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Clinical Research

Hepatoprotective Effects of Probiotic Administration on High Fat-High Fructose Diet Induced Nonalcoholic Steatohepatitis in Sprague-Dawley Rats

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Intestinal barrier function, critical for maintaining optimal gut health, is achieved primarily through mucins and tight junction proteins (i.e., zonula occludens-1 and occludin). An aberrant expression of these proteins results in increased paracellular permeability, leading to nonalcoholic steatohepatitis. Furthermore, enhanced expression of the inflammasome's nucleotide-binding domain, leucine-rich repeat-containing protein 3 (cryopyrin), and Toll-like receptor 4 are also associated with nonalcoholic steatohepatitis. To understand the role of probiotic supplementation (*Lactobacillus acidophilus*, *Bifidobacterium longum*, and *Streptococcus thermophilus*) in rats on a high-fat, high-fructose diet with nonalcoholic steatohepatitis, the expression of occludin, cryopyrin, and Toll-like receptor 4 was evaluated. A comparison of the results between the control, nonalcoholic steatohepatitis group, and probiotic-treated nonalcoholic steatohepatitis groups showed (a) a lack of any significant difference in occluding expression (P = 0.724) and (b) a significant reduction in serum Toll-like receptor 4 (P = 0.012) and cryopyrin (P = 0.025) by probiotics. Furthermore, only one rat developed nonalcoholic steatohepatitis in the probiotic group, compared to six rats in the non-probiotic group. In conclusion, there were hepatoprotective effects of probiotic administration on high-fat, high-fructose diet-induced nonalcoholic steatohepatitis in Sprague-Dawley rats.

Keywords: NASH, NLRP3, Occludin, Probiotic, TLR4

Abbreviations Used: Analysis of variance, ANOVA; High-fat-high fructose, HF-HFr; *Lactobacillus rhamnosus* GR-1, LGR-1; Lipopolysaccharides, LPS; Leucine-rich repeats, LRR; Myeloid differentiation factor 88, MyD88; Non-alcoholic fatty liver disease, NAFLD; Nonalcoholic steatohepatitis, NASH; Nuclear factor-kappa B, NF-κB; Nucleotide-binding domain and leucine-rich repeat containing protein 3 (also known as cryopyrin), NLRP3; Nucleotide oligomerization domain, NOD; Pathogen associated molecular pattern, PAMPS; Pathogen recognition receptor, PRR; Tight junction, TJ; Toll-like receptor 4, TLR4

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INTRODUCTION

NAFLD is the most prominent cause of chronic liver diseases worldwide (Younossi et al., 2019). Around 30–40% of people with NAFLD develop NASH, which increases mortality by approximately 5–10% from cirrhosis and/or liver cancer (Jasirwan et al., 2019). Fat deposition in hepatocytes can impair liver metabolism and increase insulin resistance, doubling the risk of type 2 diabetes mellitus (Byrne and Targher, 2015; Sarwar et al., 2018).

A diet high in fat is believed to be one factor associated with liver fat accumulation. A high-fat diet can also affect the intestinal microbiota composition, causing microbial dysbiosis that is closely associated with metabolic disorders, such as central obesity, insulin resistance, dyslipidemia, hypertension, and NAFLD (Flisiak-Jackiewicz and Lebensztejn, 2019; Jiang et al., 2020).

Dysbiosis is an alteration of commensal bacterial composition typically found in healthy individuals (Petersen and Round, 2014). This condition compromises the integrity of the

intestinal tight junction, resulting in bacterial antigens such as LPS and PAMPS entering the liver through portal veins (Safari and Gérard, 2019). Recognition of LPS by TLR4 has been suggested to have potential for anti-inflammatory therapies (Nijland et al., 2014).

Also, PAMPs can trigger an inflammatory response by activating inflammasomes such as NOD, LRR, and NLRP3. A prolonged continuation of such changes combined with increased fat deposition in the liver may lead to chronic inflammation and fibrosis, resulting in NASH and, later, cirrhosis (Henao-Mejia et al., 2012).

TLRs are the most investigated members of the PRR families that can recognize bacterial and viral components (Guo and Friedman, 2010). TLR signaling increases liver injury in chronic liver disease, such as NASH, and its expression results in inflammation and fibrosis (Ferreira et al., 2015). Gram-negative bacterial cells, TLR4 exclusively binds to LPS leading to the promotion of the pro-inflammatory cytokines and chemokines production (Kawasaki and Kawai, 2014).

