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Research Article
Effect of Differents Cowmilk and Soymilk (soy yogurt) Formulation on Blood Glucose Level and *Glut4* Gene Expression in Rats Soleus Muscle

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Abstract

Background and Objective: Soy yogurt is fermented soy milk and its nutrient-rich with isoflavones. Soy yogurt decreases blood glucose levels by utilizing the conversion of isoflavones. This study aimed to analyze the effect of soy yogurt on blood glucose level and *Glut4*gene expression on rats' soleus muscle. **Materials and Methods:** Twenty-five rats (eighteen-weeks old) were divided into 5 groups, e.g., the control (P_0), positive control (P_1, 100% yogurt) and three treatment groups (P_2, 50% soy yogurt+50% yogurt; P_3, 75% yogurt+25% soy yogurt and P_4, 75% soy yogurt+25% yogurt). All treatment groups were treated with different soy yogurt formula and administered for 12 weeks by gavaging. Under anesthetized, rats were sacrificed, then blood samples and soleus muscle were collected and stored at -80°C until use. RNA was extracted from soleus muscle and run for *Glut4* mRNA expression using (RT)-PCR. Data were analyzed using one way ANOVA and followed by post hoc test LSD (Least Significant Differences test). **Result:** There is no difference in rat body weight among groups after 12 weeks of soy yogurt consumption. Blood glucose levels were decreased at least 25% lower level compared to the control baseline by the various formulation of soy yogurt. Interestingly, there is a distinct pattern of *Glut4* mRNA expression level in the soleus muscle, P_1 increased the expression but not with other formulations decreased the expression (P_2, P_3 and P_4). **Conclusion:** Taken together, a different formulation of soymilk and cowmilk effectively reduces blood glucose level and modulates *Glut4*mRNA expression. In addition, a specific combination of bacteria type for fermenting soy yogurt could be a key to effectiveness for modulating blood glucose levels.

Key words: Soy yogurt, blood glucose, *Glut4,* soleus muscle

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Soybean (*Glycine max*) is a type of leguminous plant which has an important antioxidant namely isoflavones (genistein, daidzein and glycitein), phytic acid and saponins[1]. Soybeans also contain carbohydrates, phytochemicals, saponins, phytic acid and fiber. Thus, soy protein in diet might give many proven health benefits[1,2].

Soy protein or isoflavones can improve glycemic control that can increase insulin sensitivity and reduce the risk of diabetes[1]. Soy milk is a good source of protein. Soy milk and cow milk can be fermented to produce sour soy milk (soy yogurt and yogurt). Fermentation conditions will influence bioactive peptides formation. In fermented processed products, the bioactive peptides formed depend on the type of bacteria present in the starter culture and the degree of hydrolysis (fermentation time) has happened. An adequate proteolysis process is needed to facilitate the extrication of bioactive peptides from protein, in the excessive proteolysis process that will decrease its activity[3]. Fermentation is one of the efforts carried out and has been proven to increase nutritional value and improve soy milk acceptability. In addition, soy yogurt also has some benefits needed by the lactic acid bacteria fermentation process which decrease in glucose level in the bloodstream, prevent cancer, balance the digestive system and overcome fungal and bacterial infections[1,3].

Streptococcus, Lactobacillus and *Bifidobacterium* are often used as a starter for making yogurt or soy yogurt. Fermentation will change in the content of various isoflavones and β-glucosidase activity in soy milk or cow milk. It had reported formulation bacteria is important for determining the content of fermented milk. Streptococcus thermophilus as a starter had shown that it resulted a higher content of aglycone and increased beta-glucosidase activity. The quantity isoflavone content increases, this can be observed in the percentage of daidzein from 14.24 to 36.20, genistein from 6.89 to 28.80 and glycitein 2.45 to 12.44, this takes place after 24 hours of fermentation[4]. In during the fermentation process, the highest β-glucosidase activity and bioconversion of isoflavones after 12 hours is occurred in *L. rhamnosus*, this is influenced by an increase in the content of isoflavone aglycones in fermented soy milk which improve the biological function of soy milk[5].

