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Research report

Alteration of behavior and monoamine levels attributable to *Lactobacillus plantarum* PS128 in germ-free mice

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HIGHLIGHTS

- Both live and heat-killed PS128 showed no obvious toxic effects to the germ-free (GF) mice.
- Live PS128 increased locomotor activity of the GF mice.
- Live PS128 reduced anxiety-like behavior of the GF mice in the elevated plus maze.
- Live PS128 increased both dopamine and serotonin level in the striatum.

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ABSTRACT

Probiotics, defined as live bacteria or bacterial products, confer a significant health benefit to the host, including amelioration of anxiety-like behavior and psychiatric illnesses. Here we administered *Lactobacillus plantarum* PS128 (PS128) to a germ-free (GF) mouse model to investigate the impact of the gut–brain axis on emotional behaviors. First, we demonstrated that chronic administration of live PS128 showed no adverse effects on physical health. Then, we found that administration of live PS128 significantly increased the total distance traveled in the open field test and decreased the time spent in the closed arm in the elevated plus maze test, whereas the administration of PS128 had no significant effects in the depression-like behaviors of GF mice. Also, chronic live PS128 ingestion significantly increased the levels of both serotonin and dopamine in the striatum, but not in the prefrontal cortex or hippocampus. These results suggest that the chronic administration of PS128 is safe and could induce changes in emotional behaviors. The behavioral changes are correlated with the increase in the monoamine neurotransmitters in the striatum. These findings suggest that daily intake of the *L. plantarum* strain PS128 could improve anxiety-like behaviors and may be helpful in ameliorating neuropsychiatric disorders.

1. Introduction

For decades, probiotics in viable form and non-viable form have been used to normalize physiological dysfunctions such as developmental programming of epithelial barrier function, gut homeostasis, and immune responses [1–3]. In recent years, many reports demonstrated that probiotics also are capable of altering the brain and behaviors of the host via the gut–brain axis (GBA). For example, *Lactobacillus rhamnosus* JB-1 and *Bifidobacterium longum* NCC3001 both showed anxiolytic effects in mice [4,5], whereas *Lactobacillus helveticus* R0052 and *B. longum* R0175 had similar effects in rats [6]. Furthermore, it has been shown that the behavioral changes were associated with alterations in neurochemicals in the brain. As shown in studies using maternal separation to induce

Abbreviations: ALT, alanine aminotransferase; CPK, creatine phosphokinase; CREA, creatinine; DA, dopamine; EPM, elevated plus maze; FST, forced swim test; GBA, gut–brain axis; GF, germ-free; HPLC–ECD, high-performance liquid chromatography with electrochemical detection system; MRS, Man Rogosa Sharpe; NA, noradrenaline; OFT, open field test; SPF, specific pathogen-free; TG, triacylglycerol.

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depression-like behaviors in rats, administration of *Bifidobacteria infantis* 35624 was able to alleviate depression-like behaviors and reduce the level of serotonin (5-HT) and dopamine (DA) metabolites [7]. Interestingly, heat-killed *Lactobacillus brevis* SBC8803 was also able to stimulate 5-HT receptors in intestinal cells of mouse [8], suggesting that non-viable bacterial components also have the potential to regulate the GBA.

In our unpublished observations using specific pathogen-free (SPF) mice, we found that daily administration of a newly isolated *Lactobacillus plantarum* PS128 (PS128) at 10^9 CFU increased locomotor activity and reduced anxiety-like behaviors (see Supplementary data). These effects may be solely due to PS128 oral administration; however, because of the diversity and quantity of the normal commensal bacteria in the gastrointestinal tract of the SPF mice, the behavioral effects could also be a convergent result of mixing the exogenous PS128 and the indigenous gut microbes. Such an interesting finding led us to investigate whether and to what degree PS128 could produce behavioral effects. To isolate the effects purely caused by PS128 and exclude the influences and interactions of other microbes, we used germ-free (GF) mice to investigate the behavioral effect of PS128.

GF animals are microbiota-deficient and are bred in a sterile environment. These animals are a valuable tool for investigating the components of the GBA and provide a better understanding of the psychological effects of the microbiota [2]. Past studies using GF mice had demonstrated that gut microbiota are essential for normal brain development and behavior. Because of a lack of normal gut microbiota, the GF mice had displayed significantly less anxiety-like behaviors compared to control mice [9–11]. These behavioral changes were accompanied by an increased turnover rate of noradrenaline (NA), DA, and 5-HT [9]. Interestingly, postnatal colonization of commensal microbiota in the gut of GF mice led to normalization of the anxiety-like behavior and an increase in the turnover rate of the striatal monoaminergic neurotransmitter systems [9,12]. In addition, Sudo et al. reported that specific pathogen-free (SPF) mice that have normal gut microbiota and GF mice administered the probiotic bacterium *Bifidobacterium infantis* both had lower serum corticosterone (CORT) levels than did GF mice [13]. These results indicate that colonization of commensal microbiota as well as monoassociation of a probiotic bacterium could affect postnatal development of the neurochemicals and the hypothalamic–pituitary–adrenal (HPA) stress response in mice.

