Gut *Bifidobacterium longum* is associated with better native liver survival in patients with biliary atresia
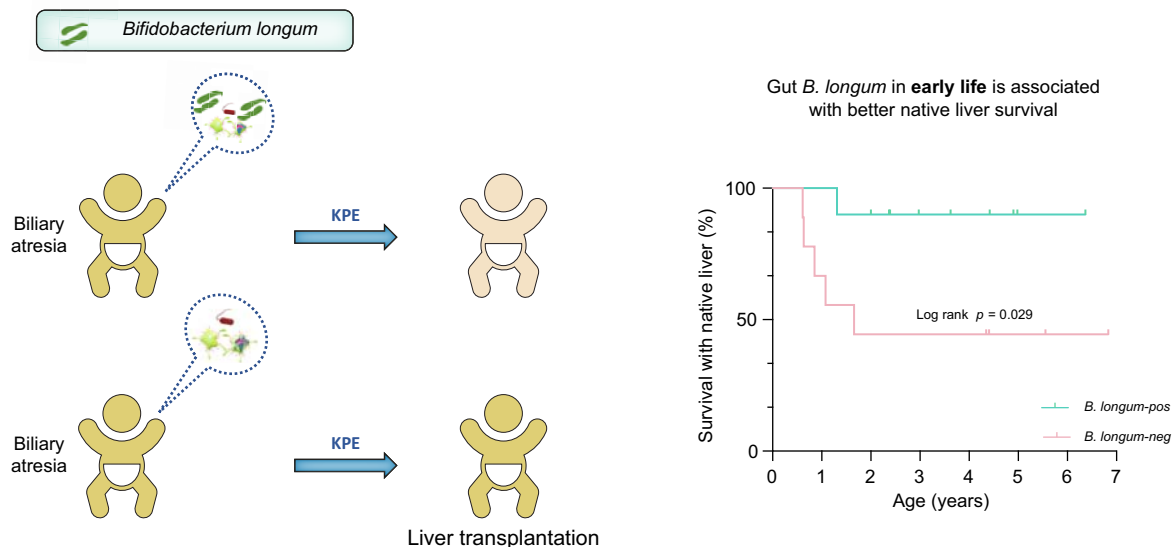
Authors

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Graphical abstract

Highlights:

- *Bifidobacterium longum*, a well-known probiotic, is significantly diminished in patients with biliary atresia.

- *B. longum* is linked to decreased gamma-glutamyltransferase, total and direct bilirubin levels post-Kasai portoenterostomy.

- Patients with early detectable *B. longum* had significantly longer native liver survival.

Impact and implications:

Bifidobacterium longum (*B. longum*) is a beneficial bacterium commonly found in the human gut. It has been studied for its potential impacts on various health conditions. In patients with biliary atresia, we found that a greater abundance of *B. longum* in the fecal microbiome is associated with improved clinical outcomes. This suggests that early colonization and increasing *B. longum* levels in the gut could be a therapeutic strategy to improve the prognosis of patients with biliary atresia.

Gut *Bifidobacterium longum* is associated with better native liver survival in patients with biliary atresia

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Background & Aims: The gut microbiome plays an important role in liver diseases, but its specific impact on biliary atresia (BA) remains to be explored. We aimed to investigate the microbial signature in the early life of patients with BA and to analyze its influence on long-term outcomes.

Methods: Fecal samples (n = 42) were collected from infants with BA before and after Kasai portoenterostomy (KPE). The stool microbiota was analyzed using 16S rRNA next-generation sequencing and compared with that of age-matched healthy controls (HCs). Shotgun metagenomic sequencing analysis was employed to confirm the bacterial composition in 10 fecal samples before KPE. The correlation of the microbiome signature with liver function and long-term outcomes was assessed.

Results: In the 16S rRNA next-generation sequencing analysis of fecal microbiota, the alpha and beta diversity analyses revealed significant differences between HCs and patients with BA before and after KPE. The difference in microbial composition analyzed by linear discriminant analysis and random forest classification revealed that the abundance of *Bifidobacterium longum* (*B. longum*) was significantly lower in patients before and after KPE than in HCs. The abundance of *B. longum* was negatively correlated with the gamma-glutamyltransferase level after KPE (*p* <0.05). Patients with early detectable *B. longum* had significantly lower total and direct bilirubin 3 months after KPE (*p* <0.005) and had a significantly lower liver transplantation rate (hazard ratio: 0.16, 95% CI 0.03-0.83, *p* = 0.029). Shotgun metagenomic sequencing also revealed that patients with BA and detectable *B. longum* had reduced total and direct bilirubin after KPE.

Conclusion: The gut microbiome of patients with BA differed from that of HCs, with a notable abundance of *B. longum* in early infancy correlating with better long-term outcomes.

Introduction

Biliary atresia (BA) is a devastating disease of progressive fibro-obliterative cholangiopathy that causes cirrhosis in early infancy.[1,2] Kasai portoenterostomy (KPE) restores the obstructed bile flow and reduces liver injury. Approximately half of patients who undergo KPE eventually develop end-stage liver disease and require liver transplantation (LT) before 2 years of age.[1] Younger age at the time of the KPE operation, lower bilirubin 3 months post-KPE, and lower gamma-glutamyltransferase (GGT) levels after KPE have been associated with improved native liver survival.[3–5] Currently, there are limited medical treatment options for BA. Management after KPE involves addressing complications such as cholangitis, optimizing nutrition, and supplementing fat-soluble vitamins.

Growing evidence has suggested that the intestinal microbiome plays important roles in health and disease.[6] The roles of the gut microbiota in BA pathogenesis and clinical course have

been implicated.[7] Studies have explored the potential benefit of a probiotic, *Lactobacillus casei rhamnosus*, in preventing cholangitis after KPE.[8,9] In an animal study, newborn mice fed butyrate had an increased abundance of *Bacteroides* and *Clostridia* in their intestinal microbiome, and an increase in fecal glutamate/glutamine metabolites rendered neonates resistant to experimental BAs.[10] In a human study, a greater abundance of *Clostridiaceae* and a lower abundance of *Enterobacteriaceae* were significantly associated with the clearance of jaundice within 6 months of age in patients with BA.[11] A recent study showed that liver damage in BA is exacerbated by interactions among enriched *Klebsiella* (*K. pneumoniae*), *Veillonella* (*V. atypica*), and *Enterococcus* (*E. faecium*), which are associated with the dysmetabolism of tryptophan and bile acids.[12]

Despite several studies implying the potential impact of the microbiome on BA, data are still insufficient to identify specific bacterial species that could affect long-term outcomes and

have therapeutic potential. In our study, the first goal was to determine the characteristics of the microbiome during the disease course of BA. The second goal was to identify the constituents of the microbiome that impact the short-term and long-term outcomes of patients with BA.

