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Effect of Probiotic Supplementation on Sprague Dawley Rat Liver Histopathology Fed by High Fat High Fructose Diet

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Abstract

Background: Nonalcoholic fatty liver disease (NAFLD) is an important cause of liver diseases. Gastrointestinal microbiota dysbiosis elicits and aggravates NAFLD into Non-Alcoholic Steatohepatitis (NASH) and cirrhosis, increasing morbidity and mortality. Microbial manipulation such as probiotic supplementation has been for to treat NAFLD and prevent reverse its progression. However, the study about the protective effect of probiotics on NAFLD is still limited.

Objective: This study aimed to evaluate the effect of probiotics on liver histopathology *Sprague-Dawley* rats which given a High-Fat-High-Fructose (HFHFr) diet

Methods: This study was a post-test-only control study group design. The samples were 21 *Sprague-Dawley* male rats in 7 – 8 weeks of age and were divided into three groups.The Control Group (C) was provided with a standard chow diet. The Non-Probiotic (NP) group was given a HFHFr diet for eight weeks. The Probiotic group (P) was given a HFHFr diet for eight weeks, and a combination of HFHFr and probiotic consisted of *Lactobacillus acidophilus, Bifidobacterium longum,* and *Streptococcus thermophilus* for the next eight weeks. Histopathological samples were obtained from liver biopsy to assess NAFLD activity score (NAS), and fibrosis stages. We analyzed the difference in histopathological results using the Fisher's Exact Test.

Results: We found a significant difference in NAFL and NAS Score between NP and P group. The P group was shown to have lower trends for NAFLD and NASH than the NP group, but not for fibrosis.

Conclusion: Probiotics supplementation reduced steatosis and inflammation of the liver

Keywords: *Probiotic; NAFLD; Fibrosis; Histopathology*
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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) has emerged as one of the most common chronic liver diseases in the paediatric population.[1] In patients with NAFLD, 16 % will develop advanced fibrosis, and 9.3% developing end-stage liver disease[2]. Non-Alcoholic Fatty Liver Disease (NAFLD) is a group of disorders characterized macrovesicular hepatic steatosis, fibrosis, and end-stage liver disease and occurs in individuals without association with alcohol consumption.[3]

Several risk factors associated with induction and development of NAFLD and Nonalcoholic Steatohepatitis (NASH), such as type 2 diabetes mellitus, dyslipidemia, and metabolic syndrome. Diets associated with those conditions including High-Fat-High-Fructose diet, will also affect liver function and development into NAFLD or NASH. High concentration of sugar and fat induces hepatic de novo lipogenesis. This type of diet also gives rise for endoplasmic reticulum stress, promoting mitochondrial dysfunction and elevating hepatocytes apoptosis. [4,5]

Alterations in gut microbiota composition and function negatively impacts the host (dysbiosis) and play causal roles in developing NAFLD.[6] As the liver receives major blood supply from the intestine, it has a particular susceptibility of intestinal microbiota metabolite.

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Dysbiosis caused endotoxins and toxic metabolite production that compromised intestinal permeability, increasing the entry of bacterial metabolites and components into the liver through the portal vein.[7] The microbiota can aggravate NAFLD through various mechanisms, one of them is by influencing short-chain FAs (SCFAs) synthesis and lipogenesis, increasing liver fat deposition and resulting in steatosis.[8] In a study in which gut microbiota collected from obese donors were transplanted into germ-free mice, the mice that received gut microbiota before donor's weight loss had higher levels of hepatic triglyceride and cholesterol than mice that received post-weight loss gut microbiota.[6] This mechanism, combined with inflammation caused by toxic microbiota product (especially lipopolysaccharide), will damage the liver, progressing into NASH, cirrhosis, or even hepatocellular carcinoma.[9]

Specific measures to "balance" the intestinal microbiota to its normal state are widely studied.[10] Studies showed the gut microbiota has a crucial role in the pathogenesis of NASH/NAFLD, support the hypothesis that microbiota manipulation is a potential and effective therapy as an alternative in the management of NAFLD/NASH.[10] Probiotics, by WHO, are defined as live microorganisms which when administered in adequate amounts.[11] In several experiments with murine models, probiotic supplementation improved liver histology, reduced liver fat, decreased serum ALT, and reduced liver fibrosis.[9] However, there was still limited data regarding the hepatoprotective effect of probiotic supplementation.

This study aims to determine the probiotic's role as protective agents against NAFLD and NASH in murine-models.

