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Gastrointestinal microbiome and cholelithiasis: Current status and perspectives

Dan WY *et al*. Gastrointestinal microbiome and cholelithiasis

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Abstract

Cholelithiasis is a common digestive disease affecting 10% to 15% of adults. It imposes significant global health and financial burdens. However, the pathogenesis of cholelithiasis involves several factors and is incompletely elucidated. In addition to genetic predisposition and hepatic hypersecretion, the pathogenesis of cholelithiasis might involve the gastrointestinal (GI) microbiome, consisting of microorganisms and their metabolites. High-throughput sequencing studies have elucidated the role of bile, gallstones, and the fecal microbiome in cholelithiasis, associating microbiota dysbiosis with gallstone formation. The GI microbiome may drive cholelithogenesis by regulating bile acid metabolism and related signaling pathways. This review examines the literature implicating the GI microbiome in cholelithiasis, specifically gallbladder stones, choledocholithiasis, and asymptomatic gallstones. We also discuss alterations of the GI microbiome and its influence on cholelithogenesis.

Key Words: Gallstone; Cholesterol gallstone; Common bile duct stone; Bile acid; Bile microbiome; Gastrointestinal microbiome

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Core Tip: Cholelithiasis is a common digestive disease that imposes significant global health and financial burdens. High-throughput screening demonstrated the relationship between bile, gallstones, and the fecal microbiome in cholelithiasis and provided evidence that gastrointestinal (GI) microbiota dysbiosis is associated with gallstone formation. We summarize the current literature, pool the available cholelithiasis-related studies about the GI microbiome, discuss the underlying mechanisms by which the GI microbiome modulates cholelithiasis, and suggest potential microbiome-targeting therapeutics for cholelithiasis prevention.

INTRODUCTION

Cholelithiasis or gallstones is a common digestive disease with a high incidence and relatively low mortality. With an overall prevalence of 11.0% in China[1], cholelithiasis is an important public health problem. Gallstone disease's prevalence ranges from 0.60 to 1.39% per year in European population surveys[2]. In the United States, 20 to 25 million adults have cholelithiasis, costing more than $6 billion annually[3,4]. Although many cholelithiasis patients are asymptomatic, approximately one-third develop biliary-pancreatic diseases, including acute or chronic cholecystitis, cholangitis, pancreatitis, and even biliopancreatic cancerous lesions. These diseases impose significant global health and financial burdens.

The study of the human microbiome, particularly the gastrointestinal (GI) microbiome, has rapidly evolved in recent decades, primarily due to the new generation of sequencing technology. The composition, diversity, and richness of microbial communities in the GI tract change during disease states. In addition to definite intestinal dysbiosis-related digestive diseases, the GI microbiome is altered in many biliary disorders, which are rarely traditionally considered microbial in etiology. A predictive model including the genera *Burkholderia*, *Caballeronia*, and *Paraburkholderia* was better able to predict cholangiocarcinoma than the tumor marker carbohydrate antigen 19-9[5]. The proportion of *Streptococcus* is proportionate to the severity of primary sclerosing cholangitis[6].

Investigations of the GI microbiome have extended to cholelithiasis. In the early 20th century, studies supported the existence of interactions between gallstones and bacteria such as *Helicobacter*. Although it has been recognized that specific bacteria contribute to gallstone formation, studies have recently shown that a complex GI microbiome rich in *Desulfovibrionales* promotes gallstone formation by regulating bile metabolism. This review examines the literature implicating the role of the GI microbiome in cholelithiasis, including the biliary, gallstone, and fecal microbiomes (Table 1). We will summarize the literature and discuss mechanisms by which the GI microbiome

modulates cholelithiasis; finally, we discuss advances in microbiome-based therapeutics.

THE GI MICROBIOME RELATED TO CHOLELITHIASIS

The biliary microbiome

Studies suggest that the healthy biliary tract is a sterile environment. With the development of sequencing technology, studies revealed the existence of a biliary microbiome. Stewart *et al*[7] detected bacteria in bile samples in nearly a third of gallstone patients. The first study to identify the existence of human biliary microbiota in the gallbladder described the composition of human biliary microbiota using 16S ribosomal RNA (rRNA) gene sequencing. The healthy biliary microbiota is dominated by *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Verrucomicrobia* at the phylum level[8]. In addition to bacteria, *Cyanobacteria* and *Spirochaetes* were also present in low amounts.

