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ORIGINAL ARTICLE

Synergistic Reduction of TNF-α by Combined Ursodeoxycholic Acid and Multi-Strain Probiotics in a Rat Model of Cholestasis

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ABSTRACT

Background: Cholestasis is a complex hepatobiliary disorder in which impaired bile flow provokes a sustained inflammatory response, with tumor necrosis factor-alpha (TNF-α) acting as a central pro-inflammatory mediator. Ursodeoxycholic acid (UDCA) is the first-line therapy, but a substantial subset of patients responds incompletely, motivating adjunctive strategies that target extrahepatic, gut-derived drivers of inflammation through the gut–liver axis.

Objective: To evaluate whether multi-strain probiotics potentiate the anti-inflammatory (TNF-α–lowering) effect of ursodeoxycholic acid (UDCA) in experimental cholestasis.

Methods: This randomised, post-test-only controlled experimental study was reported in accordance with ARRIVE 2.0 guidelines. Thirty-five male Sprague-Dawley rats were randomised into seven groups (n=5): healthy control (K1), disease control (K2), UDCA monotherapy (K3), probiotic monotherapy (K4), and three UDCA plus dose-escalating probiotic combinations (K5–K7). Cholestasis was induced by common bile duct ligation; interventions ran for 21 days. Serum TNF-α was quantified by ELISA and analysed with one-way ANOVA, Tukey HSD and Games-Howell post hoc tests, effect sizes, and dose-response regression.

Results: TNF-α differed markedly across groups ($F(6,28)=783.5$, $p<0.001$, $\eta^2=0.994$). Ligation raised TNF-α from 5.69±0.25 pg/mL (K1) to 17.88±0.43 pg/mL (K2; $p<0.001$, Cohen's $d=34.7$). Both monotherapies reduced TNF-α (K3 14.91±0.49; K4 12.50±0.32; both $p<0.001$), but combination therapy achieved significantly greater suppression, lowest in K7 (7.91±0.18 pg/mL; 55.8% reduction; 81.8% normalisation toward healthy). A significant dose-dependent decline occurred across combination groups (slope=−0.113 pg/mL per mg, $r=-0.94$, $R^2=0.89$, $p<0.001$).

Conclusion: Combined UDCA and probiotics produce a synergistic, dose-dependent reduction of TNF-α in experimental cholestasis, supporting probiotics as a rational adjunct targeting the gut–liver axis and warranting translational evaluation in cholestatic patients.

1. Introduction

Normal hepatobiliary physiology depends on the continuous, regulated flow of bile from hepatocytes through the intrahepatic and extrahepatic biliary tree to the gallbladder and, when required for digestion, into the duodenum. Any interruption of this secretory pathway produces cholestasis, defined as the partial or complete impairment of bile formation or flow. Cholestatic features are detectable in an estimated 10–20% of the general population, and defined cholestatic liver diseases reach prevalences of up to approximately six cases per 100,000 individuals, imposing a considerable and often underappreciated burden on quality of life and health-system resources [1]. Left untreated, persistent cholestasis progresses to cholangitis, biliary fibrosis, and ultimately cirrhosis, with attendant portal hypertension and hepatic failure [1,2]. In Indonesia, where access to specialised hepatobiliary care is unevenly distributed and where advanced cases are channelled through the national referral system to tertiary centres such as Dr. Kariadi General Hospital, the clinical and economic consequences of inadequately controlled cholestasis are especially pressing.

The pathogenesis of cholestasis extends well beyond a purely mechanical disturbance of bile flow to encompass a florid and self-perpetuating inflammatory process. The intrahepatic accumulation of hydrophobic bile acids is directly cytotoxic to hepatocytes and cholangiocytes and simultaneously functions as a danger signal [3]. Injured cells release damage-associated molecular patterns (DAMPs) that engage Toll-like receptors (TLRs) on resident Kupffer cells, activating the nuclear factor-kappa B (NF-κB) signalling cascade and driving the transcription of pro-inflammatory cytokines [3,4]. Among these, tumor necrosis factor-alpha (TNF-α) occupies a pivotal position: upon binding its receptors it amplifies the inflammatory response, upregulates adhesion molecules, and recruits additional immune cells to hepatic tissue, thereby

establishing a sustained inflammatory cycle [3]. TNF-α further induces hepatocyte apoptosis and necroptosis and disrupts the function of canalicular bile transporters, aggravating bile retention and closing a vicious feed-forward loop between cholestasis and inflammation [5].

Crucially, this inflammatory loop is not confined to the liver. Cholestasis alters the composition of the intestinal microbiota and compromises the integrity of the intestinal epithelial barrier, permitting translocation of bacterial products such as lipopolysaccharide (LPS) into the portal circulation, where they further activate TLR4/NF-κB signalling and cytokine production [6,7]. This bidirectional communication between the gut and the liver — the gut–liver axis — has emerged as a central organising concept in hepatology, and accumulating evidence indicates that intestinal dysbiosis and barrier failure are drivers, and not merely consequences, of cholestatic disease progression [7,8]. The recognition that gut-derived inflammation contributes materially to hepatic injury has profound therapeutic implications, because it identifies the intestinal compartment as an actionable target that conventional hepatocentric therapy leaves largely untouched [6].

Ursodeoxycholic acid (UDCA) remains the first-line and most widely accepted pharmacological therapy for cholestatic disorders [9]. A hydrophilic bile acid that endogenously constitutes only about 3% of the total bile acid pool, UDCA acts by enriching the hydrophilic fraction of cholestatic bile, rendering it less cytotoxic and thereby reducing injury to biliary epithelium, portal inflammation, and ductular proliferation; it additionally enhances bile acid secretion and exerts recognised immunomodulatory effects, including interference with NF-κB activation [9]. Despite these benefits, a clinically meaningful subset of patients responds inadequately to UDCA, continuing to experience disease progression or failing to normalise biochemical and inflammatory markers [9]. This limitation is at least partly attributable to the fact that UDCA acts predominantly on intrahepatic mechanisms and exerts little influence over the extrahepatic, gut-derived component of cholestatic inflammation, and it has motivated a sustained search for effective, safe, and affordable adjuncts, including hepatoprotective natural compounds and, increasingly, microbiota-directed therapies [10].