Materials and methods

Patients and follow-up

The study was conducted at the National Taiwan University Children's Hospital (NTUCH) from November 2015 to June 2023. Patients who were diagnosed with BA and who presented to the hospital were enrolled in the study. Fecal samples were collected from patients with BA before and after Kasai portoenterostomy (KPE) at approximately 2 months and at 6-9 months of age after informed consent was obtained from each patient. Fig. 1A illustrates the timeline of fecal sample collection and participant enrollment. The clinical data of the patients, including blood chemistry parameters such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), GGT, total bilirubin (t-bil), and direct bilirubin (d-bil) were recorded. After KPE, all patients with BA received antibiotics, including amoxicillin with sulbactam, gentamicin, and metronidazole. Antibiotics were administered for 7 to 14 days after the operation or adjusted when clinically indicated for treating acute infection. Ursodeoxycholic acid at a dosage of 10 mg/kg/day was given to patients with cholestasis.

After discharge, patients with BA underwent regular follow-ups at the outpatient clinic, with monthly visits within the first 3 months after KPE and then every 1-3 months thereafter. Clinical follow-up data were recorded for up to 7.5 years. The levels of the t-bil and d-bil 3 months after KPE were recorded and analyzed to evaluate the influence of the microbiota on the outcome of BA. The long-term outcome of patients was characterized as either surviving with native liver (SNL group) at the latest follow-up, or undergoing LT or dying before LT (LT group).

We employed two approaches in this study. The first approach was to compare the dynamic microbiome profiles of patients with BA to those of healthy controls (HCs) at different time points. Fecal samples from HCs collected at 2 and 6 months of age were used for comparisons. The second approach was to assess whether the specific microbiome during the early stages of the disease could predict outcomes, including bilirubin levels 3 months post-KPE and native liver survival during long-term follow-up. This research was approved by the Institutional Review Board (IRB) of the National Taiwan University Hospital under 201505029RINB and 201912116RIND.

Microbial genomic DNA extraction from human stools and library construction

Stool DNA was extracted using the QIAamp PowerFecal Pro DNA kit (Qiagen, Germany). The V3–V4 hypervariable region of the bacterial 16S ribosomal RNA gene was PCR amplified with KAPA HiFi Hot Start Ready Mix (2X) (Roche, Mannheim, Germany). 16S V3–V4 amplicon libraries were constructed according to the Illumina library construction protocol. After library construction, metagenomic sequencing was performed using the paired-end 2 × 300 bp Illumina MiSeq protocol (Illumina MiSeq, USA).

16S rRNA sequencing and data analysis

Bacterial 16S rRNA sequencing data were analyzed using the QIIME2 next-generation microbiome bioinformatics pipeline.[13] The paired-end raw data were denoised using DADA2 (Divisive Amplicon Denoising Algorithm 2) within the QIIME 2 plugin, which detects and corrects amplicon errors and filters out potential base errors and chimeric sequences. The taxonomies of denoised amplicon sequence variants (ASVs) were identified by a scikit-learn naive Bayes machine-learning classifier trained on the full-length 16S rRNA sequences of three databases, Greengenes, Silva, and EZBiocloud to achieve a higher classification ratio and greater accuracy at the species level.[14–17] The classification results of EZBiocloud were chosen for this study because approximately 47% of the ASVs were identified at the species level. The alpha diversity was calculated with the QIIME2 plugin alpha-group-significance and replotted with GraphPad Prism version 8.[18] The beta diversity was calculated based on PLS-DA (partial least squares discriminant analysis) using the mixOmics package in the Bioconductor database (V3.17) (https://doi.org/10.18129/B9.bioc.mixOmics) of R software (V.4.0.4).[19] The linear discriminant analysis (LDA) effect size (LEfSe) results were analyzed by using MicrobiomeAnalyst and replotted with GraphPad Prism.[13,20] Spearman correlations were calculated using the microbiomeSeq package (https://github.com/umerijaz/microbiomeSeq) of R software (V.4.0.4) and replotted with GraphPad Prism.[21,22]

