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Nitric Oxide and Inflammatory Cytokine Productions in Diabetic Rats Supplemented with Goat Milk and Soy Milk Kefir

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Abstract Diabetes has been known as an inflammatory diseases, and the certain of probiotics when administered in adequate amounts were reported have an anti-inflammatory properties. The purpose of this study were to evaluate the effect of administration of goat milk and soy milk kefir on immune responses including spleen lymphocyte proliferation, production of pro-inflammatory cytokine (TNF-α) and anti-inflammatory cytokine (IL-10), as well as peritoneal macrophage nitric oxide (NO) production in diabetic rats induced with streptozotocin-nicotinamide. Male Wistar rats were divided into five groups: negative control, positive control, diabetic rats fed goat milk kefir, diabetic rats fed kefir combination from goat milk and soy milk kefir and diabetic rats fed soy milk kefir. After 5 weeks treatment, the rats were sacrificed and sampled for spleen and peritoneal macrophages. The results showed that kefir combination from goat milk and soy milk kefir could increase lymphocyte proliferation and IL-10 production, as well as decreased TNF-α significantly ($p<0.05$) in diabetic rats. However, the effect of kefir combination showed the lowest NO production in diabetic rats compared with goat milk kefir and soy milk kefir. In conclusion, kefir combination from goat milk and soy milk may be used as biotherapeutic for type-2 diabetes mellitus by improving systemic immune responses through increasing in lympocyte proliferation and IL-10 as well as decreasing in TNF-α production.

Keywords: *goat milk kefir, soy milk kefir, lymphocyte proliferation, IL-10, TNF-α, Nitric oxide, diabetic*

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1. Introduction

The development of fermented milk products are increasing along with increasing public awareness about the importance of health care. Fermented milk has been widely used for biotherapy in many health problems, such as tuberculosis, diabetes and high cholesterol. Usually, fermented milk is made from cow milk, but it can also be prepared from other milk such as goat milk, buffalo milk and mare milk. To improve the health effects of fermented milk can be made with a variety of additional ingredients or substitute with a non-dairy ingredients, for example extracts of nuts, red or black rice, tubers or additional of dietary fiber.

Goat milk has been known to have more benefits than cow milk. The small size of fat globules, higher digestibility, and less allergic were the reasons of preferred goat milk feeding in infants over cow milk [1]. In addition, goat milk rich in medium-chain triglycerides [2], which can serve as an antimicrobial, a protective effect on the intestinal microarchitecture and immunomodulator [3,4]. Growth factor activity in goat milk is also higher than cow milk, so that may be used as a nutraceutical for gastrointestinal dissorder [5]. For economic reasons, sometimes goat milk is mixed with soy milk. Even though soy milk also has more value due to a cheaper source of high protein foods and the isoflavone contents which is important as an antioxidant.

Kefir is a probiotic, fermented by several bacteria and yeasts, encapsulated in a polyssacharide matrix, and resembles jelly grains. Kefir is also presented as its sourish product both in sugary or milky suspensions containing vitamins, aminoacids, peptides, carbohydrates, ethanol, and volatile compounds. Kefir is known to have a diverse microbial content depending on the country and fermentative substrates, which cause distinct probiotic effects [6]. Kefir consumption related to health effect was documented in various study include in improving microbial composition in gut. The effect caused by kefir

consumption in the intestinal microbiota composition may be due to a combination of factors, such as direct pathogen inhibition by acids and bacteriocin production, besides competitive pathogen exclusion in the intestinal mucosa [7]. There were increased in mice intestinal lactic acid bacteria (LAB), reduced in enterobacteria and clostridia populations after kefir administration [8].

