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Original Article

Probiotic and ursodeoxycholic acid combination improves microbial gut dysbiosis and bilirubin levels in cholestatic rats

[La combinación de probióticos y ácido ursodesoxicólico mejora la disbiosis microbiana intestinal y los niveles de bilirrubina en ratas colestásicas]

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Abstract

Context: Cholestasis is marked by a disturbance in bile flow from the liver and potentially progresses to cirrhosis. Hepatic transplantation is the mainstay of therapy in hepatic cirrhosis, which is a high-cost, time-consuming, and complex procedure. Increasing evidence regarding the major role of gut microbiota in bile acid metabolism has prompted the effort to study probiotics as a potential alternative therapeutic strategy in chronic liver disease.

Aims: To explore the effect of probiotic administration on *Bifidobacterium*, as part of the gut microbiota, and bilirubin levels in mice with cholestasis after biliary duct ligation who received standard ursodeoxycholic acid (UDCA) therapy.

Methods: Thirty-five male Sprague Dawley rats were randomly assigned into seven groups: Sham-operated rats as healthy controls, cholestatic rats as negative control, UDCA only, probiotics only, and three groups received UDCA-probiotics on a stratified dose for 21 days. The bile duct ligation (BDL) procedure was used to induce cholestasis. Biochemical analyses were conducted for direct and total bilirubin levels, while *Bifidobacterium* DNA quantification was assessed with PCR.

Results: BDL induces cholestasis, marked by a significant increase in direct and total bilirubin levels. The combination of UDCA-probiotics increased the amount of *Bifidobacterium*, with the highest dose of probiotics showing the highest amount, and the results were comparable among the other groups. The lowest direct and total bilirubin levels were found in the groups with the highest probiotics dose and UDCA combination.

Conclusions: UDCA and probiotic combination significantly improved the amount of *Bifidobacterium* and bilirubin levels in cholestatic rats.

Keywords: *Bifidobacterium*; bilirubin; cholestasis; probiotic; ursodeoxycholic acid.

Resumen

Contexto: La colestasis se caracteriza por una alteración del flujo biliar desde el hígado y puede progresar a cirrosis. El trasplante hepático es el pilar del tratamiento de la cirrosis hepática, que es un procedimiento costoso, que requiere mucho tiempo y es complejo. La creciente evidencia sobre el papel principal de la microbiota intestinal en el metabolismo de los ácidos biliares ha impulsado el estudio de los probióticos como una posible estrategia terapéutica alternativa en la enfermedad hepática crónica.

Objetivos: Explorar el efecto de la administración de probióticos sobre *Bifidobacterium*, como parte de la microbiota intestinal, y los niveles de bilirrubina en ratones con colestasis después de la ligadura del conducto biliar que recibieron una terapia estándar con ácido ursodesoxicólico (UDCA).

Métodos: Se asignaron al azar treinta y cinco ratas Sprague Dawley macho a siete grupos: Ratas operadas simuladamente como controles sanos, ratas colestásicas como control negativo, solo UDCA, solo probióticos y tres grupos recibieron UDCA-probióticos en una dosis estratificada durante 21 días. Se utilizó el procedimiento de ligadura de conducto biliar (BDL) para inducir la colestasis. Se realizaron análisis bioquímicos para los niveles de bilirrubina directa y total, mientras que la cuantificación del ADN de *Bifidobacterium* se evaluó con PCR.

Resultados: BDL induce colestasis, marcada por un aumento significativo en los niveles de bilirrubina directa y total. La combinación de UDCA-probióticos aumentó la cantidad de *Bifidobacterium*, y la dosis más alta de probióticos mostró la cantidad más alta, y los resultados fueron comparables entre los otros grupos. Los niveles más bajos de bilirrubina directa y total se encontraron en los grupos con la dosis más alta de probióticos y la combinación de UDCA.

Conclusiones: La combinación de UDCA y probióticos mejoró significativamente la cantidad de *Bifidobacterium* y los niveles de bilirrubina en ratas colestásicas.

Palabras Clave: ácido ursodesoxicólico; *Bifidobacterium*; bilirrubina; colestasis; probiótico.

