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ABOUT COVER

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AIMS AND SCOPE

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RESPONSIBLE EDITORS FOR THIS ISSUE

Basic Study

Microbiologic risk factors of recurrent choledocholithiasis post-endoscopic sphincterotomy

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Abstract

BACKGROUND

Choledocholithiasis is a severe disorder that affects a significant portion of the world's population. Treatment using endoscopic sphincterotomy (EST) has become widespread; however, recurrence post-EST is relatively common. The bile microbiome has a profound influence on the recurrence of choledocholithiasis in patients after EST; however, the key pathogens and their functions in the biliary tract remain unclear.

AIM

To investigate the biliary microbial characteristics of patients with recurrent choledocholithiasis post-EST, using next-generation sequencing.

METHODS

This cohort study included 43 patients, who presented with choledocholithiasis at the Guangdong Second Provincial General Hospital between May and June 2020. The patients had undergone EST or endoscopic papillary balloon dilation and

were followed up for over a year. They were divided into either the stable or recurrent groups. We collected bile samples and extracted microbial DNA for analysis through next-generation sequencing. Resulting sequences were analyzed for core microbiome and statistical differences between the diagnosis groups; they were examined using the Kyoto Encyclopedia of Genes and Genomes pathway hierarchy level using analysis of variance. Correlation between the key genera and metabolic pathways in bile, were analyzed using Pearson's correlation test.

RESULTS
The results revealed distinct clustering of biliary microbiota in recurrent choledocholithiasis. Higher relative abundances (RAs) of *Fusobacterium* and *Neisseria* (56.61% ± 14.81% *vs* 3.47% ± 1.10%, 8.95% ± 3.42% *vs* 0.69% ± 0.32%, respectively) and the absence of *Lactobacillus* were observed in the bile of patients with recurrent disease, compared to that in stable patients. Construction of a microbiological co-occurrence network revealed a mutual relationship among *Fusobacterium*, *Neisseria*, and *Leptotrichia*, and an antagonistic relationship among *Lactobacillales*, *Fusobacteriales*, and *Clostridiales*. Functional prediction of biliary microbiome revealed that the loss of transcription and metabolic abilities may lead to recurrent choledocholithiasis. Furthermore, the prediction model based on the RA of *Lactobacillales* in the bile was effective in identifying the risk of recurrent choledocholithiasis (P = 0.03).

CONCLUSION
We demonstrated differences in the bile microbiome of patients with recurrent choledocholithiasis compared to that in patients with stable disease, thereby adding to the current knowledge on its microbiologic etiology.

Key Words: Choledocholithiasis; Biliary tract; Microbiome; Endoscopic sphincterotomy; Recurrence; *Lactobacillus*

Core Tip: Treatment of choledocholithiasis by endoscopic sphincterotomy (EST) has become widespread, but recurrence post-EST is relatively common. In this study, we analyzed the bile microbiome of patients with recurrent choledocholithiasis. Increase in Fusobacterium and Neisseria, and the absence of Lactobacillus in bile were the key microbiologic features of recurrent choledocholithiasis. Bile microbiome imbalance might cause poor metabolism of carbohydrates and amino acids and increased glycan biosynthesis in the biliary tract, leading to disease recurrence. The microbiological features in bile could be an effective predictor for choledocholithiasis recurrence post-EST. The findings of our study will help develop new prevention strategies for post-surgery recurrence of choledocholithiasis.

INTRODUCTION

Cholelithiasis is a common and socially significant health problem worldwide, occurring in approximately 5%-22% of adults, with synchronous common bile duct stone (CBDS) in 20% of these patients[1-4]. In western countries, it is one of the leading gastrointestinal conditions that results in hospitalization [4]. Cholelithiasis with CBDS can lead to biliary obstruction, secondary cholangitis, and obstructive jaundice, endangering lives in some severe cases and often requiring surgical interventions[5]. The introduction of endoscopic treatment started a new era in the treatment of choledocholithiasis[6-9], and management by endoscopic sphincterotomy (EST) or endoscopic papillary balloon dilation (EPBD) has become widespread, replacing open laparoscopic cholecystectomy or open common bile duct exploration with choledochoscopy[10,11].

However, long-term surveys have revealed up to 39% recurrence of choledocholithiasis post-EST, and life-long follow-ups are still needed after surgery[12-14]. Recurrent choledocholithiasis post-EST involves complicated factors, including infections and biliary anatomical abnormalities[15,16]. The elimination of certain pathogens in the bile duct can significantly reduce the recurrence rate[17,18].

Therefore, further investigations into the microbiological etiology and underlying mechanisms of recurrent choledocholithiasis post-EST are crucial for its prediction and prevention in clinical practice.

Complex microbiomes in the biliary system have been observed using next-generation sequencing (NGS)[19]. In these systems, the microbiota metabolize and secrete cholesterol and bile acids; their dysfunction may cause pathophysiological defects and result in stone formation[20,21]. Unlike primary stones, secondary stones in recurrent choledocholithiasis predominately consist of more cholesterol than calcium bilirubinate[22], and their microbiological etiology remains unclear.

In this study, we investigated the microbiological etiology of recurrent choledocholithiasis using NGS to find the key pathogens associated with recurrence post-EST and their metabolic characteristics in disease relapse.

MATERIALS AND METHODS

Study participants and sample collection

Ethical compliance: Consecutive recruitment of eligible patients was carried out in the Department of Endoscopy at Guangdong Second Provincial General Hospital from May to June 2020. All experimental protocols were approved by this hospital's ethics committee (project 2019-QNJJ-14-02). The study design complied with all relevant ethical regulations in accordance with the Declaration of Helsinki and Belgian Privacy Commission. Written consent was obtained from all patients in the study.

