

## Goat Milk Yoghurt by Using Lacto-B Culture Modulates the Production of Tumor Necrosis Factor-Alpha and Interleukin-10 in Malnourished Rats

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### Abstract

Total spleen lymphocytes, lymphocyte proliferation, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and interleukin-10 (IL-10) in spleen lymphocyte culture were studied in malnourished Wistar rats fed with goat milk yoghurt. Malnourished rats were created by using standard feed restriction as much as 50% of normal rats for 21 d. Goat milk yoghurt containing three types of micro-organism e.g., *Lactobacillus acidophilus*, *Sterptococcus thermophilus* and *Bifidobacterium longum* derived from Lacto-B culture in powder form. After 21 d, the rats continued to receive restricted feeding and supplemented with goat milk yoghurt for 7 d. Total splenocytes were counted by hemocytometer. Splenocytes proliferation was expressed as stimulation index, whereas the TNF- $\alpha$  and IL-10 of spleen lymphocyte culture were measured by ELISA technique. The total number of splenocytes and stimulation index of splenocytes in moderate malnourished and normal rats supplemented with goat milk yoghurt was not significantly different. The level of TNF- $\alpha$  in the rat supplemented with goat milk yoghurt was lower ( $p < 0.05$ ) than the control group, whereas the level of IL-10 in the rat supplemented with goat milk yoghurt was higher ( $p < 0.05$ ) than the control group. In conclusion, goat milk yoghurt supplementation in malnourished rats could decrease TNF- $\alpha$  as a representation of the pro-inflammatory cytokine, while it increases IL-10 as a representation of the anti-inflammatory cytokine.

**Key words:** goat milk yoghurt, IL-10, lymphocyte proliferation, malnutrition, TNF- $\alpha$

### Introduction

Goat milk has many advantages in terms of percentage of an over-sized fat droplets smaller than cow's milk (Silanikove *et al.*, 2010). Because the goat milk fat globule has a greater surface area, and lipases in the gut are supposedly able to attack the lipids more rapidly. Almost 20% of the fatty acids of goat milk fall into the short-chain fatty acids category (C4:O to C12:O) compared with 10- 20% for cow milk. Lipases attack the ester linkages of the shorter-chain fatty acids more readily, so these differences may contribute to more rapid digestion and absorption of goat milk fat (Arora *et al.*, 2013). Some physicochemical properties of goat milk such as smaller fat globules, higher percentage of short and medium chain fatty acids, and softer curd formation of its proteins

are advantageous for higher digestibility and healthier lipid metabolism relative to cow milk (Park, 1994). Thus goat milk has been recommended for either infants, old, convalescent people (Kompan and Komprej, 2012), and goat milk-based diet (animal fat) has a beneficial effect and thus its consumption may be recommended especially in cases of malabsorption syndromes (Alferrez *et al.*, 2001). Goat milk has a nutritional value similar to that of cow milk and could be used as an alternative to cow milk for rehabilitating undernourished children (Razafindrakoto *et al.*, 1993).

The prevalence of malnutrition in Indonesia is still relatively high despite a declining trend from 2007 to 2010. The proportion of malnourished children under five in Indonesia decreased from 18.4% in 2007 to 17.9% in 2010 (Risksedas, 2010). Protein malnutrition disrupts the normal ecology of the microflora affecting strictly anaerobes, impairs host immune response and antibacterial defenses, enhances the susceptibility to infection, and leads to mucosal atrophy (Dock *et al.*, 2004). The direct relationship between malnutrition and death is mainly due to the immunodeficiency status and consequently, greater

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susceptibility to infectious agents (Franca *et al.*, 2009). The severity of the malnutrition depends on the degree of nutrient imbalance and the interaction between nutrients and the age of the host (Nunez *et al.*, 2013). Severe protein malnutrition, mainly in newborns and small children, also provokes thymus atrophy that, in turn, reduces thymus cell number and also severely affects the development of peripheral lymphoid organs. The immediate consequence of this atrophy is leucopenia, decreased CD4/CD8 ratio and increased number of immature T cells in the periphery (Savino, 2002).

The spleen has a very high blood flow, that it filters off cell-free fluid from the blood into the lymphatic system and that the rate of filtration is modulated by neuronal and humoral factors (Semaeva *et al.*, 2010). The spleen was chosen as a representative of systemic lymphoid organ to evaluate the systemic immune system status (Ramiro-Puig *et al.*, 2007).

The induction of proinflammatory and anti-inflammatory cytokines is important in determining whether the immune system is successful in providing protection against specific pathogenic organisms (Al-Bana *et al.*, 2008). Production TNF- $\alpha$  by bone marrow cells is also significantly lower in malnourished animals (França *et al.*, 2009).