There are some resemblances and dissimilarities between the biliary and GI microbiomes. The comparative metagenomic analysis demonstrated no significant differences between the GI tract and bile in the predominant phylum *Firmicutes* and the rare phylum *Fusobacteria*. However, the microbial diversity in the biliary tract is more diverse than in the GI tract[9]. The bile duct and duodenum share the core microbiota with the genus *Escherichia–Shigella*, *Fusobacterium*, and *Enterococcus*[10]. Given that the bile duct is anatomically connected to the GI tract *via* the duodenal papilla, it was hypothesized that the biliary microbiota originates from intestinal bacteria and migrates retrograde into the biliary tract. Consistent with the hypothesis, studies demonstrated that the biliary microbiota shared a compositional similarity to duodenal microbiota, and all bacteria in bile were detected in the upper GI using 16S sequencing[11,12]. It is noteworthy that the intestinal microbiome contributes to the heterogeneity of the biliary microbiome in cholelithiasis patients, despite the high prevalence of oral cavity and respiratory tract inhabitants to intestinal inhabitants[13].

Because of interactions between the biliary microbiome and metabolic disease, investigators analyzed the bacterial composition associated with cholelithiasis. A Colombian study demonstrated a predominance of *Pseudomonas spp*. in gallbladder tissue and bile[14]. To compare the difference in bile microbiome between gallbladder stone patients and healthy individuals, Molinero *et al*[8] measured bile samples using 16S rRNA sequencing. They demonstrated that the relative abundance of the family *Propionibacteriaceae* in patients with gallbladder stones was lower than in healthy controls. In contrast, the relative abundance of the family *Bacteroidaceae*, *Prevotellaceae*, *Porphyromonadaceae*, and *Veillonellaceae* was higher.

Short-chain fatty acids (SCFAs) consist of butyrate, propionate, and acetate; these are associated with inflammatory diseases and cancers and may serve as nutritional and therapeutic agents in diseases[15,16]. Evidence suggests that low levels of expression of bacteria-producing SCFAs harm the microbial balance of the biliary tract, affecting gallstone formation. There is a significantly decreased abundance of *Clostridiumsensu_stricto*, *Lachnospiraceae* _UCG-008, *Butyrivibrio*, and *Roseburia* (which produce SCFAs) at the genera level in patients with primary choledocholithiasis. Lyu *et al*[12] found that specific bacteria-producing SCFAs might participate in generating common bile duct stones.

In recent years, several groups interrogated the human biliary microbiome in recurrent bile duct stones patients, albeit in different disease states. The biliary microbiome in the recurrence of choledocholithiasis showed a significantly low diversity[17]. The composition of biliary bacteria differed significantly between primary bile duct stone patients and those with secondary bile duct stones. By contrast, several families, such as *Propionibacteriaceae*, *Sphingomonadaceae*, and *Lactobacillaceae*, were enriched in the recurrent cholelithiasis group[18]. Chen *et al*[19] analyzed the biliary microbiota of 16 patients with recurrent choledocholithiasis and 44 patients with primary choledocholithiasis. The 16S rRNA sequencing revealed a prevalence of the phyla *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* (all of which are intestinal bacteria) in the primary choledocholithiasis group. These data suggest that the

biliary microbiota might originate from gut microbiota and access the biliary tract through the duodenal papilla. Furthermore, there was substantial enrichment of the genera *Prevotella*, *Alloprevotella*, *Nesterenkonia*, and *Pyramidobacter* (without significant alterations in microbial diversity) compared with recurrent choledocholithiasis patients[19]. One study described the biliary microbiome in recurrent cholelithiasis[20]. Investigators compared the biliary microbiomes of patients with primary choledocholithiasis with those with recurrent choledocholithiasis. Consistently, the phyla *Proteobacteria* and *Firmicutes* predominated in both groups. Patients with recurrent choledocholithiasis showed less microbial biliary diversity (with reduced *Bacteroidetes* and *Actinobacteria* and enrichment of *Synergistetes* at the phylum level) than controls. A study considered 11 adults with recurrent choledocholithiasis and nine post-endoscopic removal patients to assess the characteristics of the biliary microbiome. The richness and diversity decreased in the recurrence group. The recurrence group had a higher relative abundance of phylum *Actinobacteria* and *Firmicutes* and a lower relative abundance of *Bacteroidetes* than the non-recurrence group[21].

