depression-related neurotransmitters 5-HT, DA, and NE were also detected. As shown in Fig. 9, chronic restraint stress not only changed NE levels in the PFC ($F(3,28) = 8.951$, $P < 0.001$) and hippocampus ($F(3,28) = 8.074$, $P < 0.001$). It also influenced hippocampus 5-HT levels ($F(3,28) = 6.392$, $P = 0.002$). The NE levels in the PFC ($P = 0.018$) and hippocampus ($P = 0.001$) and the 5-HT levels in the hippocampus ($P = 0.019$) were all lower in the CRS group than in the control group. While the NE levels in the hippocampus ($P = 0.006$) and 5-HT levels in hippocampus ($P = 0.002$) were both higher in the LAC group than in the CRS group. CIT administration showed significantly higher 5-HT levels in both the PFC ($P = 0.013$) and hippocampus ($P = 0.01$) than in the CRS group, but it did not affect NE levels.

Correlation

The correlations between behavioral results and biological outcomes are shown in Fig. 10. The sucrose preferences in SPT were positively correlated with hippocampus 5-HT content ($r_p = 0.603$, $P < 0.001$). The time in open arms in EPM was negatively correlated with plasma CORT ($r_p = -0.448$, $P = 0.01$) and ACTH ($r_p = -0.441$, $P = 0.019$) levels. The distance traveled in the center in OFT was positively correlated with hippocampus NE content ($r_p = 0.441$, $P = 0.012$). The time spent with new objects in ORT was negatively correlated with plasma CORT ($r_p = -0.499$, $P = 0.004$) and ACTH ($r_p = -0.405$, $P = 0.024$) levels while positively correlated with plasma IL-10 content ($r_p = 0.467$, $P = 0.008$) and hippocampus NE content ($r_p = 0.59$, $P < 0.001$).

DISCUSSION

The present study confirmed and expanded upon previous findings demonstrating behavior and cognition