Probiotics offer a mechanistically complementary approach. Live beneficial microorganisms, particularly *Lactobacillus* and *Bifidobacterium* species, restore microbial balance, reinforce tight-junction integrity, and directly modulate immune signalling, suppressing TNF-α through inhibition of the NF-κB and mitogen-activated protein kinase (MAPK) pathways [11-13]. In cholestatic liver injury specifically, *Lactobacillus acidophilus* has been shown to inhibit bile acid synthesis and promote bile acid excretion, ameliorating hepatic injury, while *Lactobacillus* strains reduce ethanol- and diet-induced hepatic inflammation and oxidative stress in other liver-injury models [11,14]. Probiotic fermentation also yields short-chain fatty acids such as butyrate that strengthen the intestinal epithelium and exert anti-inflammatory effects that reverberate along the portal circulation to the liver [8,15]. In preliminary observations by our group in the same model system, probiotic supplementation combined with UDCA restored *Lactobacillus* abundance and improved liver-function enzymes, providing a biological foundation for the present cytokine-level investigation.

Despite this compelling mechanistic rationale, important gaps remain. Most existing evidence derives from studies of single agents, from liver-injury models other than cholestasis, or from investigations that report only categorical group comparisons without quantifying the magnitude or the dose-dependence of the effect [16]. The specific question of whether adjunctive multi-strain probiotics potentiate UDCA-mediated suppression of TNF-α — the cytokine most centrally implicated in cholestatic inflammation — and whether any such potentiation follows a dose-response relationship, has not been rigorously established. Addressing this gap is of direct translational relevance: because UDCA is the established first-line therapy and probiotics are inexpensive, safe, and widely available, a demonstrable synergistic benefit would be immediately actionable in clinical practice, including in resource-constrained health systems such as Indonesia's. We therefore designed a controlled, dose-escalating experiment with healthy and disease controls to test the hypothesis that combined administration of UDCA and multi-strain probiotics reduces serum TNF-α in a rat model of common bile duct ligation more effectively than either agent alone, and to characterise, for the first time in this model, the quantitative dose-response relationship of the adjunctive probiotic component.

2. Methods

Study design and ethical approval

This study employed a randomised, post-test-only control group experimental design and is reported in full accordance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) 2.0 guidelines, the internationally accepted reporting standard for in vivo research; the equivalent clinical reporting frameworks (STROBE and CONSORT) are noted here as analogues but are not directly applicable to a controlled animal experiment. The protocol was reviewed and approved by the Medical Research and Ethics Committee, Faculty of Medicine, Diponegoro University (protocol number 49/EC/H/FK-UNDIP/VI/2023). All procedures involving animals were performed under this approval and in compliance with institutional and national guidelines for the humane care and use of laboratory animals. The experiment was conducted in the experimental laboratory facilities affiliated with the

Department of Surgery, Faculty of Medicine, Universitas Diponegoro / Dr. Kariadi General Hospital, Semarang, Indonesia.

Animals and housing

Thirty-five male Sprague-Dawley rats aged approximately two months and weighing 150–200 g were used. Only male animals were studied to eliminate the confounding influence of the oestrous cycle on inflammatory responses. Animals were housed under controlled environmental conditions at a temperature of 28.0 ± 2.0 °C with a 12-hour light/dark cycle and had free access to standard rodent chow and water ad libitum. A seven-day acclimatisation period preceded any experimental procedure to minimise stress-related variability. General health, physical appearance, and activity level were assessed before enrolment, and only animals in good health were included. Exclusion criteria comprised pre-existing illness, weight outside the specified range, or death during acclimatisation.

Sample size justification and randomization

The number of animals per group was determined using the resource equation approach, which is appropriate when the underlying variance is not known a priori and which constrains the error degrees of freedom (E = total animals − number of groups) to the recommended range of 10–20. With seven groups and five animals per group, the total of 35 animals yields E = 28, comfortably within the acceptable range while adhering to the 3Rs principle of using the minimum number of animals consistent with valid inference. Post hoc power analysis for the observed between-group effect ($\eta^2 = 0.994$) confirmed statistical power exceeding 0.99 at $\alpha = 0.05$. Animals were allocated to the seven groups by simple randomisation; eligible animals were enrolled consecutively as they completed acclimatisation to minimise selection bias. Standardised housing, single-sex selection, and randomisation were used to control for potential confounders including baseline body weight, age, and cage or litter effects. The investigator performing the ELISA quantification was blinded to group allocation.

Induction of cholestasis by common bile duct ligation

Cholestasis was induced via common bile duct ligation (CBDL) in 30 of the 35 animals; the remaining 5 animals served as the healthy control group and did not undergo ligation. The CBDL model faithfully reproduces the obstructive, inflammatory, and dysbiotic features of human cholestasis and is widely used to evaluate hepatoprotective and immunomodulatory interventions [17-19]. Prior to surgery, all animals received prophylactic antibiotic prophylaxis with cefotaxime (18 mg, intramuscular; Indofarma®, Jakarta, Indonesia). Anaesthesia was induced with ketamine hydrochloride (0.5 mL, intramuscular; Dexa Medica®, Cikarang, Indonesia). Under sterile conditions, a midline laparotomy was performed and the common bile duct was identified and ligated using 5-0 silk sutures (DemeTECH®, Miami Lakes, FL, USA). Postoperative analgesia was provided with oral ibuprofen (7 mg; Pharos®, Semarang, Indonesia) every 8 hours for 3 days to ensure adequate pain management and welfare.

Experimental groups and intervention

After randomisation and CBDL, all interventions were administered orally for 21 consecutive days. The seven groups were defined as follows: K1 (healthy control) did not undergo ligation and received a standard diet and water ad libitum; K2 (negative/disease control) underwent ligation and received a standard diet and water ad libitum; K3 (positive control) underwent ligation and received UDCA 13.5 mg; K4 underwent ligation and received probiotics 36 mg; K5 received UDCA 13.5 mg plus probiotics 18 mg; K6 received UDCA 13.5 mg plus probiotics 36 mg; and K7 received UDCA 13.5 mg plus probiotics 54 mg. Doses of UDCA and probiotics were derived from human therapeutic doses using standard allometric body-surface-area conversion for the rat. The probiotic intervention was a commercially available multi-strain formulation (L-Bio® sachet; PT Lapi Laboratories, Indonesia) containing *Bifidobacterium lactis* W51, *Bifidobacterium lactis* W55, *Lactobacillus acidophilus* W55, *Lactobacillus casei* W56, *Lactobacillus salivarius* W57, and *Lactobacillus lactis* W58. This escalating design (18, 36, and 54 mg probiotic at a fixed UDCA dose) was chosen specifically to permit assessment of a dose-response relationship. The full composition of the seven experimental groups is summarised in Table 1.