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Abbreviations: UDCA: ursodeoxycholic acid; BDL: biliary duct ligation; CFU: colony-forming units.

INTRODUCTION

Cholestasis is marked by a disturbance in bile flow from the liver to the gallbladder and duodenum (Hilscher et al., 2020). Accumulation of bile acids in the liver causes damage to the liver and can lead to liver disease (Cheemerla and Balakrishnan, 2021). Indonesia notes that the prevalence of this condition affects 20 million people, and around 20-40% of cases will develop cirrhosis (Kementrian Kesehatan Republik Indonesia, 2018). In conditions that have reached cirrhosis, transplantation is the primary choice of therapy, which requires high costs and a long time to find the donor. Currently, ursodeoxycholic acid (UDCA) is used as an alternative to transplantation to inhibit the progression of liver damage. This prompted us to explore other alternatives that could be more effective (Manns, 2013; Samant et al., 2019).

Research into gut microbiota in the medical field has increased in recent years as technology has become more advanced in understanding the complexity of this subject (Cani, 2018). Recent evidence shows that the composition and diversity of the gut microbiota are affected by host health conditions and actively influence the host (Chassaing et al., 2014). Gut microbiota plays a significant role in the homeostasis of bile acid metabolism through breaking down bile acid conjugation using bile salt hydrolase (Wahlström et al., 2016). On the other hand, bile acids are known to have antimicrobial effects, so reduced bile acid levels can potentially trigger the development of pathogenic bacteria (Islam et al., 2011). Studies in patients with chronic hepatitis B show a decrease in the ratio of *Bifidobacterium* to *Enterobacteriaceae* bacteria, reflecting the disease's severity (Liu et al., 2012).

Seeing the large role of intestinal microbiota in the pathogenesis of liver disease, several microbiota-based therapeutic strategies have been developed, including probiotics that can improve intestinal dysbiosis and facilitate the growth of bacteria that are beneficial to host health (Britton et al., 2021). Liu et al. studied the administration of probiotics in a cholestatic mouse induced by biliary duct ligation (BDL) and found that probiotics significantly improved liver inflammation, injury, and fibrosis (Liu et al., 2020). A meta-analysis by Khalesi et al. (2017) also showed that probiotic supplementation reduced elevated bilirubin levels due to cholestasis. The promising benefits of probiotics have sparked the idea of using this component as a modality to inhibit the progression of liver damage, thus proving beneficial, especially for patients awaiting liver donors (Khalesi et al., 2017). Thus, this study aims to explore the effect of probiotic administration on *Bifidobacterium* and bilirubin levels in mice with cholestasis after biliary duct ligation who received standard UDCA therapy.

MATERIAL AND METHODS

Animal study

This study is an experimental, randomized, post-test-only study with a control group and has been approved by the Medical Research and Ethics Committee at Diponegoro University (46/EC-H/KEPK/FK-UNDIP/V/2023). Thirty-five samples were required for this study based on Federer's formula. In this study, there were thirty-five male Sprague Dawley rats aged two months, weighing 200-250 g, without anatomical abnormalities or liver damage. The rats were caged individually and acclimated for 7 days with a standard diet and drink at 37°C and a bright room. Surgery procedure allocation was assigned to each rat randomly. The rats were given preoperative 18 mg cefotaxime (Indofarma, Jakarta, Indonesia) as a prophylaxis antibiotic and 0.5 mL ketamine hydrochloride (Dexa Medica, Cikarang, Indonesia) as anesthetic via intramuscular injection. Under sterile conditions, a midline laparotomy was performed, and the common bile duct was ligated with a 5-0 silk suture (DemeTECH, Miami Lakes, FL, USA) (Tag et al., 2015). Postoperative 7 mg oral ibuprofen (Pharos, Semarang, Indonesia) was given every 8 hours for 3 days to alleviate postoperative pain in the rats. Sham-operated rats underwent the same surgical procedure except for bile ductus ligation (BDL).

