RESEARCH ARTICLE

Lactococcus lactis D4 Decreases NF-κB and α-SMA in Rat Models of Obstructive Jaundice

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Abstract

BACKGROUND: Obstructive jaundice, often due to choledocholithiasis or malignancies, leads to immune suppression, intestinal damage, and bacterial translocation, worsening outcomes. Some inflammatory mediators like nuclear factor kappa B (NF-κB), alpha-smooth muscle actin (α-SMA), and interleukin-6 (IL-6) are important in this process. Current treatments remain inadequate, highlighting the need for novel approaches. Probiotics, such as *Lactococcus lactis* D4 (LLD4), may help reduce inflammation and bacterial translocation, thus offering a potential therapeutic option. This study was conducted to evaluate the effects of LLD4 on NF-κB, α-SMA, and IL-6 in obstructive jaundice rat models.

METHODS: This post-test randomized controlled study involved 15 male Wistar rats divided into three groups: sham, bile duct ligation (BDL), and BDL+LLD4 groups. The rats were maintained for 7–10 days, with the rats in BDL+LLD4 group received fermented milk containing LLD4 via gavage at a dose of 112 mg/20 gBW per day for 7 days. The expression levels of NF-κB, α-SMA, and IL-6 were analyzed using immunohistochemistry.

RESULTS: Administration of LLD4 were able to significantly reduced NF-κB expression compared to the BDL group (40.20±21.276 *vs.* 53.60 ± 20.403) in obstructive jaundice rat models. Though not significant, BDL+LLD4 group showed lower α-SMA expression compared to BDL group (58.40±14.271 *vs.* 63.20±9.16). However, administration of LLD4 did not give any significant effect on IL-6 expression.

CONCLUSION: LLD4 reduces inflammation in models of obstructive jaundice by lowering the NF-κB and α-SMA expression. This indicates that LLD4 might be potential as an adjunct therapy for reducing morbidity in obstructive jaundice cases.

KEYWORDS: obstructive jaundice, bile duct ligation, *Lactococcus lactis* D4, NF-κB, α-SMA, IL-6

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Introduction

Obstructive jaundice, marked by high morbidity and mortality, poses significant clinical challenges.(1) This condition can be classified into intrahepatic and extrahepatic obstructions, with common etiologies including choledocholithiasis, strictures, and malignancies. (2) The obstruction of bile flow disrupts the normal excretion of bile salts, leading to systemic complications such as immune suppression, intestinal mucosal damage, and bacterial translocation, which can further exacerbate patient outcomes.(3-5)

Proinflammatory cytokines, such as uclear factor kappa B (NF-κB), alpha-smooth muscle actin (α-SMA), and interleukin (IL)-6 play a crucial role in the inflammatory

response associated with obstructive jaundice. IL-6 is produced by various cells, including T cells and macrophages, and serves as a key mediator in the inflammatory process. NF-κB functions as a transcription factor that regulates the expression of multiple inflammatory genes, contributing to the inflammatory milieu.(6,7) Furthermore, the expression of α-SMA is an important marker of myofibroblast activation, indicating the progression of fibrosis within the liver, which can result from chronic inflammation.(8)

Despite advancements in understanding the pathophysiology of obstructive jaundice, effective therapeutic strategies to mitigate its complications remain limited. Current treatments primarily focus on relieving the obstruction but do not adequately address the inflammatory response or its systemic consequences. Therefore, there exists a critical gap in exploring adjunctive therapies that target the inflammatory processes associated with this condition to improve patient outcomes.(3,4)

Probiotics have emerged as promising therapeutic agents for mitigating inflammation and enhancing intestinal barrier function. Probiotics, defined as live microorganisms beneficial to the host, have shown potential in reducing inflammation and intestinal permeability.(9,10) Strains from *Lactobacillus* and *Lactococcus* species, in particular, have demonstrated the ability to reduce intestinal permeability and inflammation, which may alleviate the complications associated with obstructive jaundice.(11) In Sumatera Barat, Indonesia, *Dadiah*, a traditional fermented buffalo milk product, is rich in lactic acid bacteria with potential probiotic properties. Therefore, this study was conducted to evaluate the effects of *Lactococcus lactis* D4 (LLD4), isolated from *Dadiah*, on inflammatory mediators NF-κB, α-SMA, and IL-6 in a Wistar rat model of obstructive jaundice. By understanding the effect of this probiotic, the study seeks to provide insights into new therapeutic strategies for managing obstructive jaundice and its associated complications.