Measurement of serum TNF-α

On day 22, all animals were euthanised under overdose anaesthesia in accordance with recognised humane guidelines, and blood samples were collected for TNF-α quantification. Serum TNF-α was measured by quantitative sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. Serum samples and TNF-α standards were added to microplate wells pre-coated with specific anti-TNF-α capture antibodies and incubated to permit antigen–antibody binding; after washing and addition of substrate, optical density was read on a microplate reader at 450 nm. TNF-α concentrations were interpolated from a standard curve and expressed in pg/mL. All samples were assayed in the same run to minimise inter-assay variability, samples were distributed across the plate to avoid group-position confounding, all values fell within the validated dynamic range of the standard curve, and the operator was blinded to group allocation.

Confounder control, co-interventions, and reproducibility

Principal confounders were controlled by design and restriction: all animals were male, of the same strain,

age range, and weight range, and were housed under identical standardised conditions, with randomisation used to distribute any residual variation across groups. Because a cholestasis intervention that modulates the intestinal microbiota is sensitive to shared-environment effects, animals were managed to minimise cross-contamination between groups, and the animal (rather than the cage) was treated as the unit of analysis, consistent with the unit of randomisation. All perioperative co-interventions — prophylactic cefotaxime, ketamine anaesthesia, and the three-day postoperative ibuprofen course — were administered uniformly across every group, including controls, and were completed well before the day-22 endpoint, so that they cannot account for the between-group differences observed. Animals were monitored daily against pre-defined humane endpoints; none met an early endpoint and all completed the protocol. Individual-animal data are available to permit independent reanalysis.

Statistical analysis

Data were analysed using SPSS version 26.0 (IBM Corp., Armonk, NY, USA) with confirmatory computation in a numerical Python environment. Continuous data are presented as mean ± standard deviation (SD) with 95% confidence intervals (CI). The Shapiro-Wilk test assessed normality and the Levene / Brown-Forsythe test assessed homogeneity of variance; because the Shapiro-Wilk test has limited power at n=5, non-parametric confirmation was also obtained. Between-group differences were evaluated by one-way analysis of variance (ANOVA); the magnitude of the overall effect was quantified using eta-squared (η^2) and omega-squared (ω^2), interpreted in the context of a controlled, low-variance experimental system. Pairwise comparisons used Tukey's honestly significant difference (HSD) test, complemented by the Games-Howell procedure; pairwise standardised mean differences were expressed as Cohen's d, with the small-sample-corrected Hedges' g computed to guard against upward bias, and family-wise error across the 21 pairwise contrasts was controlled. To confirm robustness, Welch's ANOVA and the non-parametric Kruskal-Wallis test (with epsilon-squared effect size) were additionally computed. The dose-response relationship across the combination groups was modelled by ordinary least-squares linear regression of serum TNF-α on probiotic dose, reporting the slope with its 95% CI, the Pearson correlation coefficient, R^2, and the p-value, with residuals inspected for normality and homoscedasticity; the non-parametric Jonckheere-Terpstra test for ordered alternatives confirmed monotonic trend. Percentage reduction relative to the disease control and percentage normalisation toward the healthy baseline (relative to the K2–K1 range) were computed for each treatment group. A two-tailed p-value < 0.05 was considered statistically significant, and exact p-values are reported to three decimal places except where $p < 0.001$.

3. Results

All experimental animals were two-month-old male Sprague-Dawley rats with an average body weight of approximately 200 g. Before intervention, all animals were in good health, physically normal, and displayed normal activity levels, and all completed the seven-day acclimatisation period under standardised laboratory conditions with free access to food and water. Cholestasis was induced by common bile duct ligation in 30 animals, after which the animals were randomly allocated into the seven predefined groups summarised in Table 1. No animal died prematurely or was excluded; all 35 animals completed the 21-day protocol and were included in the final analysis, yielding complete data with no missing values.

Serum TNF-α differed profoundly across the seven groups, as detailed in Table 1 and illustrated in Figure 1. One-way ANOVA demonstrated a highly significant overall effect ($F(6,28) = 783.54$, $p < 0.001$), with an exceptionally large effect size ($\eta^2 = 0.994$, $\omega^2 = 0.993$), indicating that group allocation accounted for essentially all of the variance in TNF-α. The Shapiro-Wilk test confirmed normality within each group (all $p > 0.05$) and the Brown-Forsythe test confirmed homogeneity of variance ($W = 0.52$, $p = 0.790$). Robustness analyses were fully concordant: Welch's ANOVA ($F(6, 12.3) = 650.19$, $p < 0.001$) and the non-parametric Kruskal-Wallis test ($H = 33.16$, $p < 0.001$, $\varepsilon^2 = 0.970$) both confirmed the overall difference. These overall inferential statistics are consolidated in Table 3.

Table 1. Composition of the seven experimental groups and serum TNF-α levels (mean ± SD, n = 5 per group).

Group	Intervention	TNF-α (pg/mL)	95% CI	Red. (%)	Norm. (%)
K1	Healthy control (no ligation)	5.69 ± 0.25	5.38–6.00	—	—
K2	Disease control (CBDL, untreated)	17.88 ± 0.43	17.35–18.41	—	—
K3	UDCA 13.5 mg	14.91 ± 0.49	14.30–15.52	16.6	24.4
K4	Probiotics 36 mg	12.50 ± 0.32	12.10–12.90	30.1	44.1
K5	UDCA 13.5 + Probiotics 18 mg	11.98 ± 0.26	11.66–12.30	33.0	48.4
K6	UDCA 13.5 + Probiotics 36 mg	8.82 ± 0.33	8.41–9.23	50.7	74.3
K7	UDCA 13.5 + Probiotics 54 mg	7.91 ± 0.18	7.69–8.13	55.8	81.8

Notes: CBDL: common bile duct ligation; UDCA: ursodeoxycholic acid; CI: confidence interval; SD: standard deviation. Reduction is relative to the disease control (K2); normalisation is relative to the K2–K1 range.

