Iranian Journal of Microbiology

Volume 13 Number 3 (June 2021) 381-388

ORIGINAL ARTICLE

Effect of giving probiotic supplement *Lactobacillus acidophilus* La-14 as an immunomodulator to maintain a respiratory system in *Mus musculus*

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Received: February 2021**, Accepted:** May 2021

ABSTRACT

Background and Objectives: In 2020 the whole world is experiencing a pandemic condition due to infection with the SARS-CoV-2 virus which can cause the COVID-19 disease. This condition results in "Panic Buying", because everyone tries to avoid the spread and transmission of the COVID-19 disease by doing various ways, one of which is by taking additional supplements such as vitamin C and probiotic supplements.

Materials and Methods: The materials used were mice *Mus musculus* male DDY strain aged 1-2 months. Probiotic supplement *Lactobacillus acidophilus* La-14 with a viability of 1×10^8 CFU/ml. with a weight of 0.16 grams dissolved in 0.25 ml 0.9% NaCl. Vitamin C used is a commercial vitamin C tablet, weighing 0.06 grams in 0.25 ml 0.9% NaCl. Meanwhile, the feed for mice *(Mus musculus)* is a complete feed from Pokphand with the code BR1 CP511B. Lung histology preparations data were analysed descriptively and statistically through the test *Chi square* while the data on the number of lymphocytes were analysed descriptively.

Results: The histological observations of lungs of *Mus musculus* showed that in the treatment of ML, MV, and MKA test was carried out *chi square* ratio between the groups that did not have lymphocyte infiltration and those that had lymphocyte infiltration showed a significant difference ($p < 0.05$). Meanwhile, the results of the lymphocyte count showed that ML and MV treatment was higher than that of MK treatment.

Conclusion: It is suggested that the administration of probiotics can stimulate and modulate the respiratory immune system.

Keywords: *Lactobacillus acidophilus*; Vitamin C; Lymphocyte infiltration; Lymphocytes; *Mus musculus*

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INTRODUCTION

In 2020 the whole world is experiencing a pandemic condition due to infection with the SARS-CoV-2 virus which can cause the COVID-19 disease. This condition results in "Panic Buying", because everyone tries to avoid the spread and transmission

of the COVID-19 disease by doing various ways, one of which is by taking additional supplements such as vitamin C and probiotic supplements (1). Probiotic supplements are supplements that can be used to protect and maintain the health of the digestive system, especially the stomach and intestines (2).

Microorganisms have actually been widely used for a long time, both in the food and health industries. According to the Food and Agriculture Organization (FAO) and the World Health Organization (WHO), probiotic strains, which are a group of lactic acid bacteria, if consumed in sufficient quantities can provide health benefits for the body (3). The bacterial species that were first used as probiotics were *Lactobacillus acidophilus* and *Bifidobacterium bifidum* (4). Several studies state that *L. acidophilus* besides having anti-diarrhoea ability, also has a function as an immunomodulator or enhances the body's immune system so that it can prevent disease infections in both animals and humans (5, 6).

The way probiotic bacteria work can be started from the effect of their action on the immune system. *Lactobacillus* that is eaten can stimulate the activity of macrophages against several different bacterial species (7). Probiotic bacteria can affect directly or indirectly, because they can stimulate induction causing changes in macrophages. This effect is demonstrated by the different types of lymphocytes. Probiotic bacteria have the effect of modulating the immune response against harmful antigens through B lymphocytes and the production of antibodies (8).

The defence of the body's immune system starts from the skin which functions as a barrier to the invasion of antigens into the body. Then enter the physiological stage, where the body temperature and pH can respond so as to create conditions that are not suitable for these antigens. After that the next step is the antigen that has successfully entered the body can fight against the innate and acquired immune systems. The two immune systems consist of many types, one of which is lymphocytes to detect and eliminate pathogens (9).

