

The effect of two *Lactobacillus rhamnosus* strains on the blood lipid profile of rats fed with high fat containing diet

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Abstract

Diet pattern and lifestyle of modern society has triggered various diseases associated with hypertension and coronary heart disease. The main objective of this study was to investigate the effect of two probiotic lactobacilli strains (*Lactobacillus rhamnosus* SKG34 and *Lactobacillus rhamnosus* FBB42) isolated from different sources (fermented milk and feces of healthy infants), on the blood lipid profile of rats, fed with high fat containing diet. The ability of these strains to reduce the cholesterol content of rats blood was also investigated in this study. Four groups of 6 rats were fed for 28 days with high fat containing diets (HF), high fat containing diets supplemented individually either with *L. rhamnosus* SKG34 or *L. rhamnosus* FBB42, and high fat containing diets supplemented with a combination of those strains. During these treatments, the amount of food intake and the body weight gain of rats were measured. On day 28, all rats were sacrificed and the population of lactic acid bacteria (LAB) in cecal content and lipid profile of rats were determined by dilution plating method on MRS agar and CHOD-PAP enzymatic method, respectively. The results showed that administration of probiotics, either singly or in combination, was found to increase the population of LAB and this resulted in a slight decrease in the pH of the cecal content ($P > 0,05$). It was also found in this study that the probiotics *L. rhamnosus* SKG34 and *L. rhamnosus* FBB42, either applied singly or in combination, significantly lowered the total content of cholesterol, TG and HDL-c, but increased HDL-c in rats fed with high fat containing diet. Besides that, administration of *L. rhamnosus* SKG34 and *L. rhamnosus* FBB42 reduced (with equivalent results) the ratios of TC: HDL-c, TG: HDL-c, and LDL-c: HDL-c which are normally used as a predictor of cardiovascular diseases (CVD). This indicated that *L. rhamnosus* SKG34 and *L. rhamnosus* FBB42 are potential to be developed as probiotics to be used in improving blood lipid profiles.

Keywords

Probiotics

Lactobacillus rhamnosus

Lipid profiles

Cholesterol

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Introduction

It was reported by the WHO (2014) that 17.5 millions people lost their life due cardiovascular related diseases. This number was about 31% of the total global death case. Among those, approximately 7.4 million of were caused by coronary heart disease and about 6.7 million were due to stroke (WHO, 2014). In the timeline period of 1999 and 2003, it was

reported that the contribution of hypercholesterolemia on the heart attack case in western European countries and central and Eastern Europe countries were 45% and 35%, respectively (Yusuf *et al.*, 2004). The risk of heart attack in a person with hypercholesterolemia condition is three times greater than that with normal cholesterol condition. Further, it was stated that more than 75% of the reported death occurred in countries with mediocre income. This is closely associated with

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the pattern of food consumption where people in such countries tend to consume high fat containing diets with low fiber content. High fat containing foods, especially those with high content of saturated fatty acids may increase blood cholesterol levels, risk of atherosclerosis, and coronary heart disease (Grundy *et al.*, 1982; Xu *et al.*, 2006). It was also reported that foods containing fat, salt and high sugar but low in complex carbohydrates, fruits, and vegetables increased the risk of cardiovascular disease (WHO, 2003). The incident of CVD can normally be prevented through lifestyle improvements or by avoiding risk factors such as smoking, unhealthy food consumption, increased physical activity and alcohol abuse (WHO 2014). A decrease in blood cholesterol level has been one of the global concerns because high blood cholesterol level has a great contribution to the high rate of global mortality which is estimated by WHO to reach 23.6 million cases in 2030 (Xie *et al.*, 2011). Manson *et al.* (1992) reported that a decrease by 1% of the blood cholesterol level may lower the risk of CVD by 2-3%. An extensive studies conducted by the Cholesterol Treatment Trialists (CTT) Collaboration (CTT, 2010) showed that a decrease in LDL level by 2-3 mmol/L could reduce heart attacks cases by 40-50%.