As shown in Table 1 and Figure 1, common bile duct ligation produced a marked elevation of serum TNF-α in the disease control group (K2, 17.88 ± 0.43 pg/mL; 95% CI 17.35–18.41) relative to the healthy control (K1, 5.69 ± 0.25 pg/mL; 95% CI 5.38–6.00), a more than threefold increase that was highly significant ($p < 0.001$) and of extraordinary magnitude (Cohen's d = 34.7), confirming a robust inflammatory response to cholestasis. Both monotherapies significantly lowered TNF-α relative to the disease control. UDCA alone (K3) reduced TNF-α to 14.91 ± 0.49 pg/mL (95% CI 14.30–15.52; $p < 0.001$ vs K2; d = 6.4), a 16.6% reduction. Probiotic monotherapy (K4) achieved a greater reduction to 12.50 ± 0.32 pg/mL (95% CI 12.10–12.90; $p < 0.001$ vs K2; d = 14.2), corresponding to a 30.1% reduction, and was itself significantly lower than UDCA monotherapy (K3 vs K4, $p < 0.001$). The complete matrix of pairwise comparisons is presented in Table 2.

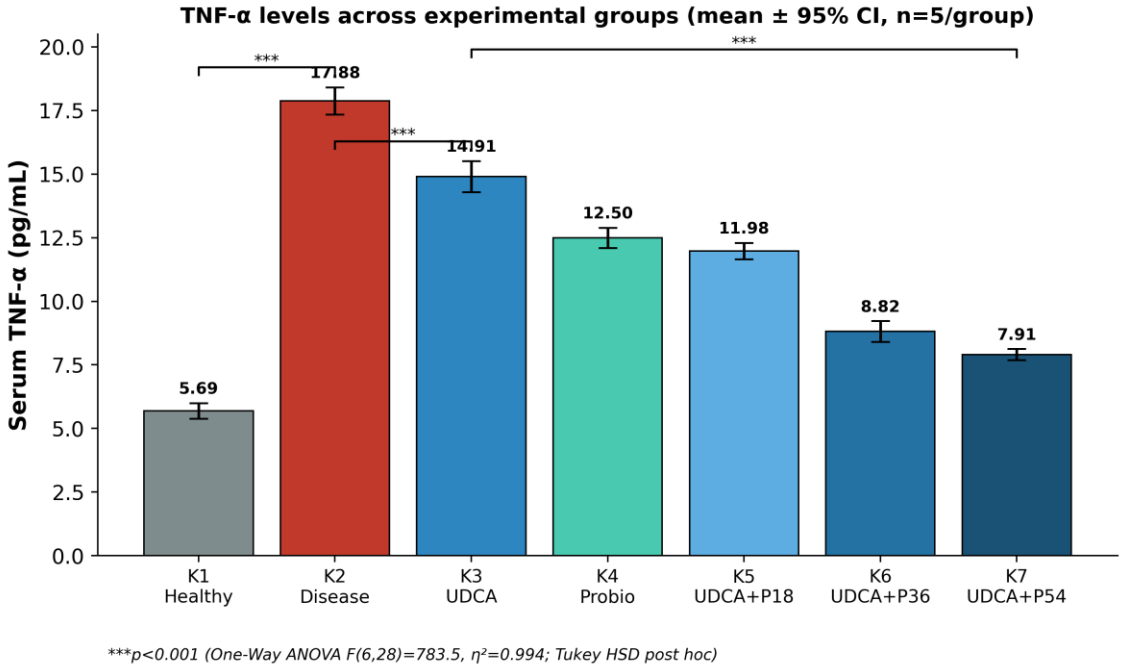

Figure 1. Serum TNF-α levels across the seven experimental groups (mean ± 95% CI). Bile duct ligation markedly elevated TNF-α (K2), while UDCA, probiotics, and their dose-escalating combinations progressively reduced it (One-Way ANOVA $F(6,28) = 783.5$, $p < 0.001$, $\eta^2 = 0.994$; ***$p < 0.001$, Tukey HSD).

The combination groups achieved the greatest suppression of TNF-α, as detailed in Table 1 and Figure 1. The lowest-dose combination (K5, UDCA + probiotics 18 mg) reduced TNF-α to 11.98 ± 0.26 pg/mL (95% CI 11.66–12.30; 33.0% reduction), a value modestly but significantly lower than probiotic monotherapy (K4 vs K5, p = 0.023). Increasing the probiotic dose produced progressive, significant further reductions: K6 (UDCA + probiotics 36 mg) reached 8.82 ± 0.33 pg/mL (95% CI 8.41–9.23; 50.7% reduction) and K7 (UDCA + probiotics 54 mg) reached 7.91 ± 0.18 pg/mL (95% CI 7.69–8.13; 55.8% reduction), the latter representing an 81.8% normalisation toward the healthy baseline. Each incremental dose step was statistically significant (K5 vs K6, $p < 0.001$, Cohen's d = 10.6; K6 vs K7, p = 0.001, d = 3.4), and every combination group differed significantly from both monotherapies (all $p < 0.001$ vs UDCA control K3; $p \leq 0.023$ vs probiotic control K4). Notably, the highest-dose combination (K7) reduced TNF-α by 7.00 pg/mL relative to UDCA monotherapy (K3), an effect of very large magnitude (Cohen's d = 19.0), as summarised in Table 3.

The dose-dependence of the combination effect was formally confirmed by regression analysis, presented in Figure 2. Ordinary least-squares modelling across the three combination groups, in which the UDCA dose was held constant and only the probiotic dose varied (18, 36, and 54 mg), demonstrated a strong, significant inverse relationship between probiotic dose and serum TNF-α (slope = −0.113 pg/mL per mg, 95% CI −0.137 to −0.089, Pearson $r = -0.94$, $R^2 = 0.89$, $p < 0.001$), indicating that each additional milligram of probiotic was associated with a reduction of approximately 0.11 pg/mL in TNF-α and that 89% of the variance in TNF-α across these groups was explained by dose. The monotonic nature of this trend was corroborated by the non-parametric Jonckheere-Terpstra test ($z = -3.96$, $p < 0.001$), and residuals of the regression were approximately normal and homoscedastic. Because standardised mean differences computed at n = 5 are subject to upward bias, the small-sample-corrected Hedges' g was also computed for the key contrasts; the correction (factor ≈ 0.90) left the qualitative conclusions unchanged (for example, the disease-

versus-healthy contrast corresponded to g ≈ 31.3). The single borderline comparison in the dataset was the low-dose combination versus probiotic monotherapy (K4 vs K5, p = 0.023); under a conservative family-wise correction this difference would not retain significance and is therefore interpreted as suggestive, whereas all other reported contrasts remained significant. The complete inferential summary is provided in Table 3.

