International Journal of Pharmacy and Pharmaceutical Sciences

Print ISSN: 2656-0097 | Online ISSN: 0975-1491 Vol 12, Issue 11, 2020

Original Article

FORTIFIED DADIH (FERMENTED BUFFALO MILK) WITH VITAMIN D3 IMPROVES INTERLEUKIN-6 AND CAECUM SHORT CHAIN FATTY ACIDS ON DIET-INDUCED OBESE RAT

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Received: 24 Jul 2020, Revised and Accepted: 02 Sep 2020

ABSTRACT

Objective: This study aims to determine the effect of fortified dadih with vitamin D3 on IL-6 expression level and the concentration of caecum SCFA in obese rats.

Methods: A total of 30 male *Sprague Dawley* rats were divided into five equal groups: healthy-control-(K-), obese-control-(K+), obese-intervention-(X1, X2, and X3). K(+), X1, X2, and X3 were in obesity conditions, which was induced by a high-fat sucrose diet (HFSD) and K(-) as a healthy-control-group. Furthermore, vitamin D3-fortified dadih at doses of 4 g/200 g-body-weight/d, dadih only at doses of 4 g/200 g-body-weight/d, and vitamin D3 only at 36 IU/200 g-body-weight/d was administered to X1, X2, and X3 groups, respectively.

Results: Treatment using fortified dadih with vitamin D3 showed significantly reduce weight gain ($p<0.05$) compare to K(+) and X2. In addition, X1 showed a decreased level of Interleukin-6 expression ($p<0.05$) than K(+), X2, and X3 groups but higher than K(-). Also, it showed the highest total SCFA, acetate, and propionate concentration ($p<0.05$). However, a moderately negative correlation was discovered between the pair of total SCFA and Interleukin-6 expression, acetate and Interleukin-6 expression, SCFA and body weight, propionate and body weight, butyrate and body weight. On the contrary, a strong positive correlation was observed between the pair of Interleukin-6 expression levels and body weight.

Conclusion: This study shows that fortified dadih with vitamin D3 from fermented foods improve the expression level of Interleukin-6 and increase the production of SCFA. Also, they improve intestinal homeostasis because of the increased SCFA production.

Keywords Obesity, Dadih, Vitamin D3, IL-6, SCFA

DOI: http://dx.doi.org/10.22159/ijpps.2020v12i11.39209. Journal homepage: https://innovareacademics.in/journals/index.php/ijpps.

INTRODUCTION

Obesity is a chronic metabolic disorder characterized by increased fat storage in the body [1, 2]. When energy consumption exceeds expenditure, 60-80% of the excess is stored as body fat [3]. Its development is not only a balance between energy intake and expenditure but also between white and brown adipose tissue in the form of thermogenesis through increased expression of mitochondria Uncoupling Protein 1 (UCP-1) [4]. At the developmental stage, enlargement of adipose tissue caused adipocyte hypertrophy [5], and this increased production of Tumour Necrotic Factor-Alpha (TNF-a), Interleukin-6 (IL-6) and Monocyte Chemotactic Protein-1 (MCP-1) [6].

Dadih is a traditional food from West Sumatera, Indonesia, and it is also known as fermented buffalo milk [7]. Some of the LAB that is usually found in dadih are *Lactobacillus Plantarum, Leuconostoc Mesenteroides, Streptococcus Faecalis, S. lactis subspecies lactis, S. cremoris, L. casei subspecies casei, and Lactobacillus casei subspecies, Rhamnosus, and Lactococcus* [7]. In addition, as a regulator of obesity, LAB helps to reduce fat absorptions, energy intake, the weight of white fat tissue, the average size of adipocytes, modulates intestinal microbiota, as well as inhibits the differentiation of 3TL-LI adipocytes, and the growth of pathogenic bacteria in the gastrointestinal tract [8–12]. Pathogenic bacteria within the gut stimulate production and secretion of Lipopolysaccharides (LPS) from intestinal epithelial cells, and they bind to cytokine receptors on hepatocytes and adipocytes to trigger the release of pro-inflammatory cytokines (TNFa, IL6, IL1b, and MCP1). However, probiotic therapy with LAB reduced the release of LPS from the intestinal epithelial cells, and it decreases the production of proinflammatory cytokines in adipose tissue [13].