Study cohort: In this study, we included 43 choledocholithiasis participants diagnosed using computed tomography (CT) or magnetic resonance cholangiopancreatography (MRCP). All patients were assessed by experienced doctors, without risk of EST-related complications[10]. Patients with a history of malignant diseases, autoimmune diseases, diabetes, structure abnormality of the biliary tract, or any exposure to antibiotics within one month were excluded from the study. All patients accepted laparoscopic cholecystectomy (LC) treatment following an EST or endoscopic papillary balloon dilation (EPBD), during the same hospitalization episode. The components of the stones were recorded according to the method of Dosch[23]. All patients received CT or MRCP examinations one week after the treatment to ensure the complete removal of stone in the biliary tract. All participants underwent at least one-year follow-up with transabdominal ultrasonography every three months, and CT or MRCP was performed once recurrence of choledocholithiasis was indicated through clinical presentations or imaging examinations. Patients were divided into stable and recurrent groups according to their disease evaluation at the end of the follow-up period.

Bile sample collection: Bile samples were collected during endoscopic treatment. The ERCP was performed to confirm the diagnosis of choledocholithiasis, followed by the EST or EPBD treatment, and the bile sample was collected through suction during the treatment. The bile samples were immediately transported to the laboratory and stored at -80 °C until extraction.

Microbiome DNA extraction and 16S rRNA gene amplicon sequencing

Bile sample (3 mL) was centrifuged at 16000 × *g* at 4 °C for 10 min, and the pellet was washed twice with phosphate-buffered saline before DNA extraction. Microbiome DNA was then extracted using the QIAamp PowerFecal DNA kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

The *16S rRNA* gene obtained from each bile sample was amplified by targeting the V3-V4 hypervariable regions using the following primers: 341F 5′-CCTACGGGNGGCWGCAG-3′ and 806R 5′-GGACTACHVGGGTWTCTAAT-3′ using the UCP Multiplex PCR Kit (Qiagen)[24]. The amplicon library was prepared using the QIAseq Ultralow Input Library Kit (Qiagen). An Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, United States) and Qubit dsDNA HS Assay Kit (Invitrogen Life Technologies, Carlsbad, CA, United States) were used to validate the library pooling. Paired-end sequencing was conducted using the MiSeq platform (Illumina, San Diego, CA, United States) with MiSeq Reagent Kit version V3 (Illumina).

Microbiome sequence curation and analysis

Trimming and quality filtering of the data were performed using the CLC Genomic Workbench version 20.0, with the Microbial Genomics Module (Qiagen). Sequences were matched to the Greengenes database version 13.5.

The amplicon sequencing, and the taxonomic and statistical analyses were performed using Calypso version 8.84[25]. Alpha diversity was determined based on Fisher's alpha index, which was assessed using the analysis of variance test. Microbial diversity was visualized using the canonical correspondence analysis based on the prognosis groups. Key taxonomic discovery analysis related to prognosis was performed using linear discriminant analysis effect size (LEfSe) at the genus level[26]. The relative abundance (RA) measurements of the genera, with biomarker significance were compared using the Wilcoxon rank test.

The core microbiome was identified as described by Ainsworth[27]. Network analysis was performed to identify the co-occurring and exclusive bacteria using Calypso[25]. Genera and orders of the bile microbiome were represented as nodes, taxa RAs as node size, and edges as positive and negative associations. Networks were generated based on the associations between both genera and orders using Pearson's correlation; nodes were colored based on their association with different prognosis groups. Only relationships with statistical significance ($P < 0.05$) were visualized in the network.

Metagenome prediction of the bile microbiome was performed using the amplicon sequencing approach in the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt)[28]. The statistical differences between the diagnosis groups were examined using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway hierarchy level using analysis of variance. Correlation analysis was carried out between the key genera and metabolic pathways in bile using Pearson's correlation test. Survival analysis of the identical microbiological risk factors was carried out using Kaplan-Meier analysis.

Statistical analyses

Except for those analyzed using Calypso, data were analyzed using GraphPad Prism v7.00 software (GraphPad, La Jolla, CA, United States). All analyses in the study were statistically significant at $P < 0.05$, and P values were adjusted using false discovery rate, Bonferroni, or area under curve correction.

RESULTS

Clinical features and prognosis of patients with choledocholithiasis one-year post-EST

Forty-three choledocholithiasis patients, who underwent LC following EST were recruited in this study and received a one-year follow-up survey. Thirteen patients had co-occurrence of cholelithiasis; the baseline clinical characteristics of the 43 choledocholithiasis patients were shown in Table 1. The stone components were recorded according to the methods of Dosch[23]; they were classified as brown pigmented stones, black pigmented stones, cholesterol stones, and mixed stones. Four recurrent cases without other complications were observed using routine ultrasonography as well as CT during the follow-up period. No significant differences were found in the clinical features or the stone components between patients with and without recurrent choledocholithiasis.

Bile microbiome characteristics in patients with choledocholithiasis

A total of 702 unique operational taxonomic units were identified in the bile of all patients with choledocholithiasis, indicating the diversity of the microbiome in bile (Figure 1). *Streptococcus* and an unclassified genus of *Enterobacteriaceae* were the most dominant genera; they were detected in the bile of 28 and 29 patients, respectively. The average RAs of *Streptococcus* and *Fusobacterium* in bile were 13.59% and 19.91%, respectively.

Key microorganisms in bile of patients with recurrent choledocholithiasis

The bile microbial structure in patients with recurrent choledocholithiasis was different from that in patients without recurrence, with lower alpha diversity ($P = 0.41$) and distinct beta diversity ($P = 0.03$; Figure 2).

LEfSe biomarker discovery analysis identified *Fusobacteriales* and *Neisseriales* as biomarkers in the recurrent group and *Lactobacillales* in the stable group at the order level (Figure 3A). The RAs of *Fusobacteriales* (56.61% ± 14.81% *vs* 3.47% ± 1.10%) and *Neisseriales* (8.95% ± 3.42% *vs* 0.69% ± 0.32%) were higher in patients with recurrent choledocholithiasis than that in stable patients post-EST ($P < 0.05$), while the RA of *Lactobacillales* was significantly lower in the recurrent group (1.48% ± 1.28%) than that in the stable group (25.04% ± 4.76%; $P < 0.05$; Figure 3B).

