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The Effect of Vitamin D3-Fortified Goat Milk Kefir on hs-CRP Levels of Type 2 Diabetic Rattus Norvegicus Rats: An Experimental Study

Pengaruh Kefir Susu Kambing Terfortifikasi Vitamin D3 terhadap Kadar hs-CRP Tikus Rattus Norvegicus DM Tipe 2: Studi Eksperimental

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Kefir, Goat Milk, Vitamin D3 Fortification, hs-CRP, Diabetes Mellitus

ABSTRACT

Background: Insulin resistance and chronic hyperglycemia in diabetes are linked to the synthesis of the inflammatory biomarker hs-CRP. Goat milk kefir and vitamin D have anti-diabetic and anti-inflammatory properties.
Objectives: Aimed to evaluate the impact of goat milk kefir fortified with vitamin D_3 on hs-CRP levels of diabetic rats.
Methods: Twenty-one male Rattus norvegicus rats were randomly divided into four groups for a 35-day study: Control (C), Diabetes Mellitus (DM), unfortified goat milk kefir treatment (P1), and vitamin D3-enriched goat milk kefir treatment (P2). Diabetes was induced via intraperitoneal injection of streptozotocin (STZ) at 65 mg/kg BW and nicotinamide (NA) at 230 mg/kg BW. Goat milk kefir was administered orally at 2 mL/200 g BW/day, with the fortified version containing 600 IU of vitamin D3 per 100 mL. Fasting blood glucose levels and serum hs-CRP were measured pre- and post-intervention using the GOD-PAP and ELISA methods, respectively. Data were analyzed using bivariate and multivariate methods in GraphPad Prism 8.
Results: There was a statistically insignificant decrease in hs-CRP levels in the P2 group (p-value=0.21) and in the P1 group (p-value=0.63), suggesting limited impact on inflammation. However, there was a statistically significant drop in blood glucose levels in the P2 group (ΔFBG -65.50±35.44 mg/dL, p-value=0.03) and in the P1 group (ΔFBG -81.63±50.07 mg/dL, p-value=0.05).
Conclusions: The reduction in hs-CRP levels indicates that vitamin D3-fortified kefir may help modulate low-grade inflammation and shows promise in managing diabetes. Future research should examine dose, duration, and sample size for better efficacy assessment.

INTRODUCTION

Diabetes Mellitus (DM) has become a serious global health emergency. Around 90% of worldwide diabetic people have type 2 diabetes, a chronic metabolic disease caused by insulin resistance and characterized by hyperglycemia condition as a result[1]. According to World Health Organization (WHO) report data in 2016, the number of global diabetic adults (aged 18 and above) increased by just under four times, from approximately 108 million in 1980 to 422 million in 2014, and is projected to grow up to 463 million with diabetes in 2019[1,2]. Meanwhile, the prevalence of diabetes mellitus in the population aged 20-79 years in Indonesia in 2019 is estimated to reach 10.7 million people and will continue to increase to 16.9 million people in 2045, making Indonesia one of the top 10 countries in the world with the highest diabetes rates[1].

The onset of type 2 diabetes mellitus is a complicated process influenced by various factors, and many aspects remain to be thoroughly studied. Nevertheless, it is clear that there is a strong relationship between obesity, inactivity, and a heightened risk of insulin resistance and type 2 diabetes. This increased risk primarily stems from stress-related inflammation in adipose tissue, ultimately resulting in the insensitivity of the insulin receptor[3]. Type 2 diabetes mellitus is associated with several serious complications, including cardiovascular diseases and a risk of death[4]. These

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complications are tied to hyperglycemia and insulin resistance in individuals with unmanaged type 2 diabetes. Hyperglycemia is a clinical symptom resulting from pancreatic β-cell destruction, interfering with the production of the hormone insulin and increasing blood glucose levels[5]. The combination of insulin resistance and prolonged hyperglycemia fosters inflammation, triggering the release of pro-inflammatory cytokines such as interleukin (IL) 1 and 6, along with Tumor Necrosis Factor-α (TNF-α). This, in turn, stimulates the synthesis of C-Reactive Protein (CRP) in hepatocyte cells, serving as an inflammation biomarker [6,7]. Increasing hs-CRP level as an inflammation marker indicates an acute phase response[8]. Typically, individuals at risk of diabetes complications or experiencing low-grade chronic inflammation have lower levels of CRP. Standard CRP tests may not detect this, necessitating the use of a high-sensitivity C-reactive protein (hs-CRP) test to assess the likelihood of cardiovascular complications[9]. Previous studies have identified a strong link between hs-CRP levels and type 2 diabetes mellitus[10]. In another study, the identification of hs-CRP as a more potent prognostic biomarker for all-cause mortality than for cardiovascular diseases may enhance the early detection and prevention of fatal diseases, extending beyond cardiovascular diseases in early type 2 diabetes mellitus[11].

