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# The potential of *Lactobacillus rhamnosus* and *Lactobacillus plantarum* isolated from goat's milk in inhibiting *Salmonella typhimurium* ATCC 14028 infections in rats

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**Abstract**

Probiotic is live non-pathogenic microorganisms that give beneficial effects on health when they are administered in adequate amounts. The objective of the study was to evaluate the influence of Lactic Acid Bacteria (LAB) isolates (*L. rhamnosus* and *L. plantarum*) as well as cheese containing the probiotics on microflora profiles, morphological profile of ileum and caecum, and immunomodulator potency by measuring lymphocyte proliferation and IgA levels in rats. Male *Sprague Dawley* rats were fed with the probiotics or cheese containing the probiotics for 10 days, infected with *S. typhimurium* for 3 days, and continued to be fed with or without the probiotics or the cheese. A total of 6 treatments were applied, which were: (pro-typ-pro, pro-typ-std, che-typ-che, che-typ-std, pro-PBS-pro, and std-typ-std). The measured variables were the number of LAB and *S. typhimurium* colonies, lymphocyte cells, and the level of SIgA. The results showed that the highest number of LAB in the ileum and caecum in probiotic fed rats (pro-typ-std followed by pro-typ-pro) as compared to the control, whereas number of *S. typhimurium* was lower. The study showed that the treatment of probiotic isolate was able to improve the number of lymphocyte during the first 10 days, during the infection of *S. typhimurium,* and post infection stage. The treatment of probiotic isolate was able to improve SIgA at the time of *S. typhimurium* intervention. In conclusion, mixed isolates of *L. rhamnosus* and *L. plantarum* and cheese containing the probiotics were able to show preventive and remedial functions during *S. typhimurium* ATCC 14028 infection, thus demonstrate the potential to be used as probiotic cultures.



## Introduction

Despite many probiotic bacteria have been known, the search for lactic acid bacteria (LAB) with probiotic characteristics from different sources is continuing. A recent review by Nuraida (2015) showed that Indonesian fermented foods were an abundant sources of LAB with probiotic characteristics. Several criteria for selecting LAB as candidate for probiotic are used, including their ability to attach to the intestine and to pass the gastrointestinal track, and show antimicrobial activity against pathogenic bacteria. Milk, in particular goat milk, is a potential source of LAB. LAB generally be found in raw milk at 20 to 30% of the total bacterial population. Delavenne *et al.* (2012) were successfully isolated LAB with antifungal properties from goat, cow, and sheep milk. Previously, Salva *et al.* (2010) reported two strains of Lactobacillus rhamnosus that possesed immunomodulatory activities.

One role of probiotic bacteria is to maintain the balance of intestinal microflora in humans or animals by means of reducing the incidence of gastrointestinal infections by pathogenic bacteria. Some strains of *Lactobacillus* have been proven to inhibit Gram-negative pathogenic bacteria by producing lactic acid and the ability to survive at low pH. According to Fayol-Messaoudi *et al.* (2005), some strains of *Lactobacillus* have been proven to inhibit the growth of serovar *typhimurium* SL144. Strain *L. casei* GG is able to: reduce the number of *S. typhimurium* at the onset of infection by releasing antimicrobial

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substances, modify the surface of *S. typhimurium* thereby reducing its ability to penetrate to the small intestine, and stimulate the immune system (Hudault *et al*., 1997).

There are two types of *Salmonella* infection in the intestines; (1) non-typhoid, and (2) typhoid fever (Trussalu *et al.*, 2004). The infection is due to the various serotypes of *S. enterica* that causes mild diarrhea, digestive tracts inflammation, and typhoid fever that may cause death. The infection of *S. typhimurium* in the digestive tract is preceded by the invasion of the Peyer's Patches and M-cell, with ileum as the main target (Hudault *et al.*, 1997).