In the current study, we assessed the safety of PS128 administration in the basic physiological processes of GF mice by observing their phenotype, tissue histology, and metabolites in the blood. In addition, we examined whether PS128 could alter the locomotion, anxiety-like, and depression-like behaviors of GF mice by using the open field test (OFT), elevated plus maze (EPM), and forced swim test (FST), respectively. Furthermore, in an attempt to probe the underlying mechanisms mediating the effects of PS128, we also measured the DA and 5-HT levels and turnover rates in various brain regions related to anxiety and examined the responses of the HPA axis in GF mice.

2. Materials and methods

2.1. Animals

Male GF C57BL/6JNarl mice (6 weeks old) were purchased from the National Laboratory Animal Center (Taipei, Taiwan). Mice were maintained in vinyl isolators in a room kept at a constant temperature ($22 \pm 1\,^{\circ}C$) and humidity (55–65%) with a 12-h light:dark cycle. The mice were fed a commercial diet (5010 LabDiet; Purina Mills, St. Louis, MO) and sterile water ad libitum. All experiments were performed in accordance with relevant guidelines and regulations and were pre-approved by the Institutional Animal Care and Use Committee of National Yang-Ming University (IACUC No. 1001102). Saline, heat-killed, or live PS128 (10^9 CFU/mouse/day) was orally administered to GF mice ($n = 10$/group) for 16 days. On day 14, all mice were weighed and then given the oral administration. After the oral administration, the mice were placed in sterile transport cages and moved to the behavioral room for acclimation. The behavioral tests started on day 15 and ended on day 16. All the behavioral tests were conducted during the light phase. On the first day we performed the OFT, EPM, and the first session of the FST. On the second day we conducted the second session of the FST (see Section 2.7). Each mouse was allowed to rest for 30 min after the second FST and then was subjected to retro-orbital blood collection. Blood samples were left at room temperature for 30 min and then centrifuged to collect serum for future use. After the blood collection, the mouse was euthanized immediately by cervical dislocation. The brain was quickly removed and was temporarily preserved on dry ice, the cecum was removed and weighed, and samples of liver, lung, and small intestine were collected for histological examinations.

2.2. Preparation of L. plantarum PS128

PS128 was first inoculated in Man Rogosa Sharpe (MRS) broth (BD Difco, MD, USA), cultured at 37 °C for 18 h, and then harvested by centrifugation at $6000 \times g$ for 10 min. To prepare live PS128, the supernatant was removed and the pellet was re-suspended with MRS broth containing 12.5% glycerol for cryopreservation. Final concentration of the live PS128 was adjusted to 5×10^9 CFU/ml and stored at −20 °C until use. To prepare the heat-killed form of PS128, the pellet described was re-suspended with saline and then incubated at 100 °C for 1 h. This heat-killed PS128 was then stored at −20 °C until use. Before use, live and heat-killed PS128 preparations were thawed and centrifuged. The supernatants were removed and replaced by saline. Then, the preparations were pre-warmed at 37 °C for 1 h and then orally administered to mice. For the control group, pre-warmed saline was used.

2.3. Blood biochemistry

The collected blood sample was centrifuged at $2500 \times g$ for 10 min at 4 °C and the serum was stored at −80 °C until use. The serum levels of alanine aminotransferase (ALT), creatine phosphokinase (CPK), creatinine (CREA), and triacylglycerol (TG) levels were determined using an automatic biochemical analyzer (HITACHI 7080; Hitachi, Tokyo, Japan).

2.4. Histopathological examination

Liver, lung, and intestine tissue samples were collected and fixed in 10% phosphate-buffered formalin. After overnight fixation, samples were prepared for paraffin section. Tissues embedded in paraffin were sectioned at 4 μm and stained with hematoxylin and eosin for histological examination under light microscope (BX-51; Olympus, Tokyo, Japan).

2.5. Open field test

Each GF mouse was placed into an arena with Plexiglas walls ($25.4 \times 25.4 \times 38$ cm) with photobeam sensors to record locomotor activities for 10 min (Tru Scan Activity System; Coulbourn Instruments, Whitehall, PA, USA). The central zone was defined as a region in the center measuring 12.5×12.5 cm. Locomotor activities were automatically recorded and analyzed by Tru Scan 2.2 software (Tru Scan Activity System, Coulbourn Instruments). To minimize the odor interference, the arena was cleaned with 70% ethanol after

each run. The illumination is 325 lux in the center and 293 lux in the peripheral.