These association studies demonstrate a correlation between the biliary microbiome and cholelithiasis; however, these studies sampled the microbiome from bile but not the bile duct epithelium.

The gallstone microbiome

Awareness of the gallstone microbiome arose *via* a circuitous route. The first microbial study of gallstone formation dates back decades; the authors found a lower prevalence of gallstones in germ-free mice[22]. In the 1990s, bacterial DNA was detected using PCR in all common bile duct stones, mixed cholesterol stones, and brown pigment stones[23], demonstrating the existence of bacteria in gallstone formation. In the 21st century, researchers found that 42% of patients had bacteria gallstone samples[7], and over 80% of the stone cores contained bacteria, primarily from the intestine[24]. These results were vital for assessing the connections between the microbiota and cholelithiasis.

According to scanning electron microscopy studies, there are bacterial biofilms on gallstone surfaces[25]. Bacterial biofilms are microbial communities attached to the surface or embedded in the matrix[26]. The typical surface-related biofilm originates from single planktonic cells attached to the surface. These cells divide and produce extracellular polymeric substances. Microbial communities then form the three-dimensional structure attached to the surface. Biofilms are environmental reservoirs for pathogens that correlate with infectious kidney stones, bacterial endocarditis, and airway infections in cystic fibrosis patients[27]. Studies found that biofilm formed by *Salmonellae* aggregates on the surface of human gallstones[28]. The appearance of gallstones led to a 4.5-fold increase in hyperbiofilm isolates. *Salmonella* spp. increased in persistence in bile *via* genetic alterations[29].

The gut microbiome

The gut microbiome in human health and diseases has received considerable attention. The intestinal microbiota is dominated by *Bacteroidetes*, followed by *Firmicutes*, *Proteobacteria*, *Actinobacteria*, and *Verrucomicrobia*[30]. Investigations of the microbial composition of cholelithiasis patients using 16S rRNA profiling identified a predominance of *Firmicutes* and, to a lesser extent, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria* at the phylum level[31-33]. These findings suggest a shift in the gut microbiome from healthy individuals to cholelithiasis patients, suggesting an association with cholelithiasis pathophysiology.

The gut microbiome of cholelithiasis patients is maladjusted. Most studies showed reduced gut microbiota diversity in cholelithiasis patients[5,32]. There are alterations of the gut microbiome in cholelithiasis patients of various statuses. Song and colleagues demonstrated that *Klebsiella*, *Roseburia*, *Collinsella*, *Dialister*, and *Enterobacerin* at the genus level were significantly decreased, whereas *Streptococcus*, *Lactobacillus*, *Dorea*, *Romboutsia*, *Fusobacterium*, and *Megamonas* were over-represented in asymptomatic gallstone patients relative to controls[33]. Wu *et al*[9] compared the gut microbiota of twenty-nine patients with gallbladder stones and thirty-eight healthy controls. They

found increased abundances of *Proteobacteria* and decreased abundances of *Faecalibacterium*, *Lachnospira*, and *Roseburia*; this was the first study to characterize gut microbiota dysbiosis in gallstone patients. A genome-wide search suggested an underlying link between the biliary tract core microbiome and the formation of cholesterol gallstones.

There have been attempts to utilize dysbiosis as a predictive and diagnostic tool or biomarker. Keren *et al*[34] described a significantly increased abundance of *Oscillospira* and a reduced abundance of *Roseburia* at the genus level in cholelithiasis patients compared with healthy controls. They suggested that the genera *Oscillospira* and *Roseburia* may be used in the early prediction or diagnosis of cholelithiasis as microbial biomarkers[34]. The relative abundance of the genus *Eubacterium*, which metabolizes and removes cholesterol, was lower in cholelithiasis patients. Line discriminant analysis effect size analysis showed that *Ruminococcus gnavus* predicted cholelithiasis[32]. These findings suggest that the gut microbiome is altered during cholelithiasis and that these changes may be valuable for diagnosis.