Chemical-based drugs have been used as a method to control blood cholesterol level. However, this method has been found to be costly and has negative effects for long-term application. Therefore, this is not an optimal way to control blood cholesterol level. Due to those situations, biological approach (such as consumption of probiotics or functional foods containing probiotics), which is cheap and safe in the long term application, has recently received a lot of scientist's attention. The idea is based on the findings of Shaper *et al.* (1963) and Mann (1974) who reported that blood cholesterol level of people in Samburu tribe and the Masai warriors in Africa decreased following consumption of milk fermented with *Lactobacillus*. The decrease was allegedly caused by the *Lactobacillus* containing in the fermented milk, and this has lead many scientists to investigate various health aspects of probiotic, *Lactobacillus* in particular.

Probiotics are living microorganisms, when administered regularly in adequate amount, they will provide their hosts with health benefit (FAO, 2002). Probiotics have been well-known to have beneficial effects to human health by maintaining the balance of beneficial bacteria residing in the gastrointestinal tract of human (Fuller, 1989). Some beneficial effects of probiotics are prevention of diarrhea (Salazar *et al.*, 2007; Pant *et al.*, 2007; Collado *et al.*, 2009),

stimulation of hosts immune system (Isolauri *et al.*, 2001; Isolauri and Salminen, 2008), prevention of colon cancers (Liong, 2008), prevention of atopic dermatitis in children (Betsi *et al.*, 2008; Torii *et al.*, 2010), having an antioxidant effect (Kim, 2006ab; Chu-Chyn *et al.*, 2009; Sekhon, 2010; Gao, 2011), and lowering blood cholesterol levels (Lee *et al.*, 2009; Ooi *et al.*, 2010; Kumar *et al.*, 2012).

A study conducted by Ha *et al.* (2006) reported that administration of probiotic *L. plantarum* CK102 was found to decrease the total cholesterol, HDL-c, and TG in rats by 27.9%, 28.7% and 61.6%, respectively. Similarly, Jeun *et al.* (2010) also found that the administration of *L. plantarum* KCTC3928 in mice lowered the TC and 33% and HDL-c level by 42% and 32%, respectively. In contrast to those found by Ha *et al.* (2006), administration of *L. plantarum* KCTC3928 in mice was found to increase the HDL-c by 35%. Several studies on the effects of probiotics, applied in humans, on blood lipid profile showed various results. Bertolami *et al.* (1999) and Naruszewics *et al.* (2002) found that probiotics could improve blood lipid profiles. However, Hataka *et al.* (2008) and Simon *et al.* (2006) and Lewis and Burmeiser (2005) found that administration of probiotic *L. rhamnosus* LC705 (10¹⁰cfu/g, 2 capsules per day) for 4 weeks, administration of *L. fermentum* 2x10⁹ cfu / capsule, 4 capsules a day for 10 days, and administration of *L. acidophilus* for 6 weeks, respective did not affect the lipid profile in human subjects. These results showed initial indication that the effect of probiotics varied and supported the assumption that beneficial effect of probiotics is strain dependent and highly affected by its origin. Based on those background it is worthed to study the hypocholesterolemic effect of the two *Lactobacillus rhamnosus* strains (*L. rhamnosus* SKG34 isolated from Sumbawa horse milk and *L. rhamnosus* FBB42 isolated from unhealthy infant feces) for further development of their potential as probiotics.

Materials and Methods

Strain and cultivation methods

Two lactobacilli, *L. rhamnosus* SKG34 and *L. rhamnosus* FBB42 were obtained from the Udayana University Culture Collections, and used in this study. The lactobacilli were grown in the Man Regosa Sharpe broth (MRS, Pronadisa Laboratorios Conda SA C / La Forja 9. 28850 Torrejon de Ardoz, Madrid, Spain) containing: 20 g dextrose, 10 g bacteriological peptone, beef extract 8 g, 5 g sodium acetate, 4 g yeast extract, 2 g dipotassium phosphate, ammonium citrate 2 g, 1 g tween 80, 0.2 g magnesium sulphate,

and 0.06 g manganese sulphate per liter medium. Amount of 50 μ L glycerol stock of *L. rhamnosus* SKG34 and *L. rhamnosus* FBB42 were cultured into 5 ml MRS broth and incubated for 48 hours under anaerobic conditions (anaerobic gas generating Pouch, Oxoid) at 37°C. One loopful of culture broth was next streaked on to MRS agar (Pronadisa) and was further incubated in anaerobic condition at 37°C for 48 hours. A single colony was then isolated and used for further studies.

