Prediction study of native liver survival in biliary atresia: Insights into the gut microbiota and vitamin K

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This certifies that the Dissertation of Kyong Ihn is approved

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Kyong Ihn

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ABSTRACT

Prediction study of native liver survival in biliary atresia: Insights into the gut microbiota and vitamin K

Background: Biliary atresia (BA) is a severe pediatric liver disease characterized by bile duct obstruction, often leading to liver failure if left untreated. We investigated the association between the gut microbiota and long-term liver transplant-free survival (LTFS) in patients with BA following Kasai hepatoportoenterostomy.

Methods: In this retrospective analysis, we included 258 patients with BA, categorized into a discovery cohort (n=206) for model training and a validation cohort (n=52) for external validation. Patients were categorized into the LTFS-Y (those who survived with native liver ≥ 2 years) and LTFS-N (those requiring liver transplantation in < 2 years) groups. Stool samples from eight patients in the discovery cohort were sequenced for the V3-V4 region of the 16S rRNA gene to identify gut microbes and infer functional pathways. A random forest classifier with significant variables between LTFS-Y and -N was used to establish a prediction model.

Results: Fourteen serological factors related to cholestasis, hepatic injury and function were significantly differentiated between LTFS-Y and -N. Upon validating the prediction model with independent data, random forest classifier with the significant factors showed 100% accuracy, and estimated prothrombin time test with an international normalized ratio was as important variable to predict the clinical outcomes. The gut environment of the LTFS-Y group was colonized with more diverse bacteria, composed of *Bacteroidales*, *Campylobacterales*, *Actinomycetales*, and *Lactobacillales*, than that of the LTFS-N group and inferred to be enriched with metabolites from vitamin K2-producing pathways. Conversely, patients in the LTFS-N group were expected to have more metabolites from tetrapyrrole producing-related pathways that are related to hepatic dysfunction.

Conclusion: The presence of vitamin K2-producing gut bacteria is associated with prolonged native liver function in patients with BA after Kasai hepatoportoenterostomy. These results indicate potential therapeutic strategies, including gut microbiota modulation and vitamin K2 supplementation, to improve clinical outcomes.

Key words : Biliary atresia, Vitamin K2, Microbiota, Liver transplant, Menaquinone

I. Introduction

Biliary atresia (BA) is a rare but severe pediatric liver disorder characterized by progressive fibrosis and obstruction of the intrahepatic and extrahepatic bile ducts, leading to cholestasis and, if untreated, liver failure[1-3]. The incidence of BA varies significantly across regions, with a notably higher prevalence in East Asia than in Western countries. For instance, Korea reports an incidence of approximately 1.06 per 10,000 live births, whereas in the United States and France, the incidence ranges between 0.65 and 0.73 per 10,000 live births[4-6].

The pathophysiology of BA is complex, involving a multifactorial interplay of genetic, environmental, and immune-mediated mechanisms that lead to bile duct injury and subsequent fibrosis[7,8]. Although the exact etiology remains elusive, early surgical intervention through Kasai hepatoportoenterostomy (HPE) is essential for restoring bile flow and delaying the progression of liver failure[9]. Despite the benefits of HPE, nearly half of the patients progress to end-stage liver disease within 5 years, requiring liver transplantation[10]. Notably, Japan has reported some of the most favorable long-term survival rates, with 63% survival at 5 years and 54% at 10 years post-HPE[11].

Identifying factors that predict native liver survival is crucial for optimizing clinical outcomes. The key prognostic indicators include patient's age at the time of HPE, postoperative bilirubin levels, and degree of liver fibrosis at the time of surgery[12]. Several studies have highlighted that the clearance of jaundice within 6 months post-HPE is a critical predictor of long-term native liver survival[10,13,14]. Patients who achieve jaundice clearance typically exhibit a more robust bile acid flow, metabolized by the gut microbiota, leading to a healthier composition of secondary bile acids[15]. Conversely, insufficient bile acid secretion results in gut microbiota imbalance and disrupts bile acid homeostasis, exacerbating disease progression. Song et al. observed that disease progression in patients with BA correlates with the dominance of bacteria such as *Klebsiella*, *Streptococcus*, *Veillonella*, and *Enterococcus*[16]. Specifically, *Enterococcus faecium* showed a positive relationship with lithocholic acid derivatives, and alterations in tryptophan metabolism were observed in patients with BA, suggesting that disrupted bile flow influences gut microbiota composition, which may, in turn, impact long-term outcomes after HPE surgery[17].

The pediatric end-stage liver disease score, which incorporates parameters such as serum

prothrombin time (PT) and international normalized ratio (INR), is widely used to assess liver function in these patients[18]. Vitamin K deficiency is a common complication of chronic cholestatic liver disease, particularly in children with BA. Vitamin K is essential for synthesizing prothrombin and other clotting factors. This deficiency is often worsened by impaired intestinal absorption, especially in cases of cholestasis, and associated with disruptions in bile acid synthesis pathways[19].

Vitamin K does not cross the placenta effectively, and the neonatal gut lacks the bacterial colonization necessary for vitamin K production before birth, resulting in low vitamin K levels in newborns[20,21]. Moreover, several patients with BA are treated with antibiotics post-HPE to prevent ascending cholangitis, which can further disrupt the colonization of healthy gut flora, including vitamin K-producing bacteria[22].

Recent research underscores the significant role of the gut microbiota in liver diseases, particularly the influence of vitamin K-producing bacteria on liver health[23,24]. Alterations in gut microbial communities, influenced by dietary vitamin K intake, have been shown to affect overall health outcomes. Additionally, different studies suggest that manipulating the gut microbiota through vitamin K supplementation could offer therapeutic benefits in the treatment of liver diseases and other conditions, such as rheumatoid arthritis[25,26].

Building on these insights, the current study aimed to characterize the gut microbiome structure in patients with BA who maintained liver transplant-free survival (LTFS) for 2 years versus those who did not. We developed a prognostic model using the random forest method to predict whether patients who underwent HPE could survive for 2 years without requiring liver transplantation. This model incorporated serological factors related to hepatobiliary health, both pre- and post-HPE, as well as temporal changes.

Furthermore, we analyzed human stool samples from both patient groups to investigate the differences in gut bacteria responsible for vitamin K production and functional pathways related to vitamin K synthesis. Through quantitative analysis of stool metabolites, we explored the potential effects of these altered products on disease progression.

2. Materials and Methods

2.1. Study population

This retrospective study included 258 infants diagnosed with BA who underwent HPE at Severance Children's Hospital between 2005 and 2024. The study protocol was approved by the Institutional Review Board (IRB) Severance Children's Hospital (approval number: 4-2019-0306).

2.2. Data collection

Clinical and laboratory data, including demographic information, laboratory test results, and clinical outcomes, were extracted from medical records. The key laboratory test results included platelet count, albumin, alkaline phosphatase (ALP), aspartate aminotransferase, alanine aminotransferase, total bilirubin (TB), direct bilirubin (DB), gamma-glutamyl transferase (GGT), and INR. These data were collected at multiple time points—at birth, before HPE, and at 3 months, 6 months, and 2 years post-HPE.

The study design is illustrated in Fig 1. The cohort was divided into two groups, including those who survived with their native liver for at least 2 years post-HPE without requiring liver transplantation in the LTFS-Y group and those who did not achieve this outcome in the LTFS-N group.

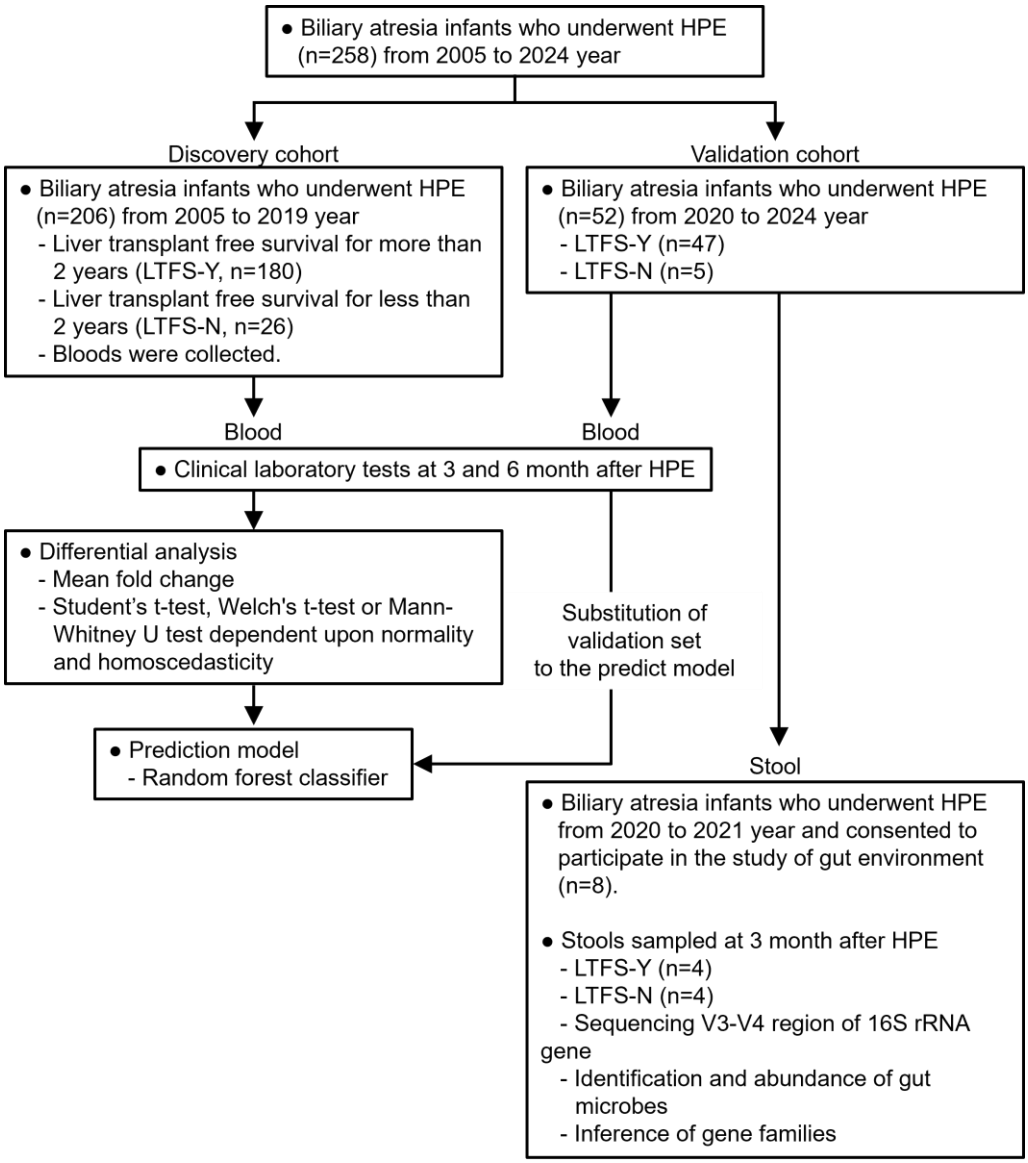


Figure 1. Schematic diagram of the study design.

