

Leukocytes Count and Lymphocyte Proliferation of Dinitrochlorobenzene Sensitized Rat Supplemented with Fermented Goat Milk

Nurliyani, Eni Harmayani, Marsetyawan HNE Soesatyo

Abstract—Goat milk has an hypoallergenic effects, and allergic diseases related to abnormal of intestinal flora. Probiotic microorganisms do exert an activity on the immune system in the skin of the individual. The purpose of this study are to determine the number of leukocyte and lymphocyte proliferation in rat supplemented with fermented goat milk (acidophilus milk and kefir) and sensitized with dinitrochlorobenzene (DNCB). Female Wistar rats 6-8 weeks olds were divided into 3 treatment groups. The first group supplemented goat milk kefir, second group acidophilus goat milk, and third group as control. During 28-day experiment, on day 15 rat sensitized with allergen DNCB on the dorsal of the body, and on day 24 was challenged with DNCB on the ear. Sampling of blood and tissue of intestinal Peyer's patch (PP) were performed on day 14 (before DNCB sensitized) and on day 28 (after DNCB sensitized). The results showed the number of neutrophils in rats supplemented with acidophilus milk was higher ($P < 0.05$) in after DNCB sensitized than before, but the lymphocyte count was lower. The number of monocytes, eosinophils, and basophils before and after DNCB sensitized have the same average for all treatments of milk fermented and control. Fermented goat milk (kefir and acidophilus milk) did not affect on rat PP lymphocyte proliferation culture supernatant, whereas the rat that had been DNCB sensitized showed higher in proliferative response to PHA mitogen ($P < 0.05$) than before sensitized. In conclusion, supplementation of acidophilus goat milk with a dose of 2.0 ml / head / day on DNCB sensitized rat, can increase the number of neutrophils that play a role in innate immunity, however it was not able to increase lymphocyte proliferation that related to adaptive immunity.

Keywords—Leukocytes, Lymphocyte proliferation, Kefir, Acidophilus milk, Dinitrochlorobenzene

I. INTRODUCTION

RECENTLY, dairy goat farming such as Ettawah Crossed Bred in Indonesia started to grow, with the vigorous promotion of the health benefits of goat's milk. Demand

for goat's milk is usually used for patients with gastrointestinal disorders and allergic to cow's milk. Demand for goat's milk continues to increase, especially in developing countries that aware the importance of prevention of disease with natural food. Goat milk hypoallergenic products not yet available in the market, which is available on the market is hypoallergenic milk products are still using ingredients from cow's milk, and soy. Positive effect of fermented milk containing probiotics, especially the health of the digestive tract associated with immunomodulatory properties, because probiotics can control the excessive activity of Th1 and Th2 cytokines. Allergic contact dermatitis (particularly Th1 / T helper 1) is a common allergic disease of the skin. Th1 cells mainly produce IL-2 and IFN- γ associated with protective immunity and DTH responses (delayed-type hypersensitivity), which can be known by the edema. Besides, the allergy can also be seen from the number of leukocytes. Contact hypersensitivity is a T cell-mediated cutaneous immune reaction to haptens. Depending upon the allergen, Langerhan cells (LC) can either bind the hapten to MHC (major histocompatibility complex) molecules on the surface directly or internally process the allergen into complete antigens. LCs then migrate via the afferent lymphatic vessels into skin-draining regional lymph nodes to present the haptened peptides to naïve T cells [1].

