RESEARCH ARTICLE

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Green Tea Yoghurt with Encapsulated *Lacticaseibacillus paracasei* E1 Improves Hepatocyte Damage in High-Fat High-Fructose Diet Mice by Reducing MDA and Increasing SOD

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Background: Obesity is a global epidemic caused by excessive body fat, which is increasing free fatty acids in the liver, causing oxidative stress and liver cell damage. Green tea yogurt with encapsulated *Lacticaseibacillus paracasei* E1 (GTY-LpE1) might have a beneficial effect in reducing liver cell damage. This study was conducted determine GTY-LpE1 effect on superoxide dismutase (SOD) expression, malondialdehyde (MDA) expression and liver histopathology in high-fat high-fructose diet (HFFD) mice.

Material and Methods: A completely randomized design (CRD) with 7 groups, including normal diet (ND) group, HFFD group, 1.3 mg/kg BW simvastatin (SIM)-administered HFFD group, 5 g/kg BW probiotic yoghurt (PY)-administered HFFD (PY), 2.5 g/kg BW GTY-administered HFFD (2.5 GTY), 5 g/kg BW GTY-administered HFFD (5 GTY), and 10 g/kg BW GTY-administered HFFD (10 GTY). The diet was given for 16 weeks, followed by oral administration of sim/yoghurt during the last 4 weeks. Mice were sacrificed and the liver was collected. SOD and MDA expression were analyzed by flow cytometry. Histopathology analysis was done by evaluating hematoxylin-eosin (HE) staining of the liver.

Result: The percentage of necrotic cells were 34.55, 34.31, and 21.95%, when treated with 2.5, 5, and 10 g/kg BW with GTY-administered HFFD, respectively, these were lower than the ones in the HFFD group (69.49%). The percentage of MDA expression were 15.55, 18.69, and 22.42%, respectively, these were lower than the ones in the HFFD group as well. The percentage of SOD expression were 9.49, 7.85, and 11.11%, respectively, these were higher than the ones in the HFFD group (3.44%).

Conclusion: GTY-LpE1 could decrease the number of necrotic cells in the HFFD mice livers and improve the hepatocyte damage by reducing MDA expression and enhancing SOD expression. GTY-LpE1 can be used as an alternative food to control obesity.

Keywords: *alginate, chitosan, encapsulation, green tea, probiotic*

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Introduction

Obesity is a chronic low-grade systemic inflammatory disorder defined by the accumulation of fat tissue in the body and potentially unfavorable health concerns.[1] Obese people have 3.5 times increased risk of fat accumulation in the liver since the liver is one of the storage areas for excess energy and food. A fatty liver is a triglyceride-rich hepatocyte. Lipolysis releases triglycerides from adipocytes into free fatty acids for energy, which are then circulated through the body. Subsequently, the released fatty acids can serve as an energy substrate for other tissues in the body. During obesity, free fatty acids enter the liver through the portal circulation, causing elevated levels of free fatty acids in the liver, which results in heightened lipid synthesis, gluconeogenesis, and insulin resistance in the liver.[2]

Reactive oxygen species (ROS) generated by elevated levels of free fatty acids in the liver are capable of causing damage to DNA and mitochondrial proteins, thereby promoting oxidative stress, inflammation, and injury to liver cells.[3] ROS is a metabolic byproduct that plays an important role in the development of obesity by participating as a regulatory factor for mitochondrial activity, promoting adipogenesis and lipogenesis, and playing an important role as an agent that regulates energy balance in hypothalamic neurons that control appetite.[4]

The changing times have resulted in changes in trends and unhealthy lifestyles, shown by the increase in obesity. If left unchecked, this can lead to serious health complications and higher medical costs.[5] One way to overcome this problem is to improve unhealthy lifestyles. Consumption of foods with high fat and sugar is one of the causes of obesity. One compound that can improve and compensate for this is antioxidant, which have been reported to be active against obesity through several molecular signaling pathway mechanisms.[6] In addition, the abundance of antioxidant sources and their easy accessibility make them potential candidate compounds for obesity control.