After surgery, five sham-operated rats were assigned to the K1 group, while 30 BDL-operated rats were randomly assigned into the other six groups: (K2) BDL, (K3) BDL + UDCA, (P1) BDL + Probiotic 36 mg, (P2) BDL + UDCA + Probiotic 18 mg, (P3) BDL + UDCA + Probiotic 36 mg, (P4) BDL + UDCA + Probiotic 54 mg, with each group included five rats. Standard diet and drink followed the same feeding pattern. UDCA was given orally at a dose of 13,5 mg based on conversion from the therapeutic dose used in humans (Parízek et al., 2016). Probiotic L-Bio (Lapi Laboratories, Indonesia), which contained 10^9 colony-forming units (CFU) of *Bifidobacterium lactis* W51, *Bifidobacterium lactis* W55, *Lactobacillus acidophilus* W55, *Lactobacillus casei* W56, *Lactobacillus salivarius* W57 and *Lactococcus lactis* W58, was given orally at a dose according to each group. All treatment was given continuously for 21 days.

Biochemical analysis

From each group, the number of *Bifidobacterium*, total bilirubin levels, and indirect bilirubin levels were examined. Data on the number of *Bifidobacterium* were obtained from the results of qPCR examination of caecum feces samples from experimental animals. Data on total bilirubin levels and direct bilirubin levels were obtained from laboratory examinations using the blood of experimental animals.

Bifidobacterium DNA extraction and quantitative PCR

Fecal samples were collected from the caecum after termination was conducted. 200 mg of cecal material was placed in a vial with glass beads and added with 300 µL of SDE 1 buffer and 20 µL of proteinase K. The mixture was homogenized for 1 min using a cell lyse homogenizer (Precellys, Bertin Equipment, Germany), and incubation was continued for 8 h at 60°C. After incubation, 100 µL of SDE 2 buffer was added, vortexed, and incubated on ice for 5 min. After centrifuging at 13,000 rpm for 5 min, the supernatant was obtained and combined with µl SDE 3 buffer. Two minutes of incubation was performed at room temperature, followed by 2 min of centrifugation at 13,000 rpm. In another step, 250 µL of supernatant was collected and added with 250 µL of cold isopropanol and 250 µL of SDE 4 buffer. The solution was vortexed and transferred to an SDE column. After drying by centrifugation at 13,000 rpm for 3 min, the resulting solution was quantified, and DNA quality was analyzed using a nanodrop spectrophotometer (MaestroNano, MaestroGen, Taiwan.).

The primer for *Bifidobacterium* in this study was designed based on deoxyribonucleic acid (DNA) primer for Lactobacillus obtained from the BLAST database. Target bacteria were diluted in 9 mL of NaCl 0.9% (from 10^9 CFU/mL) to yield 10^2 CFU/mL to 10^9 CFU/mL. After centrifugation, 300 µL of SDE 1 buffer and 50 µL of lysozyme (10 mg/mL) were added to the pellet. The DNA was extracted according to the protocol for targeted viral DNA. Reagents consisting of 2x QPCR Mastermix (Smobio, no ROX), forward-reverse primers, template DNA, and nuclease-free water, were prepared. After vortexing, the mixture was transferred to qPCR tubes, and qPCR analysis was performed using a Biorad CFX96 instrument. Melting curve analysis was used to evaluate the quality of PCR amplification, and the result was stated in Log CFU/g measurement.

Total bilirubin and direct bilirubin levels

Blood specimens were taken from the orbital venous sinus of all experimental animals after 21 days of treatment. Blood specimens were centrifuged to obtain blood serum, placed in sample tubes without anticoagulant (1 mL), frozen completely, and transported to a private laboratory in Yogyakarta. Rat blood serum was examined for total bilirubin and direct bilirubin levels using the AutoAnalyzer method with Diazo reagent.

Statistical analysis

Statistical analysis was performed using the IBM SPSS® Statistic software, version 23 (New York, Manhattan). Results are expressed as means ± SD. Statistical comparisons were conducted using the Kruskal-Wallis and Mann-Whitney tests. Differences were considered significant at $p<0.05$.

