Pengaruh pemberian susu kuda terfermentasi terhadap imunitas vaksin hepatitis A pada mencit Balb/c

Influence of fermented horse milk supplementation on immunity against hepatitis A vaccine in Balb/c mice

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Abstrak

Telah dilakukan penelitian untuk mengetahui pengaruh pemberian susu kuda terfermentasi terhadap imunitas vaksin hepatitis A pada mencit Balb/c. Penelitian ini menggunakan desain acak lengkap dengan 30 ekor mencit, yang dibagi menjadi 3 kelompok. Pada kelompok I diberikan per oral 0,4 mL/20g/BB susu kuda terfermentasi, kelompok II 0,4 mL/0,45 mg/20g/BB levamisol (kontrol positif) dan kelompok III 0,4 mL/20g/BB akuades air (kontrol negatif) setiap hari selama 46 hari, sebagai tambahan pada makanan standar. Mencit diimunisasi 3 kali secara intraperitoneal pada hari ke 7, 28, dan 42 dengan 0,65 μg/20g/BB vaksin hepatitis A. Sampel darah dikumpulkan dari *plexus retroorbitalis* dengan pipa kapiler berheparin setelah 1 minggu imunisasi pertama (hari ke 14), kedua (hari ke 35), dan 4 hari (hari ke 46) setelah imunisasi ketiga. Kemudian mencit dikorbankan untuk diisolasi limfositnya dan dilakukan kultur. Kadar IgG, IgM dan IgA dalam sera dan media kultur ditetapkan dengan metode ELISA. Hasil penelitian menunjukkan bahwa. kadar IgM (kecuali hari ke 46), IgG (subtipe IgG1 (kecuali hari ke14) , IgG2a, IgG2b, IgG3 (kecuali hari ke 35), dan IgA berbeda signifikan ($p<0,05$) dibanding dengan kontrol. Antibodi yang disekresi in vitro dalam kultur limfosit, menunjukkan hasil yang sama untuk IgA.

Dari hasil penelitian dapat diambil kesimpulan, bahwa pemberian per oral susu kuda terfermentasi dapat meningkatkan imunitas terhadap vaksin hepatitis A pada mencit Balb/c. Hasil SDS-PAGE susu kuda dan susu sapi terfermentasi, menunjukkan adanya protein 30-kDa pada susu kuda maupun susu sapi, serta protein 7,4-kDa yang hanya terdapat pada susu kuda terfermentasi.

Kata kunci : susu kuda terfermentasi, imunitas, vaksi hepatitis A, mencit Balb/c.

Abstract

It has been evaluated the influence of fermented horse milk into Balb/c mice on immunity against hepatitis A vaccine. The study employed a complete randomized design using thirty mice which were divided into 3 groups. Each group orally consumed fermented horse milk at dose 0.4 mL/20g body weight, levamisole (as positive control) at dose 0.4 mL/0.45 mg/20g body weight and distilled water (as negative control) at dose 0.4 mL/20g body weight respectively on a daily basis in addition to the standard diet for 46 days. The mice were immunized intraperitoneally (3 times) at d 7, d 28, and d 42, with 0.65 μg/20g body weight of hepatitis A vaccine. Blood

samples were collected from *plexus retroorbitalis* using heparinized capillary one week after first (d 14), second (d 35) and 4 days (d 46) after third immunizations, and then mice were sacrificed to isolate the lymphocytes. The IgM, IgG and IgA titers in the sera and the culture media were measured by ELISA.

The IgM (except d 46), IgG (subtype IgG1 (except d 14), IgG2a, IgG2b, IgG3 (except d 35), and IgA titers in the sera significantly higher ($p<0.05$) than controls. This difference was associated with enhanced in vitro antibodies secretion by cultured lymphocytes isolated from the spleen for IgA. The result suggested that orally fermented horse milk supplementation inhances immunity against hepatitis A vaccine in Balb/c mice. The SDS-PAGE showed that 30-kDa proteins were present in fermented horse milk as well as fermented cow milk, but 7.4-kDa protein present only in fermented horse milk.