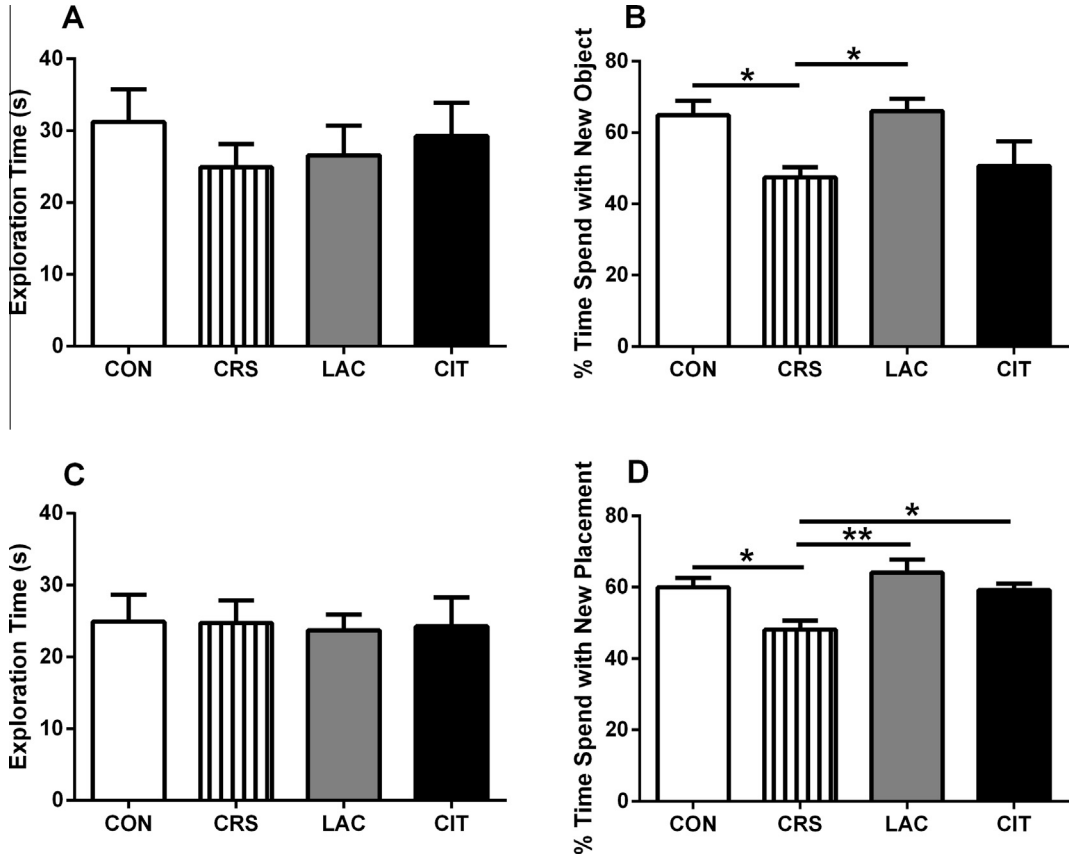

Fig. 5. *Lactobacillus helveticus* NS8 supplementation made better improvement than citalopram intervention in memory recognition tests. The behaviors of rats in object recognition test and placement recognition test are shown in the above figure. Panel A and panel B show the total time spent with the two objects and the relative amount of time exploring new object in ORT. Panel C and panel D show the total time spent in the two placements and the percentages of time exploring new placement in OPT. All values are expressed as mean ± SEM. $N = 7–8$/group. $^{*}P < 0.05$, $^{**}P < 0.01$.

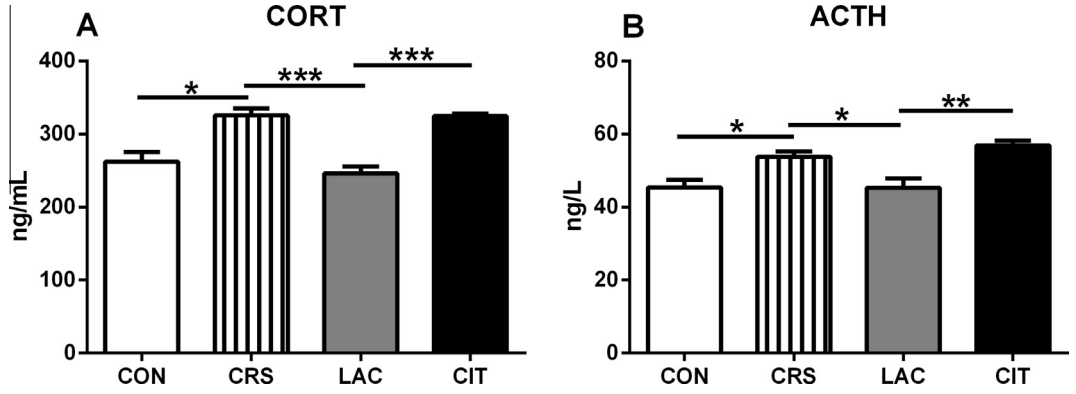

Fig. 6. *Lactobacillus helveticus* NS8 supplementation reduced plasma stress hormones content while citalopram intervention did not. Plasma CORT (Panel A) and ACTH (panel B) levels are shown in the above graphs. All values are expressed as mean ± SEM. $N = 8$/group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

changes in adult rats subjected to chronic restraint stress. After 3–4 weeks of chronic restraint stress (2–6 h/day), rats showed less body weight gain, more anxiety-like behavior (less time spent in aversive arms in EPM and less distance traveled in aversive areas in OFT), more depressive-like behavior (less sucrose solution consumption in SPT), and memory impairment (less novel object exploration time in ORT and less novel location exploration time in OPT) (Beck and Luine, 2002; Bowman et al., 2003; Ferraz et al., 2011; Huynh et al., 2011). These behavioral and cognitive aberrations were paralleled by biochemical alterations including higher levels of stress hormones and pro-inflammatory cytokines levels in plasma, lower levels of anti-inflammatory