The collected data, in the form of measurement results of the number of *Bifidobacterium*, total bilirubin levels, and indirect bilirubin levels, were processed through the process of editing, coding, entry, and cleaning data. Data were analyzed statistically with a computer program. Data on the number of *Bifidobacterium*, total bilirubin levels, and direct bilirubin levels were analyzed using data normality testing with the Shapiro-Wilk test. Normally distributed data were tested with parametric statistics with a one-way ANOVA test followed by Post Hoc LSD. Data that were not normally distributed were tested with the Kruskal-Wallis test followed by Post Hoc Mann-Whitney. A $p<0.05$ was considered significant with a 95% confidence interval.

RESULTS

All rats (n = 35) received the allocated treatment completely until termination and were all included in the final analysis.

Probiotic and UDCA combination improved *Bifidobacterium* dysbiosis

The quantitative PCR result of *Bifidobacterium* was addressed in Table 1. The results between K1 and K2 were comparable, suggesting that BDL does not induce any change in the amount of *Bifidobacterium*. However, the amount of *Bifidobacterium* was significantly increased by the administration of probiotic 54 mg and UDCA combination, compared to the other groups. Meanwhile, the amount of *Bifidobacterium* between the K1 and P3 groups was comparable (Fig. 1).

Based on the results of the Mann-Whitney test, there appears to be a difference in the number of *Bifidobacterium* between the negative control group (K2) and group P4, there is a difference between the positive control group (K3) and group P4, there is a difference between group P1 and group P4, there is a

difference between group P2 and group P4, and there is a difference between group P3 and group P4.

Probiotic and UDCA combination decreased bilirubin level

The biochemical analysis results of direct and total bilirubin were addressed in Tables 2 and 3, respectively. A comparison of direct and total bilirubin levels among the groups showed a significant result. Thus, this indicates that the BDL-only group had a significantly elevated bilirubin level and suggests that BDL induced hepatic injury. While the administration of probiotic and UDCA combination significantly improved the bilirubin levels in cholestatic rats, compared to only UDCA or probiotic administration (Figs. 2 and 3).

Based on the results of the Mann-Whitney test, in the boxplot graph, there appears to be a difference in total bilirubin levels between the healthy control group (K1) and the K2, K3, P1, P2, P3, and P4 groups, there is a difference between the negative control group (K2) and the K3, P1, P2, P3, and P4 groups, there is a difference between the positive control group (K3) and the P1, P2, P3, and P4 groups, there is a difference between the P1 group and the P2, P3, and P4 groups, and there is a difference between the P2 group and the P4 group.

Based on the results of the Mann-Whitney test, in the boxplot graph, there are differences in direct bilirubin levels between the healthy control group (K1) and groups K2, K3, P1, P2, and P3, there are differences between the negative control group (K2) and groups K3, P1, P2, P3, and P4, there are differences between the positive control group (K3) and groups P3 and P4, there are differences between group P1 and groups P3 and P4, there are differences between group P2 and groups P3 and P4, and there are differences between group P3 and group P4.

Table 1. *Bifidobacterium* count analysis.

Group	Mean ± SD (Log CFU/g) (n = 35)	Median (min-max) (n = 35)	p-value
K1	6.61 ± 3.05	5.20 (4.93 – 12.05)	0.005
K2	5.31 ± 0.48	5.34 (4.86 – 6.03)	0.431*
K3	5.05 ± 0.06	4.98 (4.84 – 5.48)	0.069*
P1	5.10 ± 0.62	5.10 (4.29 – 6.04)	0.634*
P2	5.00 ± 0.42	4.90 (4.41 – 5.46)	0.545*
P3	4.80 ± 0.67	4.85 (3.72 – 5.38)	0.267*
P4	9.31 ± 2.00	10.18 (5.80 – 10.62)	0.015

K1: Sham; K2: BDL; K3: BDL + UDCA; P1: BDL + Probiotic 36 mg; P2: BDL + UDCA + Probiotic 18 mg; P3: BDL + UDCA + Probiotic 36 mg; P4: BDL + UDCA + Probiotic 54 mg; *Normal distribution (p>0.05). UDCA: ursodeoxycholic acid; BDL: biliary duct ligation.