Some studies indicate that there are differences in the composition of intestinal flora between patients with atopic and non-atopic, as well as between industrialized countries and developing countries. If allergy sufferers infected by pathogens, the Th2 response tends to shift to Th1, which means allergy symptoms decrease or disappear. This is in accordance with the "hygiene hypothesis" or "germless theory" [2], which states that the limited exposure to pathogenic bacteria and viruses during childhood result in insufficient stimulation of Th1 cells, resulting in expansion or stimulation to Th2 cells which tend to cause allergic disease. Lately there is an alternative interpretation that supports the hygiene hypothesis, the "microflora hypothesis" [2]. This hypothesis states that cases of allergic diseases as a result of gastrointestinal microbiota disruption, because antibiotics and dietary changes in these countries. Therefore, probiotic bacteria is a candidate agent for the prevention and treatment of allergic disease with a favorable improvement immunoregulator signal [2]. Recently, it has been known that the regulation of lymphoid tissue in the digestive tract can occur by the activity of probiotics, thus allowing the lactic acid bacteria can be used for positive interaction with intestinal cells. Live lactic acid bacteria (probiotics) derived

Nurliyani, Department of Animal Product Technology, Faculty of Animal Science, Universitas Gadjah Mada, Jl. Fauna, no. 3, Bulaksumur, Yogyakarta 55281, Indonesia (corresponding author: phone +62-274-513363; fax: +62-274-521578; e-mail: nurliyani@yahoo.com)

Eni Harmayani, Faculty of Agriculture Technology, Universitas Gadjah Mada, Jl. Sosio Yustisia, Bulaksumur, Yogyakarta, Indonesia; phone/fax: +62-274-549650; e-mail: eniharmayani@yahoo.com

Marsetyawan HNE Soesatyo, Departmen of Histology and Cell Biology, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia; e-mail: marshnes@yahoo.com

from human can help restore normal function of intestinal microbes, reducing the symptoms of the disease, expressing the influence of anti-carcinogenic and anti-atherogenic [3]. Surprisingly found that probiotics do also exert an effect in an individual's body at a location distant from the region in which they colonize it. In particular, it has been found that probiotic micro-organisms do also exert an activity on the immune system in the skin of the individual. Accordingly it has been found that upon ingestion by an individual they may balance a suppression of the skin's immune system inherent to exposure to stress, such as physical, chemical or biological stress, while they may also reduce the individual's tendency to develop inflammatory and/or irritant reactions upon exposure to such a stress condition [4]. Development of anti-allergy functional foods can be based on the food effect on the number of leukocytes (neutrophils, eosinophils, basophils, lymphocytes, and lymphocytes). It is therefore important to look for functional foods that can regulate the cells in the immune system to avoid allergic reactions that involves an inflammatory reaction. This study will explore the potency of fermented goat milk (kefir and acidophilus milk) as anti-allergy through the number of leukocytes and their effects on cellular immune response through the proliferation of lymphocytes from the PP culture supernatant on DNCB sensitized rat.

II .MATERIALS AND METHODS

A. Materials

Goat milk of Etawah Crossed Bred from Indonesia, kefir grain, *Lactobacillus acidophilus*, 2,4-Dinitrochlorobenzene (Sigma-Aldrich), acetone and corn oil as allergen solvent, RPMI (Roswell Park Memorial Institute)-1640 medium (Sigma), FBS (fetal bovine serum (Gibco), penicillin-streptomycin (Gibco), fungizone (Gibco), PBS (phosphate-buffered saline), ammonium chloride, PHA (phytohaemagglutinin) mitogen (Murex), MTT (methyltetrazolium) (Sigma), sodium dodecyl sulphate (SDS), and HCl, methanol, and Giemsa solution.

B. Research Design

Female Wistar rats 6-8 weeks old, were divided into 3 groups, each group used 6 rats. Group 1) rats supplemented goat's milk kefir, 2) rats supplemented acidophilus goat milk, and 3) control (given distilled water). On day 14 of starting treatment, the rats were sensitized with a contact allergen DNCB 5% in the dorsal of the body, and 10 days later on the dorsal ear leaf were challenged with 1% DNCB [5]. The experiment was conducted during 28 days. Blood sampling and small intestine PP tissue were performed on day 14 and day 28. During the experiment rats received the standard AIN-93 diet [6]. All procedures related to animal experiment were conducted following the recommendation of the Ethical Committee of Universitas Gadjah Mada, Indonesia (Ethical Clearance Number: KE/FK/194/EC).