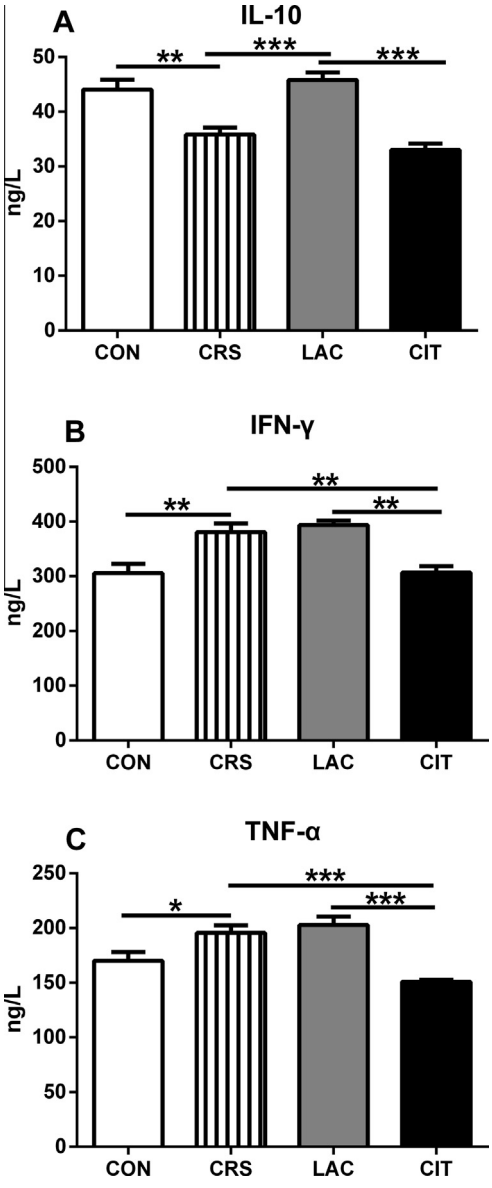

Fig. 7. *Lactobacillus helveticus* NS8 supplementation promoted plasma IL-10 release while citalopram intervention promoted IFN-γ and TNF-α release. The plasma cytokines content are shown in theabove graphs. Plasma IL-10 levels (Panel A), IFN-γ levels (Panel B) and TNF-α levels (Panel C) are shown in the three graphs given above. All values are expressed as mean ± SEM. $N = 8$/group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

cytokines in plasma (Ferraz et al., 2011; Voorhees et al., 2013), less 5-HT and NE content in the brain, and lower BDNF content (or BDNF mRNA levels) in the hippocampus (O'Mahony et al., 2011; Radahmadi et al., 2015). Most of the abnormalities resulting from chronic restraint stress were attenuated by chronic supplementation of probiotic *L. helveticus* NS8 or antidepressant CIT. These results support the current hypothesis that chronic *L. helveticus* NS8 supplementation can counteract chronic stress-induced behavioral, cognitive, and biochemical aberrations as well as many antidepressants.

The physiological and behavioral responses to chronic restraint stress may be mediated by the brain–gut–micro biota axis. The endocrine, immune, and nervous systems and each pathway of the brain–gut–microbiota axis were activated to deal with chronic stress (Dinan and Cryan, 2012; Mahar et al., 2014; Moloney et al., 2014). The long-lasting exaggeration of HPA activity, the chronic inflammation, the persistent disruption of brain neurotransmitters, and the decrease in BDNF were all detrimental to heath, driving the depression pathogenesis (Schiepers et al., 2005; Belmaker and Agam, 2008; Marin et al., 2011; Gold, 2014).

