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Original Article

Kepel (*Stelechocarpus burahol*) Synbiotic Supplementation Improves Oxidative Stress in High-Fat Diet-Fed Rats

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Abstract

Background: Synbiotic contains antioxidant that has been suggested to improve oxidative stress induced by high-fat diet (HFD) consumption. This study aimed to evaluate the effect of synbiotic supplementation consisting of kepel (*Stelechocarpus burahol*) with the addition of *Lactobacillus casei* and *L. plantarum* on oxidative stress in HFD-fed rats.

Methods: Twenty-five Wistar rats were divided into five groups (n = 5) for eight weeks of treatment. The HFD control (HFD alone) group and three different groups supplemented with three various doses of kepel synbiotic (Syn 1.2 mL, Syn 1.8 mL, and Syn 2.4 mL) were fed HFD for the first four weeks and continued supplemented kepel synbiotic for the second four weeks. Meanwhile, the normal diet (ND) control group was given regular food alone throughout the study. The serum, liver, heart, and brain oxidative stress markers were assessed.

Results: Kepel synbiotic supplementation consistently improved oxidative stress by decreasing malondialdehyde (MDA) levels and increasing superoxide dismutase (SOD) activity inhibition rate in serum, liver, heart, and brain in the HFD group compared to the ND group. This improvement effect occurred in a dose-dependent manner, increasing in higher kepel synbiotic doses.

Conclusion: Kepel synbiotics showed a beneficial effect in improving oxidative stress in the serum, liver, heart, and brain of HFD-fed rats. Supplementation of kepel synbiotic can be considered a complementary therapeutic agent in improving oxidative stress, especially due to HFD consumption.

Keywords: Oxidative stress, Rats, *Stelechocarpus burahol*, Synbiotic

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Introduction

Uncontrolled oxidative stress has been postulated to be involved in the development of various diseases (1). Oxidative stress is a state of imbalance between oxidants, such as reactive oxygen species (ROS), and antioxidants. ROS are generated by endogenous sources involving cellular biochemical activities and exogenous sources, such as high-fat diet (HFD) consumption, which contribute to deoxyribonucleic acid (DNA), proteins, and lipid membrane damage (2,3). In a controlled or physiological state, the body has a defense system through several antioxidant enzymes as compensatory mechanisms against oxidative stress, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) (1). Meanwhile, the body's antioxidant defense system is inadequate at high levels of oxidative stress, resulting in direct damage to lipids, especially polyunsaturated fatty acids. This process is called lipid peroxidation. Furthermore, many studies confirm that the lipid peroxidation process also produces various oxidation products, such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), which are mutagenic and toxic, respectively (4-6).

Synbiotics (also called symbiotic) have been considered complementary therapeutic agents to ameliorate oxidative stress (7). A synbiotic is a product consisting of prebiotics and probiotics with beneficial health-promoting effects (8). Many studies have confirmed the antioxidant effects of probiotics and synbiotics on experimental animals and humans. Lasker et al (9) showed that HFD caused oxidative stress and impaired liver function in rats, whereas yogurt supplementation improved this condition. In other studies, synbiotic supplementation (*Lactobacillus casei* + inulin) in humans successfully improved oxidative stress, indicated by low oxidative stress markers and increased antioxidant activity (10). In addition, synbiotic supplementation (*Lactobacillus plantarum* + inulin) was reported to increase antioxidant activity in the heart, including total antioxidant capacity (TAC), SOD, and GPx, along with decreased SOD levels in diabetic rats (11).

Kepel fruit (*Stelechocarpus burahol*) is a typical plant originating from Java Island, Indonesia (12). Previous studies showed better antiradical and antioxidant effects in kepel compared with vitamin C, due to the high phenol and flavonoid components (13,14). In addition,

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the application of kepel as an oral deodorant in the rat models increased *Bifidobacteriaceae* probiotics quantity, which contributed to the absorption of fecal odors and reduced *Enterobacteriaceae* pathogenic quantity within the intestinal tract (15). These findings indicate that kepel has a potential function as a prebiotic. However, no studies have evaluated kepel as a dairy product improving oxidative stress. Therefore, it is possible to combine kepel with *L. casei* and *L. plantarum* as a synbiotic product that could improve oxidative stress due to HFD. This study aimed to evaluate the effect of kepel synbiotic supplementation on HFD-fed rats.