Green tea (*Camelia sinensis* L.) is one of the plants that contain antioxidants, with catechins as the main antioxidant substance. In addition, yogurt also contains antioxidants that are good for inhibiting oxidation processes that can cause cell damage. The addition of probiotic bacteria to yogurt can increase the antioxidant content of yogurt and the balance of gut microbiota.[7] However, the addition of bacteria has a shelf life, and damage to the bacteria can occur when it passes through the stomach. Therefore, encapsulation of probiotic bacteria was carried out to help maintain viability during industrial processing and gastrointestinal transit.[8]

Previous studies have shown the ability and potential of green tea and yogurt as antioxidants that are good for the body's health separately. However, no studies have been conducted to investigate the potential of adding probiotic bacteria encapsulation and green tea into yogurt to overcome obesity-induced oxidative stress and liver damage. Therefore, this study was conducted to investigate the addition of probiotic bacteria encapsulation and green tea leaves into yogurt that might overcome obesity-induced oxidative stress and liver damage, as indicated by changes in SOD level, MDA level and liver histopathology in mice.

Materials and methods

Lacticaseibacillus paracasei E1 (LpE1), Lactobacillus bulgaricus FNCC 0040 (Lb40), and Streptococcus thermophilus FNCC0041 (St41) Cultures

LpE1 was obtained from the collection of the Microbiology Laboratory, Department of Biology, Universitas Brawijaya, Malang. Lb40 and St41 were obtained from Center for Food and Nutrition Studies, Universitas Gajah Mada, Yogyakarta. LpE1 and Lb40 were cultured in de Man Rogosa Sharpe (MRS) broth and incubated at 37°C. St41 was cultured in MRS broth medium and incubated at 45°C using a shaker at 150 rpm for 48 hours. Bacterial harvesting was done by centrifugation at 3000 rpm for 10 minutes at 4°C, then washed twice in 0.85% NaCl solution. The number of each isolate to be used was counted with a hemacytometer, at $1x10^9$ CFU/mL.

LpE1 Encapsulation

LpE1 encapsulation was carried out according to the previous research.[9] The encapsulation process consisted of 2 stages: internal coating with 1.5% sodium alginate (Cat. No. 71238; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) and external coating with 0.5% chitosan medium molecular weight (Cat. No. 448877; Sigma-Aldrich). The LpE1, alginate and chitosan mixture were spray dried and stored for further analysis.

Plain Yogurt (PY) and Green Tea Yoghurt (GTY) Preparation

Green tea leaves (*Camelia sinensis* L. var. sinensis) from Pasir Canar plantation were purchased from Bird Tea Gallery of Greenfields, Malang, East Java, was added

with 6% sucrose (w/v) and heated at 85°C for 30 minutes. Pasteurized milk was added with/without 4% green tea leaves for overnight in the refrigerator. After that, the green tea leaves were filtered to remove the debris. St41 and Lb40 (2:1) at a total of 3% and 3% encapsulated LpE1 (w/v) were mixed with milk tea/milk without tea (plain). The mixed tea was then incubated for 30 hours at 37°C. The PY and GTY was then stored at 4°C.

Animal Treatment

Healthy male *Balb/C* mice (*Mus musculus*), aged 5-6 weeks-old, weighed 20±3 g, were obtained from Prof. Nidhom Foundation Surabaya, East Java. Mice were acclimatized for 7 days before being randomly divided into two major groups: normal diet (ND) and high-fat high-fructose diet (HFFD). Mice in ND and HFFD groups were fed with diets based on American Institute of Nutrition 93-Growth (AIN-93g) and High Fat Diet 32, respectively, with slight modification (Table 1). In addition, mice in HFFD group were supplied with 10% fructose drink. The diet and drink were given for 12 weeks. After that, mice in HFFD group were administered orally with/without 1.3 mg/kg BW simvastatin (SIM), 5 g/kg BW PY (PY), 2.5 g/kg BW GTY (2.5 GTY), 5 g/kg BW GTY (5 GTY), or 10 g/kg BW GTY (10 GTY) for 4 weeks. The protocol of this animal study was approved by the Research Ethics Committee of Universitas Brawijaya (#155-KEP-UB-2023).

Table 1. Diet for ND and HFFD groups.

Composition	Group	
	ND	HFFD
Fat (%)	9.12	25.11
Protein (%)	6.23	7.15
Carbohydrate (%)	59.68	50.05
Moisture (%)	14.97	7.13
Ash (%)	10.00	10.56

Liver Isolation

At the end of the 16th week, mice were injected intramuscularly (0.05 mL) with combination of 100 mg/kg BW ketamine and 20 mg/kg BW xylazine from Ket-A-Xyl (AgroVet, Lima, Perú) and sacrificed. Liver was collected, weighed, and washed thrice with PBS. One lobe was separated, fixed with 10% neutral buffered formalin from PatChem (BBC Biochemical, Washington, US), paraffinized, sliced and stained with hematoxylin-eosin (HE). The HE staining results were analyzed for percentage (%) of hepatocyte damage (necrosis) by Olympus BX51 microscope (Evident, Shinjuku-ku, Tokyo, Japan) observation at 200x magnification in three different fields.

