— Rapid Communication —

Comparison of the Immunomodulatory Effects of Live and Heat-killed *Lactobacillus pentosus* S-PT84

Takayuki Izumo,* Masayuki Ida, Toshihiro Maekawa, Yuichi Furukawa, Yoshinori Kitagawa, and Yoshinobu Kiso

Institute for Health Care Science, Suntory Wellness Limited, 1–1–1 Wakayamadai, Shimamoto-cho, Mishima-gun, Osaka 618–8503, Japan

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Lactobacillus pentosus S-PT84, which was isolated from Kyoto pickles, enhances splenic natural killer (NK) cell activity, increases IgA production from Peyer's patch cells and has high T-helper 1 (Th1) cytokine and type 1-interferon-inducing activity. However, all of these effects were obtained by using heat-killed S-PT84. In the present study, we investigated the immunomodulatory effect of live and heat-killed S-PT84 in BALB/c mice. Unexpectedly, live S-PT84 indicated weak activity on Th1 cytokine (interleukin-12 or interferon-γ) production from splenocytes compared to heat-killed S-PT84 *in vitro*. Similar tendencies were also shown in S-PT84 having different cell wall thickness. Next, we compared the immunomodulatory effect after oral ingestion of each S-PT84. The results indicated splenic NK activity was equally enhanced by live or heat-killed S-PT84 ingestion, though heat-killed S-PT84 indicated slightly higher activity in interferon-γ production and inclination toward Th1 immunity. These results suggested that both live and heat-killed S-PT84 have an equal immunomodulatory effect in BALB/c mice. It is thought that the use of heat-killed S-PT84 is advantageous from the viewpoint of ease of handling compared to live S-PT84.

Key words —— *Lactobacillus pentosus* S-PT84, natural killer activity, interleukin-12, interferon-γ, interleukin-4

INTRODUCTION

Probiotics are usually defined as "live non-pathogenic microorganisms, which when ingested in adequate amounts confer health benefits to the host".[1] There are many reports that probiotics are effective in the maintenance of health or in the improvement of several diseases.[2,3] Isolauri reported that probiotics reinforced the different lines of gut defense, which are immune exclusion, immune elimination, and immune regulation.[4] Furthermore, Blum *et al.* cited several reports that provided molecular evidence of the beneficial effect of probiotics on intestinal immune homeostasis.[5] Therefore, probiotic-mediated immunomodulatory alterations are important beneficial effects in animals or humans.

A number of reports have shown that various beneficial effects were obtained by heat-killed or inactive lactic acid bacteria.[6,7] Neumann *et al.* reported that interleukin-12 (IL-12) or interferon-gamma (IFN-γ) production from mouse peritoneal cells or splenocytes was equally induced by live *Lactobacillus delbrueckii* and dead bacteria.[8] Recently, Adams reported that the use of dead probiotics as biological response modifiers has several attractive advantages compared to live probiotics.[9] Ueno *et al.* reported that heat-killed bacteria could maintain intestinal homeostasis and improve intestinal disorders without the need for live probiotics.[10] However, in a comparison of the immunomodulatory effect between live bacteria and dead bacteria, most reports demonstrated that live bacteria were more effective.[11–15] Therefore, it is generally well recognized that the efficacy of dead bacteria is limited compared to live bacteria.

Lactobacillus pentosus strain S-PT84 is a lactic acid bacterium of plant origin that was isolated from Kyoto pickles (*shibazuke*). Heat-killed S-PT84 can stimulate IL-12 and IFN-γ production