Previous studies have shown that T2DM and T2DM with complications are associated with vitamin D deficiency[12,13]. In a 5-year follow-up investigation involving the Asian population, it was observed that the occurrence of type 2 diabetes was markedly higher in the vitamin D deficiency group compared to both the vitamin D insufficiency group and the vitamin D sufficient group. The positive impacts of administering vitamin D on measures of insulin production and resistance, lipid profiles, and indicators of inflammation and oxidative stress may result from its influence on activating insulin receptor expression, suppressing cytokine generation, and reducing parathyroid hormone production[14]. The potential of vitamin D to impact glycemic control and the risk of type 2 diabetes is believed to stem primarily from its anti-inflammatory properties[15]. Vitamin D plays a pivotal role in improving glucose tolerance and acts as an anti-inflammatory agent, concurrently reducing hs-CRP levels in individuals with Type 2 Diabetes Mellitus (T2DM)[16]. It suppresses the expression of pro-inflammatory cytokines associated with insulin resistance and reduces nuclear factor kappa B (NF-κB), thereby inhibiting the secretion of hs-CRP in hepatocytes[17,18]. The inverse relationship between High-sensitivity C-Reactive Protein (hs-CRP) as an inflammation biomarker and vitamin D is influenced by its impact on parathyroid hormone levels, potentially resulting in reduced concentrations of hs-CRP. A one month of vitamin D3 supplementation improved vitamin D status and significantly reduced pro-inflammatory cytokines without altering serum Ca^{2+}, PO_{2-}, or lipid levels of healthy Saudi subjects[19]. Research involving children with vitamin D levels below 15 ng/mL indicated a strong association with increased CRP levels. A six-month regimen of vitamin D supplementation on obesity-related chronic inflammation subjects was observed to significantly reduce CRP levels. Vitamin D primarily exerts its anti-inflammatory effects by decreasing CRP levels and helping to maintain stable concentrations of IL-10[20]. A randomized controlled study found that vitamin D3 fortification in milk and water is more absorbable compared to other foods, such as juice[21]. Furthermore, another study on the intervention in T2DM rats for 28 days with fermented buffalo milk fortified with vitamin D3 showed significant benefits in reducing hs-CRP levels[22]. Goat milk offers potential anti-inflammatory benefits due to its oligosaccharides, which help prevent hepatocyte cell damage that can stimulate the secretion of CRP as part of the acute phase response through inflammatory cytokines[23]. Research has indicated that goat milk contains more oligosaccharides than bovine or sheep milk[24]. It also includes Medium-Chain Triglycerides (MCT) and short-chain fatty acids, both of which are easily digestible and metabolized[25]. Furthermore, goat milk has a lower lactose content than cow's milk, making it a suitable alternative for individuals with lactose intolerance[26,27]. However, it is important to note that goat milk contains less vitamin B_6, C, and D compared to other types of milk[26,28].

A recent meta-analysis has demonstrated that kefir, a fermented dairy product, has the potential to significantly reduce levels of CRP. This effect appears to be particularly pronounced in Asian populations and among individuals diagnosed with metabolic syndrome[29]. In research involving patients with Crohn's disease, Yilmaz et al. discovered that regular intake of kefir can alleviate symptoms and improve short-term quality of life, while also favorably affecting biochemical markers such as hemoglobin, CRP, and erythrocyte sedimentation rate[30]. As a functional food containing bacteria, kefir could suppress the pro-inflammatory cytokine secretion[31]. Kefir is a fermented milk product that adds kefir grains containing Lactic acid bacteria (LAB) and yeast to the milk[32]. Enriched with yeast and specific bacteria strains absent in yogurt, including *Lactobacillus helveticus*, *Lactobacillus kefiri*, *Lactococcus lactis*, *Lactobacillus kefiranofaciens*, *Leuconostoc mesenteroides*, *Acetobacter ghanensis, Leuconostoc pseudomesenteroides*, and *Acetobacter orientalis*, kefir holds the potential to offer a greater variety of probiotic bacteria for the gastrointestinal tract compared to yogurt[33,34]. Kefir could be a therapy for people with diabetes because it can lower fasting blood glucose and HbA1C levels[35]. A study about the effects of kefir combination from goat and soy milk with a 2 mL/200 g body weight dose in rat-induced diabetes by administration of streptozotocin (STZ) and nicotinamide (NA) has shown a decreased level of fasting blood glucose after 35 days of intervention[36]. Conversely, the probiotics present in kefir have the potential to regulate gut microflora, influencing the control of blood glucose and lipid levels[28,37]. Additionally, they may inhibit the release of pro-inflammatory cytokines by modulating immune and anti-inflammatory systems, thereby enhancing the production of IL-10 and TGF-β. This is essential for reducing CRP levels[38,39].