The NLRP3 inflammasome is a large intracellular multi-protein complex that consists of an inflammasome sensor molecule (typically an NLR) and adaptor proteins, such as the apoptosis-associated speck-like protein containing a caspase-recruitment domain and the precursor pro-caspase-1 (Wan et al., 2016). The NLRP3 inflammasome is essential for host immune defense against bacterial pathogens. Nevertheless, overexpression of NLRP3 can lead to inflammatory disease. It has been shown that inhibition of the NLRP3 inflammasome can ameliorate endothelial gap junction dysfunction (Chen and Vitetta, 2020).

Occludin is largely expressed in the tight junction of the cecum in many organs, including the gastrointestinal tract, where it plays a vital role in maintaining the physical barrier of the intestinal mucosa (Feldman et al., 2005; Hwang et al., 2013). An impaired expression of the occludin protein can be seen in various diseases such as inflammatory bowel disease, irritable bowel syndrome, and extra-intestinal disorders including allergy, asthma, metabolic syndrome, cardiovascular disease, and obesity (Oshima and Miwa, 2016). A high-fat diet negatively impacts gut health by disrupting the barrier system through a variety of mechanisms.

Among others, dietary fat directly modulates tight proteins and dietary fat-induced bile acid production (Rohr et al., 2020; Rose et al., 2021). In brief, the increased intestinal epithelial permeability may contribute to the inappropriate response of mucosal immune cells and the induction of inflammation through an increased transfer of food and bacterial antigens across the intestinal mucosa (Bhat et al., 2018).

As dysbiosis plays an important role in NAFLD pathogenesis, probiotic administration has been widely considered as one of the treatment options for NAFLD and NASH through rebalancing of the intestinal microbiota. Published results show that probiotics positively contribute to preventing obesity, preventing NASH progression, and reducing liver inflammation (Kobyliak et al., 2018; Chen and Vitetta, 2020). However, research regarding the effect of probiotics on intestinal mucosa integrity and their correlation with NASH is still limited. Therefore, we examined the protective effect of probiotic supplementation against impaired intestinal permeability and NASH.

MATERIAL AND METHODS

Animals

Male Sprague-Dawley rats ($n = 21$, 7–8 weeks old, weight: 120–200 g) were housed in the vivarium maintained at 23°C, 50% relative humidity, and a 12/12 light-dark cycle at the Animal Experimental Laboratory in the Faculty of Veterinary Medicine at Airlangga University Surabaya, Indonesia. After 1 week of acclimatization, the rats were divided into three groups and treated as below:

Group I: Control group receiving normal chow diet ad libitum
Group II: Non-probiotic group receiving high fat-high fructose (HF-HFr) diet ad libitum for 12 weeks
Group III: Probiotic group receiving high fat-high fructose diet ad libitum for 12 weeks followed by an additional supplement of probiotic (*Lactobacillus acidophilus*, *Bifidobacterium longum*, and *Streptococcus thermophiles*, containing 1×10^9 cfu per 200 mL for each species) for 8 weeks

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

Histological Examination

At the end of the treatment, the rats were killed by decapitation. The ileum tissues were harvested, washed with 0.9% saline, fixed in 4% formalin, and then embedded in paraffin. The paraffin-embedded tissues were cut into 5 µm sections, deparaffinized in xylene, and rehydrated with a graded series of ethanol. Immunohistochemical visualization of select antigens was performed as described elsewhere (Kim et al., 2016).

The immunohistochemical staining was used to visualize occluding expression. The presence of brown chromogen indicated occludin expression in ileal epithelial cells. The intensity of expression was evaluated by histomorphometry using a modified immunoreactive score that was the average of 10 fields of view at 400X magnification for each sample (Hofmann et al., 2008). Occludin expression was presented using the Remmele immunoreactive score (Remmele and Stegner, 1987).

We also took liver biopsies from each rat to determine the stages of NASH. A 4 µm thick liver tissue slide was taken from each rat and then stained with hematoxylin-eosin and Masson stain to see the fibrosis. The NAS score is comprised of three criteria: steatosis, inflammatory cell infiltration, and fibrosis. The NASH score was categorized into "no steatohepatitis," "possible/borderline steatohepatitis," and "definite steatohepatitis" (Kleiner et al., 2005).

Biochemical Analyses

For laboratory analyses, 3 mL of blood was clotted overnight at 4°C, and then centrifuged for 20 min at 300X *g*. The serum was collected and stored in small aliquots at −40°C until used for TLR4 and NLRP3 assays by ELISA.