Preparation of probiotic cells

Lactobacillus rhamnosus SKG34 and *L. rhamnosus* FBB42 were inoculated in 5 ml MRS broth medium, incubated statically at a temperature of 37°C for 24 hours, centrifuged at 5,000 rpm for 5 minutes at 5°C, and the supernatant was discarded. The cell mass was then washed twice with saline solution (NaCl 0.85%) and resuspended with saline to obtain bacterial cell density of approx. 10^8 cfu / ml.

Preparation of rats

Twenty-eight male Wistar rats with initial body weight of 79.2 ± 15.1 g were acclimatized for 1 week and fed ad libitum with standard diet (AOAC, 1990, Table 1) in cages with a dimension of 45 cm x 30 cm x 10 cm. After acclimatization, the 24 rats were randomly selected and used for further studies. The rats were then fed with high fat containing diet (standard diet added with 10% lard) for 2 weeks. The rats were next divided into 4 groups of 6 rats and followed by administration with high-fat feed (HF), HF and *L.rhamnosus* SKG34 (HF-SKG34); HF and *L. rhamnosus* FBB42 (HF-FBB42) and HF and a combination of the two probiotics (*L.rhamnosus* and *L. rhamnosus* SKG34 FBB42; HF-SKG34-FBB42).

The rats were administered with probiotic orally by giving 0.5 ml of cells suspension (10^8 cells/ml) using a sonde, once a day at 12:00 to 13:00 pm for 4 weeks (28 days). The body weight and the amount of diet consumed were measured daily. This study followed the ethical clearance of experimental animal used at the Udayana University.

Blood sampling

The rats were anesthetized using a mixture containing 10% ketamine and 2% xylazine analytical grade, KEPRO B.V., Holland). The blood was taken through the eyes of the rats (eye pit), put into Eppendorf tube and allowed to stand at ambient temperature for 45 minutes. The serum was obtained by centrifugation of the blood samples at 10,000 rpm, 5°C, for 30 min, and stored at -20°C until required. In the meanwhile, the content of the cecum were

Table 1. Composition of diet used in this study

Komponen	Standard Diet (g)	High Fat Diet (g)
Casein	100	100
Corn oil	80	0
Lard	0	100
Mineral mix	50	50
Vitamin mix	10	10
Cellulosa (CMC)	10	10
Water	50	50
Corn Starch	700	680

* g/kg according to AOAC (1990)

collected and diluted in saline solution (0.85% NaCl) prior to enumeration of LAB and measurement of pH.

Analysis of lipid profile

Total cholesterol (TC) of serum, high density lipoprotein-cholesterol (HDL-c) and triglyceride (TG) levels were measured by the method of CHOD-PAP enzymatic photometric test using a commercial KIT Brands DiaSys (DiaSys Diagnostic Systems GmbH AlteStrasse 9 65 558 Holzheim Germany), while the low density lipoprotein-cholesterol (LDL-c) was obtained from calculation using a formula of $LDL = TC - HDL - (TG/5)$ (Shrivastava et al., 2013).

Population of lactic acid bacteria in the cecum of rats

The population of LAB in the cecum was determined by dilution and spread plate on MRS agar. The cecum contents were removed, collected in a sterile tube, weight, and added in saline solution to obtain 2 times dilution factor. Subsequently, this suspension was further diluted to 10^{-6} . Suspensions with dilution factors of 10^{-3} - 10^{-6} (0.1 ml. each) were spread on MRS Agar medium supplemented with Bromo Cresol Purple (BCP) and incubated anaerobically for 24 hours at 37°C.