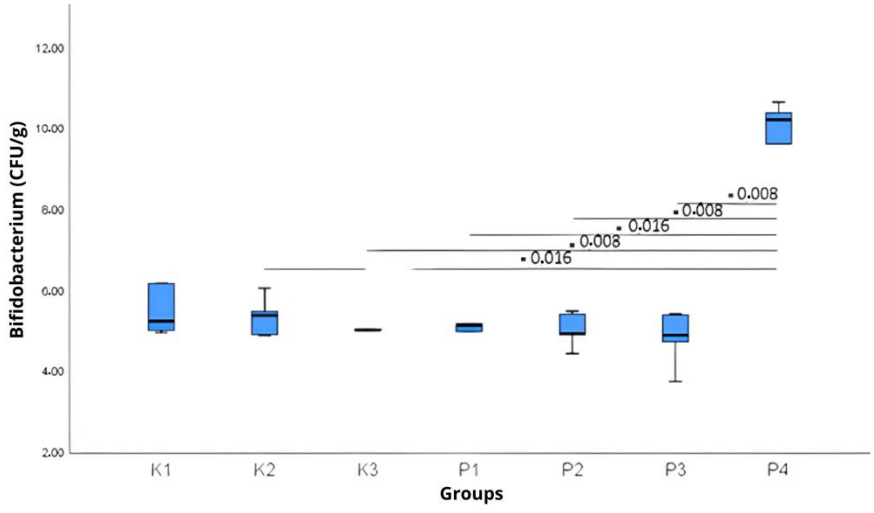

Figure 1. *Bifidobacterium* amount between each group presented in boxplots.

Data are presented as mean ± SD (n = 35). Lines connecting two groups indicate a significant difference in those groups; *significant (p<0.05).

K1: Sham; K2: BDL; K3: BDL + UDCA; P1: BDL + Probiotic 36 mg; P2: BDL + UDCA + Probiotic 18 mg; P3: BDL + UDCA + Probiotic 36 mg; P4: BDL + UDCA + Probiotic 54 mg. UDCA: ursodeoxycholic acid; BDL: biliary duct ligation.

Table 2. Direct bilirubin level analysis.

Group	Mean ± SD (mg/dL) (n = 35)	Median (min-max) (n = 35)	p-value
K1	0.33 ± 0.08	0.27 (0.27 – 0.41)	0.006
K2	1.52 ± 0.15	1.49 (1.35 – 1.76)	0.195
K3	1.00 ± 0.07	0.95 (0.95 – 1.08)	0.006
P1	0.87 ± 0.08	0.81 (0.81 – 0.95)	0.006
P2	0.62 ± 0.08	0.68 (0.54 – 0.68)	0.006
P3	0.60 ± 0.12	0.68 (0.41 – 0.68)	0.041
P4	0.49 ± 0.07	0.54 (0.41 – 0.54)	0.006

K1: Sham; K2: BDL; K3: BDL + UDCA; P1: BDL + Probiotic 36 mg; P2: BDL + UDCA + Probiotic 18 mg; P3: BDL + UDCA + Probiotic 36 mg; P4: BDL + UDCA + Probiotic 54 mg; *Normal distribution (p>0.05). UDCA: ursodeoxycholic acid; BDL: biliary duct ligation.

Table 3. Direct bilirubin level analysis.

Group	Mean ± SD (mg/dL) (n = 35)	Median (min-max) (n = 35)	p-value
K1	0.21 ± 0.08	0.15 (0.15 – 0.29)	0.006
K2	1.38 ± 0.08	1.32 (1.32 – 1.47)	0.006
K3	0.82 ± 0.08	0.88 (0.74 – 0.88)	0.006
P1	0.82 ± 0.08	0.88 (0.74 – 0.88)	0.006
P2	0.74 ± 0.10	0.74 (0.59 – 0.88)	0.284
P3	0.56 ± 0.07	0.59 (0.44 – 0.59)	0.000
P4	0.38 ± 0.13	0.29 (0.29 – 0.59)	0.048

K1: Sham; K2: BDL; K3: BDL + UDCA; P1: BDL + Probiotic 36 mg; P2: BDL + UDCA + Probiotic 18 mg; P3: BDL + UDCA + Probiotic 36 mg; P4: BDL + UDCA + Probiotic 54 mg; *Normal distribution (p>0.05). UDCA: ursodeoxycholic acid; BDL: biliary duct ligation.