Taken together, the pattern of results describes a clear hierarchy of anti-inflammatory efficacy: disease control (highest TNF-α) > UDCA monotherapy > probiotic monotherapy > low-dose combination > mid-dose combination > high-dose combination > healthy control (lowest TNF-α). The magnitude of TNF-α suppression — expressed both as reduction from the disease control and as normalisation toward the healthy baseline — is shown in Figure 3, which demonstrates that normalisation rose from 24.4% with UDCA alone to 44.1% with probiotic monotherapy and to 81.8% with the highest-dose combination. Across every analytic approach — parametric ANOVA, robust Welch's ANOVA, and non-parametric Kruskal-Wallis — the conclusions were identical, and the exceptionally large and concordant effect sizes (η^2 = 0.994; ε^2 = 0.970) indicate that the observed differences are highly unlikely to reflect chance or model-specific artefact.

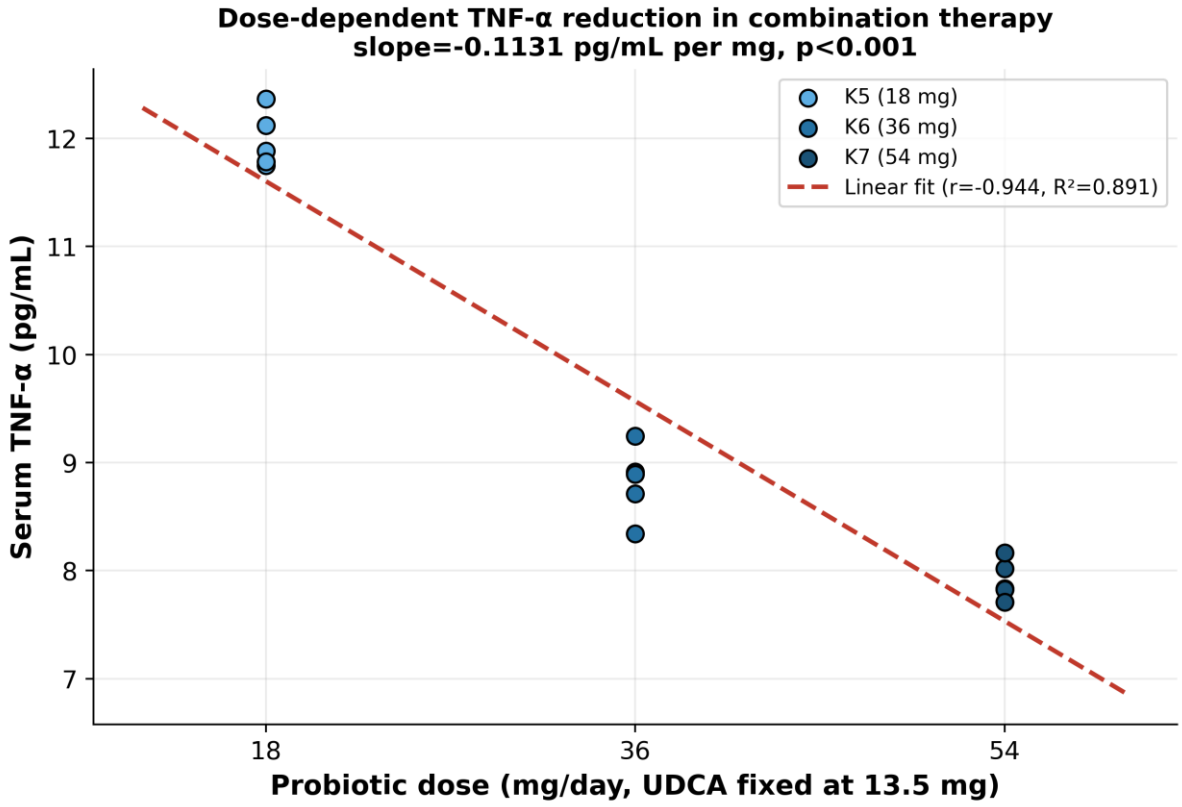

Figure 2. Dose-dependent reduction of serum TNF-α across the combination-therapy groups (K5–K7) at a fixed UDCA dose. Linear regression showed a strong inverse relationship between probiotic dose and TNF-α (slope = −0.113 pg/mL per mg, 95% CI −0.137 to −0.089, r = −0.94, R^2 = 0.89, p < 0.001).

Table 2. Pairwise post hoc comparisons of serum TNF-α between groups (p-values).

Group	Mean ± SD	K1	K2	K3	K4	K5	K6
K1	5.69 ± 0.25						
K2	17.88 ± 0.43	<0.001					
K3	14.91 ± 0.49	<0.001	<0.001				
K4	12.50 ± 0.32	<0.001	<0.001	<0.001			
K5	11.98 ± 0.26	<0.001	<0.001	<0.001	0.023		
K6	8.82 ± 0.33	<0.001	<0.001	<0.001	<0.001	<0.001	
K7	7.91 ± 0.18	<0.001	<0.001	<0.001	<0.001	<0.001	0.001

Notes: p-values from pairwise comparisons (two-tailed). All treatment groups differed significantly from both the healthy (K1) and disease (K2) controls (all p < 0.001). Overall One-Way ANOVA: F(6,28) = 783.5, p < 0.001.

Table 3. Overall inferential statistics, effect sizes, and dose-response analysis.

Analysis	Result
One-Way ANOVA	$F(6,28) = 783.54$, $p < 0.001$
Effect size (ANOVA)	$\eta^2 = 0.994$; $\omega^2 = 0.993$ (very large)
Welch's ANOVA (robust)	$F(6, 12.3) = 650.19$, $p < 0.001$
Kruskal-Wallis (non-parametric)	$H = 33.16$, $p < 0.001$; $\varepsilon^2 = 0.970$
Homogeneity (Brown-Forsythe)	$W = 0.52$, $p = 0.790$
Disease vs healthy (K2 vs K1)	$\Delta = 12.19$ pg/mL, $p < 0.001$; $d = 34.7$
UDCA vs disease (K3 vs K2)	16.6% reduction, $p < 0.001$; $d = 6.4$
Probiotics vs disease (K4 vs K2)	30.1% reduction, $p < 0.001$; $d = 14.2$
Best combination vs UDCA (K7 vs K3)	7.00 pg/mL lower, $p < 0.001$; $d = 19.0$
Highest dose (K7) normalisation	81.8% toward healthy (55.8% reduction)
Dose-response (K5–K7)	slope = −0.113 (95% CI −0.137, −0.089); $r = -0.94$; $R^2 = 0.89$; $p < 0.001$
Trend (Jonckheere-Terpstra)	$z = -3.96$, $p < 0.001$

Notes: η^2: eta-squared; ω^2: omega-squared; ε^2: epsilon-squared; d: Cohen's d; Δ: mean difference.