Measurement of pH of the cecum

The pH of the cecum was determined using a pH meter (TOA ion meter IM 40S).

Statistical analysis

The data obtained in this experiment was analyzed using one way analysis of variance (ANOVA). This analysis was then continued using Duncan's multiple range tests when the value of significant difference was < 0.05 ($p < 0.05$).

Results and Discussion

All rats in the treatment groups gained weight following administration either with high-fat containing diet or high-fat containing supplemented with probiotics, indicating that external factors

Table 2. Total diet, food intake per day and body weight gain of rats fed with high-fat diet and *L. rhamnosus*

Treatment	Total food intake(g)	Food intake/day(g)	Body weight gain(g)
HF	174.8+ 27.0 a	6.24+ 0.97 a	31.18+6.97 a
HF-SKG34	176.2+ 26.7 a	6.29+ 0.96 a	33.42+12.7 a
HF-FBB42	189.7 + 32.2 a	6.78+ 1.15 a	38.87+11.7 a
HF-SKG34-FBB42	176.8+ 31.9 a	6.31+ 1.14 a	30.90+9.30 a

The values are expressed as means \pm SD

HF = High Fat Diet, HF-SKG34 = High Fat Diet with *L. rhamnosus* SKG34, HF – FBB42= High Fat Diet with dan *L. rhamnosus* FBB42, and HF –SKG34-FBB42 = High Fat Diet with *L. rhamnosus* SKG34 and *L. rhamnosus* FBB42.

and the status of the animals were under normal conditions throughout the experiment. Therefore, any changes occurred during this study must be due to treatments applied in the study. The rat weight gain, total diets, and the amount of diets daily consumed are presented in Table 2.

The amount diet consumed per day and the body weight gain of rats after 28 days did not show significant differences ($p > 0.05$) among treatments. The administration of probiotic *L. rhamnosus* resulted in an increase in the population of LAB in the cecum of rats (Table 3), although it was not significant statistically ($p > 0.05$). The respective increase in LAB population in the rats cecum following administration of SKG34, FBB42, and the combination of SKG34 and FBB42 were 1.22, 1.16, and 1.28 times, respectively. This indicated that *L. rhamnosus* was capable to adapt and proliferate in the gastrointestinal tract of rats. The growth and the activity of the probiotic in the cecum slightly decreased the pH of the cecum. The lowest pH (pH 6.58) was detected in the cecum treated with FBB42 and with combinations of FBB42 and SKG34. This indicated that the two *L. rhamnosus* strains isolated from different origin survived and multiplied in the gastrointestinal tract of rats. This result is in line with that reported by Sujaya *et al.* (2008a) and Uni *et al.* (2012) who conducted in vitro studies on *L. rhamnosus* SKG34 (Sumbawa mare milk isolates) and *L. rhamnosus* FBB42 (infant stool isolate), in a model of digestive tract conditions. High-fat containing diets, especially those with high cholesterol and saturated fatty acid content, may increase blood cholesterol levels and cause a person to suffer from atherosclerosis (Grundy *et al.*, 1982; Xu *et al.*, 2006). In this study, administration of rats with diet containing 10% pig fat (without the presence of probiotics) for 2 weeks was found to increase the rat's blood serum by 16.91%, (from 57.50 mg / dL to 67.22 mg / dL). This indicated that the pig fat containing saturated fatty acids and monounsaturated fatty acids (MUFA) had potency to increase blood cholesterol level. According to Rohman *et al.* (2012) the main composition of fatty acids in pig fat (lard) are palmitic (20.66%), stearic

Table 3. Population of LAB and pH of the rats cecum content fed with high-fat diet and *L. rhamnosus*

Treatment	cecum pH	LAB Total (CFU/g cecum)
HF	6.64 + 0.07 a	2.40 x 10 ⁶ a
HF-SKG34	6.60+ 0.06 a	2.93 x 10 ⁶ a
HF-FBB42	6.57+ 0.04 a	2.78 x 10 ⁶ a
HF-SKG34 -FBB42	6.58 + 0.05 a	3.06 x 10 ⁶ a

The values are expressed as means \pm SD

HF = High Fat Diet, HF-SKG34 = High Fat Diet with *L. rhamnosus* SKG34, HF – FBB42 = High Fat Diet with dan *L. rhamnosus* FBB42, and HF –SKG34-FBB42 =High Fat Diet with *L. rhamnosus* SKG34 and *L. rhamnosus* FBB42.