2.6. Elevated plus maze

For assessing anxiety-like behavior, an EPM comprising two closed arms and two open arms was used for this test (height, 45 cm; full arm length, 66 cm; arm width, 10 cm; wall height of closed arm, 30 cm). The lighting in the maze was measured 472 lux in the open arms and 200 lux in the closed arms. The GF mouse was placed in the center crossed area (10 × 10 cm) at the beginning and allowed free exploration for 10 min. Mouse behaviors were recorded by a video camera mounted on the ceiling of the maze center and analyzed by a video tracking software (EthoVision; Noldus Information Technology, Wageningen, the Netherlands). With the EthoVision tracking software we calculated the time each mouse spent in the closed and open arms but not in the center crossing area during the first 5 min. We also calculated the ratio of time spent in the open arm compared to the time in the closed arm. The total distance traveled during the test period was also quantified.

2.7. Forced swim test

To evaluate the effect of PS128 on depression-like behavior, the GF mice were subjected to FST. On the first day the mouse was placed in a transparent acrylic cylinder (internal diameter, 10 cm) containing 15 cm of water to swim for 6 min. The water temperature was adjusted to 23–25 °C. On the second day, the mouse was subjected to a second swim session for 5 min. The swimming behaviors were recorded by a video camera. The video images were later analyzed by the EthoVision video tracking software (Noldus Information Technology, Wageningen, the Netherlands). Time of immobility was quantified.

2.8. Measurement of serum corticosterone levels

We used a commercial enzyme immunoassay (CORT EIA Kit; Cayman, Ann Arbor, MI, USA) to measure the CORT level in serum collected after the second day FST test. The measurements were conducted in duplication and the averaged mean was used for analysis.

2.9. Quantification of brain monoamines and their metabolites by high-performance liquid chromatography with electrochemical detection

The ice-cold brain was dissected on a filter paper placed on a glass dish on ice. Specific brain regions including the striatum, prefrontal cortex, and hippocampus were removed. The brain samples were immediately preserved in ice-cold 0.6% perchloric acid and homogenized by sonication. The homogenized samples were then centrifuged at 12,000 rpm for 20 min at 4 °C. The supernatants were filtered through a 0.22 mm polyvinylidene difluoride membrane (4 mm syringe filter; Millex-GV; Millipore, MA, USA) and preserved at −80 °C until use. Before analysis, the supernatants were thawed and properly diluted. We used high-performance liquid chromatography with electrochemical detection system (HPLC–ECD) to evaluate the levels of monoamines and their metabolites in the samples (20 μl). The HPLC–ECD system comprised a micropump (CMA-100; CMA, Stockholm, Sweden), an online injector (CMA-160), a Microtech LC-pump (Microtech Scientific, Sunnyvale, CA, USA), a BAS-4C electrochemical detector (Bioanalytical Systems, Inc., West Lafeyette, IN, USA), and a reversed-phase column (Kinetex C_{18}, 2.6 um, 100 × 2.1 mm I.D.; Phenomenex, CA, USA), as described previously [14]. The potential for the glassy carbon working electrode was set at +650 mV with respect to the Ag/AgCl reference electrode at room temperature (25 °C). The mobile phase contained 0.1 M NaH_2PO_4, 8% methanol, 0.74 mM 1-octanesulfonic acid (sodium salt), 0.03 mM ethylenediamine tetraacetic acid, and 2 mM KCl, and was adjusted to pH 3.74 with H_3PO_4. Concentrations of dopamine (DA), dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), serotonin (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) ranging from 1 to 100 ng/ml in the samples were interpolated by the standard curve obtained from standards (Sigma–Aldrich, St. Louis, MO, USA).

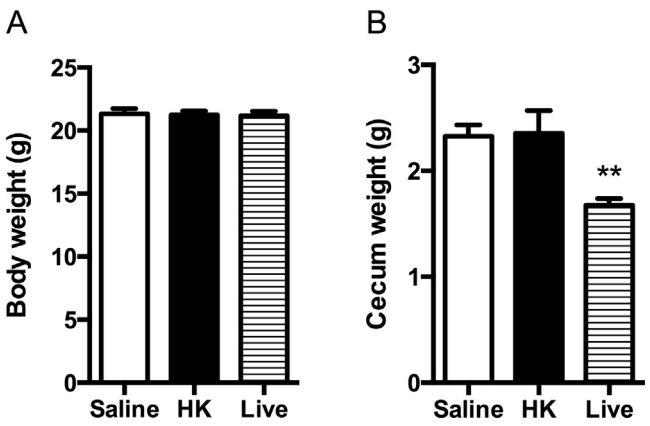

Fig. 1. Effect of *L. plantarum* PS128 on body weight and cecum weight. (A) Neither heat-killed nor live PS128 affects the body weight of the germ-free (GF) mice. (B) Cecum weight is significantly reduced in the live PS128 group. HK, heat-killed PS128; Live, live PS128; $^{**}p < 0.01$.

2.10. Statistical analysis

All data were expressed as mean ± SEM. Comparisons among the saline, HK, and live groups were performed by one-way or two-way analysis of variance (ANOVA) when appropriate. Differences between groups were determined by post hoc Tukey's pairwise multiple comparison analysis.

