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Probiotic Complex Feed Additive against LL/2 Lung Cancer in Mice

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Keywords:

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1. Abstract

Cancer is a major public health problem worldwide. Lung cancer is the top 1 for the estimated cancer-caused deaths in Taiwan in 2019. Therefore, Research and development (R&D) of novel anti-lung cancer drugs and the ideal therapeutic strategies are urgently needed and important. This study was investigated the inhibition efficacy of LL/2 lung cancer via orally administered with probiotic complex feed additive for three months in an orthotopic LL/2 lung cancer-bearing mouse model. The results showed (1) the Body Weight (BW) of mice in each group (negative control group, positive control group, and probiotic complex group) was decrease from 3rd week after tumor induction. (2) The survival rate of mice in the negative control group and the positive control group were 0%. The survival of mice in the probiotic complex group was 10%. The number of survival mice on D33 in the probiotic complex group (n = 3) was higher than the negative control group (n = 0) and the positive control group (n = 0). Until the end of this experiment (D85), one mouse in the probiotic complex group (n = 1) survived ($p < 0.05$; the negative control group vs. the probiotic complex group) and its physiological symptoms were normal. (3) The average lung weight of the negative control group was the highest, followed by the probiotic complex group and the positive control group. (4) The number of tumor nodules in lung in the positive control group and the probiotic complex group were lower than the negative control group. The number of tumor nodules in lung in the negative control group and the positive control group showed a significant difference ($p < 0.01$). (5) On 3rd week after the induction of lung cancer in each group, the values of four liver and kidney function indexes (GOT, GPT, BUN, and CRE) were all increased, especially the negative control group. (6) The histo-pathological examination (tumor cell infiltration, tumor cell mitosis, and tumor cell necrosis) of tumor in lung was performed. The probiotic complex group was better than the negative control group according to the histo-pathological examination. Based on the above test results, although the results of lung weight, lung tumor number, liver and kidney index, and histo-pathological examination, the positive control group was better than those of the probiotic complex group. However, the positive control group was administrated with the clinical anti-cancer chemotherapy drug, 5-FU which side effects caused all mice to die on D33 as same as the negative control group (all mice to die on D33). The results showed that 5-FU has slightly welled anti-cancer effect, but the side effects of 5-FU are strong, resulting in the low survival rate and shorting the survival time of mice in this experiment. On the other hand, the side effects of the probiotic complex group were better than the positive control group. Although the probiotic com-

plex group anti-tumor efficacy was slightly worse than the positive control group, but the probiotic complex group was significantly better in the lower side effects compared to the positive control group, the higher survival rate and the longer survival time than those in the positive control group. The overall efficacy and safety data showed that the probiotic complex group was significantly better in the anti-cancer choice than the positive control group. According to these results, the probiotic complex had safety and the positive effect on the inhibition of *in situ* LL/2 lung cancer in mice.

2. Introduction

Cancer is the world's major public health problem and the second leading cause of death in the United States [1]. Pointed out that there were 836,150 and 852,630 new cancer cases in men and women in the United States in 2017. The top ten cancers in men in the United States are prostate cancer (19% incidence), followed by lung and bronchial cancer (14% incidence), colorectal cancer (9% incidence), and bladder cancer (7% incidence), skin melanoma (6% incidence), renal and renal pelvic cancer (5% incidence), non-Hodgkin lymphoma (5% incidence), leukemia (4% incidence), oral cavity and throat cancer (4% incidence), liver and intra hepatic cholangiocarcinoma (3% incidence). The top ten cancers among American women are breast cancer (30% incidence), followed by lung and bronchial cancer (12% incidence), colorectal cancer (8% incidence), bladder cancer (7% incidence), thyroid cancer (5% incidence), skin melanoma (4% incidence), non-Hodgkin lymphoma (4% incidence), leukemia (3% incidence), pancreatic cancer (3% incidence), kidney and renal pelvis cancer (3% incidence). It can be seen that the incidence of lung and bronchial cancer is the second in the incidence of new cancer cases in the United States. In Taiwan, according to the Ministry of Health and Welfare's announcement of the top ten causes of death in 2019, cancer has ranked first among the top ten causes of death in Taiwan for 38 consecutive years since 1982. The top ten cancers are lung cancer, followed by liver and intra hepatic cholangiocarcinoma, colorectal and anal cancer, female breast cancer, oral cancer, prostate cancer, pancreatic cancer, stomach cancer, esophageal cancer, and ovarian cancer. Based on this information, lung cancer is very important disease in United States and Taiwan.

Lung cancer is the leading cause of death of cancer patients worldwide. Treatment failure and the main cause of death are often related to cancer metastasis [1]. Although these patients with lung cancer can be removed and treated with surgery and chemotherapy [2-4], unfortunately, even after the best chemotherapy drugs are used for treatment, the 5-year survival rate of patients with lung cancer is only 1%-49% [1, 5, 6]. Therefore, there is an urgent need to establish a suitable animal model of lung cancer, find treatment strategies and understand the cell mediators that contribute to cancer invasion and metastasis, and develop new therapeutic agents targeting these mediators, so as to assist biotechnology and medical practitioners in developing more effective inhibition of cancer proliferation and cancer metastasis.

3. Material and Methods

3.1. Reagent

5-fluorouracil (5-FU; Sigma-Aldrich Co.,USA), Zoletil 50 (50 mg/mL, Virbac Laboratories, France), TrypLE™ Express (Sigma-Aldrich), Fetal bovine serum (FBS; Gibco®, USA), Dulbecco's Modified Eagle Medium (Sigma-Aldrich), penicillin-streptomycin (Sigma-Aldrich), and probiotic complex were used in this study.

3.2. Fresh Preparation of Mice' Feed with Probiotic Complex

Firstly, three g of probiotic complex was well mixed with 500 g powdered mouse feed (probiotic complex : powdered mouse feed = 3 : 500).The fresh, mixed mouse feed with probiotic complex must be placed for 72 hours at 4°C before feeding to mice. New and fresh mouse feed with probiotic complex were be provided to mice every day.