Probiotic yoghurt intake was associated with significant anti-inflammatory effects that paralleled the expansion of peripheral pool of putative T regulatory (Treg) cells in inflammatory bowel disease (IBD) patients (Baroja *et al.*, 2007). The concept that probiotics act via the induction of regulatory cells is inherently attractive, because it seems unlikely that they could act by adequately replacing all bacteria in the intestinal microflora that are capable of causing inflammation in experimental mice/individuals susceptible to IBD-like inflammation or IBD (Giacinto *et al.*, 2005). Several different mechanism of probiotics are: 1) involves altering the composition of the intestinal microbiota by producing bacteriocins, which are products that eliminate certain bacteria, or by altering pH, which will alter the growth characteristics of certain bacteria, 2) involves altering the epithelial barrier function of the intestine, and 3) have important immunoregulatory activity, so certain probiotics and their products can activate regulatory T cells and regulatory pathways, leading to downregulation of inflammation (Sartor, 2011). The previous study by Giacinto *et al.* (2005) showed that probiotic administration was associated with an early increase in the production of IL-10 and an increased number of regulatory CD4<sup>+</sup> T cells. The increase in CD4<sup>+</sup> CD25<sup>high</sup> T cells correlated with the decrease in the percentage of

TNF- $\alpha$ - or IL-12-producing monocytes and dendritic cell (DC) (Baroja *et al.*, 2007).

Recently, yoghurt are often added by probiotics to improve health effects, and originally yoghurt is made using a starter *Lactobacillus bulgaricus* and *Streptococcus thermophilus* cultures that can be derived from the powder or liquid culture. Probiotic such as *Bifidobacterium longum* reported by the previous study (Pavlovic *et al.*, 2006) grew better in goat milk than in cow milk. According to Dock *et al.* (2004), *S. thermophilus* and *L. helveticus* enhance the recovery of gut atrophy induced by malnutrition.

In this study, yoghurt was made directly using Lacto-B powder which inoculated into the milk. Lacto-B is a non dairy powder-containing probiotic consists of *B. longum*, *L. acidophilus*, and *S. thermophilus* known as treatment for diarrhea. Probiotic strains such as *Lactobacillus* species, *Bifidobacterium* species and *Streptococcus* species have long history of safe use and are Generally Recognized As Safe (GRAS). Multi-strain and multi-species probiotics have improved functionality as compared to single strain (Dash, 2009). This study aimed to determine the effect of goat milk yoghurt which is made with culture from Lacto-B powder on total of rat splenocytes, splenocyte proliferation, levels of TNF- $\alpha$  and IL-10 in splenocyte culture supernatant of malnourished rats.

## Materials and Methods

### Goat milk yoghurt preparation

Goat milk yoghurt was prepared from goat milk of Etawah Crossedbred from Indonesia and culture of Lacto-B powder. Goat milk was pasteurized at 85°C for 30 min and cooled to 43–45°C. After cooled, pasteurized goat milk was inoculated with 3% Lacto-B powder (Novell Pharmaceutical Laboratories) containing *S. thermophilus*, *L. acidophilus* and *B. longum* and incubated at 45°C for 6 h (Dave, 1998; Sunarlim, 2009 with slight modification).

### Experimental animals

Three week old male Wistar rats weighing 25.5–41.0 g, were individually caged and housed. During 7 d the rats fed unrestricted amounts of a standard laboratory diet AIN-93G (Reeves *et al.*, 1993) and then randomly assigned to two groups: one maintained in the same conditions (control) and another group fed 50% of the intake of the control's (restricted or malnourished) (Rosso *et al.*, 1981) for 21 d. Both groups were allowed to drink water ad libitum. After 21 d, the rat were assigned to four groups: 1) Malnourished rats (MC), 2) Malnourished rats with goat milk

yoghurt (MY), 3) Normal rats (NC), and 4) Normal rats with goat milk yoghurt (NY). MC and NC groups (control without yoghurt) were given aquadest with a volume equal to the volume of treated yoghurt. Aquadest as control or yoghurt were given orally with force feeding. The dose of goat milk yoghurt was 2 mL/100 g body weight/day, whose consumption is equivalent to the children consumption of 100 mL yoghurt/day (WHO, 2006). Each group of rat using 7 replications (n=7 rats). Rat body weight was measured every 3 d. All of groups were treated for 7 d, and then were killed. Lymphocytes are isolated from rat spleen for cultured. All procedures related to animal experiment were conducted following the recommendation of Medical and Health Research Ethics Committee (MHREC) Faculty of Medicine Universitas Gadjah Mada, Indonesia (KE/FK/753/EC).