2.3. Analysis of serological factors and gut environment

Blood samples were drawn from the central or peripheral vein before HPE, and at 3 and 6 months post-HPE (Fig 2). All samples were centrifuged at 3000 rpm at 4°C for 15 min, and the supernatants were collected and stored at -80°C for clinical laboratory tests.

Stool samples were swabbed from infants with BA at 3 months post-HPE (Fig 3) using Transwab® tubes containing Liquid Transport Medium (Sigma, MW176S). The samples were immediately snap-frozen in liquid nitrogen and stored at -80°C until DNA extraction. Total genomic DNA was extracted using the Maxwell® RSC PureFood GMO and Authentication Kit (Promega, Madison, WI, USA), and subjected to sequencing for the V3 – V4 region of the 16S rRNA gene. The concentration, quantification, and quality of DNAs were determined using a UV – vis spectrophotometer (NanoDrop 2000c, Thermo Fisher Scientific, Waltham, MA, USA), QuantiFluor® ONE dsDNA System (Promega), and Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA). DNA libraries were prepared using the Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA, USA), and 8 pM of library DNA was sequenced on an Illumina MiSeq™ system employing MiSeq Reagent kits v3 (Illumina), generating de-multiplexed 300 bp paired-end reads in either direction. Raw sequence reads were trimmed using Trimmomatic[27] to exclude reads with an average quality score of less than 30. Bacterial taxa and their abundances were analyzed using Quantitative Insights into Microbial Ecology 2 (QIIME2)[28], and functional pathways were inferred by using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) 2 v2.3.0 beta[29], based on 16S rRNA gene sequencing.

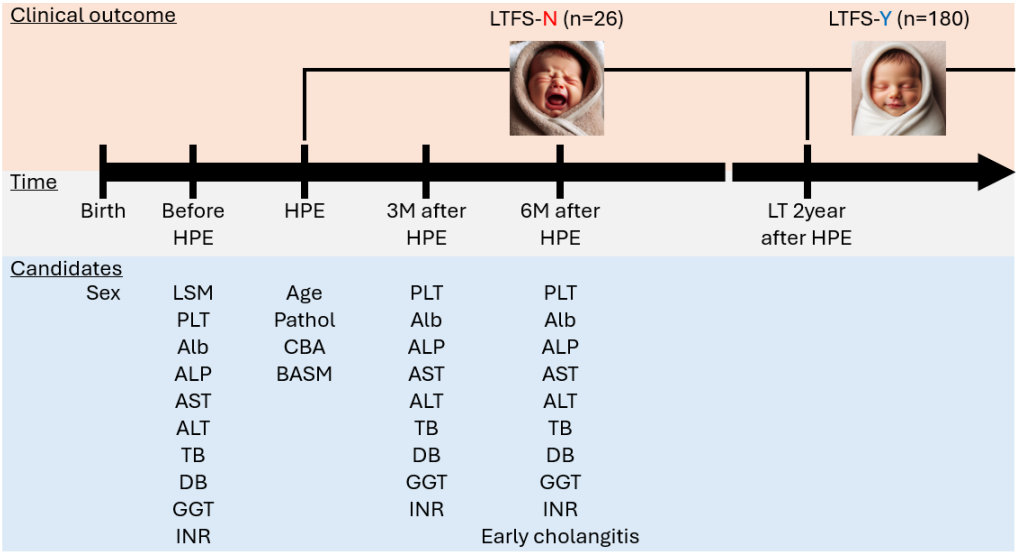


Figure 2. Timeline diagram for the study of clinical laboratory tests.

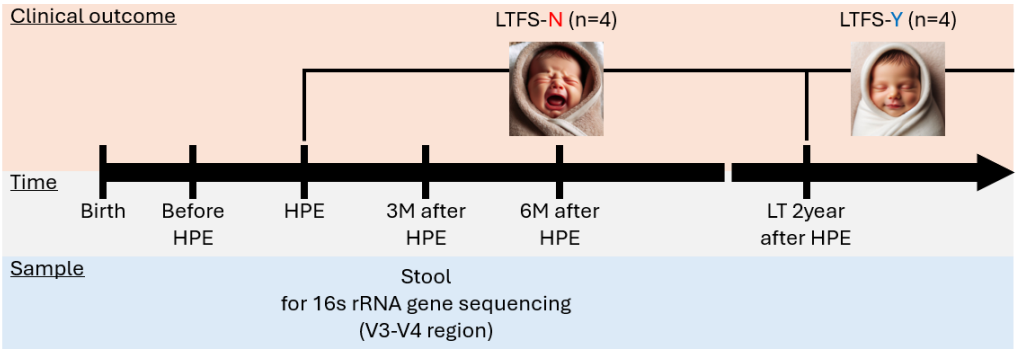


Figure 3. Timeline diagram for the study of gut environment.

2.4. Statistical analyses

All statistical analyses were conducted using SPSS software (version 28.0; IBM Corp., Armonk, NY, USA). The normality of continuous variables was assessed using the Kolmogorov–Smirnov test. For independent continuous variables, either the Student's t-test or Mann–Whitney U test was applied, depending on data distribution. Categorical variables were compared using the chi-squared test, and Fisher's exact test was used for cases with small sample sizes. Quantitative data were reported as means with standard deviations, and categorical data were expressed as counts and percentages. The 5-year overall survival was evaluated using the Kaplan–Meier method, and comparisons were made using the log-rank test.

During data preprocessing, missing values were imputed using the proximity matrix predictions from the random forest (rfImpute() of the randomForest R package). For microbiome analyses, the 16S rRNA sequence reads were rarefied to standardize sequencing depth across samples (Rarefy(depth = min()) of the GUniFrac R package). Alpha diversity within each group was measured using observed ASVs (specnumber() in the vegan R package) and Chao1 (estimate (permutations = 100) in the vegan R package) for richness and Shannon H (diversity (index = "shannon") function in the vegan R package) and inverse Simpson (diversity (index = "simpson") function in the vegan R package) for evenness. For beta diversity, the standardized sequence reads were normalized to relative abundance, and diversity between groups was visualized using non-metric dimensional scaling based on Bray–Curtis dissimilarity (metaMDS (distance = "bray") of vegan R package), with statistical significance assessed by analysis of similarity (ANOSIM) (anosim (distance = "bray," permutations = 9999) in the vegan R package).

Statistical comparisons between the two groups were conducted using the Student's t-test (t.test (var.equal = TRUE) function in the stats R package), Welch's t-test (t test (var.equal = FALSE) in the stats R package), or Mann–Whitney U test (wilcox.test (exact = FALSE, correct = TRUE) in the stats R package) for continuous variables and chi-square test (chisq.test() in the stats R package) for categorical variables. Variables that significantly differed between the two groups were used to build decision trees using the random forest machine learning algorithm (random forest (importance = TRUE, proximity = TRUE) of the random forest R package). To optimize the model, the number of trees and variables was determined based on the lowest out-of-bag (OOB) score. The ability of the random forest model to accurately predict clinical outcomes was assessed by calculating the error

rate, accuracy, sensitivity, specificity, positive predictive value, and negative predictive value using the confusion matrix () function in the caret R package. All analyses were conducted at a significance level of 5%.

3. Results

3.1. Study cohorts and clinical data analysis

A comprehensive analysis was conducted to identify the significant predictors of native liver survival in infants with BA who underwent HPE. To explore the potential causal relationships between gut–liver interactions and liver transplant-free survival, we examined gut microbiota composition and related functional pathways using 16S rRNA gene sequencing.

We included 258 patients with BA, registered between 2005 and 2024. Patients in the LTFS-N group underwent HPE at a significantly older age (71.8 ± 27.7 days) compared with those in the LTFS-Y group (59.4 ± 28.8 days, p=0.026), underscoring the critical impact of early intervention on survival outcomes (Table 1). Preoperative liver stiffness scores were higher in the LTFS-N group (18.4 ± 14.6 kPa) than in the LTFS-Y group (12.8 ± 10.1 kPa, p = 0.05), suggesting more advanced hepatic fibrosis among those with poorer outcomes. Additionally, higher ALP levels were observed in patients in the LTFS-N group (642.8 ± 250.2 IU/L vs. 533.4 ± 262.1 IU/L, p = 0.029) than in those in the LTFS-Y group, indicating more severe cholestasis before surgery.

Postoperative serological markers revealed significant differences between the two groups. TB and DB levels remained markedly elevated in patients in the LTFS-N group than in those in the LTFS-Y group at both 3 and 6 months post-HPE, indicating persistent cholestasis. For instance, at 3 months, TB level was 9.1 ± 3.2 mg/dL in the LTFS-N group compared to 1.5 ± 1.9 mg/dL in the LTFS-Y group (p < 0.001), and by 6 months, TB level was 11.4 ± 4.5 mg/dL in the LTFS-N group compared to 1.1 ± 2.1 mg/dL in the LTFS-Y group (p < 0.001). Similarly, aspartate aminotransferase and GGT levels were significantly higher in patients in the LTFS-N group than in those in the LTFS-Y group, indicating ongoing hepatic injury.

Analysis of liver synthetic functions, particularly PT (INR), also revealed distinct differences between the groups. At 6 months, patients in the LTFS-N group exhibited higher PT (INR) values (1.4 ± 0.4) than those in the LTFS-Y group (1.0 ± 0.2, p=0.001), reflecting compromised synthetic capacity in patients in the LTFS-N group. This reinforces the role of PT (INR) as a critical prognostic indicator beyond bilirubin levels.

The overall 5-year survival rate was significantly lower in patients in the LTFS-N group (76.4%) than in those in the LTFS-Y group (95.6%, p < 0.010). Moreover, patients in the LTFS-N group were

followed up for a shorter duration (71.2 ± 48.9 months vs. 91.5 ± 62.8 months, p = 0.042). These findings underscore the importance of early surgical intervention, vigilant management of cholestasis, and comprehensive monitoring of liver synthetic function in predicting native liver survival in patients with post-HPE BA.

To construct a robust predictive model and validate the findings, the data were divided into two cohorts based on registration years. The discovery cohort including 206 patients (26 from the LTFS-N group and 180 from the LTFS-Y group) from 2005 to 2019, was used to identify significant factors and build a predictive model using a random forest classifier. The validation cohort including 52 patients (5 from the LTFS-N group and 47 from the LTFS-Y group) from 2020 to 2024, was employed for external validation and further analysis of the gut microbiota. Stool samples from eight consenting patients were analyzed using 16S rRNA gene sequencing, allowing for the identification and quantification of gut microbes and the inference of functional pathways.