Hepatocyte Isolation and Immunostaining

Hepatocyte isolation was performed using perfusion methods.[10] Hepatocytes were isolated from connective tissue, filtered using a 70 µm cell strainer and then centrifuged at 2,500 rpm for 5 minutes, 10°C. After centrifugation, the hepatocytes was stained with fluorescein isothiocyanate (FITC) anti-Malondialdehyde (MDA) (Cat. No. ab27615; Abcam, Cambridge, UK,) or anti-Superoxide Dismutase (SOD)1 EP1727Y (Cat. No. ab51254; Abcam).[11] The stained samples were incubated for 20 minutes under dark conditions, resuspended with 400 µL PBS, and read by flow cytometry (FACS Calibur, BD Biosciences, Franklin Lakes, NJ, USA). The hepatocyte population was then gated and analyzed using Cell Quest Pro (BD Biosciences).

Statistical Analysis

Normality and homogeneity of data were analyzed consecutively using the Shapiro-Wilk test and Lavene's test. Then further analysis was performed with one-way ANOVA, followed by Duncan's multiple range test (DMRT).

Results

GTY-LpE1 Improved Liver Morphology in HFFD Mice

The liver of ND group was red and no fatty spots were observed (Figure 1). The liver of HFFD group was pale brown, indicating a high presence of hepatic steatosis. This study demonstrated that the liver morphology of treated group gradually improved compared to ones in the HFFD group. After GTY-administered HFFD treatment, the hepatic steatosis condition gradually improved, evident from changes in liver color and reduced steatosis.

GTY-LpE1 Decreased Necrotic Cells

The hepatocyte of ND group showed a normal cell shape, with visible cell membranes and round or oval nuclei in the center of the cell (Figure 2A). The HFFD group exhibited cell damage, which was characterized by pyknosis,

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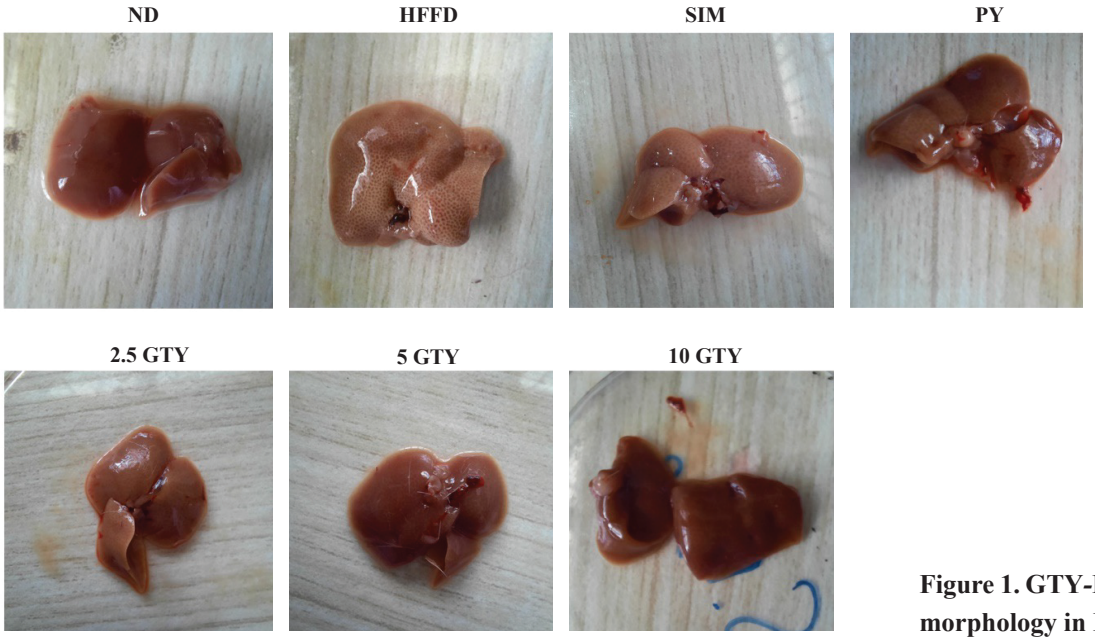

Figure 1. GTY-LpE1 improved liver morphology in HFFD mice.

karyorrhexis, and karyolitic. HFFD group (69.49±5.44%) showed approximately a 4-fold increase in necrotic cells compared with the ones in the ND group (18.17±9.12%). The SIM (34.1±3.10%), PY (25.15±6.75%), and GTY groups showed a decrease in necrotic cells than the ones in the HFFD group (Figure 2B). The necrotic cells were 34.55±3.23%, 34.31±5.7%, and 21.95±0.86% when treated with 2.5, 5, and 10 GTY, respectively.