Bile microbiological ecosystem analyses in patients with choledocholithiasis with different prognoses post-EST

Core microbiome analyses showed that *Streptococcus, Prevotella, Fusobacterium*, an unclassified genus of *Enterobacteriaceae*, and an unclassified genus of *Clostridiaceae* were the shared core genera in both the stable and the recurrent group. *Veillonella, Oribacterium, Neisseria, Leptotrichia*, and *Campylobacter* were the specific core genera in the recurrent group, while *Enterococcus, Clostridium*, and an unclassified genus of *Aeromonadaceae* were the unique genera in the stable group (Table 2). Construction of a microbiological co-occurrence network revealed a mutual relationship among *Fusobacterium, Neisseria*, and *Leptotrichia* (Figure 4A).

Additionally, *Lactobacillales, Fusobacteriales, Enterobacteriales, Clostridiales*, and *Bacteroidales* were the shared core orders in both the stable and the recurrent group. *Pasteurellales, Neisseriales*, and *Campylobacterales* were the unique core orders in the recurrent group, while *Pseudomonadales, Burkholderiales, Bacillales, Aeromonadales*, and *Actinomycetales* were the unique orders in the stable group. Co-occurrence network analyses suggested mutual enhancement among the key recurrence-related pathogens in bile

Table 1 Clinical characteristics of choledocholithiasis patients

	Stable (*n* = 39)	Relapse (*n* = 4)	*P* value
Age (yr) (range)	47 (38-64)	44 (38-46)	0.142
Sex			
Male (cases) (%)	24 (61.54)	3 (75.00)	0.626
Female (cases) (%)	15 (38.46)	1 (25.00)	
History of smoking (cases) (%)	10 (25.64)	1 (25.00)	0.978
Comorbidities			
Type 2 diabetes mellitus	4 (10.26)	1 (25.00)	0.381
Hypertension	6 (15.38)	1 (25.00)	0.620
Hyperlipoidemia	13 (33.33)	2 (50.00)	0.505
Accompanied diagnosis			
Cholelithiasis (cases) (%)	11 (28.21)	2 (50.00)	0.366
Acute cholangitis (cases) (%)	4 (10.26)	1 (25.00)	0.381
Pancreatitis (cases) (%)	1 (2.56)	0 (0.00)	-
Serum biochemical indexes			
ALT (U/L)	161.50 ± 159.11	67.75 ± 75.61	0.210
AST (U/L)	128.51 ± 151.74	34.75 ± 38.20	0.063
Total Bilirubin (μmol/L)	92.19 ± 82.97	24.23 ± 23.50	0.057
Direct Bilirubin (μmol/L)	84.92 ± 91.99	18.73 ± 23.69	0. 060
Amylase (U/L)	118.69 ± 192.30	77.50 ± 30.39	0.544
Follow-up time (d)	369.80 ± 2.67	372.00 ± 4.00	0.101
Recurrent time from EST (d)	-	208.80 ± 87.97	-
Stone components			
Brown pigment (cases) (%)	29 (74.36)	2 (50.00)	0.303
Black pigment (cases) (%)	8 (20.51)	1 (25.00)	
Cholesterol (cases) (%)	0 (0.00)	0 (0.00)	
Mixed component (cases) (%)	2 (5.13)	1 (25.00)	

EST: Endoscopic sphincterotomy.

and antagonistic relationships among *Lactobacillales*, *Fusobacteriales*, and *Clostridiales* in the ecosystem, indicating the role of probiotics in the prevention of recurrence (Figure 4B).

Functional characteristics of bile microbiome in patients with choledocholithiasis with different prognoses post-EST

The metabolites from microorganisms are the key pathogenic factors for the host; therefore, the characteristics of the metabolic pathways in bile were analyzed for deeper insight into the microbiologic etiology of recurrent choledocholithiasis post-EST. Comparative analyses of microbiological functions were carried out at the 2nd hierarchy level of the KEGG pathway. In the stable group, the bile microorganisms were active in the transcription and metabolism related to the nervous system, infectious diseases, biosynthesis of carbohydrates and amino acids; while, in the recurrent group, the microbes were active in translation, replication and repair, metabolism of cofactors and vitamins, glycan biosynthesis and metabolism, genetic information processing, energy metabolism, and biosynthesis of secondary metabolites (Figure 5).

Furthermore, correlations between the key genera in the two groups and the different metabolic pathways were analyzed to identify the influence of certain microbes on the host (Figure 6). In the bile ecosystem of the patients with recurrent disease, *Fusobacterium* and *Campylobacter* had positive correlations with the metabolism of amino acids, replication and repair, and translation ($P < 0.05$), while the unclassified genus of *Enterobacteriaceae* had a negative correlation with all the discrepant metabolic

Table 2 Core microbiome in bile of choledocholithiasis patients with different prognosis

Core microbiome	Type	Group	Recurrent. Occ	Stable. Occ
Clostridium	Unique	Stable	0	0.44
Enterococcus	Unique		0	0.44
Unclassified genus of *Aeromonadaceae*	Unique		0.25	0.49
Fusobacterium	Core	Recurrent&Stable	1	0.41
Prevotella	Core		0.5	0.56
Streptococcus	Core		0.5	0.67
Unclassified genus of *Clostridiaceae*	Core		0.75	0.49
Unclassified genus of *Enterobacteriaceae*	Core		0.5	0.69
Campylobacter	Unique	Recurrent	0.5	0.18
Leptotrichia	Unique		0.75	0.28
Neisseria	Unique		0.75	0.31
Oribacterium	Unique		0.5	0.18
Veillonella	Unique		0.5	0.38

pathways between the two groups ($P < 0.05$). *Leptotrichia* had a positive correlation with all the discrepant metabolic pathways in the bile of the stable group ($P < 0.05$). Correlation analyses indicated that in bile of the recurrent group, increased *Fusobacterium* could alter the metabolism of amino acids, replication and repair, and translation functions, leading to the formation of secondary bile stones.