3. Results

3.1. Chronic oral administration of L. plantarum PS128 showed no toxic effects in the GF mice

Lactobacillus spp. has long been used as an additive in health foods, including fermented milk drinks [15]. However, there is minimal safety data regarding long-term ingestion of lactic acid bacteria in GF mice. To rule out the possible toxicity of PS128, we administered both live and heat-killed PS128 to GF mice for 16 days and examined the physical and histological changes of GF mice. Regarding external appearances, there were no obvious changes in skin, fur, and eyes among all groups. In addition, we found no significant changes in body weights in different groups, although treatment with live PS128 significantly reduced the cecum weight compared to the saline and heat-killed PS128 groups (Fig. 1A and B; $p < 0.01$). The macroscopic appearance of major organs excised from the sacrificed mice was normal. The histological samples of liver, lung, and small intestine in each group showed no differences compared to controls (Fig. 2). To investigate whether there were adverse effects of chronic PS128 ingestion on the liver function, general metabolism, and kidney function, we measured serum levels of alanine aminotransferase (ALT), creatine phosphokinase (CPK), triacylglycerol (TG), and creatinine (CREA), respectively. We found that although there were variations, these parameters were all within the normal range and were not significantly different among different treatment groups compared to controls (Fig. 3).

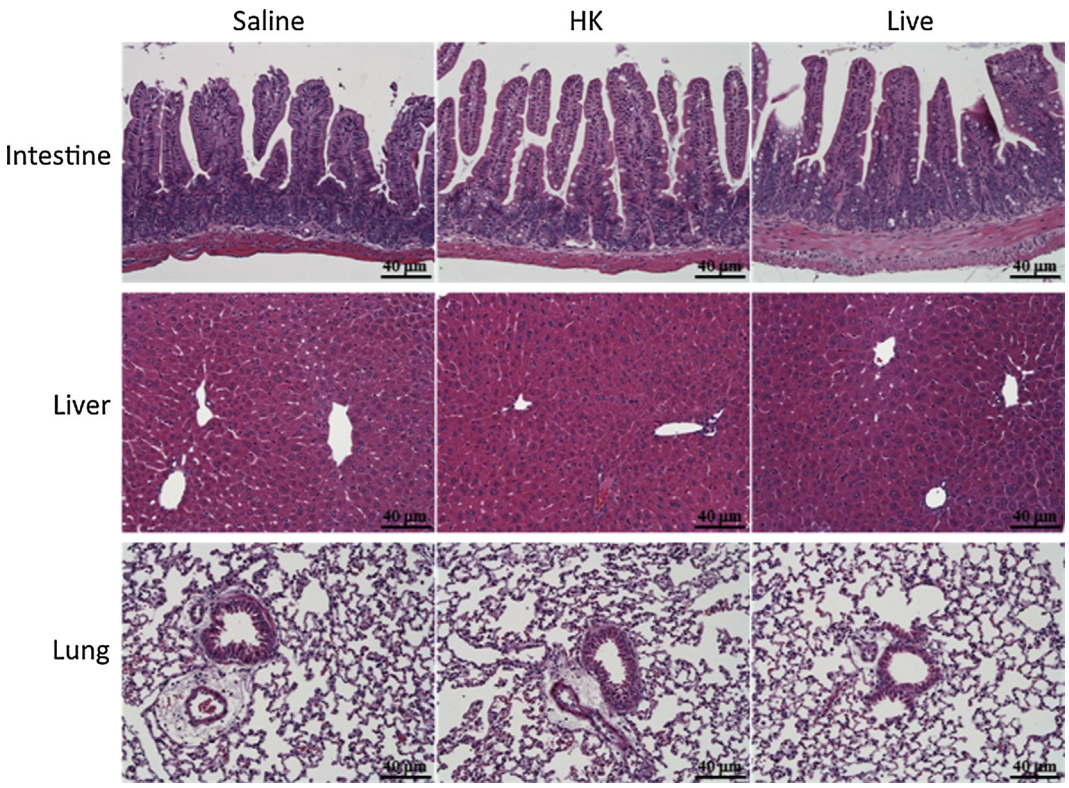

Fig. 2. PS128 has no significant effect on the organ histology of the GF mice.
Histological examinations of liver, lung, and intestine show no significant differences at the microscopic level among different treatment groups. There are no obvious adverse effects of chronic PS128 ingestion. HK, heat-killed PS128; Live, live PS128.

