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Effect of Probiotic *Lactobacillus* sp. Dad13 on Humoral Immune Response of Balb/C Mice Infected with *Salmonella typhimurium*

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Abstract

An *indigenous* strain of lactic acid bacterium (LAB) identified as *Lactobacillus* spp. Dad13 (Dad13), isolated from traditional fermented buffalo milk, was found to be potential as probiotic. The aim of this research was to study the effect of probiotic Dad13 on humoral immune response of Balb / C mice infected with *Salmonella typhimurium*. The specific objective was to find out the effect of different Dad13 consumption time (before and along with infection of *S. typhimurium*) on the humoral immune response of Balb / C mice. The experiment was conducted by *in vivo* trial on 20 males of Balb / C mice, age of 6-8 weeks, fed with AIN-93 standard diet. The mice were assigned into 4 groups. Each group received the following treatments, ie. Dad13 only, Dad13 before infection, Dad13 along with infection and Salmonella infection only. A volume of 100 µl Dad13 cell suspensions (10^{10} CFU / ml) were given by *oral forced feeding* daily for a week, at week 3 for group before infection and at week 4 for group of Dad13 only and Dad13 along with infection. *Salmonella* infection (10^{9} CFU / ml) was given once orally at week 4 to all groups except group treated with Dad13 only. The humoral immune response of Balb / C mice was detected 2 weeks after infection by measuring the titers of IgG and IgA specific from serum and mucosal intestinal liquid samples using *Enzyme-linked Immunosorbent Assay* (ELISA) method. The result indicated that humoral immune response of Balb / C mice consuming Dad13 before and along with *Salmonella* infection were significantly different ($p<0.05$). Dad13 consumption along with *Salmonella* infection increased circulated IgG and IgA as well as secretory IgA. It can be concluded that Dad13 probiotic feeding along with infection increased humoral immune response more significantly compared to that before infection.

Key words : Probiotic, *Lactobacillus* sp. Dad13, Immune response, *Salmonella typhimurium*

Introduction

Food borne Salmonellosis is still a big problem in Indonesia. *Salmonella typhimurium* is one of the pathogenic Salmonella species which causes the diseases. Besides the treatment with antibiotics, functional food such as probiotic is currently considered as an alternative way to reduce the number of diarrhea cases which are caused by food contamination. Probiotic gives health effect by improving the microbial balance of gastrointestinal tract (Perdigon *et al*., 1995; Kaminogawa *et al*., 2004). Probiotic is defined as live microorganism added to food that give benefit to host by increasing the microbial balance of intestinal (Ziemer *et al*., 1998). Another definition stated that probiotic is live microorganism which is consumed by oral and give health effect (Losada *et al*., 2002; Champagne *et al*., 2005). Some lactic acid bacteria (LAB) have been studied as potent probiotic.

Current researchs of probiotic focus on the capability of the products to increase immune system and immune response mechanism which is thought to play an important role to protect the body from any

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kind of pathogen infection. Studies on the effect of probiotic on the immune system involved the usage of experimental animal, especially mice as a model (Tejada-Simon *et al*., 1999; Sheih *et al*., 2001; Sgouras *et al*., 2003; LeBlanc, *et al*., 2004) and most of the result could be adopted to human (Perdigon *et al*., 1995). The immune evaluation is conducted on the parameter that represent innate immunity and acquired immunity, as systemic immunity or humoral immunity.

An *indigenous* strain of *Lactobacillus* spp. Dad13 (Dad13) which was isolated from traditional fermented buffalo milk (Ngatirah, 2000), was recommended as a potent probiotic. The aim of this study was to identify the effect of the Dad13 on humoral immune response of Balb/C mice infected with *Salmonella typhimurium*. The specific objective was to find out the effect of different consumption time of Dad13 (before and along with *Salmonella* infection) on the humoral immune response.

Materials and Methods

Bacterial strains, growth condition and cell suspension.

Lactobacillus spp. Dad13 obtained from Dr. Eni Harmayani (Food Nutrition Culture Collection, GMU) was maintained in MRS (Man Rogosa de Sharpe) broth medium (Oxoid) and grown to end of log phase at 37°C for 16 h, using an overnight culture. The number of cells were determined by plate count method serial dilution in 0.1% peptone water on MRS agar (Oxoid) incubated at 37°C for 24-48 h. *Salmonella typhimurium* (Food Nutrition Culture Collection/FNCC 157) was grown with agitation (120 rpm) in TSB (Tryptic Soy Broth) medium (Oxoid) to end of log phase at 37°C for 18 h, using an overnight culture. The number of cells were determined by plate count method serial dilution in 0.1% peptone water on SS (*Salmonella shigella*) agar (Oxoid) incubated at 37°C for 24-48 h. Cell suspensions of Dad13 and *Salmonella* were prepared by resuspended in sterile phosphate-buffered solution (PBS) of pH 7.0 to the desired concentration of 10^{9} CFU/ml for Salmonella and 10^{10} CFU/ml for Dad13.

