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Anti-stress effect of the *Lactobacillus pentosus* strain S-PT84 in mice

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We investigated if the orally administered *Lactobacillus pentosus* strain S-PT84 (S-PT84) might show anti-stress activity and ameliorate stress-induced immune suppression in mice. Stress of mice induced an increase in serum corticosterone and a decrease in splenic natural killer activity and in the number of splenocytes versus control mice. However, these changes were not observed in stressed mice that had been administered S-PT84. Furthermore, interleukin (IL)-12 and IL-10 production, which was downregulated in lipopolysaccharide-activated macrophages from stressed mice, was maintained at control levels in the macrophages of stressed mice that had been fed S-PT84. Interferon-γ production, which was downregulated in concanavalin A-activated splenocytes from stressed mice, tended to be maintained at control levels in stressed mice that had been fed S-PT84, although IL-4 production by these cells was not influenced by S-PT84 administration. Additionally, reduced glutathione (GSH) levels were decreased in serum and peritoneal macrophages from stressed mice versus controls, but these GSH levels were significantly higher in stressed animals that had been administered S-PT84 compared with those that had not. These results suggest that S-PT84 exerts anti-stress activity through immune modulation and/or antioxidative activity.

Key words: *Lactobacillus pentosus* strain S-PT84, stress-induced immune suppression, anti-stress, reduced glutathione (GSH)

INTRODUCTION

In modern society, people can be exposed to stress throughout their lifetime. Much research over recent decades has suggested that stress may be associated with the onset, course, and outcome of physical illness [1–3]. Furthermore, many studies have suggested that stress-induced immune suppression increases the risk of infections. Of note, Japan is becoming a super-aged society more rapidly compared with other countries, which is forcing many elderly people to work in spite of the fact that their immunity is compromised due to their age [4, 5]. In this situation, many people may be exposed to stress.

In general, it is well known that stress affects the immune system. Immune functions such as natural killer (NK) activity, phagocytosis [6], and NK T activity [7] are reported to be suppressed by restraint stress. Furthermore, skewing of the T helper type 1 (Th1)/Th2 balance towards Th2-dominant immunity has been reported to be induced by stress [8]. These physiological responses are induced by the release of corticosteroid hormones via the hypothalamic-pituitary-adrenal axis, and high levels of glucocorticoid cause apoptosis and necrosis in immature T and B cells [9, 10].

Stress also induces redox imbalance associated with reactive oxygen spices (ROS) production via numerous cellular cascades [11–13]. Gostner *et al.* [14] reviewed the key functions that ROS and other redox-active molecules fulfill in immunity. Stress is now considered as an important modulator of the immune system [15–17] and a major cause of increased risk for immune-related diseases, such as inflammatory diseases, infection, and cancer [18–20]. It is therefore important to prevent immune suppression or disturbance caused by stress.

The intake of functional foods over many years does not cure a disease but can maintain human health, and there will be a greater demand for such foods in the future. Some ingredients or components derived from food materials that show anti-stress activity have been reported. Kumquat pericarp improves both the

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suppressed plasma interferon (IFN)-γ and NK activity per splenocyte in stressed mice, and the active compound is considered to be β-cryptoxanthin [21]. Myricetin, which is a flavonol that is widely present in daily food, attenuates the depressant-like behaviors observed in mice exposed to repeated stress [22]. The probiotic bacterial strain *Lactobacillus rhamnosus* Lcr35 produces an anti-hypersensitivity activity in a restraint stress model [23]. As described above, the anti-stress effect of some food ingredients or components such as lactic acid bacteria (LAB) are becoming clear; however, it is remains unclear whether LAB can ameliorate immunological activity that is suppressed by stress.

Some LAB that are ingredients of functional foods can modulate immune responses. The mechanism by which LAB regulate immune responses is becoming increasingly clear due to the large number of studies on this subject, and application of LAB to improve human health has also been shown [24–26]. We previously found that the *Lactobacillus pentosus* strain S-PT84 (S-PT84) has the strongest interleukin (IL)-12-inducing activity among a variety of LAB isolated from Kyoto pickles [27]. Heat-killed S-PT84 modulates the Th1/Th2 balance toward Th1 dominance, exerts an anti-allergy effect [27], activates NK and NK T (NKT) cells via Toll-like receptor 2/4 pathways [28], and exerts an anti-viral infection effect through induction of IL-12, IFN-γ, and IFN-α production [29, 30].