The analysis of eight patients from the validation cohort (four from the LTFS-Y group and four from the LTFS-N group) revealed trends in preoperative and postoperative liver function markers, although significance was not reached owing to the small sample size (Table 2). Preoperatively, ALP and TB levels were slightly more elevated in the LTFS-N group than in the LTFS-Y group, suggesting more advanced cholestasis at baseline. Postoperative measurements at 3 and 6 months showed that TB, DB, and GGT levels remained elevated in patients in the LTFS-N group, reflecting persistent cholestasis and bile duct injury. Additionally, PT (INR) at 6 months was higher in patients in the LTFS-N group than in those in the LTFS-Y group, indicating compromised liver synthetic function. Albumin levels and platelet counts showed a greater decline in the LTFS-N group than in the LTFS-Y group, supporting a pattern of progressive liver dysfunction and portal hypertension.

Table 1. Patient characteristics, pre-operative/post-operative laboratory variables, and long-term survival outcomes

	LTFS-Y N = 227	**LTFS-N N = 31**	**p-value**
Sex, male	91 (40.1%)	12 (38.7%)	0.883
Age at HPE, days (SD)	**59.4 ± 28.8**	**71.8 ± 27.7**	**0.026**
Pre-op liver stiffness score (kPa)	12.8 ± 10.1	18.4 ± 14.6	0.05
Pre-op PLT $10^3/\mu L$	434.7 ± 137.5	461.2 ± 209.3	0.499
Pre-op Alb (g/dL)	3.7 ± 0.4	3.8 ± 0.4	0.376
Pre-op ALP (IU/L)	533.4 ± 262.1	642.8 ± 250.2	0.029
Pre-op AST (IU/L)	180.5 ± 154.4	205.9 ± 123.6	0.304
Pre-op ALT (IU/L)	128.3 ± 107.1	147.5 ± 121.1	0.406
Pre-op TB (mg/dL)	7.8 ± 2.6	8.4 ± 3.6	0.394
Pre-op DB (mg/dL)	5.9 ± 2.1	6.6 ± 2.9	0.2
Pre-op GGT (IU/L)	483.2 ± 348.2	546.4 ± 354.2	0.356
Pre-op PT (INR)	1.0 ± 0.2	1.0 ± 0.2	0.586
TB at 3 months (mg/dL)	**1.5 ± 1.9**	**9.1 ± 3.2**	**< 0.001**
TB at 6 months (mg/dL)	**1.1 ± 2.1**	**11.4 ± 4.5**	**< 0.001**
DB at 3 months (mg/dL)	**1.3 ± 1.6**	**7.5 ± 2.5**	**< 0.001**
DB at 6 months (mg/dL)	**0.8 ± 1.8**	**9.6 ± 2.8**	**< 0.001**
AST at 3 months (IU/L)	**126.1 ± 132.9**	**208.5 ± 75.8**	**< 0.001**
AST at 6 months (IU/L)	**110.9 ± 88.8**	**233.2 ± 95.7**	**< 0.001**
ALT at 3 months (IU/L)	129.7 ± 201.7	132.2 ± 152.8	0.936
ALT at 6 months (IU/L)	110.8 ± 148.1	127.7 ± 157.2	0.665
GGT at 3 months (IU/L)	**746.0 ± 685.2**	**1262.9 ± 996.4**	**0.008**
GGT at 6 months (IU/L)	**383.5 ± 417.9**	**703.1 ± 573.9**	**0.032**
PLT at 3 months $10^3/\mu L$	336.7 ± 134.1	294.1 ± 147.4	0.136
PLT at 6 months $10^3/\mu L$	265.2 ± 118.9	254.8 ± 118.0	0.722
Alb at 3 months (g/dL)	**4.0 ± 0.4**	**3.5 ± 0.5**	**< 0.001**
Alb at 6 months (g/dL)	**4.0 ± 0.5**	**3.4 ± 0.6**	**0.001**
ALP at 3 months (IU/L)	**473.2 ± 249.2**	**960.8 ± 615.3**	**< 0.001**

ALP at 6 months (IU/L)	**467.8 ± 271.1**	**1071.2 ± 894.2**	**0.011**
PT (INR) at 3 months	1.8 ± 11.2	1.4 ± 0.5	0.619
PT (INR) at 6 months	**1.0 ± 0.2**	**1.4 ± 0.4**	**0.001**
Five-year overall survival rate	**95.6%**	**76.4%**	**< 0.010**
Follow-up, months	**91.5 ± 62.8**	**71.2 ± 48.9**	**0.042**

Bold typeface represents significant p values, ($p < 0.05$).

LTFS: liver transplant-free survival, SD: standard deviation, HPE: Kasai hepatoportoenterostomy, PLT: platelet, Alb: albumin, ALP: alkaline phosphatase, AST: aspartate aminotransferase, ALT: alanine aminotransferase, TB: total bilirubin, DB: direct bilirubin, GGT: gamma-glutamyl transpeptidase, PT (INR): prothrombin time (international normalized ratio).

Table 2. Patient characteristics and operative outcomes of 8 validation cohort patients, pre-operative/post-operative laboratory variables

	LTFS-Y **N = 4**	**LTFS-N** **N = 4**	**p-value**
Sex, male	0 (0%)	0 (0%)	1.000
Age at HPE, days (median, range)	59.5 (34–89)	57.0 (47–72)	1.000
Pre-op liver stiffness score (kPa)	11.2 (6.2–19.4)	10.8 (7.4–16.2)	1.000
Pre-op PLT 10^3/μL	418.8 (320–595)	455 (273–541)	1.000
Pre-op Alb (g/dL)	4.1 (3.7–4.3)	4.3 (3.7–4.5)	0.486
Pre-op ALP (IU/L)	599 (405–818)	862 (556–979)	0.200
Pre-op AST (IU/L)	180 (97–463)	196 (164–391)	0.886
Pre-op ALT (IU/L)	133 (77–365)	131 (105–142)	1.000
Pre-op TB (mg/dL)	9.7 (4.8–14.1)	10.2 (9.4–10.9)	0.886
Pre-op DB (mg/dL)	7.0 (3.8–10.2)	7.1 (6.8–8.3)	0.886
Pre-op GGT (IU/L)	688 (631–761)	200 (162–890)	0.343
Pre-op PT (INR)	1.06 (0.89–1.14)	1.01 (0.93–1.10)	0.686
TB at 3 months (mg/dL)	2.2 (0.9–4.9)	4.7 (2.4–10.7)	0.200
TB at 6 months (mg/dL)	1.4 (0.2–1.8)	1.6 (1.4–8.1)	0.400
DB at 3 months (mg/dL)	1.7 (0.3–4.7)	3.8 (3.2–8.2)	0.200
DB at 6 months (mg/dL)	1.1 (0.1–1.4)	1.2 (1.0–6.6)	0.400
AST at 3 months (IU/L)	128 (79–224)	182 (136–286)	0.343
AST at 6 months (IU/L)	165 (78–218)	122 (101–164)	0.857
ALT at 3 months (IU/L)	49 (26–244)	103 (24–242)	1.000
ALT at 6 months (IU/L)	137 (66–243)	24 (18–30)	0.057
GGT at 3 months (IU/L)	1492 (552–2939)	1580 (914–2294)	1.000
GGT at 6 months (IU/L)	961 (55–1765)	1143 (832–1191)	0.857
PLT at 3 months 10^3/μL	362 (355–575)	362 (277–410)	0.886
PLT at 6 months 10^3/μL	324 (164–457)	194 (171–234)	0.400
Alb at 3 months (g/dL)	4.4 (4.2–4.7)	4.3 (4.1–4.4)	0.343

Alb at 6 months (g/dL)	4.5 (3.9–4.9)	3.4 (3.2–3.7)	0.057
ALP at 3 months (IU/L)	478 (328–652)	492 (291–947)	1.000
ALP at 6 months (IU/L)	912 (337–1291)	758 (368–872)	0.857
PT (INR) at 3 months	1.01 (0.89–1.07)	1.26 (0.98–1.42)	0.114
PT (INR) at 6 months	1.01 (0.99–1.07)	1.20 (0.99–1.61)	0.400
Follow-up, months	40 (24–42)	29 (6–35)	0.114

LTFS: liver transplant-free survival, HPE: Kasai hepatoportoenterostomy, PLT: platelet, Alb: albumin, ALP: alkaline phosphatase, AST: aspartate aminotransferase, ALT: alanine aminotransferase, TB: total bilirubin, DB: direct bilirubin, GGT: gamma-glutamyl transpeptidase, PT (INR): prothrombin time (international normalized ratio).

3.2. *Model development and validation*

Fourteen serological markers that significantly differed between the LTFS-N and LTFS-Y groups were included in the predictive model (Fig 4). The performance of the random forest classifier was assessed using OOB error rates, which were visualized across various combinations of the number of trees and variables (Fig 5A). The lowest OOB error rates, 3.40% and 3.88%, were achieved with seven different combinations, as summarized in the 2 × 2 contingency tables (Fig 5B)[30]. To optimize the model, the Euclidean distances between the misclassification of the reference and predicted outcomes were calculated, with the model employing 200 trees and two variables to balance computational efficiency and predictive performance (Fig 5C).

To evaluate the clinical applicability, the predictive model was validated using external data from 52 patients with BA in the validation cohort (Fig 6A). Remarkably, the model demonstrated 100% accuracy with no misclassifications, resulting in a 0% error rate, perfect validity, and strong predictive indices (Figs 6A, 6B, and 7). The importance of variables within the model was ranked by the mean decrease in the Gini coefficient, with DB and TB levels consistently among the top predictors (Fig 6C). However, considering the gut–liver axis, the increase in bilirubin levels likely resulted from impaired enterohepatic circulation. Consequently, PT (INR) was selected as a more appropriate downstream marker for LTFS.