Glut4 plays a role in glucose homeostasis, studies in mice with one allele of *Glut4* gene is altered has 50% reduction in *Glut4* expression in skeletal muscle, heart and fat cells. It caused mice to suffer severe insulin resistance and developing diabetes[6,7]. In normal muscle and fat cells, *Glut4* is recycled between the membrane of plasma and intracellular storage vesicles. *Glut4* differs from other glucose transporters, which is about 90% located in the intracellular when the condition is not stimulating insulin or other stimuli such as exercise. In the presence of insulin or other stimuli, the balance of this recycling process is changed to support translocation of *Glut4* from intracellular storage vesicles towards the membrane of plasma and also to transverse tubules in muscle cells so that an increase in the maximum speed of glucose transport into cells[8].

Insulin acts on the target cells by activating tyrosine kinase. Autophosphorylation of tyrosine kinase insulin receptors stimulates the catalytic activity of tyrosine kinase receptors that produce tyrosine phosphorylation from Insulin Receptor Substrates (IRSs) including IRS1, IRS2, IRS3, IRS4, Gab1 and Shc. This bond then activates the enzyme phosphatidylinositol 3 kinase (PI3K). PI3K phosphorylates specific phosphoinositides to form PIP_2 into PIP_3. PIP_3 then activates serine/threonine kinase, phosphoinositide-dependent kinase-1 (PDK1). Activation of PDK1 causes activation of Akt / protein kinase B (PKB) and protein kinases C λ and ζ (PKCλ /ζ). The signal transduction activator activates the phosphorylation of its substrate, AS160 that stimulates *Glut4* translocation (an insulin-dependent glucose-carrying protein) from the intracellular vesicle exocytosis to the cell surface so that glucose can enter the cell[9,10]. The intracellular translocation of *Glut4* into the membrane of plasma is stimulated by expression of the active form of protein kinase B or atypical isoform PKC in cell culture experiments. Both of these kinases are chemical mediators in the process of insulin stimulating *Glut4* translocation *in vivo*. Good atypical PKC isoforms have been shown that blocking their work will weaken *Glut4* movement, whereas studies of activation of blocked PKB have conflicting results. Furthermore, in muscle cells of diabetic subjects, at concentrations of ecological insulin, glucose transport stimulation has been shown to be impaired, whereas activation of PKB is normal[6,7].

Skeletal muscle is the main organ that utilizes glucose stimulated by insulin and converts it into energy so that the body has the energy to produce movement and do activities. Skeletal muscle is the main network that contributes to systemic glucose depletion in post-prandial conditions to maintain normal glycemia. Insulin has a rapid effect to induce glucose uptake and glycogen synthesis in protein-independent synthesis mechanisms. During hypoxia-related muscle contractions, the level of glycogenolysis and glucose uptake increases to prepare glucose needed for glycolytic process[10]. Muscle glucose absorption is well adjusted to ensure glucose homeostasis and for the needs of the energy

muscles that are needed quickly, but continuously[8,10]. *Glut4* and *Glut1* are expressed in skeletal muscle and modulated on the development stage and fiber types composition. *Glut4* is expressed in oxidative and glycolytic muscle fibers differentially in mice. During fetal life in mice, the dominant glucose carrier *Glut1*, with marked expression suppressed perinatally, as a consequence of changes that occur in the pre-transaction stage. In contrast, expression of skeletal muscle *Glut4* in fetal mice is very low will induce continuous *Glut4* mRNA and protein occurs in the perinatal phase[6,7]. *Glut4* is in an insulin-responsive vesicle, insulin stimulation will move these vesicles toward the cell surface and then insert *Glut4* to move into the plasma membrane, to absorb glucose. In the present study, we analyze the effect of different soy yogurt formulation on blood glucose level and its correlation with *Glut4* mRNA expression in rat soleus muscle.

