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Immunomodulatory Effects of the Stout Camphor Medicinal Mushroom, *Taiwanofungus camphoratus* (Agaricomycetes)–Based Health Food Product in Mice

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ABSTRACT: *Taiwanofungus camphoratus* is a unique medicinal mushroom endemic to Taiwan, and it is used as a folk medicine in East Asian countries. The aim of the present study was to investigate the immunomodulatory effects of "leader *Antrodia cinnamomea* capsule" (LAC), a health food product containing solid-state cultivated mycelial powder of *T. camphoratus*. For the *in vivo* studies, mice were orally administered LAC (76, 250, and 760 mg/kg b.w.) for 30 days, and its effects on cell-mediated humoral immune function were examined. The results of the concanavalin A–induced splenic lymphocyte proliferation test showed that LAC significantly increased splenic lymphocyte proliferation compared with the control. In addition, serum hemolysin analysis showed that LAC treatment significantly increased the half value of serum hemolysin (HC_{50}) in mice compared with the control. Moreover, treatment with LAC significantly increased the phagocytic index as measured by carbon clearance and natural killer cell activity. Taken together, these findings provide strong evidence that LAC can modulate immune function.

KEY WORDS: *Taiwanofungus camphoratus*, immunomodulatory effects, T lymphocytes, phagocytosis, natural killer cells, medicinal mushrooms

ABBREVIATIONS: b.w., body weight; **ConA**, concanavalin A; **DTH,** delayed-type hypersensitivity; **HBSS**, Hank's buffered salt solution; **HC_{50}**, half value of serum hemolysin; **LAC**, leader *Antrodia cinnamomea* capsule; **LDH**, lactate dehydrogenase; **MTT**, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **NK**, natural killer; **RBC**, red blood cell; **SA**, serum assay; **SRBC**, sheep red blood cell

I. INTRODUCTION

The stout camphor medicinal mushroom, *Taiwanofungus camphoratus* (M. Zang & C.H.Su) Sh.H. Wu et al. (syn. *Antrodia cinnamomea*, *A. camphorata,* Polyporales, Agaricomycetes), is endemic to Taiwan and grows on the inner cavities of age-old *Cinnamomum kanehirae* Hayata (Lauraceae) trees, which are also endemic to Taiwan.[1] *T. camphoratus* has been used as a traditional medicine by aborigines in Taiwan for many centuries. It has been widely used to promote health and to treat diseases such as liver disease, drug and food intoxication, diarrhea, and cancers.[2,3] Recently, the major components of *T. camphoratus*, including polysaccharides, terpenoids, benzenoids, and nucleic acids, have been identified.[2,4] The pharmacological functions of these compounds have been extensively explored, including regulation of the immune system[5–7] as well as anticancer,[8–10] anti-inflammatory,[11,12] hepatoprotective,[13–16] and antioxidant effects.[17–19]

The human immune system is needed to guard against infection by microorganisms and tumor growth. Abnormal immune function causes serious infection, tumor progression, autoimmune disease, and allergic

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responses.[20] A number of polysaccharides isolated from medicinal mushrooms have been widely studied for their immunomodulatory activities. For example, polysaccharides from *Ganoderma lucidum* and *Grifola frondosa* have been proven to stimulate the activity of macrophages, T lymphocytes, and cytokine secretion.[21–24] Previous studies have shown that the polysaccharide fraction of *T. camphoratus* also enhances phagocytic effects and dendritic cell maturation and promotes Th1 responses.[6,25] In addition, methyl antcinate K, a triterpenoid isolated from the fruiting bodies of *T. camphoratus*, activates dendritic cells to promote Th2 differentiation.[26] However, the immunomodulatory effect of mycelial extracts of *T. camphoratus* still needs to be clarified. However, *T. camphoratus* has been marketed as a health food product or food supplement in Taiwan. The "leader *Antrodia cinnamomea* capsule" (LAC) is a health food product containing solid-state cultivated mycelial powder of *T. camphoratus*. In the present study, we investigated the immunomodulatory effects of LAC in mice.

