Effects of probiotics and high fructose diet on De Ritis ratio, TGF-B levels, and liver histopathology of Sprague Dawley rats

By Ninung Rose Diana Kusumawati

Effects of probiotics and high fructose diet on De Ritis ratio, TGF-B levels, and liver histopathology of Sprague Dawley rats

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ABSTRACT

Background: Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease. Histopathological diagnosis has been the gold standard for diagnosing and determining the severity of non-alcoholic steatohepatitis (NASH). However, this method is invasive, especially for diagnosing NASH in children. This research aimed to study the effect of probiotics on the De Ritis ratio, TGF-B levels, and histopathological changes in rats given High Fat, High Fructose (HFHFr) diet to look at the correlation between the De Ritis ratio and liver histopathology in diagnosing NAFLD.

Method: Twenty-one male Sprague Dawley rats were included and divided into three groups. In the first group, as control only received a normal chow diet for eight weeks. The second group received an HFHFr diet for eight weeks, and the last group received HFHFr for eight weeks and a combination of HFHFr and probiotics for another eight weeks. After decapitation, we took 3 ml of serum from each rat to measure AST, ALT, and TGF-B. A 4 μm thick liver tissue slide was taken and stained with Hematoxylin – Eosin Stain for histopathological analysis. We used the Non-Alcoholic Steatohepatitis (NAS) Score for measuring liver damage progression.

Results: De Ritis ratio and TGF-B level did not significantly differ between probiotic and non-probiotic groups (p = 0.064 and 0.383), but there was a significant NAS score difference (p = 0.001) in probiotic and non-probiotic groups. This was followed by a significant correlation between the De Ritis ratio and the NAS score (r = 0.613, p = 0.003).

Conclusion: Probiotic supplementation alleviated liver damage caused by the HFHFr diet but did not successfully reduce the De Ritis ratio or improve TGF-B.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease. Its increase in prevalence and severity correlates with the rise in obesity and metabolic syndrome.[1] NAFLD is a multisystem disease affecting extrahepatic organs, and it has a long-term impact on health which extends into adulthood and causes significant morbidity and mortality.[2] NAFLD encompasses a broad spectrum of manifestations, from isolated hepatic steatosis without inflammation to advanced form non-alcoholic steatohepatitis (NASH) with histologic features of inflammation and fibrosis, which may lead to cirrhosis and end-stage liver disease.[3]

Gut dysbiosis, or imbalance in normal gut microflora, has been known as one contributor to NAFLD progression. Abnormal gut microbiota compromised intestinal epithelial junction and increased mesenteric capillary permeability, leading to the leaking of lipopolysaccharides (LPS) and other pathogen-associated molecular patterns (PAMPS) into the portal circulation.[4] This triggers hepatic inflammatory response and fibrosis in the liver. As dysbiosis plays an essential role in NAFLD pathogenesis, probiotics administration has been widely researched as one of the treatment options for NAFLD and NASH prevention, as probiotics theoretically can rebalance intestinal microbiota. It has been found that probiotics positively contribute to preventing obesity, preventing NASH progression, and reducing liver inflammation.[5,6]

On a molecular level, Transforming Growth Factor (TGF) signaling is considered one of the most critical pathways in the development of liver disease, particularly NAFLD. TGF-β signaling is a core regulator of fibrosis, activating myofibroblasts, leading to excessive ECM production and inhibition of ECM degradation.[7]

Histopathological diagnosis has been the gold standard for diagnosing and determining the severity of NASH. However, this method is invasive, especially for diagnosing NASH in children. There is a consensus that liver function enzymes, such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT), are

biomarkers that reflect disease severity in a number of chronic liver diseases.[1] The ratio of the serum activities of AST and ALT was first described by Fernando De Ritis in 1957 and has been known as the De Ritis ratio. The release of ALT and AST from liver cells to the bloodstream represents hepatocellular damage or death.[8]

This study aims to evaluate the effect of probiotics in rat models fed with High Fat High Fructose (HFHFr) on the De Ritis ratio and liver histopathological change and better analyze the De Ritis ratio in conjunction with hepatic pathological changes.