MATERIALS AND METHODS

The present study was carried out as a collaborative research work between the Faculty of Animal Husbandry and Faculty of Medicine, Universitas Padjadjaran in the period of July 2017 until June 2018.

Animal: Twenty five Wistar rats, 18 weeks old, female, were bred in the Animal Facility of PT. Bio Farma, Indonesia. Rats were kept at 24°C under a 12 hrs light, 12 hrs dark cycle (light on, 6 am to 6 pm) and 55% relative humidity, with food and water ad libitum for 80 weeks in Animal Physiology Laboratory, Physiology Division, Faculty of Medicine, Universitas Padjadjaran. Rats were divided randomly into five groups treatment. Control group which was not given the treatment as the control group (P_0), Group-1 was given treatment by 100 % yogurt (P_1), Group-2 was given treatment by 50% soy yogurt and 50% yogurt (P_2), Group-2 was given treatment by 75% yogurt and 25% soy yogurt (P_3) and Group-2 was given treatment by 75% soy yogurt dan 25% yogurt (P_4). All rats were habituated for 7 days prior to treatments. Soy yogurt or yogurt was administered by gavaging, 2 mL/day/rats for 12 weeks. Rats were sacrificed using the CO_2 chamber, blood samples were collected via cardiac puncture and soleus muscle were dissected, weighed and dipped frozen in liquid nitrogen. Soleus muscle was stored in the freezer at -80°C until use.

Materials: Formulation of soy yogurt and yogurt were produced with specific bacteria formula fermentation (Supplemental Table 1) according to the procedure published. Milk fermentation was performed at the Faculty of Animal Husbandry, Universitas Padjadjaran, Jatinangor, Indonesia[11].

Blood Glucose level

Five micromillimeter blood sample was utilized for determining blood glucose levels after 12 weeks of treatment using Rat Glucose Assay Kit (Crystal Chem, Cat. number: 81693, USA). All procedure followed manufactured protocols.

Semi-quantitative Reverse Transcript (RT) PCR: RNA of soleus muscle was isolated using Trizol reagent from Invitrogen, USA and procedures was performed according to the manufacturer's instructions. Equal amount of 50 ng RNA was amplified using Onestep PCR kit (Bioline, USA)[12]. Specific *Glut4* primers were designed with Forward: 5'GGGCTGTGAGTGAGTGCTTTC-3', Reverse 5'CAGCGAGGCAAGGCTAGA-3' and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal control of gene expression, GAPDH primer sequences as followed: Forward 5'GTTACCAGGGCTGCCTTCTC-3', Reverse 5'GATGGTGATGGGTTTCCCGT3'). *Glut4* expression results for each sample were normalized with *Gapdh* mRNA levels for integrity of the results. Equal of PCR Products was run into agarose 1,2 % gel in TAE buffer and Image was captured for density analysis using Image J software.

Supplemental Table 1: Composition of probiotics used in the study

No.	Group	Composition	Probiotics	Ratio No. bacteria
1	P_0	Control (NaCl 0.9%)	Water	
2	P_1	Cow Milk	*Lactobacillus bulgaricus*	2
			Streptococcus thermophilus	2
			Lactobacillus acidophilus	1
3	P_2	Cow Milk 75%+Soymilk 25%	*Lactobacillus bulgaricus*	2
			Streptococcus thermophilus	2
			Lactobacillus acidophilus	1
4	P_3	Cow Milk 50%+Soymilk 50%	*Lactobacillus bulgaricus*	2
			Streptococcus thermophilus	2
			Lactobacillus acidophilus	1
5	P_4	Cow Milk	*Lactobacillus acidophilus*	2
			Bifidobacterium bifidum	2

Statistical analysis: Data were analyzed with One-Way Analyze of Variance (ANOVA) and followed by LSD post hoc test by using SPSS 20.0. Statistical significance was designated at $p<0.05$. Data are expressed as mean ± standard of deviation (SD).