Shotgun metagenomic sequencing

To confirm the 16S rRNA sequencing data, we performed shotgun metagenomic sequencing in 10 patients with qualified fecal samples obtained at the pre-KPE stage. The DNA concentration was measured using a Qubit DNA BR assay kit with a Qubit 3.0 fluorometer (Thermo Fisher Scientific), and 150 ng of DNA was used for DNA library preparation with an Illumina DNA Prep kit (Illumina, Cat. No. 20060060). Briefly, DNA was fragmented to an average length of 600 bp with bead-linked transposomes, and the Read1 and Read2 sequencing primers were added simultaneously. Following post-fragmentation cleanup, PCR amplification with primers containing P5 or P7 adapters with specific indices was performed to complete library construction. After PCR, the amplified libraries were cleaned by double-size selection with sample purification beads. The quality of the purified library was assessed by capillary electrophoresis on a Qsep 100 (Bioptics) with a DNA S2 cartridge. The qualified libraries were sequenced on a NovaSeq 6000 (Illumina) at a 2 × 150 base read length, and 33 million reads (~10 Gb of data) per sample were subjected to metagenomic analysis. The raw sequence data were processed by Trimmomatic (version 0.39) to filter out low-quality reads and adapters.[23] Taxonomic profiling was performed using MetaPhlAn4 with the default parameters and the CHOCOPhlAn database (vOct22).[24,25] The results of MetaPhlAn4 were calculated by a Pavian metagenomics data explorer (https://fbreitwieser.shinyapps.io/pavian/) to obtain the relative abundances for each sample.[25]
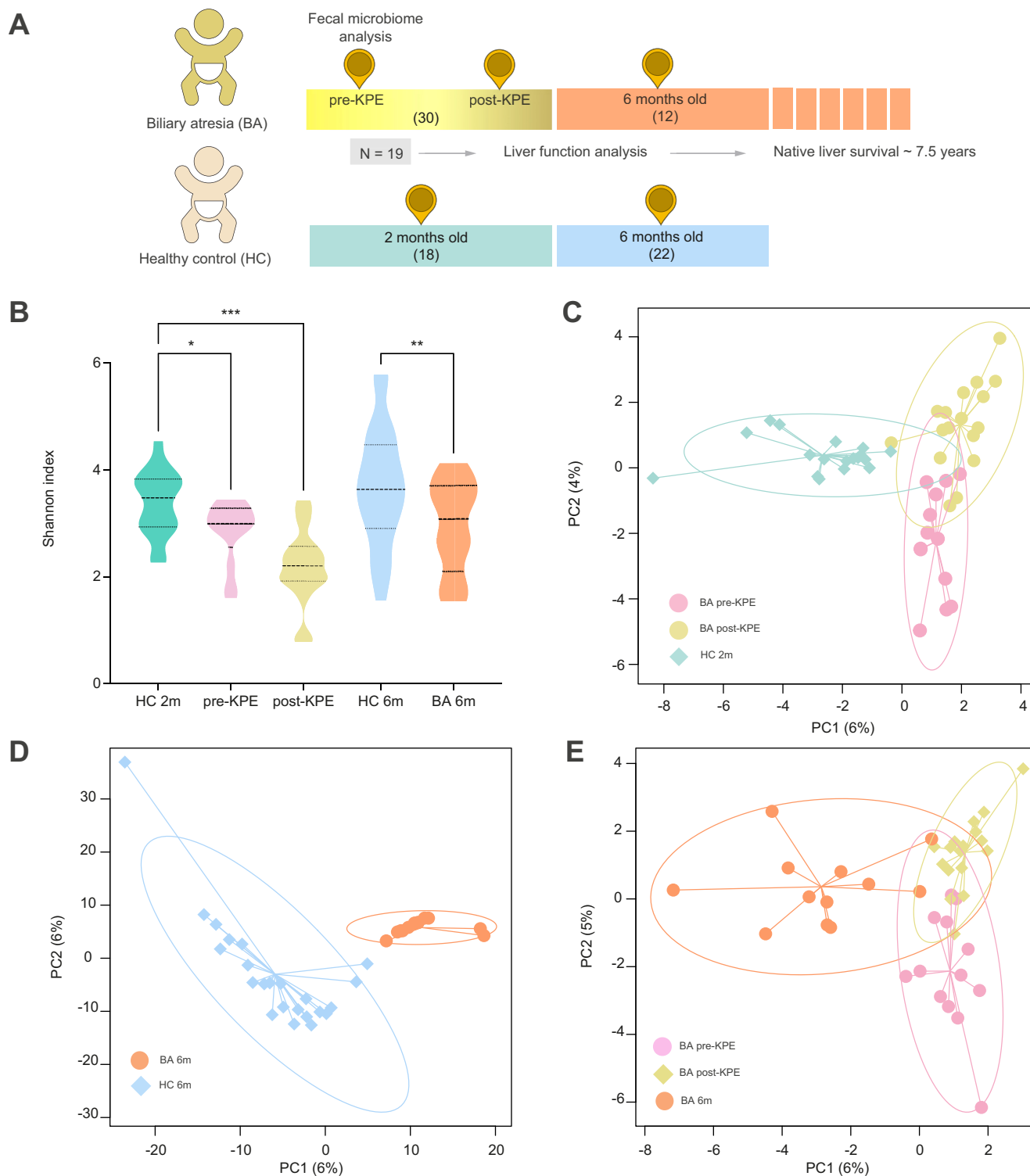
Fig. 1. Diversity and composition of the gut microbiota in patients with BA and HCs. (A) Schematic view of patients with BA and HC enrollment and fecal microbiome analysis. A total of 42 BA samples (30 and 12 samples at 2 and 6 months), and 40 from HCs (18 and 22, respectively) were analyzed. Nineteen patients with BA underwent longitudinal follow-up. (B) Shannon indices calculated at the ASV level for BA patients and HCs. * p <0.05; ***p <0.005 (one-way ANOVA followed by a two-stage step-up method of Benjamini, Krieger, and Yekutieli as a *post hoc* test); (C-E) Partial least square discriminant analysis of beta diversity of gut microbiota between patients with BA (pre-KPE, post-KPE and 6 months old) and HCs (2 months and 6 months old) at the ASV level. 3m, 3 months after KPE; 6m, 6 months old. ASV, amplicon sequence variant; BA, biliary atresia; KPE, Kasai portoenterostomy; HCs, healthy controls.

Statistical analysis

Continuous variables were compared using the Mann–Whitney *U* test unless otherwise specified. The comparison of alpha diversity between different groups was performed via one-way ANOVA followed by the two-stage step-up method of Benjamini, Krieger, and Yekutieli as a *post hoc* test. Categorical data were compared using the chi-square test or Fisher's exact test. The random forest classifications were analyzed using MicrobiomeAnalyst, which employs an ensemble of 2,000 decision trees trained on all samples classified at the species level, and then replotted with GraphPad Prism.[20,26] Native liver survival was analyzed using Kaplan–Meier analysis.

Results

The microbial community structure differed between infants with BA and HCs

First, to understand the microbiome signatures of patients with BA in comparison to HCs at different time points, the gut microbial compositions of patients with BA were compared to those of age- and sex-matched HCs using 16S rRNA analysis. This analysis included 42 fecal samples from patients with BA before and after KPE at approximately 2 months (BA-2m, n = 30) and at 6-9 months (BA-6m, n = 12). Additionally, 40 fecal samples from HCs were collected at 2 months of age (HC-2m, n = 18) and at 6 months of age (HC-6m, n = 22). There were no differences in gestational age, delivery methods, or breastfeeding between patients with BA and HCs. The baseline clinical characteristics of the participants are listed in Table 1.

We compared the alpha diversity of patients with BA to that of healthy controls at 2 months of age and 6 months of age using the Shannon index (Fig. 1B). BA pre-KPE and post-KPE significantly differed from that of HCs at 2 months of age (*p* = 0.047 and *p* = 0.028, respectively). The difference in alpha diversity between HCs and patients with BA persisted after KPE until the age of 6 months (*p* = 0.020). We also compared the alpha diversity between patients with BA at different time points. The differences were significant between pre-KPE and post-KPE, as well as post-KPE and BA-6m (*p* = 0.028 and *p* = 0.020, respectively). However, the differences between pre-KPE and BA-6m were not significant (*p* = 0.410). The beta diversity, analyzed at the ASV level using PLS-DA, showed significant differences in microbial composition between HCs and patients with BA before and after KPE (Fig. 1C). The difference between HCs and infants with BA became more apparent at 6 months of age (Fig. 1D). The fecal microbial beta diversity of infants with BA exhibited significant separation at different time points, including before and after KPE and at 6 months of age (Fig. 1E).