Microbiologic risk factor analysis of the recurrent group post-EST

Fusobacteriales and *Neisseriales* were identified as the bile biomarkers in the recurrent group and *Lactobacillales* in the stable group. Kaplan-Meier analysis was carried out to confirm whether these biomarkers can be used as independent predictive factors for recurrence post-EST (Figure 7). The statistical results revealed that patients with *Lactobacillales* in the bile were at a lower risk of recurrence post-EST ($P = 0.03$) than patients, who lacked this order in their bile.

DISCUSSION

It was assumed that the biliary system is sterile in healthy people; however, an increasing amount of NGS-supported evidence shows that bile supports a complex and abundant microbiome in healthy individuals[19,29]. The frequently identified microorganisms using traditional culture techniques are *Enterococcus*, *Klebsiella*, and *Pseudomonas*; these bacteria are active in reducing the bile acid pool and regulating bile acid metabolism[30-33]. However, the contribution of microbes to the biliary system is still unclear. NGS techniques revealed that the most common inhabitants of the biliary tract are *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Fusobacteria*, *Synergistetes*, and candidate phylum *Saccharibacteria* (*TM7*)[34]. Some of these microorganisms regulate the hydrolysis of bile acids to constituent components, cleavage of exogenous aromatic rings, deconjugation of bile acid complexes by hydrolytic enzymes, and the formation of free bile acids[35]. The disturbance of the microbiologic ecosystems in bile may lead to dysfunctional bile acid metabolism, resulting in a series of bile duct diseases[20,21]; however, the most disease-specific pathogens and their unique functions remain unknown.

Similar to the results from previous studies[29,34,36], this study revealed that the biliary tract was composed of a diversity of bacteria, and the majority of microorganisms in the bile were *Streptococcus*, *Prevotella*, *Fusobacterium*, *Enterococcus*, *Veillonella*, and *Clostridium*. *Lactobacillus* and *Lactococcus* were reported as the major genera in bile[36]; however, these two genera could only be detected in 11 patients in this study. These differences could be attributed to the differences in study designs; we included only patients with severe choledocholithiasis, who needed surgical intervention, for the analysis of microbial risk factors for disease recurrence. Another factor could be the difference in bile sampling; we chose the endoscopic route over open surgery for the collection of bile.

Endoscopic treatment such as EST can provide definitive relief to choledocholithiasis; however, the formation of gallstones will not stop unless the etiologic factors are eliminated[12,13,37]. Among all the risk factors for choledocholithiasis recurrence, only biliary infections are correctable; microbiological treatment is the most potential therapy against the recurrence of choledocholithiasis[5,15,16,38,39].
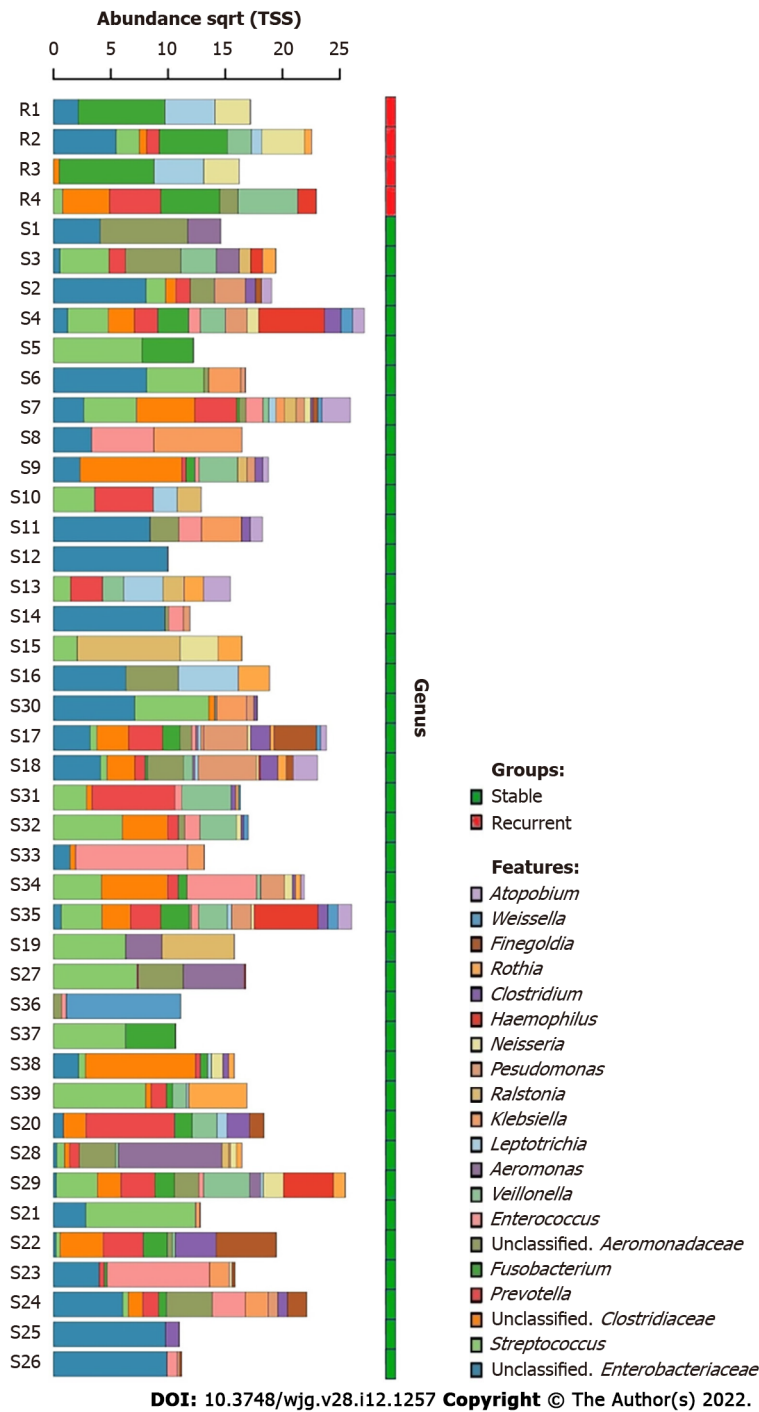
Figure 1 Dominant bacterial genera in the bile of choledocholithiasis patients. The top 20 dominant bacterial genera in bile are shown in the bar chart, the bile microbiome of recurrent choledocholithiasis patients post-endoscopic sphincterotomy are in the red group and choledocholithiasis patients without recurrence post-EST are in the green group. The genera were listed from the bottom to the top according to their relative abundance in bile samples.