II. MATERIALS AND METHODS

A. Test Samples

LACs were manufactured by Taiwan Leader Biotech (Taipei, Taiwan), and the LAC powder contained 99% solid-state cultivated mycelial powder of *T. camphoratus* and 1% magnesium stearate.

B. Animals

Specific-pathogen-free mice were obtained from the National Institutes for Food and Drug Control (Beijing, China). The animals were housed in pathogen-free cages at the National Institute of Nutrition and Food Safety of the Chinese Center for Disease Control and Prevention (Beijing, China), an Association for the Assessment and Accreditation of Laboratory Animal Care–accredited facility, The temperature was set at 21 ± 2°C, relative humidity was 55% ± 20%, and lights were on 12 hours/day. Female BALB/c mice were used to measure weight, delayed-type hypersensitivity (DTH), the half value of serum hemolysin (HC_{50}), antibody production cell assays, concanavalin A (ConA)–induced lymphocyte proliferation, natural killer (NK) cell activity, and phagocytic activity of mice peritoneal macrophage assays. In addition, female Kunming mice were used for the carbon clearance test.

C. Treatment of Animals

Forty-eight female BALB/c mice were randomly assigned to 4 groups, with 12 mice in each group. Group 1 served as a vehicle control group and received distilled water, whereas the 3 treatment groups received 76, 250, and 760 mg/kg b.w. LAC, respectively. These doses were 3, 10, and 30 times the human recommended daily intake based on body weight conversion. Test samples were prepared with distilled water and administered to animals via oral gavage once daily for 30 consecutive days (dosing volume, 0.4 mL/20 g b.w.). After treatment for 30 days, animals from each group were used for various immune function tests.

D. Organ and Body Weight Ratio

The body weight of each animal was measured prior to sacrifice. The spleen and thymus were collected from the scarified mice at the end of LAC treatment. Organ weight was then measured, and the organ-to-body weight ratio was calculated.

E. DTH Assay

At the end of LAC treatment, animals were immunized with 2% sheep red blood cells (SRBCs) (v/v, in normal saline) via intraperitoneal injection. After 4 days of immunization, the thickness of the left hind footpad was measured using calipers, and each animal was measured twice at the same site to calculate the mean value of thickness. The site of measurement was then challenged again with 20% SRBCs (20 μL) via subcutaneous injection. After 24 hours, the thickness of the left hind footpad was measured. The difference between the 2 measurements was used to evaluate the level of DTH.

F. Mitogen-Induced Splenic Lymphocyte Proliferation

To isolate the splenocytes, mice were sacrificed and sterilized with 75% ethanol. Then the spleens were removed and homogenized with Hank's buffered salt solution (HBSS) and centrifuged at 1000 × *g* for 10 minutes. The cells were suspended with RPMI 1640 medium, and cell density was adjusted to 5×10^6 cells/mL. Cell suspension (1 mL) was plated to 24-well plates with or without ConA solution (7.5 μg/mL) and incubated at 37°C with 5% CO_2 for 72 hours. At 68 hours, 700 μL medium was discarded, then 700 μL serum-free RPMI 1640 medium and 50 μL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent were added. After incubation for 4 hours, the formazan crystals were dissolved with 1 mL/well acidic isopropanol. The absorbance was measured at 570 nm, and the proliferation of splenic lymphocytes was represented as $(OD570_{\text{with ConA}} - OD570_{\text{without ConA}})$.

G. Measurement of Antibody Secreting Cells

Mice were immunized with 2% SRBCs (v/v, in normal saline) by intraperitoneal injection. Four days after immunization, mice were sacrificed to collect spleens and the spleen cell suspension was prepared. Agar solution (0.5 mL in HBSS buffer), 50 μL 10% SRBCs in serum assay (SA) buffer (v/v), and 20 μL spleen cell suspension were mixed well in a tube. The mixtures were poured on slides and incubated at 37°C in 5% CO_2 for 1.5 hours. Then complement diluted with SA buffer (1:10) was added to the slides and incubated for 1.5 hours. The hemolytic plaques were counted after incubation.

