THE EFFECT OF FORTIFIED DADIH (FERMENTED BUFFALO MILK) WITH VITAMIN D3 ON CAECUM CHOLESTEROL CONCENTRATION AND HIGH SENSITIVITY C-REACTIVE PROTEIN (hs-CRP) LEVEL IN TYPE 2 DIABETES MELLITUS RAT MODEL

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ABSTRACT
Type 2 diabetes mellitus (T2DM) may be developed by a cardiovascular complication. T2DM and its complications associated with a decrease in levels of 25 hydroxyvitamin D below normal. The level of 25-OH-D can increase and it can be gained by probiotics supplementation. Dadih is a probiotic useful as antidiabetic, antiatherosclerotic, and it can reduce serum cholesterol. Vitamin D is beneficial for T2DM since it improves insulin production, acts as an anti-inflammatory and prevents dyslipidemia thereby preventing cardiovascular disease. This research aims to investigate the effects of giving dadih-fortified-vitamin-D3 toward caecum-cholesterol-concentration and hs-CRP-levels to T2DM-induced-rats. This study used a randomized pre-post test with control group design in 30 Wistar rats divided into 5 groups, namely T1, T2, and T3-treatment-groups. T3-group was given dadih-fortified-vitamin-D3, while T1 and T2-groups were given vitamin-D3 and dadih, respectively. The control groups were healthy-control (C1), and T2DM (C2). The intervention was given through oral sonde for 28days. The variables analyzed were caecal-cholesterol-concentrations using a spectrophotometer and hs-CRP using the ELISA method. The statistical tests were used for the caecum-cholesterol-concentration and hs-CRP levels. The mean of caecum-cholesterol-concentration in the T3-group (83.68 ±1.93mg.100g⁻¹), was higher than T1 (77.99 ±1.70; p = 0.004) and C2-control (24.39 ±1.47; p = 0.0001). The mean of hs-CRP-level-post-intervention in the T3-group (4.21 ±0.41 ng.mL⁻¹), was lower than C2 (17.15 ±0.85;p = 0.0001), T1 (6.59 ±0.27; p = 0.0001) and T2 (5.43 ±0.39; p = 0.004). There is a very strong inverse correlation between the concentration of cholesterol and hs-CRP ( r = -0.979, p = 0.0001). The conclusion is dadih-fortified-vitamin-D3—intervention is better than its single intervention as an anti-inflammation which might relate to the increased caecum-cholesterol-concentration.

Keywords: T2DM; dadih; vitamin D3; caecum-cholesterol; hs-CRP

INTRODUCTION
Diabetes Mellitus (DM) as a chronic disease is marked by the increase of the amount of blood glucose that resulted from the incapability of the pancreas to produce insulin or the incapability of the human’s body to use existing insulin effectively (WHO, 2016). DM prevalence in 2014 is 422 million and increase to 425 million in 2017. In 2017, The International Diabetic Federation (IDF) informed that Indonesia belongs to ten big countries with 10.3 million of DM patients of 20 to 79 years old (IDF, 2015). Type 2 diabetes mellitus (T2DM) is the one commonly emerged. It reaches 90% of the total cases of DM (IDF, 2015). T2DM is caused by a decrease in the body responds to insulin, known as insulin resistance. The inability of pancreatic-β-cells to maintain stable glucose balance causes the increase of plasma glucose levels (hyperglycemia) (IDF, 2015; Kahn, Cooper and Del Prato, 2014).

T2DM-patients have significantly lower 25(OH)D-concentrations than healthy people (Stivelman and Retnakaran, 2012). The hypovitaminosis D is one of the causes of glucose intolerance and the decrease of insulin secretion of T2DM either directly by activating vitamin D receptors (VDR) or indirectly by calcemic hormones and inflammation (Shang and Sun, 2017).