C. Processing of Goat Milk Kefir and Acidophilus Goat Milk

Goat milk kefir was made by heating milk at 90-95° for 5-10 minutes, then cooled to room temperature (18-24°C). Milk

was inoculated with kefir grains as much as 2-8%, and fermented for 18-24 hours at room temperature. Kefir grain separated by filtration and stored at 4 ° C [7].

Goat milk acidophilus milk made heating at a temperature of 120 °C for 15 minutes, then cooled to 37-38°C. Milk inoculated with *Lactobacillus acidophilus* starter as much as 5% and incubated for 18-24 hours at 37-38°C, then cooled / stored at 4°C [8]. In this study conducted heating milk at 90 °C for 30 minutes.

Fermented milk was supplemented 2 ml / head / day for 28 days with force feeding method. Preparation of fermented milk was carried out every 7 days and stored in the refrigerator.

D. Leukocytes Count

Rat blood samples were taken with a hematocrit and additional anti-coagulant EDTA, then made preparations for blood smear on an object glass. Fixation of blood smear used absolute methanol, and then was stained with 10% Giemsa for 30 minutes. Blood smear preparations were washed with distilled water and dried. Observation and counting the number of leukocytes using microscop 100x magnification and the numbers are calculated as a percentage relative [9]

E. Lymphocyte Culture Supernatant Collection and Lymphocyte Proliferation Assay

Isolation and collection procedures of PPs lymphocyte were performed according to [10] and [11] with slight modifications. Briefly, PPs were excised aseptically from the small intestine side of the rat and placed in 10 ml of RPMI (Roswell Park Memorial Institute)- 1640 medium containing 10% FBS (fetal bovine serum: Gibco) and 2% penicillin-streptomycin (Gibco). The collected patches were washed with RPMI medium. The PP tissues were ripped with syringe tip, and also by pipetting up and down and spraying by RPMI medium a few times by using disposable syringe for releasing the lymphocytes. After releasing the lymphocytes from the PP tissue, the suspension was allowed to separate from cell debris. The supernatant was removed into conical tubes, and then the cells were counted by haemocytometer. The cell concentrations that to be cultured were 5×10^5 / ml. The lymphocytes were cultured in plate with 96 wells in RPMI medium, and were added with 5 µg / ml of PHA mitogen in each well. The volume of the lymphocyte culture was 100 µl in each well. The plate was placed into 5% CO₂ incubator for 72 hours at 37°C. Methyl tetrazolium (MTT) solution (5 mg/ml dissolved in PBS) was added 10 µl /well on microplate, and incubate for 4 hours at 5% CO₂ incubator. Ten percent SDS in 0.01 M HCl was added on microplate well (100 µl/well), and incubate overnight at room temperature. Read the optical density (OD) at 550 nm in microplate reader [11].

III. RESULTS

A. Leukocytes Count

1. Neutrophil

The average of neutrophil in rat supplemented with acidophilus goat milk was higher (P <0.05) after DNCB

sensitized compared to before sensitized. No difference of neutrophil in rat kefir supplemented and controls, before and after DNCB sensitized (Table I)

TABLE I
THE AVERAGE OF NEUTROPHIL (%)

Supplementation	Before	After	Average
Goat milk kefir	20.00 ^a	23.67 ^a	21.83
Acidophilus goat milk	21.00 ^a	36.17 ^b	28.58
Control	19.67 ^a	20.67 ^a	20.17
Average	20.22	26.83	23.52

^{a,b}Different superscripts in the same row and column showed significant difference (P<0.05).

2. Eosinophil

The average of eosinophils in rat supplemented with kefir was lower (P <0.05) in after DNCB sensitized than before, but the percentage of eosinophils was not different to the control and acidophilus milk supplementation (Table 2).

TABLE II
THE AVERAGE OF EOSINOPHIL (%)

Supplementation	Before	After	Average
Goat milk kefir	2.00 ^a	0.33 ^b	1.67
Acidophilus goat milk	0.00 ^b	0.67 ^{ab}	0.33
Control	1.33 ^{ab}	0.17 ^b	0.75
Average	1.11	0.38	0.75

^{a,b}Different superscripts in the same row and column showed significant difference (P<0.05).