Microbiome characteristics of patients with BA at different time points

The taxonomies of ASVs were identified using the naïve Bayes machine-learning classifier trained on the EZBio cloud 16S rRNA database. Fig. 2A shows the difference in the constitution of the fecal microbiota at the genus level. The dominant microbiota at 2 and 6 months of age primarily comprised *Bifidobacterium* and *Escherichia* in both the HC and BA groups. The HC group exhibited a higher proportion of these two bacteria than the BA group. Notably, the colonization of *Veillonella* and *Klebsiella* in patients with BA pre-KPE was higher than that in HCs (Fig. 2A). After receiving KPE, the level of *Bifidobacterium* decreased, while the level of *Enterococcus* increased dramatically, exceeding the levels observed in both the pre-KPE and HC-2m groups (Fig. 2A). Patients with BA were discharged from the hospital 29±9 days after KPE, and most were in stable clinical condition at 6 months of age. We found that the abundance of *Bifidobacterium* remained low in BA-6m (Fig. 2A). Taxonomic analysis at the species level revealed that the abundance of *B. longum* was significantly lower in patients with BA at the time of diagnosis (pre-KPE), post-KPE, and at 6 months of age than in age-matched HCs (Fig. 2B).

The taxonomic difference between the patients with BA and age-matched HCs was analyzed using LEfSe and random forest classification. A significantly lower abundance of *B. longum* was observed in patients with BA than in HCs before KPE, and this lower abundance persisted in the BA group after KPE up to 6 months of age, as depicted in Fig. 3A. The proportion of *Bifidobacterium breve* (*B. breve*) was lower in the BA post-KPE group, while the proportions of *Flavonifractor plautii* (*F. plautii*) and *Bifidobacterium bifidum* (*B. bifidum*) were lower in the BA-6m group. On the other hand, *Veillonella dispar* (*V. dispar*) and *Clostridium ramosum* (*C. ramosum*) predominated in the gut microbiome of the BA-6m group (Fig. 3A). The

Table 1. Baseline characteristics of patients with BA and HCs.

	BA 2m (pre/post- KPE)	HC 2m	BA 6m	HC 6m
Number	n = 19*	n = 18	n = 12	n = 22
Male:female	8:11	9:9	5:7	15:7
Gestational age, week	39 (1)	37 (2)	39 (1)	39 (1)
Birth body weight (g)	3,056 (422)	2,681 (187)	3,031 (389)	3,233 (278)
Delivery method				
Vaginal, n (%)	6 (68.4)	5 (19.2)	7 (58.3)	8 (47.1)
C-section, n (%)	13 (31.6)	21 (80.8)	5 (41.7)	9 (52.9)
Diet, n (%)				
IF	5 (26.3)	1 (5.9)	5 (41.7)	3 (25.0)
BM	14 (73.7)	16 (94.1)	7 (58.3)	9 (75.0)

2m: 2 months; 6m: 6 months.
Numbers (parentheses): means (standard deviations) or numbers (percentages).
BA, biliary atresia; BM, breast milk exclusively or breast milk mixed with infant formula; HC, healthy control; IF, infant formula; KPE, Kasai portoenterostomy.
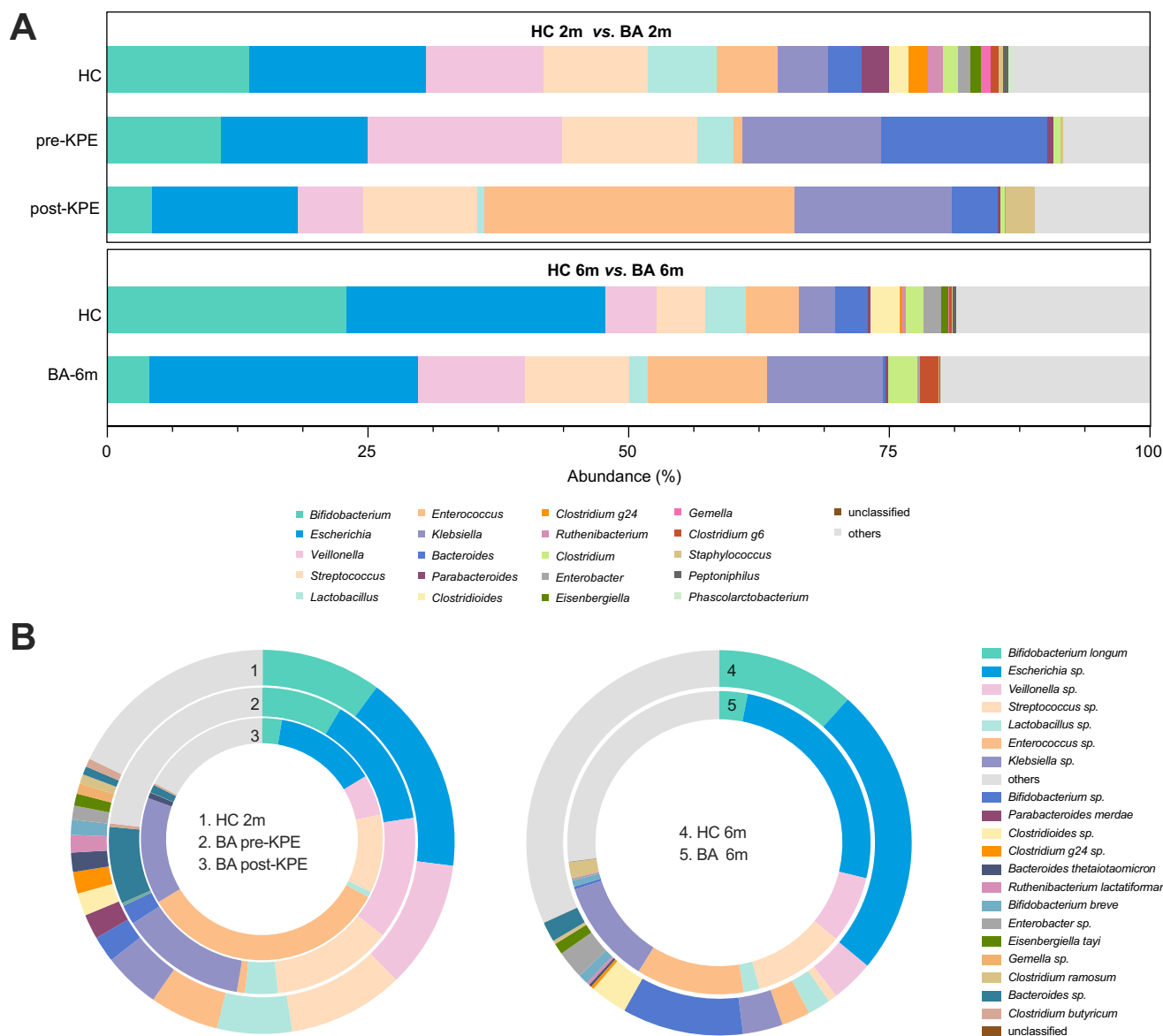*Fecal samples at pre-KPE period: n = 13, post-KPE n = 17.