Statistical Analyses

Statistical analyses were performed using IBM SPSS Statistics 25.0. The data were presented as the mean and standard deviation. In addition, the Mann–Whitney test was used to analyze the mean difference of TLR-4 and NLRP3 in the non-probiotic and probiotic

groups. We also analyzed the correlation between TLR4, NLRP3, NAS score, and occludin using Spearman's correlation test. A P-value of <0.05 was considered statistically significant.

RESULTS

The data presented in Table 1 show the effect of probiotic treatment on body weight, occluding, NLRP3, and TLR4 in rats on a control as well as on a HF-HFr diet.

The data presented in Fig. 1 show the effect of probiotic treatment (right panel) on the histopathologic changes in the liver of HF-HFr rats (middle panel). The left panel represents the control liver on a normal diet. In Fig. 1 (middle panel), the liver from HF-HFr rats shows the presence of steatosis (blue arrow), inflammatory cell infiltration (red arrow), and ballooning of cells (yellow arrow). There was a significant diminution in the distribution of abnormal cellular features in the liver of probiotic-treated HF-HFr rats (Fig. 1, right panel).

While none (0/7) of the rats on the control diet exhibited NASH, 6/7 HF-HFr rats exhibited full NASH, with 1/7 showing borderline NASH. Treatment of HF-HFr rats with probiotics resulted in a profound decrease in NASH incidence, with just 1/7 having full-blown NASH, 4/7 borderline NASH, and 2/7 NASH-free (Table 2).

The data presented in Fig. 2 show the effect of probiotic treatment (bottom panel) on the histopathologic changes in the ileum of HF-HFr rats (top-right panel). The top-left panel represents the control ileum on a normal diet. In Fig. 2, the arrows show the brown staining representing occludin expression. Occludin expression was much higher in the control group, decreased in the HF-HF rats, and was not recovered by probiotic treatment (Fig. 3).

TABLE 1 | The effect of probiotic treatment on body weight, occludin, NLRP3, and TLR4 in rats on control as well as on HF-HFr diet.

Variables	Control (n = 7)	Non-Probiotic (n = 7)	Probiotic (n = 7)
	Mean ± SD		
Weight (g)	134 ± 8.48	259.71 ± 23.49	214 ± 29.03
Length (cm)	15.57 ± 0.34	15.43 ± 0.45	15.36 ± 0.38
NLRP3 (ng/mL)	0.00 ± 0.0	1.12 ± 0.66	0.93 ± 0.14
TLR4 (ng/mL)	0.00 ± 0.0	1.41 ± 0.18	1.06 ± 0.34
Occludin	4.36 ± 1.91	3.57 ± 1.39	3.69 ± 1.23

TABLE 2 | Effect of probiotic treatment on the histological liver NAS score.

Groups	NAS Score Interpretation		
	Not NASH	Borderline	NASH
Control	7	0	0
Non-Probiotic	0	1	6
Probiotic	2	4	1

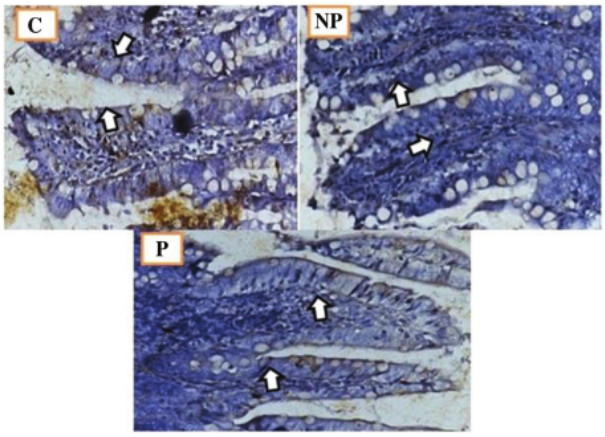

FIGURE 2 | Ileum histological biopsy image using immunofluorescence staining in 400X magnifications in control (C), non-probiotic (NP), and probiotic (P) group. The arrow shows the brown staining representing occludin expression.

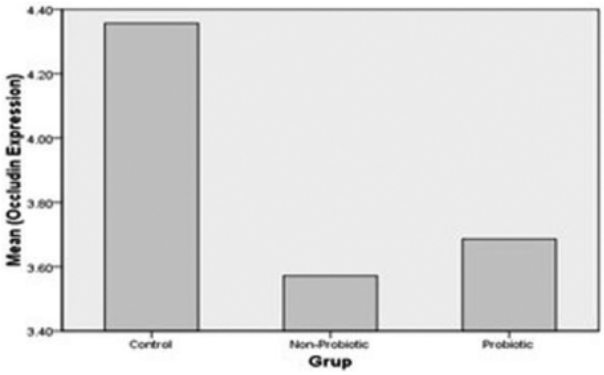

FIGURE 3 | Graph showing the mean value of occludin expression score (one-way ANOVA test, P = 0.724).

