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Comparative analysis of renal fibrosis quantification using hematoxylin-eosin and Sirius Red staining in unilateral ureteral obstruction rat models treated with *Lactococcus lactis* D4

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Abstract
Renal fibrosis represents the final common pathway for nearly all forms of progressive kidney diseases. This study evaluates the anti-fibrotic potential of *Lactococcus lactis* D4, a dadih-derived probiotic, while simultaneously comparing the diagnostic accuracy of Hematoxylin-Eosin (H&E) versus Sirius Red (SR) staining. Using 18 Sprague Dawley rats across three experimental groups (Control, UUO-induced P1, and UUO+probiotic P2), we performed detailed histopathological assessments. Results indicated that *L. lactis* D4 significantly mitigated fibrotic progression. Furthermore, comparative analysis revealed that while H&E is sufficient for identifying gross morphological changes in high-fibrosis environments, SR offers superior precision in quantifying early-stage or minimal collagen deposition. These findings provide a framework for selecting staining methods in resource-limited versus high-precision research settings.

Keywords: Chronic kidney disease, UUO, *Lactococcus lactis* D4, Sirius Red, hematoxylin-eosin, renal fibrosis

Introduction
Chronic Kidney Disease (CKD) is a global health burden characterized by the progressive loss of renal function and the accumulation of extracellular matrix (ECM) proteins, primarily collagen. This process, known as renal fibrosis, involves a complex interplay of inflammatory cytokines, oxidative stress, and myofibroblast activation (Huang *et al.*, 2023) [4]. The Unilateral Ureteral Obstruction (UUO) model is widely recognized as the gold standard for studying renal fibrosis because it rapidly mimics the hemodynamic and morphological changes seen in human obstructive nephropathy (Nørregaard *et al.*, 2023) [7].

The pathogenesis of fibrosis in the UUO model begins with mechanical stretch, followed by cellular infiltration and the release of pro-fibrotic markers like TGF-β1. These markers trigger the epithelial-to-mesenchymal transition (EMT), leading to excessive collagen deposition in the interstitial space (Nan *et al.*, 2024) [6]. Monitoring this progression is vital for evaluating therapeutic interventions, yet the choice of histological staining remains a subject of debate among pathologists and researchers (Farris & Alpers, 2014) [2].

Hematoxylin-Eosin (H&E) is the most ubiquitous staining technique in clinical pathology, providing excellent visualization of cellular morphology, nuclear detail, and inflammatory infiltrates. However, H&E lacks specificity for individual ECM components, often making it difficult to differentiate between true collagen deposition and other eosinophilic proteinaceous material (Feldman & Wolfe, 2014) [3]. In contrast, Sirius Red (SR) is a polyazo dye that specifically binds to the basic groups of collagen fibers, enhancing their natural birefringence under polarized light (Street *et al.*, 2014) [8].

Parallel to diagnostic concerns is the search for novel therapeutic agents to arrest fibrotic progression. *Lactococcus lactis* D4, a probiotic strain isolated from traditional Indonesian "dadih," has shown promise in modulating systemic inflammation. Probiotics are increasingly recognized for their "gut-kidney axis" interactions, where they reduce uremic toxins and suppress systemic pro-inflammatory cytokines such as TNF-α and IL-6 (Ihsan, Alvarino, & Etriyel, 2025) [5].

Despite the potential of *L. lactis* D4, there is a lack of comparative data on how different staining methods represent the fibrotic changes induced by this probiotic treatment. Understanding whether H&E can provide a surrogate measurement comparable to SR is crucial for clinical settings where specialized stains are not readily available (Althof, 2026) [1]. This study aims to fill that gap by providing a rigorous, computerized comparison of fibrosis percentages across varying degrees of renal damage.

2. Materials and Methods

The study was conducted using a post-test-only randomized control group design involving 18 male Sprague Dawley rats. Animals were housed in standardized conditions and divided into three cohorts: a control group (Sham laparotomy), a P1 group (UUO induction), and a P2 group (UUO + rectal *L. lactis* D4). All procedures followed the ethical guidelines for animal welfare established by the institutional review board (Andalas University Ethics Committee, 2025) [5].

The UUO procedure involved double-ligation of the left ureter to induce rapid hydronephrosis and subsequent interstitial fibrosis. For the P2 group, *L. lactis* D4 was administered rectally at a concentration of 8×10^9 CFU/mL daily for 7 days. This administration route was chosen to maximize gut-microbiota interaction and systemic absorption of anti-inflammatory metabolites (Ihsan *et al.*, 2025) [5].

Tissue processing involved fixing the left kidneys in 10% buffered formalin followed by paraffin embedding. Serial sections of 4-5 µm thickness were prepared to ensure that both H&E and SR stains were applied to virtually identical anatomical areas. This serial sectioning technique is critical for minimizing sampling bias when comparing two different histological methods (Feldman & Wolfe, 2014) [3].

Quantification was performed using ImageJ software. For H&E, fibrosis was defined by the area of pale-pink eosinophilic material in the interstitium that lacked normal tubular architecture. For SR, the software was programmed to detect the specific red intensity of collagen fibers. Five non-overlapping fields from the cortex and medulla were analyzed for each slide to ensure a representative mean (Street *et al.*, 2014) [8].