MATERIALS AND METHODS

We performed a murine-model post-test only control study group design conducted in Animal Experimental Laboratory in Faculty of Medicine Airlangga University, Surabaya, Indonesia. Bodyweight and the length of all rats were measured before the experiment. The samples were 21 *Sprague-Dawley* male rats at 7 – 8 weeks of age. After one week of acclimatization, we divided those samples into three groups. The Control Group (C) was provided with a standard chow diet for eight weeks. The Non-Probiotic (NP) group was given High-Fat-High-Fructose (HFHFr) diet for eight weeks. The Probiotic group (P) was given HFHFr diet for eight weeks, and a combination of HFHFr and probiotic supplementation consisted of *Lactobacillus acidophilus, Bifidobacterium longum,* and *Streptococcus thermophil*[6] for the next eight weeks. HFHFr diet consists of 59% of energy derived from fat, 30% from carbohydrates, and 11% from protein, and standard chow diet with 10% of energy derived from fat, 30% from protein, and 60% from carbohydrates.[12]

After treatment, all the rats were measured for post-test body weight and sacrificed. Histopathological samples were obtained from liver biopsy. We made 4µm slides of liver tissue and then stained them with routine hematoxylin-eosin and Masson stain to see the fibrosis. According to Kleiner [17]l., histopathological examination for assessing NAFLD Activity Score (NAS) is comprised of steatosis, lobular inflammation, cellular ballooning, and fibrosis. (see table 1) We further evaluate the non-alcoholic fatty liver status by using the 5% steatosis as a cut-off point. NAFLD is defined by the presence of steatosis in >5% of hepatocytes, using only the 'steatosis grade' component of NAS. We also evaluate the NAS Score into three categories: Not NASH (Score 1 – 2), Borderline (Score 3 – 4), and NASH (Score 5 – 8). We also categorized the fibrosis stage using Kleiner score criteria.[13]

We presented the data in mean and standard deviation. Histopathological data is shown in cross-tabulation. Statistical analysis was carried out using the IBM SPSS Statistics 25.0 statistical program. We measured the difference in histopathological results using the Mann-Whitney test. Significant difference marked with p-value less than 0.05

This study protocol was approved by Ethical Committee of Faculty of Medicine, Universitas Diponegoro, Semarang, Indonesia No.82/EC/H/KEPK/FK-UNDIP/V/2019.

RESULTS

In this study, 21 rats were intervened and analyzed for histopathological analysis. Anthropometric measurements were shown in table 1. We found no significant difference between pre-test and post-test body weight. ($p > 0.05$) (see table 2)

Our histopathology results (see figure 1) were presented in cross-tabulation. Our control group did not develop NAFLD and NASH, although rats in those groups developed stage I fibrosis. We also found that in the probiotic group, there were fewer subjects developing NAFLD (see table 3), NASH (see table 4), and stage II fibrosis (see table 5)

Fisher's Exact analysis showed that compared to the NP group, the P group was shown to have lower trends for NAFLD ($p = 0.001$) and NASH ($p = 0.001$). It is shown that the P group had a lower trend for the fibrosis stage, although not statistically significant. ($p = 0.500$)

DISCUSSION

In present study we found that 8 weeks of HFHFr diet was already able to induce NAFL in our NP group. This finding was in parallel with a German murine-model study that found 8 weeks of HFHFr diet increased hepatic fat deposition. The same study also discovered altered intestinal barrier function in mice given the diet.[14] A study in China found that high-fat diet induced dysbiosis in gastrointestinal microbiota and significantly increased the risk of metabolic syndrome, as shown by increased body weight, decreased insulin sensitivity, and marked dyslipidemia.[15] It was supported by the fact that in dysbiosis, the altered gut microbiota used choline for metabolism, reducing free choline and inhibiting very-low-density lipoprotein (VLDL) excretion from the liver. Combined with increased intake of fat, especially short-chained fatty acid, excessive fat deposition was inevitable.[16] We hypothesized that the HFHFr diet in our study was able to induce dysbiosis . We found no rats in P group had steatosis at the end of our treatment. This finding supported the fact that in this study, probiotic supplementation was able to reverse NAFL process. Our

Table 1. NAFLD Activity Score (NAS) Scoring System

Item	Score	Extent
	NAS Components	
Steatosis Grade	0	< 5%
	1	5 – 33%
	2	>33 – 66%
	3	> 66%
Lobular Inflammation	0	No Foci
	1	<2 foci / 200x
	2	2 – 4 foci / 200x
	3	>4 foci / 200x
Hepatocyte Balooning	0	None
	1	Few balloon cells
	2	Prominent ballooning
	Fibrosis Stage (Evaluated separately from NAS)	
Fibrosis Stage	0	None
	1	Perisinusoidal or Periportal
	2	Perisinusoidal and portal/periportal
	3	Bridging fibrosis
	4	Cirrhosis

Table 2. Anthropometrics Characteristic of subjects

Group	Baseline Length	Pre-Test Weight	Post-Test Weight	p
		Mean ± SD		
C (n = 7)	15.65 ± 0.18	146.00 ± 37.60	169.29 ± 37.46	0.128*
NP (n = 7)	15.20 ± 0.22	134.29 ± 18.62	141.00 ± 14.92	0.203*
P(n = 7)	16.00 ± 1.00	118.43 ± 2.23	123.29 ± 11.57	0.203*

*Wilcoxon Test between Pre-Test and Post Test Weight

result was in line with several studies, as probiotic supplementation showed a significant decrease in NAFLD scores, steatosis, and improvement in the liver enzyme.[17, 18]