Hypothalamic–pituitary–adrenocortical (HPA) activation is one of the most important parts of stress response (Swaab et al., 2005). Both human and murine subjects release more stress hormones after chronic stress. Long-term increases in stress hormones suggest the failure of HPA axis negative feedback under the etiology of depression (Barden, 2004; Swaab et al., 2005; Lupien et al., 2009). In the present experiment, plasma CORT and ACTH levels were both correlated with anxiety-like behavior in EPM. Probiotic supplementation has been found to regulate the HPA axis function both in early age and in adulthood (Sudo et al., 2005; Eutamene and Bueno, 2007; Gareau et al., 2007). The present results demonstrated the modulatory effects of *L. helveticus* NS8 to restore circulating CORT levels like other probiotics, such as *Bifidobacterium infantis*, *Lactobacillus rhamnosus* strain R0011 (95%) and *L. helveticus* strain R0052 (5%), *L. rhamnosus* (JB-1), and *Lactobacillus farciminis* (Sudo et al., 2005; Gareau et al., 2007; Bravo et al., 2011; Ait-Belgnaoui et al., 2012). But we also found the CORT levels of control group were higher compared to other studies (Naert et al., 2011). This was possibly because the control rats were also deprived of food and water during restraint time and food and water deprivation could increase CORT levels on their own (Tannock and Savage, 1974).

The immune system is also involved in the stress response. Sustained stress exposure can induce maladaptive inflammation (Gold, 2014). The pro-inflammatory and anti-inflammatory balance is dysregulated, and the entire immune response moves toward inflammation, as demonstrated by the increase in the release of pro-inflammatory cytokines and decrease in the release of anti-inflammatory cytokines (Schiepers et al., 2005; Rook and Lowry, 2008; Gold, 2014). Levels

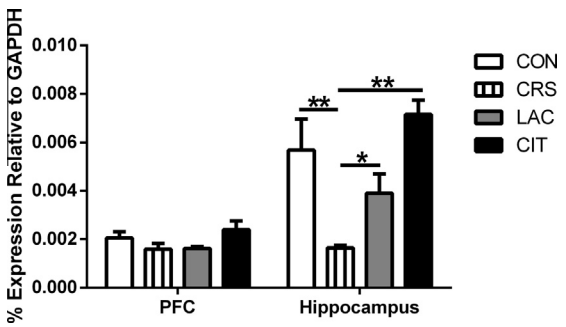

Fig. 8. *Lactobacillus helveticus* NS8 supplementation increased BDNF mRNA expression in hippocampus as citalopram intervention did. The BDNF mRNA expression in prefrontal cortex and hippocampus are shown in the above graph. All values are expressed as mean ± SEM. $N = 3$/group. $^{*}P < 0.05$, $^{**}P < 0.01$.

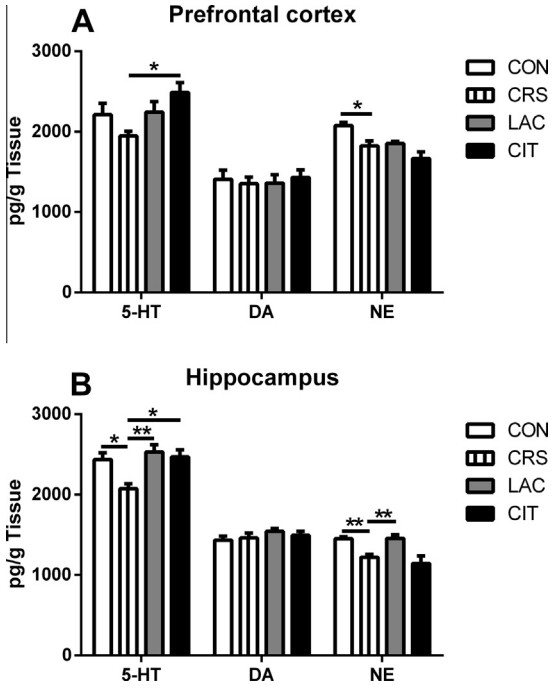

Fig. 9. *Lactobacillus helveticus* NS8 supplementation increased both 5-HT and NE content in hippocampus while citalopram intervention just increased 5-HT content. The monoamine neurotransmitters in prefrontal cortex and hippocampus are shown in the above graphs. Panels A and B show 5-HT, DA, and NE levels in the prefrontal cortex and hippocampus respectively. All values are expressed as mean ± SEM. $N = 8$/group. $^{*}P < 0.05$, $^{**}P < 0.01$.