GTY-LpE1 Reduced MDA Expression in HFFD Mice

HFFD group (33.34±2.25%) showed approximately a 2-fold higher of MDA expression compared with the ones in the ND group (14.79±3.75%) (Figure 3). The MDA expression of SIM group (12.23±1.88%) was not significantly different (DMRT's Post Hoc test, $p<0.05$) than the ones in the ND group. The MDA expression of PY (19.31±3.86%) and GTY group were significantly decrease (DMRT's Post Hoc test, $p<0.05$) than the ones in the HFFD group. The MDA expression were 15.55±2.29%, 18.69±1.89%, 22.42±2.81% when treated with 2.5, 5, and 10 GTY, respectively.

GTY-LpE1 Increased the SOD Expression in HFFD Mice

HFFD group (3.44±1.30%) showed approximately 5-fold lower of SOD expression compared to ND group (18.20±2.21%) (Figure 4). The SOD expression of SIM (6.05±2.90%), PY (10.01±1.64%) and GTY group were significantly increase (DMRT's Post Hoc test, $p<0.05$) than the ones in the HFFD group. The SOD expressions were 9.49±2.10%, 7.85±0.99%, 11.11±2.13% when treated with 2.5, 5, and 10 GTY, respectively.

Discussion

This study showed that HFFD group had more necrotic cells than the ones in the ND group. This is because the imbalance between energy intake and expenditure driven by a long-term HFFD might result in FFA accumulation, leading to steatosis and dysregulation of lipid metabolism.[12] Elevated FFA levels in the liver generates ROS, which damage mitochondria, DNA and proteins, leading to oxidative stress, inflammation, and cell injury in the liver.[4,13]. Elevated ROS levels in the liver induce intracellular alterations, including necrosis. Our results demonstrated that HFFD mice have more necrotic cells than ND mice. The results of statistical tests showed that GTY-LpE1 treated group was able to significantly reduce the number of necrotic cells.

A previous study reported that green tea microencapsulation could protect against ethanol-induced gastric injury in mice. The phenolic compounds from green tea might have a gastroprotective effect in reducing the lesion caused by ethanol, and the microencapsulation process maintains bio-accessible green tea polyphenols after simulated gastrointestinal digestion.[14] Also, fermented mixed tea was reported to prevent hepatic steatosis in HFFD rats due to microsomal triglyceride transfer protein's activation and inhibiting some proinflammatory, lipid metabolism-related, and oxidative stress genes.[15]