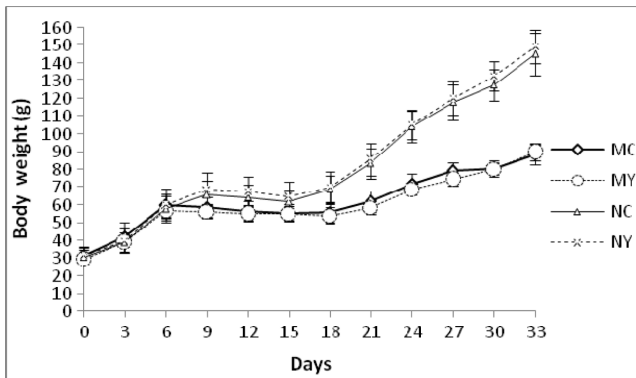
#### Lymphocyte proliferation assay

Lymphocyte proliferation was assayed according to Jain *et al.* (2008) 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  $1000\times g$  for 5 min at 40°C. Suspension was incubated with erythrocyte lysis buffer (0.17 M Tris HCl and 0.16 M  $\text{NH}_4\text{Cl}$ , 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 haemocytometer. Viable splenocytes ( $1.5\times 10^6$  cells/mL) were finally cultured in heat inactivated 10% FBS enriched RPMI 1640 medium supplemented with or without mitogen 5  $\mu\text{g}/\text{mL}$  phytohaemagglutinine (PHA) (Murex) and incubated at 37°C in a humidified atmosphere of 5%  $\text{CO}_2$  incubator for 72 h. Then, 10  $\mu\text{L}$  of methylthiazolotetrazolium (MTT) (5 mg/mL) was added in each well and incubated for 4 h at 37°C. Acidified isopropanol (100  $\mu\text{L}$  of 0.1 N HCl in anhydrous isopropanol) was added and mixed thoroughly to dissolve the dark blue crystals of formazan. Formazan quantification was performed using an Enzyme-linked immunosorbent assay (ELISA) plate reader with 550 nm. The lymphocyte proliferation was expressed as stimulation index (SI) which was calculated as the corrected ab-

sorbance of mitogen-stimulated cells divided by the corrected absorbance of unstimulated cells (Keller *et al.*, 2005).

#### Measurement of cytokine levels (TNF- $\alpha$ 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- $\alpha$  and IL-10 (eBioscience, Bender MedSystem, Vienna, Austria) were used. Assays were performed according to instruction provided by the manufacturers. Briefly, microwell plate was washed twice with Wash Buffer. The Standard was reconstituted with 250  $\mu\text{L}$  aquabidest for Standard of TNF- $\alpha$ , and 380  $\mu\text{L}$  of aquabidest for IL-10, and then prepared standard with 1:2 dilution in small tube. For TNF- $\alpha$ : 225  $\mu\text{L}$  of reconstituted standard TNF- $\alpha$  (concentration = 5 ng/mL) was pipetted into S1 tube (standard 1) containing 225  $\mu\text{L}$  Sample Diluent (concentration = 2500 pg/mL). This procedure was done for the next tubes until the concentration of the final tube (S7) was 39.1 pg/mL. The procedure for analysis of IL-10 was the same as TNF- $\alpha$  analysis. For IL-10: 225  $\mu\text{L}$  of reconstituted standard (concentration = 2000 pg/mL) was pipetted into S1 tube (standard 1) containing 225  $\mu\text{L}$  Sample Diluent (concentration = 1000 pg/mL). The S1 standard (225  $\mu\text{L}$ ) 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. Each standard (100  $\mu\text{L}$ ) was pipetted into well for standard, and 100  $\mu\text{L}$  of Sample Diluent was pipetted into well for blank. Each well for sample was filled with 50  $\mu\text{L}$  of Sample Diluent and added 50  $\mu\text{L}$  sample. Biotin conjugate solution was added into microwell plate, and covered with adhesive film. The plate was incubated at room temperature (18-25°C) for 2 h. Adhesive film was removed, and washed microwell plate 4 times with 400  $\mu\text{L}$  Wash Buffer for each well. Streptavidin-HRP solution (100  $\mu\text{L}$ ) was added into all well, and covered with adhesive film. The plate was incubated at room temperature for 1 h. Micro plate was washed 4 times, whereas washing for plate in IL-10 assay as much as 3 times, and added 100  $\mu\text{L}$  tetramethyl-benzidine (TMB) substrate into wells. The plate was incubated at room temperature for 10 min, and avoided from direct exposure to light. The enzyme reaction was stopped by pipetting 100  $\mu\text{L}$  of Stop Solution into each well. The absorbance of each microwell was read on ELISA plate reader 405 nm. Washing for plate in IL-10 assay as much as 3 times.



**Fig. 1. Body weight (g) of malnourished and normal rats.** (day 0-6: adaptation; day 6-27: restriction feeding; day 27-33: yogurt supplementation and continued restricted feeding. MC: Malnourished-control; MY: Malnourished-yoghurt; NC: Normal-control; NY: Normal-yoghurt)

### Statistical analysis

The data of total splenocytes, lymphocyte proliferation, TNF- $\alpha$  and IL-10 levels of supernatant lymphocyte culture in malnourished and normal rats supplemented with goat milk yoghurt, were analyzed by ANOVA using SPSS 12.0 (2003).

## Results

To study the effects of goat milk yoghurt containing *S. thermophilus*, *L. acidophilus* and *B. longum* on improve the immunity of malnourished rats, the goat milk yoghurt has been supplemented for 7 d after 21 d received restricted feeding and continued restricted feeding

together with goat milk yoghurt supplemented.

### Effects on body weight

The rat body weight that received 50% restricted feeding and normal rats for 21 d and after supplemented with goat milk yoghurt is showed on Fig. 1.