Materials and Methods

Animals

Twenty-five male Wistar rats aged 8-10 weeks weighing 180-220 g were provided by the Laboratory of Physiology, Faculty of Medicine of the Universitas Islam Indonesia (FM UII), Yogyakarta, Indonesia. The rats were housed in a controlled room with a temperature range from 21 to 25°C, a humidity of 40-70%, and a 12-hours light/dark cycle. They were given free access to tap water and regular food containing carbohydrates 60%, protein 16%, vitamins and minerals 21%, and fat 3%. Before starting the treatment, the rats were acclimatized for a week.

Study design

The study timeline scheme is shown in Table 1. After a week of acclimatization, rats were divided into five groups (n = 5). The HFD control (HFD alone) group and three different groups supplemented with three various doses of kepel synbiotic (Syn 1.2 mL, Syn 1.8 mL, and Syn 2.4 mL) were fed *ad libitum* HFD for the first four weeks. Afterward, synbiotic-supplemented groups were given the kepel synbiotic according to their respective doses by oral gavage for the second four weeks. Regular food was also given to all groups during this period. Meanwhile, the normal diet (ND) control group was given regular food alone throughout the study. After treatments were completed, the rats were fasted for 12 hours and sacrificed to collect blood, liver, heart, and brain tissue under general anesthesia ketamine 40 mg/kg for biochemical analysis.

HFD preparation

The HFD was formulated with a mixture of 20% regular feed, 40% quail egg yolk, and 40% duck egg yolk. Quail and duck egg yolks were boiled until half-cooked, mixed with crushed regular feed, and formed balls in small sizes. The *ad libitum* HFD was administered daily.

Kepel synbiotic preparation

Kepel fruit was purchased from a local market in Yogyakarta, Indonesia. The two lactic acid bacteria were *L. casei* NRRL B-1922 and *L. plantarum* CCRC 12251, obtained from the Center for Food and Nutrition Studies of Universitas Gadjah Mada, Yogyakarta, Indonesia. Kepel fruit was extracted by the maceration method, using methanol solvent as described in a previous study (13). The synbiotic preparation method followed a previous study with some modifications (16). The initial starter culture contained 2 ml of *L. casei* and *L. plantarum* separately in 8 mL of de Man, Rogosa, and Sharpe (MRS) broth incubated at 37°C for 24 hours. Afterward, the final starter culture contained 1 mL of the initial starter incubation result, 9 ml of kepel juice, 1 g skim milk, and 3 g glucose, then it was incubated at 37°C for 24 hours. The final kepel synbiotic was a mix of the final starter, 10 g skim milk, 3 g glucose, 100 ml kepel juice, and 4 mL of each final starter culture, incubated at 37°C for 24 hours. Accordingly, the final product was a supplemental kepel synbiotic.

Preparation of serum and tissue samples

The blood samples (2 mL) were obtained from retro-orbital venous and then centrifuged at a speed of 8000 rpm for 10 minutes to obtain serum for biochemical analysis.

One gram of fresh liver, heart, and brain tissue was homogenized with PBS (0.01 M, pH 7.4) and centrifuged for 15 minutes at 8000 rpm at 4°C. Then, the obtained supernatant was used for MDA analysis. Meanwhile, in obtaining SOD supernatant, tissue homogenization was performed in ice-cold 0.1 M Tris-HCl, pH 7.4 containing 5 mM β-ME, 0.1 mg/mL PMSF, and 0.5 % Triton X-100 then centrifuged at 9000 rpm for 5 minutes at 4°C.

Biochemical analysis

MDA is a lipid peroxidation product that reacts with thiobarbituric acid-reactive substances (TBARS). The assessment of MDA in serum, as well as liver, heart, and

Table 1. Study timeline

Animals	Preparation	Randomization	Treatment		End
Wistar rats (n = 25)	Acclimatization	ND (n = 5)	Regular feed	Regular feed	Sacrifice and assessment (n = 25)
		HFD alone (n = 5)	HFD	Regular feed	
		Syn 1.2 mL (n = 5)	HFD	1.2 mL of kepel synbiotic + regular feed	
		Syn 1.8 mL (n = 5)	HFD	1.8 mL of kepel synbiotic + regular feed	
		Syn 2.4 mL (n = 5)	HFD	2.4 mL of kepel synbiotic + regular feed	
Day		**0–7**	**8–36**	**37–65**	**66**

brain supernatant, was determined using the colorimetric method by monitoring red compound at 532 nm with a spectrophotometer based on standard assessment protocol and available reagent kits from Elabscience (Wuhan, Hubei, China). MDA levels were expressed in nmol/mL for serum and nmol/g for tissue. SOD activity inhibition rate in serum, liver, heart, and brain supernatant was assessed by colorimetric method at 450 nm using a microplate reader according to protocol and reagent kits provided by BioVision (Milpitas, California, USA). The inhibition rate of SOD activity was expressed in percent. Serum AST and ALT levels were assessed by the optimized UV-test method using reagent kits supplied by DiaSys (Holzheim, Germany) and the protocol following standard manufacturer guidelines. The unit for expressing AST and ALT levels was U/L.