of pro-inflammatory cytokines (including IL-6, TNF-α, and IFN-γ) usually increase after chronic restraint stress, and the levels of anti-inflammatory cytokines (like IL-10) tend to decrease (Ferraz et al., 2011; Voorhees et al., 2013). It was suggested that the immune regulation of probiotics maybe correlated with IL-10 (Ochoa-Reparaz et al., 2010a,b; Ohland et al., 2013). The present experiment showed *L. helveticus* NS8 increased IL-10 release while CIT decreased IFN-γ and TNF-αlevels, indicating different mechanisms underlying the two treatments. This immune regulatory effect of NS8 was consistent with that observed in other studies of *L. helveticus* strain (Ohland et al., 2013).

The hippocampus, one of several brain regions related to behavior and cognition regulation, was impaired after chronic stress (Marije aan het Rot et al., 2009). Chronic restraint stress could induce hippocampus impairment like neuronal loss, dendritic retraction, and BDNF content reduction (McLaughlin et al., 2007; Takuma et al., 2007; O'Mahony et al., 2011). While BDNF was critical for neurogenesis and synaptic plasticity, both of which were necessary to maintain hippocampus morphology and function (Marije aan het Rot et al., 2009; Lu et al., 2014). And the BDNF decrease was associated with neuronal loss and could even induce hippocampus atrophy which was very common in major depression (Belmaker and Agam, 2008) while long time antidepressant treatment could increase neurogenesis and BDNF levels (Vaidya et al., 2007; Belmaker and Agam, 2008). Moreover, hippocampus neurogenesis regulation was also connected with gut microbiota (Gareau et al., 2011). Germ-free mice exhibited increased adult hippocampus neurogenesis compared to conventionally colonized mice (Ogbonnaya et al., 2015). The BDNF content or BDNF mRNA levels reduction was possibly associated with cognition impairment and the increase in depressive-like behavior (Mehrpouya et al., 2014; Radahmadi et al., 2015). Thus the antidepressant effect (sucrose consumption increase) and spatial memory improvement (novel location exploration time increase in OPT) of *L. helveticus* NS8 and CIT was possibly reached by restoring BDNF levels in hippocampus. Many probiotics were found to have the potential of regulation BDNF levels, such as *L. helveticus* R0052 and *Bifidobacterium longum* R0175 (Ait-Belgnaoui et al., 2014; Distrutti et al., 2014). Since the BDNF content was greatly influenced by HPA activity and monoamine transmission (Vaidya et al., 2007; Mahar et al., 2014), the restoration of BDNF content maybe related to the normalized stress hormones levels and monoamine content (5-HT and NE). And the present results also demonstrated that only *L. helveticus* NS8 administration increased novel object exploration time in ORT, which might be correlated with its regulation to plasma stress hormones, IL-10 and hippocampus NE content.

5-HT and NE are crucial neurotransmitters of mood and cognition regulation. Balances of both are easily disturbed by chronic stress (Hamon and Blier, 2013). The 5-HT and NE levels in the PFC and hippocampus were decreased which might be connected with the behavioral aberrations caused by chronic restraint stress (Bowman et al., 2009; O'Mahony et al., 2011). In the present study, the hippocampus 5-HT content was correlated with depressive-like behavior in SPT while hippocampus NE content was correlated with anxiety-like behavior in OFT and recognition preference in ORT. Gut flora were found to influence the serotonergic system in the hippocampus (Clarke et al., 2013). Gut bacteria could also affect catecholamine system. They could recognize catecholamine signals and perform adaptive activities to allow their populations to flourish; this affected the host (Freestone et al., 2008; Sudo, 2014). In this way, the antidepressant effects of *L. helveticus* NS8 may be caused by the recovery of 5-HT content in the hippocampus, which was similar to the results of selective serotonin reuptake inhibitor (SSRI) therapy. Since CIT administration did not affect behavior (in OFT and ORT) and NE content, the anti-anxiety and cognition improving effects in ORT of *L. helveticus* NS8 might be related to the restoration of NE content in the hippocampus. Both Bifidobacterium strains and *LB* strains were found to reduce the rate of anxiety-like and depressive-like behavior and promote memory in both rodent models and human studies (Sullivan et al., 2009; Desbonnet et al., 2010; Bercik et al., 2011; Bravo et al., 2011; Messaoudi et al., 2011; Arseneault-Breard et al., 2012; Davari et al., 2013; Ohland et al., 2013; Tillisch et al., 2013; Ait-Belgnaoui et al., 2014; Distrutti et al., 2014). However, only one study showed probiotics to have a therapeutic effect in a depression model. This model used *B. infantis* in a rat maternal separation model (Desbonnet et al., 2010).