Therefore, investigation into the biliary microbiology characteristics of recurrent choledocholithiasis is crucial to both etiology and prevention studies. To the best of our knowledge, this is the first pilot study to investigate the microbiological risk factors in recurrent choledocholithiasis post-EST. Increased *Fusobacterium* and *Neisseria* were recurrence-related biomarkers in the bile microbiome. Furthermore, we discovered the antagonistic potentials of *Lactobacillus* and an unclassified genus of *Enterobacteriales* against *Fusobacterium* and *Neisseria*, indicating the potential use of probiotics in the prevention of recurrence post-EST.

Bacteria in bile play an active role in gallstone formation[35]. *Escherichia coli* and *Klebsiella* in bile can produce hydrolytic enzymes such as β-glucuronidase, phospholipase A[40], and conjugated bile acid hydrolase; in addition, they can cause deconjugation of bilirubin diglucuronide and precipitation of calcium bilirubinate, which ultimately leads to biliary stone formation[41,42]. We identified *Clostridium* as one of the key microorganisms in the bile microbiome, which, according to Leung *et al*[43], is a more
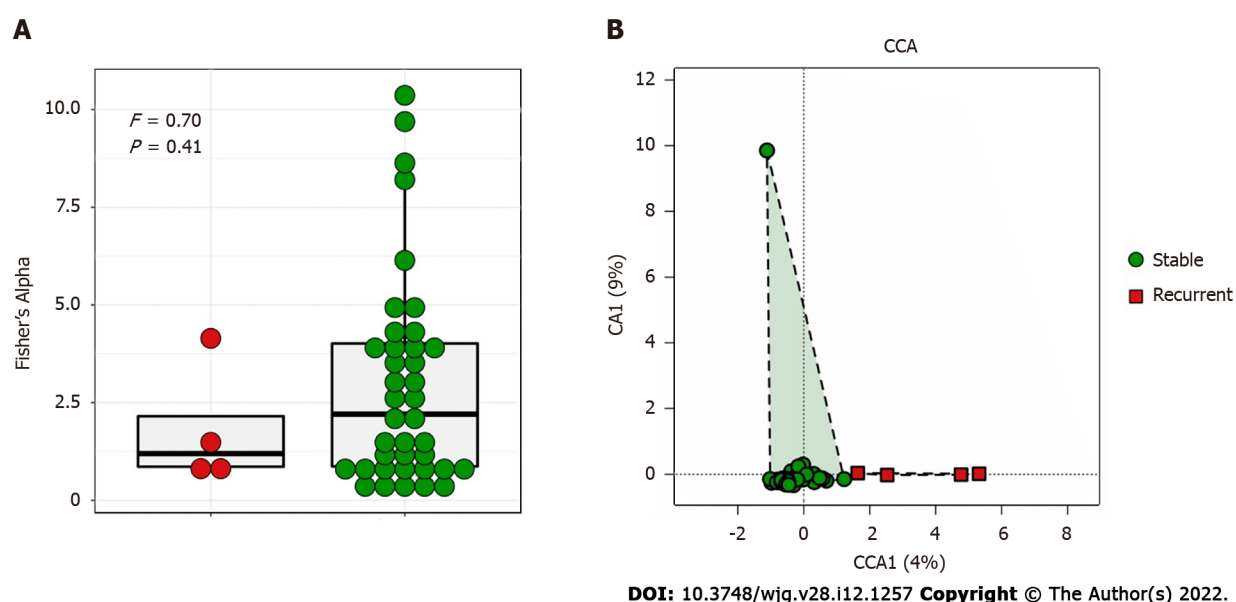
A: Comparison of alpha diversity of bile microbiome by the Fisher's Alpha Index between stable (green) and recurrent (red) choledocholithiasis patients post-endoscopic sphincterotomy (EST)

B: CCA

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Figure 2 Diversity analysis of bile microbiome of choledocholithiasis patients. A: Comparison of alpha diversity of bile microbiome by the Fisher's Alpha Index between stable (green) and recurrent (red) choledocholithiasis patients post-endoscopic sphincterotomy (EST); B: Comparison of beta diversity of bile microbiome using canonical correspondence analysis between stable (green) and recurrent (red) choledocholithiasis patients post-EST.

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Figure 3 LEfSe analysis of group-specific microbes in choledocholithiasis patients with different prognosis post endoscopic sphincterotomy. A: Colored cladogram showing microbiota with biomarker significance in choledocholithiasis patients with different prognosis post-endoscopic sphincterotomy (EST) (red for biomarkers in recurrent patients and green for biomarkers in patients without recurrence post EST); B: Relative abundance comparison of microbes with biomarker significance in choledocholithiasis patients with different prognosis post-EST. Statistical significance is expressed as [a]$P < 0.05$, [b]$P < 0.001$.

important microorganism in the deconjugation of bilirubin diglucuronide than *E. coli*, because it exhibits a 34-fold higher β-glucuronidase enzyme activity in the biliary tract. A lack of *Lactobacillus* in the bile could be a probable risk factor for choledocholithiasis, because *Lactobacillus* in bile can absorb cholesterol and reduce total serum cholesterol[44,45]. The core microbiome pattern in the bile of patients with choledocholithiasis in this study offers a more comprehensive understanding of the influence of the bile microbiome on biliary stone formation.