Standard diet AIN-93.

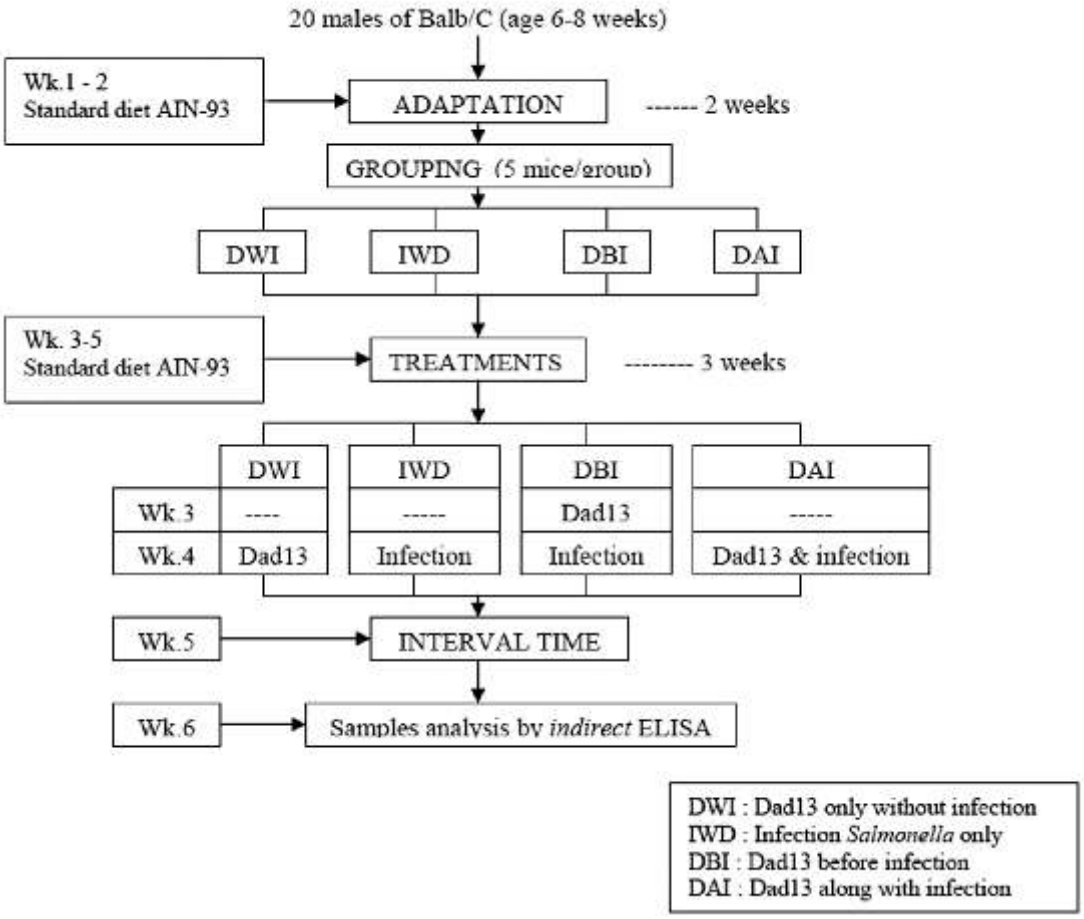
Standard AIN-93 diet for the experimental animals was prepared according to Reeves *et al.* (1993). The materials (Table 1) starting from the lowest amount added by sufficient water to dilute and mix the materials. The mixture was shaped and dried in a cabinet dryer (40-60°C).

Table 1. Composition of standard diet AIN-93 (gr/1000 gr)

No	Componen	Quantity(gram)
1	Maizena	620.7
2	Casein	140.0
3	Sucrose	100.0
4	Soybean oil	40.0
5	Cellulose	50.0
6	Mineral mix, MX AIN-93	35.0
7	Vitamin mix, VX AIN-93	10.0
8	Kholin bitartrat	2.5
9	L-cystin	1.8
Quantity		1000.0

Immune system stimulation studies.