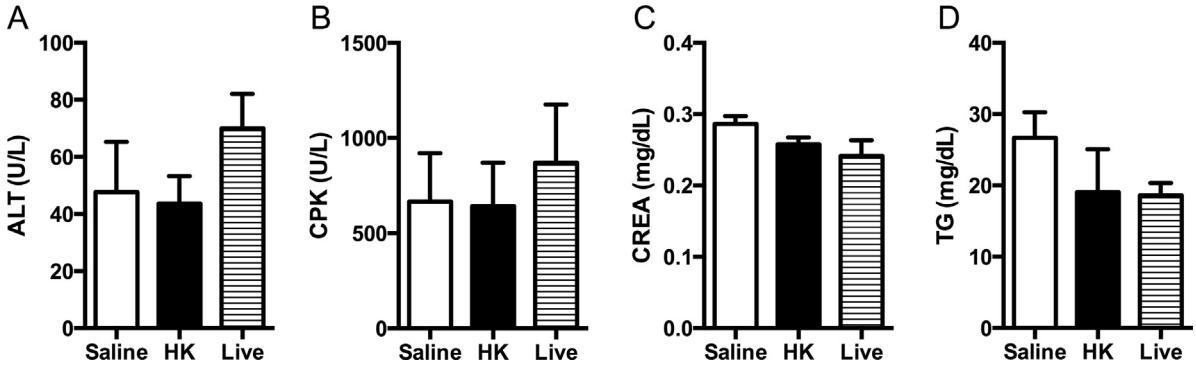

Fig. 3. PS128 has no significant effects on physiological functions of the GF mice.
The liver function, general metabolism, and kidney function are assessed by measuring the serum levels of ALT, CPK, GT, and CREA, respectively. There are no significant differences among different treatment groups. ALT, alanine aminotransferase; CPK, creatine phosphokinase; CREA, creatinine; TG, triglyceride. HK, heat-killed PS128; Live, live PS128.

These results suggested that daily oral administration of both live and heat-killed PS128 at dosages of 10^9 CFU had no toxic effects and was considered safe for the GF mice.

3.2. PS128 increased locomotor activity and reduced the anxiety-like behavior of the GF mice

To test the behavioral effects of chronic PS128 ingestion in GF mice, we first conducted OFT to examine their general activity. In the OFT we found that live PS128 administration significantly increased the total distance moved during the test period in comparison to that of the saline group, whereas the heat-killed PS128 group was not different from the saline group (Fig. 4A; $p < 0.05$). This activity increase also reflected on the increase in total moving time and reduced resting time (Fig 1B and C; $p < 0.01$). The time spent in the central area was not significantly different among different groups (Fig. 1D). We next used EPM test to assess the anxiety-like behaviors. In the EPM we did not see a clear increase in locomotor activity (Fig. 1E). However, in the live PS128 group the time spent in the closed arm was significantly reduced (Fig. 4F). The ratio of time spent in the open arm compared to the closed arm showed significant increase in the live PS128 group (Fig. 4G). In the heat-killed group the effects were not different from those of the saline group (Fig. 4E–H). Finally, we used FST to examine the depression-like helplessness behaviors in mice treated with heat-killed and live PS128. In this test we found no differences in the immobile time in both live and heat-killed PS128 groups compared to that of the saline-treated group (Fig. 4I). These results suggested that administration of live PS128 increased exploratory locomotor activity of the GF mice and reduced anxiety-like behaviors. The administration of