*To whom correspondence should be addressed: Institute for Health Care Science, Suntory Wellness Limited, 1–1–1 Wakayamadai, Shimamoto-cho, Mishima-gun, Osaka 618–8503, Japan. Tel.: +81-75-962-7049; Fax: +81-75-962-1690; E-mail: Takayuki_Izumo@suntory.co.jp

through interactions between dendritic cells and natural killer (NK) cells,[16] and can induce several beneficial immunomodulatory effects including the inhibition of allergic reaction and the prevention of virus or pathogenic microorganism infection in mice.[17–20] However, it was not known whether live S-PT84 exhibits additive effects compared to heat-killed S-PT84. Therefore, we compared the immunomodulatory effects between live and heat-killed S-PT84 in BALB/c mice. Furthermore, to investigate whether cell wall thickness, which is important factor of immunomodulatory activity in S-PT84, affects cytokine production from splenocytes, we compared the production of several cytokines in S-PT84 having different cell wall thickness.

MATERIALS AND METHODS

Animals —— BALB/c female mice, 7- to 8-weeks-old, were obtained from Japan SLC, Inc. (Hamamatsu, Japan). The animals were fed a commercial diet (AIN-93M, Oriental Yeast Co. Ltd., Tokyo, Japan) and tap water *ad libitum* and housed for 1 week at 25 ± 1°C and 60 ± 5% humidity under a 12-hr light-dark cycle before experimentation. Experiments were approved by the Animal Care and Use Committee of Suntory Holdings Limited and performed according to the Guideline for Animal Care and Use of Suntory Holdings Limited (This guideline was based on compliance with the Law for the Humane Treatment and Management of Animals, Law No. 105, 1973, as revised on June 1, 2006).

Preparation of Live and Heat-killed *Lactobacillus pentosus* S-PT84 —— S-PT84 was cultured in de Man, Rogosa, Sharpe (MRS) broth (Difco Laboratories, Detroit, MI, U.S.A.) at 30°C or 37°C for 24 hr. Cultured S-PT84 were collected by centrifugation at $9190 \times g$ for 10 min, washed twice with sterile saline, and then washed with distilled water. After cell enumeration, a portion of the S-PT84 suspension was heat-killed at 95°C for 5 min, and the remaining portion was used as live S-PT84. Both S-PT84 solutions were used in the following experiments.

Preparation of Mouse Splenocytes and Assays of S-PT84 Cytokine-inducing Activity in Mouse Splenocytes —— BALB/c mice were anesthetized with diethyl ether and sacrificed by exsanguination. The spleens were aseptically removed, and splenocytes were prepared after removal of erythrocytes. The splenocytes were suspended at 2.5×10^6 cells/well/0.5 ml of RPMI 1690 medium (Nacalai Tesque, Kyoto, Japan) with 10% fetal bovine serum (FBS, Cambrex, Charles City, IA, U.S.A.) containing in 100 U/ml of penicillin, and 100 μg/ml of streptomycin. The splenocytes were cultured in the presence of live or heat-killed S-PT84 (0.5 ml at 0.38, 1.13, 3.8 or 11.3×10^6 cells/ml) in 10% FBS-RPMI-1640 medium in a 48-well culture plate (Corning, Corning, NY, U.S.A.). Supernatants were collected after 24 hr for determination of IL-12, tumor necrosis factor-alpha (TNF-α), IFN-γ or IL-4 concentrations and stored at −80°C for further analysis.

Administration of S-PT84 and Assays of Cytokine Concentrations in Culture Supernatants —— Live or heat-killed S-PT84 solution was prepared with sterile saline as 1×10^9 cells/300 μl. Mice were orally administered 300 μl of the solutions once daily for 7 consecutive days. Subsequently, mice were anesthetized with diethyl ether and killed by exsanguination. The spleens were aseptically removed, and splenocytes were prepared after depletion of erythrocytes. 1×10^6 cells/well of splenocytes were cultured in the presence of 2.5 μg/ml concanavalin A (ConA, Sigma, St. Louis, MO, U.S.A.) in 0.2 ml of RPMI-1640 supplemented with 10% FBS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin in a 96-well culture plate (Corning). Supernatants were collected after 24 hr for determination of IFN-γ or IL-4 concentrations and stored at −80°C for further analysis.