3.3. Cell Lines and Culture Condition

LL/2 mouse lung cancer cell line(ATCC® CRL- 1642™) was purchased from ATCC (Manassas, VA 20110). Dulbecco's modified eagle medium (DMEM), FBS, and antibiotics (penicillin and streptomycin) were purchased from Sigma-Aldrich. DMEM was supplemented with 10% FBS and 1% penicillin and streptomycin. The cells (1×10^5/100 μL) were incubated at 37°C with 5% CO_2. Cells were sub-cultured with TrypLE™ Express to replace flesh media per 2-3 days when they became confluent.

3.4. Animal Care

All animal experiments and animal care were approved by the Institutional Animal Care and Utilization Committee (IACUC) of Agricultural Technology Research Institute (ATRI), Taiwan. The approval number of IACUC, ATRI was No.109026. Eight weeks old male C57BL/6 mice (n = 30; the average of body weight was 25 g) were ordered from Bio LASCO Taiwan Co. Ltd and were freely fed a standard laboratory diet and the sterile drinking water and kept on a 12-h light/dark cycle at 24-27°C and 60-70% humidity using an automatic control system in the GLP Animal Laboratory, Animal Technology Research Center, ATRI, Taiwan.

3.5. Experimental Design

All mice (n =30) were divided into three groups as the negative control group (Negative control) (n = 10), the positive control group (Positive control) (n = 10), and the probiotic complex group (Sample) (n = 10).The experimental time is 85 days. The evaluation of LL/2 lung cancer inhibitory efficacy of the probiotic complex feed additive in mice.

3.6. Establishment of an Orthotopic LL/2 Lung Cancer Model in Mice

In the LL/2 lung cancer-bearing mouse model, LL/2 cells (1×10^5/mouse in 100 μL 0.9% saline) were intravenously injected into male C57BL/6 mice (n = 10/group) through the tail vein. Later, the probiotic complex in the feed was orally administrated to the

LL/2 lung cancer-bearing mice. Mice in the probiotic complex group (Sample) were free intake with probiotic complex feed additive [1.5 g/10 g body weight (BW)/day]. The negative control group (Negative control) was free intake with normal drinking water(sterilized water) and feed without the probiotic complex through the same administration route. The positive control group (Positive control) was also free intake with normal drinking water (sterilized water) and feed without probiotic complex. Moreover, the positive control group (Positive control) was through intravenous injection with 5-FU (100 mg/kg BW; once per week). During D0-D85of the experiment, the mouse's blood in each group was collected at the experimental time points (D0, D7, D14, D21, D28, D35, D42, D49, D56, D63, D70, D77, and D84 of the experiment) and performed the detection of the blood biochemistry values (liver and kidney functional indexes). At the end of the experiment, all mice were sacrificed and dissected. The lungs of mice were collected, weighted, counted the number of tumor nodules, and performed the histo-pathological examination. The survival rate of LL/2lung cancer-bearing mice in each group was evaluated.

3.7. Histo-pathological Examination

The mouse tissue samples were embedded in 10% neutral paraffin and cut to 4 μm thicknesses by paraffin tissue slicer (Leica RM 2135). Samples were stained with hematoxylin and eosin (H&E) and examined by a senior pathological veterinarian under a light microscope.

3.8. Statistical Analysis

The data is expressed as mean ± SD for at least 3 replicates. All comparisons were made by one-way ANOVA using Graph pad Prism 6 statistical analysis software. All significant differences are reported at $^{*/\#/\&}p< 0.05$, $^{**/\#\#/\&\&}p< 0.01$, $^{***/\#\#\#/\&\&\&}p< 0.001$.

4. Results

In this experiment, we implanted the mice with lung cancer line LL/2 by tail vein injection to induce in situ lung cancer. During the test period, we gave the mice the standard drug 5-FU or probiotics complex feed additive, measured the mice' body weight, and performed clinical symptom and survival rate observation every day. Blood samples were collected once a week to monitor the blood biochemical value (GOT, GPT, BUN, and CRE). At the end of the experiment, the lung weight of mice was measured and the number of lung tumor masses was counted to evaluate the inhibitory effect of probiotics complex feed additive on lung cancer.

4.1. Change of Mice' BW

After the beginning of the experiment (D0-D85), BW of the mice was measured every day. It can be seen from the results that in the third week (D21) after lung cancer was induced, the BW of mice in each group showed a downward trend. After D33 of the experimental time, the mice (n = 10) in the probiotics complex group (Sample) were only survived. On the other hand, the mice (n = 20) in the negative control group (Negative control) and the positive control group (Positive control) were all died. Therefore, the statistics analysis of mice' BW during D0-D32 of the experimental time was performed. The results were not statistically different between the negative control group (Negative control), the positive control group (Positive control), and the probiotics complex group (Sample) (Figure 1).