H. Serum Hemolysin Assay (HC_{50})

Mice were immunized with 2% SRBCs (v/v, in normal saline) by intraperitoneal injection. Blood samples were collected after immunization for 4 days and kept at room temperature for 1 hour. Blood samples were centrifuged at 2000 × *g* for 10 minutes to collect the serum. Serum was diluted to 200-fold by SA buffer. Next, 1 mL diluted serum and 500 μL 10% SRBCs (v/v, in SA buffer) were added to the tubes, followed by the addition of 1 mL complement (diluted in SA buffer at a 1:7 ratio). SA buffer was used instead of serum for the blank control. The mixtures were incubated in a 37°C water bath for 30 minutes and then placed on ice to stop the reaction. The supernatant was collected after centrifugation at 2000 × *g* for 10 minutes, and 1 mL supernatant and 3 mL Drabkin's solution were mixed in a tube then incubated at room temperature for another 10 minutes. The positive control was prepared by adding 250 μL 10% SRBCs (v/v, in SA buffer) and 3.75 mL Drabkin's solution. The absorbance was measured at 540 nm. The value of HC_{50} was calculated as follows:

$$HC_{50} = (OD540_{\text{sample}}/OD540_{\text{half SRBC hemolysis}}) \times \text{dilution factors}$$

I. Carbon Clearance Test

Mice were intravenously injected with diluted India ink via the tail vein. Blood samples were collected at 2 (t_1) and 10 (t_2) minutes after injection, then 20 μL blood was mixed with 2 mL Na_2CO_3. The absorbance was measured at 600 nm and Na_2CO_3 solution was used as the blank. Finally, mice were sacrificed to collect and weigh the liver and spleen. The phagocytic index (a) was calculated as follows:

$$k = (\lg OD_1 - OD_2)/(t_2 - t_1)$$

$$a = [\text{body weight}/(\text{liver weight} + \text{spleen weight})] \times \sqrt[3]{k}$$

J. Measurement of Phagocytic Activity of Mouse Peritoneal Macrophages

Mice were intraperitoneally injected with 5 mL HBSS buffer and massaged gently for 10 minutes. The mice were sacrificed and peritoneal macrophages were collected. Peritoneal macrophage fluid (0.5 mL) was transferred onto a slide and an equal volume of 20% chicken red blood cells (RBCs) was added and mixed well. Then the slide was placed in an enamel box with wet gauze and incubated at 37°C for 30 minutes. The slide was washed with normal saline to remove nonadherent cells. After drying, the slide was fixed with methanol and then stained with Giemsa solution, washed with distilled water, and dried again. One-hundred macrophages were counted on each slide, and the phagocytic index was calculated as the number of chicken RBCs phagocytosed by macrophages divided by the number of counted macrophages.

K. Measurement of NK Cell Activity

The YAC-1 target cells were subcultured for 24 hours prior to the assay. Cells were washed with HBSS buffer 3 times, and the cell concentration was adjusted to 1×10^5 cells/mL with RPMI 1640 medium containing 10% calf serum. The spleen cell suspension was prepared as described above, and the cell number was adjusted to 5×10^6 cells/mL. The ratio of effector cells (spleen cells) to target cells was 50:1. Effector cells (100 μL) and target cells (100 μL) were added to 96-well plates. Effector cells (100 μL) and culture medium (100 μL) were added to a target-cell spontaneous lactate dehydrogenase (LDH)–release control well. Effector cells (100 μL) and 1% NP40 (100 μL) were added to the target-cell maximum-release control well. The plates were incubated at 37°C with 5% CO_2 for 4 hours then centrifuged at 1500 × *g* for 5 minutes. The supernatant (100 μL) was transferred to new 96-well plates, and 100 μL LDH substrate (0.05 M sodium lactate, 0.66 mM nitro-tetrazolium chloride, 0.28 mM 5-methylphenazinium methosulfate, 1.3 M NAD, and 0.2 M Tris-HCl buffer, pH 8.2) was added to each well. After incubation for 10 minutes, 30 μL HCl solution (1 mol/L) was added in each well to stop the reaction. The absorbance was measured at 490 nm and NK cell activity was calculated as follows:

$$\text{NK cell activity (\%)} = (OD_{\text{Sample}} - OD_{\text{Target cell spontaneous release control}})/(OD_{\text{Target cell maximum-release control}} - OD_{\text{Target cell spontaneous release control}}) \times 100$$

L. Statistical Analysis

The results are presented as the mean ± SD of 3 independent experiments. Statistical analyses were performed using one-way analysis of variance and Dunnett's test using SPSS software. A P value < 0.05 was considered statistically significant.