A 50% restricted feeding resulted in moderate malnutrition, because the weight gain of malnourished rats in this study after malnutrition period for 21 d only 30.56% of normal rats. After restriction feeding period, the malnourished rats have lower ( $p<0.05$ ) body weight than the normal rats (Table 1).

Body weight of rats after 7 d received goat milk yoghurt and continue restricted feeding given in Table 2.

Based on Table 1 and 2, increasing of body weight of malnourished rat supplemented with goat milk yoghurt was higher (near twice:  $89.40-73.93=15.47$  g) than the control ( $88.16-78.77=9.39$  g) rats. However, the body weight of malnourished rats significantly lower ( $p<0.05$ ) than the normal rats (Table 2).

### Effect on total splenocytes

To study the immunity of malnourished rats, splenocytes counts were quantified. The results showed that the average of splenocytes counts of malnourished and normal rats treated with goat milk yoghurt was not significantly different with the control rats (Table 3).

### Effects on lymphocyte proliferation

Measurement of the proliferative response of lympho-

**Table 1. Body weight (g) of rats after 21 d of restriction feeding and normal rats before supplemented with goat milk yoghurt**

Rats	Before supplemented goat milk yoghurt	Control	Average
Malnourished (Restricted feeding)	73.93 $\pm$ 3.48 <sup>a</sup>	78.77 $\pm$ 5.03 <sup>a</sup>	76.35 $\pm$ 4.86 <sup>a</sup>
Normal	119.79 $\pm$ 9.28 <sup>b</sup>	117.61 $\pm$ 10.15 <sup>b</sup>	118.70 $\pm$ 9.41 <sup>b</sup>

Superscript with the different letters indicates significantly different ( $p<0.05$ ).

**Table 2. Body weight (g) of malnourished and normal rats treated with goat milk yoghurt**

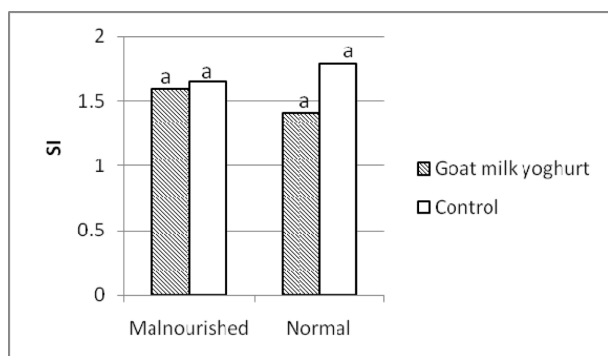
Rats	After supplemented Goat milk yoghurt	Control	Average
Malnourished	89.40 $\pm$ 4.56 <sup>a</sup>	88.16 $\pm$ 5.97 <sup>a</sup>	88.78 $\pm$ 5.14 <sup>a</sup>
Normal	148.92 $\pm$ 9.27 <sup>b</sup>	144.67 $\pm$ 11.86 <sup>b</sup>	146.80 $\pm$ 10.46 <sup>b</sup>

Superscript with the different letters indicates significantly different ( $p<0.05$ ).

**Table 3. Splenocytes count ( $\times 10^6$ ) of malnourished and normal rats treated with goat milk yoghurt**

Rats	Goat milk yoghurt	Control	Average
Malnourished	43.00 $\pm$ 18.04 <sup>a</sup>	32.93 $\pm$ 11.25 <sup>a</sup>	37.96 $\pm$ 15.36
Normal	38.71 $\pm$ 8.90 <sup>a</sup>	47.07 $\pm$ 17.32 <sup>a</sup>	42.89 $\pm$ 13.92
Average	40.85 $\pm$ 13.85	40.00 $\pm$ 15.83	40.42 $\pm$ 14.60

Superscript with the same letter indicates not significantly different.



**Fig. 2.** Stimulation index (SI) of splenocytes of malnourished and normal rat treated with goat milk yoghurt. (superscript with the same letter indicates not significantly different)

cytes is the most commonly used technique for evaluating cell-mediated immune response. Quantitative analysis of proliferative response involves measuring the number of cells in culture in the presence and absence of a stimulatory agent such as an antigen or a mitogen. Concanavalin A (Con A) and PHA stimulate T cells (Meydani and Ha, 2000).

Lymphocyte proliferation that was expressed as an stimulation index of lymphocytes in malnourished and normal rat was not significantly different. Furthermore, no significant changes were also observed in stimulation index of lymphocytes of rat treated with goat milk yoghurt for 7 d compared to the control in normal and malnourished rat (Fig. 2).

The average of lymphocyte stimulation index in the malnourished and normal rats supplemented with goat milk yoghurt was  $1.62 \pm 0.68$  and  $1.60 \pm 0.72$ , respectively.