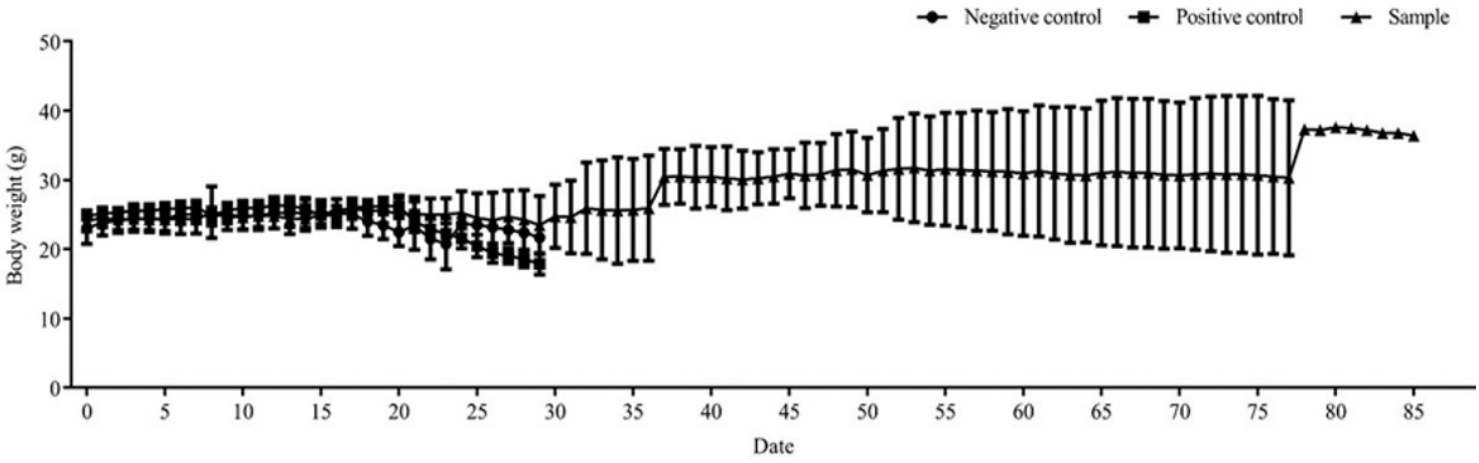

Figure 1: The change of mice' body weight. Data expressed as mean ± SD. The negative control group (Negative control); the positive control group (Positive control); the probiotic complex group (Sample).

4.2. Changes of Mice' Survive

After the beginning of the experiment (D0-D85), the number of surviving mice in each group was observed daily. The results showed the survival rate of the negative control group (Negative control) is 0% (0/10), the survival rate of the positive control group (Positive control) is 0% (0/10), and the survival rate of the sample group (probiotic complex feed additive) was 10% (1/10) at the end of the experiment (D85). Comparing the survival rate changes between 3 groups, the negative control group and the sample group showed a significantly difference ($p < 0.05$); the positive control group and the sample group compared with the non-significant difference ($p > 0.05$); the positive control group and the negative control group compared with the non-significant difference ($p > 0.05$) (Table 1). Based on the analysis of the daily survival rate of the experiment, mice in the negative control group and the positive control group were all dead (n = 20) on D33 of the experiment. On the same tome (D33), 3 mice survived in the sample group and its survival rate was 30% (3/10). Furthermore, one mouse survived until the end of the experiment (D85) (Figure 2).

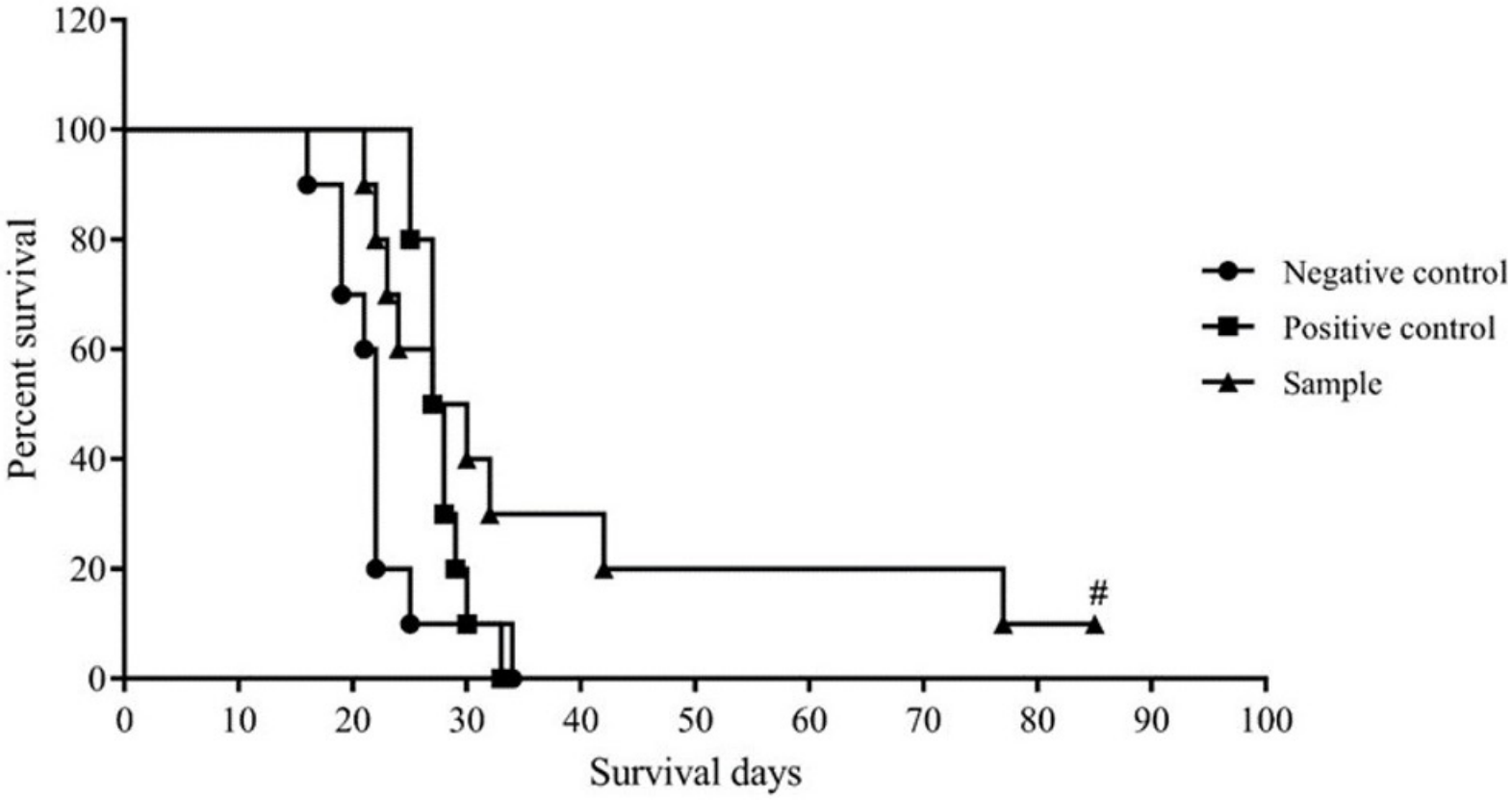

Figure 2: Survival rate of mice in each group. The survival rate of the negative control group (Negative control) is 0%, the survival rate of the positive control group (Positive control) is 0%, and the survival rate of the probiotic complex group (Sample) is 10%. The negative control group (Negative control); the positive control group (Positive control); the probiotic complex group (Sample). Negative control vs. Sample ($^{\#}p < 0.05$)

Table 1: The survival rate of each group at the end of the experiment. The negative control group (Negative control); the positive control group (Positive control); the probiotic complex group (Sample). $^{*}p < 0.05$ (Negative control vs. Sample).