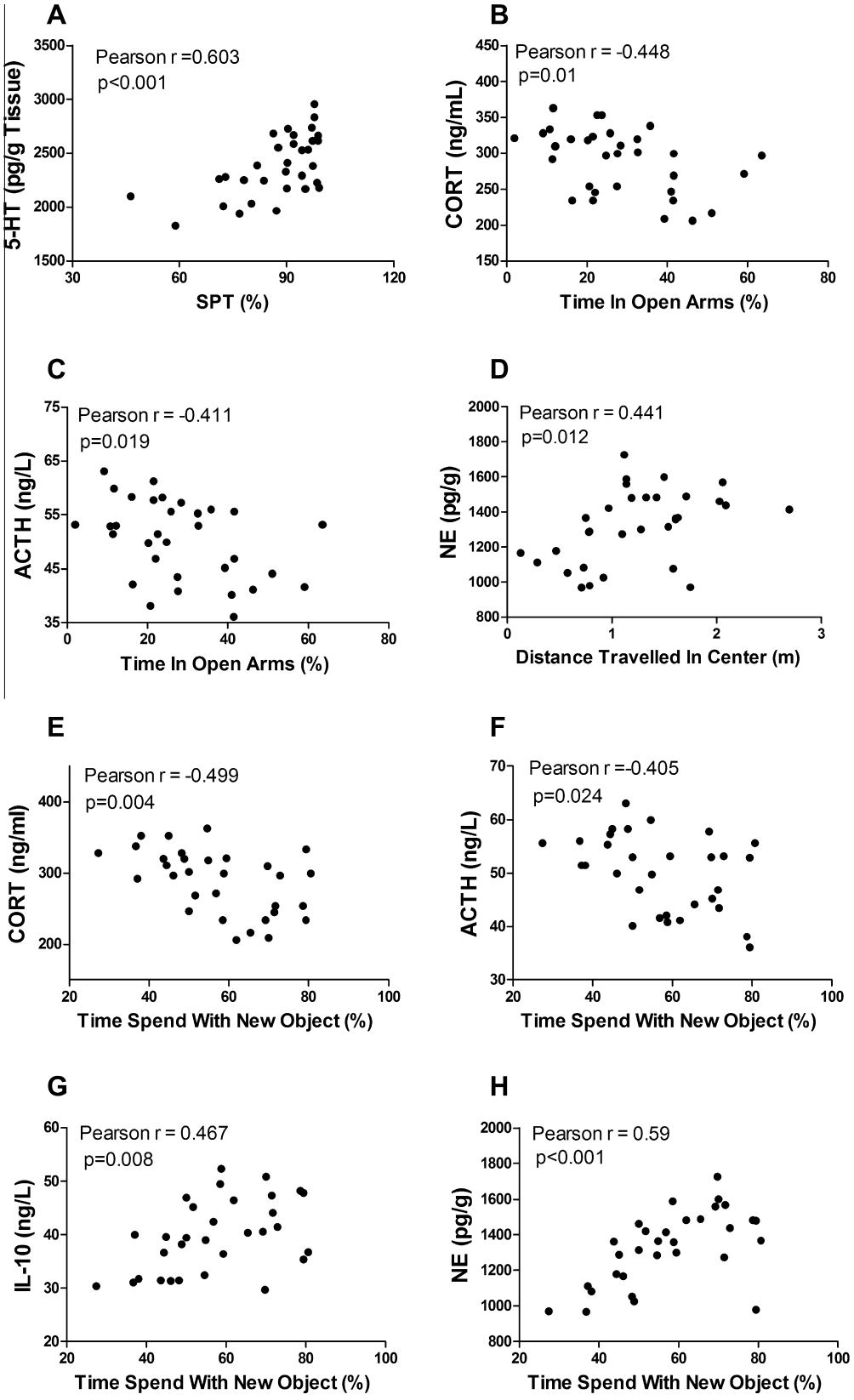

Fig. 10. The behavioral results were correlated with relevant biological outcomes. Panel A shows the correlation between sucrose preferences and hippocampus 5-HT content. Panel B and panel C show the correlation between time in open arms in EPM and plasma CORT and ACTH levels. Panel D shows the correlation between distance traveled in center in OFT and hippocampus NE content. Panel E, panel F, panel G and panel H show the correlation between time spend with new object in ORT and plasma CORT, plasma ACTH, plasma IL-10 and hippocampus NE content, respectively.

Although the behavior modulation effect has been described in detail, the physiological and biochemical mechanisms under therapeutic conditions remain unclear.

The reduction in 5-HT synthesis is widely thought to play a causative role in the etiology of major depression. Increasing 5-HT content in synaptic cleft is one of the commonest targets in depression treatment (Marije aan het Rot et al., 2009). CIT is a frequently used antidepressant aiming at inhibiting the reuptake of 5-HT without influence in other neurotransmitters (Hyttel, 1982). In the present study, CIT treatment increased sucrose preference in SPT, improved placement recognition in OPT, decreased IFN-γand TNF-αlevels in plasma, improved hippocampus BDNF mRNA expression and increased 5-HT content in the PFC and hippocampus, curing most of the abnormalities in chronic restraint stress depression model. The similar behavioral and biochemical correction effect of *LB* treatment indicated the 5-HT system was also involved in its therapy. *L. helveticus* NS8 treatment was demonstrated to regulate 5-HT and its synthesis in the hyperammonemia rat (Luo et al., 2014). In the present study, the restoration of 5-HT content may be related to the enhancement of tryptophan availability (Desbonnet et al., 2008a; Borre et al., 2014a). The brain 5-HT content is positively correlated with its precursor tryptophan levels. However, tryptophan can also degrade into kynurenine. This