Modulated intestinal microbiota with probiotics is a promising approach to improving host health since it is protected from infection and disease as well as produces vitamins and Short Chain Fatty Acids (SCFAs) [14, 15]. In the intestinal lining, it contributes to energy metabolism through the production of SCFAs, which are usually produced from the fermentation of fibers, proteins, and peptides [16]. The end products of this reaction are acetate, propionate, and butyrate. Cell culture studies showed that these products can change the function of adipose tissue by reducing the production of proinflammatory molecules and stimulating adipogenesis. In addition, oral administration of butyrate influences weight control through increased energy expenditure and fat oxidation in obese rats [17].

Vitamin D is an important regulator of immunity, composition and function of intestinal microbial, and metabolic processes in the body [18–20]. The deficiency of vitamin D leads to several diseases such as obesity, diabetes, cardiovascular disease, and metabolic syndrome [21]. Research showed a relationship between its deficiency and obesity since obese individuals tend to have a low 25 hydroxyvitamin D (25(OH)D) serum level [22–24]. Furthermore, the fortification of dadih with vitamin D3 is expected to increase its intake in obese people. The active form of vitamin D, 1,25-dihydroxyvitamin D3 (1,25(OH)2D3) inhibits IL-6 secretion by suppressing phosphorylation of Akt and signaling of Nuclear Factor Kappa B (NF-kB) [25]. Yogurt is a fermented milk product similar to dadih, and when fortified with vitamin D, it caused weight loss in obese patients with type 2 diabetes mellitus [26].

This study aimed to determine the effect of fortified dadih with vitamin D3 on IL-6 expression level and concentration of caecum SCFA in obese rats.

MATERIALS AND METHODS

Dadih preparation

100 ml buffalo milk was pasteurized at 72 °C for 15 s [27] and cooled until 30 °C, then vitamin D3 was added at a dose of 900 IU. Pasteurized milk was poured into a clean bamboo tube and then covered with banana leaves (fig. 1). The tube was placed at room temperature for two days. The dadih is stored at-18 °C (frozen) for future uses (fig. 2).

Fig. 1: Fortified dadih during spontaneous fermentation

Fig. 2: Fortified dadih after fermentation

Research design and experimental-animals

This research was a true-experiment study using a randomized post-test with control group design only. The animal used was male *Sprague Dawley* rats, aged 8–12 w, weighing 150–200 g. They were acclimatized at the laboratory of the Center for Food and Nutrition Studies at Gajah Mada University, Yogyakarta. In addition, they were placed in individual stainless-steel cages at regulated temperatures (21 °C) and housed in a cleaned and germ-free place. The experimental animals were fed with 20 g/d of the Comfeed II standard-diet (7% fat) during the non-HFSD and intervention period. Also, they received ad-libitum water during the experiment, and animal care was performed following the Guidelines of the Central Laboratory for Food and Nutrition Studies.

Thirty rats were divided into five groups, which were obese-control-(K+), obese-intervention-(X1, X2, and X3) and healthy-control-(K-)-groups. The obese-condition was induced by HFSD for 28 d, and after this period, the X1, X2, and X3-groups were treated daily with vitamin D3-fortified dadih at doses of 4 g/200 g-body-weight/d, dadih without fortification at doses of 4 g/200 g-body-weight/d, and vitamin D3 at doses of 36 IU/200 g-body-weight/d, respectively. The blood sample and caecum digesta were obtained at the end of the intervention. Furthermore, IL-6 level and SCFA concentration were analysed by ELISA (EliKine™, China) and gas chromatography, respectively. This study was approved by the Health Research Ethics Commission (KEPK) of the Faculty of Medicine Diponegoro University, Semarang through ethical clearance No. 129/EC/H/KEPK/FK-UNDIP/X/2019.

Serum IL-6 determination

The determination of serum IL-6 was performed using ELISA-Kit according to the manufacturer's instructions (EliKine™, China). Also, 100 µl of standard and serum of each sample were transferred into well plates, which were sealed with a cover and incubated at room temperature for 2 h. After incubation, the wells were aspirated and washed by filling them with washing buffer (250 µl), repeating the process twice for a total of three washes. 100 µl of diluted Mouse IL-6 detected antibodies within the well and incubated at room temperature for 1 h. Thereafter, the wells were aspirated and washed by filling them with washing buffer (250 µl), repeating the process twice for a total of three washes. Similarly, 100 µl of the working dilution of Streptavidin-HRP was added to each well. In addition, the plate was sealed with a cover and incubated at room temperature for 30 min in the dark, and the wells were aspirated and washed by filling them with washing buffer (250 µl), repeating the process twice for the total of three washes. 100 µl of HRP substrate (TMB) was also added and sealed with a cover. Furthermore, it was incubated at room temperature for 15 min in the dark. Finally, 50 µl of stop solution was added, and the color turned yellow immediately. The sample's absorbance was further read by ELISA Reader at 450 nm.