In this study, we tested the hypothesis that S-PT84, which has potent immunomodulatory activity, might be effective for ameliorating stress-induced immune depression. The stress used in this study was restraint stress of mice, which was used as a physical stress. Corticosterone levels in serum were measured as a marker of stress. Splenic NK activity, IL-12 and IL-10 production by macrophages, and IFN-γ and IL-4 production by splenocytes were measured as parameters of immune activity. Reduced glutathione (GSH) levels in serum and macrophages were also measured as indicators of oxidative damage. We found that S-PT84 has an anti-stress activity through an immune modulating and/or antioxidative activity.

MATERIALS AND METHODS

Preparation of heat-killed LAB

The *L. pentosus* strain S-PT84 was cultured in deMan, Rogosa and Sharpe (MRS) broth (Difco Laboratories, Detroit, MI, USA) at 35°C for 48 hr. Cultured bacteria were collected by centrifugation, washed twice with sterile saline, and heat killed at 95°C for 5 min. Heat-killed bacteria were washed with distilled water, freeze-dried, and used in the following experiments.

Animals and treatments

Male C57BL/6 mice (7 weeks of age) were purchased from Charles River Laboratories Japan, Inc. (Shizuoka, Japan). These animals were housed at a temperature of 25 ± 1°C and humidity of 60 ± 5% under a 12 hr light-dark cycle and were provided with a commercial diet (CE-2) and tap water *ad libitum* for 1 week before experimentation. S-PT84 (final dose: 0.075%) was added to an AIN-93 M diet (maintenance formulation) (Oriental Yeast Co., Ltd., Tokyo, Japan), which was then given to these mice. The control group was given the AIN-93 M diet alone. Mice were fed the AIN-93 M diet with or without 0.075% S-PT84 for 1 week and were then restrained for 17 hr according to the methods of Iwakabe *et al.* [8]. The control mice were left in a cage for 17 hr without food or water. Two milliliters of 4.05% thioglycollate medium (Difco) were injected intraperitoneally into mice at the time of peritoneal macrophage collection 4 days before in vitro assay. All experiments were approved by the Animal Care and Use Committee of Suntory Holdings, Ltd. (Osaka, Japan), and the Guidelines for Animal Care and Use of Suntory Holdings, Ltd., were followed. The procedures were such that the animals did not suffer unnecessarily.

Measurement of corticosterone

The serum corticosterone level was determined using a Corticosterone ELISA Kit for Rats and Mice (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's instructions.

Measurement of NK activity [27]

Yac-1 cells, which are target cells of NK cells, were maintained in RPMI-1640 (Nacalai Tesque, Kyoto, Japan). For assay, the cells were suspended in phosphate-buffered saline (PBS, Nissui Pharmaceutical Co., Tokyo, Japan) (1×10^6 cells/ml) containing 40 μg/ml of 3, 3′- dioctadecyloxacarbocyanine perchlorate (DiO; Sigma, St. Louis, MO, USA) and were incubated at 37°C for 10 min. The stained cells were then washed with RPMI-1640 and resuspended in 10% fetal bovine serum (FBS)-containing RPMI 1640 (5×10^4 cells/ml). The DiO-stained target cells (100 μl), splenocytes at various concentrations (100 μl), and 25 μg/ml of propidium iodide (PI; Molecular Probes, Eugene, OR, USA) were added to 96-well round-bottomed microplates (BD Biosciences, Franklin Lakes, NJ, USA) that were then centrifuged at 200 × *g* and cultured at 37°C in 5% CO_2 for 4 hr. The rate

of spontaneous cell death was determined by culturing the target cells alone without adding effector cells. After the reaction, the DiO^+PI^+ cells (dead target cells) were counted by using an Epics XL flow cytometer (Beckman Coulter). NK activity was calculated according to the following formula:

NK activity (%) = $(DiO^+PI^+) / (DiO^+PI^- + DiO^+PI^+) \times 100$ – rate of spontaneous cell death (%).

Ex vivo assessment of cytokines

Peritoneal macrophages (2×10^6 cells/ml) that were collected as described above and splenocytes (5×10^6 cells/ml) were each cultured in RPMI-1640 medium (Nacalai Tesque, Kyoto, Japan) containing 10% FBS in 24-well microplates (BD Biosciences). Macrophages were cultured with lipopolysaccharide (LPS, from *Escherichia coli* 055; 100 ng/ml; Wako Pure Chemical industries, Osaka, Japan) for 48 hr, and splenocytes were cultured with Concanavalin A (Con A; 2.5 µg/ml, Wako) for 24 hr at 37°C. The culture supernatant was collected, and IL-12 (p70) and IL-10 production by macrophages and IFN-γ and IL-4 production by splenocytes were measured by using an OptEIA ELISA kit (BD Pharmingen, San Diego, CA, USA).