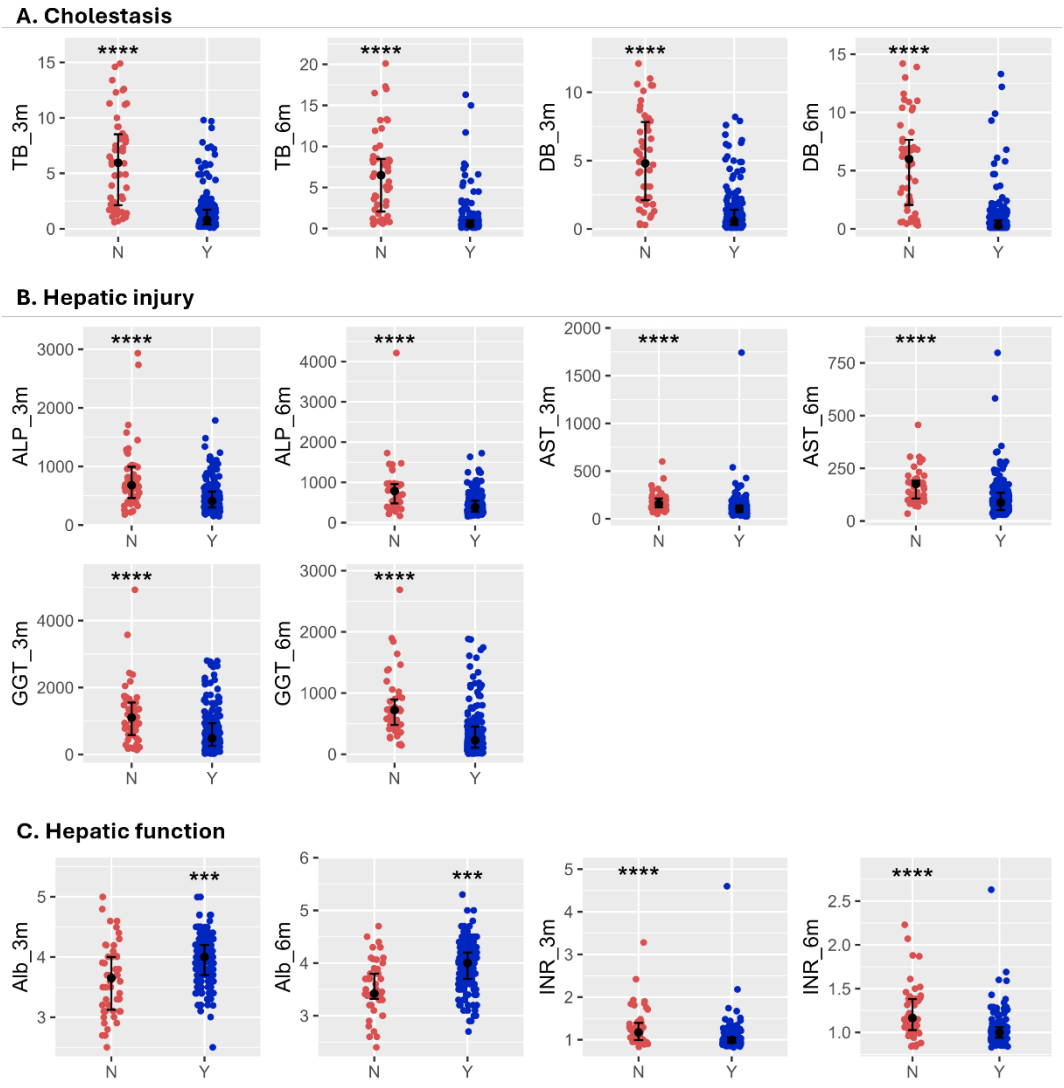
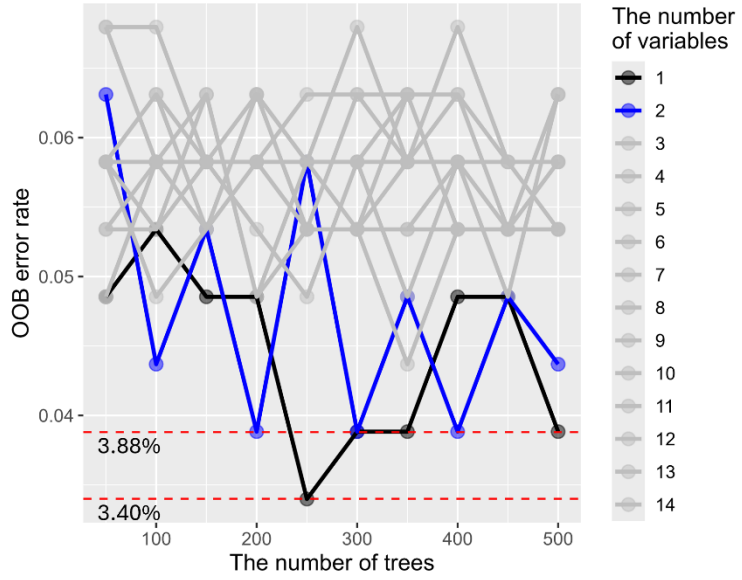


Figure 4. Serological factors significantly differentiating clinical outcomes.

A. Error rates from varied number of trees and features



B. Analysis of diagnostic accuracy

T: 200 V: 2	RF: N	RF: Y	Error	T: 350 V: 1	RF: N	RF: Y	Error
Ref: N	22	4	0.154	Ref: N	22	4	0.154
Ref: Y	4	176	0.022	Ref: Y	4	176	0.022
T:250 V: 1	RF: N	RF: Y	Error	T: 400 V: 2	RF: N	RF: Y	Error
Ref: N	21	5	0.192	Ref: N	21	5	0.192
Ref: Y	2	178	0.011	Ref: Y	3	177	0.017
T: 300 V: 1	RF: N	RF: Y	Error	T:500 V: 1	RF: N	RF: Y	Error
Ref: N	19	7	0.269	Ref: N	20	6	0.231
Ref: Y	1	179	0.006	Ref: Y	2	178	0.011
T: 300 V: 2	RF: N	RF: Y	Error				
Ref: N	21	5	0.192				
Ref: Y	3	177	0.017				

C. The optimization of number of trees and variables

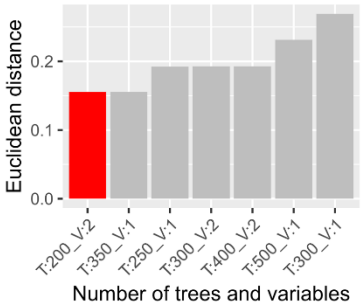
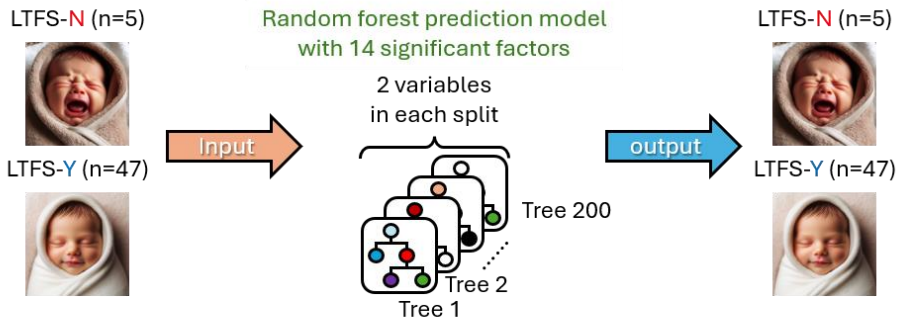


Figure 5. Optimization of random forest classifier using the number of trees and variables.

A. Validation of the prediction model with an independent data set



B. Analysis of diagnostic accuracy

Contingency table				Validity and predictive value	
N=40		Random forest			
		LTFS-N	LTFS-Y		
Reference	LTFS-N	5	0	Error rate	0%
				Accuracy	100%
				Sensitivity	100%
				Specificity	100%
	LTFS-Y	0	47	PPV	100%
				NPV	100%

C. Variable importance

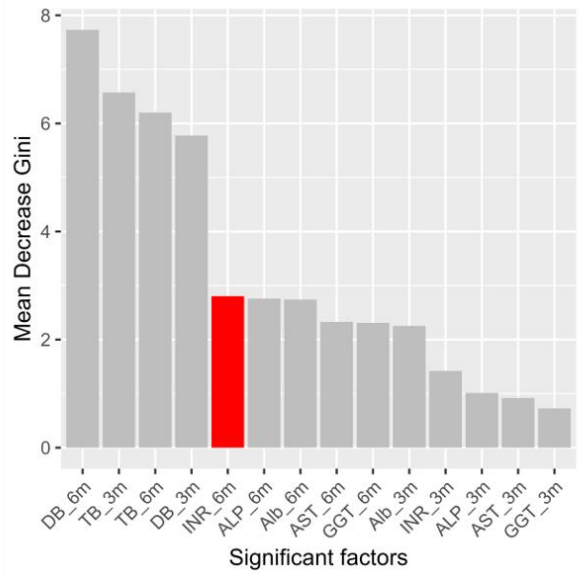


Figure 6. Validation of the prediction model with external data.

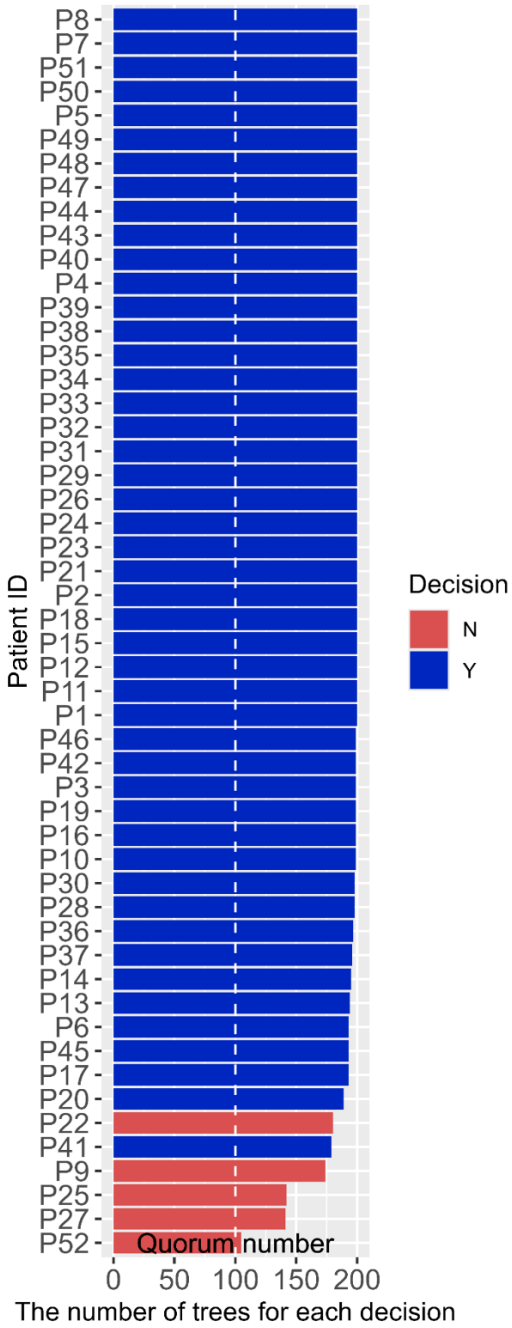


Figure 7. Clinical outcomes predicted by random forest classifier using external data.