#### Effect on TNF- $\alpha$ level in splenocyte culture supernatant

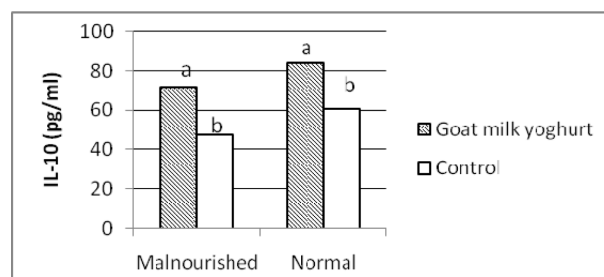
Levels of TNF- $\alpha$  was significantly decreased ( $p < 0.05$ ) in supernatant of cultured splenocytes from rat received goat milk yoghurt for 7 d compared to the control rat (Fig. 3), whereas TNF- $\alpha$  levels in malnourished and normal rat were not significantly different. The average of TNF- $\alpha$  in rats supplemented with goat milk yoghurt was  $19.90 \pm 5.87$  pg/mL, whereas in the control rat was  $25.01 \pm 4.08$  pg/mL.

#### Effect on IL-10 level in splenocyte culture supernatant

Goat milk yoghurt that supplemented in malnourished and normal rats increased level of IL-10 in spleen lymphocyte culture significantly ( $p < 0.05$ ) (Fig. 4). The average of IL-10 rats supplemented with goat milk yoghurt was



**Fig. 3.** Levels of TNF- $\alpha$  (pg/mL) in supernatant splenocytes culture of malnourished and normal rat treated with goat milk yoghurt. (superscript with the different letter indicates significantly different)



**Fig. 4.** Levels of IL-10 (pg/mL) in splenocytes culture supernatant culture of malnourished and normal rat treated with goat milk yoghurt. (superscript with the different letter indicates significantly different)

$78.05 \pm 23.72$  pg/mL, whereas in the control rat was  $53.80 \pm 18.79$  pg/mL.

## Discussion

Malnutrition causes decreasing the weight depending on degree of malnutrition. According to Cortés-Barberena *et al.* (2008), the mild malnutrition or first degree when the weight deficit reached 10-25%; moderate or second degree when the weight deficit reached 25-40%; and severe or third degree when the weight deficit was greater than 40% of that of age-matched control rats. Increasing of rat body weight in this study after receiving goat milk yoghurt was nearly twice when compared to the control rat 7 d before. This is accordance with the previous study (Prada *et al.*, 2007), in the rats treated with low protein diets for 60 d, 30 d of recovery feeding showed that body weight was twice when compared to that observed in the group one month before and no significant difference in proteinemia, albuminemia, circulating free fatty acid (FFA) levels and liver lipid content between the groups of low protein diets and normal protein. The low

of body weight in malnourished rats in this study also similar to the other previous study using rats 5 wk old with 55% dietary restricted (Hubert *et al.*, 2000) and protein restricted (Prada *et al.*, 2007). Decreasing this body weight gain through by reducing the intake of calories or other nutrients per unit body mass (Hubert *et al.*, 2000). The children on goat milk outgained the cow milk children in bodyweight over the 2-wk trial period and fat absorption tended to be better in the goat milk children (Razafindrakoto *et al.*, 1993).

Splenocytes counts of moderate malnourished and normal Wistar rats in this study similar to the previous study (Cortés-Barberena *et al.*, 2008), total splenic lymphocytes from normal Wistar rats was not significantly different with the moderate malnourished rats, but was significantly different between normal or moderate and severe malnourished rats. Maybe moderate malnutrition are not causing spleen damage that produces lymphocytes, but in the case of severe malnutrition occurs spleen damage. This possibility supported by the study effect of severe malnutrition in Wistar rats showed that malnutrition plays an important role in thymus and spleen atrophy by increasing the rate of spontaneous apoptosis, and indicate that severe malnutrition is associated with a significant increase of spontaneously apoptotic cells in the thymus (9.8-fold) and spleen (2.4-fold). These alterations may contribute to the immune suppression found in malnourished organisms (Ortiz *et al.*, 2008).

The total number of lymphocyte from normal rats was higher than the diabetic rat ( $260 \pm 23$  vs.  $123 \pm 25$ )  $\times 10^6$ /spleen, because that lymphocytes are more prone to injury in the diabetic state (Chi *et al.*, 1982). In this study, the number of lymphocytes in normal or malnourished rat was lower (Table 3) than the lymphocytes in the previous study by Chi *et al.* (1982). This differences may be due to differences of age and species of rats, because lymphocytes number may also decrease with age, whereas splenic morphology is affected by species, age and genetic (Cesta, 2006), that also effects on lymphocytes number. According to Cheung and Nadakavukaren (1983), that age related to cellularity changes (cells/g of tissue) of the spleen of rats and the spleen increase in weight with age. In this study, source of lymphocytes using Wistar rats and previous study by Chi *et al.* (1982) using mixed of Wistar and Sprague Dawley rat lymphocytes.