We found that eight weeks of probiotics supplementation significantly reduce the NAS Score criteria (from NASH to Borderline) on all rats in the P group. This finding was in line with a previous study that significantly reduced inflammatory markers and steatosis following probiotic supplementation.[19] Interestingly, despite the non-NAFLD status of all the P group rats, the liver histology is still classified with borderline, signifying some degree of inflammation. The key factors that play a role in converting mild fatty liver to NASH have not yet been determined. Some key factors include oxidative stress, lipid peroxidase, reactive metabolites, adipose tissue products, and gut microbiota dysbiosis.[20] These evidence were supported by a clinical trial in NASH patients treated with antibiotics. The patients' NASH degree were significantly lowered followed by microbiota change detected in stools.[21] We hypothesized that dysbiosis induced by HFHFr diet might develop NASH, and eight weeks of probiotic supplementation only managed to reduce the steatosis, resulting in the 'Borderline' result in our probiotic group's NAS Score. We thought that our probiotic supplementation duration was not long enough to decrease the inflammatory process, as shown in other studies that supplied probiotics for a longer period.[19]

In addition, our study discovered that although insignificant, probiotic supplementation can decrease liver fibrosis. Another study also found that probiotic improved liver histology, reduced liver fat, decreased serum ALT, and reduced liver fibrosis.[9] Furthermore, another research found that probiotics supplementation also decreased the number of proinflammatory cytokines that also had a role in fibrosis.[22] We suggested that in this study, a significant decrease of NASH and fibrosis were mediated by this mechanism.

Interestingly, our study did not find any significant weight increase after the HFHFr diet. This finding was different from another study, as the samples were able to have a significant weight gain in the same amount of time.[14] We thought that the main trigger for liver damage (and resolution) in our samples was mainly caused by dysbiosis. Bacterial overgrowth and increased translocation of bacteria to the portal and systemic circulation, increased lipopolysaccharide (LPS) levels, increased endotoxin, and activation of proinflammatory signals (TNF-α and IL-6) are seen in various chronic liver diseases, including NAFLD.[23] Probiotics supplementation improved gut mucosa permeability, as showed by increased expression of occluding and claudin-1 expression as vital markers of tight-junction integrity.[24]

There were limitations of this study. The rats were fed *ad libitum,* so there were no data regarding daily/mean calorie intake for each group. Measurement of metabolic and inflammatory markers, as well as gut microbiota on each rat was not done in this study. We suggested a standardized calculation of dietary intake in each rat for better understanding the food dose in each group. For better understanding of the pathophysiological process, we advised in future studies to include the measurement of gut microbiota and other related biomarkers.

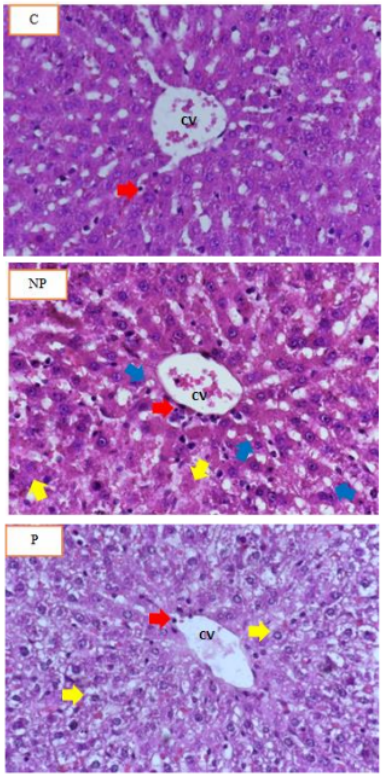

Figure 1. Liver histopathological slides from each group (H.E. stained, 200x
C = Control, NP = Non-Probiotic, P = Probiotic, Red Arrow = Inflammatory cell infiltration, Blue Arrow = Steatosis, Yellow Arrow = Ballooning)

Table 3. Steatosis Grade Interpretation According to Group

		Steatosis Grade Interpretation		P
		Not NAFL	NAFL	
Group	**C**	7	0	
	NP	0	7	**0.001***
	P	7	0	

*Fisher's Exact Test

Table 4. NAS Score Interpretation According to Group

		NAS Score Interpretation			P
		Not NASH	Borderline	NASH	
Group	**C**	7	0	0	
	NP	0	0	7	**0.001***
	P	0	7	0	

*Fisher's Exact Test

Table 5. Fibrosis Stage According to Group

		Fibrosis Stage		P
		Stage 1	Stage 2	
Group	**C**	7	0	
	NP	6	1	0.500*
	P	7	0	

*Fisher's Exact Test

CONCLUSION

In conclusion, eight weeks of probiotic supplementation reduced NAFLD incidence and decreased NAS score in *Sprague-Dawley* rats induced by the HFHFr diet. The results of this study are expected to provide new insights for alternative medical treatment for patients with NAFLD. However, it is necessary to do further research on humans as subjects, with a longer probiotics supplementation time for more significant results, and consider the probiotics choice.

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