Methods

Study Design

This experimental laboratory study utilized a post-test-only randomized control group design, conducted on male Wistar rats aged 10–16 weeks, with body weights ranging from 160 to 250 g. The subjects were divided into three groups: sham, bile duct ligation (BDL), and BDL+LLD4 groups. The sham group underwent laparotomy, without bile duct ligation or cutting. The BDL group underwent laparotomy with common bile duct ligation and cutting, whereas the BDL+LLD4 group received bile duct ligation followed by daily administration of LLD4 via gavage at a dose of 112 mg/20 gBW per day for 7 consecutive days. All animals were monitored and kept healthy for 7–10 days post-surgery. The protocol of this study was approved by the Ethics Committee of Faculty of Medicine, Universitas Andalas (No. 368/UN.16.2/KEP-FK/2024).

Animal Preparation

Fifteen male Wistar rats bred at the Biomedical Laboratory of the Faculty of Medicine, Universitas Andalas, were included as the subjects for this study. The sample size of five rats per group was determined based on WHO recommendations for animal experiments. Rats were individually housed in ventilated cages on a 12-hour light-dark cycle, with unrestricted access to food and water. Health monitoring was performed daily, with a one-week acclimatization period before the study. The study intervention lasted for five months and included animal care, experimental procedures, and histological examinations.

Preparation of Obstructive Jaundice Rat Model

Under aseptic conditions and isoflurane anesthesia, bile duct ligation was performed using 5-0 silk sutures. The bile duct ligation procedure was performed to create a rat model of obstructive jaundice. Rats were anesthetized to ensure the animal was unconscious and free from pain. After anesthesia, the abdominal area was cleaned and sterilized. A small midline abdominal incision was made to access the hepatobiliary system. The common bile duct (*ductus choledochus*) was carefully identified and ligated using a sterile surgical suture to prevent bile flow from the liver to the intestines. After ligation, the abdominal incision was closed with sutures, and the animal was monitored during the recovery period. The ligation of the bile duct resulted in the obstruction of bile flow, leading to the accumulation of bilirubin in the bloodstream, which caused jaundice. Seven days post-surgery, liver tissue samples were collected from the left lobe and fixed in 10% formalin for histological analysis.

Preparation and Administration of LLD4

LLD4 was obtained from the fermentation process of *Dadiah*. Once the bacteria had grown, a lactic acid bacteria suspension of material containing bacteria was taken from the agar media and incubated.(12) The suspension isolate was put into a microtube in a dose of 112 mg/20 gBW and given to the BDL+LLD4 group for 7 consecutive days.

This process was done at the Laboratory of Animal Product Technology, Faculty of Animal Science, Universitas Andalas.

Immunohistochemistry (IHC)

Five µm of rats' liver tissue sections were stained with hematoxylin and eosin for histomorphometric analysis to assess NF-κB, α-SMA, and IL-6 expressions through IHC. Following deparaffinization, rehydration, and antigen retrieval, primary antibodies were applied. A biotinylated secondary antibody and DAB (3,3'-diaminobenzidine) facilitated visualization, with hematoxylin counterstaining. The stained slides were then analyzed using ImageJ (National Institutes of Health, Bethesda, MD, USA) to quantify the positive areas relative to the total tissue area. To ensure the accuracy and reliability of the IHC results, the interpretation of the stained slides was performed by an expert in anatomical pathology. This approach minimized the potential for bias, as the analysis was conducted by a trained professional with specialized knowledge in histological assessment.

Results

Rats in the BDL and BDL+LLD groups receive bile duct ligation to create obstructive jaundice model. The rats began to exhibit clinical signs of jaundice, such as yellowing of the skin and sclera, within 3-5 days after the procedure. The full development of jaundice and associated hepatic changes, including inflammation and fibrosis, occurred within one week following the ligation.

LLD4 Administration Reduced NF-κB Expression

NF-κB expression was also detected by brown staining (black arrows) in the cytoplasm of the hepatocytes (Figure 1A, 1B, and 1C). All groups, including the sham, BDL, and BDL+LLD4 groups, showed positive staining in the hepatocyte cytoplasm. The mean NF-κB expression was higher in the BDL group than in the sham group (53.60±20.40 *vs.* 14.80±9.31), while there was a decrease in the BDL+LLD4 group compared to the BDL group (40.20±21.28) (Figure 1D). One-way ANOVA results revealed significant differences in NF-κB expression among the experimental groups (Figure 2D) (p=0.015). The post-hoc LSD test demonstrated a significant increase of NF-κB levels in the BDL group and a significant decrease in the BDL+LLD4 group compared to the sham group (p<0.05).