There is differences in intestinal microbiota composition between healthy and diabetes individu, and usually obese people with insulin resistance were characterized by an altered composition of gut microbiota, particularly an elevated Firmicutes/Bacteroidetes ratio compared with healthy people [9]. The crucial role of gut microflora are maintaining the intestinal barrier integrity, sustaining a normal metabolic homeostasis, protecting the host from infection by pathogens, enhancing host defense system and even influencing the nervous system in type-2 diabetes mellitus (T2DM) [9]. However, the damage microbial ecology can result in a number of disease condition that include allergy, inflammatory bowel disease (IBD), obesity, cancer and diabetes [10]. In T2DM, microflora has an important contribution to low-level inflammation in the onset of T2DM, and to the further development of T2DM through inflammatory components [9]. Therefore, in order to obtain the benefecial effect for health, is needed to maintain a balance in gut microflora, so that the metabolit products would be balance.

Cytokines are a small molecule proteins that are secreted by cells and primarily involved in host responses to disease or infection and any involvement with homeostatic mechanism. Some cytokines promote inflammation (pro-inflammatory cytokines), whereas some cytokines suppress the activity of pro-inflammatory cytokines (anti-inflammatory cytokines). Other functions of cytokines are the control of cell proliferation and differentiation, the regulation of angiogenesis and immune responses [11,12].

Insulin resistance is a major characteristic of T2DM and is causally associated with obesity. While, inflammation plays an important role in obesity-associated [13]. Type-2 (non-insulin-dependent) diabetes mellitus is associated with increased blood concentrations of markers of the acute-phase response, including the main cytokine mediator of the response [14]. In subjects with obesity-related insulin resistance, are reported that local expression of TNF-α and plasma IL-6 are higher [15]. In addition, TNF- α may play a potentially important pathophysiological role in human obesity and non insulin dependent diabetes mellitus (NIDDM) [16].

Although the IL-10 was originally produced by Th2 cells, but is now known to be made by a variety of cell types. Based on a new data show that IL-10 produced by effector Th1 cells helps limit the collateral damage caused by exaggerated inflammation [17]. Different from TNF-α and IFN-γ, IL-10 is considered primarily an inhibitory cytokine, important to an adequate balance between inflammatory and immunopathologic responses [18]. Interleukin -10 is an anti-inflammatory cytokine with lower circulating levels in obese subjects, and acute treatment with IL-10 prevents lipid-induced insulin resistance [13]. The low production capacity of IL-10, a centrally operating cytokine with strong anti-inflammatory properties, associates with the metabolic syndrome and type 2 diabetes in old age [19]. Interleukin -10 increases insulin sensitivity and protects skeletal muscle from obesity-associated macrophage infiltration, increases in inflammatory cytokines, and their deleterious effects on insulin signaling and glucose metabolism. So that anti-inflammatory cytokine can be use in the treatment of T2DM [13]. Histopathology performed on pancreatic tissue demonstrated that treatment with IL-10 reduced the severity of insulitis, prevented cellular infiltration of islet cells, and promoted normal insulin production by beta cells. Taken together these results indicate IL-10 suppresses the induction and progression of autoimmune pathogenesis [20]. A previous study showed that water kefir slightly lowered pro-inflammatory cytokine TNF-α and increase anti-inflammatory cytokine IL-10 in blood serum of diabetic rats induced with STZ [21]. While, the information of kefir combination from goat milk and soy milk on inflammatory cytokines production in splenocyte culture is still limited.

Nitric oxide (NO) biosynthesis has been reported to occur in a variety of cell types including endothelial cells, vascular smooth muscle cells, adrenal gland cells, Kupffer cells, cerebellar neurones, macrophages and polymorphonuclear leukocytes (PMN) [22]. Macrophage activation and dysfunction underlie development of type-1 DM (insulin dependent diabetes mellitus, IDDM) and are associated with complications in type-1 and type -2 DM (non-insulin dependent diabetes mellitus, NIDDM), respectively [23].