Statistical analysis

Statistical analysis was carried out using IBM SPSS Statistics 26 (Chicago, Illinois, USA). Results are represented as mean ± standard deviation (SD). The one-way ANOVA test followed by Tukey's post hoc, or Kruskal-Wallis followed by Mann-Whitney U as post hoc was used to examine comparisons between groups. A *P* value lower than 0.05 was considered statistically significant.

Results

Systemic oxidative stress

Four weeks of HFD administration caused systemic oxidative stress. This condition is reflected by significantly ($P<0.001$) increased levels of serum MDA (Figure 1A) and decreased serum SOD activity inhibition rate (Figure 1B) in the HFD group compared to the ND group. Furthermore, the synbiotic supplementation in all doses suppressed systemic oxidative stress by significantly ($P<0.001$) decreasing MDA levels and increasing serum SOD activity inhibition rate compared to the HFD group. Additionally, administering synbiotics at a dose of 2.4 mL showed the best improvement of oxidative stress, characterized by the lowest MDA levels and highest SOD activity among treated groups.

Liver function and oxidative stress

The HFD also disrupts liver function and causes oxidative stress.There was a significant increase in liver MDA levels ($P<0.001$; Figure 2A), a decrease in SOD activity inhibition rate ($P<0.001$; Figure 2B), and an increase in AST ($P<0.01$; Figure 2C) and ALT levels ($P<0.001$; Figure 2D) in HFD group compared to the ND group. Statistically significant improvement of these assessed biomarkers indicated that the synbiotic supplementation improves liver function and oxidative stress. Moreover, the higher dose of synbiotic showed the most favorable improvement effect.

Oxidative stress in the heart

Oxidative stress also occurred in rats' heart tissue due to HFD administration. A significant increase ($P<0.001$) in heart MDA levels (Figure 3A) in the HFD group compared to the ND group was observed. Simultaneously, the heart SOD activity inhibition rate (Figure 3B) was decreased significantly ($P<0.001$) in the HFD group compared to the ND group. The synbiotic supplementation in different doses improved oxidative stress by decreasing heart MDA levels and increasing heart SOD activity inhibition rate significantly ($P<0.001$) compared to the HFD group. Furthermore, the 2.4 mL dose of synbiotic resulted in the highest reduction in heart MDA levels and the highest heart SOD activity compared to other doses.

Brain oxidative stress

Figures 4A and 4B showed that administration of HFD increased brain MDA levels significantly ($P<0.001$) following decreased brain SOD activity inhibition rate in the HFD group compared to the ND group. Oral supplementation of synbiotics in all doses decreased MDA levels ($P<0.001$) compared to the HFD group. Furthermore, the brain SOD activity inhibition rate increased significantly ($P<0.001$) in the Syn 1.8 and Syn 2.4 mL groups and ($P<0.01$) in the Syn 1.2 mL group compared to the HFD group. The Syn 2.4 mL group showed the best improvement effect in ameliorating oxidative stress.

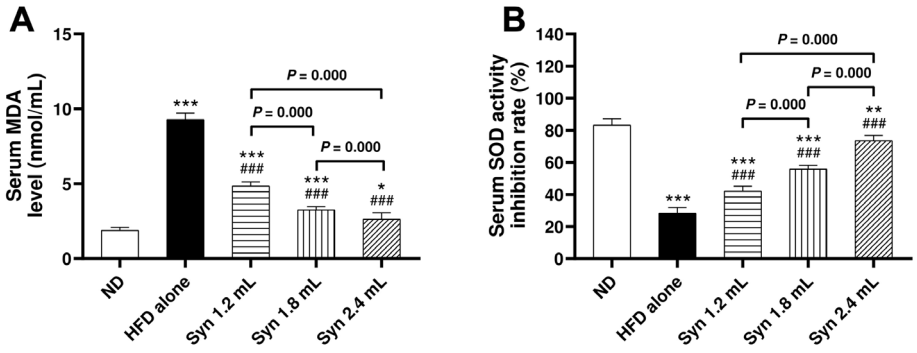

Figure 1. Effect of kepel synbiotic supplementation on serum oxidative stress markers: (A) MDA and (B) SOD. ND: normal diet; HFD: high-fat diet; MDA: malondialdehyde; SOD: superoxide dismutase. Data were expressed as the mean ± SD. Data were analyzed using one-way ANOVA followed by Tukey's post hoc test. * $P<0.05$; ** $P<0.01$; *** $P<0.001$ compared with ND group. ### $P<0.001$ compared with HFD alone group.