METHODS

Research Design

This true experimental study with a completely random design was carried out in Animal Experimental Laboratory in the Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, East Java, Indonesia. This research was approved by the Ethical Committee of the Faculty Medicine, Universitas Diponegoro, Semarang, Indonesia (No.82/EC/H/KEPK/FK-UNDIP/V/2019).

Twenty-one male *Sprague-Dawley* rats aged 49 – 56 days weighing 110 – 200 grams each were included in this study. We excluded rats that were sick, shown by the exudate from body orifices and the rats being inactive. All rats were acclimated for one week at room temperature ranging from 20°C to 24°C and given a normal (chow) diet and water *ad libitum*. After acclimatization, we randomly divide these rats into three groups:

a. The control (C) group was provided with a normal chow diet for eight weeks.
b. The non-probiotic (NP) group was given with High Fat and High Fructose (HFHFr) diet for eight weeks.
c. The probiotic (P) group was given the HFHFr diet for eight weeks, and for extra eight weeks, the group was provided with the combination of the HFHFr diet and the probiotic solution containing *Lactobacillus acidophilus, Bifidobacterium longum,* and *Streptococcus thermophiles*.

By the end of the treatment for each group, all rats were decapitated. Blood and histological sample were taken after decapitation.

Laboratory Analysis

We took 3 milliliters of blood from each rat, kept the sample overnight at -40ºC temperature, and then centrifuged it for 20 minutes at 1000 rpm. We then took the serum for laboratory parameter measurement. AST and ALT levels were measured using an enzymatic method, and then AST levels were divided by ALT levels to get AST/ALT ratio. We used an enzyme-linked immunosorbent assay (ELISA) test using the Abcam Rat TGF beta 1 ELISA kit to measure the TGF-β levels.

Histopathological Analysis

A 4 µm thick liver tissue slide was taken from each rat. From each liver, we made two tissue samples. Those samples were stained with hematoxylin-eosin for NAS-Score analysis and Masson stain to see the fibrosis. According to Kleiner et al., the NAS Score comprises three criteria: steatosis, lobular infiltration of inflammatory cells, and hepatocyte ballooning.[9] By adding the score from the three components, the final result will be divided into not steatohepatitis (score 1 – 3), possible/borderline (4 – 5), and definite steatohepatitis (6 – 8).[9]

Statistical Analysis

Results are expressed as mean and standard deviation from each group. Statistical analysis was performed using IBM SPSS Statistics 25.0. De Ritis ratio, TGF-β levels, and NAS Score mean the difference between non-probiotic and probiotic groups were analyzed with Mann-Whitney analysis. We also measured the correlation between those three variables using Spearman's Rho analysis. P-values <0.05 were considered statistically significant.

RESULTS

Our samples characteristic are presented in Table 1. There were not any dropout rats in this study.

Liver biopsy results are shown in Figure 1 and Figure 2. None of the rats in the control group had a NAS score of 3 or higher in this study. As in the NP group, all rats had undergone NASH because the NAS Score was 5 or higher. In the probiotic

group, six rats had a NAS Score of 3, and one rat had a NAS of 4. NAS Score was statistically different between NP and P groups (p = 0.001; Mann-Whitney Test).

Among the 3 groups, the De Ritis ratio was significantly different in all groups. (p = 0.007, Kruskall Wallis Test). Further investigation showed that the De Ritis ratio was not significantly different in NP and P groups. (p = 0.064; Mann-Whitney Test, see Figure 3).

In the control groups, TGF-β levels were not detected as they were secreted lower than the detection threshold, so we provided the data as zero. TGF-β level was not significantly different between NP and P group. (p = 0.383; Mann-Whitney Test, see Figure 4).