Nitric oxide bioavailability is low in diabetes mellitus, either due to hyperglycemic cause an increase in reactive oxygen species (ROS) via glucose auto-oxidation in various tissues, leading to high oxidative/nitrosative stress [24] or decreasing NO production from L-arginine by the NO synthases (NOS) [25]. Production of large quantities of NO via the up-regulation of iNOS can have a variety of effects, which may be detrimental or beneficial depending on the amount, duration and anatomical site of synthesis. The benefecial effect of iNOS-induced NO has been found to exert a direct antimicrobial effect. NO appears to be of particular importance in host defense against intracellular pathogens, and perhaps in the maintenance of microbial latency. NO may act in concert with reactive oxygen species to damage microbial DNA, proteins, and lipids [26]. The negative effect of NO, iNOS-mediated NO production may occasionally become part of a dysregulated immune response, resulting in chronic inflammatory disorders [27]. This is because intravascular monosit/macrophages and lymphocytes can infiltrate into islets of Langerhans, resulting inflammatory products and initiate further immune mechanisms leading to the specific destruction of islet β –cells and then initiation of insulitis process [28]. Information of NO production in diabetic is unclear. According to study by [23] showed that NO production in peritoneal macrophage was increase in type-2 diabetic rats, whereas according to [24] showed that NO bioavailability in diabetic is low.

The purpose of this study was to evaluate the effect of administered of goat milk kefir, soy milk kefir and kefir combination from goat milk and soy milk on pro-inflammatory (TNF-α) and anti-inflammatory (IL-10) cytokines in splenocyte culture and nitric oxide production in peritoneal macrophage in diabetic rats induced with STZ-nicotinamide.

2. Materials and Methods

2.1. Kefir Preparation

Preparation of soy milk according to [29] with slight modifications. Whole soybeans were washed and soaked overnight in distilled water. After decanting the water, the soaked soybeans were mixed with 3 times their weight of distilled water, blended and filtered.

Goat milk and soy milk were pasteurized for 10 min at 90°C. The pasteurized milk were devided into 3 groups of kefir combination from goat milk and soy milk (100:0, 50:50, and 0:100) and cooled at room temperature. Kefir grains was inoculated into pasteurized milk as much as 2%, and incubated at room temperature for 18 hours [29]. After separating the grains and stirring, kefir samples were stored at 4°C for use in experimental animals.

2.2. Animal and Diets

Male Wistar rats 8-12 weeks old were individually cage and housed. During 7 days the rats were fed unrestricted amounts of a standard laboratory diet AIN-93G [30] and were then randomly assigned into five groups (n=6 per group): 1) negative control (normal rats); 2) positive control (untreated diabetic rats); 3) diabetic rats fed goat milk kefir; 4) diabetic rats fed kefir combination from goat milk and soy milk; and 5) diabetic rats fed soy milk kefir. In group 4, kefir was prepared from combination of goat milk and soy milk 1:1. For rat model of type- 2 diabetes was done by induction with streptozotocin-nicotinamide (STZ-NA) [31]. Diabetic condition was prepared by injection with streptozotocin (STZ) of 60 mg/kg b.wt, i.p, and after 15 min the rats were injected with nicotinamide (NA) of 120 mg/kg b.wt, i.p (prepared in fresh in 0.1 M citrate buffer pH 6.3). The rats had diabetic, if 7 d after induction, the fasting blood glucose was more than 126 mg/dL [31]. The dose of goat milk kefir was 2 ml/200 g b.wt/day orally, for 35 d experiment with force feeding. In negative control, the rats were given 2 ml phosphate-buffered saline (PBS). Furthermore, the rats were sacrificed by being given ketamine (80 mg/kg) anesthesia, and peritoneal macrophages were sampled for NO analysis, whereas spleen were sampled for lymphocyte proliferation assay and analyses of cytokines. All procedures related to animal experiment in this study were approved by Medical and Health Research Ethics Committee (MHREC), Faculty of Medicine Universitas Gadjah Mada, Indonesia (Approval Number: KE/FK/907/EC).