The immunomodulatory effect of Dad13 was determined by *in vivo* trial on Balb/C mice infected with *Salmonella typhimurium* (Unit for Experimental Animal Development, Integrated Research and Testing Laboratory-GMU). Twenty males of Balb/C mice (age of 6-8 weeks, 10-19 g weight each) were obtained from Biotechnology Research Centre-GMU. The mice were acclimated for 2 weeks and divided randomly into 4 groups. Each group (5 mice) received the following treatments : Dad13 only (DWI); Dad13 before infection (DBI); Dad13 along with infection (DAI) and *Salmonella* infection only (IWD) (Figure 1). Throughout the experiment the mice were fed with standard diet 5 g/day and water *ad libitum* (Kusumawati, 2005).



A volume of 100 µl (10^{10} CFU / ml) of Dad13 cell suspensions were given daily by oral forced feeding for a week, at week 3 for group of Dad13 before infection (DBI) and at week 4 for group of Dad13 only (DWI) and Dad13 along with infection (DAI). Salmonella infection (10^{9} CFU / ml) was given once orally at week 4 (day 23) to all groups except group treated with Dad13 only (DWI).

Indirect ELISA

The humoral immune response of Balb / C mice was detected 2 weeks after infection by measuring the titers of IgG and IgA specific to *Salmonella sp.* from serum and mucosal intestinal liquid samples using *indirect* ELISA (*Enzyme-linked Immunosorbent Assay*) method. Some stages were done for ELISA, starting from antigen preparation, antibody isolation, and detection of IgG / IgA specific using *indirect* ELISA (Ausubel *et al.*, 1995).

Antigen preparation

Total crude protein *S. typhimurium* was used as an antigen. The antigen was prepared by centrifugation and sonication of bacterial culture. A portion of 100 ml Salmonella culture in TSB medium was centrifuged (1200 g) at 4°C for 10 min. Cell pellet was resuspended in sterile PBS of pH 7.0 and centrifuged. The pellet was washed 3 times. Then the pellet was resuspended in 2

ml of PBS and sonicated 5 times for 30 s. Then the supernatant was removed and stored at -20°C until used as coating for ELISA (Ausubel *et al.*, 1995; Adytianingsari, 2004).

Antibody isolation

Approximately 0.5 ml of mice blood samples were bleed from retroorbital using micro-hematocrit. All mice were subsequently sacrificed by cervical dislocation and aseptically dissected. The entire stomach was dissected and the intestine (before and after *caecum*) which contained *Peyer's patches* was recovered (Adytianingsari, 2004). The distance from first *Peyer's patch* until *caecum* was approximately 25 cm, so the intestine was cut in the distance of 30 cm from *caecum*. Antibody samples were prepared from serum and mucosal intestinal liquid. Serum samples were recovered from centrifugation (1000 g) of blood samples at 4°C for 15 min. Serum was stored at -20°C. Mucosal intestinal samples were flushed with 5 mL of PBS pH 7.0 and particulate material was removed by centrifugation (10000 g, for 10 min, at 4°C). The remaining supernatant fluid was stored in triplicate at -20°C. Both serum and intestinal fluid were used to measure the antibody IgG / IgA concentration (LeBlanc *et al.*, 2004).

Detection of specific IgG/IgA

Firstly, *checker board* ELISA was done to calibrate and optimize ELISA procedure of crude protein of *S.typhimurium* antigen and antibody from samples (serum and intestinal liquid) for measuring IgG / IgA concentration. *Checker board* gave result on *cut off point* value upon dilution of samples. The *cut off point* value was used as a basis for samples detection by *indirect* ELISA. Antigen protein *S. typhimurium* (100 µl / well) in buffer coating of 0.2 M carbonatebicarbonate buffer (pH 10.6) was coated on 96-well flat-bottomed microplates (Maxishop, Nunc) with concentration 5 µg / ml. The microplates were incubated at 37°C over night in water bath. The plates were then washed 3 times with 200 µl / well washing buffer (0.15 M NaCl; 0.05 TritonX-100; 0.02% NaN3) and blocked with 200 µl / well for 1 h at 37°C with PBS of pH 7.0 containing 1% BSA. Plates were washed 3 times with 200 µl / well washing buffer and incubated for 1 h at 37°C with 100 µl / well primer antibody of serum (diluted 1 / 10 in incubation buffer) or intestinal liquid (without dilution), both of them were added in duplicate. The control was conducted by replacing samples with 100 µl / well incubation buffer. Then plates were washed again 3 times with 200 µl / well washing buffer and incubated for 1 h at 37°C with 100 µl / well *goat anti mouse* IgG / IgA *alkaline phosphatase* conjugated (diluted at 1 / 3000). After incubation, plates were washed 3 times with 200 µl / well washing buffer and added with 150 µl / well buffer substrate (4-*Nitrophenil phosphate* in substrate buffer pH 9.6) then incubated for 30 min at 37°C. The optical density (OD) was read at 405 nm using ELISA reader (Benchmark-Bio-rad).