3.3. Gut microbiota diversity and functional pathways

Given the critical role of prothrombin in blood coagulation and its synthesis in the liver, it has been hypothesized that vitamin K-producing bacteria in the gut are significantly associated with long-term liver survival in patients with BA. Our analysis revealed that the LTFS-Y group exhibited higher levels of gut bacterial richness (measured by ASVs and Chao1) and evenness (measured by Shannon H and Simpson indices) than the LTFS-N group (Fig 8A). Nonmetric multidimensional scaling ordination further confirmed significant differences in gut bacterial composition between the two groups (Fig 8B).

To further explore the gut bacterial lineages associated with survival status, significantly different taxa (Fig 9) were visualized using a cladogram (Fig 10). The intestinal tracts of patients in the LTFS-Y group were notably highly populated with bacterial lineages such as *Bacteroidetes (Prevotellaceae* and *Porphyromonadaceae* families*), Epsilonproteobacteria (Campylobacterales* order*), Actinomycetales,* and *Lactobacillales* (Fig 10). No bacterial taxa were found to be highly abundant in the LTFS-N group. Although the low taxonomic resolution of the V3–V4 region sequencing limited the identification of specific species, several bacterial lineages, including *Lactococcus lactis, Leuconostoc lactis*, and *Lactobacillus fermentum*[31] of the *Lactobacillales* order, and *Prevotella buccae*[32], were identified as potential vitamin K producers based on previous literature.

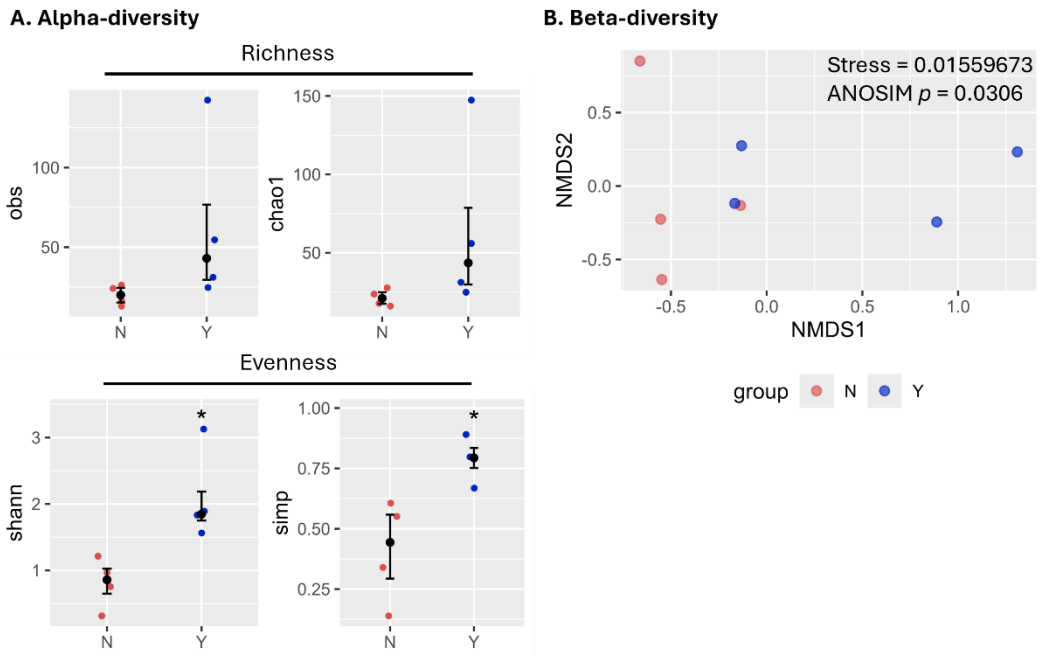


Figure 8. Diversity of gut microbiota.

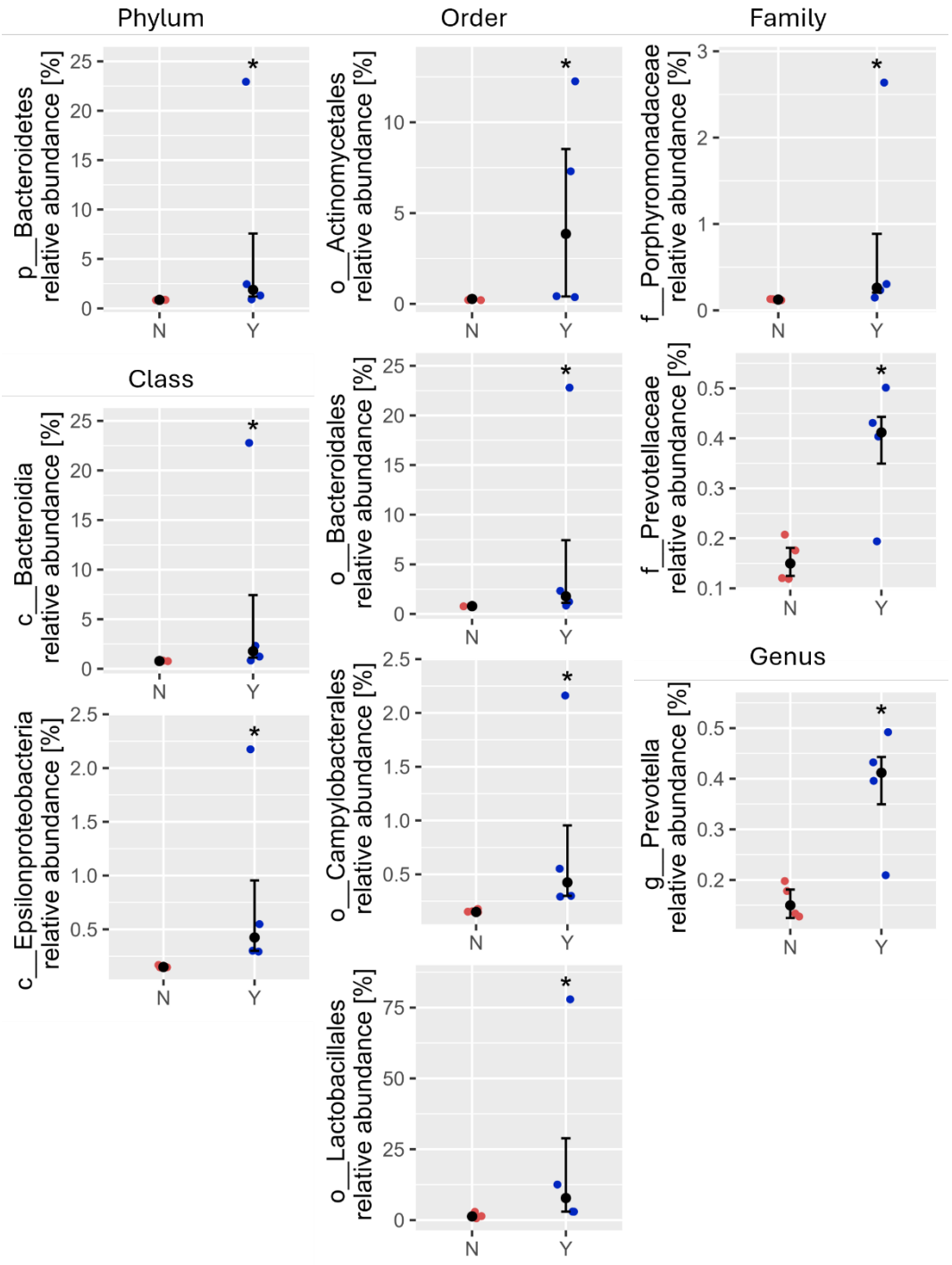
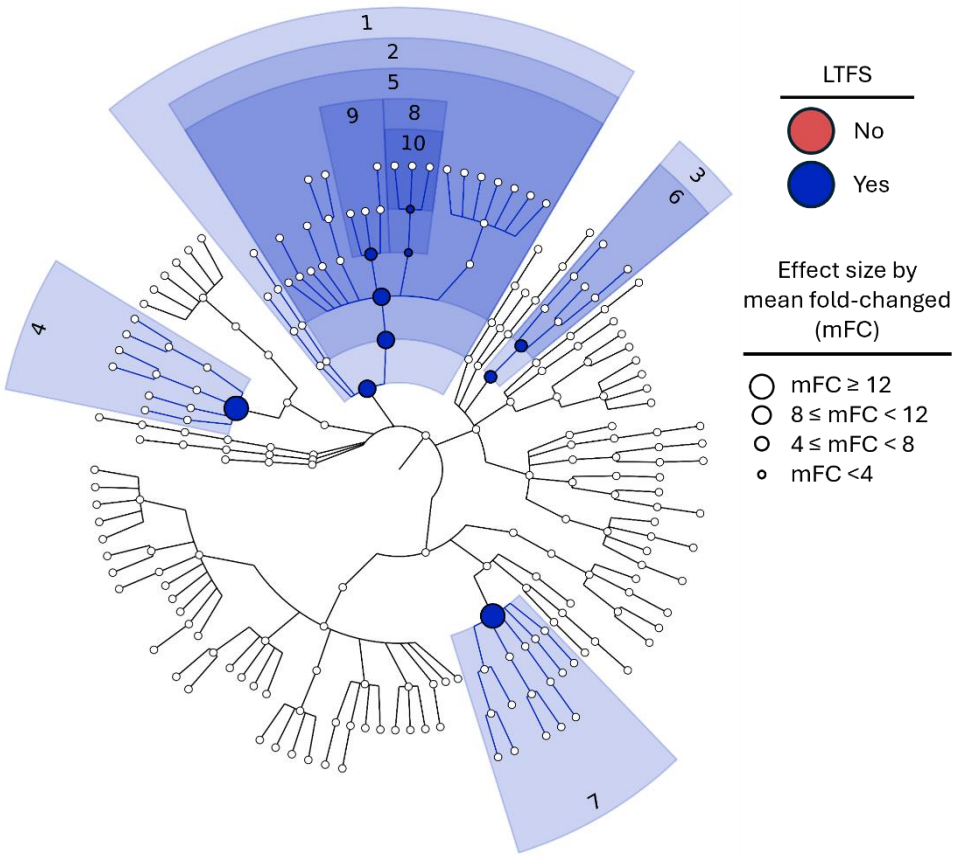


Figure 9. Significant bacterial taxa in the LTFS-N and -Y groups.



More abundant in LTFS-N

Not detected

More abundant in LTFS-Y

[1]p__Bacteroidetes|[2]c__Bacteroidia|[5]o__Bacteroidales|[8]f__Prevotellaceae|[10]g__Prevotella

[1]p__Bacteroidetes|[2]c__Bacteroidia|[5]o__Bacteroidales|[9]f__Porphyromonadaceae

[3]c__Epsilonproteobacteria|[6]o__Campylobacterales

[4]o__Actinomycetales

[7]o__Lactobacillales

Figure 10. Significant bacterial lineages significantly associated with clinical outcomes.