III. RESULTS

A. Effects of LAC on Body and Organ Weight in Mice

Prior to the investigation, different doses of LAC (76, 250, and 760 mg/kg b.w.) were tested in normal mice to detect any physiological or psychological abnormalities. No mortalities were observed in the control group or the LAC experimental group. No behavioral abnormities were observed in any of the experimental groups. The initial and final body weights of LAC treatment groups were not statistically significant compared with the control group. The body weight gains in the LAC treatment groups were not significantly altered compared with the control mice (Table 1). In addition, spleen- and thymus-to-body weight ratios in the LAC treatment groups were also not significantly different from the control group (Fig. 1).

B. Effects of LAC on Cellular Immunity in Mice

To evaluate the effects of LAC on cellular immunity, DTH assays and splenic lymphocyte proliferation tests were conducted in mice treated with various doses of LAC for 30 days. SRBC-immunized mice showed increasing footpad swelling as a DTH reaction. There were no significant differences in the level of footpad swelling between any of the LAC treatment groups and the vehicle control (Fig. 2A). Results of the ConA-induced splenic lymphocyte proliferation test showed that LAC at a dose of 760 mg/kg b.w. markedly increased splenic lymphocyte proliferation to 125.6% ± 5.87% compared to the vehicle control (Fig. 2B). These results suggest that a high concentration of LAC has effects on cellular immunity in mice through stimulation of the proliferation of splenic lymphocytes.

C. Effects of LAC on Humoral Immunity in Mice

Hemolytic plaque formation and the serum hemolysin level were used to evaluate whether LAC affected humoral immunity in mice. The results of hemolytic plaque formation showed no obvious difference between the LAC-treated groups and the control (Fig. 3A). In a serum hemolysin assay, the HC_{50} value was markedly increased in mice treated with LAC. However, a significant increase in HC_{50} was observed in the 76 and 760 mg/kg treatment groups (176.33 ± 38.71 and 152.16 ± 53.82, respectively) compared to the control mice (99.48 ± 41.02) (Fig. 3B).

D. Effects of LAC on Phagocytic Activity in Mice

The rate of carbon clearance and the phagocytic ability of macrophages against chicken RBCs were used as an index of phagocytosis to evaluate the effect of LAC on phagocytic activity in mice. As shown in

TABLE 1: Body Weight and Weight Gain

Group	Dose (mg/kg b.w.)	Initial Body Weight (g)	Final Body Weight (g)	Weight Gain (g)
Control	0	18.7 ± 0.8	21.6 ± 1.1	2.9 ± 0.4
LAC	76	18.9 ± 0.7	21.5 ± 1.3	2.6 ± 0.8
LAC	250	18.7 ± 0.9	21.6 ± 1.6	2.9 ± 0.8
LAC	760	18.8 ± 0.8	21.4 ± 1.4	2.6 ± 0.9

Results are presented as the mean ± SD.

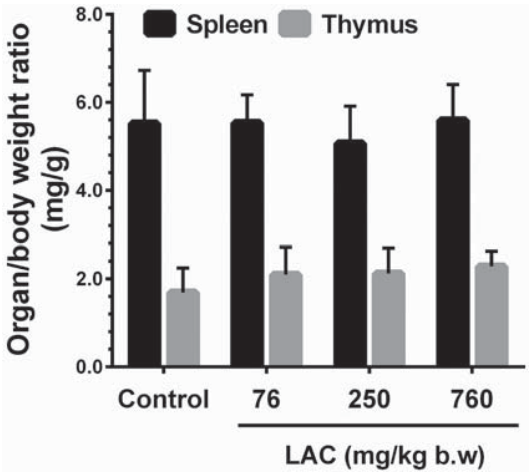

FIG. 1: Effect of LAC on spleen- and thymus-to-body weight ratios in mice. Forty-eight mice were randomly divided into 4 groups, with 12 mice in each group. The control group received distilled water, whereas the LAC treatment groups were administered 76, 250, and 760 mg/kg b.w. LAC once a day for 30 days. After the end of treatment, the mice were sacrificed and body weight, spleen weight, and thymus weight were measured. All results are presented as the mean ± SD.

