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The effect of *Moringa oleifera* Lam leaf extract fermented by *Lactobacillus plantarum* on the expression of B220⁺ and CD11b⁺ cells in mice infected with *Salmonella typhi*

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Abstract. Defense against pathogens is essential for survival and it is controlled by innate and adaptive immunity. Phagocytosis is a complex mechanism that involved the activation of macrophages and its cytokines, stimulated T cells, then activation of B cells. *Moringa oleifera* plants are thought to have immunostimulatory properties due to its complex nutrients and phenolic acids and flavonoids contents as phytochemicals. *Lactobacillus plantarum* is able to produce lactic acid as the final product to reform carbohydrates, hydrogen peroxide, and bacteriocin as antimicrobial substances, which lead to inhibit pathogenic bacteria. This experiment aims to evaluate the CD11b⁺ and B220⁺ population after *Salmonella typhi* infection with/without administration of *M.oleifera* leaves extract. *Salmonella typhi* was labeled with carboxyfluorescein succinimidyl ester (CFSE) to determine phagocytosis activity. This experiment used was a complete randomized factorial pattern design. Mice were divided into two groups, namely the without treatment group and treatment group mice (fermented red *M.oleifera* leaves extract dose 84 mg/kg BW) and infected by *Salmonella typhi*. Data analysis was confirmed with the one-way ANOVA test followed by Tukey test (p<0.05). The results showed that fermented red *M.oleifera* leaves extract can increase the number of CD11b⁺ and B220⁺ cells at the time of 2 hours and 4 hours.

Keywords: B cells, macrophage, *Moringa oleifera*, *Salmonella typhi*, *Lactobacillus plantarum*

1. Introduction

Salmonella typhi is a Gram-negative facultative intracellular anaerobe that one of the leading causes of acute gastroenteritis, which is characterized by inflammatory diarrhea [4,23,33,36]. Prevention is anticipating the infection of *Salmonella typhi* by increasing the innate and adaptive immunity [30]. Cellular immunity is the body's foremost defense in the face of attacks by various microorganisms, therefore it can provide a direct response [38]. One way to kill intracellular bacteria is by increasing macrophage activation, T cell stimulation and cytokines released by macrophages associated with phagocytic processes and killing microbes [1]. Macrophage cells play an important role in the body's defense system because of the ability of bacterial phagocytosis and produce various inflammatory



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mediators [32]. The results of phagocytosis will be in the form of protein fragments which are then presented to T cells for the formation of antibodies [40]. After the process of antigen elimination, antibodies are produced by B cells [32]. *Moringa oleifera* leaves used for many medicinal uses and can be used either as supplemental nutrition or as medicine [12]. This plant has been proved to have immunomodulatory effects because it contains nutrients and complex phytochemicals with phenolic acids and flavonoids [3,27,42].

Lactobacillus plantarum used as fermented increases protein content, the bioavailability of nutrients, improves the shelf life of food products [2,14]. *Lactobacillus plantarum* produces lactic acid as the final product of carbohydrate metabolism, hydrogen peroxide and bacteriocins as antimicrobial agents [15]. The increase in total flavonoids during fermentation is thought to be due to the activity of lactic acid bacteria which during fermentation process, lactic acid bacteria produce enzymes that can release phenolic compounds contained in *Moringa oleifera* leaves so that they can add phenol groups to flavonoid compounds [20].

Flavonoids as immunostimulants can provide intracellular stimuli such as macrophages and T cells to work better and can eliminate incoming infections [42]. The growth of pathogenic bacteria such as Salmonella and Escherichia coli can be inhibited by antimicrobial substances and acid compounds formed [2,7,10,14,39].