3.4. Functional Pathway Analysis

Based on the 16S rRNA gene sequencing, functional pathways within the microbial community were predicted using PICRUSt2. The gut environment of patients in the LTFS-Y group was enriched with metabolites from the menaquinone (vitamin K2) producing pathways (e.g., superpathway of menaquinol-8 biosynthesis II and 5,8-dihydroxy-2-naphthoate biosynthesis II) (Figs 11A and 12), thiol-producing pathways (e.g., mycothiol biosynthesis) (Figs 11B and 12), lactic acid-producing pathways (e.g., heterolactic fermentation and bifidobacterium shunt) (Figs 11C and 13), and degradation pathways (e.g., catechol degradation I and D-galactose degradation I) (Figs 11D and 14). Conversely, patients in the LTFS-N group exhibited an enrichment of metabolites associated with tetrapyrrole biosynthesis, including those from the adenosylcobalamin (vitamin B12) salvage pathway (Figs 11E and 15).

Notably, menaquinone-producing pathways emerged as the top functional pathways by effect size, highlighting their potential role in post-HPE hepatobiliary physiology regulation (Fig 16). The significant elevation of *Lactobacillales* in the patients in the LTFS-Y group, along with lactic acid production via heterolactic fermentation and the *Bifidobacterium* shunt pathways, suggests a possible long-term antifibrotic effect and maintenance of hepatic function in these patients[33]. *Actinomycetales*, known to produce mycothiol, a glutathione ortholog with antioxidant and anti-inflammatory properties, appeared prominently in the LTFS-Y group[34]. However, the specific beneficial effects of mycothiols in patients with BA require further investigation. Conversely, high levels of tetrapyrrole may contribute to hepatic pathogenesis; acute hepatitis and liver cirrhosis can be accompanied by high adenosylcobalamin (vitamin B12) serum levels, and heme converted from uroporphyrinogen-III may induce chronic liver damage[35,36].

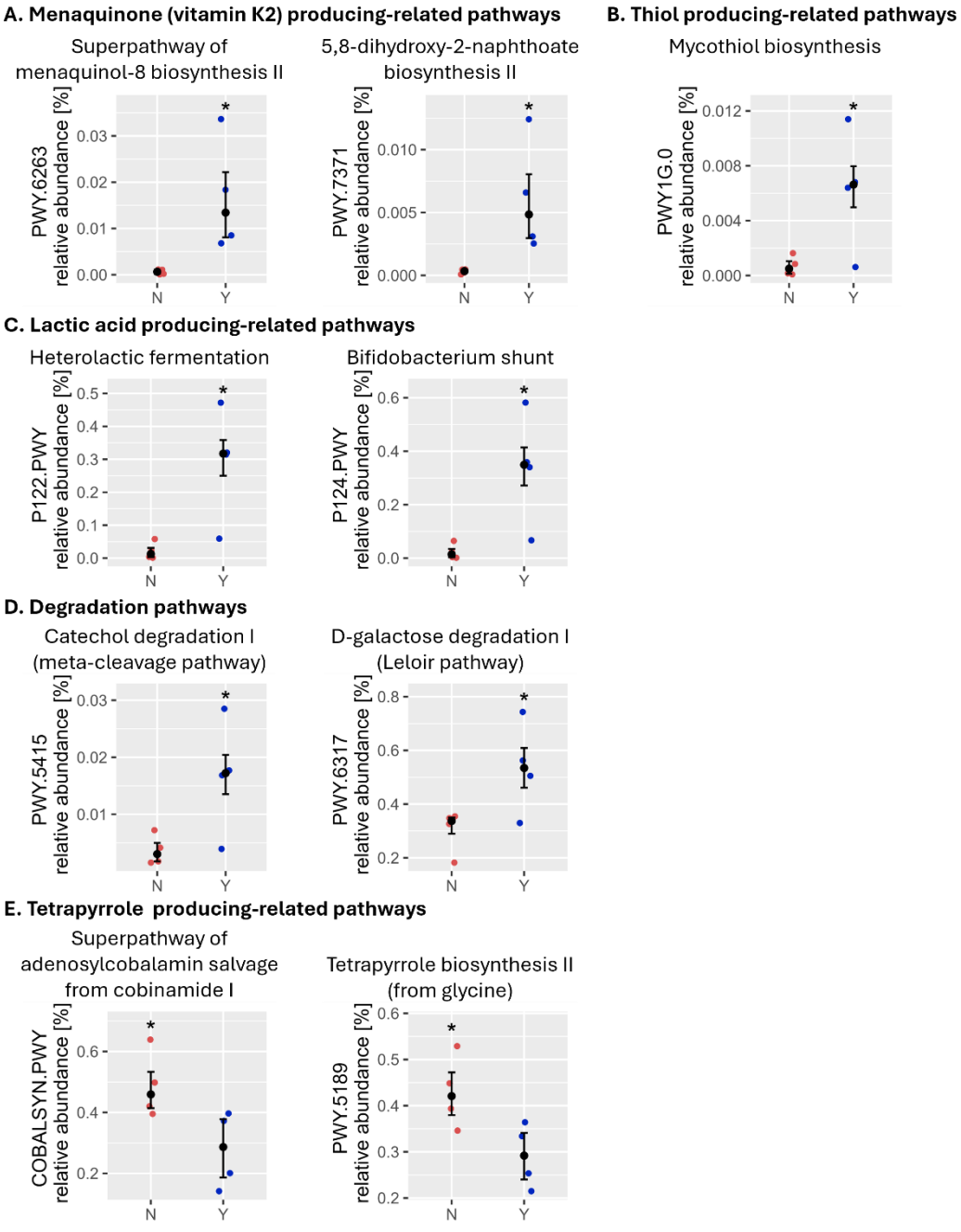


Figure 11. Significant functional pathways inferred based upon microbial community.

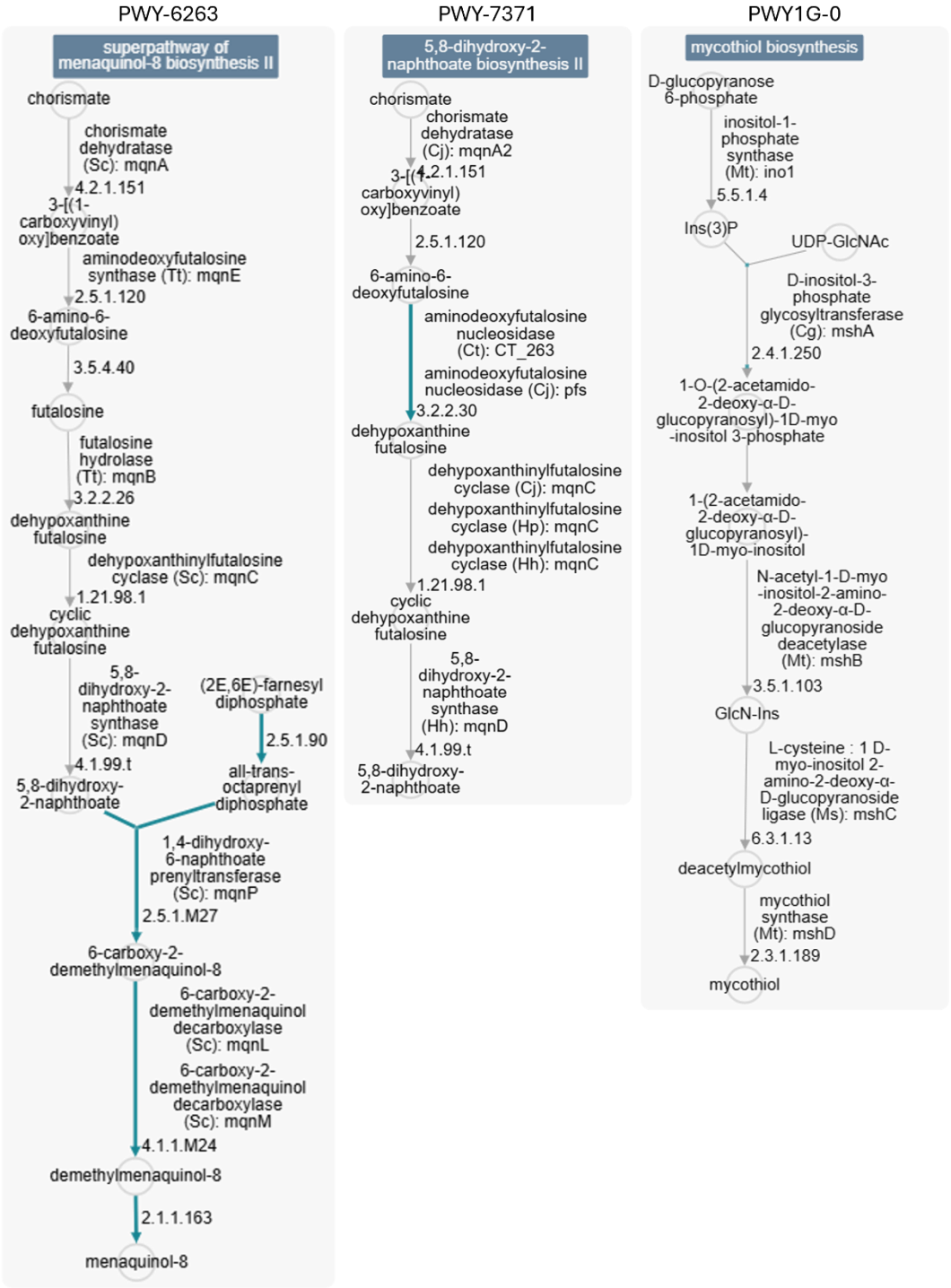


Figure 12. Vitamin K2 producing-related and mycothiol biosynthesis pathways.