According to Casey *et al.* (2007), there are four ways probiotic defense against pathogens; the antagonistic properties resulted from antimicrobial substances, the ability to compete against pathogenic bacteria for making attachment on intestinal mucosa, the ability to compete against pathogenic bacteria for nutrients, and the improvement of host's immune system. Hence, the purpose of this study is to test the effectiveness of probiotic isolates and cheese containing *L. rhamnosus* and *L. plantarum* originated from goat's milk on the profile of intestinal microflora and their potency as immunomodulator in rat.

## Materials and Methods

*Treatments preparation*

In vivo test was performed using male *Sprague Dawley* rats, 6 weeks of age, and 120-140 g in weight. Bacterial culture containing *L. rhamnosus* and *L. plantarum* isolated from goat's milk was proven to stand on low pH, bile salts, have antagonistic properties against several pathogenic bacteria, and be able to stick to the intestinal mucosa. American Type Culture Collection (ATCC) pathogenic bacteria which were obtained from the culture collection of Department of Food Science and Technology, Bogor Agricultural University, i.e. *Salmonella typhimurium* (ATCC 14028). The culture of *S. typhimurium* ATCC 14028 was prepared with sterilized Phosphate Buffer Saline (PBS) and contained 8 log CFU $mL^{-1}$. This culture was ready to be administered into the rats at the infection stage.

Bacterial culture containing *L. rhamnosus* and *L. plantarum* was grown in de Man Rogosa and Sharpe Broth (MRSB, Difco) medium for 24 hours, and then centrifuged at 5000 rpm for 10 minutes. The media was prepared according to Kim *et al.* (2001). The culture then was diluted with PBS to obtain colonies of 8 log CFU $mL^{-1}$. Bacterial culture containing Gram-negative pathogenic *S. typhimurium* ATCC 14028 was grown in Tripton Soy Broth (TSB, Difco) for 24 hours, and then centrifuged at 5000 rpm for 10 minutes. The culture then was diluted with sterile PBS to obtain concentration of log 8 CFU $mL^{-1}$ and be ready to be administered orally to rat at infection stage. Soft cheese was produced from goat's milk with mixed cultures of *L. rhamnosus* and *L. plantarum*. Cheese containing log 8 CFU $g^{-1}$ colonies was fed to the rat 1g/d every morning before being given any feed.

Testing the effectiveness of probiotic bacteria against *S. typhimurium* was carried out using the procedures described by deLeBlanc *et al*. (2010). The experiment was divided into 3 stages, initial stage (day 1 to 10), infection stage (day 11 to 13) and final stage (day 14 to 23). Rats were given probiotic (pro), cheese (che), standard feed (std), and infected with *S. typhimurium* (typ). Treatment ¬pro, rats were given probiotic orally 1 mL/rat (log 8 CFU $mL^{-1}$ LAB) followed by standard feed. Treatment che, each rat was given 1g fresh cheese containing log 8 CFU $g^{-1}$ LAB followed by standard feed. Treatment std, rats were given standard feed only. Treatment typ, rats were infected with S. typhimurium ATCC 14028 (log 8 CFU $mL^{-1}$). There were 6 groups of treatments that applied, each group consisted of 9 rats. Pro-typ-pro: rats were given probiotic at initial stage, infected with *S. typhimurium* at infection stage, and given probiotic at final stage. Pro-typ-std: rats were given probiotic at initial stage, infected with *S. typhimurium* at infection stage, and given standard feed at final stage. Che-typ-che: rats were given cheese at initial stage, infected with *S. typhimurium* at infection stage, and given cheese at final stage. Che-typ-std: rats were given cheese at initial stage, infected with S. typhimurium at infection stage, and given standard feed at final stage. Std-typ-std: rats were given standard feed at initial stage, infected with *S. typhimurium* at infection stage, and given standard feed at final stage.