Furthermore, functional analysis indicated that the loss of transcription and metabolic abilities, and increased function of translation, replication and repair, metabolism of cofactors and vitamins, glycan biosynthesis and metabolism, genetic information processing, energy metabolism, and biosynthesis of other secondary metabolites could lead to recurrent choledocholithiasis. Most of these microbiologic functions were caused by the increased abundance of *Fusobacterium* and *Leptotrichia* and the loss of an unclassified genus of *Enterobacteriales*. However, little is known about the specific health-related functions of the metabolites of these microbes in the bile, and these important metabolic pathways require further research.
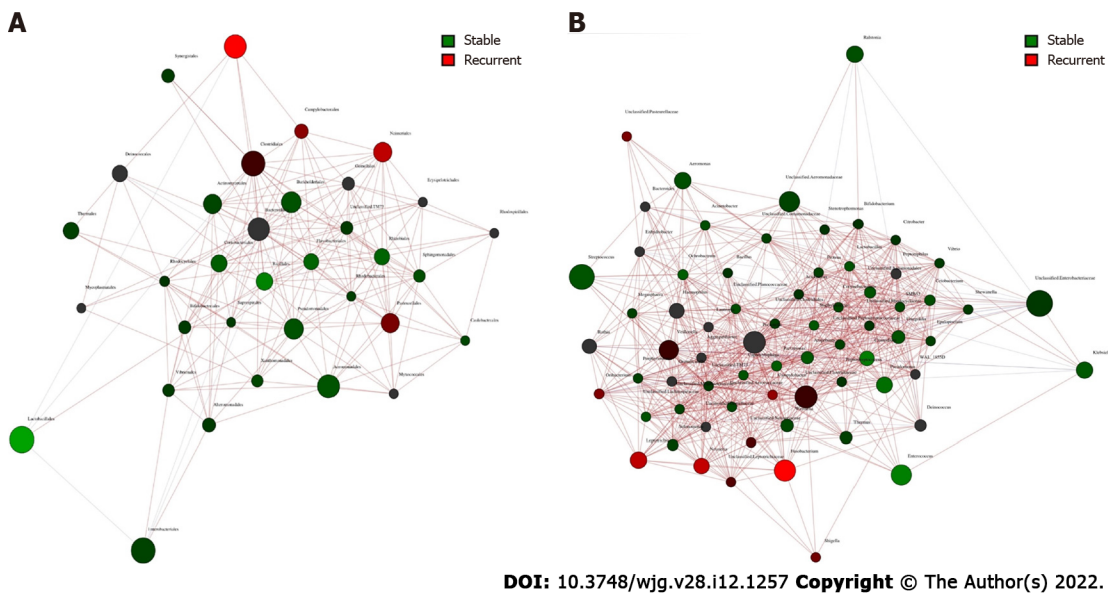
Figure 4 Co-occurrence network analysis of bile microbiome of choledocholithiasis patients with different prognosis post endoscopic sphincterotomy. A: Co-occurrence and disease-specific bacterial interactions at the order level. Order was presented as nodes (stable group specific order in green and recurrent group specific order in red), order abundance was presented as node size, and edges were represented based on their association tested using Pearson's correlation (positive inter-node correlations in blue, negative inter-node correlations in red); B: Co-occurrence and disease-specific bacterial interactions at the genus level. Genus was presented as nodes (stable group specific genus in green and recurrent group specific genus in red), genus abundance was presented as node size, and edges were represented based on their association tested using Pearson's correlation (positive inter-node correlations in blue, negative inter-node correlations in red).
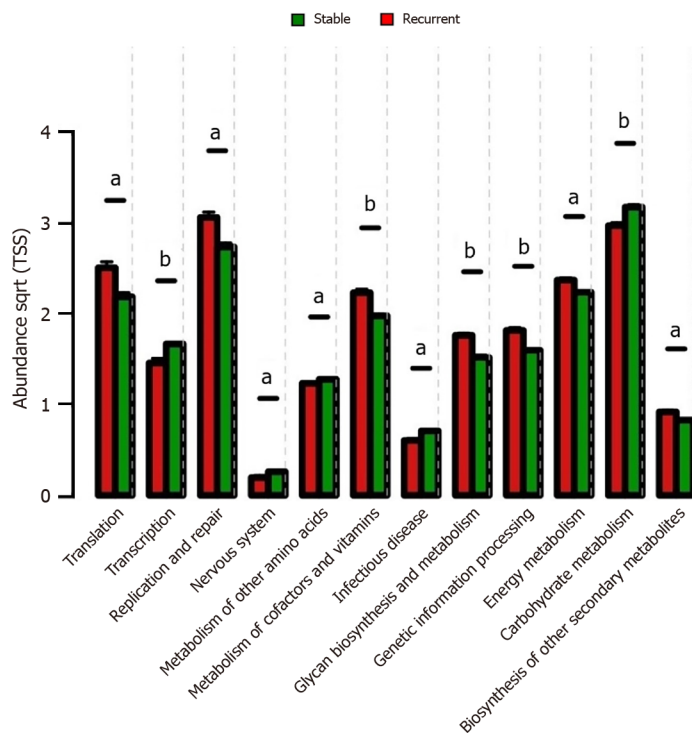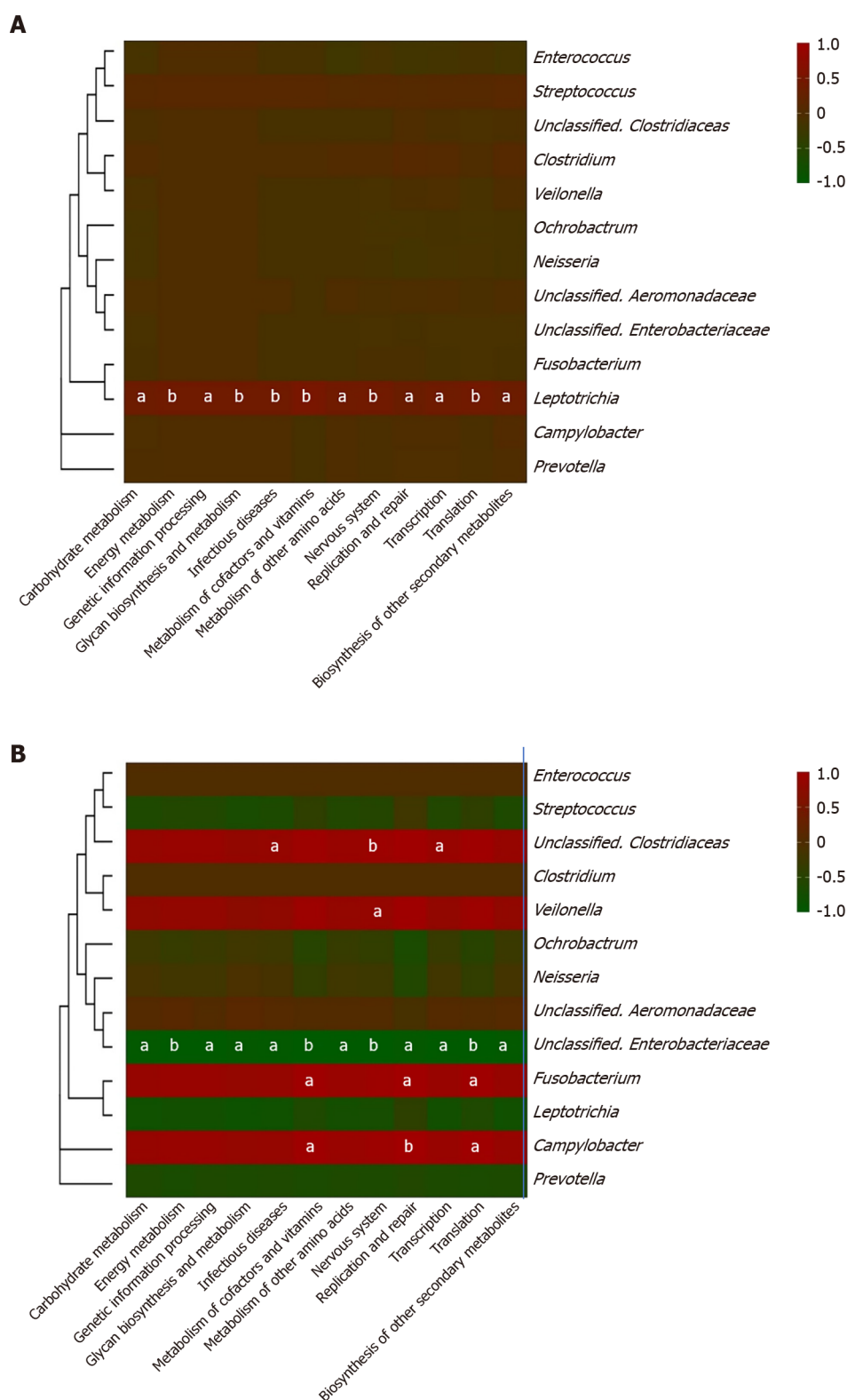
Figure 5 Comparison of microbial function prediction of bile microbiome of choledocholithiasis patients with different prognosis post endoscopic sphincterotomy. Functional analysis was performed at the 2nd hierarchy level of the Kyoto Encyclopedia of Genes and Genomes pathways in the bile microbiome of choledocholithiasis patients. Wilcoxon test was applied to the comparison of each category of microbial function; those with significant differences are shown in the bar chart. Statistical significance is expressed as [a]$P < 0.05$, [b]$P < 0.01$.