ELISA for Determination of Cytokine Concentrations —— IL-12, IFN-γ, TNF-α and IL-4 concentrations in culture supernatants were determined by sandwich enzyme-linked immunosorbent assay (ELISA) using the following kits: BD OptEIATM Set Mouse IL-12 (p70), IFN-γ, TNF-α (Mono/Poly) and IL-4 ELISA (BD Biosciences, San Jose, CA, U.S.A.), respectively, according to the manufacturer's recommended protocol.

Measurement of Splenic NK Activity after Administration of S-PT84 —— Yac-1 target cells were suspended in phosphate-buffered saline (PBS, Nissui Pharmaceutical Co, Tokyo, Japan) at a density of 1×10^6 cells/ml and stained with 40 μg/ml of 3,3′-dioctadecyloxacarbocyamine perchlorate (DiO, Sigma) for 10 min at 37°C. The stained cells were washed once with PBS and resuspended in RPMI-1640 with 10% FBS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin at a

density of 5×10^4 cells/ml. Then 100 µl of the DiO-stained target cell suspensions and 100 µl suspensions of 2×10^7, 1×10^7 or 5×10^6 cells/ml of effector cells (splenocytes) and 25 µg/ml of propidium iodide (PI, Molecular Probes, Eugene, OR, U.S.A.) were added to 96-well U-shaped microplates (BD Biosciences). The plates were centrifuged at $200 \times g$ and incubated at 37°C in 5% CO_2 for 2 hr. Spontaneous cell death was determined by incubating target cells without effector cells. After incubation, DiO^+PI^+ cells were analyzed using an Epics XL (Beckman Coulter, Brea, CA, U.S.A.). NK activity was calculated according to the formula:

NK activity (%) = (DiO^+ PI^+)/(DiO^+ PI^- + DiO^+PI^+) × 100 – spontaneous lysis (%)

Statistical Analysis —— Data are presented as the mean ± standard error (S.E.). Significant differences in values were determined using the Student's *t*-test for individual group comparisons, and a two-way analysis of variance (ANOVA) for NK activity comparison. *p*-Values less than 0.05 were considered significant.

RESULTS

Effect of Live or Heat-killed S-PT84 on Cytokine Production from Mouse Splenocytes

To compare the immunomodulatory effect of live and heat-killed S-PT84 *in vitro*, we determined cytokine production from splenocytes after stimulation with each bacterium. As shown in Fig. 1A, IL-12 production from splenocytes was