Spearman's correlation test showed that the De Ritis ratio was not correlated with TGF-β but was moderately correlated with the NAS score (Table 2).

DISCUSSION

In our study, the De Ritis ratio (AST/ALT ratio) were significantly different in all group. De Ritis ratio was not significantly different in Non-Probiotic (NP) and Probiotic (P) groups. De Ritis ratio was moderately correlated with NAS score. Contrary, probiotic supplementation was shown to reduce inflammation and steatosis in this study, as shown by the NAS score being significantly lower in the P group.

NAFLD refers to fat accumulation in the liver, starting as simple steatosis, which then progresses to non-alcoholic steatohepatitis (NASH), liver fibrosis, and cirrhosis. Although the initial stage is usually mild, NASH is a potentially serious condition; as many as 25% of patients with NASH may develop cirrhosis.[10]

Serum AST and ALT increased with increasing body weight, but the increase in ALT was more dominant than AST. Most cases of elevated ALT can be attributed to overweight (body mass index [BMI] 25 kg/m2) and obesity (BMI 30kg/m2).[10]

In NASH patients, the AST/ALT ratio is <1, mainly in morbidly obese patients. It corresponds to our study in which AST/ALT ratio is 0.415 in the NP group and 0.408 in the Probiotic group. Although not significantly different, the increase in AST/ALT ratio in the non-probiotic group

Table 1. Subjects' Characteristics.

Variables	Control (n = 7)	Non-Probiotic (n = 7)	Probiotic (n = 7)
	Median (minimum-maximum)		
Weight (gram)	122 (119 – 202)	141 (112 – 159)	119 (115 – 121)
Length (cm)	15.6 (15.5 – 16.0)	15.2 (15.0 – 15.5)	16.0 (15.0 – 18.0)
AST (ng/mL)	30.1 (25.0 – 30.6)	21.8 (20.5 – 22.8)	20.8 (16.8 – 21.5)
ALT (ng/mL)	78.5 (75.8 – 79.8)	50.7 (47.8 – 52.7)	50.6 (47.8 – 51.2)
De Ritis ratio	0.383 (0.32 – 0.40)	0.415 (0.41 – 0.47)	0.408 (0.35 – 0.42)
TGF-β (ng/L)	0.000 (0.00 – 0.00)	1259 (1179 – 1334)	1310 (1185 – 1524)
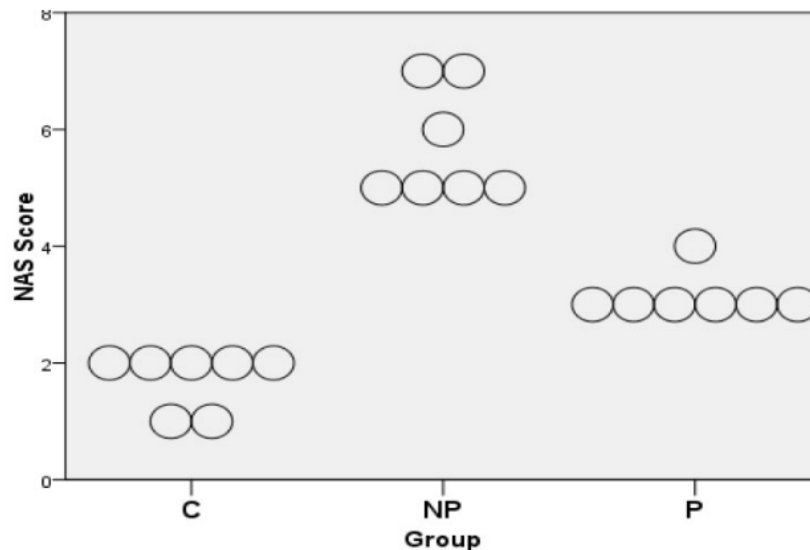
Figure 1. NAS Score Results (C = Control; NP = Non-Probiotic; P = Probiotic).
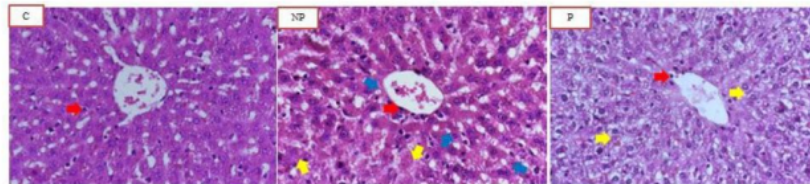
Figure 2. Liver Histopathology Results (C = Control; NP = Non-Probiotic; P = Probiotic). Blue arrows point at steatosis, Red arrows show lobular inflammation, and yellow arrows show ballooning degeneration.