Group	Number	Number of survival mice	Survival rate
Negative control	10	0	0%
Positive control	10	0	0%
Sample	10	1	10%*

4.3. Comparison of Each Group Mice' Lung Weight and Tumor Mass Number

At the end of the experiment (D85), all mice were sacrificed, and the mice in each group could find tumor masses in the lungs (Figure 3). The lungs of the mice were taken out to weigh and count the number of tumor masses. The results were presented that [1] Comparison of lung weights in each group: the negative control group (Negative control) and the positive control group (Positive control) are significantly different ($p < 0.01$).The probiotic complex group (Sample) and the positive control group (Positive control) showed a statistically significant difference ($p < 0.05$) (Figure 4A). [2] Comparison of the number of lung tumor mass in each group: The number of lung tumor mass in the negative control group was significantly higher than that in the positive control group ($p < 0.05$) (Figure 4B).

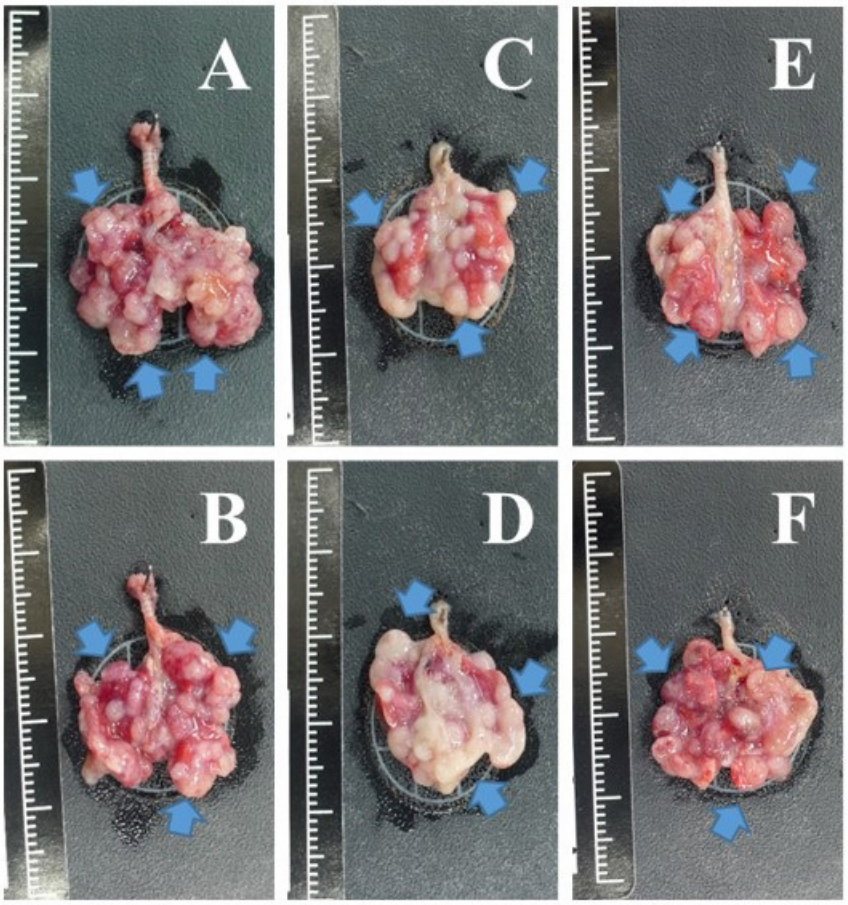

Figure 3: Tumor growth status of mice in lung in each group. (A) The negative control group: the anterior of mouse's lung. (B) The negative control group: the posterior of mouse's lung. (C) The positive control group: the anterior of mouse's lung. (D) The positive control group: the posterior of mouse's lung. (E) The probiotic complex group: the anterior of mouse's lung. (F) The probiotic complex group: the posterior of mouse's lung. The blue arrow reported the tumor mass of lung. The negative control group (Negative control); the positive control group (Positive control); the probiotic complex group (Sample).

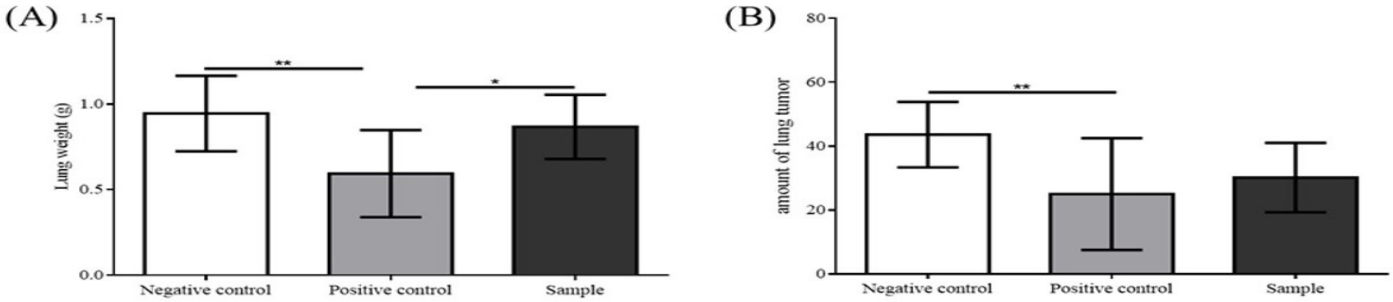

Figure 4: Analysis of lung weight and the number of tumor mass in lung in each group. (A) Analysis of lung weight. (B) Analysis of lung tumor mass number. The data showed mean ± SD. All significant differences were reported as $^{*}p < 0.05$ and $^{**}p < 0.01$. The negative control group (Negative control); the positive control group (Positive control); the probiotic complex group (Sample).