(10.91%), oleic (39.13%) and linoleic acid (19.56%). Hypocholesterolemic properties of probiotics to reduce incidence of global problem in coronary heart diseases have received serious attention in the recent years. The use of chemical-based drugs to treat patients with high blood cholesterol level has been found to have some undesired bad side effects. Besides that they are unaffordable by many people. Therefore, application of probiotics seems to be preferable because they provides indirect effects by modulating and stimulating gut microbiota of rats/humans or by affecting the metabolic pathways in human and animal bodies holistically. .

Adminsitration of probiotics to rats fed with high fat containing diet was found to affect their serum lipid profile. The total cholesterol level of rats serum (TC), HDL-c, LDL-c, and TG in the four groups following feeding with high fat containing diet without and with probiotic are shown in Table 3. As shown in Table 3 that the serum TC, HDL-c, LDL-c, and TG content of rats treated with high fat containing diet combined with probiotics (HF-SKG34, HF-FBB42, and HF-SKG34-FBB42) were statistically significant ($P < 0.05$) when compared to those fed with high fat containing diet only (HF treatment groups). However, within the groups treated with high fat containing diet in combination with probiotics (HF-SKG34, HF-FBB42, and HF-SKG34-FBB42), no significant different statistically was observed ($p > 0.05$). All probiotics were found to lower the content of TC, LDL-c, TG, but improved the level of HDL-c. When

Table 4. Blood lipid profile of rats fed with high-fat diet and *L. rhamnosus*

	Treatments			
	HF	HF-SKG34	HF-FBB42	HF-SKG34-FBB42
TC (mg/dL)	74.28 + 9.8 a	64.18 + 7.54 b	62.23+ 6.07 b	64.39 + 9.07 b
TG(mg/dL)	40.15+8.17 a	28.58+5.27 b	31.82+6.22 b	30.85+4.06 b
HDL-c(mg/dL)	44.14+7.20 b	52.10+6.33 a	48.35 + 5.42 ab	52.07 + 4.44 a
LDL-c(mg/dL)	22.11 + 6.11 a	6.36+ 2.74 b	7.52 + 4.65 b	5.15+ 4.91 b
Arteriosclerosis Index				
TC/HDL-c	1.70+ 0.21 a	1.23 + 0.06 b	1.29+ 0.10 b	1.23 + 0.09 b
TG/HDL-c	0.93 +0.23 a	0.56+0.17 b	0.66+0.13 b	0.59+0.07 b
LDL-c/HDL-c	0.51+0.18 a	0.12+0.05 b	0.16+0.11 b	0.11 + 0.09 b

The values are expressed as means±SD

a,b Mean values within a row with different letters differ significantly ($p < 0.05$, $n = 6$).

HF = High Fat Diet, HF-SKG34 = High Fat Diet with *L. rhamnosus* SKG34, HF -FBB42= High Fat Diet with dan *L. rhamnosus* FBB42, and HF -SKG34-FBB42 = High Fat Diet with *L. rhamnosus* SKG34and *L. rhamnosus* FBB42

compared to the HF treatment group, the HF-SKG34 reduced the TC, TG, and LDL-c by 13.60%, 28.82%, and 71.22%, respectively, but increased the HDL-c by 15.29%. A slightly better result was shown by the treatment group of HF-FBB42 which lowered TC, TG, and LDL-c by 16.22%, 20.76%, and 66.01%, respectively, but it increased the HDL-c by 8.71%. When these 2 probiotics were combined in a high fat containing diet, a decrease of TC, TG and LDL-c by 13.32%, 23.17%, and 72.18%, respectively was observed. In this treatment, the HDL-c value increased by 15.23%.