All rats were maintained for a total of 23 days and the rats in each treatment were dissected after the first 10 days of the experiment (initial stage), then 3 days post infection (infection stage), and finally after 10 days of the latest stage of the experiment. The initial stage of the experiment (day 1 to 10) was conducted with the aim to determine the effects of *L. rhamnosus* and *L. plantarum* administration in rats. The infection stage (day 11 to 13) was aimed to test the resistance of *L. rhamnosus* and *L. plantarum* from isolates and fresh cheese against *S. typhimurium*. The final stage (day 14 to 23) was aimed to investigate the effects of administering *L. rhamnosus* and *L. plantarum* through isolates and fresh cheese after *S. typhimurium* infection.

The measured variables were number of LAB,

*S. typhimurium*, lymphocytes, and absorbance value of SIgA. All treatment protocols have been approved by the Committee of Ethics, Department of Health, Republic of Indonesia No: KE.01.02/EC/06H/2011.

*Quantitative test of lactic acid bacteria*

Total LAB was determined following the conventional pour plate procedures described by Ortolani *et al*. (2007). As wide as 1 $cm^2$ of dissected ileum and caecum was washed 3 times with PBS solution to remove its contents. Samples were crushed and homogenized in sterile plastics, and then 9 mL of sterile Butterfield's phosphate-buffered was added. After several dilutions, LAB was grown in MRSA at 37$^o$C for 48 hours. Colonies was counted using a colony counter.

*Quantitative test for* S. typhimurium

Total count of *S. typhimurium* was determined by the procedures described by Thushani *et al.* (2003). Sample preparation was similar to that for LAB, but after several dilutions, the bacteria were pour-plated in Xylose-Lysine Deoxycholate Agar (XLDA) medium and incubated at 37°C for 24 hours. Each determination was done in duplicate.

*Number of lymphocyte cells ex vivo in rat*

The rats were dissected at the end of the experiment, and the lymph was taken. The lymph was placed on a sterile plate containing sterile PBS and washed with sterile Roswell Park Memorial Institute (RPMI)-1640 medium. The lymph was mashed, placed in a sterile plate and then added with 5 mL RPMI-1640. By using a sterile syringe, the fluid of the mashed lymph was placed in a sterile plastic tube and centrifuged at 1500 rpm for 15 min. After the supernatant was removed, the precipitate was soaked in sterile ammonium chloride (NH4Cl) 0.85% and incubated for 2 min. The precipitate was added with 3 mL sterile RPMI-1640 and re-centrifuged at 1500 rpm for 10 min. Afterward, the precipitated cells were washed with 5 mL RPMI-1640 and re-centrifuged at 1000 rpm for 10 min. Another 2 mL of RPMI-1640 was added to the cells and several dilutions were performed. Cell suspension was mixed with trypan blue in a ratio of 1:1 (v:v). The counting was carried out using a microscope at 400 times magnification. The number of live cells was counted in 2 large boxes of hemocytometer (each has 16 small boxes) and the cells per mL of suspension were calculated by this formula: number of cells $mL^{-1}$ = average number of cells in each area × $10^4$ × dilution factor, and 104 equal to one area (Gill *et al.*, 2000).

*Test for secretory immunoglobulin-A (SIgA)*

The procedures of Roller *et al*. (2004) were used to test for SigA with slight modification. Fluid samples were taken from the intestinal mucosa of ileum and caecum. Samples were washed with PBS solution and then centrifuged at 5000 rpm for 15 minutes. Supernatant was stored in Eppendorf tubes and stored at -20°C until ready for use. A sample of 100 µL was pipetted into wells containing 100 µL sodium bicarbonate ($NaHCO_3$), and then incubated overnight at 4°C. Samples were washed 3 times with PBS-Tween and blocking was done with FBS-Tween, followed by incubation at 37°C for 60 min. Samples were washed again 3 times with PBS-Tween. Goat anti-rat antibody of 100 µL was coated on the wells. The antibody was previously been diluted with solvent PBS-Tween 20 (1:50 $vv^{-1}$). Samples and antibody were incubated at 37°C for 60 minutes. The next step was done by washing it with PBS-Tween. A total of 100 µL peroxidase antibody produced in rabbit (diluted 1:10000 in PBS-Tween) was added to the wells and incubated again at 37°C for 60 minutes. Peroxidase substrate, 100 µL tetramethylbenzidine, was added and then the wells were placed in a dark room for 30 minutes. Enzyme reaction was stopped by adding 100 µL of 1 mol/L $H_3PO_4$. The amount of SIgA was determined by measuring the wavelength at 450 nm.