Immunomodulators work at various levels of the body's immune system. Therefore, various types of drugs have been developed which selectively inhibit or intensify immune cell populations and subpopulations. Immunomodulators cause measurable changes in immune function. Immunomodulators affect cells that produce dissolved mediators such as cytokines (10). Immunosuppressants can inhibit the immune response in organ transplantation and autoimmune diseases such as pemphigus, lupus and / or allergies (11, 12). Meanwhile, immunostimulants increase the immune response in infectious diseases, tumors, primary or secondary immunodeficiency, and changes in antibody transfer (13). Immunomodulators can work specifically and not specifically. The specific action of immunomodulators affects the immune system due to the presence of certain antigens or immunogens, with selective specificity for the immune response (10). Immunomodulation is selective when stimulation is interpreted as an immune reaction to one or several antigens, for example the response to vaccine administration (14). The non-specific action of immunomodulators is used to stimulate or suppress immune responses, without directing stimulated cell activity to specific antigens. They are divided into three types: type I, acting on the normal immune system; type II, works on a suppressed immune system; and type III, acting on a normal functioning immune system and immunosuppression.

Based on the results of previous research, it was stated that *L. acidophilus* can be used to stimulate the body's immune system. However, until now there has never been any testing of *L. acidophilus* ability to increase the respiratory immune system. Therefore, this research aimed to examine the ability of *L. acidophilus* for infiltration that occurs in the lungs and the number of lymphocyte cells. Therefore, the results of this research can later be used as a reference to increase immunity in the respiratory tract, especially during the Covid-19 pandemic.

MATERIALS AND METHODS

Place and time of research. The research and development place of care and intervention for experimental animals was carried out at Pusvetma of Surabaya, while microscopic observations of the results were carried out at the Microbiology Laboratory in Faculty of Health Sciences, Muhammadiyah University of Surabaya. This research was conducted from October to December 2020.

Tools and materials. The tools used 1 ml syringe, 3 ml syringe, Olympus CX 23 microscope, mortar and pestle, Sonde, SV-490C brand Digital Scales. The materials used were mice *Mus musculus* male DDY strain aged 1-2 months. Probiotic supplement *L.*

acidophilus La-14 with a viability of 1×10^8 CFU/ml. with a weight of 0.16 grams dissolved in 0.25 ml 0.9% NaCl. Vitamin C used is a commercial vitamin C tablet, weighing 0.06 grams in 0.25 ml 0.9% NaCl. Meanwhile, the feed for mice *(Mus musculus)* is a complete feed from Pokphand with the code BR1 CP511B.

Data collection technique. The type of research was research true experimental with Post Test Only Control Group Design, which used experimental mice *(Mus musculus)* aged 1-2 months as research objects. There were three treatments used in this research, namely the first treatment with provision of feed and suspension probiotic supplements *L. acidophilus* of 0.25 ml of La-14 (ML)/ mouse, the second treatment with feeding and suspension of vitamin C (MV) as much as 0 , 25 ml / mouse and the third treatment was a control treatment (MK) which was only given feed without being given probiotic supplements of *L. acidophilus* La-14 or vitamin C in the research object. The treatment was carried out every day for 7 days of observation.

Observation of lymphocyte infiltration. The observation was made by making histological preparations of lungs *Mus musculus* using Haematoxylin Eosin (HE) staining. Furthermore, the results of the preparations were observed for the presence or absence of lymphocyte infiltration as seen from the infiltration rate in the histological lung preparations. The presence of lymphocyte infiltration on lung preparations indicated an immune response.

Observation of the number of lymphocytes. The observation of the number of lymphocytes was done by taking blood from the tail. Then the staining was done by using the Wright method, after it was carried out differential counting per 100 cells to count the number of leukocytes, one of which was lymphocytes. Observation of the number of lymphocytes on a reading of differential counting per 100 cells showed a marker of an immune response.

Data analysis. Lymphocyte infiltration data were analysed using the *Chi-Square* Test. The variables observed were on a categorical scale (nominal / ordinal). The results of the research were considered significant if the p value was <0.05. While the data on the number of lymphocytes in the differential counting reading per 100 cells were analysed descriptively which was presented in graphical form.