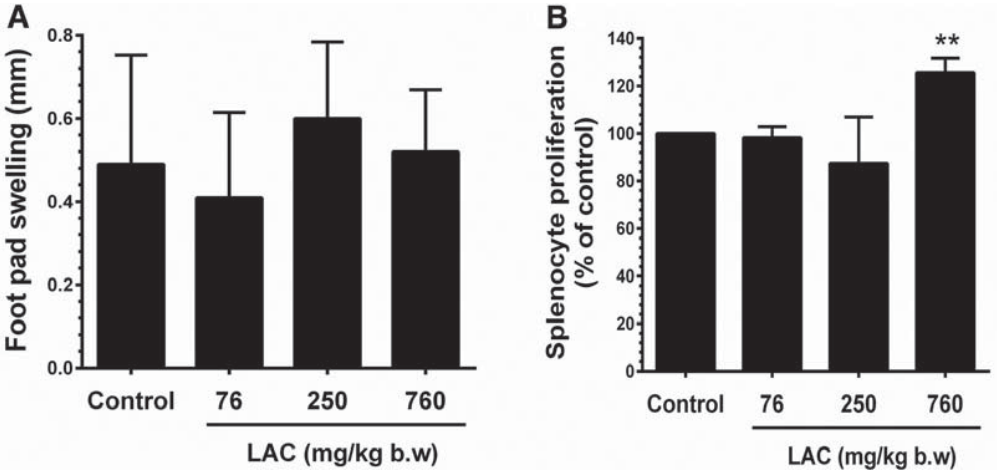

FIG. 2: Effect of LAC on DTH and splenocyte proliferation. (A) Mice were immunized with 2% sheep red blood cells via intraperitoneal injection as stated in the materials and methods. After 24 hours, the thickness of the left hind footpad was measured. (B) After immunization with 2% SRBCs, mice were sacrificed and the spleen was removed and the splenocytes were isolated. Cultured splenocyte proliferation was measured by the MTT assay. All results are presented as the mean ± SD. $^{**}P < 0.01$ compared to control.

Fig. 4A, the carbon clearance rate, termed the phagocytic index, increased in all LAC treatment groups. However, a significant increase in the phagocytic index was observed at a dose of 760 mg/kg b.w. (5.28 ± 1.2), which was significantly higher than that of the control group (4.51 ± 1.25). However, we observed no obvious difference in the phagocytic activity of peritoneal macrophages to chicken RBCs between the LAC treatment groups and the control (Fig. 4B).