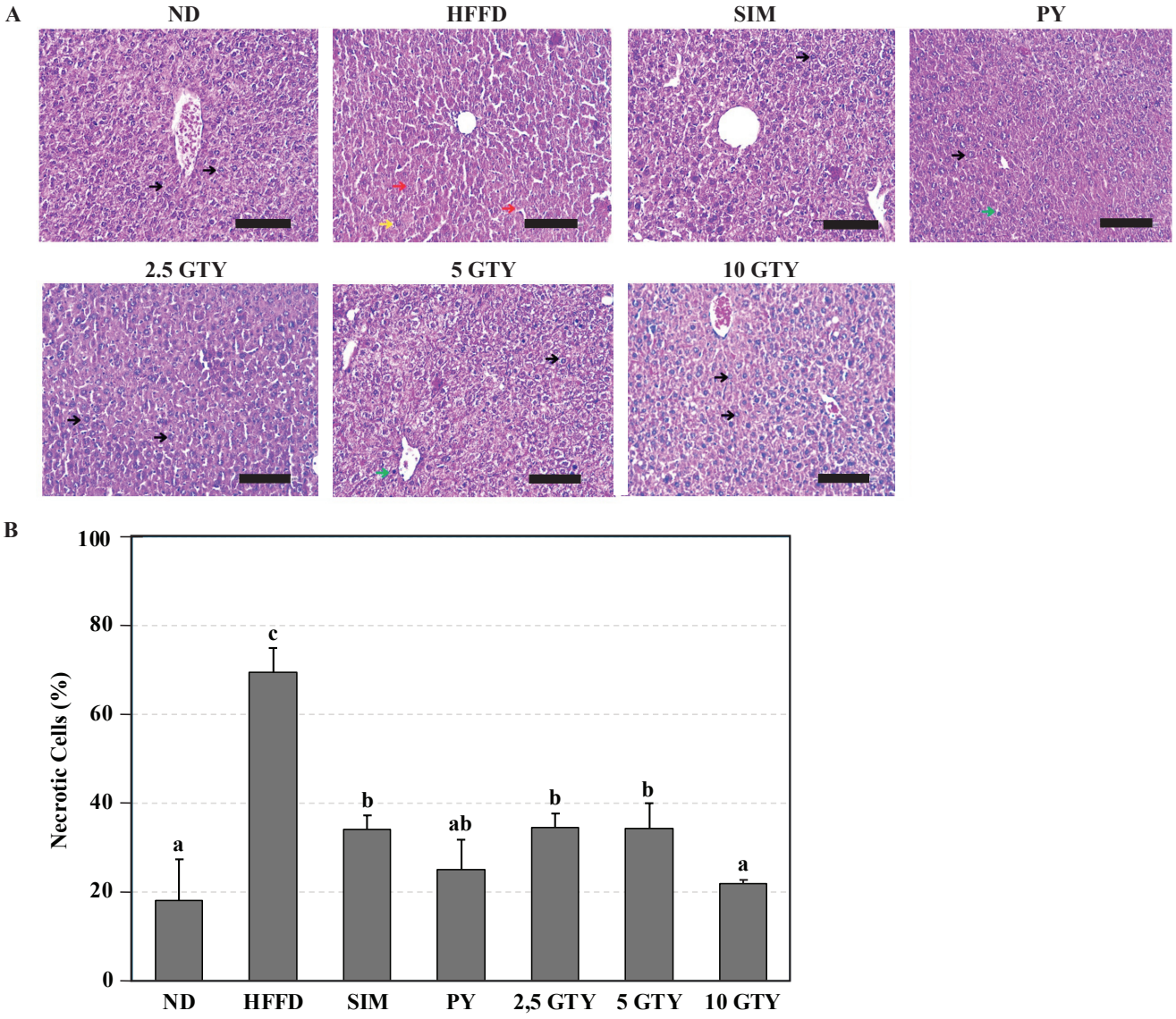

Figure 2. Overview of hepatocyte change caused by HFFD. A: Hepatocyte in each group at 200× magnification and 100 µm scale (black line). B: Number of necrotic cells in each treatment. Necrotic cell counting was performed in three different fields of view in each treatment. →: Normal; →: Karyolysis; →: Karyorrhexis; →: Pyknosis. Data are presented as mean and standard deviation. Different letters indicate a significant difference between groups based on the DMRT test.

This study aims to determine the effect of GTY-LpE1 administration on the expression of SOD, MDA, and liver damage due to obesity in mice. The results of this study are in accordance with previous studies which stated that in oxidative stress conditions in HFFD mice, the production of MDA which is the end product of lipid peroxidation, increases in the liver of HFFD mice, while the activity of antioxidant enzymes such as SOD decreases in the liver of HFFD mice.[16,17] Therefore, the expression of MDA and SOD may serve as indicators of the severity of HFFD mice.

Interestingly, a previous study reported that green tea fermentation by L. *paracasei* NTU101 increased the content of epigallocatechin gallate (EGCG), epicatechin gallate (ECG), and chlorogenic acid.[18] The presence of *L. paracasei* in yoghurt has positive effects on the digestive tract health, which can affect the body's antioxidant system and the balance gut microbiota through the release of short-chain fatty acid (SCFA), which plays important role in assisting metabolism and protecting the immune system.[7,19] Yogurt has good antioxidant content to inhibit the oxidation that can cause cell damage.[20] Therefore, giving PY can reduce the necrotic cells, MDA, and increase SOD.

Administration of simvastatin in this study was able to reduce the number of necrotic cells, reduce MDA expression