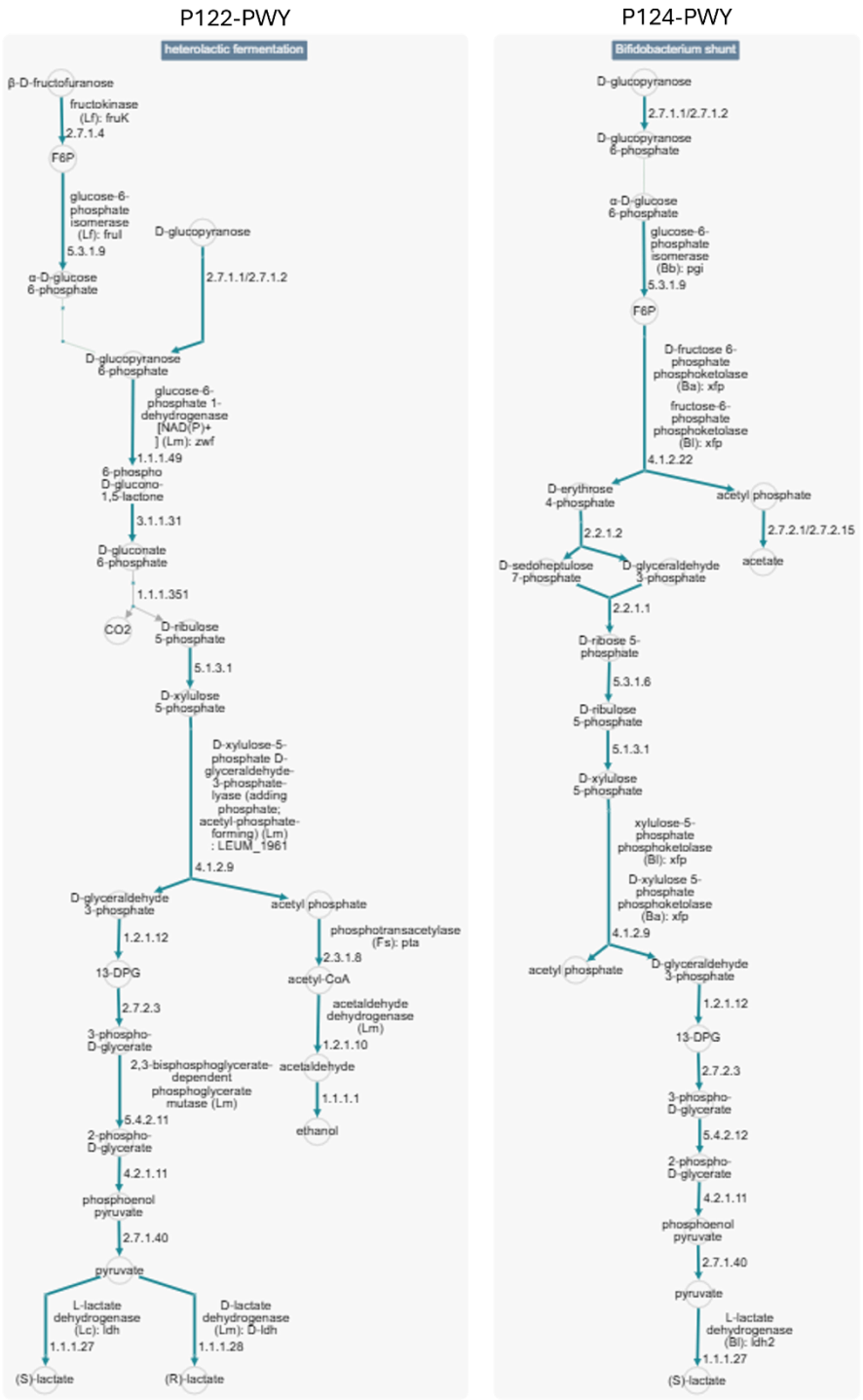


Figure 13. Lactic acid producing pathways.

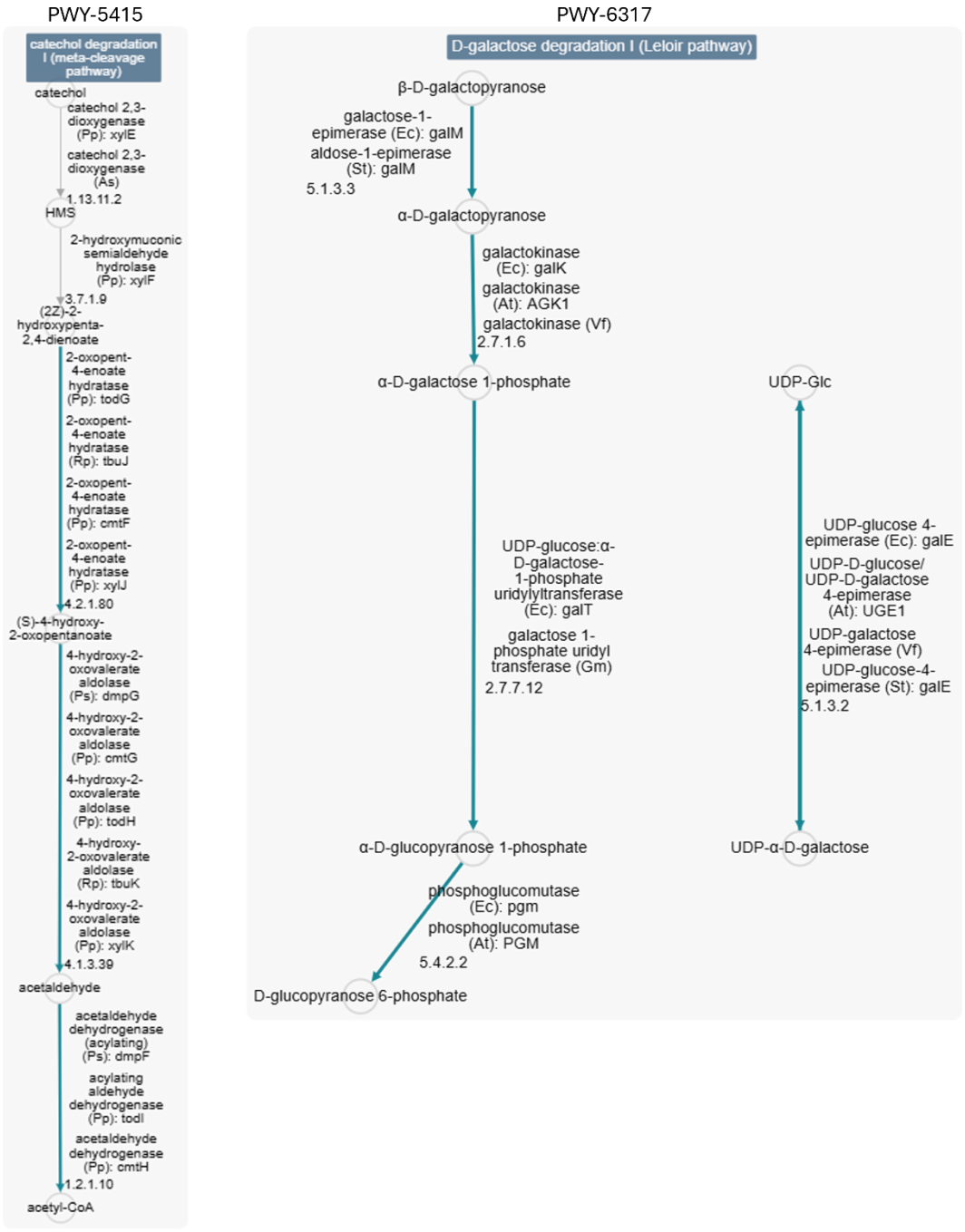


Figure 14. Degradation pathways.

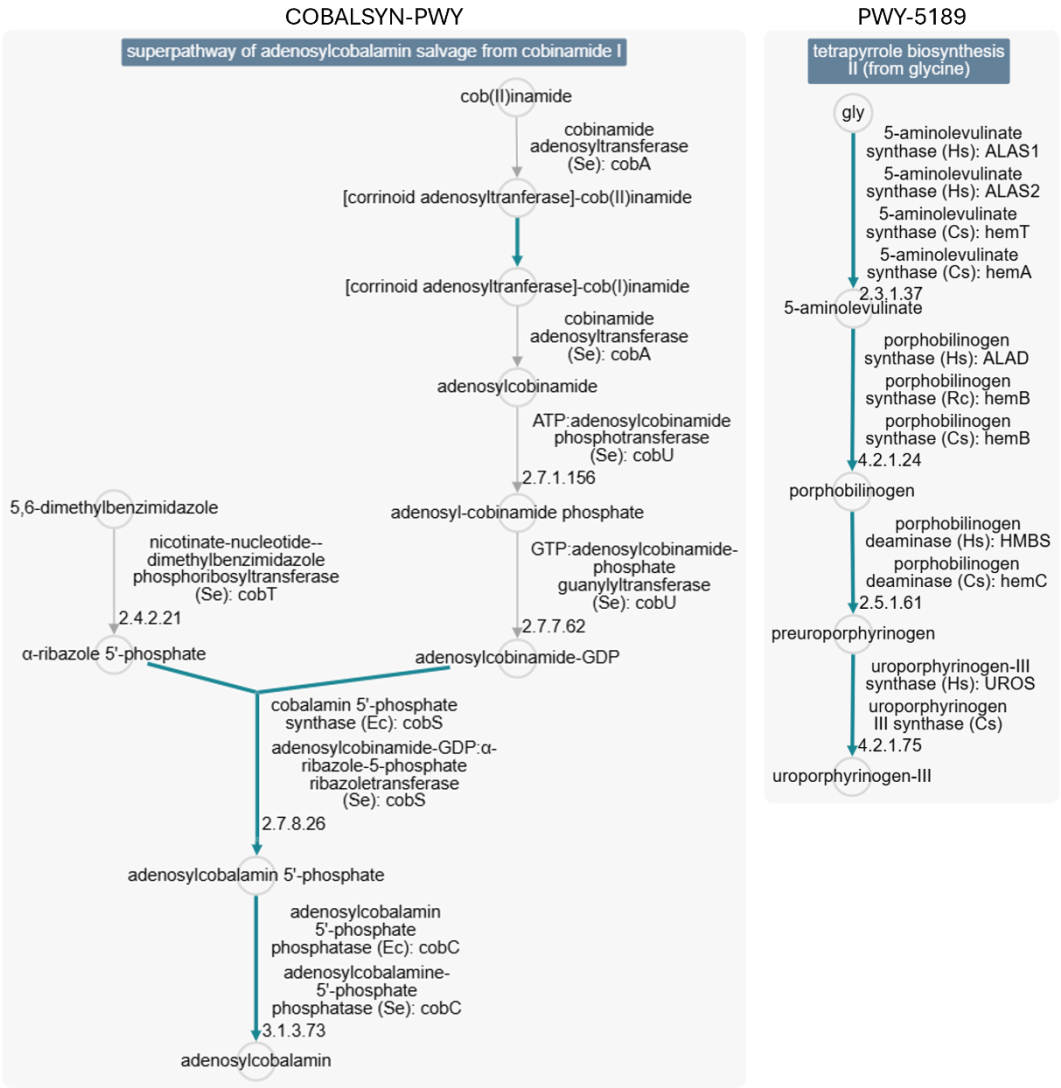


Figure 15. Tetrapyrrole producing pathways.