2.3. Lymphocyte Proliferation Assay

Before isolation of spleen lymphocytes, the spleens were weighed. Lymphocyte proliferation was assayed according to [32] with slight modifications. Spleen was removed and placed in 10 ml of RPMI 1640 (Sigma) media containing 10% fetal bovine serum (FBS) (Gibco) and 2% penicillin-streptomycin (Gibco). The spleen was washed with RPMI media. Aseptically collected spleen tissues were gently teased with sterile needles and forceps to release splenocytes into the RPMI 1640 (Sigma) media containing 10% FBS and 2% penicillin-streptomycin. Tissue suspensions were allowed to stand for 2 min to sediment large tissue clumps. The upper portion containing splenocytes was collected and centrifuged at 1000 × *g* for 5 min at 40°C. Suspension was incubated with erythrocyte lysis buffer (0.17 M Tris HCl and 0.16 M NH4Cl, pH 7.2) for 1 min and washed twice by centrifuging as above with RPMI 1640 medium. Cell viability was checked by trypan blue (0.4% solution) and counted by hemacytometer. Viable splenocytes ($1.5x10^6$cells/ ml) were finally cultured in heat inactivated 10% FBS enriched RPMI 1640 medium supplemented with or without mitogen 5 µg/ml phytohaemagglutinine (PHA)(Murex) and incubated at 37°C in a humidified atmosphere of 5% CO_2 incubator for 72 h. Then, 10 µl of methylthiazoletetrazolium (MTT) (5 mg/ml) was added in each well and incubated for 4 h at 37°C. Acidified isopropanol (100 µl of 0.1N HCl in anhydrous isopropanol) was added and mixed thoroughly to dissolve the dark blue crystals of formazan. Formazan quantification was performed using an ELISA plate reader with 550 nm. The lymphocyte proliferation was expressed as stimulation index (SI) which was calculated as the corrected absorbance of mitogen-stimulated cells divided by the corrected absorbance of unstimulated cells [33].

2.4. Analyses of Cytokines (TNFα- and IL-10)

Splenocytes were cultured as described above, and supernatant was collected after 72 h to analyze cytokine levels. Rat specific ELISA kits for measurement of TNF –α and IL-10 (eBioscience, Bender MedSystem, Vienna, Austria) were used. Assays were performed according to the instruction provided by the manufacturers. Briefly, microwell plate was washed twice with Wash Buffer. The Standard was reconstituted with 250 µl aquabidest for Standard of TNF–α, and 380 µl of aquabidest for IL-10, and then prepared standard with 1: 2 dilution in small tube. For TNF–α: 225 µl of reconstituted standard TNF–α (concentration = 5 ng/ml) was pipetted into S1 tube (standard 1) containing 225 µl Sample Diluent (concentration= 2500 pg/ml). This procedure was done for the next tubes until the concentration of the final tube (S7) was 39.1pg/ml. The procedure for analysis of IL-10 was the same as TNF–α analysis. For IL-10: 225 µl of reconstituted standard (concentration =2000 pg/ml) was pipetted into S1 tube (standard 1) containing 225 µl Sample Diluent (concentration = 1000 pg/ml). The S1 standard (225 µl) was transferred into S2 standard tube (concentration = 500 pg/ml), and continue this procedure was done for the next tubes until the concentration of the final standard (S7) was 15.6 pg/ml. The next procedures according to instruction in ELISA kits using biotin conjugate, streptavidin-horseradish peroxidase (HRP), and tetramethyl-benzidine (TMB) substrate.

2.5. Isolation of Macrophage and Culture

Rats were sacrificed by being given ketamine (80 mg/kg) anesthesia, and placed in the supine position, and then the abdomen skin were opened and sprayed with 70% ethanol for desinfection. Ten ml cold RPMI medium was injected into peritoneum cavity using a syringe. After gentle abdominal massage, about 30 ml of peritoneal fluid was extracted using the same syringe and transferred to 50-ml sterile polypropylene tubes on ice, and then were centrifuged at 1200 rpm for 10 min at 4°C. Supernatants were removed and added 1-2 ml complete RPMI medium into the precipitate (pellet). A 20 µl aliquot was stirred and added 930 µl RPMI medium and 50 µl trypan blue (total

1.0 ml), and the number of viable cells were counted with a hemocytometer. The counted cells were resuspended to a concentration of 10^6 cells /ml. Cells suspension (200 µl) were cultured into each well in steril microplate 24 wells, and incubated in 5% CO_2 at 37°C for 24 h [34].