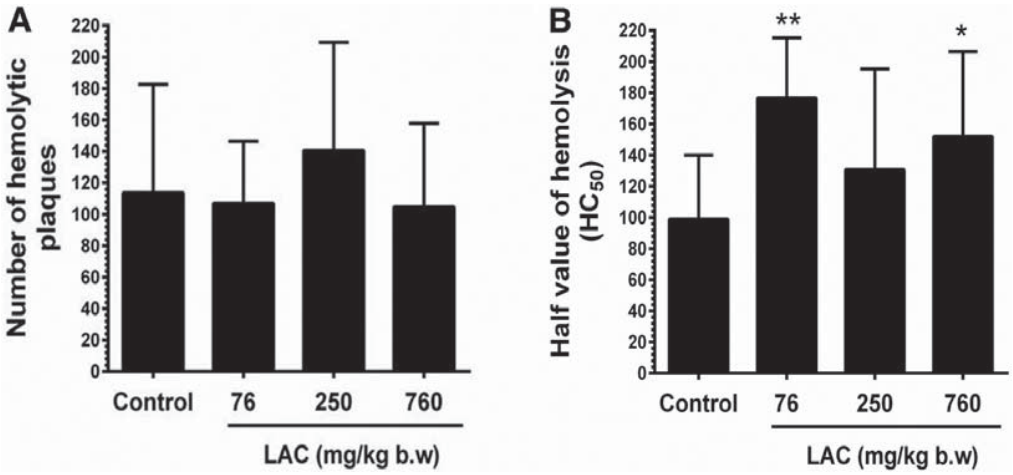

FIG. 3: Effects of LAC on humoral immunity in mice. (A) Mice were immunized with SRBCs and concomitantly treated with various doses of LAC. Four days after immunization, the plaque-forming cell assay was enumerated in spleen cells. (B) After immunization, the LAC concentration required for 50% hemolysis was calculated. All results are presented as the mean ± SD. $*P < 0.05$; $**P < 0.01$ compared to control.

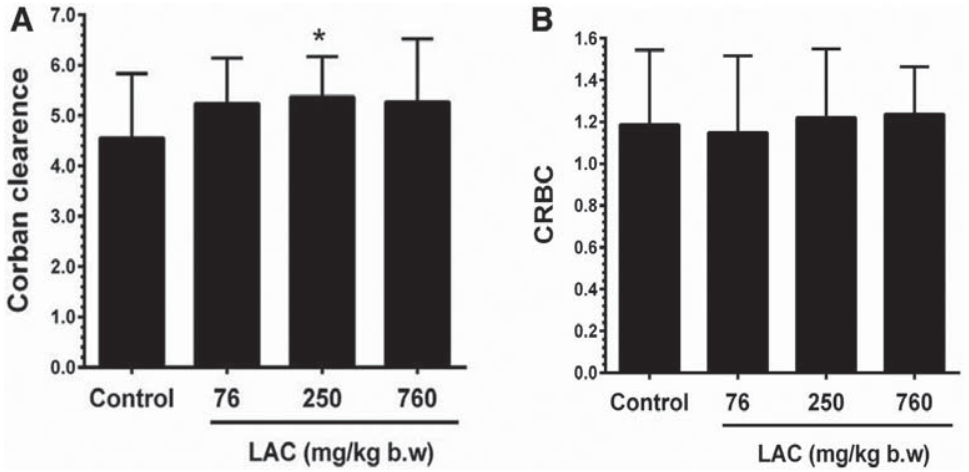

FIG. 4: Effect of LAC on phagocytic activity of mononuclear macrophages. (A) Mice were intravenously injected with diluted India ink via the tail vein. Blood samples were collected at 2 (t_1) and 10 (t_2) minutes after injection. Blood was mixed with Na_2CO_3 and the absorbance was measured at 600 nm. The phagocytic index was calculated with the weight of the liver and spleen. (B) Mice peritoneal macrophages were isolated and peritoneal macrophage fluid was prepared. Then 0.5 mL peritoneal macrophage fluid was mixed with 20% chicken RBCs and coated in glass slides. After they were fixed with methanol and then stained with Giemsa solution, 100 macrophages were counted on each slide and the phagocytic index was calculated by the number of chicken RBCs phagocytosed by macrophages/number of counted macrophages. All results are presented as the mean ± SD. $*P < 0.05$ compared to the control.

E. Effects of LAC on NK Cell Activity

Mice were orally administrated LAC at doses of 76, 250, and 760 mg/kg b.w., and NK cell activity was assessed after treatment for 30 days. As shown in Fig. 5, LAC at a dose of 76 mg/kg b.w. (54.63% ± 3.76%) significantly enhanced NK cell activity compared to the control (52.26% ± 4.19%).