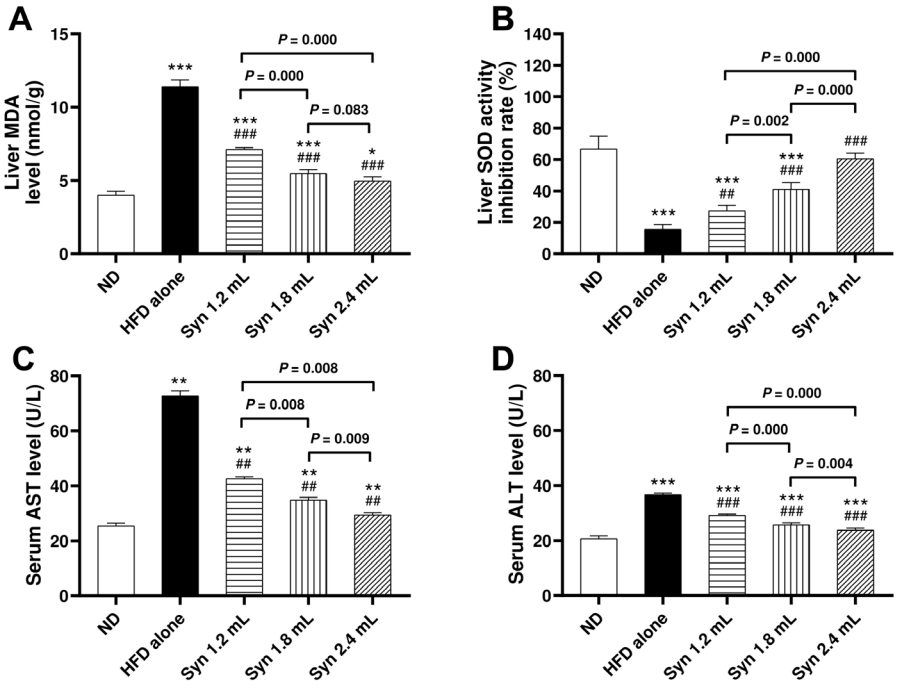

Figure 2. Effect of kepel synbiotic supplementation on liver oxidative stress and function markers (A) MDA, (B) SOD, (C) AST, (D) ALT. ND: normal diet; HFD: high-fat diet; MDA: malondialdehyde; SOD: superoxide dismutase; AST: aspartate transaminase; ALT: alanine transaminase. Data were expressed as the mean ± SD. Data were analyzed using one-way ANOVA followed by Tukey's post hoc test or nonparametric data using Kruskal-Wallis followed by the Mann-Whitney U test. * $P<0.05$ ** $P<0.01$; *** $P<0.001$ compared with ND group. # $P<0.05$; ## $P<0.01$; ### $P<0.001$ compared with HFD alone group.