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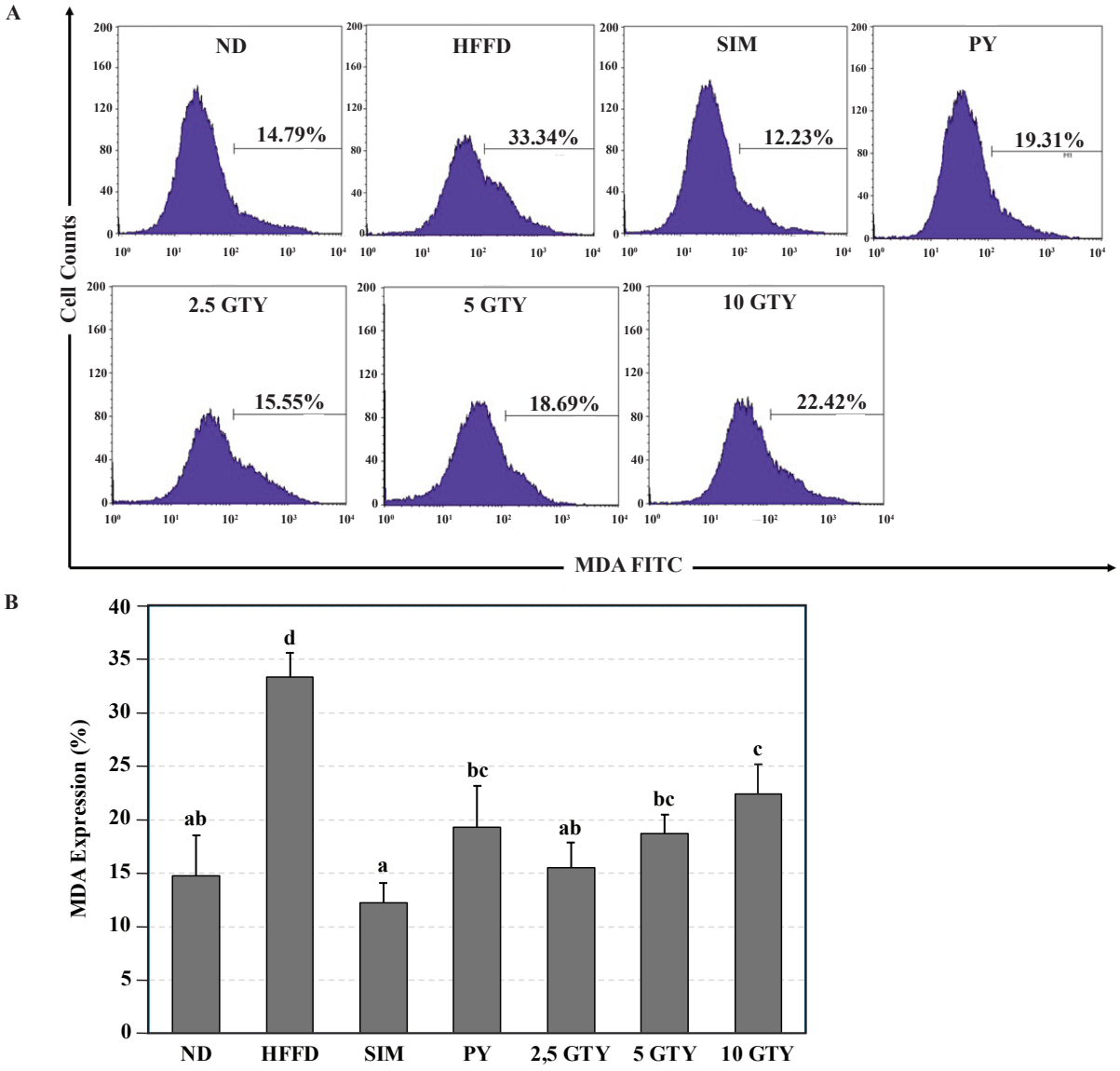

Figure 3. MDA expression in the liver of ND or HFFD group. A: Flow cytometry results. B: The results are presented as mean and standard deviation. Different letters indicate significant differences between groups based on the DMRT test.

and increase SOD expression. Simvastatin was able to convert 3-hydroxy-3-methylglutaryl-coenzyme A (HMG CoA) to L-mevalonate through competitive inhibition of the active site of HMG CoA reductase, which resulted in decreased cholesterol synthesis and increased expression of LDL receptors in the liver.[21] Decreased cholesterol levels will reduce the occurrence of fatty liver, reduce lipid deposition in adipocytes and hepatocytes in the liver, and anti-inflammatory effects that can reduce inflammation that is often associated with obesity.[22] Moreover, green tea polyphenols have been reported to reduce body fat.[22] which is the increase of the green tea polyphenols by LpE1 in the current study might potentially reduce obesity caused by lipid accumulation. Another study reported that *L. paracasei* NTU101 decreased serum and liver total cholesterol in the hypercholesterolemia hamster model.[23] A decrease in total cholesterol might lowering oxidative stress due to lipid peroxidation.[24] Therefore, administration of GTY-LpE1 is able to reduce MDA expression and increase SOD expression.