LLD4 Administration Reduced α-SMA Expression

The expression of α-SMA was observed by brown staining (black arrows) in the cytoplasm of hepatic sinusoidal stellate cells. Positive staining was present in the cytoplasm of stellate cells in the sham, BDL, and BDL+LLD4 groups (Figure 2A, 2B, and 2C). Mean α-SMA expression was slightly higher in the BDL group (63.20±9.16) compared to the sham group (59.80±5.39). But administration of LLD4 treatment in the BDL+LLD4 group, showed a small decreased (58.40±14.27) compared to the BDL group. One-way ANOVA results indicated no significant differences in α-SMA expression among the experimental groups (Figure 3) (p=0.754). Though not significant, but administration of LLD were able to reduce the α-SMA in obstructive jaundice rat model.

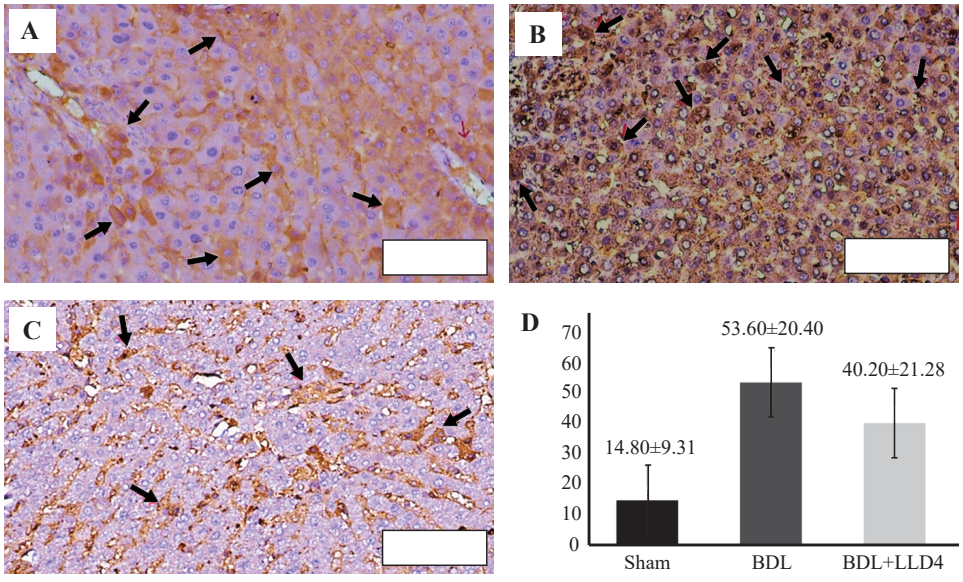

Figure 1. NF-κB expression in the sham, BDL, and BDL+LLD4 groups. IHC expression of NF-κB in the liver tissue of test animals was indicated by brown staining in the hepatocyte cytoplasm (black arrow). A: sham group. B: BDL group. C: BDL+LLD4 group. White bar: 20µm. D: Comparison graph of NF-κB IHC results, with significant mean difference (p=0.015, One-way ANOVA).

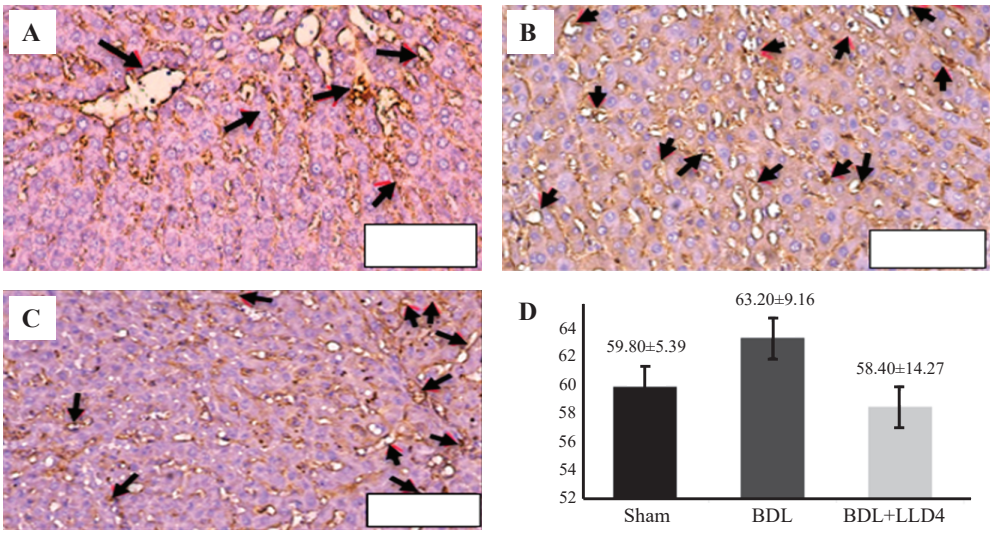

Figure 2. α-SMA expression in the sham, BDL, and BDL+LLD4 groups. IHC expression of α-SMA in the liver tissue of test animals was indicated by brown staining in the cytoplasm of stellate cells (black arrow). A: sham group. B: BDL group. C: BDL+LLD4 group. White bar: 20μm. D: Comparison graph of α-SMA IHC results, with no significant mean difference (p=0.754, One-way ANOVA).