Lactic acid bacteria (LAB) of goat milk yoghurt in this study was  $9.1 \times 10^8$  CFU/mL and Bifidobacteria was  $2.0 \times 10^6$  CFU/mL. Supplementation this goat milk yoghurt for 7 d did not effect on lymphocyte number in spleen of nor-

mal and malnourished rats. This results similar to the previous study by Galdeano and Perdigon (2006), *L. casei* with dose of  $10^8$  CFU/mouse/day, that fed for 7 d did not influence the T-cell population of lamina propria in the mice small intestine.

There was no different between splenocyte proliferation response in moderate malnourished and normal rats fed goat milk yoghurt, because of the possibility of moderate malnutrition in rats no significant damage of the spleen as occurs in severe malnutrition. Giving yoghurt with a dose of 2 mL/day that containing LAB  $9.1 \times 10^8$  CFU/mL and bifidobacteria  $2.0 \times 10^6$  CFU/mL have no effect on proliferative response in normal and malnourished rats. First, this is because in normal (healthy) rats, yogurt supplementation is only used for the maintenance, and there is a homeostasis in the immune system and also there was no significant damage to the spleen in moderate malnutrition. Tolerance and homeostasis are maintained by specialized subsets of lymphocytes. Subsets of CD4<sup>+</sup> T cells have drawn most of the attention so far, and there are several phenotypes, depending on the type of cytokines or surface molecules they express (Corthesy *et al.*, 2007). Second, this possibility to occur spleen lymphocyte proliferation response is required a certain dose and duration of administration of probiotics and optimal mitogen concentration as well. According to Kirjavainen *et al.* (1999), *L. rhamnosus* GG (LGG) and *Propionibacterium freudenreichii* subsp. *shermanii* JS (PJS) have specific dose and duration-dependent immunomodulatory effects on the proliferative activity of B and T lymphocytes and may also reduce lymphocyte sensitivity to the cytotoxic effects of lectin mitogens. A significant decrease in basal lymphoproliferation (by 32 to 42%) was observed with PJS treatment after the 3- and 7-d periods; however, this activity returned to control levels after 14 d of treatment, which also resulted in significantly enhanced T-cell proliferation at optimal and supraoptimal ConA concentrations (by 24 to 80%). The 14 d LGG treatment also enhanced the latter activity (by 119%). In this study using 5  $\mu$ g/mL of PHA mitogen-specific T cell proliferation alone, so it is not stimulated B cells, and the PHA dose may also not optimal to induce lymphocyte proliferation response. Study by Palacios *et al.* (2007) using 60  $\mu$ g/mL T-cell mitogen PHA, showed that *in vitro* lymphocyte proliferation stimulated by T-cell mitogens decreased with age in free-living vertebrate.

After supplemented with goat milk yoghurt, the average of IL-10 was higher (Fig. 4) than TNF level (Fig. 3). The results showed that goat milk yoghurt containing *L. aci-*

*dophilus*, *S. thermophilus* and *B. longum* tend to induce anti-inflammatory cytokine in malnourished and normal rat. This results similar to the previous study using probiotic *E. coli*, that the probiotic strain was able to downregulate the increased levels of TNF- $\alpha$  both in the intestine from colitis rats, and in plasma and lungs in mice treated with LPS (Arribas *et al.*, 2009). In addition, the ability of this probiotic bacterium *E. coli* to reduce TNF- $\alpha$  production in inflammatory conditions was also demonstrated in the trinitrobenzene sulfonic acid (TNBS) model of rat colitis or when it was evaluated in the dextran sodium sulfate (DSS)-induced colitis in mice (Grabig *et al.*, 2006). TNF- $\alpha$  producing cells in the thymus increased significantly in the malnourished control group compared with the wellnourished control group, and no significant differences were observed among the test groups, milk, probiotic fermented milk, and bacterial-free supernatant, where the values obtained were similar to the wellnourished control group (Nunez *et al.*, 2013). According to study by Galdeano *et al.* (2011), the administration of probiotic fermented milk as a dietary supplement during the re-nutrition process in a murine immunodeficiency model by malnutrition showed could be a good adjuvant diet to improve the gut and systemic immune response for the protection against Salmonella infection (Galdeano *et al.*, 2011). This probiotics interact with immunocompetent cells using the mucosal interface and modulate locally the production of proinflammatory cytokines (Borrueal *et al.*, 2002). These proinflammatory cytokines are responsible for the presence of a chronic systemic inflammatory state, which shares numerous characteristics with the "acute-phase response" found in critically ill patients (Mantovani *et al.*, 2004). Practically, measurement of circulating levels of TNF- $\alpha$  would enable not only to identify malnourished patients in a more rapid and cost-effective way, but would also help to select those who might benefit from some type of nutritional intervention. Usually, malnourished patients had higher values of IL-1 and TNF- $\alpha$  (16.7 and 28.0 pg/mL) (Correia *et al.*, 2007).