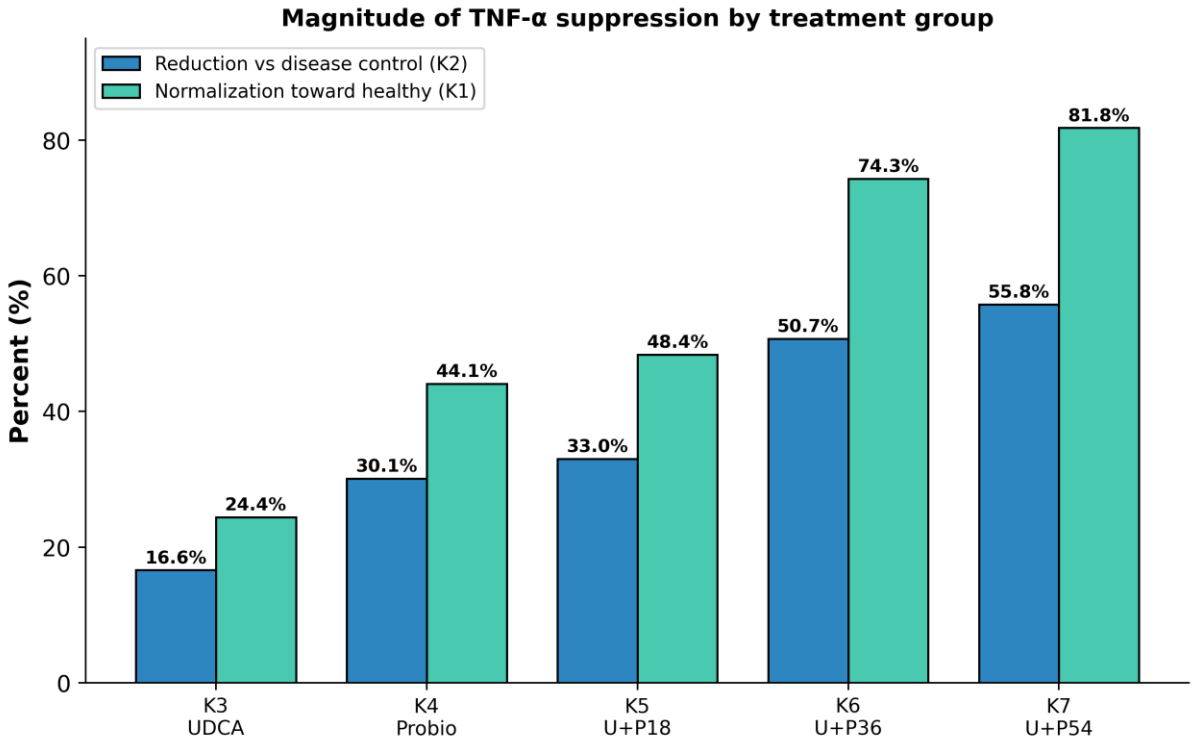

Figure 3. Magnitude of TNF-α suppression by treatment group, expressed as percentage reduction relative to the disease control (K2) and percentage normalisation toward the healthy baseline (K1). Combination therapy produced the greatest, dose-dependent effect, with K7 normalising TNF-α by 81.8%.

4. Discussion

Cholestasis is increasingly recognised not merely as a disorder of bile flow but as a complex inflammatory condition in which immune dysregulation plays a central pathogenic role [1,3]. Among the mediators involved, TNF-α has emerged as a pivotal cytokine linking hepatocellular injury, bile-acid dysregulation, and fibrogenesis; elevated TNF-α concentrations are consistently observed in cholestatic conditions and contribute to hepatic stellate cell activation and fibrosis [3,5]. The present study, conducted at the Department of Surgery of Universitas Diponegoro / Dr. Kariadi General Hospital, was designed to determine whether adjunctive multi-strain probiotics potentiate the anti-inflammatory efficacy of UDCA in a controlled, dose-escalating experimental model, and to characterise the dose-response relationship of the probiotic component.

The principal quantitative findings, detailed in Table 1 and Figure 1, are unambiguous. Common bile duct ligation produced a more than threefold elevation of serum TNF-α relative to healthy controls, with an effect size (Cohen's d = 34.7) that leaves no doubt about the robustness of the induced inflammatory response.

Against this background, both monotherapies produced significant reductions, but combination therapy was consistently and substantially superior, and the effect was strongly dose-dependent, with the highest-dose combination normalising TNF-α by more than four-fifths toward the healthy baseline. The overall effect size ($\eta^2 = 0.994$) and the concordance of parametric, robust, and non-parametric analyses lend considerable confidence to these conclusions.

The marked elevation of TNF-α in the disease control group is mechanistically consistent with the established pathophysiology of cholestasis. The accumulation of toxic bile acids and the disruption of intestinal barrier integrity facilitate translocation of endotoxins such as lipopolysaccharide into the portal circulation, where LPS activates Toll-like receptor signalling and drives NF-κB-dependent transcription of pro-inflammatory cytokines including TNF-α [4,15]. This cascade underscores the critical role of the gut–liver axis in amplifying inflammation during cholestasis and reinforces the view that intestinal dysbiosis is a driver, rather than a passive consequence, of disease progression.[6,8] The experimental separation of hepatic and intestinal contributions afforded by our seven-arm design allowed these mechanisms to be probed directly.

Treatment with UDCA alone significantly reduced TNF-α, reaffirming its role as the cornerstone therapy for cholestatic disorders. Beyond its well-established effects on bile-acid hydrophilicity and hepatocyte protection, UDCA exerts immunomodulatory effects that attenuate inflammatory signalling, including interference with NF-κB activation [9]. However, as shown in Figure 3, UDCA monotherapy reduced TNF-α by only 16.6% and normalised it by only 24.4% toward the healthy baseline — the smallest effect among the active treatments. This comparatively modest response is consistent with clinical observations that a subset of patients does not fully normalise inflammatory markers on UDCA alone [9,10], and it is mechanistically explicable: UDCA acts predominantly on intrahepatic processes and exerts limited influence over the extrahepatic, gut-derived component of cholestatic inflammation. This limitation defines precisely the therapeutic space that adjunctive probiotics are positioned to fill.