*Data analysis*

Statistical analysis for SIgA was done using analysis of variance followed by Duncan's Multiple Range Test for post-hoc using SPSS 17 software. Descriptive analysis was performed for data of LAB, *S. typhimurium*, and lymphocyte.

## Results

*Number of LAB in the ileum and caecum*

The ability of LAB to stick on intestinal mucosa is very important to maintain the digestive tracts ecosystem. Probiotic isolates that were given to the animals for 10 days resulted in higher LAB population than control, but it is not the same when the animals were given probiotic-containing cheese (Table 1). the administration of LAB in the form of isolates was more efficient than that of probiotic-containing cheese.

Results indicate that the population of LAB in the rat's caecum which were given probiotic isolate and probiotic-containing cheese prior to infection is higher than the control group. Probiotics are able to protect the caecum from *S. typhimurium* infection. The animals given probiotics at post-infection

Table 1. Number of LAB in the ileum and caecum (log CFU $g^{-1}$)

| Sampling location | Day of Sampling | Treatment Group | | | | |
|---|---|---|---|---|---|---|
| | | pro-typ-pro | pro-typ-std | che-typ-che | che-typ-std | std-typ-std |
| Ileum | 10 | 6.22±0.62 | 6.78±0.2 | 5.58±0.21 | 5.47±1.34 | 5.75±0.68 |
| | 13 | 6.05±0.14 | 6.30±0.38 | 5.45±0.05 | 5.10±0.47 | 4.34±0.27 |
| | 23 | 6.23±0.80 | 5.45±0.37 | 6.12±0.71 | 5.57±0.63 | 4.98±0.92 |
| Caecum | 10 | 5.56±0.17 | 6.32±0.56 | 6.07±0.49 | 5.97±0.77 | 5.36±0.17 |
| | 13 | 6.30±0.61 | 6.69±0.04 | 6.24±0.24 | 6.10±0.67 | 4.60±0.44 |
| | 23 | 6.29±0.62 | 6.04±0.21 | 6.33±0.40 | 5.74±0.48 | 5.32±0.31 |

10: LAB administration time till day 10 (early treatment); 13: *S. typhimurium* infection at day 11 to 13 (48 hours), 23: repair time from infection until day 23 (continued treatment).

Table 2. Number of *S. typhimurium* in the ileum and caecum (log CFU $g^{-1}$)

| Sampling location | Day of Sampling | Treatment Group | | | | |
|---|---|---|---|---|---|---|
| | | pro-typ-pro | pro-typ-std | che-typ-che | che-typ-std | std-typ-std |
| Ileum | 10 | Nd | Nd | Nd | Nd | Nd |
| | 13 | 3.22±0.29 | 3.10±0.01 | 3.68±0.21 | 3.60±0.17 | 4.59±0.20 |
| | 23 | Nd | 3.07±0.06 | 1.45±1.02 | 3.13±0.73 | 4.48±0.07 |
| Caecum | 10 | Nd | Nd | Nd | Nd | Nd |
| | 13 | 3.80±0.05 | 3.61±0.25 | 3.47±0.03 | 3.53±0.13 | 4.81±0.22 |
| | 23 | Nd | 0.80±1.38 | Nd | 2.94±0.07 | 4.92±0.62 |

10: LAB administration time till day 10 (early treatment); 13: S. typhimurium infection at day 11 to 13 (48 hours), 23: repair time from infection until day 23 (continued treatment).