Goat milk yoghurt containing *L. acidophils*, *S. thermophilus* and *B. longum* that supplemented in malnourished rats could increase the level of IL-10 in splenocytes culture supernatant and also in normal rats. This result similar to the previous study (Nunez *et al.*, 2013) using mice that received probiotics fermented milk containing *L. delbrueckii subsp. bulgaricus* ( $10^8$  CFU/mL) and *S. thermophilus* ( $10^8$  CFU/mL) and the probiotic bacterium *L. casei* DN-114-001 ( $10^8$  CFU/mL) during re-nutrition period showed significantly increased number of IL-10 produc-

ing cells in the thymus. The increases of IL-10 can contribute to the decrease in cellular apoptosis observed after re-nutrition.

Probiotics in fermented milk was the most effective re-nutrition supplement to improve the histology of the thymus, decreasing cellular apoptosis in this organ (Nunez *et al.*, 2013). Other previous study reported the effects of *E. coli* Nissle 1917 on the secretion of the cytokine IL-10 in Con A-activated splenocytes obtained from mice after LPS-induced septic shock (Arribas *et al.*, 2009). That effects are dose dependent, which was shown after performing additional experiments with different doses of the probiotic *E. coli* strain Nissle 1917 ( $10^6$ - $10^9$  CFU per mice) (Hockertz, 1997).

IL-10 is secreted by T lymphocytes, and monocytes play a central role in downregulating inflammatory cascades by suppressing the secretion of proinflammatory cytokines such as TNF- $\alpha$  (Kuhn *et al.*, 1993; Van Furth *et al.*, 1996). IL-10 also inhibits the production of chemokines by leukocytes and down regulates the expression of intercellular adhesion molecule 1 and chemoattractant proteins by endothelial cells, thus inhibiting leukocyte migration to the site of infection. In turn, TNF- $\alpha$  can induce the production of IL-10 upon stimulation with endotoxin (Van Furth *et al.*, 1996).

In this study, goat milk yoghurt using Lacto-B powder directly, which consists of mixture three types of bacteria as much as  $1 \times 10^9$  CFU/mL. The total of LAB in the goat milk yoghurt was  $9.1 \times 10^8$  CFU/mL and bifidobacteria was  $2.0 \times 10^6$  CFU/mL. This number of lactic acid bacteria still in the range according to Nunez *et al.* (2013). Anti-inflammatory effects such as stimulation of IL-10-producing cells, are strain-dependent traits, and their effectiveness also depends on the concentrations used and the method of administration. Oral administration of a probiotic mixture that consisted of *B. longum* Bar 33 and *L. acidophilus* Bar 13 prevented inflammation and mucosal ulcerations in a trinitrobenzene sulfonic acid- (TNBS-) induced colitis mouse model (Roselli *et al.*, 2009). This protection was associated with an upregulation of IL-10 that caused an inhibition of the TNBS-induced increase of the CD4<sup>+</sup> population, downregulation of IL-12, and a different pattern of Foxp3<sup>+</sup> CD4<sup>+</sup> CD25<sup>+</sup> Treg cells in the intraepithelial and lamina propria lymphocytes (Mengheri, 2008). In other probiotic mixture containing *L. acidophilus*, *L. casei*, *L. reuteri*, *B. bifidum*, and *S. thermophilus* induced both Tcell and B-cell hyporesponsiveness and down-regulated T helper (Th) 1, Th2, and Th17 cytokines without apoptosis induction in mice. It also induced gener-

ation of CD4<sup>+</sup> Foxp3<sup>+</sup> Tregs from the CD4<sup>+</sup>CD25 population and increased the suppressor activity of naturally occurring. Conversion of T cells into Foxp3<sup>+</sup> Tregs is directly mediated by regulatory dendritic cells (rDCs) that express high levels of IL-10, TGF- $\beta$ , COX-2, and indoleamine 2,3-dioxygenase. The therapeutic effect of the probiotics is associated with enrichment of CD4<sup>+</sup> Foxp3<sup>+</sup> Tregs in the inflamed regions (Kwon *et al.*, 2010). The forkhead family protein Foxp3 (forkhead box P3) is a transcription factor highly expressed in CD4<sup>+</sup> Tregs. It is a regulator of T-cell tolerance and is necessary for the development and function of Tregs (Ziegler, 2006).

The other previous study showed that a yoghurt produced with a pool of potentially probiotic LAB strains was effective in inhibiting the propagation of a dimethylhydrazine- (DMH-) induced colon cancer in mice by increasing the number of IL-10-secreting cells, increasing cellular apoptosis and decreasing procarcinogenic enzymes (deLeBlanc and Perdigon, 2010). By increasing IL-10 levels and in consequently decreasing inflammatory cytokines such as TNF- $\alpha$  and IFN- $\gamma$ , some LAB can prevent the appearance of local inflammatory diseases and could be used as an adjunct therapy with conventional treatments (de LeBlanc *et al.*, 2011).

Components of food that is consumed, naturally have the potential to interact with a variety of lymphoid cells throughout the gastrointestinal tract. Protein administered orally, has been known to interact with specific secondary lymphoid organs such as tonsils and Peyer's patches. Complex interactions that occur on the side (lymphoid organs) is crucial for the development of the specific immune response. Several factors can influence the interactions, including the form of solids or liquids from food component and oral exposure methods (Sfeir *et al.*, 2004). Immunomodulating requires intact or active peptides that are able to achieve immunocompetent cells. Most of the proteins are absorbed through the apical membrane of epithelial cells, which do not bind to the receptor will undergo lysosomal degradation (Perdue, 1999).