Fig. 2. Taxonomic differences between patients with BA and HCs. (A) Relative microbial compositions (average) at the genus level in patients with BA and HCs. (B) Relative microbial compositions (average) at the species level in patients with BA and HCs after taxonomic identification. BA, biliary atresia; HCs, healthy controls.

results of random forest classification indicated significant differences in *Akkermansia muciniphila*, *B. longum*, *Clostridium paraputrificum*, *Clostridium perfringens*, and *B. breve* levels between the BA-pre-KPE and HC groups (Figs. S1A and B), and these differences also were present in the BA post-KPE group (Figs. S1A and C). There was also a significant difference in the abundance of *Streptococcus gallolyticus* between the BA-post-KPE and HC groups (Fig. S1A).

Early gut microbiome and outcomes of BA

In the second approach, we assessed whether a specific microbiome during the early stages of the disease could predict outcomes. We explored the potential impact of *B. longum*, *B. breve*, *C. ramosum*, *V. dispar*, and *C. perfringens* on the outcomes of patients with BA. *B. bifidum*, *F. plautii*, *A. muciniphila*,

C. paraputrificum, and *S. gallolyticus* were undetected in most of the BA samples and were not subjected to further analysis. Spearman correlation analysis was used to estimate the associations between bacterial abundance and clinical parameters. Elevated *B. longum* levels were significantly correlated to reduced GGT levels (p <0.05, Fig. 3B), implying a potential protective role against bile duct injury. On the other hand, an elevated level of *C. perfringens* was found to be correlated with increased alkaline phosphatase levels, suggesting a greater likelihood of harmful effects in the biliary epithelium.

Next, we investigated the effect of the potentially beneficial bacterium *B. longum* on the outcomes post-Kasai operation. Among the 22 patients with BA who underwent long-term follow-up, 9 received LT at 11.2 months of age (range 6 to 20 months). We compared the clinical parameters between patients in the LT and SNL groups. The age, sex, gestational age,

delivery method, diet, and clinical data before KPE were comparable (Table 2). The t-bil and d-til 3 months after KPE were significantly higher in patients with BA who required liver transplantation ($p < 0.005$).

When comparing the taxonomic differences between the SNL and LT groups (Fig. 4A), LEfSe analysis revealed a higher abundance of *B. longum* in the pre-KPE and post-KPE periods (Fig. 4B). At 6 months of age, LEfSe analysis revealed no representative microbiome signature, including *B. longum*. This was further shown by significantly higher relative abundance of *B. longum* in early infancy (pre-KPE) within the SNL group compared to the LT group, which was not evident at 6 months (Fig. 4C). Fig. 4D illustrates the dynamic changes in *B. longum* abundance in serial samples from selected individual patients. *B. longum* was not detected in any of the LT patients before or after KPE, indicating delayed *B. longum* colonization.

Gut *B. longum* is associated with the normalization of bilirubin and longer native liver survival

We next sought to investigate the role of *B. longum* in infancy and its correlation with long-term BA outcomes. Patients with detectable *B. longum* in the fecal microbiome before 6 months of age were classified as the *B. longum-pos* group (n = 10), and those with undetectable *B. longum* were classified as the *B. longum-neg* group (n = 9). The gestational age, sex, delivery method, and age at KPE were comparable between the two groups, as were the t-bil and d-bil levels before KPE (Table S1). Only the *B. longum-pos* group had significantly reduced t-bil and d-bil levels 3 months post-KPE (Fig. 5A,B). Notably, we found a significantly lower LT rate for patients with BA in the *B. longum-pos* group than in the *B. longum-neg* group (hazard ratio: 0.16, 95% CI 0.03-0.83, $p = 0.029$; Fig. 5C). These results suggest that establishing *B. longum* during the early life of patients with BA is associated with favorable long-term outcomes. Interestingly, all the patients in the *B. longum-pos* BA group were exclusively or partially breastmilk-fed, while only 44.4% of the patients in the *B. longum-neg* BA group were breastmilk-fed ($p = 0.011$, Table S1). In addition, *B. longum* was detected in most breastmilk-fed and partially breastmilk-fed patients, while *B. longum* was absent in patients fed infant formula (Fig. 5D). Survival did not differ between patients who consumed breast milk and those who consumed infant formula ($p = 0.098$, Fig. S2).

Ten of the original 42 fecal samples that met the quality control criteria for shotgun metagenomic sequencing during the pre-KPE period were selected to confirm the results obtained by 16S rRNA sequencing. Of these 10 patients, 6 survived with a native liver, and 4 received LT. The richness of *B. longum* was higher in patients from the SNL than the LT group (Fig. S3A). The outcomes were analyzed by grouping the patients based on identifying *B. longum* by shotgun metagenomics. Patients with detectable B. longum (*B. longum-pos-shotgun,* n = 4) in fecal samples before KPE had significantly reduced t-bil and d-bil levels 3 months post-KPE (Figs. S3B and C). In contrast, bilirubin levels did not significantly decrease in the undetectable group (*B. longum-neg-shotgun,* n = 6). Patients in the *B. longum-pos-shotgun* group exhibited a lower LT rate than those in the *B. longum-neg-shotgun* group, but the difference was not significant.

Discussion

In this study, we comprehensively illustrated the distinct bacterial communities between patients with BA and age-matched HCs throughout the first 6 months of life. Our analysis encompasses the periods before KPE, after KPE, at 6 months of age, and at longitudinal follow-up to preschool age, providing a holistic view of the BA microbiome signatures in early life and their impact on patient outcome. The microbiome alpha and beta diversity of patients with BA was significantly different at various time points, including before and after KPE, as well as at 6 months of age. These differences were observed not only within the BA group but also when comparing patients with BA to HCs, indicating that the microbial composition in patients with BA undergoes dynamic changes over time and differs from that of healthy individuals. Most importantly, *B. longum* was deficient throughout the disease course of BA and its presence in early infancy was correlated with long-term outcomes.
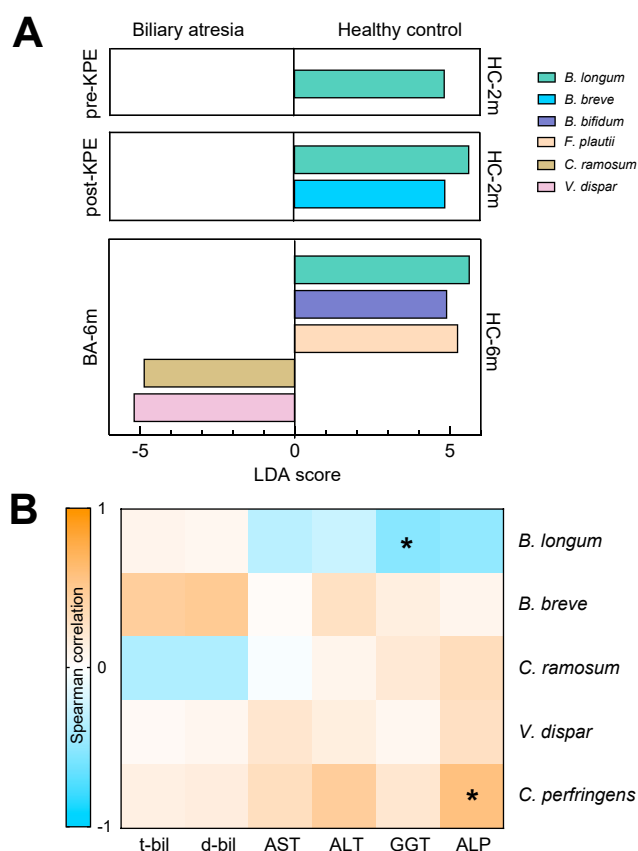