CHARACTERISTIC MICROBIOME BASED ON GALLSTONES COMPONENTS

Cholesterol gallstones

Gallstones are organic matrixes of cholesterol crystals, calcium bilirubinate, mucin, and proteins in the gallbladder or biliary tract[35]. Based on the major constituents, gallstones are classified as cholesterol gallstones (> 90%) or pigment gallstones (< 10%)[36]. Cholesterol gallstones form mixed or pure cholesterol gallstones. More than 80% of gallbladder stones are composed of pure cholesterol. Cholecystolithiasis originates primarily from the gallbladder and is composed of cholesterol or mixed gallstones, primarily cholesterol or black pigment stones. Several groups independently analyzed the human microbiome of cholelithiasis patients with different components. Bacterial diversity and several functional bacterial species of cholesterol-rich gallstones significantly decreased compared to those of pigment gallstones. These species include *Akkermansia muciniphila*, *Prevotella spp.*, *Bifidobacterium adolescentis*, *Alistipes spp.*,

Bacteroides spp., *Dorea spp.*, *Methanobacteria*, *Methanobrevibacter smithii*, *Ruminococcus spp.*, and *Faecalibacterium prausnitzii*[37]. There were various compositions of the gut microbiome in the mice in which a lithogenic diet-induced cholesterol gallstones. There was reduced richness, α diversity, the proportion of *Firmicutes*, and the ratio of *Firmicutes* to *Bacteroidetes* in the lithogenic diet group[38]. These findings suggest that alterations in the gut microbiome may play an essential role in forming cholesterol gallstones.

Helicobacter spp. were reported to participate in the formation of murine cholesterol gallstones. Mice fed a lithogenic diet and infected with various enterohepatic *Helicobacter spp.* showed a significantly higher prevalence of cholesterol gallstones than uninfected controls[39]. In humans, a retrospective cohort study demonstrated that patients with gallstone disease were at increased risk of *Helicobacter pylori* (*H. pylori*) infection[40].

Pigment gallstones

Pigment gallstones include brown pigment stones or black pigment stones. Brown pigment stones are associated with biliary tract infections. By contrast, black pigment stones are common in patients with hemolytic anemia, cirrhosis, and cardiac valve replacements. These findings were supported by human and animal research, which found changes in human microbiota in cholesterol gallstones. Nevertheless, there has been little attention paid to pigment gallstones. Notably, a pilot study indicated that the gallstone microbiome might participate in developing pigment stones[41]. The genera *Klebsiella* and *Enterococcus* (involved in bacterial biofilm formation) were dominant in pigment stones. These results suggest the participation of a gallstone microbiome in cholelithiasis. Kim and colleagues compared the biliary microbiota in patients with pigment common bile duct stones to other causes of biliary obstruction; there was enrichment of the genus *Enterococcus* in patients with common pigment bile duct stones[42]. These findings suggest a possible association between *Enterococcus* and pigment stone formation.

THE POTENTIAL MICROBIOTA-RELATED TRIGGERS IN CHOLELITHIASIS

The GI microbiome induces gallstone formation by regulating bile acid (BA) metabolism

Gallstones are formed when there is an imbalance of biliary cholesterol homeostasis. The primary pathophysiological defect in gallstones is the supersaturation of cholesterol in bile. Other factors include genetic factors (particularly lithogenic gene 1 and mitochondrial DNA variant), hepatic hypersecretion, gallbladder motility function obstacle, cholesteric phase transition, excessive secretion and accumulation of mucin, and excessive cholesterol[43,44]. Neutrophil extracellular DNA traps are involved in human gallstone formation and growth[45]. This evidence suggests the involvement of the GI microbiome in cholelithiasis. The alteration of the host GI microbiome modulates gallbladder motility and inflammation (especially mucin content), inhibiting cholesterol cholelithogenesis. The prevalence of gallstones in germ-free mice was higher than in specific pathogen-free mice[46], suggesting an underlying role of the GI microbiome in cholesterol cholelithogenesis.

Like gastric secretions and hydrochloric acid, bile is a bactericidal agent in the GI system[47]. The human gut microbiome is highly capable of transforming BAs. The oxidation of 3α-, 7α-, or 12α-hydroxyl groups on the steroid core, catalyzed by hydroxysteroid dehydrogenases, were the most prevalent BAs transformations[48]. A study found that 43 isolates of 41 species can modify human unconjugated BAs in vitro[48]. The gut microbiome chemically modifies primary BAs (*e.g.*, dehydroxylation) to affect physiology. The modified BAs are considered "secondary" BAs. The 7α-dehydroxylation of primary BAs into secondary BAs is responsible for the GI microbiota.