2.6. Nitric oxide Analysis

Nitrite (NO_2^-), which is a stable end product of NO, was measured by a colorimetric assay based on the Griess reaction. Briefly, 50 µl culture supernatants were pippeted into well sample in microplate, and added 50 µl reagent Griess. The plate was incubated for 10 min at room temperature, and then the absorbance 550-590 nm was determined using a microplate reader. The concentration of NO_2 was calculated using a standard curve established with sodium nitrite in range 0 – 100 µM. Solution for standard curve was made using 10 mM nitrit standard (69.0 mg sodium nitrit in 100 ml deionzed water as stock solution). Griess reagent was made from mixture of Griess A and Griess B (1:1). Griess A was prepared by 0.1 g N-(1-naphthyl) ethylenediamine dihydrochloride into 100 ml deionized water, whereas Griess B prepared by sulfanilamide into 100 ml 5% (v/v) orthophosphoric acid [35,36].

2.7. Statistical analysis

All the data from this study were expressed as mean ± standard deviation. Two Way ANOVA followed by Duncan's Multiple Range Test (DMRT) were used for statistical analyses, and p-values of less than 0.05 ($p<0.05$) indicated significant differences. Statistical analyses were performed by using the SPSS version 17.

3. Results and Discussion

3.1. Spleen Weight and Splenocytes Number

Spleen weight of diabetic rats (induced with 60 mg STZ/kg b.wt and 120 mg NA/kg b.wt) that fed three kinds of kefir and untreated diabetic rats were not significantly different. Only the weight of spleen in diabetic rats fed combination of kefir from goat milk and soy milk have a close weight with the negative control rats. The total number of splenocyte of diabetic rats fed combination of kefir from goat milk and soy milk were higher than diabetic rats fed soy milk kefir and positive control rats (Table 1). The results study were similar to a previous study by [37], that diabetic rats induced with 45 mg STZ/kg b.wt and fed *Hibiscus sabdariffa* Linn extract showed decreased in spleen weight and total number of splenocytes compared with the control rats. Different from other study by [38], that diabetic rats induced with 65 mg STZ/kg b.w have a higher spleen weight compared with the control rats, but the change in spleen weight can be normalized by the combined treatment of Rutin and Symilarin contributing to their synergistic anti-inflammatory and anti-oxidative properties. However, a previous study by [39] showed that the weight of spleen was not influenced by the STZ treatment. Doze differences of STZ as well as an addition of NA in diabetic induction may cause differences in response to spleen.

Table 1. The average of spleen weight and splenocyte count in rats with different treatment

Treatment groups	Spleen weight (g)	Splenocyte number (x 10^6 cell/ml)
Negative control	0.7033±0.06154^a	33.00±0.74^{ab}
Positive control	0.4960±0.19217^b	13.16± 0.50^a
Goat milk kefir	0.5020±0.12218^b	25.50± 0.63^{ab}
Goat - soy milk kefir	0.5683±0.14825^{ab}	39.88± 2.79^b
Soy milk kefir	0.4157±0.18492^b	16.25± 0.77^a

Different letters (a, b) in the same column indicate significant difference ($P<0.05$).

The decreased in spleen weight and the number of splenocytes in diabetic rats in this study due to the toxic effect of STZ. STZ –induced diabetic rats has been known as immunosuppressed through lymphopenia mechanisms which is caused by damage to DNA resulting in irreversible impairment of splenocyte function and viability [40]. Thus, the splenocytes abrogated their capacity to proliferate [41]. Kefir combination from goat milk and soy milk could normalize spleen weight and total number of splenocyte in diabetic rats near the normal rats. This was possible because of the synergistic effect of bioactive components derived from goat milk and soy milk as well as the probiotics in kefir. Study in healthy human subjects, showed that consumption of fermented goat milk with lactobacilli strain enhanced total antioxidative activity [42]. Free isoflavones from fermented soy milk showed antioxidant and anti-inflammatory properties [43].