Key words : fermented horse milk, immunity, hepatitis A vaccine, Balb/c mice.

Introduction

A number of studies have noted the immunomodulatory properties of probiotic organisms, especially the lactobacilli, Lactic acid bacteria (LAB) and other probiotic organisms in fermented milks (yogurt) appear to be beneficial in the treatment of certain diarrheas, as well as in the stimulation of immune function. Consumption of yogurt with *Lactobacillus casei*, *Lacidophilus and* mixtures of several LAB, as well as bacterial cell lysates, increases indices of immune respon, e.g., the numbers of immunoglobulin (Ig)A-producing cells and macrophages, IgG titers, and specific antibody responses to antigenic challenges compared with animals not consuming LAB (Isolauri *et al*., 1993, Sanders 1993, Thoreux and Schmucker 2001). Non bacterial milk components and components produced from milk fermentation also may contribute to the immunostimulatory activity of yogurt. Peptides and free fatty acids generated by fermentation have been shown to enhance the immune response (Takahashi *et al*. 1993).

In Indonesia, yogurt, especially fermented horse milk is used as an alternative or as a supplementation for the treatment of infection diseases by decreasing immunity response. Ediati *et al*. (2004) have proposed that consumption of horse milk inhances immunity against hepatitis A vaccine in Balb/c mice. With few exceptions, milk and yogurt have similar vitamin and mineral compositions. During fermentation, vitamins B12 and C are consumed and folic acid is produced. Although milk and yogurt have similar compositions, some minerals, eg, calcium, are more bioavailable from yogurt than from milk. In general, yogurt also has less lactose and more lactic acid, galactose, peptides, free amino acids, and free fatty acid than milk (Meydani and Ha, 2000).

Measurement of the proliferative response of lymphocytes 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. However, to measure antigen-specific proliferation, the host should be exposed to the antigen before the cells are stimulated with that antigen *in vitro* (Meydani and Ha, 2000).

A substance must possess the following characteristics to be immunogenic: (a) foreigness, (b) high molecular weight, (c) chemical complexity, and, in most cases, (d) degradability. In general, compounds that have a molecular weight less than 1 kDa are not immunogenic; those of molecular weight between 1 kDa and 6 kDa may or may not be immunogenic; and those of molecular weight greater than 6 kDa are generally immunogenic (Benjamini *et al*. 2000).

Levamisole is a drug that was previously used for eliminating intestinal parasites in animals. Levamisole can classified as both an immunoregulator and immunostimulator. It affects humoral and cell mediated immunity. Levamisole affects T-cells to a greater degree than B cells, leading to cutaneous reactivity to delayed type hypersensitivity antigens and

improvement in helper, suppressor, and cytotoxic T-cell functions (Anonymous, 2001).

Although the perception persists that consuming fermented milk has beneficial effects on the treatment of decreasing immune response diseases, there are few studies which examine the effects of fermented horse milk consumption. Therefore, this study was aimed to determine the influence of fermented horse milk supplementation on immunity against hepatitis A vaccine in Balb/c mice and for comparative immunity purposes study based on the molecular weight of proteins, SDS-PAGE was performed in fermented horse milk and fermented cow milk.

Methodology

Materials

The study used fermented horse milk (Susu Kuda Sumbawa, packaged by PT. Mega Utama Perkasa). It has properties of yellowish**,** watery, sour (pH 3-3.5), 1.65-1.70% fat, 2.15-2.2% protein, 1.23% casein, 7.5x105 cells/mL lactic bacteri), fermented cow milk (Yakult) , hepatitis A vaccine (Havrix 720, SmithKline, Beecham), levamisole, ELISA kits (Sigma) for measuring IgG, IgM and IgA, RPMI-1640 (Sigma), sodium bicarbonate (Sigma), HEPES (Sigma), fetal cow serum (Gibco), Penstrep (Gibco), Fungizone (Gibco), TRIS base (Sigma), ammonium chloride (E.Merck), Turk reagent, ethanol, chloroform, destilled water, bidestilled water and the materials for SDS-PAGE performed. Male mice Balb/c, 3 months old (± 20g), from Bio-Sciences Laboratory of Gadjah Mada University were used as animal model through out this study.