This experiment was conducted to analyze the effect of *Moringa oleifera* leaves fermented by *Lactobacillus plantarum* on the increase in phagocytic activity of macrophages and B220⁺

2. Materials and Methods

2.1. Materials

M. oleifera leaves is obtained from Pamekasan, Madura, East Java, Indonesia. *M. oleifera* leaves were taken from the Moringa tree aged 3-12 months. *S. typhi* as obtained from the Laboratory of Microbiology, Faculty of Medicine, Brawijaya University. *L. plantarum* FNCC 0137 was obtained from Food Center and Nutrition Studies (CFNS or Pusat Studi Pangan dan Gizi), Gajah Mada University. PE/Cy5 conjugated rat anti-mouse B220 and FITC-conjugated rat anti-mouse CD11b (Biollegend, USA) for immune analysis using flow cytometry was obtained from the Laboratory of Animal Physiology, Structure and Development, Brawijaya University.

2.2. Extraction and Fermentation of *M. oleifera* leaves (MOL)

Collected plant material was air-dried for 3 days and continued to dry at 40 °C for 3 h in the oven. The dried leaves were stored at room temperature before further analysis. The dried MOL was milled in a blender and sieved 100 mesh. The MOL powder was macerated with 70% ethanol for 72 h. The slurry was then filtered through Whatman paper No.1. The solvent was evaporated to dryness in a rotary evaporator at 50 °C [13]. The concentrated extract was inoculated with 10⁸ CFU/g of *L. plantarum* and incubated at 37 °C for 120 h [39]. The fermented MOL was added 10% sucrose and 5% NaCl and then freeze-dried [16].

2.3. Animal experiments

Six-week-old female mice (20-30 g) were housed in a controlled room (25°C, RH 60%) and with a 12 h light 12 h dark cycle. Animals accessed freely to feed and water at all times. After 1 week of acclimation, they were randomly assigned to 4 groups (7 animals/group). Control non-treatment (2 hours and 4 hours) were orally gavaged with distilled water every day. Test groups animals (2 groups: 2 hours and 4 hours) were pre-treated of non-fermented or fermented MOL (with doses 84 mg/kg BW/day, respectively) for four weeks, prior to *S. typhi* was labeled with CFSE to injected intraperitoneally (0.5 mL/10g BW) with a concentration of 10⁸ CFU/mL (except a group of control non treatment). The protocol of the animal experiment was approved by the ethical committee on animal experiments of Brawijaya University, Malang, Indonesia (No: 829-KEP-UB).

2.4. CFSE Staining of *S.typhi*

S. typhi bacteria were put into a microtube and centrifuged (12,000 rpm, 10 minutes, 4 °C). The obtained pellets then washed with PBS and re-centrifuged. The bacteria contained in pellets then stained with 50µl CFSE: PBS solution in a ratio of 1:20. Bacteria that have been colored with CFSE-FITC [17], then incubated at 37 °C for 30-60 minutes in dark (microtube wrapped with aluminum foil). After incubation, the bacteria then centrifuged (12,000 rpm, 10 minutes, 4 °C). The pellets obtained then washed with PBS 2 times then continued to be centrifuged again. The bacteria are then killed by heating at 60 °C for 30-60 minutes. The bacteria were then washed with PBS and measured at 620 nm (OD 0.35). The bacteria then opsonized using a 500 µl fetal calf serum (FCS) serum and incubated at 37 °C for 30 minutes. The next stage is centrifugation with the same speed as before, and the supernatant was aspirated, then the CFSE-stained bacteria were intraperitoneally injected into mice [34].

2.5. Analysis of CD11b and B220 cells

Mice were killed using chloroform, and the abdomen was sprayed with 70% alcohol then 10 mL of PBS is injected into the peritoneal lining. The abdomen was tapped for 10 seconds and the peritoneal fluid was obtained using a syringe then put into a propylene tube and added with PBS until the volume reaches 3 mL. The homogenates were centrifuged at 2500 rpm for 5 minutes at 10 °C. After centrifugation, the supernatant was removed while the pellet was added with 1.5 mL PBS, then resuspended using vortex. Homogenates were taken 50 µL and put in 500 µL PBS. The homogenate then centrifuged at 2500 rpm for 5 minutes at 10 °C, and the pellet was obtained for antibody CD11b⁺ and B220⁺ staining. Data obtained from Flow Cytometry were analyzed using BD Cellquest Pro™ software.