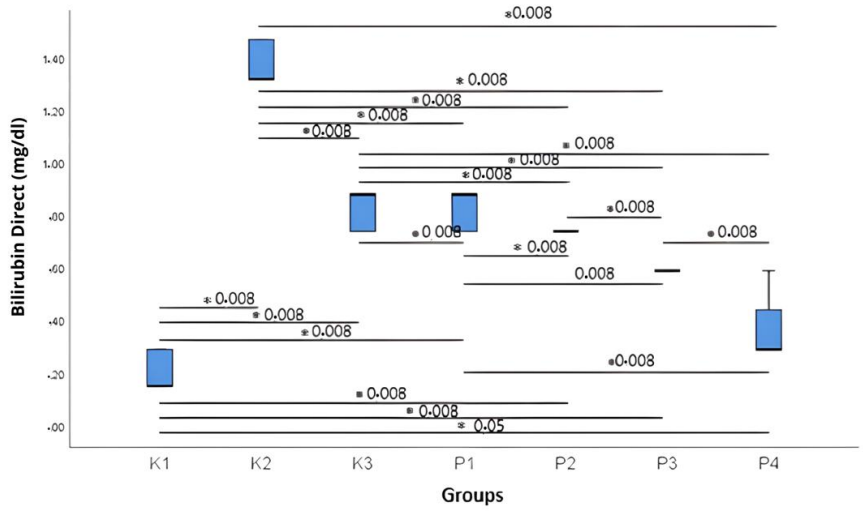

Figure 2. Direct bilirubin levels between each group.

Data are presented as mean ± SD (n = 35). Lines connecting two groups indicate a significant difference in those groups; *significant (p<0.05).

K1: Sham; K2: BDL; K3: BDL + UDCA; P1: BDL + Probiotic 36 mg; P2: BDL + UDCA + Probiotic 18 mg; P3: BDL + UDCA + Probiotic 36 mg; P4: BDL + UDCA + Probiotic 54 mg. UDCA: ursodeoxycholic acid; BDL: biliary duct ligation.

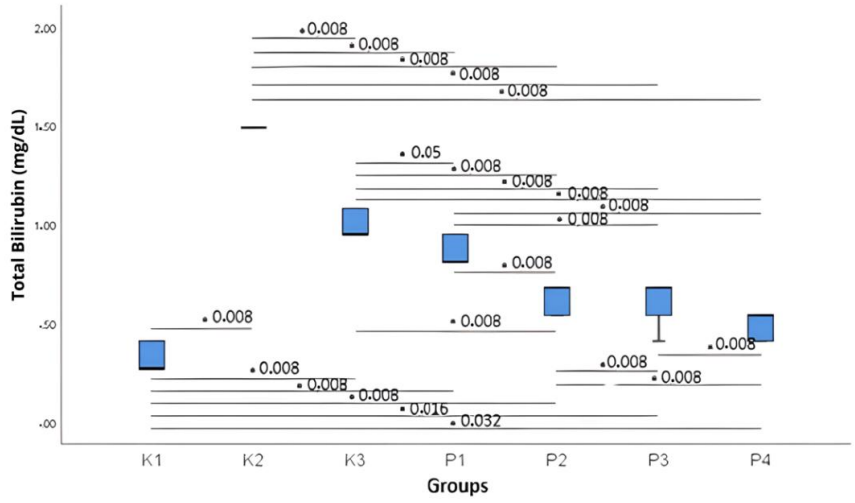

Figure 3. Total bilirubin levels between each group.

Data are presented as mean ± SD (n = 35). Lines connecting two groups indicate a significant difference in those groups; *significant ($p<0.05$).

K1: Sham; K2: BDL; K3: BDL + UDCA; P1: BDL + Probiotic 36 mg; P2: BDL + UDCA + Probiotic 18 mg; P3: BDL + UDCA + Probiotic 36 mg; P4: BDL + UDCA + Probiotic 54 mg. UDCA: ursodeoxycholic acid; BDL: biliary duct ligation.