Vitamin D acts as an anti-inflammatory and immunomodulatory of various diseases like obesity, cardiovascular, and diabetes (Jafari et al., 2015). Vitamin D is also able to proliferate and stimulate T cells. It is also able to reduce pro-inflammatory mediators like C-Reactive Protein (CRP) (Wu et al., 2014). Generally, fortification of vitamin D is done to dairy products such as yogurt.
MATERIAL AND METHODOLOGY

Dadih-fortified-vitamin-D₃ preparation and intervention

Dadih was made from buffalo milk from farms in the Gadut area, West Sumatra Province. Buffalo milk was pasteurized at temperature 72 °C for 15 seconds. The milk was then cooled to 30 °C and added 900 IU of vitamin-D₃ then put into a bamboo tube and then it covered with banana leaves for two days of fermentation. The bamboo used was betung bamboo with a diameter of 4 – 5 cm, a length of 20 cm, and a volume of 100 mL (Ranieri et al., 2009). Dadih-fortified-vitamin-D₃ was given orally using a feeding tube given to experimental Wistar rats. The dosage was 4g.kg⁻¹ BW.d⁻¹ (Kusuma et al., 2015).

Research design and experimental animals

This research was a true-experimental study with a randomized pre-post test with a control group design. Animals used were male Wistar rats, aged 8 weeks, the weight was 150 – 200 g. They were acclimatized at the laboratory of the Center for Food and Nutrition Studies at Gajah Mada University, Yogyakarta. The rats were placed in individual stainless-steel cages at regulated a temperature of 21 °C. The rats were fed with 15 g.d⁻¹ of the Comfeed II standard diet during the non-HFD period and intervention. Rats received ad libitum water during the experiment. Animal laboratory guidelines used were from the Central Laboratory for Food and Nutrition Studies, Gajah Mada University (UGM), Yogyakarta.

The T2DM intervention (T1, T2, and T3). The T2DM condition was indicated by body weight (BW) decreased, hyperglycemic status, hyperinsulinemic status, increased CRP levels in T2DM Wistar rats. The research was conducted on 30 rats divided into five groups which were the healthy control (C1), T2DM control (C2), and T2DM intervention (T1, T2, and T3). The T2DM condition was induced by HFD, STZ, and NA. After a week of acclimatization, rats were conditioned in T2DM with oral HFD 15 g.d⁻¹ for 14 days then injected intraperitoneally with STZ 45 mg.kg⁻¹ rat BW and NA 110 mg.kg⁻¹ rat BW. T2DM condition was indicated by fasting blood glucose serum >250 mg.dl⁻¹ (Gheibi, Kashfi and Ghasemi, 2017). The T3-group was then treated with dadih-fortified-vitamin-D₃ in doses of 4 g.200g⁻¹ BW.d⁻¹ every day for the intervention of 28 days, while T1 and T2-groups were given vitamin-D₃ 36 IU.d⁻¹ and dadih 4 g.200g⁻¹ BW.d⁻¹, respectively (Kusuma et al., 2015; Jafari et al., 2015). This study was approved by the Health Research Ethics Diponegoro University Semarang through ethical clearance No. 140/EC/H/KEPK/ FK-UNDIP/XI/2019.