It is necessary to fortify vitamin D3 on goat milk kefir to increase its nutritional content because the vitamin D content in goat milk is low[28]. Based on the previous study, the optimal dose of vitamin D_3 solubility

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is 600 IU[40]. The Recommended Dietary Allowance (RDA) and The Indonesian Dietary Recommendation (AKG) 2019 also recommend that the average daily dose of vitamin D_3 for adults between the ages of 19 and 70 is 15 mcg (600 IU)[41]. Thus, the present study was conducted to investigate the effects of goat milk kefir fortified with 600 IU of vitamin D_3 on hs-CRP levels of *Rattus norvegicus* diabetes mellitus rats.

METHODS

A true experimental study was conducted using a pre-post control group design from December 2019 to January 2020. The in vivo experiments took place at the Animal Trial Laboratory at the Faculty of Medicine, Universitas Diponegoro. Fasting blood glucose was assessed at the Regional Health Laboratory in Semarang City, Central Java Province. Meanwhile, hs-CRP serum levels were evaluated at the GAKI Laboratory of Diponegoro National Hospital, and vitamin D3-fortified goat milk kefir was prepared at the Center for Nutrition Research Laboratory of Universitas Diponegoro. In this study, hs-CRP levels served as the dependent variable, while the administration of vitamin D3-fortified goat milk kefir was the independent variable. Control variables included the strain of the subjects (*Rattus norvegicus*), and factors such as age, sex, body weight, diet, handling practices, housing environment, and the hygiene and sanitation of the cages.

Samples of this study were male rats (*Rattus norvegicus*) Wistar strain from Farhan Farm Mouse, Semarang. Rats were selected due to their physiological similarities to humans, adaptability, ease of handling, docility, omnivorous nature, resilience to intervention without impacting estrogen hormones, rapid metabolic capabilities, low susceptibility to vomiting, ease of control, and the potential to minimize bias. Additionally, serum retrieval through the retro-orbital plexus is relatively straightforward in rats[42]. Inclusion criteria encompassed male Wistar strain rats (Rattus norvegicus) aged 8 to 12 weeks, with a body weight ranging from 180 to 200 g. Criteria included overall health, normal behavior, and activities, absence of defects or complicating conditions, and a fasting blood glucose level exceeding 140 mg/dL. Additionally, samples meeting exclusion criteria involved those with a body weight dropping below 180 g during the experiment, mortality, or noticeable changes in behavior such as weakness and/or loss of appetite.

The sample size was counted using the Federer formula (1997), with a 10% dropout, resulting in 28 samples and seven samples in each group. Initially, subjects were placed in the individual cage for seven days at a temperature of 22 – 25° with humidity of 40- 70%, 12:12 light-dark cycle, and given the diets with BR-2 and ad libitum feeding. The cages were cleaned daily, and the subjects' body weight was recorded weekly. Following the adaptation period, the samples were randomly assigned to four groups, i.e., control (C), diabetes mellitus (DM), diabetes treated with unfortified goat milk kefir (P1), and diabetes treated with vitamin D3-fortified kefir (P2), with seven rats in each group. The technique used was a simple randomized method, with each cage numbered from 1 to 28. The selection process involved a lottery organized in the order of the control, DM, P1, and P2 groups. Samples in the DM, P1, and P2 groups had been fasted overnight before diabetes-induced by injection of 230 mg/kg Body Weight (BW) of nicotinamide (NA), which was prepared in a 0.9% sodium chloride solution. After a 15-minute interval, the samples received an injection of 65 mg/kg BW of streptozotocin (STZ) in a 0.1M sodium citrate buffer, adjusted to a pH of 4.5. This particular dosage combination has been standardized in a mouse model of type 2 diabetes and is designed to mimic the progression of T2DM in humans. The resulting condition leads to higher blood glucose levels and a more stable diabetic state, which makes it suitable for testing the long-term therapeutic effects of kefir feeding in this study[43]. Fortification dose of vitamin D_3 was 600 IU/100 mL kefir based on the previous study[40]. Samples (C, DM, P1, and P2 groups) had been fasted overnight before fasting blood glucose and hs-CRP levels pre-intervention measurement from blood samples which were taken via retro-orbital plexus using hematocrit tubes as much as around 3 mL fifteen days after diabetes induction. The same technique was also used to get the post-test serum samples. Samples were diabetic if fasting blood glucose levels had reached >140 mg/dL[44].