3. Basophil

As shown in Table 3, fermented goat milk (kefir and acidophilus), that supplemented in rat has no effect on the number of basophils. Basophils were not detected in treated and control rats.

TABLE III
THE AVERAGE OF BASOPHIL (%)

Supplementation	Before	After	Average
Goat milk kefir	0	0	0
Acidophilus goat milk	0	0	0
Control	0	0	0
Average	0	0	0 ^{ns}

ns: non significant

5. Lymphocyte

The average number of lymphocytes in rat supplemented acidophilus milk after DNCB sensitized was lower than the before (Table 4)

TABLE IV
THE AVERAGE OF LYMPHOCYTE (%)

Supplementation	Before	After	Average
Goat milk kefir	74.33 ^a	72.50 ^a	73.42
Acidophilus goat milk	75.33 ^a	60.50 ^b	67.92
Control	75.00 ^a	75.50 ^a	75.25
Average	74.88	69.50	72.19

^{a,b}Different superscripts in the same row and column showed significant difference (P<0.05).

6. Monocyte

The average number of monocytes in rat treated kefir and acidophilus milk before and after sensitized with DNCB did not differ significantly, with an average of 3.53% (Table 5).

TABLE V
THE AVERAGE OF MONOCYTE (%)

Supplementation	Before	After	Average
Goat milk kefir	3.67	3.50	3.58
Acidophilus goat milk	3.67	2.67	3.17
Control	4.00	3.67	3.83
Average	3.78	3.28	3.53 ^{ns}

ns: non significant

B. Lymphocyte Proliferation

Figure 1 showed that rat in after DNCB sensitized have a higher proliferative response to PHA (P <0.05) than before sensitized, which means that DNCB may induce cellular immune response or a Th1 response (T-helper 1). However, supplementation of fermented goat milk (kefir and acidophilus milk) have no effect on PP lymphocyte proliferation in culture supernatant of DNCB sensitized rat.

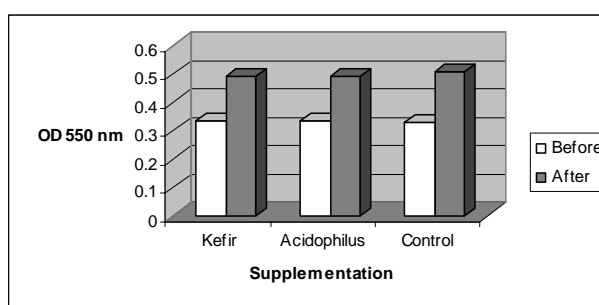


Fig. 1 Lymphocyte proliferation of rat supplemented with goat milk kefir and acidophilus goat milk and DNCB sensitized

IV. DISCUSSION

Acidophilus milk was able to increase the number of neutrophils in DNCB sensitized rat (Table 1). Unlike previous studies, neutrophil Wistar rats supplemented with *Lactobacillus plantarum* 10⁷ cfu / ml with 0.6 and 1.0 ml of volume was not significantly different from controls, while the number of neutrophils decreased significantly in rats fed the bacteria with 0.3 ml [12]. Likewise the research results that have been done by [13] showed that Wistar rats supplemented probiotic suspension of 10⁹ cfu / ml with a volume of 1 ml were not significantly different from the control. Neutrophils play a major role in the process of phagocytosis of microbial pathogens during the first few hours after entering the tissue [14]. They can ingest particles and microorganisms and kill them. Each event of the phagocytic attack results in the formation of a phagosome. The reactive oxygen species is trapped within the phagosome along with the secreted hydrolytic enzymes [15]. Phagocytic cells is one of the innate immune system, is the first line of defense against microbial disease, especially intracellular pathogens [16], tissue trauma or any inciting inflammatory signal [17]. The average neutrophil in adult female Wistar rats ranging from 8-24% [14]. In this study, supplementation of acidophilus goat milk in DNCB sensitized rat can increase the percentage of rat neutrophil exceed the normal range. This indicates the occurrence of acute inflammation. In the control rat and kefir