2.6. Statistical analysis

Data were expressed as the mean ± standard deviation (SD). The relative number (%) of B220⁺ and CD11b⁺ cells, were analyzed by one-way ANOVA followed by Duncan's Multiple Range Test (DMRT) to calculate the statistical significance between groups, p-value <0.05 was considered significant. Statistical analysis was performed by Microsoft Excel for Windows.

3. Result and Discussion

CD11b / CD18 / CD11c are complement receptors 3 and 4 expressed by macrophages [18]. Macrophages have a key role in innate immunity and also initiate specific defense mechanisms (adaptive immunity). Macrophages will phagocytose the antigen then present it at its MHC II to stimulate lymphocytes and other immune cells response [1,32].

The fermented extract of *M. oleifera* gave an increase in the relative number of B220⁺ cells, and an increase in macrophage phagocytosis at 2 and 4 hours compared to the non-treatment group (Figure1). The fermented MOL extract increases the number of B220⁺ cells is possible due to the presence of flavonoids, and pathogen injection in the form of *S. typhi* which can stimulate lymphocyte cell proliferation. The increase in total flavonoids in the fermentation process might be caused by enzymatic activity produced by microbes that trigger the overhaul of polyphenol compounds into simpler molecules to increase the total number of flavonoids. Lactic acid from *L.plantarum* can increase total phenol by producing beta-glucosidase enzymes through the biotransformation process of primary and secondary metabolites that can increase the bioactive component of polyphenols [20].

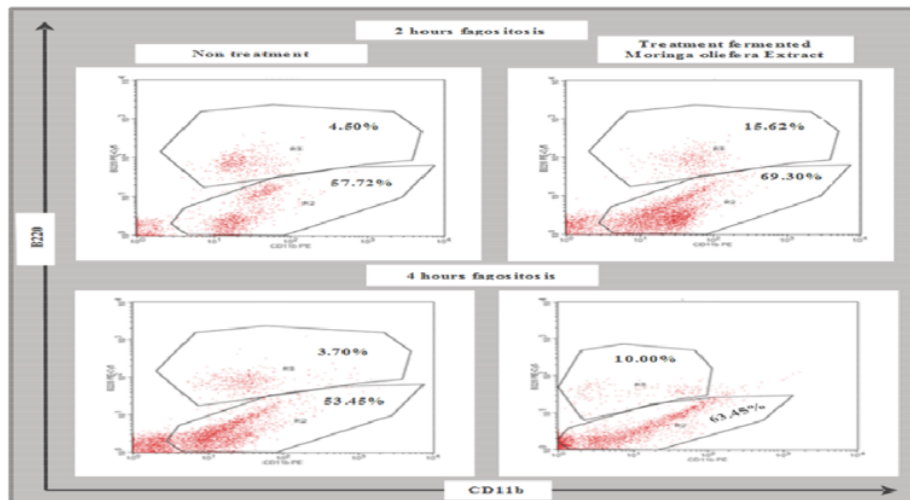
The increase in total flavonoids from MOL extracts after the fermentation process is thought to be due to β-glucosidase activity which is the key to changing the glucoside bonds to improve the quality of the product during the fermentation process [5,9,10]. Flavonoids are chemical compounds that can increase IL-2 production and increase lymphocyte cell proliferation. The lymphocyte cell proliferation is stimulated by the presence of antigens, mainly regulated by the influence of IL-2, on IL-2 receptors on the surface of the receptor. *L. plantarum* contained in MOL extract also acts as a protection against the body's immune system by stimulating Toll-like receptors (TLRs) that produce cytokines IL-6 which induce the development of B cells to produce Ig [21]. An increase in the number of B cells circulating in the body's circulation will increase the production of antibodies [40].

In the second hour results of the relative number of CD11b⁺ cells in the MOL fermentation treatment group obtained higher results than the non-treatment group, as well as in the 4th-hour treatment group the relative number of CD11b⁺ cells expression was higher than the non-treatment group. Although the relative number CD11b⁺ cells at the 4th hour decreased compared to the 2nd hour, it was still above the non-treatment group at the 2nd and 4th hour.