Probiotic monotherapy produced a distinctly greater anti-inflammatory effect than UDCA, reducing TNF-α by 30.1% (Table 1, Figure 3). This finding highlights the importance of microbiota modulation in the pathophysiology of cholestasis and is congruent with a large body of evidence that probiotics act through several complementary mechanisms [11,13]. *Lactobacillus* strains suppress pro-inflammatory cytokines including TNF-α by inhibiting key signalling pathways such as NF-κB and MAPK, and in cholestatic liver injury specifically, *Lactobacillus acidophilus* ameliorates hepatic injury by modulating bile acid synthesis and excretion [11,14]. The multi-strain formulation used here, combining *Bifidobacterium* and *Lactobacillus* species, is well suited to exert these effects across multiple pathways simultaneously.

Probiotics also play a critical role in maintaining intestinal barrier function, increasingly recognised as a key determinant of systemic inflammation. Disruption of tight-junction integrity permits translocation of microbial products, perpetuating a cycle of inflammation; probiotic-derived signals and metabolites such as butyrate reinforce the epithelial barrier and suppress NF-κB activation, thereby preventing increased intestinal permeability and subsequent endotoxemia [8,11]. This mechanism is particularly relevant in cholestasis, where impaired bile flow alters gut microbiota composition and predisposes to barrier dysfunction, and it provides a coherent explanation for the superiority of probiotic-containing regimens [6,8].

The most notable finding of this study is the enhanced and dose-dependent reduction of TNF-α in the combination groups (Figure 1, Figure 2), indicating a synergistic interaction between UDCA and probiotics. This synergy arises from the complementary targeting of both the hepatic and intestinal components of the disease: while UDCA primarily mitigates hepatocellular injury and bile-acid toxicity, probiotics address the upstream drivers of inflammation by restoring microbiota balance and reducing endotoxin-mediated immune activation [7,20]. This dual mechanism effectively interrupts the bidirectional amplification loop of the gut–liver axis, producing a more pronounced anti-inflammatory effect than either agent can achieve alone. The quantitative dose-response relationship shown in Figure 2 — a strong, statistically significant linear decline in TNF-α with increasing probiotic dose ($r = -0.94$, $R^2 = 0.89$, $p < 0.001$) — substantially strengthens the biological plausibility of the effect and moves the evidence beyond a simple categorical comparison.

Two interpretive caveats should accompany this synergy claim. First, the mechanistic model advanced here — complementary hepatic and intestinal action converging on NF-κB-dependent TNF-α transcription — is strongly supported by the literature but is not directly demonstrated by the present data, which measured a single downstream output rather than intestinal permeability, portal endotoxin, hepatic NF-κB activation, or microbiota composition; the mechanism is therefore best regarded as a well-founded hypothesis consistent with our results. Second, the term synergy is used here in the sense of a greater-than-monotherapy, dose-dependent enhancement rather than in the strict pharmacological sense established by isobolographic analysis, which the present design was not constructed to test formally; the dose-response evidence nonetheless provides reasonable support for a genuine interaction between the two agents.

The dose-dependence itself carries mechanistic significance. Higher probiotic doses may enhance colonisation by beneficial bacteria, increase the production of anti-inflammatory metabolites, and more effectively suppress pro-inflammatory signalling, consistent with reports that probiotics reduce TNF-α and NF-κB activity and improve hepatic inflammation in a manner related to strain and dose [21]. At the same time, probiotic effects are strain-specific and context-dependent, which may contribute to the heterogeneity of results across the wider literature and which argues for careful characterisation of formulation and dose in future translational work.

Our results align closely with, and extend, the international literature. Experimental studies employing the bile duct ligation model have consistently reported elevated hepatic and systemic inflammation following obstruction, and interventions that dampen TLR4/NF-κB signalling reduce this response [4,15]. Reports of probiotic and combination interventions in liver-injury models describe reductions in inflammatory markers comparable in direction to those observed here, including combined probiotic and pharmacological regimens that improve hepatic outcomes [22]. What distinguishes the present study is the direct, head-to-head comparison of monotherapy and combination therapy within a single dose-escalation framework, which allows the synergy and its dose-dependence to be quantified rather than merely inferred; the further 25 percentage points of reduction achieved beyond probiotic monotherapy at the highest probiotic dose (Figure 3) is, to our knowledge, not previously demonstrated with this quantitative resolution in a cholestasis model.

The biological plausibility of the synergy is further supported by the convergent mechanisms of the two agents on the same final common pathway. Both UDCA and *Lactobacillus*-derived signals ultimately converge on suppression of NF-κB-dependent transcription of TNF-α, but they do so from different anatomical and biochemical starting points: UDCA from within the hepatocyte and biliary compartment, and probiotics from within the intestinal lumen and epithelium [9,12,13]. Simultaneous engagement of a shared downstream node from two upstream directions provides a coherent molecular explanation for an effect that exceeds either monotherapy, and the additional contribution of short-chain fatty acids and restored bile-acid homeostasis offers plausible supplementary routes by which higher probiotic doses could yield progressively greater benefit [11,15].

Integration of these findings with our preliminary observations reinforces the mechanistic link between microbiota modulation and inflammation. The earlier demonstration that probiotic administration increased *Lactobacillus* abundance and improved liver-function enzymes in the same model provides a biological basis for the TNF-α reductions observed here. Restoration of a beneficial microbiota plausibly reduces dysbiosis-associated endotoxemia and modulates bile-acid signalling, ultimately decreasing inflammatory activation; because dysbiosis has been implicated in both the initiation and the progression of cholestasis, these results reinforce the concept that targeting the gut microbiota is a rational and potentially high-yield therapeutic strategy [6,23].