Measurement of reduced GSH level

Serum samples to be used for GSH measurement were prepared by adding 5-sulfosalicylic acid (5%) to them and then centrifuging them at 8,000 × g for 10 min; their supernatants were used for analysis. For measurement of the GSH level of peritoneal macrophages, 80 µl of 10 mM HCl were added to the macrophages (1×10^6 cells), which were then frozen and defrosted three times. Subsequently, 20 µl of 5% 5-sulfosalicylic acid were added, and the supernatants were collected after centrifugation of the samples (8,000 × g for 10 min). Serum and macrophage GSH levels were measured by using a total Glutathione Quantification Kit (Dojindo Laboratories, Kumamoto, Japan).

Statistical analysis

Data are presented as means ± SE. The significance of differences in values was tested by one-way ANOVA, followed by Tukey's test as a *post hoc* test. Values of $p<0.05$ were considered to be statistically significant.

RESULTS

Anti-stress effect of the L. pentosus strain S-PT84

Serum corticosterone levels were measured as an indicator of the stress levels of the mice. The serum corticosterone level was increased by restrain stress, and S-PT84 administration significantly ameliorated this stress-induced increase in corticosterone (Fig. 1).

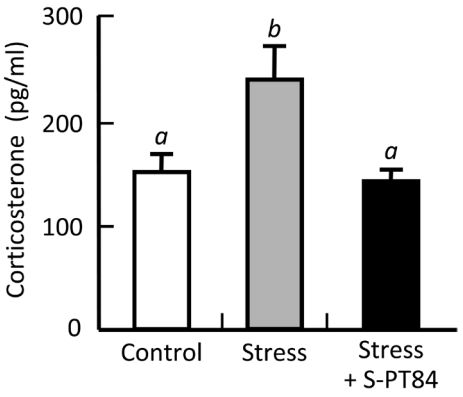

Fig. 1. Effect of oral administration of the *L. pentosus* strain S-PT84 on stress-induced changes in corticosterone in C57BL/6 mice.

Corticosterone levels in the sera of mice that were fed an AIN-93 M diet with or without S-PT84 for 1 week and then restrained were measured as described in MATERIALS AND METHODS. Data are presented as the mean ± standard error (SE) of five mice. Different letters (*a* and *b*) indicate significant differences between groups ($p<0.05$).

Effect of S-PT84 administration on stress-induced immune suppression

The effect of S-PT84 administration on restraint stress-induced effects on splenic immune function was then investigated by assay of the number of splenocytes, of splenic NK activity, and of splenocyte Con A-induced IFN-γ and IL-4 production levels. The number of splenocytes was reduced by stress, but this reduction was not as great in stressed mice that had been fed S-PT84 (Fig. 2A). Splenic NK activity against Yac-1 target cells was significantly downregulated by stress, but this downregulation was ameliorated by S-PT84 feeding (Fig. 2B). IFN-γ production from Con A-activated splenocytes was reduced in stressed mice versus controls, but this reduction was not as great in stressed mice that had been fed S-PT84 (Fig. 2C). S-PT84 administration did not significantly change the stress-induced reduction in Con A-induced splenocyte IL-4 production (Fig. 2D).

To investigate the effect of S-PT84 administration on stress-induced effects on innate immune activity, we investigated IL-12 and IL-10 production by LPS-activated peritoneal macrophages isolated from these mice. Both IL-12 and IL-10 production by LPS-activated macrophages were downregulated by stress, and the decrease in IL-12 production was much greater than that