SCFA determination

The SCFA determination was performed using gas chromatography, and a total of 0.05 g of caecal content was acidified with 0.05 ml of sulphuric acid. Furthermore, SCFA was extracted with 0.6 ml of diethyl ether and centrifuged at 14000 r. p. m. for 30 s. One microliter of the organic phase was injected directly into the capillary column (Nukol) of the gas chromatography, which was combined with a flame ionization detector. The initial temperature was 80 °C while the final was 200 °C. Nitrogen was used as a carrier gas and sample quantification was performed using a calibration curve for acetic, propionic, and butyric acid. A standard curve was performed for the quantitation of acetic, propionic, and butyric acid in the sample [28]. The total SCFA is the amount of acetate, propionate, and butyrate [29].

Chemicals and reagents

Sulphuric acid and diethyl ether were purchased from Sigma-Aldrich Co, St Louis, MO, USA. Vitamin D3 and ELISA Kit were purchased from Wellness Inc., USA, and Abbkine Inc., Wuhan, Hubei, China, respectively.

Statistical analysis

The results were expressed as mean±SD (for normally distributed data), which was otherwise manifested as a median (min-max). The statistical, the difference was analyzed using One-Way Analysis of Variance (ANOVA) followed by post hoc Bonferroni for normally distributed data. In addition, the Kruskal Wallis test followed by Mann-Whitney-U-test was used (SPSS 16). Spearman's correlative test was used to analyze the correlation between variables, and statistical analyses were performed by a computer. Likewise, the differences and correlations were considered significant at p-value<0.05 as well as 95% confidence intervals, and its strength was determined by r value.

RESULTS AND DISCUSSION

Body weight

The processed data were obtained from 30 *Sprague Dawley*-rats, which were divided into each group consisting of 6. There was an increase in body weight during the HFSD period in groups K (+), X1, X2, and X3 (they had p = 0.001; table 1). Furthermore, the body weight in K (-) increased even though it only received a standard feed. The weight gain of those that received HFSD was higher than K (-) that received a standard feed. However, bodyweight increase during the HFSD period was caused by a higher proportion of fat and

sucrose. Therefore, K (+), X1, X2, and X3 groups have higher weight gain than K (-). The consumption of high-energy foods such as fat and sugar are the main factors that cause obesity [30]. Also, previous studies have suggested that HFSD increased body weight to 51% fat and 24% lean mass due to changes in composition [30].

After intervention of fortified dadih, dadih without fortification, and vitamin D3, K (+) gained more body weight compared to K (-), X1, X2, and X3 groups. A statistical difference was obtained on the pre-post-intervention change (Δ) of rat-BW across the groups (Kruskal Wallis test; p = 0.001) followed by a Mann Whitney U-test between two-groups. However, there was no significant difference between X1 and X3 groups compared with K (-) (p=0.545, p=0.789, table 1). In addition, it was reported that fortified dadih with vitamin D3 reduced body weight gain in obese rats since it contained LAB. Several studies have shown that LAB and vitamin D3 can reduce gain in body weight and epididymal fat mass [8, 31]. Similarly, research on the administration of *Lactobacillus Plantarum Strain Ln4* reduced weight gain in mice [32]. Vitamin D has been reported to have a link with obesity as some studies showed that its concentration is low in obese individuals [33, 34]. Furthermore, its deficiency causes a decreased amount of intestinal calcium and phosphorus absorption, which increases PTH levels [35]. This increase has been hypothesized as an independent predictor of obesity. One hypothesis suggested that high physiological levels of PTH will increase calcium influx to adipocytes. In addition, it leads to increased lipogenesis and reduced catecholamine-induced lipolysis as a mechanism to increase fat storage [36].