Subsequently, after meeting the inclusion criteria, subjects were placed in the individual cage for 35 days at a temperature of 22 - 25ºC with humidity of 40-70%, 12:12 light-dark cycle. Samples in control and DM groups (C and DM) were only fed a standard diet (BR-2) and water ad libitum. At the same time, the P1 group was given a standard diet and unfortified goat milk kefir, and the P2 group was given a standard diet and vitamin D_3-fortified goat milk kefir, each at a dose of 2 mL/200 gBW/day for 35 days, based on previous studies. Fasting blood glucose and hs-CRP serum levels were measured following a 35-day intervention. The fasting blood glucose was assessed using Glucose Oxidase-Peroxidase Aminoantipyrine (GOD-PAP) method, with measurements taken at a wavelength of 500 nm. The GOD-POD method works linearly up to 500 mg/dl. It has good precision, with a coefficient of variation (CV) of 0.7% to 1.4%, and an average deviation of −0.97%. It is a straightforward, quick, cost-effective, and sensitive technique for routine colourimeters[45]. The assessment of hs-CRP levels was using a colourimetric rat hs-CRP sandwich by Enzyme-Linked Immunosorbent Assay (ELISA) method with sample serum and was read using spectrophotometry at a wavelength of 450 nm±2 nm. The ELISA technique detects low levels of High-sensitivity C-Reactive Protein (hs-CRP) with a sensitivity of 0.094 ng/mL and a range of 0.156 to 10 ng/mL[46]. It shows high precision (CVs below 10%) and recovery rates of 80% to 120%[47]. The method is validated for hs-CRP quantification in rodent serum and plasma, with minimal cross-reactivity with other proteins.

The collected data were pre- and post-test data of body weight, fasting blood glucose levels, and hs-CRP serum levels. The data were then analyzed using GraphPad Prism 8. Shapiro-Wilk test was used to analyze data normality. Differences between pre-test and post-test fasting blood glucose levels and serum hs-CRP were tested bivariate with a paired t-test if data were normally distributed and a Wilcoxon test if data were not normally

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distributed. Moreover, differences in all groups were analyzed using One-Way ANOVA or the Kruskal-Wallis test, depending on the normality distribution of the data (95% CI and p-value of 0.05). This study obtained an ethical clearance No.04/EC/H/FK-UNDIP/I/2020 (dated January 20, 2025) from the Health Research Ethics Commission (KEPK) of the Faculty of Medicine, Universitas Diponegoro (dr. Kariadi Regional Hospital, Semarang City, Central Java Province, Indonesia).

RESULTS AND DISCUSSIONS

Subjects' Initial Characteristics

According to Table 1, after diabetes induction to subjects in DM, P1, and P2 groups, it can be seen that there was a normal distribution of body weight before intervention and insignificant differences among groups (p-value=0.20). Samples in the control group had normal fasting blood glucose levels before intervention (96.98 mg/dL) since STZ-NA was not delivered. Meanwhile, the figure in diabetes groups (DM, P1, and P2) had reached >140 mg/dL, and the mean±SD of the P1 group was the highest (196.0±46.63 mg/dL). There was a significant difference between the control group and DM, P1, and P2 groups (p-value=<0.001). In addition, hs-CRP serum levels of the control samples group reached the highest among all of the groups (mean±SD=2.48±0.542), although they were not in the diabetes group, and the hs-CRP serum level of all of the samples groups was still in a normal range (<3.0 mg/L)[48]. The post-hoc test showed an insignificant difference among all groups (p-value 0.6040).

Table 1. Baseline Characteristics of Samples

Indicators	Groups				p-value
	Control (C)	Diabetes Mellitus (DM)	Diabetes with unfortified goat milk kefir treatment (P1)	Diabetes with vitamin D_3-fortified goat milk kefir treatment (P2)	
Body Weight (g)	276.8± -23.20	266.1±29.00	255.8±19.29	291.8±18.84	0.202
Fasting Blood Glucose (mg/dL)	96.98±19.27[a]	155.2±16.34[b]	196.0±46.63[b]	181.8±40.32[b]	<0.001*
hs-CRP serum (mg/dL)	2.48±0.524	2.09±0.575	1.88±1.13	2.20±0.66	0.604

Value is shown as Mean±SD; *p-value<0.05; analyzed using One-Way ANOVA. Different notations (a, b) in the same row indicate significant differences found in the post-hoc test

Body Weight Characteristics after Intervention

Turning to the body weight, as detailed in Figure 1, all groups showed an increase during the intervention, but it was not statistically significant (p-value ΔBW=0.89). In contrast, samples in the P1 group reached the highest of approximately 25.75 g (ΔBW of P1). The One-Way ANOVA test after intervention shows a significant increase among groups (p-value=0.04), in which there was a significant difference between the DM group and the P2 group based on the post-hoc test using Tukey HSD (p-value=0.04).