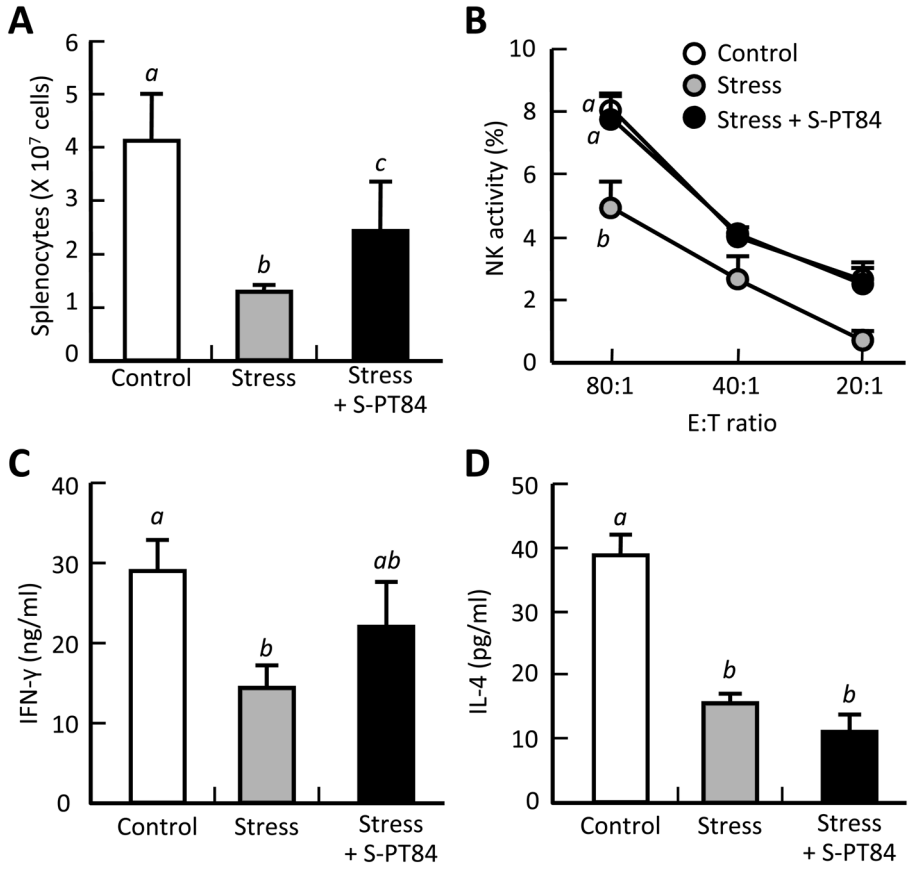

Fig. 2. Effect of oral administration of the *L. pentosus* strain S-PT84 on stress-induced immune compromisation of splenocytes.

Splenocytes were collected from mice that were fed an AIN-93 M diet with or without S-PT84 for 1 week and then restrained. The number of splenocytes (A), splenic NK activity against target YAC-1 cells (effector:target ratio = 80:1, 40:1, and 20:1) (B), and IFN-γ (C) and IL-4 (D) production levels in splenocytes cultured with Con A (2.5 μg/ml) for 24 hr were measured as described in MATERIALS AND METHODS. Data are presented as the mean ± SE of five mice. Different letters (*a*, *b* and *c*) indicate significant differences between groups ($p<0.05$).

in IL-10 production (Fig. 3A and B, respectively). In the S-PT84-fed mice, there was no stress-induced decrease in cytokine production by activated macrophages, and cytokine production was maintained at control levels.

Effect of S-PT84 feeding on stress-induced changes in GSH levels in serum and macrophages

To determine the oxidative stress induced by stress, GSH levels in serum and macrophages were measured. The serum GSH level was decreased by stress, but this decrease was not as great in stressed S-PT84-fed mice compared with mice that had not been fed S-PT84 (Fig. 4A). The GSH level in peritoneal macrophages was also significantly decreased by restraint stress; however, in the S-PT84-fed group, instead of decreasing with stress, the GSH level was higher than the control level (Fig. 4B).

DISCUSSION

Stress is a modern social problem for people, and it is therefore important to control both the mind and the body in order to overcome stress. One of the problems caused by stress is immune suppression [15–17]. In the present study, we examined if oral administration of S-PT84, which has been shown to have immune function-enhancing activity, particularly activity that enhances Th1 immunity [27–30], could ameliorate stress-induced immune suppression. Administration of S-PT84 administration to mice suppressed restraint stress-induced corticosterone elevation (Fig. 1). We consider that this