These findings carry tangible implications for clinical hepatobiliary practice. UDCA is affordable and widely available, and multi-strain probiotics are inexpensive, safe, and already in broad use; a combination that meaningfully augments anti-inflammatory efficacy without introducing significant toxicity would be readily deployable, including within resource-constrained settings. In the Indonesian context, where cholestatic disease is managed across a tiered referral system and where the national health-insurance scheme (BPJS Kesehatan) governs access to and reimbursement of therapy, a low-cost adjunct that improves the response to a first-line drug is particularly attractive. The expertise of the Department of Surgery at Universitas Diponegoro / Dr. Kariadi General Hospital in hepatobiliary disease positions it well to translate these experimental observations into carefully designed clinical evaluation, in alignment with national clinical guidelines and specialty-society recommendations.

From a translational and safety standpoint, the profile of the intervention is favourable. UDCA has a long record of clinical safety, and multi-strain probiotic formulations of the type used here are generally regarded as safe, with an established tolerability profile; serious adverse events are rare and largely confined to severely immunocompromised or critically ill populations. The combination therefore represents a low-risk, low-cost addition to an existing standard of care rather than a novel pharmacological entity requiring extensive de novo safety characterisation, which substantially lowers the barrier to clinical translation. Nonetheless, the appropriate human-equivalent dosing, the optimal strain composition, and the durability of the anti-inflammatory effect over longer treatment periods all remain to be defined, and the possibility of a plateau in the dose-response relationship beyond the doses tested here cannot be excluded from the present data.

The methodological rigour of the present analysis also deserves comment. By reporting effect sizes and confidence intervals alongside p-values (Table 3), by confirming the primary result through parametric, robust, and non-parametric routes, and by formally modelling the dose-response relationship (Figure 2), the analysis moves beyond binary significance testing and provides a quantitatively richer evidence base. The concordance of these approaches, together with the completeness of the dataset (no missing values and no premature deaths), lends the conclusions a robustness that supports their use as a foundation for subsequent translational work.

Several strengths of this study merit emphasis. The seven-arm, dose-escalating design with both healthy and disease controls permitted simultaneous assessment of disease induction, monotherapy, combination therapy, and dose-response within a single, internally consistent experiment. The analysis was rigorous and comprehensive, incorporating effect sizes, confidence intervals, robustness checks, and formal dose-response modelling, and the outcome assessment was blinded. The use of a standardised, commercially available multi-strain formulation enhances reproducibility and translational relevance, and the study was conducted and reported in accordance with ARRIVE 2.0 guidelines.

Several limitations must nonetheless be acknowledged, and the two most consequential are placed first. First, the study rests on a single inflammatory marker: while TNF-α is mechanistically central, it is only one node of a complex cytokine network, and the absence of complementary mediators (for example IL-6, IL-1β, and the anti-inflammatory IL-10) precludes full characterisation of the immunological profile of the intervention; the TNF-α reduction should therefore be read as a mechanistic signal rather than as proof of a comprehensive anti-inflammatory effect. Second, the absence of histopathological analysis prevents direct assessment of tissue-level inflammation, apoptosis, ductular reaction, and fibrosis — the endpoints that ultimately determine clinical benefit — so the cytokine findings require histological corroboration. Third, TNF-α was measured at a single time point, which cannot capture the dynamic evolution of inflammation or distinguish accelerated resolution from a shifted steady state. Fourth, the study used a controlled rat model of complete biliary obstruction in a single genetic background and facility; while this maximises internal validity, the very features that produced such large, consistent effects differ markedly from the heterogeneous, often partial cholestatic disease seen clinically, so extrapolation must be cautious and the exceptional effect sizes should not be expected in human populations. Fifth, microbiological confirmation of intestinal colonisation and markers of barrier function and endotoxemia were not obtained in this cytokine-focused analysis, so the microbiome-mediated component of the proposed mechanism remains inferential. Sixth, the small per-group sample ($n = 5$), although justified by the resource equation and appropriate for a low-variance model, yields imprecise variance estimates and limits the power to characterise the shape of the dose-response beyond a monotonic trend; no dose plateau was reached, so the therapeutically optimal dose cannot be identified from these data. Future studies should incorporate a broader cytokine panel, histopathology, serial sampling, microbiome and barrier-function endpoints, a formal factorial design to test synergy, replication across strains and facilities, and ultimately clinical validation to define optimal strains, doses, and treatment durations.

Taken together, this study provides compelling evidence that combining UDCA with multi-strain probiotics offers a more comprehensive therapeutic approach to cholestasis by simultaneously targeting hepatic injury and gut-derived inflammation. The findings support a shift in the conceptual management of cholestasis toward combination strategies that address multiple pathogenic pathways of the gut–liver axis, and they provide a quantitative, dose-resolved foundation for the translational development of probiotic adjuncts.

5. Conclusion

In a controlled, dose-escalating experimental model of common bile duct ligation-induced cholestasis, the combination of ursodeoxycholic acid and multi-strain probiotics reduced serum TNF-α significantly and dose-dependently, achieving substantially greater anti-inflammatory efficacy than either agent alone (overall $F(6,28) = 783.5$, $p < 0.001$, $\eta^2 = 0.994$; dose-response $r = -0.94$, $p < 0.001$), with the highest-dose combination normalising TNF-α by more than 80% toward the healthy baseline. These results demonstrate a synergistic, dose-dependent interaction consistent with complementary hepatic and intestinal mechanisms operating across the gut–liver axis. For Indonesian clinical practice, and for the hepatobiliary programme at Universitas Diponegoro / Dr. Kariadi General Hospital in particular, the findings support the rational evaluation of low-cost, safe multi-strain probiotics as an adjunct to UDCA in cholestatic disease. Future research should validate these observations using broader cytokine panels, histopathological endpoints, serial sampling, and microbiome analyses, and should progress toward well-designed clinical trials to identify optimal probiotic strains, doses, and treatment durations.

Declarations

Ethical Approval

The Medical Research and Ethics Committee, Faculty of Medicine, Diponegoro University approved this study (protocol number 49/EC/H/FK-UNDIP/VI/2023). All procedures were conducted in full compliance with the ARRIVE 2.0 guidelines and with institutional and national regulations for the humane care and use of laboratory animals.

Author Contributions

M.H.N. contributed to conceptualisation, methodology, investigation, formal analysis, and writing of the original draft; E.P. contributed to conceptualisation, supervision, and review and editing of the manuscript; and S.A.P. contributed to methodology, supervision, and review and editing of the manuscript. All authors have read and approved the final version of the manuscript.

Conflict of Interest
The authors declare no conflict of interest.

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Data Availability
The individual-animal data supporting the findings of this study are available from the corresponding author upon reasonable request.

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