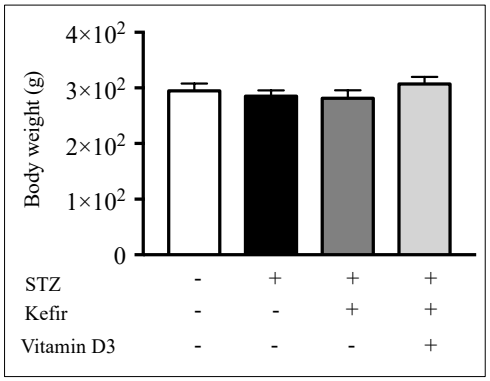

Figure 1. Body weight characteristic of the subjects after intervention

Our findings indicate that all groups gained weight during the intervention, although this increase was not statistically significant, as illustrated in Figure 1. This observation corroborates previous research indicating that a 35-day kefir intervention positively affected body weight in diabetic rats subjected to streptozotocin (STZ) and nicotinamide (NA) induction[49]. Specifically, administering STZ at 65 mg/kg body weight and NA at 230 mg/kg body weight aimed to a non-obese type 2 diabetic condition[43]. One possible explanation for the observed weight gain in diabetic rats is the phenomenon of polyphagia, a common symptom of diabetes, which results in heightened food intake[50,51]. Behavioral assessments during the study indicated that diabetic rats exhibited alterations in dietary habits following diagnosis. These modifications, combined with restricted physical activity due to individual confinement in cages, may have contributed to an energy imbalance,

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subsequently leading to weight gain. This underscores the significance of modifiable risk factors, such as dietary habits and physical activity, in the progression of type 2 diabetes[52]. In contrast, the weight gain observed in the control, P1, and P2 groups may be attributed to normal glucose homeostasis. The P1 and P2 groups, in particular, demonstrated improvements in glucose regulation, as evidenced by a reduction in fasting blood glucose levels. This suggests that the combined effects of kefir and vitamin D3 supplementation may contribute to enhanced metabolic health, potentially improving energy balance and preventing excessive weight loss commonly seen in diabetic conditions.

Overall, the significant increase in body weight observed in the P2 group compared to the DM group underscores the potential benefits of vitamin D3-fortified kefir in mitigating weight loss associated with diabetes. These findings warrant further investigation into the mechanisms underlying the effects of fortified kefir on body weight and metabolic regulation.

The significant increase in body weight observed in the P2 group compared to the DM group highlights the potential of vitamin D3-fortified kefir in reducing diabetes-related weight loss. This suggests that fortified kefir may help support weight maintenance in diabetic conditions. Further research is needed to explore the underlying mechanisms involved. Understanding these mechanisms could offer insights into its role in metabolic regulation.

Fasting Blood Glucose Characteristics after Intervention

Regarding Fasting Blood Glucose (FBG) levels as detailed in Figure 2, it can be seen that the control and DM group increased by about 16.20 mg/dL and 52.96 mg/dL, respectively, from 96.98±19.27 mg/dL and 155.2±16.34 mg/dL in pre-intervention phase to 113.2±8.592 mg/dL and 208.1±103.2 mg/dL successively after 35 days (ΔFBG), but not statistically significant (p-value=0.07 and 0.47 successively). Administration of streptozotocin (STZ) 65 mg/kgBW and 230 mg/kgBW nicotinamide (NA) caused pancreatic cell apoptosis, leading to relative insulin deficiency and consequently increasing fasting blood glucose levels [53]. Those mechanisms may occur in the DM group, where the FBG levels keep rising until the intervention phase's end, although it was not statistically significant. The administration of a high dose of NA was postulated to confer enhanced protection against pancreatic β-cell damage induced by STZ injection[43]. In contrast to our study, an increase in fasting blood glucose levels in DM control rats over a period of 35 days in Nurliyani's study were resulted from the diabetes induction using an STZ dose of 45 mg/kgBW and NA 120 mg/kgBW[36].