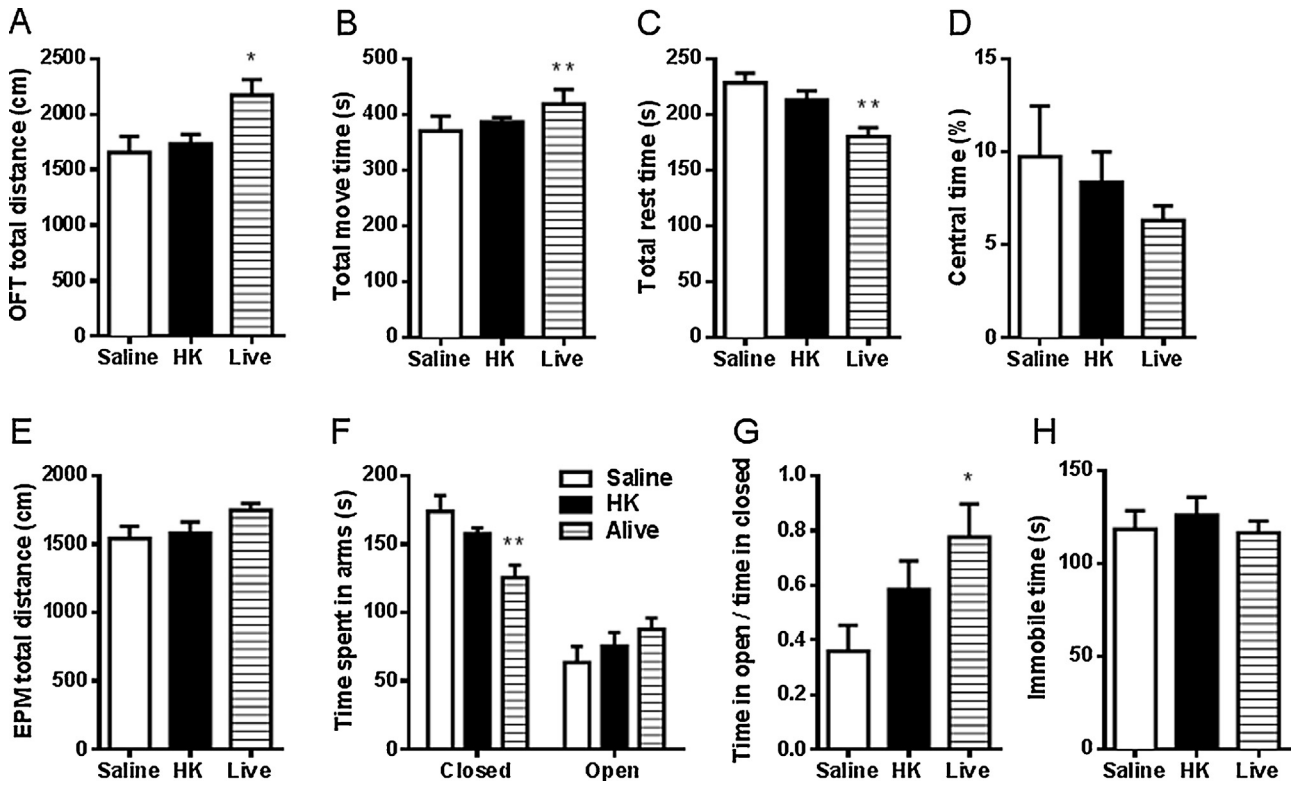

Fig. 4. Behavioral assessments of GF mice.
(A–C) Results of OFT show that live, but not heat-killed, PS128 administration increases locomotor activity of the GF mice as shown by the increase in total distance moved (A), total move time (B), and reduced total rest time (C). (D) Time spent in the center area in OFT is not different. (E–G) EPM test results. (E) There is no change in total distance moved in arms. (F) The time spent in the closed arms is significantly reduced in the live PS128 group. (G) The ratio of time spent in the open arm compared with time spent in the closed arm is significantly increased in GF mice treated with live PS128. (H) Results of FST show that the immobile time is not different in different treatment groups. HK, heat-killed PS128; Live, live PS128. $^{*}p < 0.05$; $^{**}p < 0.01$.

live PS128 reduced the time spent in the closed arms and increased the time spent in the open arms (Fig. 4F and G), which was similar to the results seen in the SPF mice (Supplementary data).

3.3. Live PS128 increased concentrations of DA, 5-HT, and their metabolites in the striatum

The behavioral tests indicated that PS128 treatment had an anxiolytic effect on the GF mice. We further explored the potential underlying mechanisms by examining neurochemical changes in the GF mice after chronic PS128 treatment. The concentrations of DA, 5-HT, and their metabolites were measured in brain samples taken from striatum, prefrontal cortex, and hippocampus. The results showed that live PS128, but not heat-killed PS128, significantly elevated DA, homovanillic acid (HVA), 5-HT, and 5-hydroxyindoleacetic acid (5-HIAA) concentrations in the striatum compared to that of the control group (Table 1; DA, $p < 0.05$; HVA, $p < 0.01$; 5-HT, $p < 0.001$; 5-HIAA, $p < 0.001$). However, we did not observe significant changes in the prefrontal cortex or the hippocampus. The DA and 5-HT turnover rates did not change in the brain areas tested (Table 1). These results indicated that treatment by live PS128 increased DA and 5-HT concentrations, specifically in the striatum area, which could potentially affect the locomotor and anxiety-like behaviors.

3.4. PS128 has no effect on the HPA axis

We further examined the HPA axis function of PS128-treated GF mice by measuring serum glucocorticoid concentrations in blood samples collected 30 min after swim stress (FST). The results showed that the concentrations of glucocorticoid were not different among different treatment groups, indicating the live PS128 treatment had no effect on the HPA axis function in the GF mice (Fig. 5).

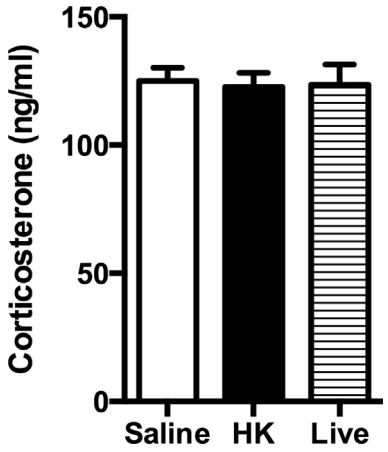

Fig. 5. PS128 has no effect on the HPA axis.
Blood samples collected 30 min after the second day FST test show that the serum CORT level increases due to the swim stress is not different among the different treatment groups. HK, heat-killed PS128; Live, live PS128.