IL-6 Expression between Groups were Not Significantly Differrent

IL-6 expression was identified by brown staining (black arrows) in the cytoplasm of the hepatocytes. The sham group exhibited no staining in the hepatocyte cytoplasm (Figure 3A), while the BDL and BDL+LLD4 groups showed positive staining (Figure 3B and 3C). There was elevated IL-6 level in the BDL (50.60±24.83) and BDL+LLD4 (64.80±29.49) groups (Table 1), indicating that the bile duct ligation process increased the IL-6 expression as the inflammation marker. One-way ANOVA results revealed no significant differences in IL-6 expression among the three experimental groups (Figure 3D) (p=0.079), suggesting that administration of LLD does not give any effect on IL-6 expression.

Discussion

NF-κB, a key transcription factor in the inflammatory response, is crucial in this study as a marker of inflammation. The results of this study demonstrated significant differences among the groups, with the BDL group showing a marked increase in NF-κB expression compared to both the sham and BDL+LLD4 groups. NF-κB remains inactive when bound to cytoplasmic inhibitor of κB (IκB). Cellular damage leads to IκB phosphorylation and degradation, activating NF-κB, which translocates to the nucleus to initiate the production of inflammatory cytokines.(13) Bile duct obstruction disrupts hepatic reticuloendothelial cell function and compromises the intestinal mucosal barrier,

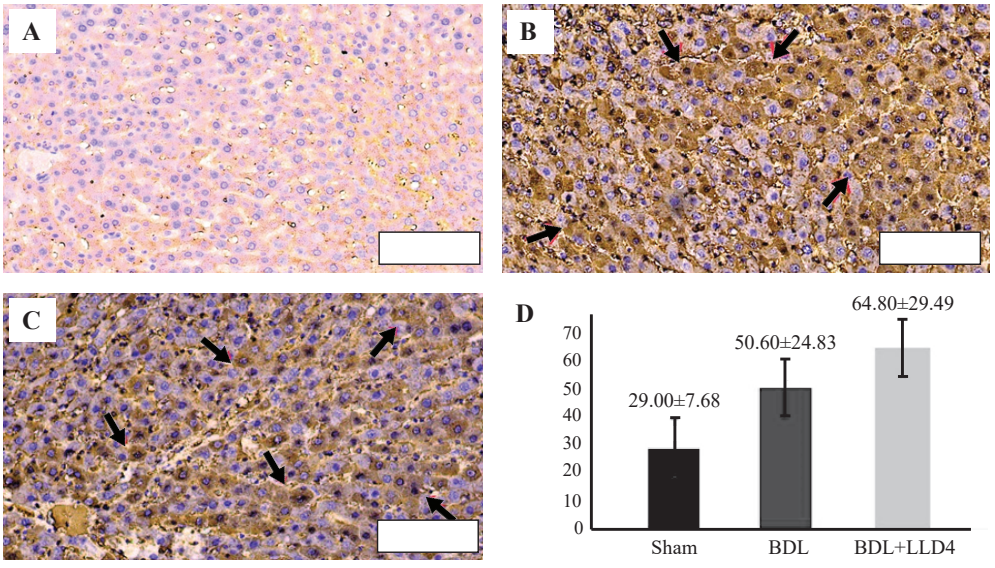

Figure 3. IL-6 expression in the sham, BDL, and BDL+LLD4 groups. IHC expression of IL-6 in the liver tissue of test animals was indicated by brown staining in the cytoplasm of stellate cells (black arrow). A: sham group. B: BDL group. C: BDL+LLD4 group. White bar: 20μm. D: Comparison graph of IL-6 IHC results, with no significant mean difference (p=0.079, One-way ANOVA).

leading to increased production of pro-inflammatory cytokines. This suggests that total bile duct obstruction predisposes as a damaging factor.(14,15) The results of current study demonstrated significant differences among the three groups, with LLD4-treated groups showing a marked reduction in NF-κB expression compared to the BDL group. The observed reduction in NF-κB expression aligns with previous studies indicating that probiotics such as *Lactobacillus* can mitigate inflammation by inhibiting various signalling pathways and enhancing epithelial barrier function. For example, lipopolysaccharides (LPS) activate Toll-like receptor 4 (TLR4) and the NF-κB pathway.(16)