Figure 6 Heatmap of correlation between the core microbiome and key metabolic pathway in choledocholithiasis patients. Thirteen core genera of bile microbiome and their correlations with the twelve discrepant metabolite pathways in different prognosis groups were analyzed using Pearson correlation analysis. The Pearson correlation coefficient between the genus and the metabolic pathway was calculated and shown in colored matrix; red represents a positive correlation, while green represents a negative correlation. A: Matrix heatmap shows the correlations between different genera and metabolite pathways in choledocholithiasis patients without recurrence post-endoscopic sphincterotomy (EST); B: Matrix heatmap shows the correlations between different genera and metabolite pathways in recurrent choledocholithiasis patients post-EST. Statistical significance is expressed as [a]*P* < 0.05, [b]*P* < 0.01.
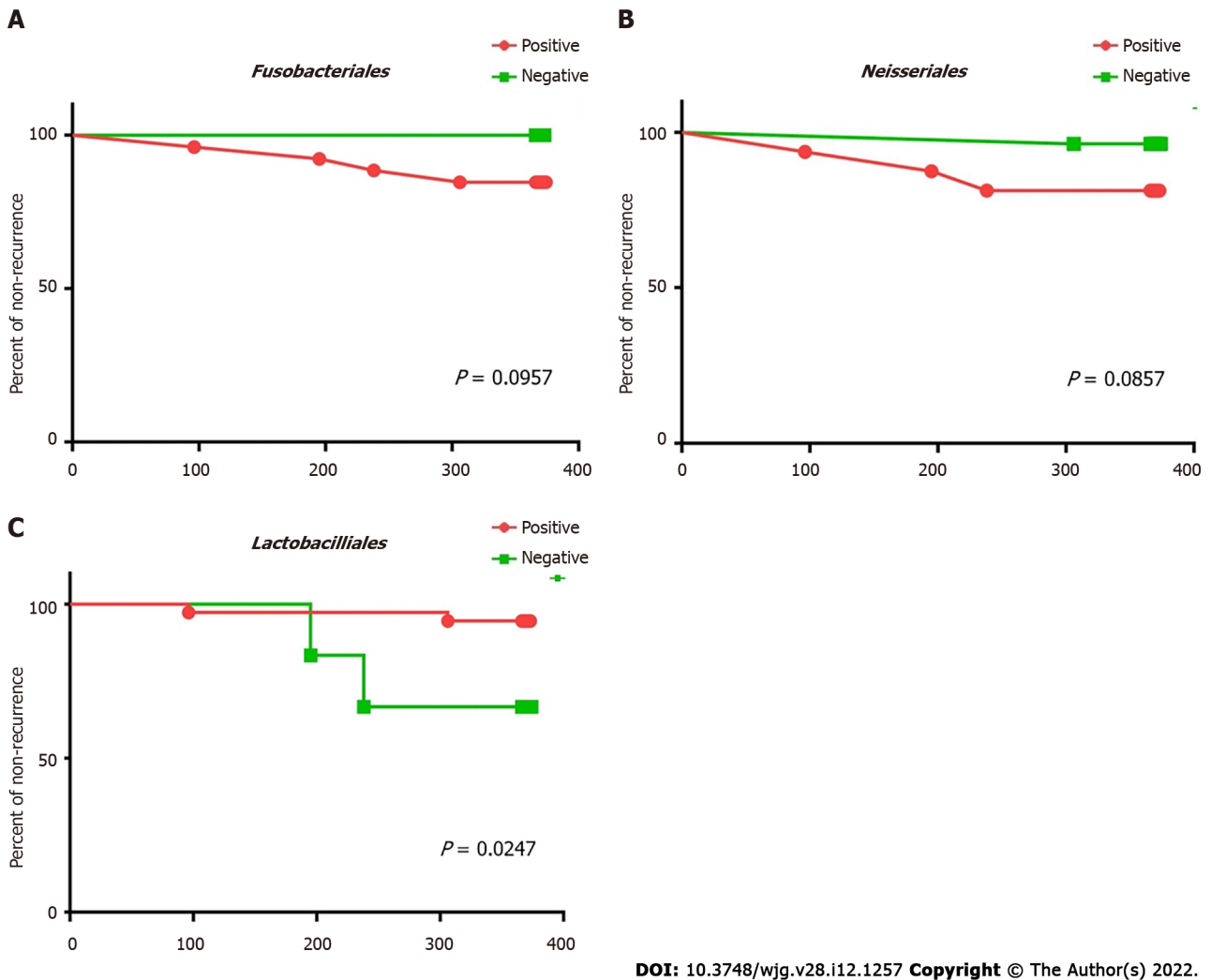
Figure 7 Kaplan-Meier analysis of recurrent time post endoscopic sphincterotomy with different microbiologic risk factors. A: Kaplan-Meier analysis of recurrent time post-endoscopic sphincterotomy (EST) between choledocholithiasis patients with (red) and without (green) *Fusobacteriales* in bile; B: Kaplan-Meier analysis of recurrent time post-EST between choledocholithiasis patients with (red) and without (green) *Neisseriales* in bile; C: Kaplan-Meier analysis of recurrent time post-EST between choledocholithiasis patients with (red) and without (green) *Lactobacillales* in bile.