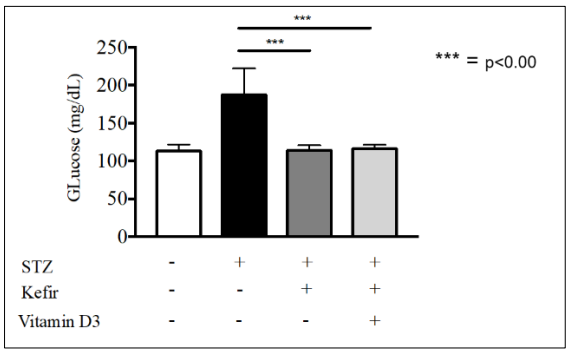

Figure 2. Subjects' glucose levels after intervention

On the other hand, samples in the P1 and the P2 group experienced a statistically significant decrease by about ΔP1 -81.63 g/dL and ΔP2 -65.50 mg/dL, respectively, from approximately 196.0±46.63 mg/dL and 181.8±40.32 mg/dL (diabetes condition) to 11.4.4±6.183 mg/dL and 116.3±5.123 mg/dL successively (normal condition) after 35 days of intervention (p-value=0.05 and 0.03 serially). Additionally, there was a statistically significant difference in ΔFBG between the DM group and the P1 group (p-value=0.001) and between the DM and the P2 group (p-value=0.01). Moreover, turning to the after-intervention data, the Kruskal-Wallis test showed a significant difference in FBG levels among groups after 35 days of intervention (p-value=0.001). Dunn's Multiple Comparison post-hoc test showed a significant difference between the control group and the DM group (p-value=0.01) and between the DM and the P1 group (p-value=0.03). Our findings align with those of Alsayadi et al., who demonstrated that a 35-day water kefir intervention resulted in significant differences in fasting blood glucose reduction between the diabetic control group and the kefir treatment group. However, no significant differences were observed among groups receiving kefir at various concentrations[49].

Previous studies have shown that a chronic increase in FBG levels initiated free radical growth, elevating oxidative stress and inflammation[54,55]. Probiotic bacteria stimulate glycogen absorption in the liver and raise the antioxidant status to reduce free radicals. Probiotics in kefir have been identified as beneficial agents for enhancing antioxidant capacity and improving insulin secretion, which aids in glycemic control. These probiotics reduce Malondialdehyde (MDA) levels, a marker of lipid peroxidation, and suppress pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α[56]. This mechanism is supported by Lee and Kang[57], indicating that probiotics like Lactobacillus and Bifidobacterium can suppress MDA levels by 0.61 to 0.68 times. By lowering oxidative stress and inflammation, they protect pancreatic β-cells from further damage and allow for partial regeneration, improving insulin secretion[35,58]. In addition, probiotic bacteria effectively

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reduce glucose absorption in the intestinal tract by producing a Glucose-dependent Insulinotropic Polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), which induces glucose absorption in muscle cells[35]. This effect is supported by a previous study which found that consuming 1 mL of kefir daily for 10 weeks improved glucose absorption in muscle cells, which contributed to a reduction in insulin resistance[59]. Bioactive components of exopolysaccharides (EPS) produced by lactic acid bacteria of kefir affect insulin release, glucose homeostasis, and suppress glucose toxicity via adenosine monophosphate (cAMP), protein kinase A activation, and Ca^{2+} ion sensitivity cycle, resulting in glucose levels reduction[60,61].

Our findings showed that vitamin D_3-fortified kefir had a slightly more significant effect on decreasing the hyperglycemia condition of the P2 samples (p-value=0.03) compared to samples in the P1 group with kefir without fortification (p-value=0.05). Rats in the P2 group were given vitamin D3-fortified goat milk kefir at a dose of 600 IU/100 mL kefir (15µg/100mL), and for every 200 g of body weight, received 2 mL fortified kefir. This fortification dose of vitamin D_3 matched a previous study, as well as the results of our study have similarities to prior studies[62,63]. A systematic review and meta-analysis study has shown that vitamin D has more practical effects on improving serum 25(OH)D and reducing insulin resistance if given in large doses and for a short period to patients with vitamin D deficient, non-obese patients, or patients with a baseline of optimal glycemic control[64]. Vitamin D_3 has played an indirect role in the normalization of calcium extracellular to ensure calcium can enter through cell membranes and mediated activation of β-cell-dependent calcium endopeptidase, cleaving proinsulin to form insulin, and elevating glucose transport to the muscle cells[17,65]. According to Mitrašinović-Brulić et al., administering STZ led to elevated levels of MDA, which were associated with heightened oxidative stress. Treatment with Vitamin D_3 notably decreased MDA levels, indicating that Vitamin D_3 may have a beneficial impact in protecting cell membranes from oxidative damage[66]. Besides, vitamin D_3 has directly stimulated insulin receptor expression in the peripheral tissue to increase insulin activity in glucose transport[67].

In conclusion, the observed reductions in glucose levels in this study are likely due to the combined effects of kefir's probiotics and vitamin D3's role in regulating insulin secretion and glucose metabolism. This synergy highlights the therapeutic potential of vitamin D3-fortified kefir. Overall, it may serve as an effective intervention for managing hyperglycemia in the future research.