Fig. 3. Bacterial differences between patients with BA and HCs and their correlations with clinical parameters. (A) LDA effect size was used to identify taxa that significantly differed between patients with BA and HCs. Taxa with *p* <0.05 and LDA > 4.8 were considered significant (Kruskal–Wallis test, followed by Wilcoxon test and LDA). (B) Spearman correlations between the fecal abundance of *B. longum*, *B. breve*, *C. ramosum*, *V. dispar*, and *C. perfringens* and the clinical parameters of patients with BA post-KPE. The correlations of *B. bifidum*, *F. plautii*, *Akkermansia muciniphila*, *Clostridium paraputrificum*, and *Streptococcus gallolyticus* were not detected due to the absence or extremely low abundance of these bacteria in almost all of the BA post-KPE samples. * *p* <0.05 by Spearman correlation test. BA, bile acids; *B. bifdum*, *Bifidobacterium bifidum*; *B. breve*, *Bifidobacterium breve*; *B. longum*, *Bifidobacterium longum*; *C. perfringens*, *Clostridium perfringens*; *C. ramosum*, *Clostridium ramosum*; *F. plautii*, *Flavonifractor plautii*; *V. dispar*, *Veillonella dispar*; HCs, healthy controls; KPE, Kasai portoenterostomy; LDA, linear discriminant analysis.

Table 2. Characteristics of patients with BA who underwent LT/died or survived with a native liver.

	Survived with native liver	LT or death	*p* value*
Number	n = 13	n = 9	
Male:female	8:11	5:4	0.666
Follow-up duration (years)	4.4 (2.0-6.8)	5.5 (2.4-8.2)	0.142
Gestational age (weeks)	39 (36-40)	39 (37-39)	0.530
Birth body weight (grams)	2,930 (2,480-3,210)	2,950 (2,547-3,406)	0.570
Delivery method			
Vaginal, n (%)	9 (69.2)	6 (66.7)	0.628
C-section, n (%)	4 (30.7)	3 (33.3)	
Diet			
Exclusive IF, n (%)	2 (15.4)	4 (44.4)	0.155
Breast milk, n (%)	11 (84.6)	5 (55.6)	
KPE (days)	31 (11-119)	50 (13-79)	0.385
KPE hospitalization days	27 (18-39)	32 (21-61)	0.149
Transplant age (months)	–	11.2 (6-20)	–
Mortality, n (%)	–	1 (11.1)	–
Before KPE			
t-bil	8.2 (3.3-10.2)	8.5 (6.4-11.3)	0.968
d-bil	5.2 (2.4-6.2)	4.5 (3.6-4.7)	0.721
3 months after KPE			
t-bil	0.5 (0.2-2.0)	7.1 (0.6-29.6)	0.0007
d-bil	0.2 (0.1-1.1)	7.7 (0.3-18.2)	0.0006

d-bil, direct bilirubin; IF, infant formula; KPE, Kasai portoenterostomy; t-bil, total bilirubin.
*Mann–Whitney *U* test for continuous variables and Fisher's exact test for categorical variables.
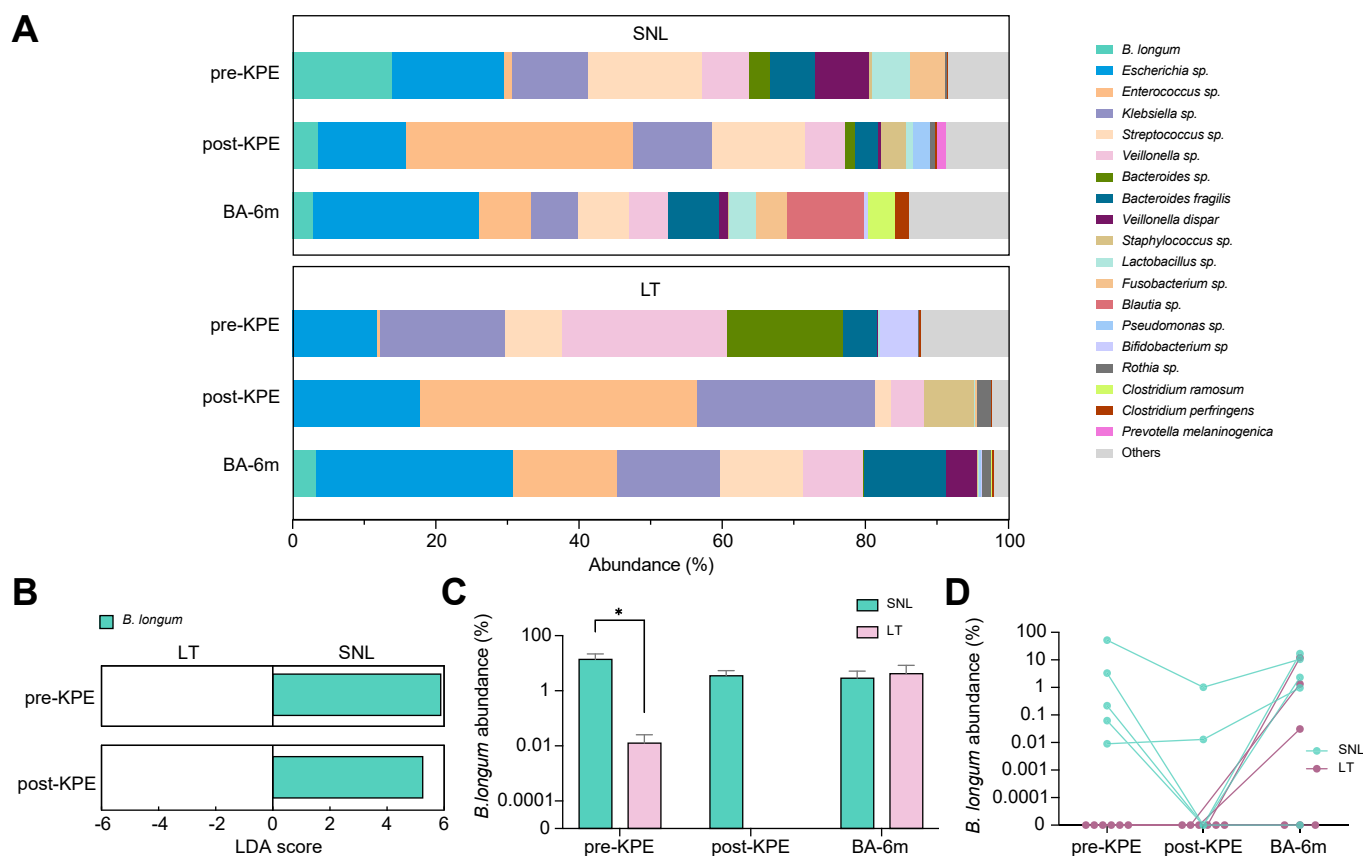