Determination of biochemical markers

The blood sample was taken first and at the end of the intervention, fasting blood glucose was taken through the retroorbital plexus. Blood glucose determination using GOD-PAP (BIOLABO SA). Determination of blood glucose after enzymatic oxidation by glucose oxidase. Blood samples were collected in a centrifugation tube and centrifuged 4000 rpm in 15 minutes (HerauesSepatech, Biofuge 15). Caecum-cholesterol-concentration determination using the Lieberman Burchand method and analyzed by Spectrophotometer (Optima SP. 300). The sample's absorbance was further read by a spectrophotometer at 680 nm (Plummer, 1971).
hs-CRP levels were determined using an enzyme-linked immunosorbent assay (ELISA) kit (Fine Test, Wuhan Fine Biotech, China) according to manufacturer instructions (Fine Test, 2019). Briefly, Reagen adds samples 100 µL of the properly diluted sample into test sample wells. The plate sealed with a cover and incubated at 37 °C for 90 minutes. After incubation, the plate was rinsed 3 times with wash buffer and let the Wash Buffer stay in the wells for 1 – 2 minutes each time. 100 µL of Biotin-labelled antibody working solution into the above wells (standard, test sample, and blank wells), sealed with a cover, and incubate at 37 °C for 60 minutes. After incubation, remove the cover and wash the plate 3times with Wash Buffer. 100 µL of SABC (Streptavidin Conjugate) Working Solution added into each well, sealed with a cover the plate, and incubated at 37 °C for 30 minutes. After incubation Remove the cover and wash the plate 5times with Wash Buffer. 90 µL TMB Substrate added into each well, sealed with a cover the plate, and incubated at 37 °C for 10 – 20 minutes in dark. After incubation, 50 µL Stop Solution added into each well. The color will turn yellow immediately. The absorbance of samples in well plates was read by ELISA Reader at 450 nm (Zenic).

**Statistical analysis**

Data were analyzed using version 21 of the statistical package for the Social Sciences (SPSS). The results were expressed as mean ±SD (for normally distributed data) otherwise it expressed as median (min-max). Statistical difference was analyzed by using One-Way Analysis of Variance (ANOVA) followed by Post Hoc Bonferroni for normally distributed data, otherwise, the Kruskal-Wallis test followed by Mann Whitney test was used. The Differences before and after intervention were analyzed using a Paired t-test for normally distributed data, otherwise the Wilcoxon test. Pearson’s correlative test was used to analyze the correlation between variables, and statistical analyses were performed by a computer. The differences and correlations were considered significant at \( p <0.05 \) and 95% confidence intervals (CI). The strength of correlations was determined by the \( r \) value.
RESULTS AND DISCUSSION

The data processed in this study was obtained from 29 Wistar rats, which are divided into each group consisting of 6 rats. The use of dadih in this study referred to the kefir study. Kefir intervention. the study of T2DM patients indicates that LAB in kefir contributes to the regeneration of pancreatic beta cells and then it increases the ability to produce insulin. Further, insulin facilitates the glucose absorption into adipose and liver that glycogenesis and lipogenesis occur. This process might contribute to increasing the weight of T2DM receiving kefir (Ostadrahimi et al., 2015).

The blood glucose levels are collected in all intervention groups (T1, T2, and T3). Before the intervention, all T2DM-rats showed significantly higher blood glucose than C1-group (Post Hoc Bonferroni test T1, p = 0.0001; T2, p = 0.0001; T3, p = 0.0001, Table 1). The blood glucose before the intervention also showed that there was no difference between the intervention group (p >0.05). The blood glucose levels decreased significantly after treatment in all treatment groups (paired t-test; p<0.05). The post-intervention of blood glucose showed that all treatment group was significantly lower than C2-group (Post Hoc Bonferroni test T1, p = 0.0001; T2, p = 0.0001; T3, p = 0.0001). Additionally, those of treatment groups remained higher than C1-group (Post Hoc Bonferroni test T1, p = 0.0001; T2, p = 0.0001; T3, p = 0.003). This indicated that all interventions used improved glycemic status, although it had not reached a healthy yet at a group level. The post-intervention blood glucose level in the T3 group had the lowest level among intervention-groups and significantly different than the T1 group. Intervention with dadih-fortified-vitamin-D3 was proved that the glucose level was able to decrease better than the single intervention (vitamin D3).

The pre-post intervention change (Δ) of blood glucose levels was significantly different among the five groups (Kruskal-Wallis test, p = 0.0001). Results of the Mann Whitney test showed the significant differences in blood glucose levels (p <0.05) between the intervention groups (T1, T2, T3) compared to the C1-group. The negative Δ blood glucose level of T3 was bigger than T1 and T2, and this was significantly different. This gave an additional finding that dadih-fortified-vitamin-D3-intervention was not only better than the single intervention (vitamin-D3) but also dadih.