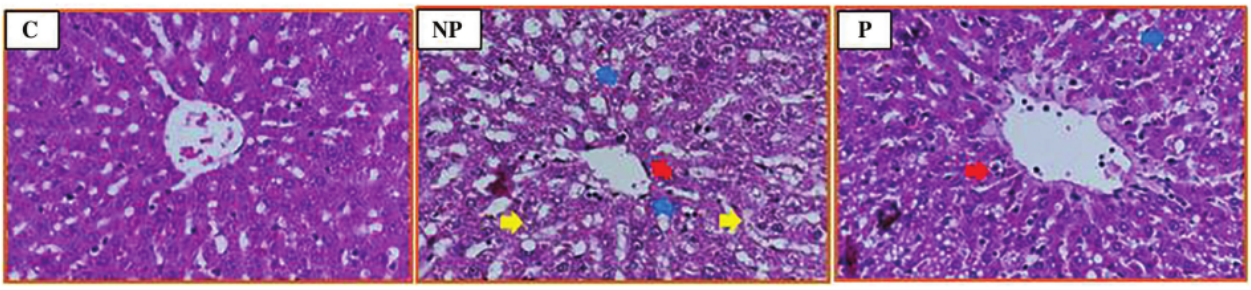

FIGURE 1 | Liver biopsy images using HE staining in 400X magnifications in control (C), non-probiotic (NP), and probiotic (P) group. The blue arrow shows the steatosis, the red arrow shows inflammatory cells infiltration, and yellow arrow indicates ballooning.

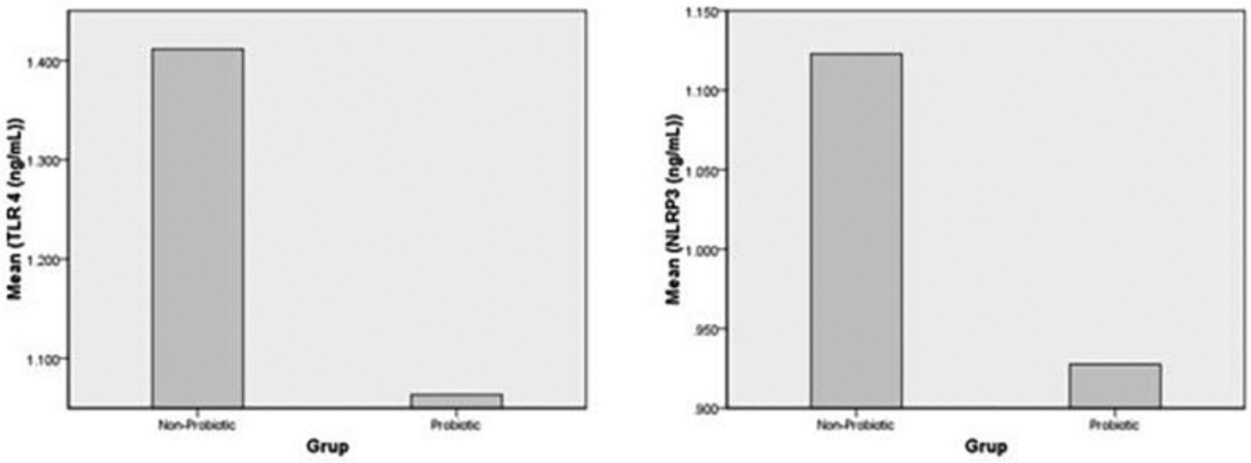

FIGURE 4 | Graph showing the mean difference of (a) TLR4 (P = 0.012) and (b) NLRP3 (P = 0.025) in NP and P groups.

TABLE 3 | Correlation between TLR4, NLRP3, NAS score, and occludin.
[#]Spearman's RHO correlation analysis (P < 0.05).