3.2. Stimulation Index of Splenocyte Proliferation

In this study showed the stimulation index of lympocyte proliferation in diabetic rats fed goat milk kefir or combination of goat-soy milk kefir were higher than diabetic rats fed goat milk kefir and soy milk kefir, even though statistically not significant (Table 2). This means kefir combination that containing probiotics has a sinergistics effect derived from goat milk and soy milk components, so that increased in lymphocyte proliferation. A previous study showed that probiotic dahi and –isolated *L. acidophilus*- fed mice could increase splenocyte proliferation compared with mice fed milk and control [44,45]. Proliferation of lymphocytes is the first indication of immune system activation by antigen [45]. In untreated diabetic rats (positive control) showed lower lymphocyte proliferation compared with normal rats, although not significantly different. However, lymphocyte proliferation in rats fed goat milk kefir and kefir combination from goat milk and soy milk have higher lymphocyte proliferation than untreated diabetic rats. The low stimulation index of splenocyte proliferation in untreated diabetic rats in this study due to hyperglycaemia reduced cell viability and induced apoptosis through oxidative stress in lymphocytes. This indicate that hyperglycemia can directly affect lymphoid cell growth [46]. This is in accordance with the results of previous study in type-2 DM, that the percentage of apoptosis in peripheral blood lymphocytes was more than that measured in normal subjects. The high apoptosis in DM are also through increased intracellular Ca, mitochondrial dysfunction (mitochondria apoptosis pathway), and changes in intracellular fatty acid metabolism [47]. In type- 2 DM, probiotics reduce the inflammatory response and oxidative stress, as well as

increase the expression of adhesion proteins within the intestinal epithelium, reducing intestinal permeability. Such effects increase insulin sensitivity and reduce autoimmune response [48].

Table 2. The average of stimulation index (SI) of splenocyte proliferation in rats with different treatment

Treatment groups	Stimulation index
Negative control	1.3203±0.12846[a]
Positive control	1.0197±0.64523[a]
Goat milk kefir	1.7938±0.23622[b]
Goat - soy milk kefir	2.1803±0.50179[b]
Soy milk kefir	1.7846±0.83595[ab]

Different letters (a, b) in the column indicate significant difference (P<0.05).

3.3. Level of IL-10 and TNF-α in Splenocyte Culture

The untreated diabetic rats have a low in IL-10, but have a high in TNF-α. The rats fed kefir combination from goat milk and soy milk have higher (p<0.05) IL-10 than goat milk kefir or soy milk kefir, but they have a lower TNF- α in supernatant splenocyte culture than the rats fed soy milk kefir (Table 3). This study indicate these cytokines could affect systemically in type-2 diabetic rats and this is in accordance with study by [49]. The probiotic *L. kefiri* treatment was reported could reduce the expression pro-inflammatory mediators and increased IL-10 locally [50]. According to [49], induction of TNF-α messenger RNA expression was observed in adipose tissue from four different rodent models of obesity and diabetes. Inflammatory cytokines play some role in the pathogenesis of diabetes, not only affect insulin resistance but may also contribute directly to beta-cell apoptosis and beta-cell failure, ultimately leading to type-2 diabetes [15]. Diabetes mellius is generally characterized by overproduction of the proinflammatory cytokine TNF-α, whereas action of the latter is modulated by the anti-inflammatory cytokine IL-10 [51], where IL-10 showed neutralization increases TNF-α production, which implies that IL-10 suppresses such proinflammatory cytokines [52]. A previous study showed that *Lactobacillus reuteri* GMNL-263(Lr263) may reducing insulin resistance as well as hepatic steatosis formation in high-fructose-fed rats and suggested that it may be a promising therapeutic agent in treating type- 2 diabetes. After Lr263 feeding, the decreased activities of hepatic antioxidant enzymes in rats with high fructose diets was dramatically reversed, and elevated TNF-α in adipose tissue was markedly decreased [53]. Kefir is a natural probiotics, where the probiotics can help regulate inflammation in a number of ways by indirect and direct effects. In indirect effect is through maintaining or repairing epithelial barriers, enhancing production of short chain fatty acids with anti-inflammatory properties (e.g., butyrate) as well as increasing synthesis of antimicrobial peptides that influence inflammation resolution pathways in the mucosa. While, direct effects by binding innate immune system receptors and triggering pathways that affect the production of cytokines associated with inflammation [54].