RESULTS

Observation results of lymphocyte infiltration. The results of lymphocyte infiltration through pulmonary histology observations obtained were then classified into several levels. Levels of lymphocyte infiltration included Level 0: there was no lymphocyte infiltration in the tissue, Level 1: there were lymphocyte cells scattered with <10 lymphocyte cells in each field of view, Level 2: there were lymphocyte cells scattered with> 10 lymphocytes in each field of view, Level 3: in some areas there was a dense collection of lymphocyte cells, Level 4: there was a dense diffusion of lymphocyte cell infiltration, Level 5: almost the entire stroma of the cancerous tissue had lymphocyte cell infiltration, Level 6: the entire stroma of the cancerous tissue contained a dense collection of lymphocyte cells (15).

Comparison of the ML treatment, the ratio of the infiltration rate of Level 0 and Level 1 was 1: 1, the MV treatment ratio of the infiltration level of Level 1 and Level 2 is 0: 1, and the treatment of the ratio of the infiltration rate of Level 1 and Level 2 were 0: 1. (Fig. 1). Based on the analysis of the *chi square* test, ratio between the TTI (infiltration level 0) and TI (infiltration level 1 and level 2) groups, there was a significant difference (p <0.05). The difference was shown by count> Table with a count value of 8 and a Table value of 3.84. In the treatment of giving probiotic supplements *L. acidophilus* La-14 from a total of 6 samples given the treatment, 3 samples of mice were identified as not having lymphocyte infiltration, and from 3 other samples identified as having infil-

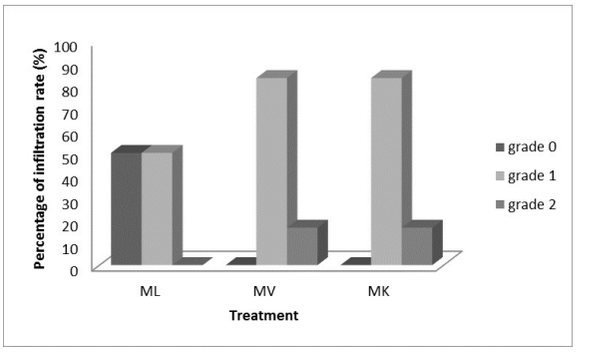

Fig. 1. Percentage of lymphocyte infiltration in lungs of *M. musculus* in each treatment

tration. Whereas in the treatment with vitamin C and control treatment from a total of 6 samples in all of these treatments, it was identified that lymphocyte infiltration was the same as the control treatment.

The observation result of microscopic was on the histological preparations of mice lungs of *(M. musculus)* with a 100× in the treatment with magnification of the objective lens probiotic supplementation *L. acidophilus* La-14 can be observed that in Fig. 2 ML1, ML2 and ML4 there was no lymphocyte infiltration or level 0, and in Fig. 2 ML3, ML5, and ML6 occurred lymphocyte infiltration in level 1. In the treatment with vitamin C that can be observed that in Fig. 3 MV1, MV2, MV3, MV4, and MV5 occurred level 1 lymphocyte infiltration, and in Fig. 3 MV6 was occurred lymphocyte filtration in Level 2. In the control treatment, the results showed that in Fig. 4 MK3 was occurred lymphocyte infiltration in level 2, and in Fig. 4 MK1, MK2, MK4, MK5, and MK6 was occurred lymphocyte infiltration in level 1.

The difference in the occurrence of lymphocyte infiltration in pulmonary histologic preparations was due to the addition of supplements as a marker of modulation of immune response. The response was characterized by lymphocyte morphology and the number of lymphocyte infiltrations in the histological pulmonary preparations in each treatment which was indicated by a red arrow in the image.