Hs-CRP Levels Characteristics after Intervention

According to hs-CRP levels depicted in Figure 3, all groups decreased slightly from the beginning to the end of the given intervention period. The DM group had the lowest levels, experiencing a statistically significant decrease (p-value=0.02) by about -1.00±0.79 mg/dL (Δhs-CRP mean±SD), from around 2.09±0.58 mg/dL to 1.09±0.59 mg/dL. The Kruskal-Wallis test showed no significant difference in hs-CRP levels after intervention among groups (p-value=0.20). Despite having no significant difference, it showed that vitamin D_3-fortified goat milk kefir decreased more hs-CRP levels of samples in the P2 group (p-value=0.21) than samples in the P1 group with unfortified goat milk kefir (p-value=0.63).

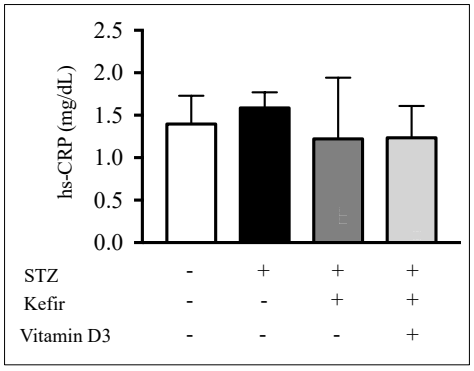

Figure 3. hs-CRP levels after intervention

Figures 2 and 3 depict that hs-CRP levels in the P1 and P2 groups decreased when fasting blood glucose levels declined to normal. The improvement in this condition was likely due to goat milk kefir, both fortified (P2) and without fortification (P1), which is in line with a previous study[68]. Additionally, the fortification with vitamin D3 may contribute to lowering hs-CRP levels. This is supported by studies that found a positive correlation between reductions in hs-CRP and blood glucose levels in rats that were fed dadih fortified with 900 IU of vitamin D3 at a dosage of 4 g per 200 g of body weight daily for 28 days. In contrast to the dose used in our study, this study induced diabetes with an STZ dose of 45 mg/kgBW and NA 110 mg/kgBW[22]. However, our findings differ from those of that study, which reported a significant decrease in hs-CRP levels. It is important to note that insulin resistance stimulates inflammation, which increases pro-inflammatory cytokines that lead to the production of CRP in the liver[6,69]. Oligosaccharides contained in goat milk have a beneficial effect on preventing inflammation[70]. On the other hand, kefir is a natural probiotic containing lactic acid bacteria that modulates the immune system to produce anti-inflammatory cytokines such as IL-10 and reduced IL-6, TNF-α, IFN-γ, and hs-CRP levels[71-73]. Research conducted by Wisudanti in 2017 demonstrated a significant increase

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in IL-10 in subjects who consumed kefir at concentrations of 0.5%, 1%, 2%, and 5% over four days[74]. In the innate immunity system, probiotics kefir play an essential role as a ligand for toll-like receptors (TLRs), which influenced signalling pathways of NF-κb, mitogen-activated protein kinase (MAPK), phosphoinositide-3-kinase-protein kinase B/Akt (PI3K-PKB/Akt), dan peroxisome proliferator-activated receptor γ (PPAR-γ), thus downregulated pro-inflammatory cytokines expression (IL-12, IL-6, and TNF-α) which trigger the liver to produce CRP[75,76].

Declining hs-CRP levels of the P1 and P2 groups in this study were insignificant. That may be caused by the protective effect of nicotinamide 230 mg/kg BW induction causing moderate diabetes so that pro-inflammatory cytokines such as TNF-α and IL-1β remained under detectable value[77]. In contrast, a previous study has shown that hs-CRP levels in men and women who had just been diagnosed with diabetes for about six months went up to >3.0 mg/L[18]. Research conducted by Medany et al[78], also indicated that administering STZ at a dose of 65 mg/kg body weight along with nicotinamide at 230 mg/kg body weight resulted in elevated fasting blood glucose levels and hs-CRP in rats from the diabetes control group compared to those in the healthy control group after eight weeks. Furthermore, the dapagliflozin supplementation, administered over eight weeks, demonstrated a significant decrease in serum fasting blood glucose levels compared to the diabetes control group. However, there was no significant difference in the reduction of hs-CRP levels between the two groups. A notable reduction in hs-CRP levels was observed in the group receiving a combination of atorvastatin and dapagliflozin when compared to the diabetes control group and those who received only dapagliflozin. This suggests that the combined interventions aimed at improving glucose and lipid profiles had a more pronounced effect on reducing hs-CRP levels. Conversely, another previous study showed similar results as ours, where hs-CRP levels in rats induced diabetes with STZ-NA without treatment showed a slight increase to around<2 mg/L after 28 days of study[79].