treatment indicates no occurrence of acute inflammation. The average number of eosinophils in Wistar rat was 0.75% (Table 2). In normal adult female Wistar rats ranging from 0-4% [14], while eosinophils in humans is only 1-4% of blood leukocytes (range in normal 5000-9000 mm³), which can phagocytized antigen-antibody complex [18]. According to [19], yogurt containing *Lactobacillus acidophilus* may decrease eosinophilia. *Lactobacillus plantarum* that supplemented in Wistar rat did not affect the number of eosinophils [12]. In some circumstances eosinophils rather than neutrophils predominate in acute inflammation. This tends to occur with parasitic worms, against which neutrophils have little success, or with a response involving the antibody IgE. Eosinophils release several proteins, such as major basic protein, which are often effective against parasites. Eosinophils also release several regulatory molecules that increase endothelial permeability. Eosinophils are also linked to certain types of allergies [20]. Basophils in this study was not found either in rats fed fermented milk as well as controls (Table 3). This is consistent with previous research conducted by [12], *Lactobacillus plantarum* that was supplemented in rat has no effect on the number of basophils, and the average number of 0%. Similarly, according to [21], that the basophils were detected very low in uninfected rat, which is only 0.06% or 1 / 1600 of leukocytes), and increases in the highest amount after 13 days of the initial infection of approximately 4.5% of total leukocytes (80-fold increase compared with normal rat). Basophils also increases when there is sensitizing antigen / allergen, but if that happens allergic type I (IgE-mediated) [22], [12]. Because in this study did not use rat that were infected, and rat also induced by allergen contact (including type IV), then the number of basophils did not increase.

Differences in lymphocyte counts between this studies (Table 4) and previous studies may be due to differences in types of bacteria are supplemented and also differences in doses. Results of the research conducted by [12], lymphocytes treated rats increased significantly after each using *Lactobacillus plantarum* suspension (10⁷ cfu / ml) and *Zygomonas mobilis* (10⁹ cfu / ml). In this study were given in the form of fermented milk, which is likely lower than the dose in the form of cell suspension. The main function of lymphocytes is the formation of humoral and cellular immunity [23]. The range of the number of lymphocytes in normal adult female rat is 70-89% [14]. There was no decrease in lymphocyte in the control rat and kefir treatment.

There was no difference the number of monocytes between control and treated rat (Table 5). The results were consistent with previous research results that were supplemented *Lactobacillus plantarum* in rat has no effect on the number of monocytes [12]. According to [14], the number of monocytes in normal adult female rats ranged 1-6%. Monocytes are responsible for front line defense in the tissue againsts foreign agents, including allergens and microbes. The number of monocytes in this study did not increase despite the sensitizing allergen. It was made possible by a dose of fermented milk is quite low. Fermented milk supplemented 2 ml / head / day doses are thought to be relatively low so as not to increase the proliferation of lymphocytes in allergic rat. In contrast to previous studies using healthy rat and suspensions of bacteria

directly. The proliferative responses of spleen cells to concanavalin-A and lipopolysaccharide mitogen were significantly enhanced in mice given different lactic acid bacteria (LAB). Spleen cells from mice given *L. rhamnosus*, *L. acidophilus* or *B. lactis* also produced significantly higher amounts of interferon-gamma in response to stimulation with concanavalin A than cells from the control mice [24]. In this study which has sensitized rat showed higher proliferation than before DNCB sensitized. This indicates that contact hypersensitivity induced by DNCB is an antigen specific responses of T cell, whereas blastogen response are non specific in nature. The epidermal Langerhan cell and T lymphocyte play a pivotal role in contact hypersensitivity [1]. Blastogenic responses of lymphocyte do not always correlate with other indicators of cell-mediate immune response, such as contact hypersensitivity [25].

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