The result of the One-way ANOVA test showed that there was a significant difference in caecum-cholesterol-concentration among groups in this study (p = 0.0001). Caecum-cholesterol-concentrations of T1, T2, and T3-groups was significantly higher than C2-groups (Table 2; Post Hoc Bonferroni test; p = 0.0001 in each group). These indicated that vitamin-D3, dadih, and dadih-fortified-vitamin-D3 the treatments contribute to the increased of caecum-cholesterol- concentrations of T2DM-rats. Interestingly, those of T2 and T3-groups were significantly higher than the T1-group (p = 0.0001 and p = 0.004). Also, those of the T2-group were significantly higher than the T3-group (p = 0.003). Therefore, dadih-intervention might have a predominant-effect in increasing caecum-cholesterol-concentration in T2DM-rats. Probiotics contribute to reducing cholesterol absorption in the intestine. This is based on the yogurt study. Probiotics assimilate cholesterol and get the cholesterol into the membrane of probiotic cells. As a result, the cholesterol absorption in the intestine reduces and the cholesterol will get into the cecum to be disposed of with feces (Ejtahed et al., 2011; Nuraida, 2015). Probiotics can reduce cholesterol concentrations in vitro through the conversion of cholesterol into coprostanol by cholesterol reductase. This condition leads to the cholesterol excretion in feces increases, while cholesterol in the plasma decreases (Lye, Rusul and Lione, 2010). Vitamin D mechanisms used in increasing caecum-cholesterol-level have not been elucidated. However, vitamin D involves the decline of blood cholesterol levels. Vitamin D inhibits HMG-CoA reductase, an enzyme function in endogenous cholesterol biosynthesis and it can also de-conjugate bile acids in the intestine, and subsequently, it reduces cholesterol concentration (Kane et al., 2013; Ostadrahimi et al., 2015). The caecum-cholesterol-concentration in those treatment-groups (T1, T2, and T3) at the post-intervention was significantly lower than healthy C1-group (p = 0.0001, p = 0.006, and p = 0.0001). This suggests that improving interventions remain open for the benefit of T2DM-individuals.

All of the treatment-groups had lower hs-CRP levels at the post-intervention, and these differences were significant (Table 1; Wilcoxon test, p = 0.028 in each group). All of the treatment-groups had lower hs-CRP levels than C2-group at the post-intervention. The negative delta (Δ) of hs-CRP levels was bigger in those receiving treatments than C2-group (Mann Whitney test; T1, p = 0.006; T2, p = 0.004 and T3, p = 0.004). All of these showed that all interventions used in this study were associated with the reduction of blood hs-CRP levels. The post-intervention of hs-CRP levels in the T3-group (Mean ±SD; 4.21 ±0.41 ng.mL⁻¹), was lower than C2 (17.15 ±0.85; p = 0.0001), T1 (6.59 ±0.27; p = 0.0001) and T2 (5.43 ±0.39; p = 0.004). Thus, dadih-fortified-vitamin-D3 intervention might have a better effect than its single intervention to decrease blood hs-CRP levels in T2DM-rats. Dadih contains LAB and the previous studies show that LAB in fermented food involves mechanisms to reduce blood-CRP-levels. LAB induces the increase of production of several anti-inflammatory cytokines, and the decrease of COX-2 expression, an enzyme that catalyzes the production of prostaglandins from arachidonic acid, in which it stimulates cell proliferation and inflammatory processes (SaediFard, Djafarian and Shab-Bidar, 2020). LAB balances the gut microbiota therefore, it inhibits the production and secretion of LPS. Decreased of LPS in the intestinal epithelium causes a decrease in proinflammatory cytokines (Wirawati et al., 2019). Probiotics from dadih can also produce SCFA in the colon, while this SCFA can reduce the synthesis of CRP enzymes in the liver (Asemi et al., 2013).