Instruments

ELISA reader (BioRad), CO2 Incubator (Heraeus), Cooled Centrifuge (Sigma), Inverted Microscope (Olympus), Hemositometer (Nebauer), Electrophoresis (Bio-Rad).

Experiments

The study employed a complete randomized design, using thirty mice and were divided into 3 groups.

Diets. Mice were acclimated for one week before the experiment. They were divided into 3 groups of 10 mice each as follows, consumed once a day orally with : fermented horse milk at dose 0.4 mL/20g body weight, levamisole (as positive control) at dose 0.4 mL/0.45 mg/20g body weight and distilled water (as negative control) at dose 0.4 mL/20g body weight respectively on a daily basis in addition to the standard diet for 46 days. Every week the weight of the mice were under controlled to evaluate their conditions troughout the experiment.

Immunization. Mice were immunized intraperitoneally (3 times) at d 7, d 28, and boosted d 42, with 0.65 μg/20g body weight of hepatitis A vaccine.

Serum collection and preparation of cell suspensions. Blood samples were collected from *plexus retroorbitalis* using heparinized capillary one week after first (d 14), second (d 35) and 4 days (d 46) after last immunizations, and then the mice were food deprived overnight, sacrificed by chloroform to isolate the lymphocytes.

After overnight incubation at 4ºC and centrifugation at 1000 x *g* for 15 min, the serum was separated and divided in aliquots. All samples were deep frozen until analysis. Cell suspensions were prepared from spleen by teasing the tissue in RPMI-1640 medium. The suspensions were centrifuged at 2500 x *g* for 15 min, the cells were washed 3 times and suspended in RPMI-1640 complete media.

In vitro cell cultures. Cell suspensions prepared from spleen ($1x10^4$ cells/well) were incubated in complete media RPMI in 96-well round bottom culture plates for 24 h and 48 h at 37ºC in a 5% CO_2 environment.

Detection of antibodies by ELISA. Microtiter wells were coated with hepatitis A vaccine as antigen (1.25 μg/mL), and incubated sequentially with 20 mg/mL bovine serum albumin, 100 μL of serially diluted serum or culture supernate, biotinylated goat anti-rat IgM, IgG (IgG1, IgG2a, IgG2b, IgG3) or IgA (2.5 μg/mL) coupled with HRP (Horse Radish Peroxydase), and OPD (o-phenylenediamine) as the substrate. Mice fed the vehicle alone were used as negative controls. The OD (Optical Density) values detected by ELISA reader have a correlation with the concentration of antibody .

Protein molecular weight determinations. For comparative study the molecular weight of proteins from fermented horse milk and fermented cow milk were estimated according to the method described by Weber & Osborn (1969) using standard protein markers (Bio-Rad). After electrophoresis, as well as after gel staining and destaining, the position of the separated proteins was recorded.

Statistical analysis. All results are expressed as the mean ± SD. Statistical analyses were performed using two-ways ANOVA, followed by Student-Newman-Keuls test. Differences were considered significant when $p \leq 0.05$.

Result and Discussion

There was no significant difference in the pattern of mice body weight change between the controls and fermented horse milk consumed within each age group (Figure 1). No diarrhea, loss of appetite or discomfort was observed during experiment. These indicate it was no health problems through out of the experiment.