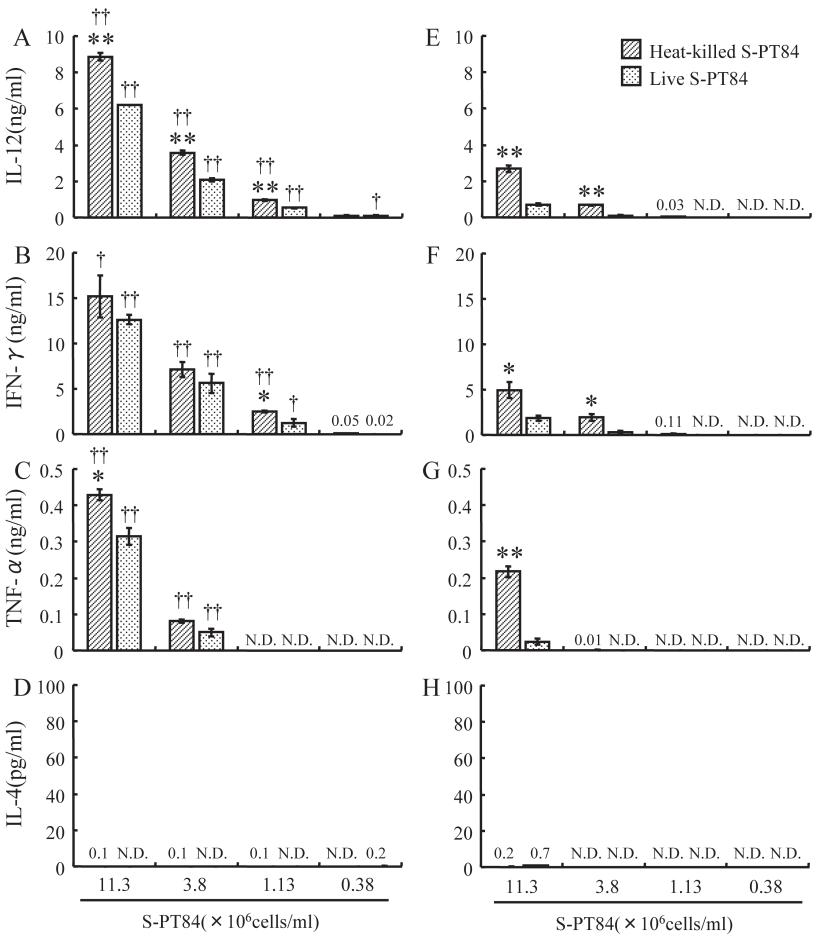

Fig. 1. IL-12, IFN-γ, TNF-α or IL-4 production from Splenocytes *in vitro*

Splenocytes were cultured in the presence of live or heat-killed S-PT84 (0.38, 1.13, 3.8 or 11.3×10^6 cells/ml, A-D: 37°C-cultivated S-PT84, E-H: 30°C-cultivated S-PT84) and culture supernatants were collected after 24 hr. IL-12 (A, E), IFN-γ (B, F), TNF-α (C, G) and IL-4 (D, H) concentrations in culture supernatants were measured by ELISA. The values represent mean ± S.E. (n = 3). Statistically significant differences between live and heat-killed S-PT84 groups: $^*p < 0.05$, $^{**}p < 0.01$. Statistically significant differences between 37°C and 30°C S-PT84 groups: $^\dagger p < 0.05$, $^{\dagger\dagger}p < 0.01$. N.D., not detected.

Table 1. Change in Average Body Weight in Mice during the Experimental Period

	Day 1	Day 8	δ value
	AVE ± S.E.	AVE ± S.E.	AVE ± S.E.
Control	17.2 ± 0.3	17.4 ± 0.3	0.2 ± 0.3
Heat-killed S-PT84 group	17.2 ± 0.3	17.5 ± 0.2	0.3 ± 0.3
Live S-PT84 group	17.2 ± 0.2	17.4 ± 0.2	0.3 ± 0.3

Data are expressed as mean ± S.E. (n = 8).

dose-dependently induced by live or heat-killed S-PT84 stimulations. However, IL-12 production was higher with heat-killed S-PT84 than live S-PT84, and statistically significant except for the low dose ($p < 0.01$). Similarly, IFN-γ or TNF-α production was induced by live or heat-killed S-PT84 stimulations (Fig. 1B and 1C). On the other hand, IL-4 production was not induced by S-PT84 (Fig. 1D).

Next, we measured cytokine production from splenocytes exposed to 30°C-cultivated S-PT84 (characterized by a thinner cell wall compared to those cultivated at 37°C). IL-12, IFN-γ and TNF-α productions were dose-dependently induced by live or heat-killed S-PT84; heat-killed S-PT84 showed higher induction than live S-PT84 (Fig. 1E, F and G). However, 30°C-cultivated S-PT84 indicated significantly lower cytokine production compared to 37°C-cultivated S-PT84 (Fig. 1).

Effect of Oral Administration of Live or Heat-killed S-PT84 on Body Weight Change

As shown in Table 1, the average body weight at day 1 and day 8 did not significantly differ between the three groups. During the experiment, average body weight was slightly increased in all groups, and abnormal changes were not observed.