4. Discussion

We used the GF mouse model to study the impact of chronic administration of PS128 on the anxiety-like and depression-like behaviors and the corresponding monoamine neurotransmitter changes in related brain regions. We first examined the safety con-

Table 1
Alteration of neurochemicals by PS128.

	Striatum			Prefrontal cortex			Hippocampus		
	Saline	HK	Live	Saline	HK	Live	Saline	HK	Live
Monoamines and metabolites									
DA	8265 ± 2003	7906 ± 1904	10,809 ± 1281*	77.9 ± 19	74.9 ± 17	74.6 ± 26	40.3 ± 13	35.7 ± 8.7	50.6 ± 19
DOPAC	367 ± 58	385 ± 95	448 ± 51	31.6 ± 5.6	39.9 ± 8.7	39.0 ± 9.5	24.6 ± 7.1	18.2 ± 3.2	24.8 ± 4.4
HVA	658 ± 112	642 ± 76	848 ± 81**	170 ± 24	172 ± 23	187 ± 21	93.5 ± 15	79.8 ± 11	87.8 ± 6.7
5-HT	424 ± 94	453 ± 58	659 ± 79***	415 ± 44	468 ± 25	458 ± 65	422 ± 104	441 ± 27	467 ± 56
5-HIAA	238 ± 33	242 ± 24	370 ± 20***	175 ± 34	180 ± 20	197 ± 25	352 ± 88	305 ± 25	363 ± 43
Turnover ratio									
DOPAC:DA	0.0405 ± 0.0065	0.0479 ± 0.0072	0.0418 ± 0.0030	0.454 ± 0.11	0.508 ± 0.14	0.563 ± 0.15	0.490 ± 0.081	0.523 ± 0.065	0.501 ± 0.10
HVA:DA	0.0775 ± 0.0033	0.0836 ± 0.016	0.0793 ± 0.0062	2.30 ± 0.69	2.50 ± 0.45	2.67 ± 0.70	2.21 ± 0.63	2.15 ± 0.27	1.89 ± 0.54
5-HIAA:5-HT	0.563 ± 0.080	0.562 ± 0.099	0.560 ± 0.032	0.425 ± 0.11	0.383 ± 0.055	0.455 ± 0.076	0.753 ± 0.12	0.699 ± 0.032	0.773 ± 0.10

Statistically significant values are highlighted in grey. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared to the saline group. DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, 5-hydroxytryptamine or serotonin; HVA, homovanillic acid, HK, heat-killed PS128; Live, live PS128.

cerns of chronic administration of PS128. In the current study, 16-day administration of live or heat-killed PS128 did not cause death or produce any clinical signs of toxicity. There was no significant change in the body weight or in the histology of various excised organ tissue samples (Figs. 1 and 2). The serum concentration of several biochemical markers for accessing the general metabolism and functions of liver and kidney was also within the normal range in the live PS128 group compared to that of controls (Fig. 3). This chronic consumption safety is similar to that of a variety of *Lactobacillus* species reported previously, including *Lactobacillus acidophilus*, *Lactobacillus pentosus*, *L. plantarum*, and *Lactobacillus reuteri* [16–18]. Interestingly, we found a significant reduction in the cecum weight in the live PS128 group versus the other groups (Fig. 1B). It is well-known that gut microbiota can modulate various host metabolic reactions, including regulating the production of different metabolites such as bile acids, choline, and short-chain fatty acids [19]. In addition, complex carbohydrates such as dietary fibers can be digested in the colon by gut microorganisms and subsequently fermented into short-chain fatty acids such as *n*-butyrate, acetate, and propionate; these compounds are known to have neuro-active properties [2]. We speculate that the changes in gut metabolites produced by live PS128 might be responsible for the reduced cecum weight in the PS128 group.

In the behavioral tests aimed at examining the general activity and anxiety-like and depression-like behaviors, we found chronic administration of live PS128 significantly increased the locomotor activity and reduced anxiety-like behaviors of GF mice (Fig. 4). Recent studies using GF mice had shown that GF mice exhibited increased locomotor activity in OFT and reduced anxiety-like behaviors in the EPM and light–dark box test relative to specific pathogen-free mice [9–11]. Apparently GF conditions alone could produce changes in motor and anxiety-like behaviors. In the present study we demonstrated that chronic live PS128 ingestion also enhances motor activity in GF mice compared to saline-treated GF mice, suggesting this bacterium further enhances the motor activity in GF mice. However, one weakness of our study was that we did not conduct similar tests using SPF mice. Thus, the degree of enhancement is difficult to compare with other studies. Nevertheless, there is a clear increase in the locomotor activity in GF mice after live PS128 treatment, and this enhancement might be related to our observed increases of DA contents in the striatum (Table 1).