DISCUSSION

Cholestasis is a condition where bile flow from the liver is disrupted due to various etiologies (Hilscher et al., 2020). More and more recent findings regarding the role of intestinal microbes in liver pathology conditions have become the basis for conducting research on the administration of probiotics in conditions of hepatic cholestasis (Hilscher et al., 2020). Intestinal microbes inhabit the normal intestinal flora and play a major role in bile acid metabolism (Parizek et al., 2016). Cholestasis conditions have been shown to alter the components and numbers of these microbes, with one of those affected being *Bifidobacterium* species (Parizek et al., 2016).

No difference was found in the number of *Bifidobacterium* between the healthy and negative control groups in our study, indicating that liver damage due to BDL did not cause a significant change in the number of *Bifidobacterium*, similar to the findings of Liu et al. (2012) that showed no difference in *Bifidobacterium* between cirrhosis patients and healthy controls (Liu et al., 2012). Our study showed that giving UDCA and 54 mg probiotic resulted in the highest number of *Bifidobacterium*, which is thought to be due to the mechanism of regulation of the normal intestinal flora by probiotics (Achufusi et al., 2022). A study by Xiong et al. showed that the number of *Bifidobacterium* in patients with hepatic cholestasis increased after the administration of probiotics (Xiong et al., 2021). Increasing the number of *Bifidobacterium* was found to be beneficial for the host, where research by Wu et al. (2020) showed that giving *Bifidobacterium* supplementation to neonates with cholestasis can shorten treatment time and improve outcomes (Wu et al., 2020). Probiotics are known to be able to reverse intestinal flora dysbiosis and improve inflammatory responses in mice. Maintaining the intestinal epithelial barrier is also an effect of probiotics, which regulate mucosal immune function and produce metabolites beneficial to the host. Intestinal dysbiosis in patients with liver disease is characterized by overgrowth of potentially pathogenic bacteria. Administration of probiotics has been shown to relieve the suppression of FXR in the gut, leading to increased FXR-FGF15 signaling to reduce the synthesis of bile acids, suppress the pathogenic bacteria growth, and alleviate cholestasis (Jiao et al., 2018).

Elevated bilirubin levels in our BDL-only group, compared with healthy control, indicates that BDL induced hepatic injury and is in line with Tag et al. (2015), who found that BDL induces hepatic inflammatory injury and fibrosis due to obstructive cholestasis and was marked by an increase in hepatic injury serum markers such as the enzymes ALT, AST, and total bilirubin. Combining UDCA and probiotics reduces direct and total bilirubin levels compared to UDCA or probiotic therapy alone, with a probiotic dose of 54 mg producing the lowest bilirubin levels. The mechanism of probiotics in improving liver injury and fibrosis still needs to be understood entirely. Liu et al. found that administration of probiotics significantly reduced direct bilirubin levels in mice with significant liver injury and fibrosis. Probiotic therapy significantly decreased hepatic CYP7A1 expression, associated with increased hepatic concentrations of CDCA, an FXR agonist, and reduced concentrations of T-β-MCA, an FXR antagonist. This condition causes an increase in hepatic FXR activity, which will reduce bile acid levels and prevent their accumulation in the liver (Liu et al., 2020).

This study has some limitations. Our study assessed the efficacy of BDL and the effects of UDCA and probiotics based on markers of bilirubin levels. It can be considered to carry out a histopathological examination of a liver biopsy to visualize the damage and its therapeutic effect directly. This study also only

assessed the impact of the intervention on the 22nd day, so, during the 21 days of intervention, the study did not assess the development of therapeutic effects resulting from UDCA and probiotics.

CONCLUSION

UDCA and probiotic combination significantly improved the amount of *Bifidobacterium* and bilirubin levels in cholestatic rats.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Concepts or ideas	x	x	x	x		x	
Design	x	x	x		x	x	x
Definition of intellectual content	x			x			x
Literature search	x	x		x	x	x	x
Experimental studies	x	x	x	x			
Data acquisition	x		x	x		x	
Data analysis	x	x	x	x		x	
Statistical analysis	x	x			x	x	
Manuscript preparation	x				x	x	x
Manuscript editing	x	x			x	x	x
Manuscript review	x	x	x	x	x	x	x

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