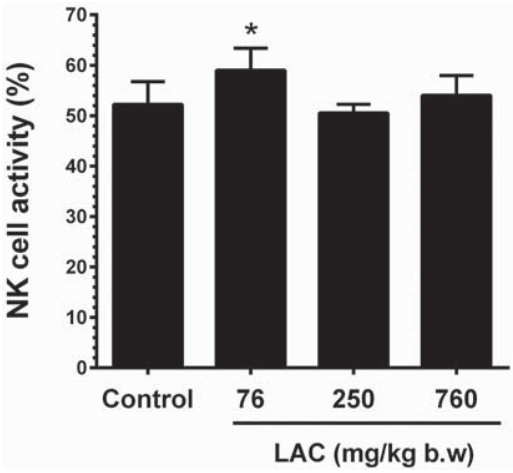

FIG. 5: Effect of LAC on NK cell activity. Forty-eight mice were randomly divided into 4 groups, with 12 mice in each group. The control group received distilled water, whereas the LAC treatment groups were administered 76, 250, and 760 mg/kg b.w. LAC once a day for 30 days. After treatment for 30 days, the mice were sacrificed and the spleen cells were isolated. The effector cells (spleen cells) were mixed with target cells (YAC-1) at a ratio of 50:1, then the LDH assay was performed to evaluate NK cell activity. All results are presented as the mean ± SD. *$P < 0.05$ compared to the control.

IV. DISCUSSION

In this study, LAC was orally administered to mice for 30 days and increased ConA-induced splenic lymphocyte proliferation, the serum hemolysin level, the rate of carbon clearance in the bloodstream, and NK cell activity. These results suggest that LAC stimulates cellular and humoral immune function in mice. The cellular immunity mostly involved the activation of T cells, phagocytes, and NK cells against pathogen invasion, tumor cells, and transplant cells.[20] ConA is a lymphocyte mitogen that selectively triggers T-cell proliferation.[27] Our study results show that LAC at a dose of 760 mg/kg b.w. significantly increased ConA-induced splenic lymphocyte proliferation. Therefore, LAC was able to stimulate T-cell proliferation. However, there was no significant change in footpad swelling thickness between the control and LAC groups. Monocytes/macrophages or the reticuloendothelial system were involved in clearing particles from the bloodstream.[28,29] For the carbon clearance test, the ink-containing carbon particles were injected into mice treated with LAC, and the results show that the phagocytic index as measured by the carbon clearance test increased. The index of the 250 mg/kg LAC group increased statistically significantly compared to the control. These results suggest that LAC can stimulate the phagocytic activity of monocytes/macrophages in mice. Additionally, 76 mg/kg LAC markedly increased NK cell activity. According to these findings, LAC had the effect of stimulating cellular immunity in mice.

Humoral immunity is mediated by antibodies produced by B lymphocytes and is involved in antigen interaction with B cells. In this study, the effect of LAC on serum antibody production was also tested, and the results show that the value of HC_{50} increased in mice treated with LAC. Especially in the low- and high-dose groups, the value of HC_{50} markedly increased compared to the control, but there was no significant difference in hemolytic plaque formation. Therefore, the oral administration of LAC increased the level of serum hemolysin, and LAC might be involved in regulating humoral immune function in mice.

Recent evidence has revealed that *T. camphoratus* is a potent immunomodulatory agent against parasitic diseases, cancers, and allergic diseases. In recent studies, *T. camphoratus* exhibited the ability

to enhance phagocytic activity in monocytes and human polymorphonuclear neutrophils and to induce T-cell proliferation. Polysaccharides and adenosine might be the active constituents of *T. camphoratus* that contribute its immune system modulatory function.[6,25] Liu et al.[30] demonstrated that administration of *T. camphoratus* polysaccharides prevented asthma in mice through induction of Th1 responses and suppressed the allergen-related Th2 responses. In addition, the polysaccharide extracts from the mycelia of *T. camphoratus* enhanced Th1 development in T1/T2 doubly transgenic mice and inhibited infection of *Schistosoma mansoni* infection.[31] Consistent with these findings, our results show that LAC had stimulatory effects on cellular and humoral immune functions in mice. However, further studies are needed to clarify the mechanisms involved. Finally, the results of this study support the notion that LAC has effects on modulating immune function and may confer benefit as a human dietary supplement.

V. CONCLUSION

Treatment with LAC significantly induced splenic T-lymphocyte proliferation and increased the phagocytic index, as measured by carbon clearance rate and NK cell activity in mice. Treatment with LAC also significantly increased HC_{50} values. Taken together, these findings strongly suggest that LAC has modulatory effects on cellular and humoral immune functions *in vivo*.

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