The results of the fermentation of MOL by *L. plantarum*, one of which can provide the effect of increasing the total protein and bioavailability of iron for food products [31,39]. *L. plantarum* contained in MOL extract increases IL-10 synthesis and macrophage secretion from inflamed colon [19]. IL-10 is a cytokine produced by many cells, including Th2 cells, dendritic cells, monocytes, B cells, keratinocytes, and regulatory T cells. IL-10 has an anti-inflammatory effect and mainly plays a role in inhibiting the Th-1 response [28].

The decrease in total sugar in the extract of fermented MOL is thought to be due to the sugar contained in MOL, sucrose, fructose, and glucose [25], used by lactic acid bacteria in the growth process. In the process of fermentation, bacterial metabolism that uses glucose as a growth nutrient occurs, then the glucose is converted to lactic acid so that the total sugar goes down [11,22]. Lactic acid and used by monocytes and macrophages as energy to increase oxygen consumption during phagocytosis. This oxygen is used by macrophages to increase the production of hydrogen peroxide used in oxidative processes [1,10]. Macrophages and neutrophils will convert oxygen molecules into ROS (Reactive Oxygen Species), assisted with IFN- γ and TLRs will destroy microbes [1]. One of the cellular immune responses is the production of Nitric Oxide (NO) from L-arginine by macrophages, which used to kill intracellular bacteria [41].

Macrophages can be activated by bacterial LPS (lipopolysaccharides), damaged body cells, or by IFN- γ . If macrophages are activated, the gene transcription that induces inducible nitric oxide synthase (iNOS) will increase, so that it will produce more NO from L-arginine. NO will act as an immunoregulatory agent with the capability of killing Salmonella [1].



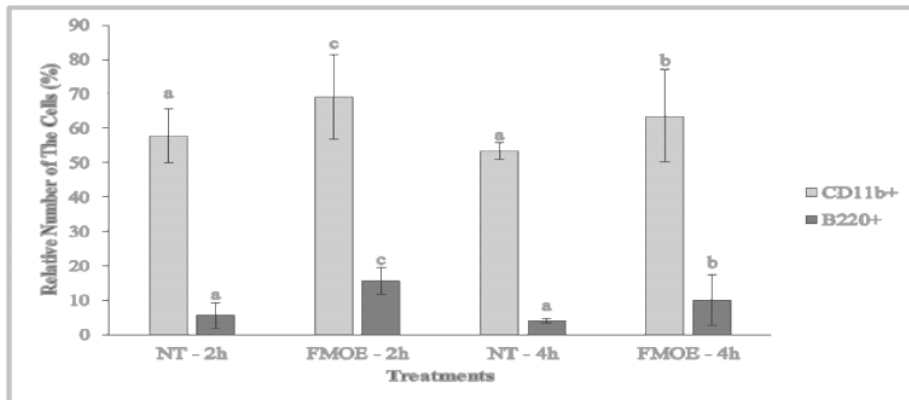


Fig.1. The Result of Flow cytometry analysis of B220⁺ and CD11b⁺ cells. a) The dot plot of the relative number of CD11b⁺ and B220⁺ cells for 2 hours and 4 hours. b) CD11b⁺ and B220⁺ cells expression were represented as mean \pm SD (n=4 for each group). NT 2h =Non treatment 2hours; FMOE 2h=Fermented MOL; NT 4h= Non-treatment 4hours; FMOE 2h=Fermented *Moringa oliefera* Extract 4 hours

And the presence of increased flavonoid compounds as immunostimulants can also provide intracellular stimulation such as macrophage cells and T cells to work better and can eliminate incoming infections [8]. Lymphocytes are one of the main producers of IFN- γ , with the recruitment of natural killer cells to the site of infection [6]. IFN activates macrophages, so macrophages will experience increased phagocytic activity. This will cause macrophages to kill bacteria faster. Macrophage activation will be followed by increased hydrolytic enzymes present in the cytoplasm. Flavonoids also have a mechanism of action by activating NK cells to stimulate IFN production [42]. IFN- γ is the main cytokine MAC (Macrophage Activating Cytokine) which will activate macrophages and stimulate phagocytic activity [37].

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