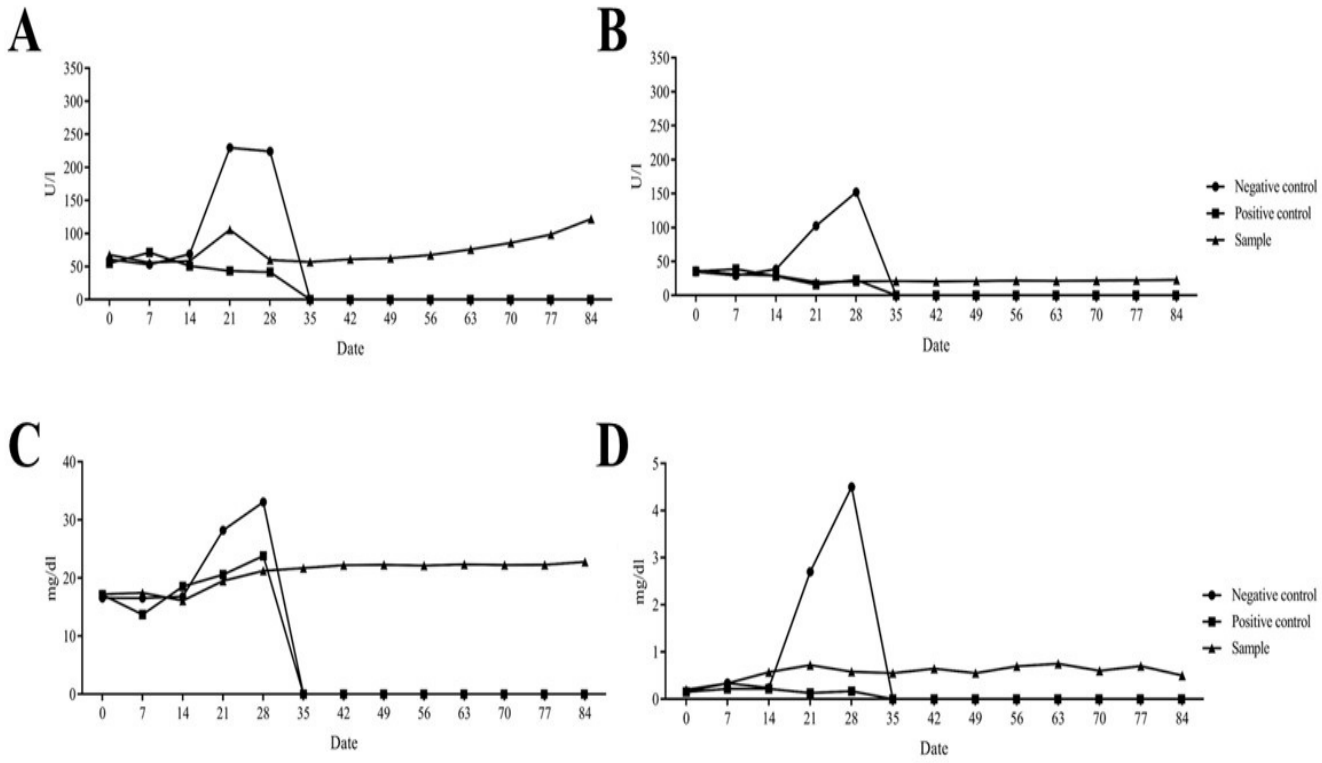

Figure 5: Analysis of mice's liver and kidney indexes in each group on D0-D84 of experiment. (A) The changes of GOT value. (B) The changes of GPT value. (C) The changes of BUN value. (D) The changes of CRE value. The data was expressed as mean ± SD. Glutamic-oxalocetic transaminase (GOT); Glutamic-pyruvic transaminase (GPT); Blood urea nitrogen (BUN); Creatinine (CRE).

4.4. Analysis of Mice' Blood Biochemical Values in Each Group

Mice in each group were collected blood and detected blood biochemical values as GOT, GPT, BUN, and CRE. (Figure 5) was showed that the change of the blood biochemical values on D0-D84 of experiment. The mice in the negative control group (Negative control) and the positive control group (Positive control) were dead on D33 of experiment, only the probiotic complex group (Sample) could be collected blood sample and detected the blood biochemical values. (Figure 6) was presented that the change of the blood biochemical values on D0-D21 of experiment for the negative control group (Negative control), the positive control group (Positive control), and the probiotic complex group (Sam-

ple). Results were showed that (1) the changes of GOT index: At D21 of the experiment, they GOT value of the negative control group (Negative control) was significantly higher than that of the positive control group (Positive control) and the probiotic complex group (Sample) ($p < 0.001$), respectively. The GOT value of the probiotic complex group (Sample) was significantly higher than the positive control group (Positive control) ($p < 0.05$). (2) The changes of GPT value: At D21 of the experiment, the GPT value of the negative control group (Negative control) was significantly higher than the positive control group (Positive control) and the probiotic complex group (Sample) ($p < 0.001$), respectively. In addition, the GPT value of and the probiotic complex group (Sample) and the positive control group (Positive control) was equivalent and no significant difference between the groups ($p > 0.05$). (3) The changes of BUN value: At D7 of the experiment, the BUN values of the probiotic complex group (Sample) and the negative control group (Negative control) were nearing ($p > 0.05$). However, the BUN value of the probiotic complex group (Sample) was significant higher than the positive control group (Positive control) ($p < 0.001$). At D21 of the experiment, the BUN value of the negative control group (Negative control) was significantly higher than the positive control group (Positive control) and the probiotic complex group (Sample) ($p < 0.001$), respectively. However, there was no statistical difference between the probiotic complex group (Sample) and the positive control group (Positive control) ($p > 0.05$). (4) The changes of CRE index: At D14 of the experiment, the CRE value of the probiotic complex group (Sample) was significantly higher than the negative control group (Negative control) ($p < 0.01$) and the positive control group (Positive control) ($p < 0.001$), respectively. Also, there was a significant difference in the CRE value of the negative control group (Negative control) compared with the probiotic complex group (Sample) and the positive control group (Positive control) at D21of the experiment ($p < 0.001$), respectively (Figure 6).