According to hs-CRP levels after intervention in Figure 3, our findings show that the P2 group experienced more decline (p-value=0.21) than the P1 group (p-value=0.63), although it was insignificant. It was probably associated with vitamin D_3-fortification goat milk kefir treatment in the P2 samples. This study proves that vitamin D_3-fortified goat milk kefir can decrease hs-CRP levels after 35 days of intervention, although it was not statistically significant. These results were consistent with other studies indicating that vitamin D_3 did not have a significant effect on hs-CRP levels in patients with chronic kidney disease (CKD)[80]. Additionally, previous research has demonstrated that kefir supplementation over a 3-week period did not lead to a reduction in hs-CRP levels[81]. Another study suggested that kefir may have a decreasing effect on hs-CRP levels when compared to unfermented milk after 12 weeks. However, this reduction was found to be statistically insignificant.[82] It may be because the hs-CRP level in those groups before intervention was not really high (<5 mg/L), therefore it could not show the maximum benefit of vitamin D_3 fortification[83]. These findings were in agreement with results from a study by Qinna NA, which revealed that total antioxidant status, inflammatory biomarkers, and other complete blood count parameters (excluding HCT) were not significantly changed in STZ-NA-induced rats after 42 days[84]. Hs-CRP levels of 2 mg/L, on the other hand, are linked to an increased risk of cardiovascular events and mortality[4].

On the other hand, an insignificant decrease in the P2 subjects' hs-CRP levels was likely due to renal toxicity caused by STZ administration affecting vitamin D_3 activation, inhibiting the maximum effect of vitamin D_3-fortified goat milk kefir treatment[53,85]. Vitamin D_3 regulated calcium binding vitamin D-dependent protein called Calbindin-D_{28k}, which has played a crucial role in inhibiting free radical formation to protect the β-cell from cytokine-mediated death[86,87]. The Calbindin-D_{28k} can also affect insulin release via calcium regulation in the β-cell pancreas[88]. Vitamin D_3 also has an anti-inflammatory effect through inhibition of NF-κB via the interaction mechanism vitamin D receptor (VDR)-inhibitor NF-κB (Iκb-α), which blocked the formation of TNFα-induced Iκb kinase (IκK) complex and, therefore, voided Iκb-α phosphorylation[89]. Another prior study has shown that vitamin D treatment can enhance VDR activity[90]. A plunge in hs-CRP levels of the DM subjects (p-value=0.02) in this study may be because subjects have higher levels of endogenous antioxidant status than other groups[91]. The obesity and diabetes status in the DM group and the treatment groups (P1 and P2) may also be impaired TLR-4 pathway activation, decreasing pro-inflammatory cytokine production such as IL-1β, IL-6, TNF-α, influencing CRP secretion[92]. However, this study did not assess endogenous antioxidant status and vitamin D levels.

Our study has several limitations that could introduce bias in the results. We conducted experiments with 21 male Wistar rats divided into four groups (Control, DM, P1, P2). The small number of rats in each group (n=4-7) limits the statistical power and may miss subtle but significant effects. Involving male rats minimizes hormonal variability but excludes the potential impact of sex-related biological differences on inflammation and glucose metabolism, which introduces selection bias and affects the generalizability of our findings. We set specific criteria for diabetic rats to meet fasting blood glucose levels (>140 mg/dL) before intervention, but inherent variations in baseline glucose and hs-CRP levels could still sway outcomes, despite randomization efforts. Rats with substantial weight loss, behavioral changes, or those that died were excluded from the analysis. This exclusion may create survivorship bias, potentially underestimating the negative effects of diabetes or the treatments applied. While we maintained consistent cage conditions and diets, variations in handling could still influence stress and inflammatory responses. Lastly, using rats from a single supplier (Farhan Farm Mouse) raises concerns about genetic uniformity, which may limit the study's external validity.

CONCLUSIONS

In conclusion, the intervention with vitamin D3-fortified goat milk kefir of 2 mL/200 g BW (a dose of 600 IU/day of vitamin D_3) can reduce hs-CRP levels by about

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0.83 mg/L in male *Rattus norvegicus* type 2 diabetes mellitus rats, although not statistically significant (p-value=0.21). However, it significantly reduced approximately 65.50 mg/dL of fasting blood glucose levels (p-value=0.03). This implies that kefir can maintain normal blood glucose levels in diabetic rats, regardless of whether it is unfortified or fortified with vitamin D3.

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AUTHOR CONTRIBUTIONS

FZ, BP: conceptor, investigation, methodology; BP, MA, AR: supervision, writing review and editing; BP, AF, YE: formal analysis; YE: formal analysis, resources; FZ, BP: writing original draft.

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