IL-6 expression level

The post-intervention-IL-6-levels was significantly different between the groups (p=0.000; table 1). Its levels of X1 was significantly different with K (+), X2 and X3 groups (p=0.000, p=0.000, and p=0.003, respectively; table 1), while the mean values were lower compared to X2 and X3. This result showed that fortified dadih with vitamin D3 reduced the production of proinflammatory cytokines compared to those without fortification and vitamin D3.

Obesity, which causes inflammation, is characterized by increased circulation of inflammatory cytokines and adipokines [37]. Various pro-inflammatory and anti-inflammatory factors such as adipokines, leptin, adiponectin, resistin, as well as cytokines and chemokines, such as TNF-α, IL-6, and MCP-1 are produced and released by adipocytes [38].

Lactic Acid (LA) as the main metabolite of LAB reduces the production of inflammatory cytokines. Furthermore, it decreases pro-inflammatory cytokines especially TNF-α and IL-6 by reducing phosphorylation of IκBα since it inhibits the activation of NF-κB [39].

As shown in several studies, some LABs similar to dadih may reduce the production of pro-inflammatory cytokines. Research on the administration of *L. curvatus HY7601* and *L. Plantarum KY1032*, which was obtained from the Korean traditional fermentation of cabbage reduced the expression of IL-6 gene in rat epididymal fat [13]. Administration of *Lactobacillus casei strain Shirota* supplements to the experimental animals also reduced its production [40].

An active metabolite of vitamin D, 1,25(OH)2D inhibits proinflammatory cytokine production (IL-1β, IL-6, IL-8, IL-12), reduces inflammatory activity in adipocytes and inflammation in visceral fat tissue [34]. In addition, it suppresses the ability of T cell and monocyte proliferation as well as its stimulation and decreases proinflammatory cytokines such as C Reactive Protein (CRP), TNFα, IL 6, IL-1, and IL-8, and increases anti-inflammatory types such as IL-10. This metabolite a ligand for the vitamin D receptor (VDR) [41], which inhibits NFkB activation and signaling [42]. *In vitro* study also showed a link between the absence of VDR and increased activity of NF-kB [43]. However, inflammatory signaling was mediated by the NFkB pathway since it increased the expression of proinflammatory cytokines such as IL-6 and TNF-α [44].

SCFA concentration and ratio

The post-intervention total of SCFA, acetate, and butyrate concentration was significantly different between groups (p=0.029, p=0.013, and p=0.022, respectively; table 1). Their concentrations of X1 were significantly different with K (+) (p=0.016; p=0.016; and p=0.016; table 1, respectively) but not with K (-) group (p=0.109; p=0.150; and p=0.262; table 1, respectively). Similarly, their concentrations were significantly different from the X3 group but not with X2. The mean of total SCFA, acetate, and propionate concentration of X1 was higher compared to other groups. This result showed that fortified dadih with vitamin D3 is better at producing SCFA compared to those without fortification and vitamin D3.

Furthermore, the ratio of acetate, propionate, and butyrate was significantly different between groups (p=0.001, p=0.001, and p=0.001, respectively; table 1). There are several references to this ratio, such as 60:20:20 and 60:25:15 [45, 46]. The difference depends on the type of substrate, diet, and composition of the intestinal microbiota [46, 47].

The production of SCFA by microbial fermentation of dietary fiber was associated with various health benefits, including improvements in body composition, energy homeostasis, lipid profile, and weight loss by stimulating several hormonal and nerve pathways, such as appetite control, increased oxidation, and muscle thermogenesis as well as liver tissue [17]. Its effect on body weight and food intake occurs through G-protein coupled receptors (GPR, GPR41, and GPR 43). GPR41 and GPR 43 are known as a Free Fatty Acid Receptor 3 (FFAR3) and 2 (FFAR2), respectively. FFAR2 is expressed on immune cells and primarily reacts to acetate and propionate, while FFAR3 is mostly present in adipocytes and preferentially binds to propionate and butyrate. Their activation increases the synthesis and signaling of satiety hormones such as GLP-1 and PYY, causing decreased appetite and increased energy expenditure [48].

The correlation between variables

The Spearman test showed that a moderate correlation was obtained between variables. A moderate negative correlation was observed between total SCFA and IL-6-levels (p=0.021; r=-0.419; table 2). Furthermore, it was observed between acetate and IL-6-levels (p=0.027; r=-0.403; table 2) as well as between body weight and each of total SCFA, propionate, butyrate (p=0.020; r=-0.423, p=0.037; r=-0.383, p=0.008; r=-0.473, respectively; table 2). Also, a very strong positive correlation was observed between body weight and IL-6-levels (p=0.000; r=0.802; table 2).