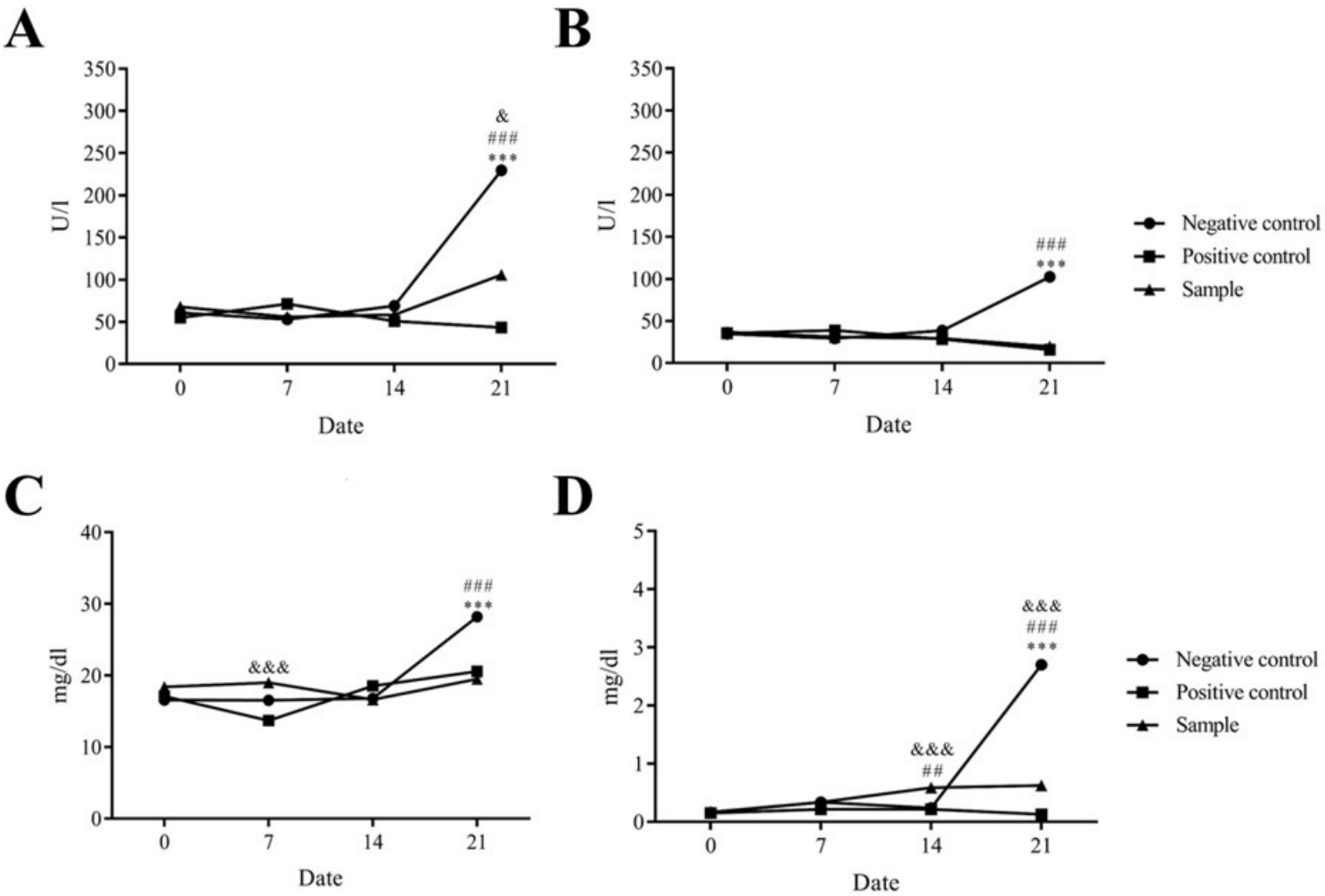

Figure 6: Analysis of mice's liver and kidney indexes in each group on D0-D21 of experiment. (A) The changes of GOT value. (B) The changes of GPT value. (C) The changes of BUN value. (D) The changes of CRE value. The data was expressed as mean ± SD. All significant differences were reported at $^{*/\#/\&}p < 0.05$, $^{**/\#\#/\&\&}p < 0.01$, $^{***/\#\#\#/\&\&\&}p < 0.001$. Symbol * indicated the comparison of negative control group and positive control group. Symbol # indicated the comparison of negative control group and sample group. Symbol & indicated the comparison of positive control group and sample group. Glutamic-oxalocetic transaminase (GOT); Glutamic-pyruvic transaminase (GPT); Blood urea nitrogen (BUN); Creatinine (CRE).