RESULTS AND DISCUSSION

Difference soy yogurt formulation effects on weight changes: Fermented milk, yogurt and soy yogurt are believed as a health drink. Potential mechanisms of action of yogurt include an increase in body fat loss, a decrease in food intake and increase in satiety, a decrease in glycemic and insulin response, altered gut hormone response, replacement of less healthy foods and altered gut microbiota[13]. Surprisingly, different formulation fermented soy and cow milk combination did not induce a significant change in Rat's body weight after 12 weeks of treatment (Fig. 1).

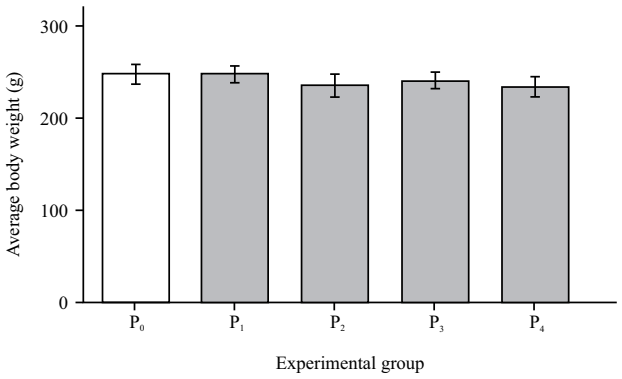

Fig. 1: Different bacteria composition and soy/cow milk formulation in fermentation does not change Rat's body weight. Data is presented as average with standard of deviation (SD). White bar represents control group and black bars represent treatment group (P_1, P_2, P_3 and P_4), *Significant was considered with $p<0.05$)

Different soy yogurt formulation reduces on blood glucose level: Different soy yogurt formulation decreased blood glucose level baseline level Treatment groups reduced the average baseline of blood glucose level around 25% compared to control group (Fig. 2). Group P_2 showed the lowest blood glucose compared to another group, it reflected the specific formulation of percentage cow and soy milk with bacteria combination plays an important role in reducing blood glucose levels. Details comparison average mean of blood glucose level between experimental groups was statistically tested and presented in Table 1.

Alteration of glucose levels after treatment with a different formulation of soy yogurt might be determined by specific protein resulted from the fermentation process. Beside protein composition like isoflavones also may take some role. The isoflavones play as important compounds in soy yogurt by inhibiting the enzyme α-glucosidase which plays a role in the entry of glucose into the intestinal lumen and this potentially reduced blood glucose levels in the bloodstream. Isoflavones bind to the active binding site of glucose in the α-glucosidase enzyme so that the enzyme was unable to digest the substrate. *In vitro* studies show that pure isoflavones (*genistein, daidzein*) had inhibitory activity against the α-glucosidase enzyme[4,14]. Isoflavone glycosides, g*enistein, daidzin* and *glicitin* also released the sugar portion and are converted into isoflavone aglycones, *genistein, daidzein* and *glycitein* by protease[15,16]. In addition, after the fermentation process, many organic compounds were broken down into smaller molecules by microbial enzymes and then exert

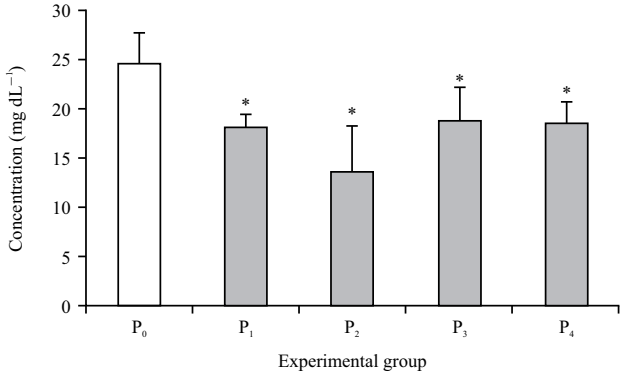

Fig. 2: Different bacteria composition and soy/cow milk formulation in fermentation decreases blood glucose level after 12 weeks treatment. Data is presented as average with standard of deviation (SD). White bar represents control group, and black bars represent treatment group (P_1, P_2, P_3 and P_4), *Significant was considered with $p<0.05$)