The genus *Clostridium* appears to be active in 7α-dehydroxylation. A study identified other strains, including *Bacteroides vulgatus*, *Bifidobacterium adolescentis*, and *Roseburia intestinalis* at the phylum level[48]. The abundance of 7α-dehydroxylating bacteria significantly increased[32] and was 42-fold higher in gallstone subjects than in gallstone-

free contrlos[49]. Antibiotic treatment significantly reduced 7α-dehydroxylation activity and cholesterol saturation[50], suggesting a possible pathogenic role for 7α-dehydroxylating bacteria. Hu *et al*[51] found that patients with cholesterol gallstone disease showed significant enrichment of *Desulfovibrionales* at the order level compared with gallstone-free controls. Following fecal transplantation from gallstone patients into mice, the mice showed a higher prevalence of gallstones; this study suggested *Desulfovibrionales* as a microbial trigger contributing to gallstone formation.

These studies elucidated the underlying mechanisms of the biliary microbiome on gallstone formation. *Desulfovibrionales* were enriched in cholelithiasis patients. The bacterial overgrowth shifts the biliary microbiome to a cholelithogenesis phenotype. The GI microbiome, rich in *Desulfovibrionales*, induces the formation of cholesterol gallstones by regulating hepatic BA metabolism in several ways[51]. First, the secondary BAs in the cecum increase with the number of 7α-dehydroxylating bacteria. Second, the biliary microbiome regulates the expression of hepatic farnesoid X receptor (FXR)-CYP7A, inhibiting synthesis. Third, a specific microbiome promotes intestinal cholesterol absorption and secretion of canalicular cholesterol into bile[51].

Lipopolysaccharide upregulates mucins via several pathways

Mucin is a glycoprotein with high molecular weight. It protects and lubricates the ducts and lumens in vivo. Mucins are classified according to their structural characteristics as secreted gel-forming mucins, soluble mucins, and trans-membrane mucins. The gel-forming mucin known as MUC5AC and the trans-membrane mucin known as the multifunctional protein MUC4 participate in cell signaling due to differential expression in normal and pathophysiological conditions. For example, the abnormal expression of MUC4 and MUC5AC was detected in biliary tract cancer, whereas it is rarely detected in healthy biliary tract[52]. Yoo *et al*[53] reported that concentrations and gene expression of MUC3 and MUC5B were significantly overexpressed in a cholesterol stone group than in normal controls. Patients with gallbladder stones were subdivided according to the density of gallstones into an isopycnic group and a calcified group. The enriched

expression of MUC4 was detected, and the proportion of bacteria (especially gram-positive bacteria) was positively associated with the expression of MUC4 in the calcification group[54]. Because of the function of MUC4 in adhesion, a high concentration of MUC4 may be beneficial for bacterial growth and subsequently modulate gallstone formation and calcification.

Mucin hypersecretion should be considered a requirement in gallstone formation[43]. Studies found that MUC5AC plays an essential role in hepatolithiasis formation and recurrence; there was increased expression of MUC5AC and MUC2 in hepatolithiasis patients. In another investigation, the effect of MUC5AC on hepatolithiasis formation was elucidated. MUC5AC was upregulated through several pathways. Lipopolysaccharide (LPS), a major surface component of the gram-negative bacteria, upregulated MUC5AC expression in biliary epithelial cells. LPS significantly upregulated the expression of prostaglandin E_2 (PGE_2). The expression of MUC5AC and MUC2 mRNA was induced by exogenous PGE_2. The agonist of EP4, a G-protein coupled receptor, significantly increased MUC2 and MUC5AC expression. $P38^{MAPK}$ mediates PGE_2/EP4-induced MUC2 and MUC5AC upregulation. PGE_2 induces MUC2 and MUC5AC expression through the EP4/p38MAPK pathway[55]. LPS promoted epidermal growth factor receptor activation by increasing the secretion of transforming growth factor-α, resulting in overexpression of MUC5AC. By contrast, the LPS-induced MUC5AC overexpression was abolished by inhibiting tumor necrosis factor-α converting enzyme activity[56]. Mucin hypersecretion in bile may result from the upregulation of MUC genes and result in a higher bile viscosity, retaining cholesterol crystals in the biliary tract[53].

