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Research report

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

Wei-Hsien Liu[a], Hsiao-Li Chuang[b], Yen-Te Huang[b], Chien-Chen Wu[a], Geng-Ting Chou[a], Sabrina Wang[d,**], Ying-Chieh Tsai[a,c,*]

[a] Institute of Biochemistry and Molecular Biology, National Yang-Ming University, Taipei 11221, Taiwan
[b] National Laboratory Animal Center, National Applied Research Laboratories, Taipei 115, Taiwan
[c] Probiotic Research Center, National Yang-Ming University Business Center of Industry-Academia Liaison, Taipei 11221, Taiwan
[d] Institute of Anatomy and Cell Biology, National Yang-Ming University, 155, Section 2, Linong Street, Taipei 11221, Taiwan

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.

* Corresponding author at: Institute of Biochemistry and Molecular Biology, National Yang-Ming University, 155, Section 2, Linong Street, Taipei 11221, Taiwan. Fax: +886 228264843.
** Corresponding author.
E-mail addresses: sabrina@ym.edu.tw (S. Wang), tsaiyc@ym.edu.tw (Y.-C. Tsai).

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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