In this study, it was found that MDA expression in the GTY group was higher compared to the SIM group, even at the highest dose of GTY (10 GTY). When compared to the SIM group with MDA expression closest to the ND group, this is because simvastatin is a drug that is designed to treat obesity by lowering cholesterol levels, therefore simvastatin

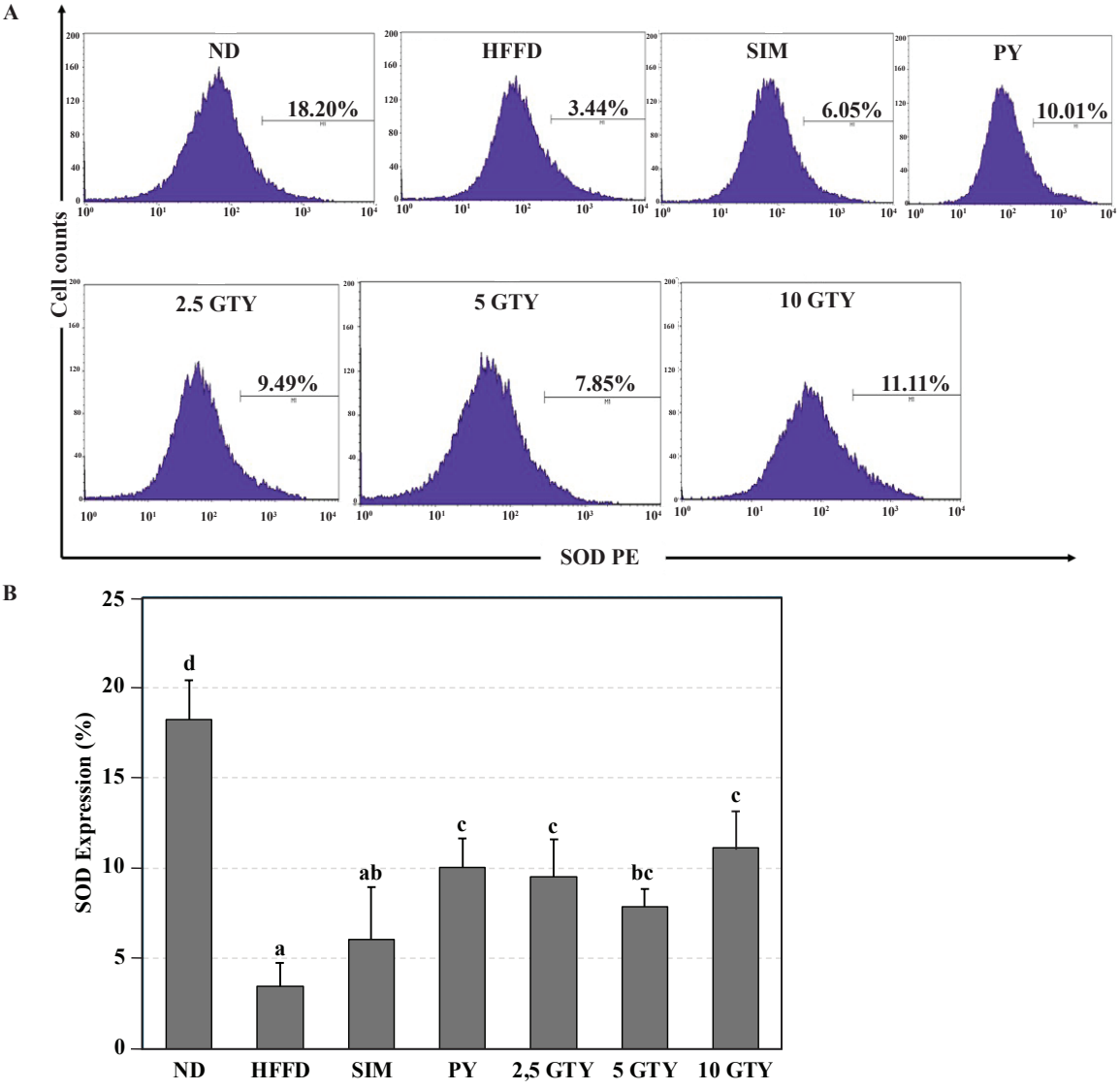

Figure 4. SOD expression in the liver of ND or HFFD group. A: Flow cytometry results. B: The results are presented as mean and standard deviation. Different letters indicate significant differences between groups based on the DMRT test.