Table 3. The average of IL-10 and TNF-α of splenocyte culture in rats with different treatment

Treatment groups	IL-10 (pg/ml)	TNF-α (pg/ml)
Negative control	273.44 ±139.72[ab]	120.84±69.02[ab]
Positive control	58.44±80.16[a]	236.44±88.98[c]
Goat milk kefir	176.64±168.76[a]	71.84±28.00[a]
Goat - soy milk kefir	452.88±367.40[b]	66.90±50.52[a]
Soy milk kefir	134.71±142.80[a]	198.72±147.22[bc]

Different letters (a, b,c) in the same column indicate significant difference (P<0.05)

3.4. Total Number of Macrophage and NO Production

Untreated diabetic rats have highest in macrophage number compared to other treatment groups, even though statistically not significant (Table 4). This result different from a previous study that peritoneal macrophage number in type -2 diabetic rats were increased significantly after treated with *Andrographis paniculata* leaves extract for 7 days [55]. This differences result in macrophage number may be caused by differences in immunostimulant components and dosage that administered to the rats. In a previous study the *Andrographis paniculata* leaves was in concentrated extract, so that it has higher activity to stimulate macrophage. Conversely, in kefir that mentioned above was not in concentrated extract components.

Table 4. The average of macrophage number and nitric oxide in rats with different treatment

Treatment groups	Macrophage ($x10^6$cell/ml) [ns]	Nitric oxide (µM)
Negative control	2.70±0.08	3.28±2.67[ab]
Positive control	4.53±0.21	4.00±0.32[b]
Goat milk kefir	2.22±0.10	8.02±2.74[c]
Goat - soy milk kefir	1.75±0.04	1.41±0.96[a]
Soy milk kefir	2.60±0.15	5.19±3.43[bc]

Different letters (a, b, c) in the same column indicate significant difference (P<0.05)
ns : not significant.

Production of nitric oxide in diabetic rats fed goat milk kefir or soy milk kefir was higher (p<0.05) than the rats fed kefir combination from goat milk and soy milk (Table 4). This indicate if goat milk and soy milk were mixed in kefir preparation, it will cause antagonistic effect which decrease in NO production in diabetic rats. According to [56], biological signaling pathways like NF-κB, mitogen-activated protein kinase (MAPK), Akt/PI3K and PPARγ are targets for probiotics or their products. Thus, their metabolic products from goat milk and soy milk in kefir fermentation can interact so that affect on signaling pathway which can reduce in NO production. The NO production in rats fed kefir combination was lower than the normal rats, even though statistically not significant. The low NO production below the normal rats may cause the low in immune response againts pathogen, since the NO has a role in adaptive and innate immunity.

Different from study by [57], diabetic rats fed goat milk kefir showed lower iNOS expression in hepar compared with diabetic rats fed kefir combination (from goat milk - soy milk) or soy milk kefir. The iNOS expression in diabetic rats fed goat milk kefir was similar to the normal rats, whereas iNOS expression in diabetic rats fed kefir

combination and soy milk kefir was higher than the normal rats. However, in the present study showed that diabetic rats fed kefir combination (not goat milk kefir) have a lowest NO production in peritoneal macrophage than other kefir treatment. *O*rgan differences might be able to affect the expression of iNOS gene that also affects the NO production.

Tumor necrosis factor-α activates macrophages to express inducible nitric oxide synthase (iNOS) and inhibit pathogen replication by releasing a variety of effector molecules including nitric oxide (NO). Furthermore, NO mediates an early step of the signal transduction pathway, inducing the innate immune response upon natural infection [58],[59]. In this study, the rats fed kefir combination from goat milk and soy milk produces the lowest TNF-α (Table 3), so that the NO production was low (Table 4). However, the function of NO in immune system too difficult for uniform describe due to protective and toxic effect of NO are frequently seen in parallel [60], so that there is no simple, uniform picture of the function of NO in the immune system. Its striking inter- and intracellular signaling capacity makes it extremely difficult to predict the effect of NOS inhibitors and NO donors, which still hampers therapeutic applications.