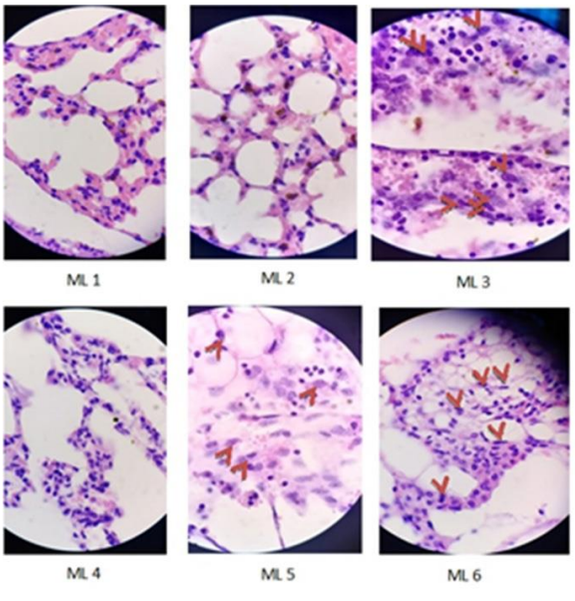

Fig. 2. Histology of lungs *M. musculus* in treatment *Lactobacillus acidophilus*, microscopic observation with 100× magnification of objective lens.

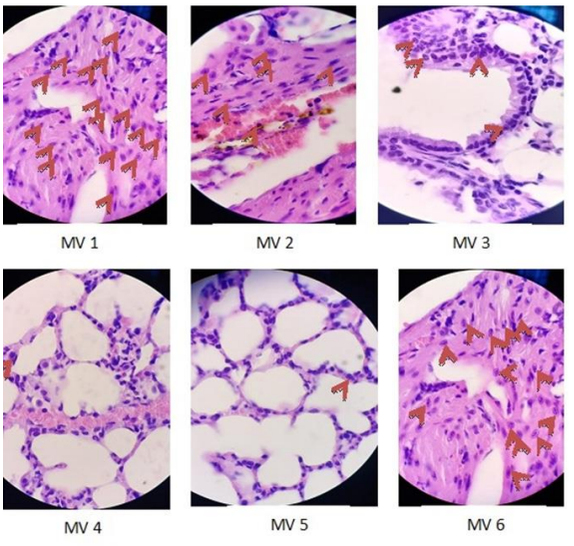

Fig. 3. Histology of lungs *M. musculus* in Vitamin C treatment, microscopic observation with 100×magnification of objective lens

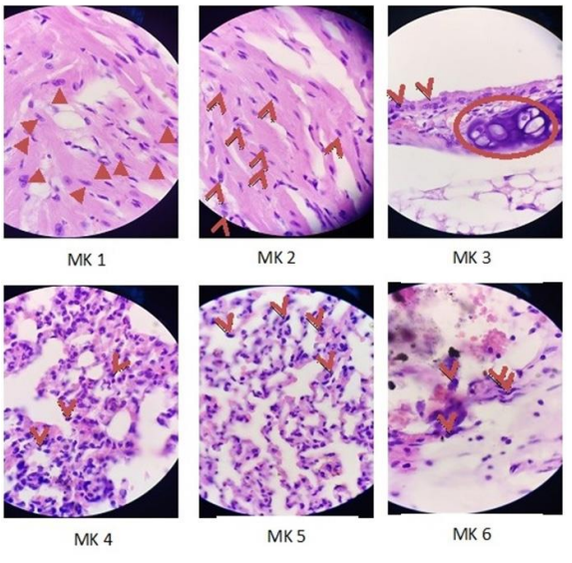

Fig. 4. Lung histology in control treatment samples, microscopic observation with 100× magnification of objective lens

The observation result of the number of lymphocytes. In this research as many as 6 samples of mice *(M. musculus)* were used in each treatment. The examination was carried out on the 7th day in which each mouse was carried out a reading of differential counting per 100 cells from the peripheral blood cells in the tail. The ML treatment resulted in lymphocyte counts with an average number of around 46 cells. In MV treatment, lymphocyte counts were obtained

with an average number of around 42 cells. Whereas in MK treatment, the lymphocyte count was 27 cells. From the three treatments, it can be observed that the highest number of lymphocytes was found in ML treatment, while the lowest number of lymphocytes is in treatment MK (Fig. 5). The difference in the number of lymphocytes in the control and treatment indicated that the presence of both probiotic supplements and vitamin C supplements could increase the lymphocyte count compared to those without receiving any supplements.