Effect of Oral Administration of Live or Heat-killed S-PT84 on Cytokine Production from Splenocytes

To compare the immunomodulatory effect of live or heat-killed S-PT84 *in vivo*, we measured Th1 and Th2 cytokine production from mouse splenocytes after 7 days ingestion of each bacterium. As shown in Fig. 2A, IFN-γ production from splenocytes was significantly increased in the heat-killed S-PT84 ingestion group compared to the control group. In the live S-PT84 ingestion group, a non-significant increase in IFN-γ production was observed. No difference was observed between live and heat-killed S-PT84. IL-4 production from splenocytes was similar in all groups (Fig. 2B). The ratio of IFN-γ to IL-4 was used as index of Th1/Th2 balance, and the ratio was significantly increased by heat-killed S-PT84 ingestion (Fig. 2C). In the live S-PT84 group, a non-significant increase in Th1/Th2 ratio was observed (Fig. 2C, $p < 0.054$).

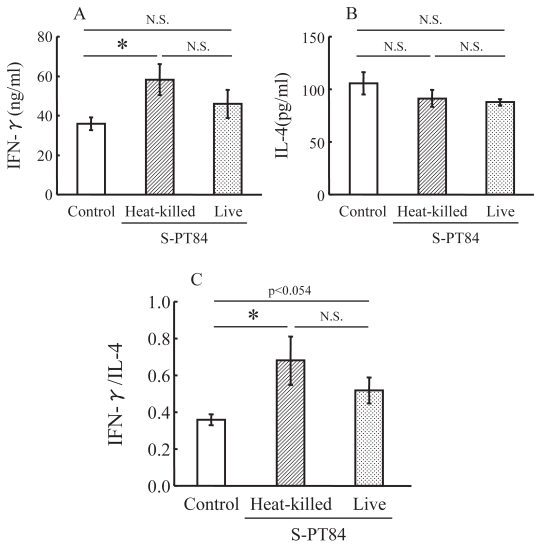

Fig. 2. IFN-γ and IL-4 Production from Splenocytes of Control or S-PT84-treated Mice

Live or heat-killed S-PT84 was orally administered to BALB/c mice for 7 days. Splenocytes were then collected and treated with concanavalin A (ConA; 2.5 ug/ml) for 24 hr. IFN-γ (A) and IL-4 (B) concentrations in culture supernatants were measured by ELISA. The ratio of IFN-γ to IL-4 was calculated as an index of Th1/Th2 balance (C). The values represent mean ± S.E. of 8 mice. Statistically significant differences between control and heat-killed S-PT84 groups: $^*p < 0.05$.

Effect of Oral Administration of Live or Heat-killed S-PT84 on Splenic NK Activity

To compare the immunostimulatory effect of live and heat-killed S-PT84 *in vivo*, we measured splenic NK activity after 7 days ingestion of each bacterium. As shown in Fig. 3, splenic NK activity was significantly increased by live or heat-killed S-PT84 ingestion. No significant difference was shown between live and heat-killed S-PT84, however heat-killed S-PT84 was slightly higher than live S-PT84.