3. Results

Quantitative analysis of the H&E slides showed that the P1 group reached the highest mean fibrosis percentage at 30.85±7.26%. This was significantly higher than the control group, which showed a baseline of 20.66±6.17%. In the P2 group, the administration of *L. lactis* D4 successfully reduced the eosinophilic fibrotic area to 18.14±6.59%, demonstrating a significant therapeutic effect (Althof, 2026) [1].

Sirius Red staining provided a more distinct visualization of collagen fibers, which appeared as sharp red lines against a yellow background. The P1 group exhibited a collagen density of 28.68±3.27%, while the P2 group showed a drastic reduction to 7.11±1.14%. Statistical comparison between P1 and P2 using SR revealed a p-value of <0.001, highlighting the potency of the probiotic intervention (Althof, 2026) [1].

When comparing the two methods directly, a significant discrepancy was noted in the P2 group (minimal fibrosis). H&E recorded an average of 18.14% while SR recorded only 7.11% (p=0.006). This suggests that in healthy or recovering tissues, H&E may "over-diagnose" fibrosis by staining cytoplasmic proteins or basement membranes that are not actually collagenous (Farris & Alpers, 2014) [2].

In the P1 group, however, where fibrosis was massive and structural destruction was evident, the difference between H&E (30.85%) and SR (28.68%) was not statistically significant. This indicates that in cases of severe, late-stage renal damage, H&E staining correlates closely with specialized collagen stains, as the bulk of the eosinophilic material is indeed accumulated ECM (Althof, 2026) [1].

4. Discussion: The success of *Lactococcus lactis* D4 in reducing fibrosis markers confirms the existence of a potent gut-kidney axis modulation. Probiotics like *L. lactis* secrete short-chain fatty acids (SCFAs) that inhibit histone deacetylases, thereby reducing the activation of pro-fibrotic genes in the kidney. This systemic effect bypasses the need for direct renal delivery, making it a highly attractive non-invasive therapeutic strategy (Ihsan *et al.*, 2025) [5].

The molecular mechanism behind the observed reduction in P2 likely involves the downregulation of the TGF-β1/Smad3 signaling pathway. Previous studies on *L. lactis* have demonstrated its ability to inhibit NF-κB activation, which is the primary driver of the inflammatory surge following ureteral obstruction. By quenching the inflammatory "fire," the probiotic prevents the subsequent "scarring" characterized by collagen deposition (Nan *et al.*, 2024) [6].

The comparative aspect of this study sheds light on the limitations of H&E staining. Because Eosin is a non-specific acidic dye, it stains any basic protein, including cytoplasmic components and fibrin. In the P2 group, the higher values in H&E likely reflect cellular edema or non-fibrotic protein accumulation rather than actual collagenous scar tissue. This "noise" in H&E staining makes it less ideal for high-precision quantification in early-stage CKD (Feldman & Wolfe, 2014) [3].

Sirius Red, due to its sulfonic acid groups, reacts specifically with the basic groups of collagen molecules. This specificity allows for a much cleaner digital separation of the "signal" (fibrosis) from the "background" (cells). The significantly lower values found in P2 when using SR suggest that the probiotic treatment was even more effective at reducing actual collagen than the H&E images initially suggested (Street *et al.*, 2014) [8].

However, the finding that H&E and SR yielded similar results in the P1 (massive fibrosis) group is of high clinical importance. In late-stage disease, the parenchymal architecture is so distorted that the interstitial space is dominated by thick bundles of collagen. At this stage, the lack of specificity in H&E becomes less of a hindrance because the majority of the eosinophilic space is, in fact, occupied by fibrosis. This justifies the use of H&E in routine diagnostic screenings where SR is not available (Farris & Alpers, 2014) [2].

Furthermore, the baseline fibrosis of ~20% in the sham group (Control) warrants discussion. Theoretically, a healthy kidney should have minimal collagen deposition. However, Sprague Dawley rats are known to exhibit age-related focal glomerulosclerosis and interstitial changes. Additionally, the physiological stress of the initial laparotomy and the 7-day observation period may contribute to a mild background inflammatory response (Nørregaard *et al.*, 2023) [7].

Finally, the integration of computerized image analysis (ImageJ) in this study reduces the subjectivity inherent in visual scoring systems like the Banff classification. By utilizing pixel-counting algorithms, we provided an objective numerical value that can be compared across different studies. This methodological rigor is essential for the reproducibility of results in pharmacological trials involving probiotics (Street *et al.*, 2014) [8].

5. Conclusion

This study concludes that *Lactococcus lactis* D4 has a significant anti-fibrotic effect on the kidneys of UUO rat models, as confirmed by both H&E and Sirius Red staining. While Sirius Red is the superior method for precise collagen quantification particularly in cases of minimal or early-stage fibrosis H&E staining is a reliable surrogate for assessing massive fibrosis in advanced stages of the disease. These results support the potential of dadih-derived probiotics as a complementary therapy for chronic kidney disease and provide a validated framework for histological assessment in varied clinical settings.

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