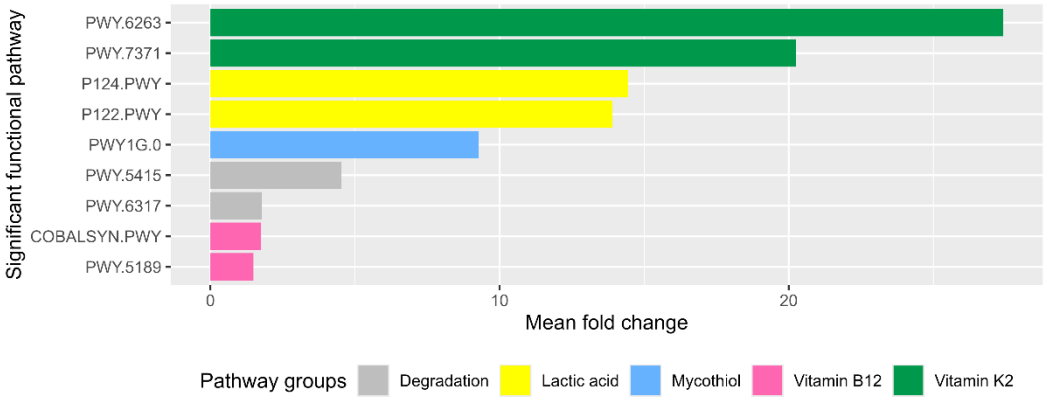


Figure 16. The effect size of significant functional pathways.

4. Discussion

A growing body of research has highlighted the role of the gut microbiome in the pathogenesis of the hepatobiliary system; the significant alteration of gut microbial communities via impaired bile flow and consequent disruption of bile acid homeostasis has been demonstrated in patients with cholestasis, e.g., obstructive jaundice, secondary biliary cirrhosis, and BA[37-39]. The clinical results were recapitulated with experimental studies; *Klebsiella pneumoniae*, *Veillonella atypica*, and *Enterococcus faecium* can exacerbate bile acid-induced hepatocyte damage, and microbiota-derived metabolites including bile acids, short-chain fatty acids, tryptophan, and indole derivatives, are reduced in the pathogenesis of metabolic disorders[15,16,40]. In BA, dysbiosis is further aggravated by prolonged antibiotic therapy for recurrent cholangitis, leading to additional disturbances in microbiota balance[41,42].

In this study, using a random forest model, we identified key predictors of LTFS, of which direct and total bilirubin levels are well-established diagnostic as well as prognostic markers of cholestasis in BA. Although bilirubin is widely recognized as a primary indicator of BA, we emphasized the use of PT (INR) as an indicator to understand the effect of gut environment on hepatic synthetic function. This marker reflects the capacity of the liver to synthesize clotting factors, often severely compromised in advanced liver disease. Our model demonstrated strong predictive accuracy with an OOB error rate of 3.40%, underscoring its clinical utility. Including PT (INR) alongside bilirubin levels allows for a more comprehensive assessment of liver function beyond traditional markers of cholestasis, enhancing the prognostic evaluation of patients with BA. Validation with an independent data achieved a 100% correct classification rate, indicative of the high accuracy of the prediction model and potential of integrating multiple serological factors in clinical practice.

Our findings highlight the potential role of the gut microbiota, particularly vitamin K2-producing bacteria in the long-term survival of patients with BA who underwent HPE. Patients in the LTFS-Y group were expected to have a gut environment enriched with metabolites associated with menaquinone (vitamin K2) synthesis, such as menaquinol-8 biosynthesis II and 5,8-dihydroxy-2-naphthoate biosynthesis II. These pathways have been known to contribute to hepatobiliary physiology, hepatoprotection and liver function[43,44]. Conversely, patients in the LTFS-N group were expected to have elevated levels of metabolites linked to tetrapyrrole biosynthesis, including adenosylcobalamin (vitamin B12) salvage pathways, which have been implicated in hepatic

pathogenesis, such as acute hepatitis and cirrhosis[45]. The absence of vitamin K2-producing bacteria may exacerbate liver injury, accelerate the progression to liver failure, and increase the requirement for transplantation.

Additionally, the significant presence of *Lactobacillales* and elevation of heterolactic fermentation and bifidobacterium shunt pathways in LTFS-Y group suggest that lactic acid produced by these bacteria may confer long-term anti-fibrotic effects, and help maintain hepatic function after HPE. Bacteria belonging to *Actinomycetales* family have been known to produce mycothiol, a glutathione-like compound with antioxidant and anti-inflammatory properties[46-50]. Coherently, our data showed significant increase of *Actinomycetales* and upregulation of mycothiol biosynthesis pathway in the LTFS-Y group. Thus, further investigation of mycothiol-producing bacteria is warranted to develop therapeutic intervention for patients with BA.

Vitamin K plays a crucial role in coagulation as a cofactor for γ-glutamyl carboxylase, which activates clotting factors and other vitamin K-dependent proteins[51]. Additionally, vitamin K2 (MK-4) serves as a ligand for the nuclear receptor PXR, influencing the expression of genes involved in bile acid metabolism and anti-inflammatory and anti-fibrotic responses[52]. Vitamin K deficiency is common in cholestatic liver disease, leading to prolonged PT due to impaired hepatic synthesis of clotting factors and altered bile acid pathways, potentially accelerating the progression of liver disease. Although vitamin K supplementation has been suggested to reduce fibrosis and improve outcomes, evidence remains inconsistent. Our findings build on the existing literature by emphasizing the importance of gut microbiota, particularly vitamin K2-producing bacteria, in modulating the composition of the gut microbiota and inflammation, thereby influencing long-term liver function.

Given the observed association between gut microbiota composition and long-term liver health in patients with BA, modulating gut microbiome can be a promising therapeutic interventions to minimize the chance of liver transplant; probiotic supplementation, dietary modifications to increase the abundance of vitamin K2-producing bacteria, or even direct vitamin K2 supplementation.

Although this study presented promising findings, it has some limitations that must also be acknowledged. The relatively small sample size, especially in the validation cohort, may have limited the generalizability of our findings. Moreover, while significant associations were observed between certain gut microbiota and clinical outcomes in patients with BA, the observational nature of the study prevented us from asserting a direct causal relationship. Longitudinal studies with a

large sample size are needed to validate these associations and further investigate the potential mechanisms linking the gut microbiota to liver health in BA. Additional variables, such as antibiotic use, diet, and genetic factors, which may have influenced the outcomes, were not fully controlled. Stool samples were collected for 3 months post-HPE in accordance with the study's prospective protocol; however, the meaningful differences in PT (INR) observed at 6 months highlight a temporal limitation, as this discrepancy restricts direct correlations between stool-derived data and subsequent liver function outcomes. In the Republic of Korea, an initial intramuscular dose of 1 mg vitamin K is mandatorily administered at birth, with no routine follow-up supplementation, as per national protocols. The same regimen was provided to all study participants. The pharmacokinetics of vitamin K varies by form; K1 exhibits a short half-life (1.5–3 h), while the half-life of K2 form ranges from approximately 1 h (MK-4) to 72 h (MK-7)[53]. Therefore, any potential impact of oral vitamin K on the gut microbiota is likely to be minimal. These limitations underscore the need for further research to comprehensively explore the longitudinal effects of vitamin K and the gut microbiota on liver health outcomes in patients with BA[25].

5. Conclusion

Our study highlights the role of the gut microbiota, particularly vitamin K2-producing bacteria, in the long-term survival of patients with BA post-HPE. These findings pave the way for new therapeutic strategies focusing on gut microbiota modulation to improve clinical outcomes in BA. Future research should prioritize large-scale, multicenter studies to validate these results and explore the therapeutic potential of probiotics, prebiotics, and vitamin K2 supplementation in this patient population. Further investigation into the specific roles of gut-derived metabolites, such as mycothiol and lactic acid, in BA could provide additional insights into disease management and therapy optimization.

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Abstract in Korean

담도폐쇄증 환자에서 비타민 K 생성 장내 미생물군의 역할과 자가 간 생존율 예측 모델 구축

배경: 담도폐쇄증(Biliary atresia, BA)은 영유아에서 발생하는 심각한 간 질환으로, 담관 폐색으로 인해 적절한 치료를 받지 않으면 간부전으로 진행할 수 있다. 본 연구는 Kasai 간문부-공장 문합술(Kasai hepatoportoenterostomy, HPE) 후 장내 미생물군, 특히 비타민 K2 생성 박테리아와 장기 자기 간 생존 여부(Liver Transplant-Free Survival, LTFS)의 연관성을 조사하는 것을 목적으로 한다.

방법: 본 연구는 258명의 BA 환자를 대상으로 후향적 분석을 수행하였으며, 모델 훈련을 위한 discovery 코호트(n=206)와 외부 검증을 위한 validation 코호트(n=52)로 구분하였다. 환자들은 LTFS-Y(이식 없이 2년 이상 생존)와 LTFS-N(2년 이내 간 이식 또는 사망)으로 분류되었다. Discovery 코호트 내 8명의 환자에게서 대변 샘플을 채취하고 16S rRNA 유전자 V3-V4 영역을 분석하여 장내 미생물과 기능적 경로를 확인하였다. 랜덤 포레스트(random forest) 기법을 통해 모델을 최적화하였으며, 오차율은 3.40%까지 감소하였다.

결과: LTFS-Y 그룹은 menaquinol-8 생합성 등 비타민 K2 생성 경로가 풍부하며, LTFS-N 그룹에 비해 장내 미생물 다양성이 높았다. 주요 혈청 인자인 직접 빌리루빈 및 총 빌리루빈은 LTFS 여부를 예측하는 데 중요한 요소로 나타났으며, 외부 검증(n=52)에서 모델의 정확도는 100%로 확인되었다. LTFS-N 환자들은 간 기능 장애와 관련된 테트라피롤 생합성 경로가 증가되었고, 이 경로는 담도염, 간경변증, 급성 간염 등과 관련된 간 병리학적 변화를 시사한다. 또한, LTFS-Y 그룹에서는 Lactobacillales, Actinomycetales와 같은 유익한 장내

미생물이 증가하여, 이들 미생물은 장기적인 간 기능 유지와 항섬유화, 항염증 효과를 제공할 수 있는 경로를 활성화하는 것으로 확인되었다.

결론: 본 연구는 비타민 K2 생성 장내 박테리아가 BA 환자의 자가 간 기능 유지와 밀접한 연관성을 가질 수 있음을 시사한다. 이 결과는 비타민 K2 생성 장내 미생물군의 조절이나 비타민 K2 보충을 통해 BA 환자의 자가 간 생존율을 높일 수 있는 치료 전략으로의 가능성을 제시한다. 그러나 이와 같은 연관성을 더 확고히 하고, 기저 메커니즘을 깊이 이해하기 위해 추가적인 연구가 필요하다.

핵심되는 말 : 담도폐쇄증, 비타민 K2, 장내 미생물군, 간이식, 메나퀴논