(up to day-23) have higher LAB population in the caecum than those given standard feed. Giving more probiotics post-infection increases LAB population and as well as the recovery of the caecum.

*Number of S. typhimurium in the ileum and caecum*

Because *S. typhimurium* was not detected in both ileum and caecum before the infection (Table 2), our results indicate that ileum and caecum is not natural habitat for *S. typhimurium*. The presence of this pathogen in the gastrointestinal tracts is due to food infection, which causes indigestion, inflammation, and even typhoid fever. After infection (day-13), *S. typhimurium* was immediately found in the ileum and caecum of all animals. However, data show that the population of *S. typhimurium* in rats which was given probiotics through isolates or cheese was lower than that of the control group. This shows that the presence of *L. rhamnosus* and *L. plantarum* in the gut is able to inhibit the growth of the pathogenic bacteria.

*Number of Lymphocytes*

The presence of bacteria in the digestive tracts is affected by the antagonistic mechanism between bacteria and the body immune system. LAB plays an important role in protecting mucous membranes and then enhancing the immune system. The amount of lymphocytes which can be used to represent body immune system is presented in Table 3.

Table 4 shows all of the treatment given to 10 days resulted in a higher number of lymphocytes compared to the control group. Giving probiotic isolates and cheese containing probiotic isolates can improve the body immune system. LAB stimulates the activity of cell immunity both non-specific and specific. After the infection of *S. typhimurium* occurred, the number of lymphocytes increased in all groups compared to the control group. The increasing number of lymphocytes was in line with the declining number of *S. typhimurium* after the infection stage and the increasing number of LAB until day-23 in ileum and caecum. This shows that probiotic isolates and cheese containing probiotic isolates are able to restore the body's immunity by increasing the number of lymphocytes.

*Secretory immunoglobulin A (SIgA)*

Immunoglobulin A (IgA) is the major immunoglobulin which is found in the intestinal mucosa and then called as the secretory IgA (SIgA). SIgA act as first line of defense against intestinal antigens, as SIgA is more resistant to proteolytic enzymes and not affected the inflammatory response. The results of SIgA absorbance obtained from the rat's ileum and caecum are shown in Table 4.

Table 4 shows that at the 10$^{th}$ days of initial

Table 3. Number of lymphocytes in the lymph (106 cell $mL^{-1}$)

| Day of Sampling | Treatment group | | | | |
|---|---|---|---|---|---|
| | pro-typ-pro | pro-typ-std | che-typ-che | che-typ-std | std-typ-std |
| 10 | 99 ±47 | 57 ±5.7 | 140±93 | 74 ±6.4 | 57±13 |
| 13 | 230±8.5 | 45± 34 | 27 ± 21 | 18 ±8.1 | 6.8±1.6 |
| 23 | 370±28 | 79 ± 59 | 190±15 | 120±71 | 15±2.1 |

10: LAB administration time till day 10 (early treatment); 13: S. typhimurium infection at day 11 to 13 (48 hours), 23: repair time from infection until day 23 (continued treatment).

Table 4. Absorbance values of secretory immunoglobulin A (SIgA) at OD 450 nm

| Sample | Day of sampling | Treatment group | | | | |
|---|---|---|---|---|---|---|
| | | pro-typ-pro | pro-typ-std | che-typ-che | che-typ-std | std-typ-std |
| ileum | 10 | 0.089±0.0C | 0.096±0.0 | 0.112±0.00ª | 0.102±0.00ª | 0.101±0.00ª |
| | 13 | 0.114±0.0C | 0.119±0.0 | 0.086±0.00ª | 0.090±0.00ª | 0.095±0.00ª |
| | 23 | 0.102±0.0C | 0.093±0.0 | 0.078±0.00ª | 0.090±0.00ª | 0.085±0.00ª |
| caecum | 10 | 0.097±0.0C | 0.112±0.0 | 0.101±0.01ª | 0.085±0.00ª | 0.104±0.01ª |
| | 13 | 0.087±0.01 | 0.083±0.0 | 0.089±0.00ª | 0.101±0.00ª | 0.096±0.00ª |
| | 23 | 0.097±0.0C | 0092±0.0( | 0.079±0.00ª | 0.084±0.00ª | 0.086±0.00ª |