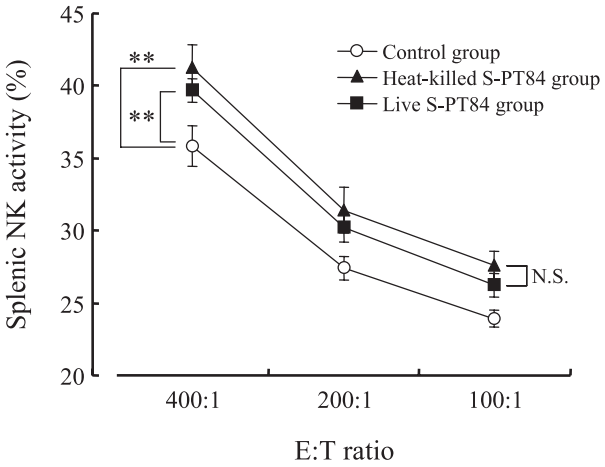

Fig. 3. NK Activity of Splenocytes from Control or S-PT84-treated Mice

Live or heat-killed S-PT84 was orally administered to BALB/c mice for 7 days. Splenocytes were then collected and NK activity was evaluated. The ratios of effector cells to target cell (E : T ratio) were 400 : 1, 200 : 1, and 100 : 1 in the present experiments. The values represent mean ± S.E. of 8 mice. Statistically significant differences between control and S-PT84 groups: $^{**}p < 0.01$.

DISCUSSION

Several reports have indicated that probiotics or lactic acid bacteria promote the immunomodulatory effects, anti-infectious activity, or epithelial barrier function in the gut.[21–23] Heat-killed S-PT84 has also been shown to enhance immune function as well as preventing virus or pathogenic microorganism infection in mice.[18–20] In this study, we compared the immunomodulatory effects between live and heat-killed S-PT84. The results of the present study showed that heat-killed S-PT84 was more effective in inducing IL-12 or IFN-γ production than live S-PT84 *in vitro*. A similar phenomena was shown in both 30°C- (thinner cell wall compared to 37°C) and 37°C-cultivated S-PT84. Sashihara *et al.* reported that the IL-12-inducing activity of heat-killed bacteria was higher than of live bacteria if cultured with immune cells under a neutral pH condition, but that IL-12-inducing activities of live and heat-killed bacteria were equal if bacteria were cultured under a low pH condition.[24] They concluded that these phenomena might be induced by the modification of peptidoglycan conformation or degradation of cell wall structure via autolysis. Some reports, including our study, have indicated that the cell wall is an important component for IL-12 production from immune cells.[25–27] In this study, S-PT84 cells were cultured with splenocytes under a neutral pH condition; therefore, the immunostimulatory cell wall component might have been degraded by enzymes in live S-PT84. We have reported that IL-12 production from mouse macrophages was increased by thicker cell walls of *Lactobacillus*.[27] Cell wall thickness is an important factor for several cytokine (IL-12, IFN-γ and TNF-α)-inducing activities of S-PT84 in mouse splenocytes, as shown in Fig. 1A and 1E. These results confirm the importance of cell wall thickness in Th1 cytokine-inducing activity of S-PT84.

The results of the present study show that IFN-γ production and IFN-γ/IL-4 ratio (Th1/Th2 balance) were significantly enhanced by heat-killed S-PT84 ingestion compared to the control group, but a significant difference was not shown with live S-PT84 (Fig. 2A and 2C). Therefore, heat-killed S-PT84 has a slight additive Th1 cytokine-inducing activity compared to live S-PT84 *in vivo*. However, it was suggested that these differences in Th1 cytokine production does not sufficiently reflect enhanced innate immunity, because no difference was shown in both S-PT84 ingestion on splenic NK activity (Fig. 3). Therefore, it is thought that the immunomodulatory effect is nearly equal with both live and heat-killed S-PT84.

Land *et al.* have reported that probiotics can cause invasive disease in children.[28] Furthermore, Boyle *et al.* have reported that populations at risk for probiotic sepsis are those with immunodeficiency and premature infants.[29] These reports have suggested that probiotics should be used with caution in certain populations. Conversely, heat-killed bacteria have a very low risk for invasive disease, are easily handled and are highly stable. Therefore, it is thought that we can safely and easily use heat-killed S-PT84 if an immunomodulatory effect is desired.

In conclusion, both live S-PT84 and heat-killed S-PT84 had equivalent immunomodulatory effects in BALB/c mice. Additionally, cell wall thickness was an important factor in the Th1 cytokine-inducing activity of both S-PT84. Further animal and human studies are necessary to evaluate in detail the effects of both live S-PT84 and heat-killed S-PT84 against immune function.

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