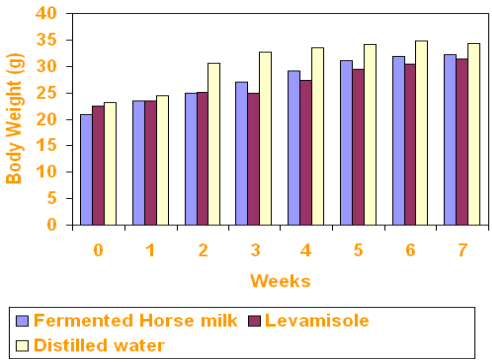

Figure 1. The pattern of mice body weight, recorded weekly between the controls and fermented horse milk supplementation

From the result of antibodies measurement in serum, there was a significant difference ($p<0.05$) between the group of fermented horse milk supplementation and the controls for the IgM secretion of d 14 and d 35, but not for d 46 (Table I). No IgM detectabled at d 46, following with the IgM secretion in the cultured cell (Table II). This result in according with the pattern of antibody response on hepatitis A infection described in Seehan (1997). IgM anti-HAV antibody is present at the onset of symptoms and reaches a maximum titer in 1 to 3 weeks.

There was also a significant ($p<0.05$) increasing difference for all subtype of IgG secretion of d 14, d 35 and d 46 (except subtype IgG1 at d 14 and IgG3 at d 35) compared to the controls (Table I).

IgG is the predominant immunoglobulin in blood, lymph, cerebrospinal and peritoneal fluids. The IgG subclasses designated as IgG1, IgG2 (IgG2a, IgG2b), IgG3, and IgG4. The half-life of IgG is approximately 23 days, except for the IgG3 with a half-life of 7 days. It can be pointed from Table I, the titer antibody of all sub classes IgG at d 35 significant decreased than that of d 14 and d 46. This phenomenon is known as mentioned in Benjamini *et al.* (2000), that during a primary and secondary antibody response, a latent phase occurs in which there is no detectable circulating antibody. The latent phase is followed by a gradual rise (primary response, 2 weeks), plateau, gradual rise (secondary response, 1 week) and final decline of the antibody titer for IgG. IgG secretion in the cultured lymphocytes (Table II) was not affected by diet because there were no differences between cells isolated from controls and the group of fermented horse milk supplementation, except IgG2a.

IgA is the major immunoglobulin in external secretions such as saliva, mucus, sweat, gastric fluid, and tears. It can be pointed from Table I, the titer of IgA in the serum were lower than that of IgM at d 14 and d 35, IgG at d 46, but higher in the cultured lymphocytes (Table II). There was a significant ($p<0.05$) increasing difference for secretion of IgA in the serum at d 14, d 35, and d 46 (Table I), following with the IgA secreted in the cultured lymphocytes of h 24 and h 46 compared with the negative control (Table II). The result related with the report of Mitchell and Galun (2002), that systemic IgG responses were higher in the mice immunized intraperitoneally of hepatitis A vaccine, while IgA responses higher in the cultured lymphocytes.

For comparative immunity purposes study on corresponding of molecular weight of proteins from fermented horse milk and cow milk were also carried out. The electrophoretic SDS-PAGE patterns of fermented horse milk and cow milk using standard protein markers were shown in Figure 2. On the basis of protein bands of SDS-PAGE in fermented horse milk and cow milk, they were appeared similar, especially that of 30-kDa proteins. The difference was that, 7.4-kDa protein present only in fermented horse milk. As we know, the molecular weight of greater than 6-kDa were generally immunogenic. It was suggested that the use of fermented horse milk was better than fermented cow milk as an alternative or as a supplementation for the treatment of infection diseases in which decreasing immunity.