Variables	Occludin (*n* = 21)
TLR4	r = −0.105[#] P = 0.652
NLRP3	r = −0.116[#] P = 0.617
NAS Score	r = 0.085[#] P = 0.714

DISCUSSION

The observation of a reduced incidence of NASH in probiotic-treated rats on a high-fat, high-fiber diet is consistent with the results of other studies regarding the use of probiotics in preventing NAFLD progression. For example, 7-month treatment with *Bifidobacterium infantis* (a probiotic) and milk oligosaccharides (a prebiotic) effectively prevented NASH in mice by reducing hepatic fat accumulation and increasing ursodeoxycolic acid in the liver and serum (Jena et al., 2018). Ursodeoxycolic acid, the 7b-epimer of chenodeoxycholic acid, is a substance used for managing liver diseases (Angulo, 2002). In another study, mice treated for 18 weeks showed that probiotics reduced liver fibrosis in mice fed high-fat, choline-deficient diet (Cortez-Pinto et al., 2016).

Intestinal homeostasis is largely dependent on the careful maintenance of the intestinal barrier, a physical boundary that separates the host from noxious luminal microbes and compounds. The maintenance of intact barrier function is largely dependent upon paracellular tight junctions and associated proteins such as occludin. Herein, we observed higher occludin expression in the ileum of control rats on a standard diet that decreased when rats were placed on a HF-HFr diet to induce NASH, although we did not find any significance.

The treatment of NASH-expressing rats with a mixture of probiotics (*Lactobacillus acidophilus*, *Bifidobacterium longum*, and *Streptococcus thermophiles*) showed a tendency for a rise in occludin expression. Our observations here are consistent with reports of improvements in the quality of the tight junction, intestinal microbiota, gene expression of tight junction proteins, and immunomodulatory activity by probiotics (Chang et al., 2020; Rose et al., 2021).

TLR-4 was significantly expressed in NAFLD and activated by LPS in the portal vein circulation caused by leaky gut. This condition further mediated the inflammatory process and the development of NASH (Bessone et al., 2019). Some pri research with TLR4 mutant (TLR4mut) rats has shown a causal role for TLR4 in the pathogenesis of chronic inflammatory liver diseases (Ye et al., 2012).

TLR4 activation caused a significant development of steatohepatitis induced by a methionine-choline-deficient diet in rats and also to a marked decrease in fructose-induced hepatic steatosis (Rivera et al., 2007; Spruss et al., 2009). This study found that TLR-4 serum levels were inversely correlated with occludin, although not significantly. We thought the increased fat in our study might induce activation of TLR-4 from the HF-HFr diet and not from the gut-epithelial disruption (Carpino et al., 2020).

In addition, we found significantly lower NLRP3 levels in the probiotic group compared with the non-probiotic group. NLRP3 and expression of its components were remarkably higher both in murine models and in humans with NASH (Yu et al., 2022). Furthermore, lack of NLRP3 expression in gene knockout mice leads to hepatic steatosis, hepatocyte inflammation, and fibrogenesis.

These results suggest that the NLRP3 inflammasome might have an important role in NASH development (Wan et al., 2016). Several studies have shown that proper probiotic supplementation can repair gut damage and reduce inflammation. A recent study has revealed that *L. johnsonii* L531 inhibits the activation of the inflammatory signaling pathway TLR4/MyD88, NF-kB, and NLRP3 and successfully maintains the integrity of the tight junction (Chen et al., 2021).

A study by Shan et al. (2022) has shown that probiotic LGR-1 inhibits NLRP3 activation by protecting the tight junction. These

mechanisms of action include protection at tight junctions, preventing K+ influx, and inhibiting NLRP3 activation and pyroptosis. The cytoprotective effect of LGR-1 depends on protection from tight junctions (Liu et al., 2022).

Additionally, a negative correlation between NLRP3 and occluding was observed, although that was not significant. We suggested that the bacterial toxin caused by dysbiosis was not the only factor associated with NLRP3 expression. NLRP3 was triggered by stress signals that can cause increased cell permeability, lysosome rupture, or mitochondrial damage (Groslambert and Py, 2018).

There were limitations to this study. We only managed to measure one parameter of tight junction integrity (occludin), as there were other proteins expressed that can represent intestinal tight-junction integrity, such as claudin. We also did not measure or differentiate the intestinal microbiota in our study. Further studies incorporating these variables will help to better understand the probiotic's protective effect on intestinal mucosa.

CONCLUSIONS

In conclusion, we found that TLR-4 and NLRP3 levels were markedly lower in the probiotic group than in the non-probiotic group. However, there is no significant correlation between occluding, TLR4, NLRP3, and NAS-score. Therefore, further study is required to enhance our understanding of the role of NLRP3 and NASH in gut epithelial integrity.

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CONFLICT OF INTEREST DECLARATION

The authors state that there are no conflicts of interest to disclose.

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