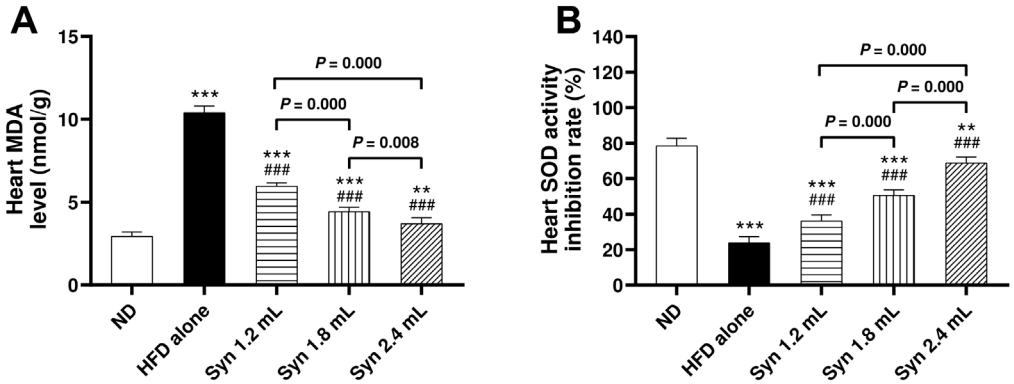

Figure 3. Effect of kepel synbiotic supplementation on heart oxidative stress markers (A) MDA and (B) SOD. ND: normal diet; HFD: high-fat diet; MDA: malondialdehyde; SOD: superoxide dismutase. Data were expressed as the mean ± SD. Data were analyzed using one-way ANOVA followed by Tukey's post hoc test. ** $P<0.01$; *** $P<0.001$ compared with ND group. ### $P<0.001$ compared with the HFD alone group.

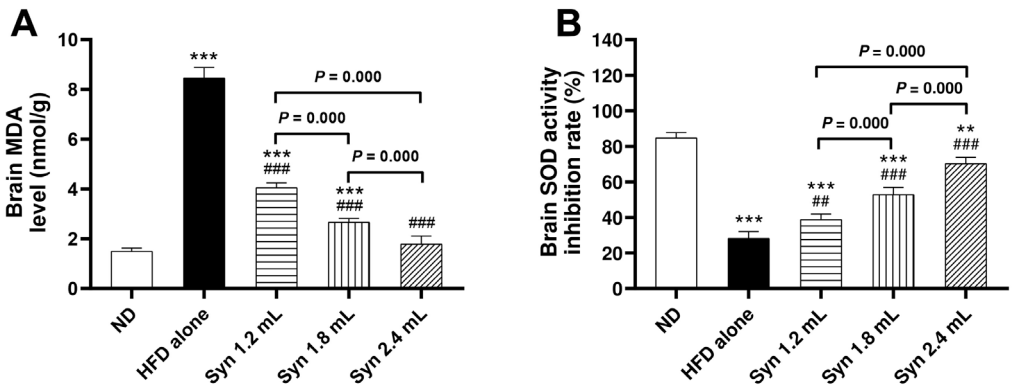

Figure 4. Effect of kepel synbiotic supplementation on brain oxidative stress markers (A) MDA and (B) SOD. ND: normal diet; HFD: high-fat diet; MDA: malondialdehyde; SOD: superoxide dismutase. Data were expressed as the mean ± SD. Data were analyzed using one-way ANOVA followed by Tukey's post hoc test. ** $P<0.01$; *** $P<0.001$ compared with ND group. ## $P<0.01$; ### $P<0.001$ compared with HFD alone group.

Discussion

The current study demonstrated that HFD formulated with quail and duck egg yolk mixed with regular foods successfully caused oxidative stress. It is plausible because quail and duck eggs have higher fat content, particularly in the yolk, compared with other poultry eggs (17,18). HFD is well known as a major risk factor for inducing ROS and excessive inflammation response in developing many diseases (1,19,20). Moreover, our study has proven the potential of kepel as a prebiotic proposed in previous studies. Although prebiotics and probiotics independently have antioxidant effects, combining the two may produce a better and synergistic effect to confer health benefits. We observed that supplementation of a synbiotic composed of kepel with the addition of *L. casei* and *L. plantarum* for four weeks improved oxidative stress in the serum, liver, heart, and brain of HFD-fed rats. This improvement effect showed a dose-dependent manner in which the higher dose of the synbiotic had a better improvement effect. To the best of our knowledge, this is the first study revealing the improvement effect of kepel, as a synbiotic dairy product, on oxidative stress in HFD-fed rats.

High serum or plasma MDA levels reflect oxidative stress at the systemic level, usually accompanied by lower levels of antioxidant enzymes such as serum SOD activity. Systemic oxidative stress plays a role in developing cardiovascular diseases, metabolic disorders, liver dysfunction, brain damage associated with cognitive impairment, and cancer (1). A previous study in dyslipidemic rats demonstrated that HFD causes oxidative stress and is alleviated by probiotic *L. fermentum* DALI02 in a dose-dependent manner, which is in line with our study (6). A systematic review and meta-analysis of randomized controlled trials also suggested that probiotic and synbiotic supplementation showed increased antioxidants (TAC and GSH) in diabetic patients (7). Consistent with existing evidence, our study proves that HFD administration causes oxidative stress reflected by increased serum MDA and decreased serum SOD in the HFD-fed group. Furthermore, kepel synbiotic supplementation containing *L. casei* and *L. plantarum* improves systemic oxidative stress.