Certain microorganisms in bile could predict the time taken before disease recurrence post-EST, and this was evaluated. The existence of *Lactobacillales* is crucial for predicting recurrence time post-EST, because patients with *Lactobacillales* in their bile had a longer progression-free time post-EST than patients without *Lactobacillales*. Therefore, the examination of *Lactobacillales* existence in bile at the time of endoscopic examination could help doctors identify high-risk patients, who are likely to have early choledocholithiasis recurrence post-EST.

The limited number of early recurrent choledocholithiasis patients could have introduced a bias in statistical analysis and could have limited the generalizability of the prediction model in this study. Furthermore, the diagnosis of choledocholithiasis recurrence relied mainly on the imaging examinations; we could have missed some stones which were invisible in the CT, underestimating the rate of choledocholithiasis recurrence. The molecular mechanisms of microorganisms underlying the recurrence post-EST was based on the PICRUSt model. Verification experiments such as analyzing the correlation between the bile microbiome and the stone composition, and animal experiments to ascertain the preventive effects of *Lactobacillus* in choledocholithiasis recurrence are warranted.

CONCLUSION

The microbiological characteristics of bile from patients with recurrent choledocholithiasis post-EST indicated that an increase in *Fusobacterium* and *Neisseria* are potential biomarkers for the identification of high-risk patients in the first EST. It elucidated the role of microbial metabolites in the underlying etiology of choledocholithiasis. A co-occurrent network of the biliary bacterial community was constructed. Potential preventive therapy against recurrent choledocholithiasis through supple-

mentation with *Lactobacillus* and maintenance of the balance of the microbial systems could be promising. These findings could help doctors better understand the etiology of recurrent choledocholithiasis and develop better monitoring and treatment strategies against recurrence post-EST.

ARTICLE HIGHLIGHTS

Research background

Choledocholithiasis is a common and socially significant health problem worldwide, and endoscopic sphincterotomy (EST) has become widespread in treating choledocholithiasis; however, recurrence post-EST is relatively common. The bile microbiome has a profound influence on the recurrence of choledocholithiasis; however, the key pathogens and their functions are not fully elucidated.

Research motivation

To determine the microbiologic risk factors of recurrent choledocholithiasis post EST.

Research objectives

To investigate the biliary microbial characteristics of the recurrent choledocholithiasis post-EST, using next-generation sequencing.

Research methods

This cohort study included 43 choledocholithiasis patients who had undergone EST were followed up for over a year. They were divided into either the stable or recurrent groups and comparison of their bile microbiome was carried out through next-generation sequencing. Resulting sequences were analyzed for core microbiome and statistical differences between the microbiologic compositions and functions. Correlation between the key genera and metabolic pathways in bile, were analyzed using Pearson's correlation test.

Research results

The results revealed distinct clustering of biliary microbiota in recurrent choledocholithiasis, in which higher relative abundances (RAs) of *Fusobacterium* and *Neisseria* and the absence of *Lactobacillus* were observed in the bile of the recurrent patients. Microbiological co-occurrence network revealed a mutual relationship among *Fusobacterium*, *Neisseria*, and *Leptotrichia*, and an antagonistic relationship among *Lactobacillales*, *Fusobacteriales*, and *Clostridiales*. Functional analysis revealed that the loss of microbiologic transcription and metabolic abilities may lead to the choledocholithiasis recurrence. Furthermore, the prediction model based on the RA of *Lactobacillales* in the bile was effective in identifying the risk of recurrent choledocholithiasis.

Research conclusions

We concluded the microbiologic differences in the bile of recurrent choledocholithiasis patients post EST, thereby adding to the current knowledge on its microbiologic etiology.

Research perspectives

The findings of our study will help develop new prevention strategies for post-surgery recurrence of choledocholithiasis.

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FOOTNOTES

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