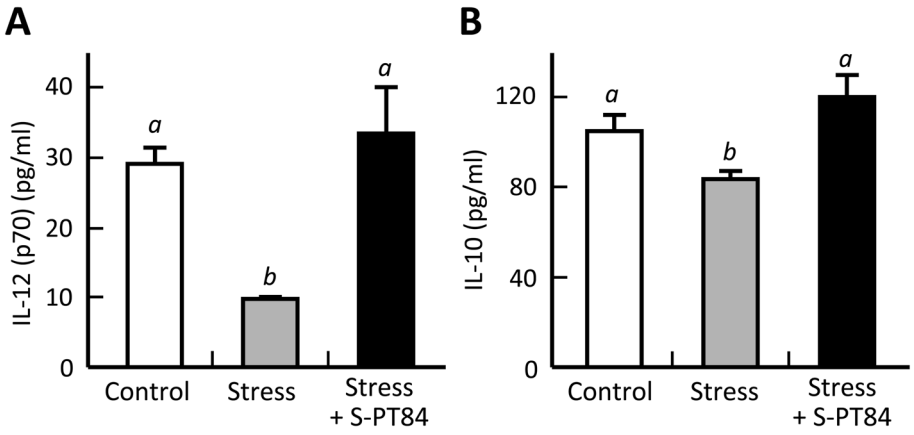

Fig. 3. Effect of oral administration of the *L. pentosus* strain S-PT84 on IL-12 and IL-10 production by macrophages in stressed mice.

Macrophages were collected from mice that were fed an AIN-93 M diet with or without S-PT84 for 1 week and then restrained. The cells were then cultured with LPS (100 ng/ml) for 48 hr. IL-12 (p70) (A) and IL-10 (B) levels in the collected culture supernatants were measured. Data are presented as the mean ± SE of five mice. Different letters (*a* and *b*) indicate significant differences between groups ($p<0.05$).

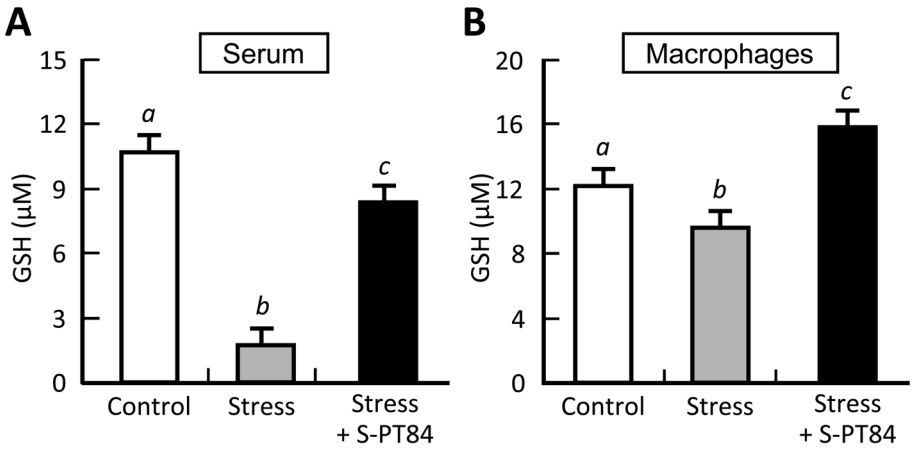

Fig. 4. Effect of oral administration of the *L. pentosus* strain S-PT84 on stress-induced down regulation of GSH in serum and peritoneal macrophages of the mice.

Serum and peritoneal macrophages were collected from mice that were fed an AIN-93 M diet with or without S-PT84 for 1 week and then restrained. The GSH level in serum (A) and macrophages (B) was measured as described in MATERIALS AND METHODS. Data are presented as the mean ± SE of five mice. Different letters (*a*, *b* and *c*) indicate significant differences between groups ($p<0.05$).

effect of S-PT84 is due to immune modulating (Figs. 2 and 3) and antioxidative (Fig. 4) activities. Oral administration of S-PT84 ameliorated the stress-induced decrease in splenic NK activity (Fig. 2B), and we consider that this effect of S-PT84 is due to the higher splenic IFN-γ/IL-4 ratio in S-PT84-administered stressed mice than that in stressed non-S-PT84-fed mice (Fig. 2C and 2D).

The stressutilized in this paper was applied according to the design described in a previous report by Iwakabe *et al.* [8]. That previous report indicated thatapplication of restraint stress to mice significantly decreased Th1 immunity but did not significantly change Th2 immunity. Our results differed from those of this previous report in that we found that such stress decreased both Th1 (IFN-γ) and Th2 (IL-4) cytokine production by Con A-activated splenocytes by more than 50% (Fig. 2C and 2D).