Vitamin D can provide a protective effect by reducing sensitivity to the circulating-CRP (Zakharaova et al., 2019). Vitamin D does not only decrease CRP but also decreases proinflammatory cytokines including TNF-α, IL-6, IL-1β (Mirhosseini et al., 2017). Vitamin D weakens the expression of proinflammatory cytokines involved in insulin resistance and regulates NF-KB activities.
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Table 1 BodyWeight, BloodGlucose and hs-CRP Level.

<table>
<thead>
<tr>
<th>Groups</th>
<th>C1</th>
<th>C2</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight of Intervention (g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>182.5</td>
<td>165.5</td>
<td>165.0</td>
<td>174.50</td>
<td>160.50</td>
<td>0.004</td>
</tr>
<tr>
<td>Δ</td>
<td>(172.0-211.0)a,b,c,d</td>
<td>(150.0-182.0)a,b,c,d</td>
<td>(145.0-173.00)a,b,c,d</td>
<td>(150.0-180.00)a,b,c,d</td>
<td>(148.00-170.00)a,b,c,d</td>
<td>0.0001</td>
</tr>
<tr>
<td>p</td>
<td>0.027</td>
<td>0.027</td>
<td>0.080</td>
<td>0.046</td>
<td>0.026</td>
<td></td>
</tr>
</tbody>
</table>

| Blood Glucose Level (mg.dL⁻¹) |      |      |      |      |      |    |
| Pre   | 70.67 ± 1.52b,c,d,e | 288.04 ± 4.46a | 284.21 ± 2.66a | 286.14 ± 2.02a | 289.52 ± 4.02a | 0.0001 |
| Δ     | (1.21-2.96)a,b,c,d,e | (1.99-4.44)a,b,c,d,e | (-189.25-180.35)b,c,d,e | (-209.11-194.36)b,c,d,e | (-215.49-204.67)b,c,d,e | 0.0001 |
| p     | 0.0001 | 0.0001 | 0.0001 | 0.0001 | 0.0001 |    |

Table 2 Caecum-Cholesterol-Concentration Test.

<table>
<thead>
<tr>
<th>Rat Group</th>
<th>Caecum Cholesterol Concentration (Mean ±SD) (mg.100g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>94.57 ±2.67 b,c,d,e</td>
</tr>
<tr>
<td>C2</td>
<td>24.39 ±1.47 a,c,d</td>
</tr>
<tr>
<td>T1</td>
<td>77.99 ±1.70 a,b,d</td>
</tr>
<tr>
<td>T2</td>
<td>89.34 ±3.20 a,b,c</td>
</tr>
<tr>
<td>T3</td>
<td>83.68 ±1.93 a,b,c</td>
</tr>
</tbody>
</table>

Note: Alphabetical superscripts showed a significance level of: a: p < 0.05 compared as C1; b: p < 0.05 as compared to C2; c: p < 0.05 as compared to T1; d: p < 0.05 as compared to T2; e: p < 0.05 as compared to T3.

Table 3 Correlation studies of caecum-cholesterol-concentration and hs-CRP levels with other biochemical markers.

<table>
<thead>
<tr>
<th>hs-CRP levels</th>
<th>Blood Glucose Level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
</tr>
<tr>
<td>Caecum-cholesterol-concentration</td>
<td>-0.979</td>
</tr>
<tr>
<td>Blood Glucose Level</td>
<td>0.988</td>
</tr>
</tbody>
</table>

Vitamin D can also improve the composition and function of intestinal microbiota, reduce pathogenic bacteria, and increase bacterial diversity. Another study in Rotterdam shows that Vitamin D deficiency is prevalent in overweight and obese children and adolescents (Zakharaova et al., 2019). Additionally, the higher vitamin D levels are associated with the lower of CRP-levels in T2DM-patients (Liefard et al., 2015). However, the study has not proved a causal relationship between vitamin D and CRP-level. The recent study used interventions of vitamin D3 and didelph-forfified-vitamin-D3 was strengthening the role of vitamin D3 to lessen CRP-level.