Statistical analysis

The data were subjected to analysis of variance with SPSS software version 10. LSD (*Least Significant Difference*) was used to compare the least square means of all treatments with $\alpha<0.05$. Results were expressed as the mean of log titer antibody (Loon and Veen, 1980).

Results and Discussion

The effect of Dad13 consumption on Balb / C mice in this research was focused on humoral immune response which is mediated by antibody. The titers of IgG and IgA as detected by indirect ELISA (Ausubel *et al.*, 1995).

The result indicated there was an increase of circulating IgG of Balb / C mice as an effect of treatment with probiotic *Lactobacillus* Dad13 (Table 2). However, there were no significantly difference ($p>0.05$) of IgG serum among 3 treatment groups : Dad13 only without infection (log titer = 2.4), Dad13 along with infection (log titer = 2.5), and *Salmonella* infection only (log titer = 2.0). Group which consumed Dad13 before infection (log titer = 1.7) was significantly different ($p<0.05$) with group consumed Dad13 along with *Salmonella* infection. It showed that humoral immune response of Balb / C mice was affected by different consumption time of Dad13.

Table 2. Log10 titer IgG circulation Balb / C mice

Treatment Group	Log10 titer circulated IgG*
Dad13 only without infection (DWI)	2.4[b]
Infection Salmonella only (IWD)	2.0[a]
Dad13 before infection (DBI)	1.7[c]
Dad13 along with infection(DAI)	2.5[b]

*Different supercript showed significantly different ($\alpha<0.05$) between grup.

The difference on humoral immune response might related with the factor of different value of imunogenic factor of antigen which entering the body of Balb / C mice, between Dad13 cell and *Salmonella* cell. *Lactobacillus* Dad13 belong to Gram positive bacteria. Peptidoglycan is part of 50% of all cell wall of Gram positive bacteria. The main component of cell wall bacteria is mostly as *teichoic* and *theichuronic acid* with percentage until 50% dried cell weight of cell wall and 10% of total dried cell. Teichoic acid is the main component of outer antigen (Ag) from Gram positive species (Brooks *et al.*, 2001). The capability of probiotic to stimulate immune system is very related with amount of peptidoglycan and lypopolisaccharide from cell wall composition (Widodo, 2003). That is the reason why Dad13 as probiotic was given in the form of cell suspension in media and not in the form of supernatant.

While *Salmonella* cell was Gram negative bacteria. This kind of bacteria have thinner peptidoglycan layer than Gram positive. Cell wall of Gram negative bacteria consist of 3 components, ie. lipoprotein, outer membrane and lipopolysaccharide (LPS). Lipoprotein is dominated protein of Gram negative with function to stabilize outer membrane and stick on peptidoglycan. Outer membrane is composed of LPS molecule which called as endotoxin of Gram negative bacteria due to its capability to stick on the outer cell and flowing out when cell lysis. Polysaccharide is the biggest part of Ag (Brooks *et al.*, 2001). Due to this reason *Salmonella* infection was conducted orally by giving *Salmonella* cell suspension which was still composed of antigenic and virulent cell wall.

The two groups of DBI (Dad13 before infection) and DAI (Dad13 along with infection) received both of Dad13 and *Salmonella* infection with different time of Dad13 consumption and resulted on significantly different of humoral immune response. It was indicated that the different time of giving probiotic gave effect to the immune system. Giving two types of antigens in the same time result in giving bigger immune response, better than giving two antigens in the different time, one by one. Two particles of antigen have bigger molecule weight than just one antigen. Klein cit Nurliyani (2003) stated that protein immunogenicity was effected by its molecule weight, the larger molecule weight of protein the more immunogenic.