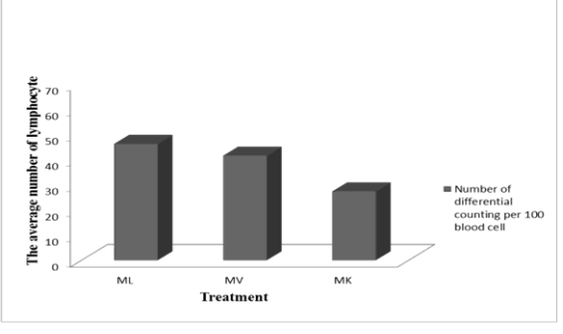

Fig. 5. The average number of lymphocyte cells observed by the peripheral blood smear of each experimental animal in each treatment with a differential counting reading per 100 cells

DISCUSSION

The immune system consisted of the natural and adaptive immune systems which were interrelated. The presence of an immune response was marked by the response of lymphocyte infiltration to the tissue and differences in the number of lymphocytes in each treatment. The results showed that there were differences in the treatment of probiotic supplements *L. acidophilus* La-14 and vitamin C, compared with a control group.

Lymphocyte infiltration in the lungs. The results showed that the infiltration rate in the provision of probiotic supplements was *L. acidophilus* La-14 included in levels 0 and 1 less than vitamin C administration and controls included in levels 1 and 2. Infiltration was a systemic disorder affecting the cells. The cells that were originally healthy which resulted in many metabolites accumulating in the cells excessively. At first, metabolites were found in cells but then these metabolites damage the cell structure (14). The incidence of lymphocyte infiltration can be related to an inflammatory reaction, because normally the body can remain exposed to other substances that have the potential to trigger inflammation from the environment. Therefore, it was hoped that the consumption of additional supplements can protect the body by increasing the immune system.

The treatment of probiotic supplementation *L. acidophilus* La-14 showed an anti-inflammatory or anti-inflammatory ability as an immunosuppressant which was part of the immunomodulatory ability. Probiotics were able to shift an allergic or inflammatory reaction so that they can reduce the rate of inflammation similar to antihistamine drugs. In addition, probiotics can also normalize the body barrier in respiration, regulate inflammatory mediators that also increase the development of the immune system, and stimulate an immune response (16). Apart from acting as immunosuppressants, probiotic bacteria can also strengthen the immune system because of the help of mucus (17). The adherence of probiotics to the mucus was caused by a protein substance that was owned by the probiotic. This substance was called “mucus-binding protein” (mucus binding protein), which was found in higher numbers in lactic acid-producing bacteria, with the presence of this protein probiotic bacteria can attach to the mucus and interact with the host (18).

Administration of vitamin C in cases of immune enhancement is usually associated with antioxidant abilities. Antioxidants in vitamin C are included in the group that acts as free radical scavenger by breaking ROS bond. Vitamin C used in this research was pure vitamin C which was very acidic. Organic vitamin C derived from natural ingredients was better than inert vitamin C for maintaining the immune system (19). Therefore, the organic vitamin C from natural ingredients or pure vitamin C supplements as ascorbic acid can be more effective when consumed with a nutritionally balanced diet compared to only taking vitamin C supplements without considering the intake of other nutrients to increase and maintain the body's immunity. Vitamin C supplements alone can only be effective when there were free radicals. Therefore, they can immediately react with free radicals. This results in the treatment of lymphocyte infiltration still occurring even though it was still at levels 1 and 2. In the same control treatment with vitamin C treatment, there was lymphocyte infiltration in lung tissue at levels 1 and 2. These results

indicated that in normal conditions without additional supplement treatment, the body responds to the disturbance by the occurrence of an inflammatory process characterized by lymphocyte infiltration.