For the occurrence of the immune responses depends on the interaction with the active component with the immunocompetent cells, and immunogenicity of LAB in intestinal depends on degree of interaction with lymphoid tissue (Meydani and Ha, 2000). According to Sfeir *et al.* (2004), complex interactions between protein and immunocompetent cells is crucial for occurrence the specific immune response. Active component in yoghurt consists of bacterial and non-bacterial components that can role as immunomodulators (Meydani and Ha, 2000). Cell surface

components in lactic acid bacteria, such as peptidoglycan, lipoproteins, and diacylated lipopeptide, act as ligands for immune cells that bind to Toll-like receptor (TLR) 2 and TLR1 or 6 heterodimers on the macrophage cell surface and then stimulate nitrite oxide (NO) production (Itoh *et al.*, 2012).

The LAB are Gram-positive bacteria and their cell walls comprise a complex mixture of glycolipids, lipoproteins and phosphorylated polysaccharides embedded in a thick layer of peptidoglycan (PGN), a polymer of  $\beta$ -(1,4)-linked N-acetylglucosamine and N-acetylmuramic acid, cross-linked by short peptides. TLR2 has been shown to recognize lipoteichoic acid (LTA), lipoarabinomannan, lipoprotein (LP) and PGN, and is likely to be involved in the recognition of LAB. Lactic acid bacteria act as immunoregulators through interaction of lipoprotein with TLR2 and as immunostimulators through interaction of peptidoglycan with nucleotide-binding oligomerization domain-2 (NOD2). As dendritic cell (DC) are potent stimulators of naive T cells and express several pattern recognition receptors (PRRs), they serve as an important link between the microbiota and polarization towards T helper type 1 (Th1), Th2- or regulatory T-cell-dominated environments. Cell surface-expressed and intracellularly expressed PRRs collectively recognize lipid, carbohydrate, protein and nucleic acid structures that are broadly expressed by different groups of microorganisms (Zeuthen *et al.*, 2008).

Recognition of microbial components by TLRs initiates signal transduction pathways, which triggers expression of genes. These gene products control innate immune responses and further instruct development of antigen-specific acquired immunity. Individual TLRs play important roles in recognizing specific microbial components (Takeda and Akira, 2005).

The presence of LAB is thought to be essential for yoghurt to exert immunostimulatory effects but components of nonbacterial yogurt, such as whey protein, short peptides, and conjugated linoleic acid (CLA), are believed to contribute to yoghurt's beneficial effects as well. It is proposed that the LAB that survive through the gastrointestinal (GI) tract, whether intact or modified, can bind to the luminal surface of M cells. LAB-bound M cells reaching to the dome region of Peyer's patch (PP) cells stimulate local immune response, which can further activate the local immune system, resulting in stimulation of the local and the systemic immune response (Meydani and Ha, 2000). Whey protein had activity as an anti-allergy, anti-inflammatory, immunomodulatory and antioxidant. One of the proteins in the milk whey fraction which has the broadest



range of biological activities related to the body's defense system, is lactoferrin, which among other acts as immunomodulator (Shimazaki *et al.*, 1998; Zimecki and Kruzel, 2000). Study by Ward *et al.* (2002), showed that lactoferrin had been known inhibit allergen-induced skin inflammation in both mice and humans, most likely secondary to TNF- $\alpha$  production. The potency of probiotic bacteria to regulate immune responses is a complex interaction between the host immune system and different bacterial compounds, including chromosomal DNA and cell wall components as well as soluble metabolites (Corthesy *et al.*, 2007).

The other non-bacterial component in goat milk yoghurt was prebiotics oligosaccharides. According to Wu *et al.* (2006), goat milk showed rich in sources of prebiotic oligosaccharides that resemble milk oligosaccharides (breast milk) (Bode, 2006), and in contrast to other prebiotics such as inulin or frukto short-chain oligosaccharides (Lara-Villoslada *et al.*, 2006). The similarities found between human and goats' milk oligosaccharides in terms of the presence of neutral (galactosyl-lactose and lacto-N-hexaose) and sialylated (3-, 6-sialyl-lactose and disialyl-lactose) structures suggest that the latter may, to some extent, carry out or resemble physiological activities described for the former (Martinez-Ferez *et al.*, 2006). Goat milk oligosaccharides also have anti-inflammatory effects in rats with experimental colitis and may be useful in the management of inflammatory bowel disease (Daddaoua *et al.*, 2006).

In conclusions, supplementation of goat milk yoghurt in malnourished rats could decrease the pro-inflammatory cytokine, while it increases the anti-inflammatory cytokine, represented by TNF- $\alpha$  and IL-10 levels in the splenocyte culture supernatant, respectively. However, that goat milk yoghurt feeding does not give any effect on total splenocyte numbers and splenocyte proliferation.

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