Different superscripts in the same row indicate significantly different ($p<0.05$). 10: LAB administration time till day 10 (early treatment); 13: *S. typhimurium* infection at day 11 to 13 (48 hours), 23: repair time from infection until day 23 (continued treatment).

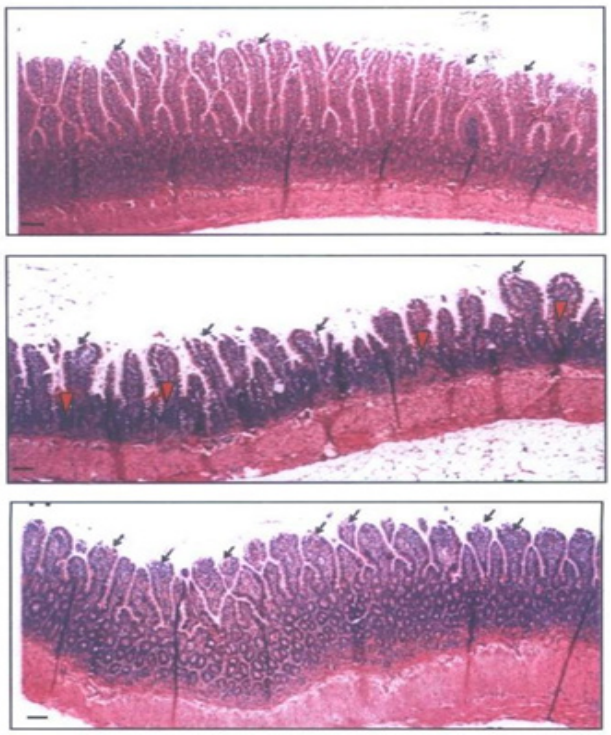

Figure 1. Ileal villi of the rats during the infection of *S. typhimurium*. The rats were dissected at the 13th day of the treatment. These tissue samples were colored using Hematoxylin-Eosin (HE). (↙) releasing of epithelial cells, (▾) goblet cells proliferation, (-) bar scale 50 µm. a) Ileal villi during the infection of *S. typhimurium* with probiotic isolates feeding b) Ileal villi during the infection of *S. typhimurium* with cheese containing probiotic isolates feeding c) Ileal villi during the infection of *S. typhimurium* with standard feeding.

treatment, the absorbance value of SIgA in ileum of cheese containing probiotic isolates group (che-typ-che) was increasing, compared to the control. The increasing of absorbance value is due to the LAB is protected by the cheese matrix and fat during passing the digestive tract. At the infection stage of *S. typhimurium*, probiotic isolates treatment (pro-typ-pro) was able to increase absorbance value of SIgA in ileum and it was significantly different ($p<0.05$) compared to the control.

Giving LAB isolates at 10 days initial treatment increased the absorbance value of SIgA in caecum than the control. However, there is no influence of probiotic isolates administration to SIgA in caecum at the infection and post-infection treatment.

## Discussion

Infecting the animals with *S. typhimurium* at day-10 to day-13 decreased the number of LAB in the rat's ileum in all treatments. Giving more probiotic isolates and cheese after infection until day-23 increased the number of LAB, which was not the same with the other treatments. The decreasing number of LAB during pathogen infection is in line with Arief *et al.* (2010) which stated that the intervention of EPEC K.1.1 caused a decrease in the number of LAB and with Hudault *et al*. (1997) which stated 5 days post-infection of *S. typhimurium* caused a decline in the number of LAB in the small intestine.