SCFA prevented the production of LPS from gram-negative bacterial cell membranes, and it has been linked to endotoxemic metabolism, inflammation, insulin resistance, adiposity, and liver fat [49]. Furthermore, SCFA reduces systemic inflammation through molecular signaling pathways by activating G-protein coupled receptor 41/43 and inhibition of the histone deacetylase (HADACs) [50, 51].

The effect of SCFA on body weight and food intake occurs through G-protein coupled receptors (FFAR3 and FFAR2). Their activation increases the synthesis and signaling of satiety hormones such as GLP-1 and PYY, causing decreased appetite and increased energy expenditure [48]. Butyrate prevents obesity by activating β3 adrenergic receptors in white adipose tissue. Acetate plays a role in regulating appetite in the brain, increasing fat oxidation, improving glucose homeostasis, and inflammatory status [52]. However, its role in the regulation of appetite, fat, and weight gain remains controversial. Besides, a previously conducted study has reported its influence on central appetite regulation and reduced food intake [53]. Other studies showed the opposite effect, causing an increase in ghrelin secretion, hyperphagia, and obesity in rats [54]. These conflicting results may be related to metabolic status and the location of its administration [52]. Similar to these results, acetate does not have a significant correlation with body weight. In humans and mice, propionate is associated with preventing the accumulation of hepatic lipids through the suppression of genes involved in fatty acid synthesis [52].

Table 1: The effect of fortified dadih with vitamin D3 on body weight, IL-6 and SCFA

Groups Marker	K(-)	K(+)	X1	X2	X3	p[1]
Body Weight after HFD administration (gr)						
Pre	181.67±5.2	186.83±5.19	184.5±5.24	181.5±3.72	184.5±8.24	0.468
Post	202.17±5.38[b,c,d,e]	220.33±5.04[a]	220.5±4.03[a]	217.5±3.98[a]	220±7.09[a]	0.001
Δ	20.5±1.04[b,c,d,e]	33.5±3.88[a]	36.0±1.67[a]	36.0±0.63[a]	35.5±1.37[a]	0.001
p	0.001	0.001	0.001	0.001	0.001	
Body Weight of intervention (gr)						0.005
Pre	201.0 (196-211)[b,c,d,e]	221.5 (211-225)[a]	220.0 (215-226)[a]	218.0 (212-223)[a]	220.0 (212-231)[a]	0.001 0.001
Post	225.0 (220-234)[b,c,d,e]	256.5 (248-261)[a,c,d,e]	244.0 (240-249)[a,b]	245.5 239-250)[a,b]	243.0 (237-254)[a,b]	
Δ	23.5 (23.0–25.0)[b,d]	35.0 (35.0-37.0)[a,c,d,e]	24.5 (23.0-25.0)[b]	27.0 (27.0-28.0)[a,b]	24.0 (23.0-25.0)[b]	
p	0.026	0.023	0.026	0.023	0.024	
IL-6 (pg/ml)						
Post	66.81±6.23[b,c,d,e]	176.53±8.21[a,c,d,e]	85.66±9.08[a,b,d,e]	127.12±1.16[a,b,c,e]	106.6±6.86[a,b,c,d]	0.000
Total SCFA (mmol/kg)						
Post	26.11 (3.23-33.54)	15.56 (11.86-32.57)[c,d]	32.07 (24.12-40.83)[b,e]	20.09 17.57-40.64)[b]	22.04 (16.43-28.32)[c]	0.029
Acetate (mmol/kg)	12.35	7.14	15.78	9.6	8.39	0.013
Post	(1.37-18.15)	(5.73-15.21)[c,d]	(11.71-21.02)[b,e]	(8.21-19.21)[b]	(6.44-11.11)[c]	
Propionate (mmol/kg)	9.25	6.12	11.11	6.71	8.15	0.093
Post	(1.13-11.66)	(4.21-12.47)[c]	(8.09-14.11)[b]	(5.97-13.70)	(5.82-10.41)	
Butyrate (mmol/kg)	4.24	2.3	5.04	3.78	5.6	0.022
Post	(0.73-5.96)	(1.92-4.89)[c,d,e]	(4.32-5.44)[b]	(3.20-7.73)[b]	(4.17-6.80)[b]	
The ratio of Acetate (mmol/kg) Post	48.60±5.35	46.19±1.71[e]	49.63±1.63[e]	47.32±1.03[e]	39.18±1.34[b,c,d]	0,001
Ratio of Propionate(mmol/kg) Post	33.73±2.84[b]	38.43±1.76[a,c,d]	34.32±1.72[b]	33.87±0.54[b]	36.38±1.07	0.001
The ratio of Butyrate (mmol/kg) Post	17.63±5.36	15.36±0.67[d,e]	16.06±3.01[e]	18.79±0.83[b,e]	24.43±1.04[b,c,d]	0.001