Based on the results obtained, it was shown that the treatment of probiotic supplementation *L. acidophilus* La-14 can increase the body's immune system. This was indicated by the higher number of lymphocyte cells than the control and not much different from vitamin C administration in general. The effect of giving probiotic supplements *L. acidophilus* La-14 in the first mice had the effect of increasing the number of lymphocytes as an immune response. Probiotic bacteria may have beneficial effects and modulate the immune response against potentially harmful antigens via B lymphocytes and antibody production. For example, *L. rhamnoses* GG given to children with acute gastroenteritis increases the humoral non-specific immune response, which was characterized by increased secretion of Ig G, Ig A and Ig M from circulating lymphocytes. *Bifidobacterium bifidum* increase the antibody response to ovalbumin, and yogurt containing *L. acidophilus, L. bulgaricus, S. thermophilus, B. bifidum* and stimulate IgA response to cholera toxin in mice (8).

Capacity of probiotics in one of which increases the protective response was the stimulation of immunity (20). This had great potential benefits to the immune system which can stimulate non-specific immunity (21). Probiotics affected non-specific immunity, this included two systems, namely a system that worked with antibodies secreted by B lymphocytes (humoral immunity) and other operating systems through T lymphocytes directly (cell-mediated immunity). Some researchers also reported that the consumption of lactic acid bacteria (LAB) group Lactobacillus can improve the immune system of cellular and humoral such as population increase and proliferation of lymphocytes (6). On the other research also stated that the administration of *L. acidophilus* FNCC 005 in mice by EPEC affect immunomodulatory properties, namely increasing the amount of immunoglobulins (5). Lactic Acid Bacteria (LAB) *L. acidophilus* FNCC 005 has potential as an antidiarrheal and increases the immune system immunomodulators (5).

Various mechanisms that may be involved as a result of giving probiotics, not only affected locally but also systemically. The mechanisms and effects that were occurred also in accordance with the bacterial strain used whether a single strain or a combination strain, because the function and efficacy (ability) of probiotic bacteria in both in vivo and in vitro experiments are strain specific (the efficacy of one probiotic strain can not be generalized to probiotics with other strains (22, 23). One of the beneficial properties possessed by probiotics increased the immune status of the host by inducing the cells involved in innate immunity (24). The immunomodulation mechanism by probiotic bacteria by activating Il-6 was a clonal expansion of IgA B lymphocytes (23). There was also an association with increased production of antibodies such as IgM, IgG and decreased IgE secretion. This was consistent with the theory that giving probiotics that can increase the innate immune response and stated that symbiotic stimulate innate and adaptive immune responses (25). The mechanism of action of probiotics was also highly dependent on the types of cells involved in both the adaptive and innate immune responses (26).

Vitamin C, which had been known to increase the body's immune system (27). The treatment of mice also had the same effect, namely increasing the number of lymphocytes as in the treatment of probiotic supplements *L. acidophilus* La-14. Vitamin C interferes in the body's biochemical functions, including the synthesis of amino acids, helps the absorption of iron and stimulates the activity of antibodies and immune system cells. The immune system in question that was formed from white blood cells, bone marrow and lymph tissue such as leukocytes, B lymphocytes (B cells) and T lymphocytes (T cells) which were involved in immunity (28). *In vitro* studies had shown that incubation of vitamin C with lymphocytes increased lymphocyte proliferation or cell division, increased antibody formation, and also provided resistance to various stimuli of cell death. Furthermore, vitamin C appeared to have an important role in the differentiation of T-cell development and maturation (29). Whereas in the control treatment that took only regular feeding, the number of lymphocyte did not increase significantly compared to treatment with probiotic supplements of *L. acidophilus* La-14 and vitamin C.

CONCLUSION

Probiotic supplementation of *L. acidophilus* La-14 in *Mus musculus* had a role in inducing immune cells so that it can stimulate and modulate the respiratory

immune system, namely by the presence of lymphocyte infiltration and a higher number of lymphocytes than without consumption of probiotic supplements. This meant that the consumption of probiotic supplements can also improve the respiratory immune system in humans.

ACKNOWLEDGEMENTS

The author would like to thank the PP Muhammadiyah Diktilitbang Council for financial assistance through the Funding and Implementation of the Batch 4 Covid-19 Research Grant Program to Dita Artanti and Members with contract number 0887.122 / I.3 / D / 2020.

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