The same phenomenon on IgG circulation was observed. Humoral immune response of two different Dad13 consumption time (before and along with

Salmonella infection) were also significantly different ($p<0.05$) in two other parameters, circulating IgA from serum and secretory of IgA from intestinal fluid (Tabel 3 and 4). Consumption of Dad13 for a week without infection (100 ml, 10^{10} CFU / ml / day) increased circulated IgG / IgA and secreted IgA as compared to feeding Dad13 before *Salmonella* infection (100 ml, 10^{9} CFU / ml).

Table 3. Log10 titer IgA circulation Balb / C mice

Treatment Group	Log10 titer circulated IgA*
Dad13 only without infection (DWI)	2.3b
Infection Salmonella only (IWD)	2.3b
Dad13 before infection (DBI)	1.7a
Dad13 along with infection(DAI)	2.3b

*Different supercript showed significantly different ($a<0.05$) between grup.

Table 4. Log10 titer IgA secretion Balb / C mice

Treatment Group	Log10 titer secreted IgA*
Dad13 only without infection (DWI)	1.2ab
Infection Salmonella only (IWD)	1.4b
Dad13 before infection (DBI)1.0a	
Dad13 along with infection(DAI)	1.4b

*Different supercript showed significantly different ($a<0.05$) between grup.

Feeding of Dad13 before infection did not give result as preventive agent from Salmonella. Dad13 might only colonize temporary in the intestine, so that there was no enough barrier to prevent Salmonella colonization that given after Dad13 consumption. The pathogenicity of Salmonella might also affect on decreasing of Ig concentration of Balb / C mice. According to Tejada-Simon *et al.* (1999), mice fed with yogurt supplemented with *Lactobacillus acidophilus* and *Bifidobacteria* and infected with Colera-toxin gave response on IgG serum, but not significantly different from control.

Effect of increased of IgG specific *Salmonella* during the research was detected in the serum 2 weeks after infection. Birge (1992) stated that immune response by antibody secretion needs time interval for its mechanism. An antigen that gave in the first time would induce primary immune response, by secreting specific antibody to an antigen. Increasing of the specific antibody molecule lasted for a few days, reached the peak in the 10-20 days, then decreased to lowest concentration after 30 days. IgG is dominant antibody in the secondary response and build the barrier to fight bacteria and virus (Brooks *et al*., 2001). IgG is the main component in serum imunoglobulin, with the molecule weight of 160,000 Dalton. Its concentration is 13 mg / ml and as 75% component of all imunoglobulin (Baratawidjaja, 2002). IgG consists of 4 sub class (IgG1 until IgG4) and have half time of 23 days (Brooks, *et al*., 2001).

Feeding Dad13 only to Balb / C mice gave effect on increased IgG and this effect increased significantly if in the same time infection was also given to the mice. This indicated that Dad13 could act as stimulator of IgG serum but not as preventive agent. The increased of IgA (serum and intestinal fluid) was not detected as the effect of Dad13 consumption compare to group treated with *Salmonella* infection within 2 weeks after infection. Dad13 could not act as stimulator as well as preventive agent of IgA serum and IgA secretion. IgA is found in the two forms, IgA serum and IgA secretion which is the biggest part. The molecule weight of IgA is 165,000 Dalton (Baratawidjaja, 2002). IgA consists of 2 sub class (IgA1 and IgA2) and half time of 14 days (Brooks *et al*., 2001).

Oral feeding of Dad13 as probiotic firstly might gave initial induction on humoral immune response of stomach mucose of Balb/C mice, then within 14 days after *Salmonella* infection, Ig precursor might have circulated in the blood which ended in lamina propria. In the blood, IgG plasm cell were existed in bigger number than IgA plasm cell. This agreed with the result of this research, circulated IgG titer (log titer = 1.7-2.5) was higher than circulated IgA (log titer = 1.7-2.4) and secreted IgA (log titer = 1.0-1.4). The asumption that LAB increased local immunity of gastrointestinal by induction/infection (Puri *et al.*, 1996) was not truly observed in this research using *indigenous* strain of Dad13 after 14 days of infection. Dad13 could not act as preventive agent as well as stimulator agent of sIgA (secretion IgA) mucose intestinal fluid Balb/C mice. Some factors related with optimum doses of consumption, strain-*host* specific, individu variability also time secretion of antibody were still in questioned.

Conclusion

Consumption of *Lactobacillus* Dad13 before and along with *Salmonella typhimurium* infection give significantly different ($p<0.05$) result. Dad13 consumption along with *Salmonella* infection increased circulating IgG and IgA also secretory IgA. Feeding of Dad13 probiotic along with infection increased humoral immune response more significantly compare to that before infection.

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