Fig. 4. Differences in bacterial composition between patients in the SNL group and those in the LT group. (A) Relative microbial compositions (average) at the species level in patients with BA in the SNL or LT group at different time points. (B) LDA effect size identified differences in bacterial composition between the SNL and LT groups in early life. No significant differences in bacterial composition were observed at BA-6m. Taxa with *p* <0.05 and LDA >4.8 were considered significant (Kruskal–Wallis test, followed by Wilcoxon test and LDA). (C) Relative abundance of *B. longum* at different time points. **p* <0.05 by the Mann–Whitney *U* test. (D) Abundance of *B. longum* in serial samples from individual patients. *B. longum*, *Bifidobacterium longum*; KPE, Kasai portoenterostomy; LDA, linear discriminant analysis; LT group, liver transplant or death group; SNL group, SNL, survive with native liver group.
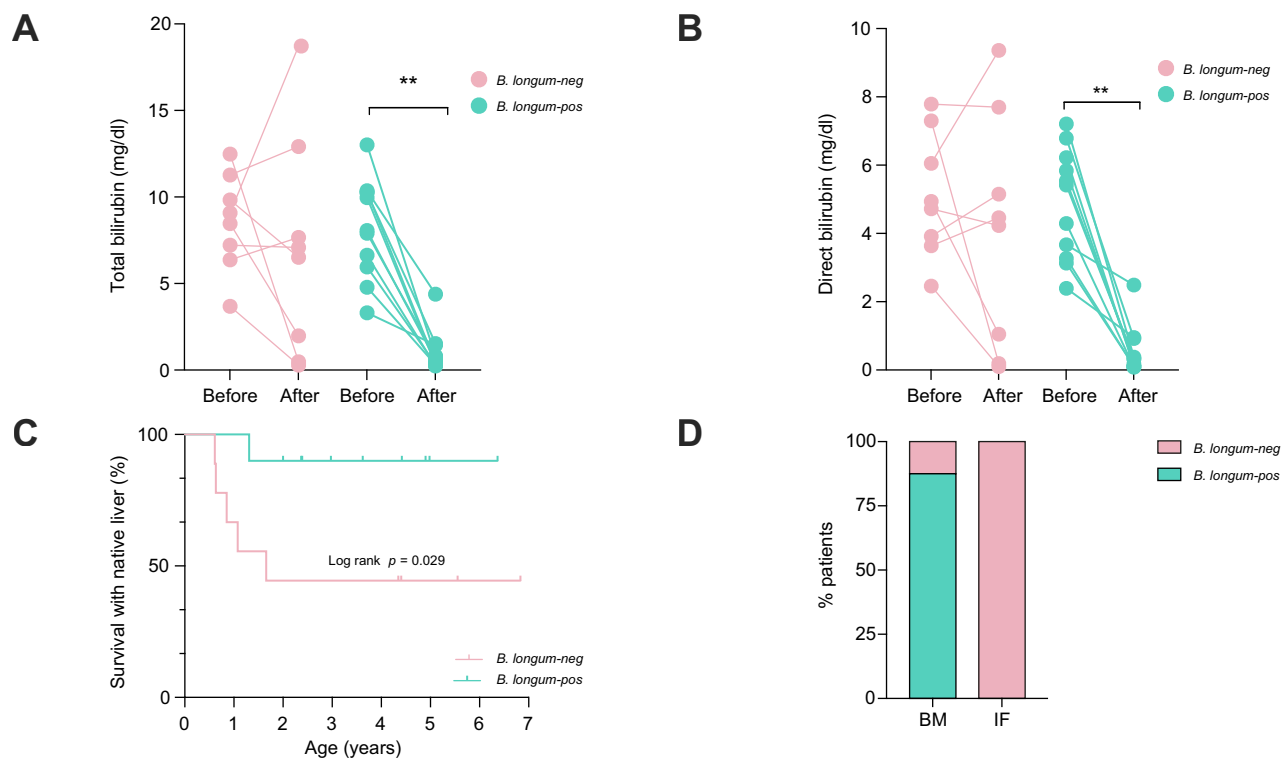
Fig. 5. Outcomes of patients with BA with *(B. longum-pos)* and without *(B. longum-neg)* detectable gut *B. longum*. (A and B) Dynamic changes in the serum t-bil and d-bil levels in patients with BA before KPE and 3 months after KPE. **p <0.005 according to the Mann–Whitney U test. (C) Proportion of patients with BA who survived with a native liver (Kaplan-Meier curves and log-rank test). (D) Feeding methods influence the presence or absence of *B. longum* in the gut microbial constitution of neonates with BA. BA, biliary atresia; *B. longum*, *Bifidobacterium longum*; BM, exclusive breast milk or mixed breast milk and infant formula; d-bil, direct bilirubin; IF, infant formula; KPE, Kasai portoenterostomy; t-bil, total bilirubin.

Previous studies have provided findings on the microbial composition in patients with BA at fragmented time points in early life.[11,27,28] A reduction in *Bifidobacterium* in patients with BA has been consistently observed since the 20th century using culture-based methods, and recent molecular studies have further supported this finding.[11,12,27–29] Our results are consistent with previous studies that assessed the microbiota at the genus level, demonstrating higher levels of *Streptococcus*, *Klebsiella*, *Enterococcus*, and *Veillonella*, as well as lower levels of *Bifidobacterium*, *Blautia*, and *Lachnospiraceae*, in patients with BA than in healthy controls.[11,12,28] However, research on the specific species of *Bifidobacterium* involved in BA and their roles has remained elusive. Identifying *Bifidobacterium* species is essential because their metabolic diversity enables distinct adaptations, integration, and interactions with the host microbiota.[30,31] At the species level, our study revealed a notable reduction in *B. longum*, *A. muciniphila*, *C. paraputrificum*, and *C. perfringens*, as well as decreased levels of *B. breve* and *S. gallolyticus* at 2 months of age and decreased levels of *F. plautii* at 6 months of age in patients with BA compared with age-matched HCs. We also noted an increase in *V. dispar* and *C. ramosum* abundance at 6 months of age.