4.5. Histo-pathological Examination of Lung Tissues in Mice

The lung tissues of mice were embedded in the paraffin and were sliced. The lung tissue slices were stained with H&E to perform the histo-pathological examination. Three items, tumor cell infiltration, tumor cell mitosis, and tumor cell necrosis, were observed by a senior pathological veterinarian. Results were presented that (1) Tumor cell infiltration: The area of tumor cell infiltration was evaluated under an optical microscope at 40× visual fields. According to the severity, this item was divided into 5 levels, respectively: Grade 0: no disease; Grade 1: slight severity (1%-25%); Grade 2: mild severity (26%-50%); Grade 3: moderate severity (51%-75%); Grade 4: most severity (76%-100%). (2) Tumor cell mitosis: The level of the tumor cell mitosis is vigorous and highly positively correlated with the severity of the disease. The number of tumor mitosis was observed and recorded under an optical microscope at 400× visual fields. (3) Tumor cell necrosis: The aggressiveness and growth of lung cancer cells are rapid, and the near-central area of the tumor mass is susceptible to necrosis for insufficient vascular and nutrient supply. The level of tumor cell necrosis was evaluated under an optical microscope at 12.5× visual fields. The data showed that (1) Tumor cell infiltration: To compare the tumor cell infiltration for each group. The negative control group (Negative control)was the most serious than the positive control group(Positive control) and the probiotic complex group (Sample). (2) Tumor cell mitosis: The negative control group (Negative control) was the most active tumor cell mitosis, followed by the probiotic complex group (Sample), and the positive control group (Positive control) was less mitosis. (3) Tumor cell necrosis: The negative control group (Negative control) was the most serious tumor cell necrosis, followed by the probiotic complex group (Sample), and the positive control group (Positive control) was less tumor cell necrosis (Figures 7-8).

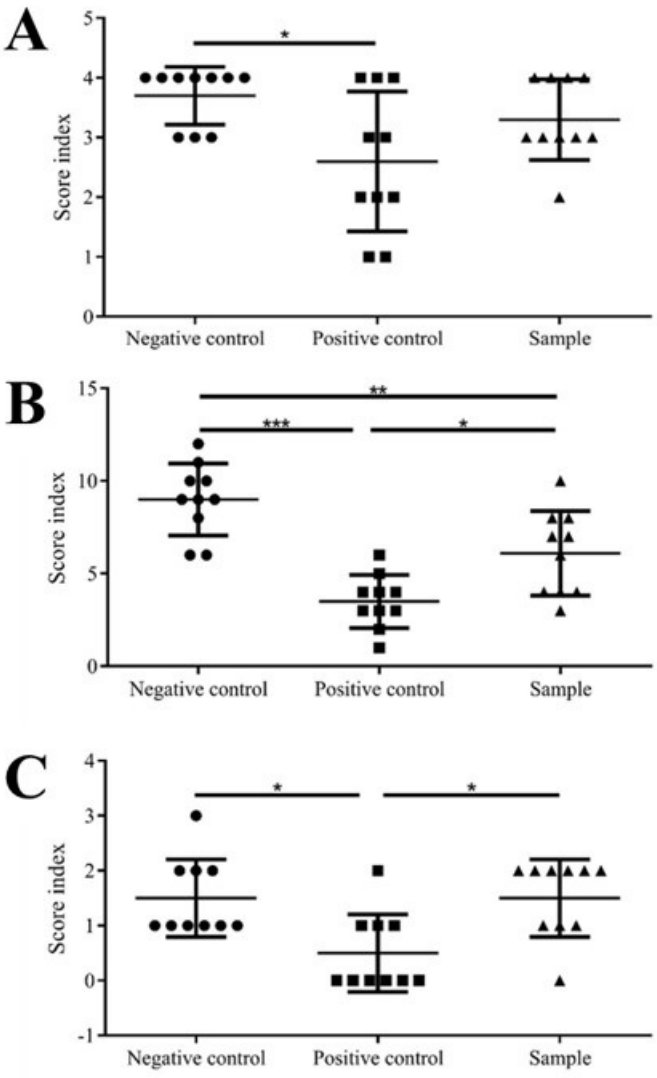

Figure 7: Analysis of the histo-pathological examination of lung tissue. (A) The score of the tumor cell infiltration was scored for the negative control group, the positive control group, and the probiotic complex group (Sample). There are 5 grades as Grade 0: no disease; Grade 1: slight severity (1%-25%); Grade 2: mild severity (26%-50%); Grade 3: moderate severity (51%-75%); Grade 4: most severity (76%-100%). (B) The score of the tumor cell mitosis was calculated for the negative control group, the positive control group, and the probiotic complex group (Sample). The number of cell divisions was calculated under the 400× microscope. (C) The score of the severity score of tumor cell necrosis was scored for the negative control group, the positive control group, and the probiotic complex group (Sample). There are 4 grades as Grade 0: no disease; Grade 1: mild; Grade 2: moderate; Grade 3: severe. The data presented mean ± SD. All significant differences compared with the negative control were reported at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