β-glucuronidase and phospholipase accelerate the precipitation of calcium bilirubinate

Bacterial enzymes, including β-glucuronidase (GUS) and phospholipase (PL), which are related to bacterial proliferation and severe infections[7], contribute to cholelithogenesis. Some bacteria produce exogenous GUS, including *Escherichia coli* and *Salmonella enterica*. GUS induces the hydrolysis of bilirubin diglucuronides to produce unconjugated

bilirubin, resulting in the precipitation of calcium bilirubinate[57]. PL hydrolyzes lecithin to water-insoluble free fatty acids and lysophospholipids, enhancing the precipitation of calcium salts and mucin secretion from the biliary epithelium[58]. Nearly one-third of the cultured strains of cholesterol gallstone could secrete GUS and PLA_2[59]. GUS and PLA_2 levels in *Pseudomonas aeruginosa* (*P. aeruginosa*) strains were highest in culturable strains, suggesting that *P. aeruginosa* was involved in gallstone pathogenesis.

Helicobacter species induce gallstone formation by precipitating calcium

In addition to Desulfovibrionales, *Helicobacter spp.* are essential mediators during gallstone formation. *Helicobacter* infections (especially with *H. pylori*) positively correlate with the prevalence of chronic cholecystitis and cholelithiasis[60]. *H. pylori* is the primary pathogenic agent of chronic gastritis, gastric ulcer, and gastric cancer.

Helicobacter spp. play a unique role in the formation of murine cholesterol gallstones. Belzer *et al*[61] identified an underlying mechanism of gallstones by testing the ability of different *Helicobacter spp.* to precipitate calcium. Urease-positive *Helicobacter spp.* precipitate calcium, while urease-negative *Helicobacter* species cannot[61]. This finding suggests that gallstone formation may be induced by *Helicobacter spp.*, which precipitate calcium directly *via* urease activity. Nevertheless, there is scant evidence to support causality in mechanisms for the GI microbiome contributing to cholelithogenesis as described above (Figure 1), and the mechanistic links between pathobionts and cholelithiasis formation require further exploration.

INFLUENCE OF HOST FACTORS ON THE MICROBIOME ASSOCIATED WITH CHOLELITHIASIS

Diet and lifestyle

The interactions and mutual influences between diet and GI microbiota are well known. Nevertheless, there is little information about the interactions between diet and biliary microbiota. The relationship between diet, biliary microbiota, and cholelithiasis is intricate. A case-control study compared the diet and biliary microbiota of patients and

healthy people with cholelithiasis to identify potential associations. The authors found that the intake of dairy products was inversely associated with the relative abundance of the phylum *Bacteroidetes*, the family *Bacteroidaceae*, and the genus *Bacteroides* in bile. In contrast, seafood and meats were positively associated with the relative abundance of the family *Pasteurellaceae*[62].

Age

Epidemiological studies indicated that age, gender, pregnancy, rapid weight loss, excessive obesity, and diabetes are the primary risk factors for gallstones[63]. The prevalence of gallstone disease progressively increases with age[35]. Age impacts the composition of the human microbiome, potentially *via* the influence of health conditions, medication use, and lifestyle factors[64]. A retrospective study investigated positive bile samples from patients with biliopancreatic system diseases and found that age was positively associated with gram-negative bacterial infections and negatively related to gram-positive bacterial infections in bile[65].

History of cholecystectomy

Guidelines recommend endoscopic sphincterotomy (EST) and stone extraction for treating bile duct stones patients and cholecystectomy as the first-line treatment of symptomatic cholelithiasis[66,67]. Cholecystectomy alters the communication between the bile and intestine, altering the BA metabolism pathway and the intestinal microbiota. Several studies explored the effect of cholecystectomy on the GI microbiota. One study compared the composition of gut microbiota before and after cholecystectomy. Post-cholecystectomy patients showed significant enrichment in the phylum *Bacteroidetes*[34] and a reduction in the genus *Faecalibacterium*[68]. Other studies compared the gut microbiota of volunteers with and without cholecystectomy. Compared to controls, post-cholecystectomy patients had a lower relative abundance of the genera *Prevotella*, *Desulfovibrio*, *Barnesiella*, *Paludibacter*, and *Alistipes*[69] and a higher relative abundance of the *Blautia obeum* and *Veillonella parvula*, which are members of the phylum

Firmicutes[70]. Reductions of *Candida albicans* and enrichments of *Candida glabrata* and *Aspergillus unassigned* were also reported[71]. These findings suggest that cholecystectomy affects the GI microbiota.