The Spearman test on all data from T2DM rats at the end of the study showed that a very strong positive correlation was observed between hs-CRP levels and blood glucose levels (Table 3), while a very strong negative correlation was found between caecum-cholesterol-concentration either with hs-CRP levels or blood glucose levels.

A very strong negative correlations was observed between caecum-cholesterol-concentration and hs-CRP-levels (r = -0.979, p = 0.0001, Table 3). Caecum cholesterol is the cholesterol that is secreted by the body and will be excreted with fecal. An increased concentration of cholesterol excreted from the body will reduce the bile acids returning to the hepatic cycle, and subsequently, the cholesterol in blood circulation will be consumed for synthesizing new bile acids (Kumar et al., 2012; Kusuma et al., 2015). The very strong inverse relation between caecum cholesterol and hs-CRP levels may be mediated by the decline circulating cholesterol levels which relate to the decline in inflammation.

The increase of fatty acids or triglycerides in adipose tissue will cause enlargement in adipose tissue and activate the HF-1 gene. This gene increases the expression of Jun kinase and IκB kinase and triggers phosphorylation of IκB and activates NFκB. It will trigger the expression of proinflammatory cytokines such as TNF-α and IL-6. This
increase of cytokines will trigger the liver to secrete CRP (Qatanani and Lazar, 2007). Plasma CRP levels are significantly correlated with high triglycerides and low HDL. CRP is synthesized in adipose tissue, the amount will increase in a state of obesity which will ultimately cause insulin resistance and diabetes (Devaraj, Singh and Jialal, 2009).

A very strong negative correlation was observed between caecum-cholesterol-concentration and blood-glucose-levels ($r = -0.988, p = 0.0001$). Glucose levels increase in groups that had low caecum-cholesterol-concentration. T2DM is associated with changes in the composition of intestinal microflora, namely an increase in the number of gram-negative bacteria and a decreased proportion of Firmicutes. The individuals with T2DM, therefore, have lipid metabolism abnormalities (Ejtahehd et al., 2011).

Lipid metabolic disorders caused by insulin resistance affect Hydroxymethyl Gluturate Coenzym-A (HMG-CoA) reductase and lipid metabolic pathways. Several studies have shown that insulin can affect the production of apolipoprotein in the liver and regulate the activity of lipase which causes dyslipidemia in diabetes mellitus (Ozder, 2014). Excessive accumulation of fat in adipose tissue causes macrophage infiltration and increases the production of proinflammatory cytokines which contributes to the development of atherosclerosis (Pei et al., 2017).

A very strong positive correlation was found between hs-CRP-levels and blood-glucose-levels ($r = 0.988, p = 0.0001$). This correlation showed that increase glucose levels relate to enhanced hs-CRP-levels. Hyperglycemia causes damage to all body tissues and affects the chronic inflammatory response such as hs-CRP. Also, hyperglycemia in cells causes damage to the mitochondria. It will increase Reactive Oxygen Species (ROS) so that the amount of free radicals increase in the body. Furthermore, hyperglycemia also increases the synthesis of diacylglycerides (DAG) which causes activation of Protein Kinase C (PKC) and ultimately changes the expression of various genes that will damage blood vessels. Increased activity of PKC leads to activation of NF-$\kappa$B and it induces various pro-inflammatory cytokines such as TNF-$\alpha$ and IL-6 which trigger the liver to produce CRP (Nowotny et al., 2015).

CONCLUSION

Dadih-fortified-vitamin-D$_3$-supplementation has a better effect than either dadih or vitamin-D$_3$ in decreasing blood-glucose and hs-CRP levels. Dadih-fortified-vitamin-D supplementation has better effect than vitamin-D$_3$ in increasing caecum-cholesterol-concentration T2DM-rat. Both hs-CRP andglucose levels have very strong inverse relationship with the caecum-cholesterol-concentrations.

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