However, although the reasons for the different results between our study and that previous study are unclear, we consider that our results suggest that the stress model that we established is a working model that can be used for examination of stress-induced immune suppression. In the present study, S-PT84 feeding ameliorated all parameters of stress-induced immune suppression that were measured in this stress model, except for the stress-induced decrease in production of IL-4, a Th2 cytokine. S-PT84 is a LAB that was identified in a screening in which the intensity of IL-12 induction in macrophages and some immunomodulating effects *in vivo* were confirmed [27]. Therefore, IL-12-inducing activity might be one of the mechanisms by which S-PT84 ameliorated stress-induced immune suppression.

Many studies have demonstrated that the Th1/Th2 balance is closely related to the reduced GSH level within antigen-presenting cells such as macrophages and that a GSH regulates IL-12 production [31–34]. Stress induces oxidative stress and decreases the GSH level in some organisms and cells, which causes damage to cells such as leukocytes [35] and hepatocytes [36], as well as skin damage [37]. Therefore, the much larger decrease in the production of IL-12 induced by stress (Fig. 3) as compared with that in IL-10 in LPS-activated macrophages might be caused by a restraint stress-induced GSH decrease. Maintenance of the IL-12-producing ability of macrophages in stressed mice by feeding them S-PT84 was partially mediated by S-PT84-induced negation of the stress-induced GSH decrease (Fig. 4). Goyal *et al.* reported that orally administered *L. rhamnosus* GG increased the levels of endogenous antioxidants (*e.g.*, GSH) against oxidative stress caused by *Giardia intestinalis* in the intestine [38]. *L. salivarius* increased the amount of GSH in the liver of an LPS-treated mouse [39]. In addition, liver damage induced in a chronic alcohol-fed mouse was prevented by *L. rhamnosus* through an effect on endogenous antioxidants such as GSH and superoxide dismutase [40]. These reports support the notion that S-PT84 negated the stress-induced GSH decrease; however, the exact mechanism by which LAB increase GSH remains to be elucidated. As far as we know, there has been only one report regarding this mechanism, in which *L. plantarum*, a member of the same genus as *L. pentosus*, was reported to increase plasma GSH levels by activating the protein kinase C pathway [41]. However, little is known regarding how LAB affect not only plasma GSH but also immunocompetent cells in the body after their oral intake. These mechanisms as well as absorption and/or distribution of LAB should be further investigated to elucidate the immunomodulatory effects of LAB.

The stress response is very complicated. In general, stress enhances sympathetic nerve activity and induces glucocorticoid production. Increased sympathetic nerve activity decreases NK activity [42, 43]. We recently demonstrated that oral administration of S-PT84 suppresses sympathetic nerve activity in the mouse spleen [44]. We therefore consider that one of the mechanisms behind the immunomodulatory effects of S-PT84, such as enhancement of NK activity, involves sympathetic nerve activity. On the other hand, glucocorticoids also lower immune functions [9, 10, 45]. Several reports concluded that restraint stress-induced changes in immune functions were caused by an increased blood glucocorticoid level [46]. A recent study showed that induction of corticosterone production under stress is due to c-FOS induction [47], which is followed by oxidative damage as shown by the presence of reactive oxygen species, lipid peroxidation, and a decrease in the GSH/GSSG ratio [48]. Tran *et al.* also reported that exposure to far-infrared rays protects against a stress-induced increase in c-FOS induction, oxidative burdens, and serum corticosterone level via induction of glutathione peroxidase [48]. However, in the present study, despite the fact that the corticosterone level was suppressed (Fig. 1) and NK activity (Fig. 2B) and IFN-γ inducible activity (Fig. 2C) were kept at control levels by S-PT84, the number of splenocytes was increased and was not kept at the control level (Fig. 2A). Thus, although it is considered that one mechanism of S-PT84 amelioration of stress-induced immune suppression might be by S-PT84 induction of the cellular redox system, the existence of another mechanism is implied by our data. To the best of our knowledge, no report has suggested that LAB directly regulate glucocorticoid production. In the present study, S-PT84 ameliorated restraint stress-induced corticosterone elevation and immune suppression, supporting the above potential mechanism of S-PT84 effects. Details regarding such a mechanism should be clarified in future studies.

In conclusion, *L. pentosus* strain S-PT84 showed anti-restraint stress activity and, in particular, ameliorated stress-induced suppression of the immune system. The mechanism of amelioration mechanism involved may be associated with S-PT84 induction of GSH in serum and macrophages. The detailed mechanisms by which LAB affect cellular redox or autonomic nerves and by which LAB compromise the immune system need to be examined in future studies.

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