The results of this study also indicated that LLD4 administration slightly reduced α-SMA expression in the BDL+LLD4 group compared to the BDL group. Bile duct ligation induces inflammation and activates hepatic stellate cells (HSC), which play a pivotal role in liver fibrosis during obstructive jaundice. Transformation of activated HSCs from fat-storing cells to myofibroblasts leads to increased type I collagen production. The α-SMA expression serves as a marker of therapeutic outcome in obstructive jaundice. Lower α-SMA percentages correlate with improved jaundice clearance, supporting our findings that LLD4 reduces α-SMA expression compared non-treated obstructive jaundice group.(17)

In current study, increase of IL-6 were seen in BDL and BDL+LLD4 groups, indicating accumulation of inflammation after the bile duct ligation process. However, there were no significant different of IL-6 expression after the LLD4 administration. Obstructive jaundice are known to affects the bile ducts and liver cells, leading to systemic complications, including oxidative damage mediated by reactive oxygen species (ROS).(9) The presence of portosystemic shunts exacerbates endotoxin entry into systemic circulation, resulting in elevated inflammatory cytokines such as IL-6.(18,19) While probiotics, such as lactic acid bacteria, are typically associated with reduced IL-6 and other pro-inflammatory markers, some studies suggest that probiotic administration may actually increase IL-6 expression in liver injury models.(20) Conversely, other research suggests that IL-6 may function as an anti-inflammatory agent, thus promoting anti-inflammatory responses.(21) IL-6's regenerative and anti-inflammatory functions operate through classic signaling, while its pro-inflammatory effects are mediated via trans-signaling, underscoring its dual role in various pathological conditions.(22)

While research on LLD4 in obstructive jaundice is limited, probiotics have shown potential in improving liver function by producing metabolites that protect the intestinal barrier, preventing significant increases in permeability post-intervention, thereby reducing excessive inflammatory responses.(23) Research indicates that *Lactococcus lactis* may ameliorate liver damage by increasing forkhead box protein P3 (FoxP3), a regulatory T cell marker that modulates immune and inflammatory responses. This probiotic also enhances lymph node count in Peyer's patches and boosts IL-10 expression, an important anti-inflammatory cytokine.(24) *Lactococcus lactis* has unique properties that distinguish it from other probiotics, particularly for the treatment of obstructive jaundice. It produces antimicrobial peptides, such as nisin, which are effective against various pathogens, helping to maintain gut microbiota balance and reduce secondary infection risks. It also generates enzymes like arginine deiminase (ADI) and alpha-mannosidase (α-MAN), which assist in restoring the balance of disturbed gut microbiota. Moreover, *Lactococcus lactis* produces short-chain fatty acids (SCFAs), including butyrate, which exhibit anti-inflammatory effects and strengthen the intestinal mucosal barrier.(12,25,26)

Unfortunately, this study only consists of small sample size that may affect data distribution. This study also lacks investigation of other pro-inflammatory and anti-inflammatory cytokines, such as IL-10, IL-1β, and tumor necrosis factor-alpha (TNF-α). Additionally, it could not determine whether IL-6 acts as a pro-inflammatory or anti-inflammatory cytokine. Furthermore, this study did not compared LLD4 with other probiotic strains and did not include a detailed analysis of the specific dosage optimization for LLD4 in clinical settings. Future research should focus on evaluating the effectiveness of various optimized doses of LLD4 to ensure its efficacy and safety in therapeutic applications, utilizing larger sample sizes, exploring longer study durations with increased dose variations, assessing the bioavailability of LLD4, confirming liver fibrosis with Sirius Red staining, comparing LLD4 with other probiotic types, and exploring additional inflammatory and anti-inflammatory cytokines to deepen the understanding of LLD4's mechanism.

Conclusion

LLD4 reduced inflammation and liver damage in a rat model of obstructive jaundice, by lowering the NF-κB, and α-SMA expression. This suggest that probiotic LLD4 might serve as an adjunct therapy to decrease morbidity and mortality in patients with obstructive jaundice.

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Authors Contribution

AS conceptualized and designed the study, led the research process, and drafted the manuscript. AV provided guidance and oversight throughout the study, as well as contributed to the interpretation of the results. ED and HH were responsible for data acquisition and collection, while MI performed data analysis and contributed to result interpretation. All authors reviewed and provided critical revisions to the manuscript, approving the final version for submission.

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