We observed a negative correlation between the abundance of *B. longum* in the gut and the levels of GGT in patients with BA, supporting a potential protective role of *B. longum* in liver function. In line with this, a rat model of biliary obstruction demonstrated that combining *B. longum* with other probiotics alleviated pathological damage caused by biliary obstructions.[32] Furthermore, a recent study revealed that patients with BA whose gut microbiome was dominated by *Bifidobacterium* exhibited lower levels of AST and bilirubin.[33] Another study using a rat model of D-galactosamine-induced acute liver injury demonstrated that *B. longum* protected against acute liver failure by reducing the serum levels of AST, GGT, and bilirubin and mitigating liver inflammation and gut dysbiosis.[34] *B. longum* is a well-studied species of beneficial bacteria known for its ability to colonize the human gut and contribute to various aspects of gut health and immune function.[35–37] These findings collectively highlight the potential beneficial effects of *B. longum* in patients with BA. In addition, we found a positive correlation between *C. perfringens* and alkaline phosphatase, which indicates an adverse role. However, we did not find a negative correlation between *C. perfringens* and survival in patients with BA. For other bacteria, we did not find a correlation with biochemical markers or native liver survival.

One important novel finding in our study is that the presence of *B. longum* early in life was associated with improved native liver survival in patients with BA. To our knowledge, we have the most extensive follow-up data regarding microbiome studies of BA. We also confirmed the data using 16S rRNA sequencing and shotgun metagenomics. In a previous study by Tessier *et al.*, *B. breve* was associated with good bile flow in 8 patients with BA at 6 months of age.[27] Wessel *et al.* demonstrated that patients with BA who experienced clearance of jaundice at 6 months post-KPE exhibited increased abundances of *Veillonellaceae* and *Clostridiaceae* in their gut

microbiota.[11] Zhang *et al.* reported that *Bifidobacterium* was the most abundant genus in the non-cholangitis group, while *Klebsiella* was predominant in the cholangitis group.[38] Previous studies have focused primarily on the associations between the microbiota and short-term outcomes. Our data revealed that patients who exhibited detectable *B. longum* at an early disease stage had lower t-bil and d-bil levels 3 months after KPE. We did not detect a correlation between *B. breve* and bilirubin levels 3 months after KPE or native liver survival in our study. The discrepancies between studies may be due to the limited patient numbers in each study and the different patient populations.[39] The roles of *B. longum* and *B. breve* can be further explored using larger sample sizes in patients of different geographic backgrounds. Our findings provide an expanded perspective, contributing to the existing knowledge base. These findings may facilitate the development of more targeted management strategies for improving long-term outcomes in patients with BA. In view of future clinical applications, a faster and more cost-efficient bacterial detection strategy, such as 16S rRNA sequencing or real-time PCR, is preferable for longitudinally detecting specific species in patients with BA.

Notably, all patients in the *B. longum-neg* group were exclusively fed infant formula. The relative abundance of *B. longum* at birth in breastmilk-fed patients with BA was comparable to that in healthy individuals (14% *vs*. 15% *B. longum*), while *B. longum* was undetectable in non-breastmilk-fed patients. Many studies have shown that breastmilk feeding can increase the abundance of *B. longum* in the gut.[40] Moreover, breastmilk feeding ameliorates infant parenteral nutrition-associated liver disease.[41] One recent study showed that breast milk increases the abundance of *B. longum* in the gut to produce indole lactic acid, which stimulates CD4+ T cells to secrete IL-22, suppressing liver inflammation.[42,43] Breastfeeding has also been shown to benefit the host immune system by providing nutrients, specifically human milk oligosaccharides, to *Bifidobacteria* early in life.[39,44] Since the native liver survival of breastfed patients was not significantly better than that of patients fed infant formula, the factor contributing to better native liver survival may be attributed to the effects of *B. longum* on the gut microenvironment and immune system, thereby benefiting the liver through the gut-liver axis. A larger patient population and clinical trials are anticipated to evaluate the effect of *B. longum* as a novel therapy to improve the outcome of patients with BA.

The limitation of this study is that the main results were obtained from 16S rRNA analysis, which may not be ideal for species-level identification. Despite this, we have made the greatest efforts to use updated bioinformatic methods to increase the resolution of 16S rRNA sequence data to the species level by using a scikit-learn-based naive Bayes machine-learning classifier.[18] In addition, we conducted a shotgun metagenomic sequencing analysis on available fecal samples, and the results were in line with our main findings. Future studies with more robust methods for confirming the dynamic changes in *B. longum* throughout the course of BA may provide better evidence for understanding its role in modulating associated outcomes.

Our study provides comprehensive insights into the differences in gut microbial composition between patients with BA and HCs during the first year of life. The abundance of *B. longum* in patients with BA and its association with post-KPE liver function and native liver survival provide a perspective for investigating the potential role of *B. longum* in the progression of BA. Further research and clinical trials are needed to explore the therapeutic potential of *B. longum* in patients with BA.

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Abbreviations

ALT, alanine aminotransferase; *A. muciniphila, Akkermansia muciniphila;* AST, aspartate aminotransferase; ASV, amplicon sequence variants; BA, biliary atresia; *B. bifidum, Bifidobacterium bifidum; B. breve, Bifidobacterium breve; B. longum, Bifidobacterium longum; C. paraputrificum, Clostridium paraputrificum; C. perfringens, Clostridium perfringens; C. ramosum, Clostridium ramosum;* d-bil, direct bilirubin; *F. plautii, Flavonifractor plautii;* HCs, healthy controls, KPE, Kasai portoenterostomy; LT, liver transplant; LT group, liver transplant or death group; LEfSe, linear discriminant analysis effect size; SNL group, survive with native liver group; GGT, gamma-glutamyltransferase; *S. gallolyticus, Streptococcus gallolyticus;* t-bil, total bilirubin; *V. dispar, Veillonella dispar.*

Conflicts of interest

The authors declare that there are no conflicts of interest.

Please refer to the accompanying ICMJE disclosure forms for further details.

Data availability statement

Due to confidentiality and informed consent agreements, the raw sequencing files are available upon request to the corresponding author: hueyling@ntu.edu.tw. The data that were used are confidential.

Supplementary data

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