process can be catalyzed by either indoleamine-2,3-dioxygenase (IDO) or tryptophan-2,3-dioxygenase (TDO). Inflammation can accelerate IDO activity and CORT can accelerate TDO activity. Both can facilitate tryptophan catabolism through kynurenine pathway, rendering tryptophan less available (Le Floc'h et al., 2011; Maes et al., 2011; O'Mahony et al., 2014). Improving inflammation and reducing CORT release can decrease tryptophan consumption, leaving more tryptophan available for synthesis of 5-HT (Le Floc'h et al., 2011; O'Mahony et al., 2014). It was possible that *L. helveticus* NS8 restored hippocampus 5-HT levels by increasing tryptophan availability by regulating the immune response (enhancing IL-10 release) and HPA axis function (reducing CORT and ACTH levels).

Orally administered *L. helveticus* NS8 can modulate host behavior and biochemical aberrations through all the three pathways of the brain–gut–microbiota axis. By reducing CORT release to regulate the function of the HPA axis, increasing anti-inflammatory cytokine IL-10 levels to correct the immune imbalance, and restoring 5-HT, NE, and BDNF levels to mitigate brain injury, *L. helveticus* NS8 supplementation normalized most of the behavioral and cognitive abnormalities caused by chronic restraint stress. The anti-depression effect may relate to the restoration of hippocampus 5-HT content and BDNF mRNA expression. The anti-anxiety effect may correlate with the reduction of plasma stress hormones release and the restoration of hippocampus NE content. The cognition improvement effect in ORT may connect with the reduction of plasma stress hormones release and the restoration of plasma IL-10 content and hippocampus NE content. All of these pathways are key physiological mechanisms in depression treatment, indicating that *L. helveticus* NS8 may be a suitable alternative therapy for depression. The present data suggest that probiotics may regulate host behavior and biochemistry through many pathways and that these pathways may be coordinated with each other.