showed more advanced fibrosis than in the probiotic group. In concordant with the previous study, the AST/ALT ratio was not significantly different across fibrosis stages.[10]

This study found a significant decrease in NAS scores after eight weeks of probiotic treatment. This finding was in line with another study on the NASH animal model. Twelve weeks of high fat and high sucrose diet with the combination of probiotics significantly reduced hepatic steatosis, necrosis, and degeneration; when compared with the non-probiotic groups.[11] Probiotics worked by competing against pathogenic bacteria in the dysbiotic gut microenvironment and improving intestinal tight junction. This mechanism protects the liver against the leakage of proinflammatory molecules that could worsen the NAFLD.[12]

In this study, we did not find any significant difference in TGF-β levels between NP and P group. In our study, the De Ritis ratio was not correlated with TGF-β. Among the proinflammatory cytokines, transforming growth factor-β (TGF-β) is the main pro-fibrogenic

cytokine that plays an important role in developing liver fibrosis. TGF-β will activate Hepatic Stellate Cell (HSC) Elevation, promote the differentiation of myofibroblasts (MFB), and induce excessive extracellular matrix (ECM) production in the liver. TGF-β is a key pro-fibrogenic cytokine involved in the pathogenesis of liver fibrosis. Over-expression or overproduction activates HSCs and induces ECM synthesis in the liver. TGF-β induces collagen deposition in mice in an in vivo study and human HSC in an in vitro study.[13,14] TGF- can induce collagen deposition, profibrogenic markers, and anti-inflammatory cytokines in human LX-2 cells by activating TGF-β signaling pathways, autophagy, and apoptosis. A study revealed that pretreatment with probiotics had a beneficial effect on LX-2 cells in response to TGF-β. In addition, probiotics can reduce collagen deposition, pro-fibrogenic factors, and inflammatory cytokines/chemokines in LX-2 cells.[14]

The insignificant difference in TGF B levels in the NP and P groups in our study may be influenced by different strains and the duration of probiotic administration. In the previous study, Kanmani P and Kim H used *Lactiplantibacillus plantarum*, *Lactobacillus brevis*, and *Weissella cibaria* strain, while in our study, using *Lactobacillus acidophilus, Bifidobacterium longum,* and *Streptococcus thermophiles*. These strain differences may have contributed to the different study results.[14]
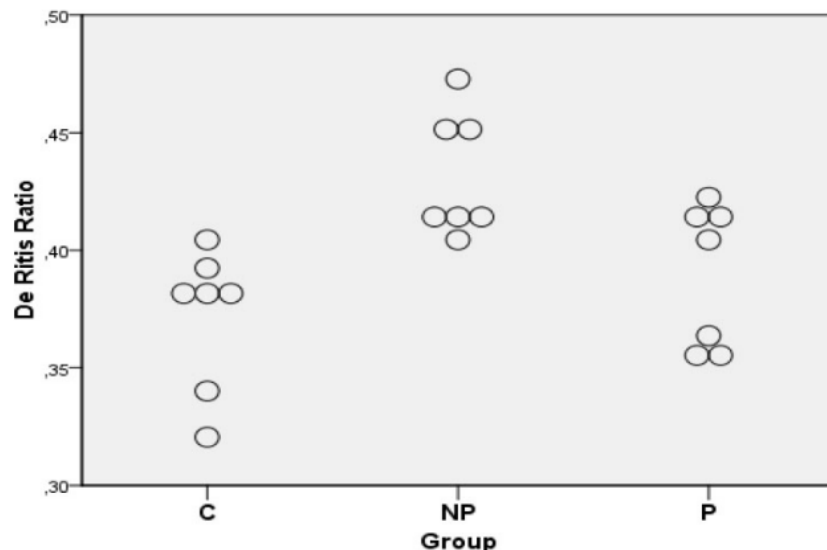
This study had some limitations. We did not measure the gut microbiota composition of each rat, so we could not identify the degree of dysbiosis. We also gave the rats' an ad libitum diet; therefore, the intake for each rat could not be measured. We suggested that in further study, every rat's nutritional intake should be monitored. Gut microbiota analysis can