There are two mechanisms of probiotic effect on the production of NO, which is indirect and direct mechanisms. In indirect mechanism through the short chain fatty acid (SCFA) (especially butyrate) as signaling molecule which can block nuclear factor-kappa B (NF-κB), where NF-κB is proinflammatory transcription factor. Thus, butyrate is an anti-inflammatory [56]. In addition, butyrate is also an important inhibitor of histone deacetylase (HDAC) and that important to regulate expression of numerous genes including those that encode key inflammatory mediators, i.e., NO, IL-6 and IL-12 [61].

Direct mechanism by using components on cell surface (peptidoglycan, lipoproteins, and diacylated lipopeptide), probiotics have direct effects on the inflammatory response. This direct effect by acting as ligands for specific members of a large family of innate immune system receptors termed Toll-like receptors (TLRs), thereby influencing important signaling pathways. The signaling pathway including NF-κB, mitogen-activated protein kinase (MAPK), phosphoinositide-3-kinase-protein kinase B/Akt (PI3K-PKB/Akt), and peroxisome proliferator-activated receptor gamma (PPARγ) [56,62]. Toll-like receptors (TLRs) are a major class of pattern recognition receptors (PRRs) that are present on intestinal epithelial cells (IECs) and immune cells which are involved in the induction of both tolerance and inflammation [63].

According to [56], strains of probiotics induvidually can effect IECs, macrophages, dendritic cells and lymphocytes. In IECs, the expression of inducible NO synthase (iNOS), the critical enzyme in the synthesis of NO, is synergistically stimulated by bacterial lipopolysaccharide (LPS) and IFNγ or by the combination of TNFα and IFNγ at the transcriptional level [64].

In macrophages, NO is synthesized by the inducible isoform of nitric oxide synthase (iNOS), which catalyzes the conversion of L-arginine to L-citrulline and NO, in response to various stimuli such as lipopolysaccharide (LPS), IFNγ, TNF-α, and IL-1β [65]. In a previous study showed that NO production stimulated by heat-killed *Enterococcus faecalis* TH10 (hk-TH10) is mediated through the TLR2-TLR1/6 pathway. Activation in hk-TH10-stimulated murine macrophage RAW264 cells might be good for the health of the host because an excessive amount of NO induces inflammatory diseases. TLR2-TLR1/6 signaling enhances the phosphorylation of MAPKs and IKBα, and thereby activates transcription factors such as AP-1 (activator protein-1), ELK1, and NF-κB [66] to enhance or suppress activation and influence downstream pathways [67]. Subunits of transcription factor NF-κB interact with poly (ADP-ribose) polymerase (PARP)-1 in the nucleus and then both bind to DNA to modulate gene expression [68]. These transcription factors are activated or upregulated by peptidoglycan, lipoproteins, and diacylated lipopeptide, which bind to the iNOS promoter and enhance NO production [66]. In type-2 DM mice, the increased NF-κB activity impairs vascular function by PARP-1-, Sp-1-, and cyclooxygenase -2 (COX-2) -dependent mechanisms [69].

4. Conclusion

This study have demonstrated when the goat milk kefir substituted with soy milk during fermentation process, then there will be a synergistic effect that can normalize the weight of spleen, increases the proliferation and the number of splenocytes, increases the production of anti-inflammatory cytokines (IL-10) and decreases pro-inflammatory cytokines (TNF-α) in type-2 diabetic rats. Therefore, the kefir combination may be use as functional food for the treatment of type-2 DM. However, the kefir combination has antagonistic effects that can reduce the production of nitric oxide in type-2 diabetic rats. Further research is required to determine the mechanism of the effect of various kinds of kefir against NO production in macrophages of diabetic type-2 rats, through analyses of bioactive components in each kefir treatment and gene expression that encodes for the NO production.

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