Table 1: Comparison average mean of blood glucose level between experimental groups

Comparison of blood glucose level	p-value*
P_0 vs. P_1	0.004*
P_0 vs. P_2	0.000*
P_0 vs. P_3	0.009*
P_0 vs. P_4	0.009*
P_1 vs. P_2	0.039*
P_1 vs. P_3	0.703
P_1 vs. P_4	0.851
P_2 vs. P_3	0.018*
P_2 vs. P_4	0.035*
P_3 vs. P_4	0.863

*p-value <0.05 is considered as significant, *Post hoc* test is utilized the LSD test

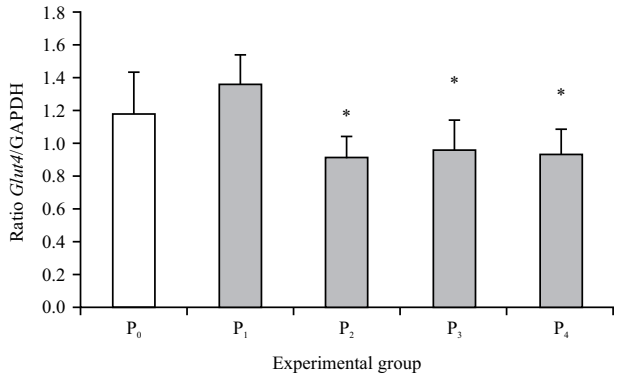

Fig. 3: Different bacteria composition and soy/cow milk formulation in fermentation (P_2, P_3 and P_4) decreases *Glut4* mRNA expression in soleus muscle after 12 weeks treatment. Data is presented as average with standard of deviation (SD). White bar represents control group, and black bars represent treatment group (P_1, P_2, P_3, and P_4), *Significant was considered with $p<0.05$)

Table 2: Significant and Comparison of average *Glut4* mRNA levels between treatments

Comparison of mRNA *Glut4* level	p-value*
P_0 vs. P_1	0.139
P_0 vs. P_2	0.039*
P_0 vs. P_3	0.067
P_0 vs. P_4	0.064
P_1 vs. P_2	0.001*
P_1 vs. P_3	0.002*
P_1 vs. P_4	0.003*
P_2 vs. P_3	0.789
P_2 vs. P_4	0.910
P_3 vs. P_4	0.889

Value p = value of significance/significance; *: *Post hoc* test further with the LSD test, Significance value $p<0.05$

various physiological effects. Therefore, we explore a different combination of bacteria for fermented soymilk. Type of microorganisms enhances biofunctional properties by increasing the level of aglycone isoflavones and peptides[3,14]. Lactic acid bacteria in soy yogurt would produce a compound that will fight pathogenic microbial infections in the body, such as infections due to the fungus Candida albicans and the bacterium *Helicobacter pylori*. This compound was a compound with low molecular weight either in the form of proteins or short peptides that have the activity of inhibiting or killing microbes (antimicrobial)[2,3,17]. Thus, it had supported and convinced our finding that different formulation of soy yogurt could decrease by 25% rats' blood glucose level after 12 weeks of administration (Fig. 2).

Difference soy yogurt formulation effects on *Glut4* gene expression in soleus muscle: Our data showed a potential link that decrease of blood glucose might directly or indirectly correlated with the role of *Glut4*. *Glut4* is very closely related to glucose metabolism and involved in insulin. *Glut4* expression is regulated in human red skeletal muscle cells that express excessive glycogen phosphorylase. It was regulated in a state of relative insulin deficiency, such as fasting. *Glut4* is not down-regulated by fasting in white skeletal muscle, insulin levels were sufficient to directly regulate the expression of this transporter only under certain cellular conditions. *Glut4* had an important role in regulating the transport of glucose-stimulated by insulin. In addition, *Glut4* is very influential in insulin-resistant conditions[18].