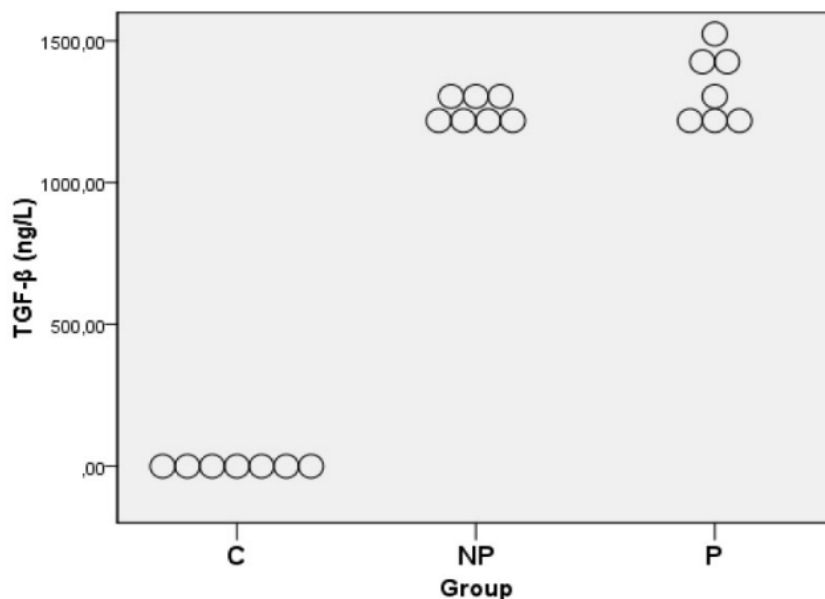also be done to measure the degree and the event of dysbiosis.

CONCLUSION

Our study found that probiotics can prevent the histopathological progression of NAFLD. This study also found an improvement in the De Ritis ratio, although not significant. As for TGF-β, there was no improvement with probiotics. Further research is needed to ascertain the effect of the probiotic strains used in each study.

CONFLICT OF INTEREST

The authors report no conflicts of interest in this work.

ETHICAL CLEARANCE

This research was approved by the Ethical Committee of the Faculty of Medicine, Universitas Diponegoro, Semarang, Central Java, Indonesia (No.82/EC/H/KEPK/FK-UNDIP/V/2019).

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AUTHOR CONTRIBUTIONS

All authors have the same contribution in writing the report on the results of this study, from the stage of proposal preparation, data search, and data analysis to the interpretation of research data and presentation of the final report.

Figure 3. De Ritis ratio result (C = Control; NP = Non-Probiotic; P = Probiotic).

Figure 4. TGF-β result (C = Control; NP = Non-Probiotic; P = Probiotic).

Table 2. Correlation Between TGF-β, NAS Score and De Ritis ratio.

Variable	De Ritis ratio (n = 21)
TGF-β (ng/L)	r = 0.292*
	p= 0.198
NAS Score	**r = 0.613***
	p = 0.003

Spearman's correlation test; significant p< 0.05

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