It is well-documented that the administration of DA or its analogs into the striatum or nucleus accumbens could stimulate locomotor activity in animals [20,21]. Therefore, the enhanced locomotor activity could be attributed to the enhancement in striatal DA neurotransmission. Past studies have demonstrated that GF mice exhibited increased DA concentration and elevated DA turnover rate in the striatum compared to SPF mice [9]. Thus, the effects of chronic live PS128 ingestion on locomotor activity we observed might be the additional change over an already enhanced DA concentration and turnover. Nevertheless, because we did not observe changes in DA turnover rate between GF mice treated with saline and those treated with live PS128, the effects of PS128 seem to be a direct increase in the concentration of DA in striatum (Table 1). Again, we would need to conduct experiments using SPF mice to clarify the effects on DA transmission caused by the GF condition alone. Although the gut microbiome can generate many neurotransmitters and neuromodulators, for example, *Lactobacillus* spp. and *Bifidobacterium* spp. produce GABA, *Candida* spp., *Streptococcus* spp., *Escherichia* spp., and *Enterococcus* spp. produce 5-HT, and *Bacillus* spp. may produce DA in the host intestine [2], we think the mechanism underlying the increased striatum DA is not due to the direst production of DA in the gut because DA cannot freely pass the blood–brain barrier to enter the brain. In addition, the increase in DA level is only seen in the striatum; it is restricted in the nigrostriatal pathway of DA transmission. Interestingly, it had been demonstrated that ingestion of *L. rhamnosus* could regulate emotional behavior via the vagus nerve [4]. Chronic impairment of the vagus nerve function leads to reduction of DA activity in the striatum [22]. Thus, we speculate that the increase in striatum DA concentration we observed is also mediated via the vagus nerve activity. This hypothesis awaits further study to substantiate the theory.

The GF mice in the live PS128 group showed significantly reduced time spent in the closed arms, and the ratio of the open arm compared to the closed arm is also increased in the EPM test, suggesting an anxiolytics effect of PS128 (Fig. 4). In addition to locomotion, altered neurotransmission in the striatum is also implicated in anxiety-like behavior [9]. Our data have shown that in addition to DA, the levels of striatal 5-HT and its metabolite 5-HIAA were also significantly increased in the GF mice treated with live PS128 (Table 1). The serotonergic system has long been linked to anxiety, and the reduction in 5-HT transmission is associated with anxiety [23]. The increase in 5-HT and its metabolite could contribute to the reduction of anxiety-like behavior in the EPM (Fig. 4E and F). However, in our study the striatal DA is also significantly increased; therefore, it is also possible that both DA and 5-HT contribute to the reduced anxiety-like phenotype. Several complex interactions between DA and 5-HT have been described in the striatum [24]. For example, in vivo studies have shown that striatal 5-HT1B receptors facilitate nigrostriatal DA release [25–28]. Human imaging studies also demonstrate that 5-HT2A and 5-HT1A receptor agonist psilocybin increases activity in the nigrostriatal DA pathway [29]. Thus, the effects of PS128 on the nigrostriatal pathway might be the result of multiple regulations and require further study to clarify.

Elevated HPA axis activity is a well-known effect of the absence of gut microbiota [2]. Also, a previous study indicated that while

the absence of normal gut microbiota caused defects in HPA axis coordination, early colonization of commensal or beneficial bacteria could reverse these defects and improve HPA axis function [13]. Moreover, a full reversal in the adult offspring only occurred when their GF mothers were inoculated with specific bacterial strains before giving birth [13]. In our study, however, we found that neither live nor heat-killed PS128 affected the serum corticosterone, indicating that PS128 has no effect on the HPA axis (Fig. 5). It is possible that the lack of effects of live PS128 on corticosterone level is due to the relatively late administration timing (i.e., at the age of 6 weeks) used in this study.

Previous studies have shown that administration of live *L. reuteri* for 9 days could prevent the visceral pain induced by colorectal distension, and dead *L. reuteri* also showed a similar effect [30]. In addition, the surface exopolysaccharide of Bifidobacteriales has been shown to facilitate commensal–host interactions through host immune modulation [31]. These studies imply that the structural components of probiotics may also play an important role in regulating the physiology of the host. In the present study, the administration of heat-killed PS128 did not show any obvious physical, behavioral, or neurochemical changes in the GF mice, suggesting the effects of PS128 were not mediated by the structural components of the probiotic.

5. Conclusions

In summary, the present study demonstrates that live PS128 is safe for chronic ingestion in GF mice. The chronic PS128 ingestion causes an increase in locomotor behavior and a reduction in anxiety-like behaviors. Also, the DA and 5-HT concentrations in the striatum are increased in the GF mice treated with live P128. These findings indicate that through the GBA, PS128 has the potential to serve as an anxiolytic agent to regulate the motor functions and the mood of the host.

Author contributions

WSL, HLC, CCW, and YCT designed the study. WSL, HLC, CCW, and GTC performed the experiments. WSL, SW, and HLC analyzed and interpreted the data. WSL, HLC, CCW, SW, and YCT wrote the manuscript.

Competing financial interests

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbr.2015.10.046.

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