Although we achieved meaningful and improving results in the present study, it was still an exploratory study and the experimental design was not perfect. Firstly, for animals grouping, although previous researches demonstrated that *L. helveticus* NS8 treatment had only beneficial effects (Luo et al., 2014) and CIT was a good antidepressant (Hyttel, 1982; Desbonnet et al., 2010), it would be better if both treatments had their own control groups. Secondly, for behavior tests, the EPM could be executed after the OFT. Although 5 min of OFT was often used for anxiety-like behavior measurement (Bellani et al., 2006; Babri et al., 2014), the locomotor activity data would be more accurate if the test lasted longer than 5 min. Although the recognition memory tasks were useful measurements in animal spatial memory and non-spatial memory (Bowman et al., 2006; Luine, 2015), other cognition tests with longer time and more trials could be used in future study. Thirdly, for animal numbers, although eight rats per group were effective in the present study, more rats would be better to reduce individual errors and to find treatment differences. Lastly, for sex differences, in animal experiments, the behavior of adult female rats was influenced by estrus cycle while the male rats' behavior was relatively stable (Marcondes et al., 2001; Sayin et al., 2014). But the female rats should not be ignored since male and female rats made different responses to certain stressful procedures (Bowman et al., 2003, 2006, 2009; Dalla et al., 2005). Thus further studies should find out whether there are sex differences in this treatment. In a word, more studies are needed to illustrate how *L. helveticus* NS8 treatment works in depression and whether it works for other kinds mental disorders.

CONCLUSION

In summary, this study provides preliminary evidence that chronic treatment with probiotic *L. helveticus* NS8 can have anxiolytic and antidepressant effects, promote cognition, decrease plasma CORT and ACTH levels, modulate pro-inflammatory and anti-inflammatory balance, and restore 5-HT, NE, and BDNF content in the hippocampus, inducing an effect similar to that of SSRI. Ever since Logan proposed probiotics as an adjuvant therapy for major depression in 2005 (Logan and Katzman, 2005), many scientists have thought about this issue deeply (Cryan and Dinan, 2012; Rook et al., 2012, 2014; Borre et al., 2014a; Dash et al., 2015). Dinan et al. (2013) proposed 'psychobiotics' to emphasize the potential therapy effects of probiotics in mental illness (Dinan et al., 2013). In the present study, NS8 showed the potential as one of the 'psychobiotics' in treatment of depression. So far, there are several studies exploring and speculating on how gut bacteria influence brain and behavior (Forsythe et al., 2010; Cryan and O'Mahony,

2011; Al-Asmakh et al., 2012; Cryan and Dinan, 2012; Montiel-Castro et al., 2013; Farmer et al., 2014; Fond et al., 2014; Tillisch, 2014; Wang and Kasper, 2014), even extending the treatment to other mental disorders such as autism spectrum disorder, obsessive–compulsive disorder, bipolar disorder, and schizophrenia (Critchfield et al., 2011; Hsiao et al., 2013; Severance et al., 2013; Kantak et al., 2014; Savignac et al., 2014, 2015). Most of these studies are either reviews or related experimental studies of physiological diseases comorbid with depression (Sullivan et al., 2009; Arseneault-Breard et al., 2012; Saulnier et al., 2013). Direct clinical and preclinical studies of depression are very scarce. These data confirm and demonstrate the hypothesis that probiotic supplementation may be an effective and safe therapy for chronic-stress-induced depression.

AUTHORS' CONTRIBUTIONS

All authors listed have contributed to the work. LS participated in designing the experimental protocol, data collection, statistical analysis and writing the manuscript. JF designed and supervised the study and revised the manuscript. LS, LJ, LW, WXL, and DYF carried out behavioral test and sample collection. WT, HX, and JF provided administrative, technical, or material support. All authors read and approved the final manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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