Table I. Measured the Optical Density (OD) detected by ELISA method in correlated with antibody secretion in the serum

Class/ Subclass	Consumption	Optical Density ± SD		
		d 14	d 35	d 46
IgM	Fented ermHorse milk	0.643 ± 0.143	0.622 ± 0.193	0.038 ± 0.009
	Levamisole	0.418 ± 0.036	0.456 ± 0.055	0.053 ± 0.007
	Distilled water	0.348 ± 0.167	0.400 ± 0.026	0.023 ± 0.014
IgG1	Fermented Horse milk	0.031 ± 0.010	0.195 ± 0.004	0.946 ± 0.006
	Levamisole	0.209 ± 0.014	0.214 ± 0.006	0.446 ± 0.007
	Distilled water	0.155 ± 0.014	0.163 ± 0.013	0.187 ± 0.012
IgG2a	Fermented Horse milk	0.393 ± 0.014	0.124 ± 0.006	0.629 ± 0.005
	Levamisole	0.059 ± 0.009	0.097 ± 0.014	1.207 ± 0.121
	Distilled water	0.030 ± 0.006	0.051 ± 0.004	0.115 ± 0.012
IgG2b	Fermented Horse milk	0.440 ± 0.008	0.271 ± 0.004	0.945 ± 0.013
	Levamisole	0.264 ± 0.008	0.210 ± 0.003	0.337 ± 0.027
	Distilled water	0.045 ± 0.006	0.194 ± 0.007	0.383 ± 0.019
IgG3	Fermented Horse milk	0.451 ± 0.003	0.077 ± 0.004	0.317 ± 0.003
	Levamisole	0.088 ± 0.007	0.101 ± 0.012	0.163 ± 0.026
	Distilled water	0.062 ± 0.003	0.087 ± 0.009	0.077 ± 0.009
IgA	Fermented Horse milk	0.254 ± 0.023	0.276 ± 0.045	0.376 ± 0.082
	Levamisole	0.273 ± 0.057	0.285 ± 0.040	0.301 ± 0.053
	Distilled water	0.247 ± 0.086	0.248 ± 0.59	0.253 ± 0.083

Table II. Measured the Optical Density (OD) detected by ELISA method in correlated with antibody secretion in the lymphocyte cultured

Class/ Subclass	Consumption	Optical Density ± SD	
		h 24	h 48
IgM	Fermented Horse milk	0.081 ± 0.002	0.022 ± 0.013
	Levamisole	0.073 ± 0.036	0.056 ± 0.055
	Distilled water	0.048 ± 0.016	0.040 ± 0.026
IgG1	Fermented Horse milk	0.081 ± 0.002	0.085 ± 0.002
	Levamisole	0.073 ± 0.006	0.066 ± 0.002
	Distilled water	1.432 ± 0.036	1.324 ± 0.070
IgG2a	Fermented Horse milk	0.481 ± 0.003	0.500 ± 0.007
	Levamisole	0.363 ± 0.023	0.422 ± 0.019
	Distilled water	0.281 ± 0.020	0.231 ± 0.021
IgG2b	Fermented Horse milk	0.043 ± 0.002	0.045 ± 0.001
	Levamisole	0.044 ± 0.002	0.043 ± 0.002
	Distilled water	0.043 ± 0.002	0.041 ± 0.001
IgG3	Fermented Horse milk	0.112 ± 0.005	0.132 ± 0.007
	Levamisole	0.104 ± 0.004	0.105 ± 0.002
	Distilled water	0.102 ± 0.002	0.101 ± 0.002
IgA	Fermented Horse milk	0.431 ± 0.037	0.475 ± 0.109
	Levamisole	0.392 ± 0.019	0.406 ± 0.022
	Distilled water	0.231 ± 0.018	0.243 ± 0.023