Organ damage due to the oxidative stress process induced by HFD results in hepatocyte damage and liver dysfunction. In the current study, an increase in liver MDA levels, serum AST and ALT enzymes, as well as a decrease in liver SOD activity in HFD-fed rats were observed. Existing scientific evidence demonstrates that HFD causes dyslipidemia, then triggers lipotoxicity, accumulation of lipids in the liver, and increased infiltration of inflammatory cells in the liver, which develops non-alcoholic fatty liver disease (9,21). Furthermore, this process is associated with oxidative stress and *de novo* lipogenesis, in which an increased supply of intrahepatic lipids contributes to insulin resistance (22). We found that kepel synbiotic supplementation improved liver function. Similarly, yogurt supplementation for eight weeks in HFD-fed rats demonstrated improved liver function and oxidative stress, reflected by decreased liver dysfunction and oxidative stress markers and increased antioxidant enzymes (9). Thus, this finding indicated that kepel synbiotic supplementation improves antioxidant activity associated with liver dysfunction prevention.

Inflammatory responses and oxidative stress have been postulated to be responsible for structural and functional deficits in the heart that develop cardiac tissue fibrosis and cardiomyocyte apoptosis (11,23,24); an HFD may cause these conditions (23). In our study, HFD administration caused oxidative stress in the heart. This finding is corroborated by a previous study reporting that HFD induces cardiotoxicity and promotes cardiac injury in rats involving increased heart MDA and decreased antioxidant enzymes such as GSH, SOD, CAT, and GPx in the heart (23). In addition, we observed increased heart antioxidant activity by kepel synbiotic supplementation. Accordingly, a previous study showed that *L. plantarum* + inulin synbiotic supplementation increased the heart's antioxidant activity in diabetic rats (11). Another similar study demonstrated that *L. plantarum* + inulin increased heart serotonin and BDNF receptor expression with decreased apoptosis, interstitial fibrosis, and heart perivascular (25). Therefore, kepel synbiotic supplementation has been shown to improve oxidative stress in the heart associated with preventing cardiac dysfunction and fibrosis in HFD-fed rats.

The brain is particularly vulnerable to oxidative stress-induced damage because it lacks an adequate antioxidant system and is rich in polyunsaturated fatty acids (26). It is plausible that HFD administration induces brain oxidative stress marked by increased MDA levels and decreased SOD activity. A previous study by Langley et al. (27) demonstrated that HFD administration in mice reduces the number of oligodendrocyte progenitors in the brain and spinal cord. In addition, AMPK-SIRT1-PGC1α signaling decreases in the spinal cord, and the 4-HNE marker of oxidative stress increases, leading to demyelinating, neuropsychiatric and cognitive disorders. Kepel synbiotics containing *L. casei* and *L. plantarum* increase antioxidants to improve brain oxidative stress characterized by increased brain SOD activity and decreased MDA levels. This process may occur through the proposed mechanism gut-brain axis. In this concern, a previous study reported that synbiotic supplementation decreased MDA levels and increased SOD activity in the rats' hypothalamus (28). A recent randomized controlled trial study also declared that synbiotic supplementation for 12 weeks improved oxidative stress associated with migraine by improving TAC and reducing nitric oxide levels (29). This finding indicates that kepel synbiotic improves brain oxidative stress through increasing

antioxidant enzymes.

Apart from the beneficial effect of kepel synbiotics, this study has some limitations due to the absence of a group treated by prebiotics or probiotics alone. Thus, we suggest adding these two groups to investigate how much the effect of kepel as a prebiotic is in improving oxidative stress in future research.

Conclusion

Results of this study showed that kepel synbiotic supplementation increased antioxidant activity which improves oxidative stress in the serum, liver, heart, and brain of HFD-fed rats. This study suggests that kepel synbiotic supplementation can be considered a complementary therapeutic agent in improving oxidative stress due to HFD. In addition, the kepel plant, which is little cultivated currently, can be improved and developed into a medicinal plant mainly to prevent diseases.

Author Contributions

Conceptualization: Naufal Arif Ismail, Alfian Novanda Yosanto.
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Validation: Naufal Arif Ismail, Nur Aisyah Jamil.
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Writing – review & editing: Naufal Arif Ismail.

Conflict of Interests

The authors declared that there is no conflict of interest.

Ethical Approval

The Ethics Committee of the FM UII approved this study procedure (Number: 31/Ka.Kom.Et/70/KE/V/2019).

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