The growth of *S. typhimurium* was completely inhibited when the rats were continuously given probiotics at post-infection stage. Data show that no pathogenic bacteria were detected at day-23 in rats that were continuously given probiotics (pro-typ-pro and che-typ-che). When probiotics were no longer given at post-infection stage (pro-typ-std and che-typ-std), the pathogenic cells were detected at low number. These results indicate that the existing probiotics (given before and during infection) are able to retard the growth of *S. typhimurium* ATCC 14028. On the contrary, rats without probiotics (std-typ-std) showed high population of *S. typhimurium* ATCC 14028 at day-23. The effect of *S. typhimurium* ATCC 14028 infection to the histological performance of the rat's ileum can be seen at Figure 1.

The ability of LAB to protect the caecum against *S. typhimurium* infection was described by Hudault *et al.* (1997); (1) decreasing the number of *S. typhimurium* with the onset of antimicrobial substances produced by LAB, (2) modifying the surface of *S. typhimurium* which will reduce penetration in the small intestine, and (3) stimulating the immune system.

*S. typhimurium* infection resulted in higher lymphocyte number in probiotic isolates and cheese containing probiotic isolates groups than the control group. An indicator of improved immune system is the increased number of lymphocytes. The body responds to *S. typhimurium* by activating the innate immune system through cell phagocytosis, and then killing the pathogens. The increasing ability of phagocytosis kills pathogenic bacteria, thereby increase the immune system.

Bujalance *et al*. (2007) stated that *L. plantarum* acts as immunomodulator by improving the response of lymphocytes in impaired immune system. *L. plantarum* 2C12 and *L. acidophilus* 2B4 could improve immune status in rats which were infected with EPEC K1.1 on the $7^{th}$, $14^{th}$, and $21^{st}$ day. Giving probiotics could improve immune status by increasing the number of lymphocytes from 106 to 107-108 cells (Ariel *et al.*, 2010).

Probiotic isolates treatment is able to induce SIgA which is capable in binding S. typhimurium with the result that prevents antigen activation. SIgA binds the antigen by minimizing commensal bacteria and preventing the formation of systemic antigen. Providing LAB isolates after *S. typhimurium* infection increases the absorbance value of SIgA in ileum. These results demonstrate an important role of SIgA in preventing internalization of *S. typhimurium*

The survival of probiotics will activate the mucosal immune system and be manifested by the increasing value of SIgA absorbance (Tsuji *et al.*, 2008). Tsuji *et al.* (2008) stated that probiotics in cheese increased production of IgA cells in the small intestine and lamina propria of the rat's colon. The role of SIgA as innate immunity which is normally produced is to against gastrointestinal infections (Favre *et al*., 2005). These results are consistent with Salva *et al.* (2010) who stated that the provision of *L. rhamnosus* CRL1505 and *L. rhamnosus* CRL1506 significantly increased IgA compared to the control. An immune mechanism due to the infection of *S. typhimurium* was the activation of intestinal local immune system induced by *L. rhamnosus* CRL1505 and *L. rhamnosus* CRL1506. Immunoglobulin A (IgA) protects the mucosa to against any pathogenic microorganisms present in the gut (Macpherson *et al.*, 2000).

## Conclusion

Administration of probiotics, either through isolates or fresh cheese containing the respective probiotics, inhibits the growth of *S. typhimurium* in the digestive tracts. The maximum inhibition of probiotics against *S. typhimurium* is obtained when the probiotics are continuously given on the pre-, during, and post-infection of *S. typhimurium* (pro-typ-std followed by pro-typ-pro). The number of lymphocytes increased in all groups compared to the control group. The probiotic isolates group (pro-typ-pro) increased the absorbance value of SIgA in ileum during the infection of *S. typhimurium* compared to the control and in caecum during the first 10 days before infection compared to the control. Damaged villi due to the infection of *S. typhimurium* are indicated because of the release of intestinal epithelial cells.

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