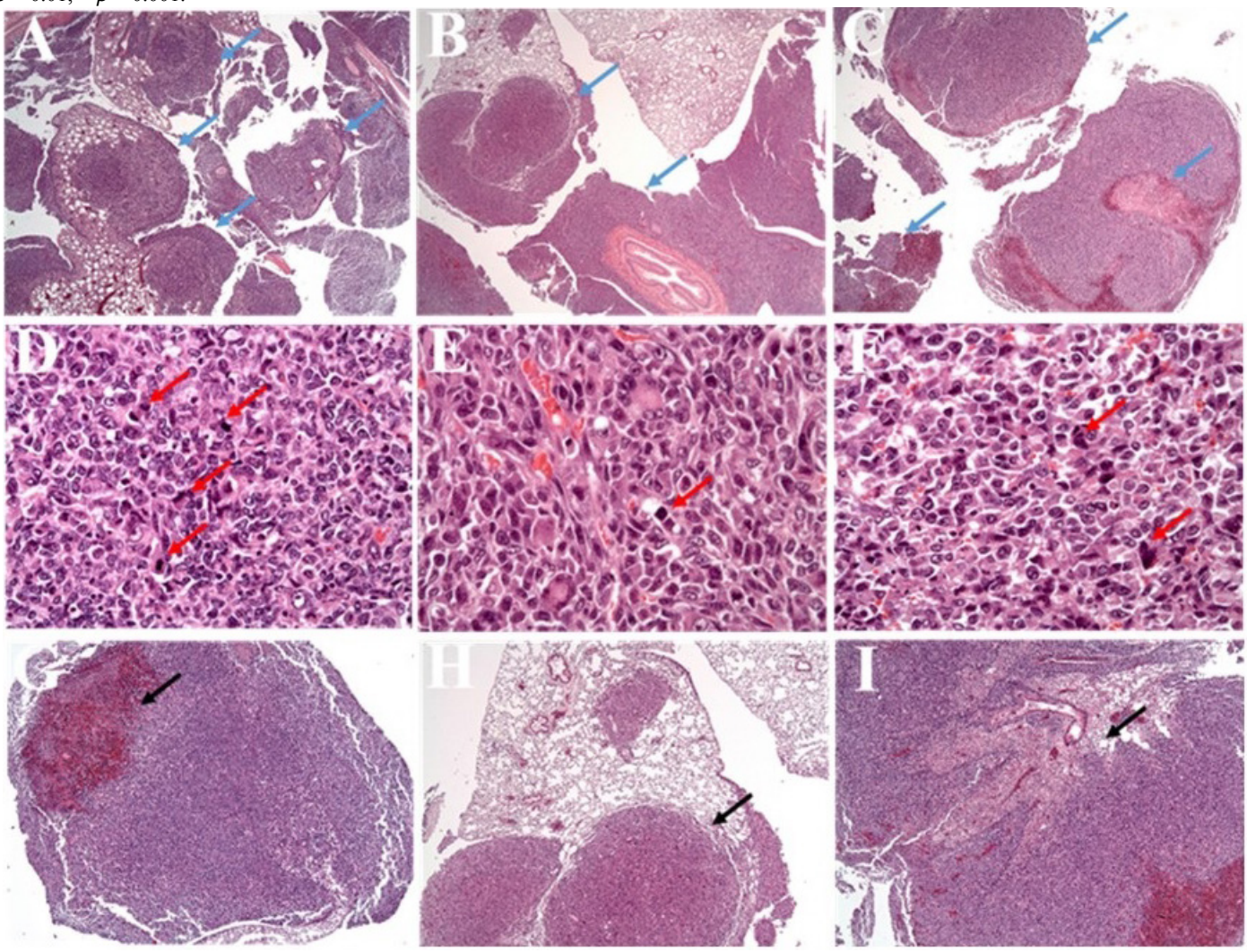

Figure 8: Histo-pathological examination of mouse's lung tissues. (A) The tumor infiltration status of the negative control group. (B) The tumor infiltration status of the positive control group. (C) The tumor infiltration status of the probiotic complex group (Sample). (D) The cell mitosis status in the negative control group. (E) The cell mitosis status of the positive control group. (F) The cell mitosis status of the probiotic complex group (Sample). (G) The tumor necrosis status of the negative control group. (H) The tumor necrosis of the positive control group. (I) The tumor necrosis status of the probiotic complex group (Sample). Blue arrow represented tumor infiltration area. Red arrow represented cell mitosis. Black arrow represented tumor necrosis.

5. Discussion

The important causes of cancer like living habits, genes, and environment etc. According to the statistic report in Taiwan, cancer has been the top 10 cause of death for many years. According to the latest 2019 reports, the top 5 cancers among Taiwanese are lung cancer, liver and intra hepatic cholangiocarcinoma, colorectal and anal cancer, female breast cancer, and oral cancer. In 2019, WHO lists cancer as one of top 10 threats to public health. Nearly, 10 million people die of cancer each year worldwide. Additionally, some reports presented that the global cancer cases will be increase to 60% in 2040. There may be nearly 29.4 million new cases of cancer each year [7-8]. According to the latest global cancer incidence rate published by the Organization for Economic Cooperation and Development (OECD), Taiwan's cancer incidence rate is 296.7 per 100,000 population, ranking 10th among 45 countries in the world [7-8]. Therefore, R&D of anti-tumor drugs is very need for these patients with cancer.

The treatment of lung cancer depends on the type of cancer cell,

the degree of metastasis, and the patient's physical condition. The surgical therapy, chemotherapy, radiation therapy, targeted therapy and palliative care were common treatment methods [9-16]. For most patients with early-stage Non-Small-Cell Lung Carcinoma (NSCLC), surgical removal of the tumor will be selected, and radiation therapy will be performed at the resection site to reduce the risk of possible recurrence. Small Cell Lung Cancer (SCLC) is usually treated with Prophylactic Cranial Irradiation (PCI), chemotherapy and/or radiation therapy, or early SCLC is surgically removed, followed by radiation therapy [17-22]. In addition, the targeted therapy is becoming more and more important for the advanced lung cancer.

Cancer is a major public health problem in the world. It is currently the first leading cause of death in Taiwan. Therefore, the establishment of the suitable cancer-bearing animal models and therapeutic strategies for development of more effective treatments for inhibition, not only of proliferation, but also of cancer metastasis is urgently needed [17]. In this study, LL/2 murine lung cancer cell line was NSCLC type. We presented the successful establishment of an orthotropic allograft NSCLC-bearing mouse model. We hope this NSCLC-bearing mouse model will be applied to research and develop the novel anti-cancer drug in the future. On the other hand, probiotic complex feed additive was firstly applied to therapy LL/2 murine lung cancer-bearing experimental mice. After one month oral administration of probiotic complex feed additive in C57BL/6 mice, the results were showed that the probiotic complex has a positive effect on inhibiting LL/2 lung cancer growth *in situ* in mice.

6. Conclusion

This study was investigated the inhibition efficacy of LL/2 lung cancer via orally administered with probiotic complex feed additive for three months in an orthotopic LL/2 lung cancer-bearing mouse model. These results in each group were showed as BW of mice, the survival rate of mice, the average lung weight of mice, the number of tumor nodules in lung of mice, four index values of liver and kidney function of mice, and the histo-pathological examination (tumor cell infiltration, tumor cell mitosis, and tumor cell necrosis) of tumor in lung. Based on these results, although the probiotic complex group was slightly worse than the positive control group, but the probiotic complex group was significantly better in side effects compared to the positive control group, the survival rate and survival time of the probiotic complex group were significantly higher and longer than those in the positive control group. The overall efficacy and safety data showed that the probiotic complex group was significantly better in again LL/2 lung cancer than the positive control group. The probiotic complex had safety and the positive effects on the inhibition of *in situ* LL/2 lung cancer in mice.

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