Five groups of rats (n=6 for each group) consists of K(-): control healthy and K(+): control Obese; X1: Fortified Dadih Treatment; X2: Dadih Treatment; X3: Vitamin D3 Treatment; Δ: changes between pre and post value; differences between the groups of rats were analyzed using ANOVA (BW HFD, IL-6, Ratio of Acetate, Propionate, and Butyrate) Kruskal Wallis (BW intervention, Total SCFA, acetate, propionate, butyrate); p: value between pre and post-treatment were analyzed using Paired t-test/Wilcoxon test; alphabetical superscripts showed a significance level of [a]: p<0.05 compared as K(-); [b]: p<0.05 as compared to K(+); [c]: p<0.05 as compared to X1; [d] p<0.05 as compared to X2; [e] p<0.05 as compared to X3. The data were written as mean±SD for normally distributed data and median (Min-Max) when data were not normally distributed; p[1]: value between all of the groups were analyzed using ANOVA if data are normally distributed and Kruskal Wallis if data are not normally distributed.

Table 2: Correlation between SCFA concentration, IL-6 levels, and body weight after fortified dadih with vitamin D3 intervention

	IL-6		Body weight	
	r	p	r	p
Total SCFA	-0.419	0.021*	-0.423	0.020*
Acetate	-0.403	0.027*	-0.359	0.052
Propionate	-0.355	0.054	-0.383	0.037*
Butyrate	-0.286	0.126	-0.473	0.008*
Body Weight	0,802	0.000*	-	-

Thirty-five of rats were analysed, statistical significance calculated by Spearman test *Value are statistically significant at p<0,05

The expansion of adipose tissue in obesity caused high IL-6 in blood circulation [55]. The expression of white adipose tissue and plasma IL-6 correlate with increased body mass, waist circumference, and free fatty acid levels [56]. In addition, increased expression of IL-6 Receptor (IL-6R) and IL-6 in adipose tissue was positively correlated with clinical indicators of obesity, such as body mass index and fat percentage [55].

Considering the results, it is reasonable to conclude that fortified dadih with vitamin D3 significantly reduces IL-6 expression level and increases the production of SCFA. However, the IL-6 expression level was not significantly different from healthy-control since it was lower than obese-control, dadih without fortification, and vitamin D3 groups. Furthermore, the total SCFA, acetate, and propionate concentration of fortified dadih with vitamin D3 groups were higher compared to others.

CONCLUSION

Fortified dadih with vitamin D3 reduces weight gain, improves the expression level of IL-6, and increases the production of SCFA. Also,

they improve intestinal homeostasis because of the increased SCFA production. The strong correlation between IL-6 and body weight indicated that the improvement of IL-6 related to the reduction of body weight gain.

ACKNOWLEDGMENT

The authors are grateful to The Faculty of Medicine, Diponegoro University

FUNDING

This research was funded by The Faculty of Medicine, Diponegoro University (Grant Number: 1160/UN7.5.4.2.1/PP/PM/2020).

AUTHORS CONTRIBUTIONS

Sari Bema Ramdika: Conceived the study question and the study design, conducted of data collection, data analysis and interpretation, and writing the manuscript.

Endang Mahati: Designed the study, supervised data collection and data analysis, contributed to data interpretation, and review and editing the manuscript.

Anang Mohamad Legowo: Designed the study, contributed to data interpretation, and review and editing the manuscript.

Muflihatul Muniroh: Designed the study, contributed to data interpretation, and review and editing the manuscript.

Yora Nindita: Designed the study, contributed to data interpretation, and review and editing the manuscript.

Diana Nur Afifah: Managed the experimental processes, supervised data collection and data analysis, contributed to data interpretation, and writing the manuscript.

CONFLICT OF INTERESTS

The authors declare no conflict of interest

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