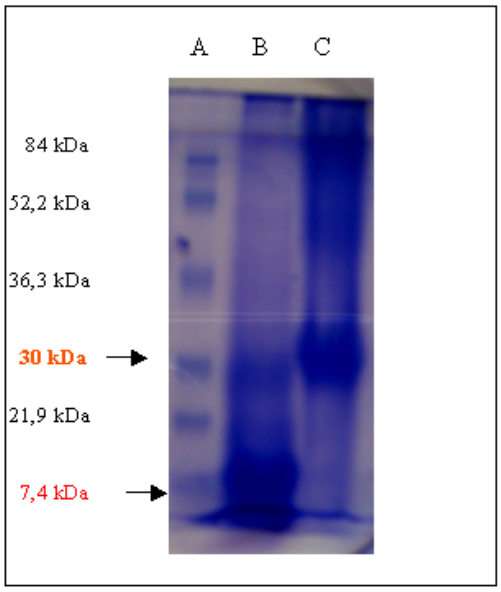

Figure 2. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE) pattern of standard protein markers (A), fermented horse milk (B), fermented cow milk (C).

Conclusion

The study concluded that orally fermented horse milk supplementation, inhances immunity against hepatitis A vaccine in Balb/c mice, especially that systemic IgG responses were higher in the mice immunized intraperitoneally, while IgA responses higher in the cultured lymphocytes. Based on the molecular weight analysis of SDS-PAGE pattern in fermented horse milk and fermented cow milk, the influence of fermented horse milk was better than fermented cow milk on immunity.

Acknowledgments

The authors thank Puji Astuti for reviewing the manuscript, Tri Setyawati and Atin Suprihatin for technical assistance.

The Paper has been presented in Malaysian Pharmaceutical Society (MPS) – UITM Pharmacy Scientific Conference 2004 6th – 8th August 2004, Kuala Lumpur Malaysia

References

Anonymous, 2001, *About Biologic Therapy Levamisole*, http://www.biologictherapy.org.

Benjamini, E., Coico, R., and Sunshine, G. (2000). *Immunology. A short course*, 4th ed., Willey-Liss, A John Wiley & Sons, Inc.m Publication, New York), 42;75

Ediati, S., Rumiyati, Ninisita, S.H., Rahel, R.T.A. and Reny, H.A. (2004), Horse Milk Enhances Immunity Against Hepatitis A vaccine in Balb/c mice, The Proceedings of 2nd Life Sciences Postgraduate Conference : *Life Sciences*: *The Power to Explore, Discover and Stimulate*, 1-4th April 2004, USM, Penang, Malaysia.

Isolauri, E., Kaila, M., Arvola, T., Majamaa, H., Rantala, I., Virtanen, E., and Arvilommi, H., 1993, Diet during Rotavirus Enteritis Affects Jejunal Permeability to Macromolecules in Suckling Rats, *Pediatr.Res.* 33: 548-553

Meydani, S.N. & Ha, W.K. (2000). Immunologic Effects of Yogurt. *AJCN* 71 (4): 861-872.

Mitchell, L.A. and Galun, E., 2000, Rectal immunization of Mice withhepatitis A Vaccine Induces Stronger Systemic & Local Immune responses than Parenteral Immunization, *Vaccine*, 21 : 1527

Sanders, M.E., 1993, Effect of Consumption of Lactic acid Cultures on Human Health, In: *Advances in Food and Nutrition Research* (Kinselia J.E., ed.), Academic Press, San Diego, CA, 67-130

Sheehan, C., 1997, Clinical Immunology, *Principles and Laboratory Diagnosis*, 2nd ed., Lippincott, Philadelphia,pp. 26;249-250

Takahashi, T., Oka, T., Iwana, H., Kuwata, T. and Yamamoto, Y., 1993, Immune Response of Mice to Orally Administered Lactic acid Bacteria. *Biosci Biotechnol Biochem,* 57:1557-60

Thoreux, K. and Schmucker, D.L., 2001, Kefir Milk Enhances Intestinal Immunity in Young but Not Old Rats, *Jour.Nut.*, 131 : 807-812

Weber, K. and Osborn, M., 1969, The Reliability of Molecular Weight Determination Dodecylsulfate Polyacrylamide Gel Electrophoresis, *J.Biol.Chem.* 244: 4405-441