is more significant and faster in reducing MDA expression and increasing SOD. While GTY is a food or supplement that only aims to control or as a prevention, not to treat, the effects of which are usually simpler and require consistent and long-term consumption to see substantial changes. In addition, previous studies have stated that simvastatin is able to show antioxidant activity that is not dependent on lipids, which is significantly able to reduce plasma concentrations of F2-isoprostanes and MDA associated with experimental hypercholesterolemia, as well as reduce the concentration of superoxide anion (O_2^-).[25]

In the results related to SOD expression, it was found that in the 5 GTY group there was a decrease in SOD expression and an increase in the 10 GTY group. Generally, many believe that high doses will provide better effects in treatment. To some extent this statement is true, but if sufficient concentrations to overcome the problem cannot be achieved, then damage that is not affected by treatment will grow rapidly to fill the gap vacated when some damage has been overcome.[26] The existence of varying effects on the results obtained can be attributed to a combination of optimal stimulation at lower doses, temporary suppression due to regulatory feedback at medium doses, and adaptive responses leading to new upregulation at higher doses. This relationship between dose administration and response obtained shows the complexity of the interaction between

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bioactive compounds, probiotics, and the body's antioxidant defense system. Therefore, determining whether a low-dose, medium-dose, or high-dose strategy is most appropriate will depend on the effects of each option on health and on considerations of the drug, its formulation, and its pharmacokinetics and pharmacodynamics. So the right dose does not have to be the highest dose.[26]

An increase in SOD as an antioxidant is followed by a decrease in lipid peroxidation. SOD is an important antioxidant defense system in protecting cells from oxidative damage mediated by free radicals.[27] Obesity was reported to reduce the mRNA expression and activity of antioxidant enzymes such as SOD in white adipose tissue.[17] MDA is one of the highly reactive products produced by increased lipid peroxidation.[28] In this study, HFFD group have higher MDA expression than the ones in the ND group, this result is in accordance with previous studies which stated that green tea reduces oxidative stress on the liver by increasing hepatic catalase and glutathione peroxidase activity, which reduced hepatic steatosis.[29] Green tea polyphenols could increase SOD expression and inhibit endoplasmic reticulum stress.[30] SOD is an enzymatic antioxidant that works by converting superoxide anion (O_2^-) into hydrogen peroxide (H_2O_2), which is more stable.[31]

In green tea, there is epigallocatechin gallate (EGCG), which activates antioxidant enzymes by triggering Nrf2 signaling. Nrf2 is a basic leucine zipper redox-sensitive transcription factor that controls the redox state of cells under harmful stress.[32] Under oxidative stress, a conformational change in Kelch-like ECH-associated protein (Keap)1 releases its binding to Nrf2, allowing Nrf2 to enter the nucleus and activate antioxidant gene expression via antioxidant response elements (AREs).[33] EGCG interacts with Keap1's cysteine residues, promoting Keap1's dissociation from Nrf2's DLG domain. In the nucleus, Nrf2 forms heterodimers with Maf proteins, binding to AREs to activate antioxidant enzymes.[34]

Based on the findings of this study, GTY-LpE1 is suggested as a potential treatment for preventing oxidative stress caused by HFFD. Some limitations of this study should be acknowledged. First, this study used the Lee Index to determine obesity groups in experimental animals, which is a very popular and easy to perform method. However, the Lee Index only measures body weight in relation to body length and does not account for the distribution of fat in different body parts. As a result, animals with large muscle mass but low body fat content may be incorrectly categorized as obese. Therefore, it is necessary to measure the levels of HDL and LDL in ND and HFFD mice to obtain more information about changes in lipid profiles. Second, this study only used MDA as a biomarker of oxidative stress and SOD as a biomarker of antioxidant activity and did not assess other biomarkers. Therefore, further research is needed with more varied biomarkers to obtain more accurate results and to gain a deeper understanding of the mechanism of action of GTY-LpE1.

Conclusion

GTY-LpE1 could improve the hepatocyte damage in HFFD mice by reducing MDA expression and increasing SOD expression. In addition, GTY-LpE1 could be an alternative of functional foods that are useful for restoring the oxidative stress caused by HFFD.

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Authors Contributions

MR, YDJ, AS, MFA, and SNA involved in conceptualization, research planning and funding. DNF, RI, BNAF, SAK, EA, NAMS involved in data analysis and writing the manuscript. SOW and WB contributed to the validation of histology data. MR, SOW, WB, YDJ, AS, MFA, SNA, DNF, RI, BNAF, SAK, EA, and NAMS conducted the experiments, discussed the results and provided critical revisions.

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