The results of this study showed that probiotics *L. rhamnosus* SKG34 and *L. rhamnosus* FBB42 appeared to improve the blood lipid profile of rats fed with high fat containing diet. Similar results were also reported by Ha *et al.* (2006); Jeun *et al.* (2010) and Ooi *et al.* (2010) who found the effectiveness of probiotics to improve lipid profile both in the *in vitro* and *in vivo* experiments. Administration of *L. rhamnosus* SKG34 and *L. rhamnosus* FBB42 clearly reduced the negative effects of saturated fatty acids and mono unsaturated (MUFA) contained in lard. An increase in risk of atherosclerosis indicated by the ratios of TG: HDL-c and LDL-c: HDL-c. These ratio shave often been used as a predictor of atherosclerosis (da Luz *et al.*, 2008). Our study (Table 4) showed that *L. rhamnosus* played an important role to lower the risk of atherosclerosis.

The mechanism by which the *L. rhamnosus* SKG34 and *L. rhamnosus* FBB42 in lowering cholesterol levels and improving lipid profiles is still unclear. However it is suspected to be due to the role of those probiotics to modulate the growth and the fermentative products of the intestinal microbiota of rats. Four mechanisms by which probiotics lowering the cholesterol level in the blood have been well documented. These include (1) the ability of probiotic bacteria to assimilate the cholesterol molecules in the

small intestine. In this case, the cholesterol will bind on membrane or cell wall of the probiotic during growth and therefore will result in a decrease in the absorption of cholesterol from the intestine to the blood (Brashears *et al.*, 1998; Anderson and Gilliand, 1999; Kimoto *et al.*, 2002); (2) the capability of probiotic microbes to enzymatically deconjugate bile acid using bile salt hydrolase (BSH). In the conjugated form, the bile acid will mostly dissolve so that only small portion is absorbed in the intestine, thus most is excreted through feces. The absorbed cholesterol will then be used to synthesize new bile acids (which is a homeostatic response), resulting in a decrease in serum cholesterol level (Brashears *et al.*, 1998; Yazid *et al.*, 1999); (3) conversion of cholesterol to coprostanol by cholesterol reductase of *lactobacillus* strains (Lye *et al.*, 2010), and (4), products of lactobacilli fermentation process in the form of short chain fatty acids that can inhibit the synthesis of cholesterol in the body (Gilliand *et al.*, 1985).

Previous studies conducted by Sujaya *et al.* (2008a) and Uni *et al.* (2012) showed that *L. rhamnosus* SKG34 and FBB42 were able to deconjugate glycodeoxycholic acid (GDCA). Besides that a decrease in the cholesterol level was suspected to be related to growth and the effect of the individual or combination fermentation process of the *L. rhamnosus* strains administered to the animals. Administration of *L. rhamnosus* SKG34 and *L. rhamnosus* FBB42 had a great contribution to increase the population of LAB in the cecum of rats, either as a result of administered probiotics or as a synergic effect of the probiotic to increases the indigenous LAB of the gastrointestinal tract of rats. As the LAB grow in the gastrointestinal tract, more acids, including short chain fatty acids (SCFAs), are produced and result in a slight decrease of the cecum pH. This acidic condition would cause precipitation

of bile so that most of this cholesterol is excreted through the feces. The rest which is absorbed, is used to synthesis bile to replace the excreted one. Some phenomena may also occur synergistically in this study to result in a reduced cholesterol level and an improved lipid profile in rats serum.

Conclusion

It can be concluded from this study that *L. rhamnosus* SKG34 and *L. rhamnosus* FBB42 were found to be effective to manage lipid profile in serum by decreasing total cholesterol, TG, LDL-c and increasing HDL-c, indicating that these two strains have possibility to be developed as potential probiotics to decrease incident of arteriosclerosis and to prevent coronary heart disease (CVD).

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