Zucker diabetic rats (ZDF/drt) and diabetic SHR/N-cp rats. ZDF/drt rats have reduced *Glut4* protein levels in adipose tissue. In addition, both ZDF/drt and SHR/N-cp rats have reduced levels of *Glut4* in skeletal muscle. In ZDF/drt rats, the *Glut4* protein was reduced to a greater extent in skeletal muscle enriched with red glycolytic fibers compared to skeletal muscle enriched with white oxidative fibers[19]. The experimental model that was widely used in insulinopenic diabetes, was streptozotocin-induced diabetic rats. This model showed intact insulin resistance of the body and reduces the expression of *Glut4* protein in skeletal and cardiac muscles.

Interestingly, the difference of soy yogurt formulation alters differently *Glut4* mRNA expression level in the soleus muscle. All group treatment group beside P_1, (P_2, P_3 and P_4) decreased small but significant the *Glut4* gene expression level in the soleus muscle (Fig. 3). *Glut4* mRNA expression of soy yogurt treatment P_2, P_3 and P_4 were lowered by 15-18% compare to control whereas P_1 showed that there is no changes after treatment for 12 weeks. Significant comparison each group was presented in Table 2. There are several factors which might explain the different effect of soy yogurt formulation on *Glut4* mRNA expression. First of all, this effect need to be studied further since in our study only soleus muscle had been explored, it seem that muscle fiber type could give a specific response. Secondly, fermented protein type as result from different bacteria formulation (Supplemental Table 1) or milk based will altered and partially affect the blood glucose regulation without involving *Glut4* regulation. It had been reported that the effect of diabetes was depend on the type of skeletal muscle fiber and RNA expression level could be not pararel with its protein levels due to post transcription modification. In contrast, *Glut4* protein was reduced in white skeletal muscle fiber, along with

an increase in unchanged *Glut4* mRNA levels and *Glut4* transcription. Therefore, role of insulin as a potential regulatory factor would determine the expression of *Glut4*[18,19]. Thirdly, the posttranscriptional process of modification will determine *Glut4* mRNA expression in the soleus muscle, therefore the mRNA expression could be different and contrast with the protein level pattern. Our limitation is information about *Glut4* protein level after treatment 12 weeks. Molecular regulation provided cell control over structure and function and the basis for cell differentiation, morphogenesis and the versatility and adaptability of each organism. It could be function as a substrate for evolutionary change, because control of the time, location and amount of gene expression could have a profound effect on the function of genes in cells or in multicellular organisms[19,20]. The activity of transcription factors was further modulated by intracellular signals that cause post-translational protein modification including phosphorylation, acetylation, or glycosylation and those changes will affect the ability of transcription factors to bind, directly or indirectly to the promoter DNA, to recruit RNA polymerases, or to support the extension of newly synthesized RNA molecules. There was a significant effect of the specific effects of non-DNA sequences on transcription[18]. This effect was referred to as epigenetic and involves high-order structure of DNA, specific non-sequence DNA binding proteins and chemical modification of DNA. In general, the epigenetic effect of changing the accessibility of DNA was into proteins thus modulating transcription[7].

However, we did not explore the type of proteins and metabolites after combination soy and cow milk fermented. In addition, we were not able to count the glycogen content in the liver and muscle for confirming glucose utilization and involvement of the glucose signaling pathway. Further, these data will clarify the role of fermented milk in regulating blood glucose levels and its detailed molecular mechanism.

CONCLUSION

Different combinations of bacteria and percentage formulation of cow milk and soy milk reduce blood glucose level and modulate *Glut4* expression in soleus muscle. Percent combination Soy and cow milk and combinations of bacteria for the fermentation process might be an important factor for modulating blood glucose levels.

SIGNIFICANCE STATEMENT

This study discovers the potential importance of percentage combination soy/cow milk and bacteria composition during the fermentation process for modulating blood glucose levels. It showed that 12-week treatment could modulate blood glucose baseline and *Glut4* expression. In addition, it is important to understand specific bacteria combinations for the fermentation of soy or cow milk. Our data support the potential of fermented soymilk and cowmilk's consumption might be very beneficial for health purposes.

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