History of EST

EST is an invasive procedure recommended for treating bile duct stones. When comparing the biliary microbial composition of choledocholithiasis patients with or without a history of EST, significant differences were found between these groups; the genus *Pyramidobacter* showed positive associations with previous EST[72]. After EST, sphincter of Oddi laxity (SOL) resulted in duodenal content flow into the bile duct, altering the biliary microbiome. In two case-control studies, cholelithiasis patients with and without SOL significantly differed in the biliary microbiome[73, 74]. Compared with those without SOL, cholangiolithiasis patients with SOL had increased phylotypes of family, including *Desulfovibrionaceae* and *Shewanellaceae*, and a larger abundance of *Bilophila* and *Shewanella algae*[73]. Compared with those without SOL, there was an enrichment of *Rhizobiaceae* in choledocholithiasis patients with SOL[74]. These findings suggest that SOL after EST plays a pivotal role in the bile duct microenvironment of cholelithiasis patients.

THE POTENTIAL MICROBIOME PREVENTIVE TARGETS IN CHOLELITHIASIS

Management of cholelithiasis depends on the gallstone location. Patients with gallbladder stones are usually treated with cholecystectomy and medical dissolution, whereas patients with extrahepatic bile duct stones are usually treated with EST or endoscopic papillary balloon dilation. Despite many strategies for cholelithiasis, efficacious methods of prevention are still needed. Oral administration of ursodeoxycholic acid, statins, and ezetimibe can prevent gallstones[75]. Given the cost-benefit ratios, oral administration of ursodeoxycholic acid or statins is not recommended for cholelithiasis prevention [36]. In recent years, the GI microbiome has emerged as one of the critical regulators of gallstone formation. Therefore, regulation of

the host GI microbiome might be a method to prevent cholelithiasis. Some prevention strategies might include *Lactobacilli*, nanoscale iron sulfide (nFeS), and Astragalus polysaccharide.

Lactobacillus

Lactobacillus species are among the most common probiotics and are associated with health benefits, including antimicrobial activity and tumor suppression[76]. Indeed, many intervention studies using *Lactobacillus* showed promising results[77], including on cholesterol, triglyceride, and low-density lipoprotein levels[78]. Investigators demonstrated the preventive effects of *Lactobacillus* on gallstone formation in a murine model based on the probiotic's hypocholesterolemic properties. The mechanism by which *Lactobacillus* targets the GI microbiome and attenuates cholesterol gallstones was elucidated. Oh and colleagues found that *Lactobacillus acidophilus* ATCC 43121 had a hypocholesterolemic effect by decreasing the expression of 3-hydroxy-3-methylglutaryl-coenzyme A reductase in the liver, which in turn reduces the expression of MUC5AC and MUC5B in the gallbladder[79]. Ye and colleagues found that *Lactobacillus reuteri* CGMCC 17942 and *Lactobacillus plantarum* CGMCC 14407 contribute to BA redistribution through the activation of the FXR pathway[80]. These findings suggest that supplementation with *Lactobacilli* might prevent cholesterol gallstone formation. However, further clinical trials are needed to develop this approach as a probiotic supplement to prevent human cholelithiasis.

nFeS

Antimicrobial clinical management with bacteria or bacterial biofilms may benefit patients with gallbladder stones; nFeS is a nanomaterial with high levels of antibacterial efficacy. The evidence suggests that oral administration of nFeS supernatants significantly reduces bacterial activity and disrupts biofilm structure, inhibiting gallstone formation[81]. In a mouse model of cholelithiasis, oral administration of nFeS supernatants resulted in approximately twice the antibacterial efficacy of oral

ciprofloxacin. Moreover, nFeS significantly cleared gallbladder stones compared with controls.

Astragalus polysaccharide

Astragalus polysaccharide is a natural macromolecule extracted from a standard traditional Chinese medicine known as the "Astragalus," which has immunomodulatory, anti-inflammatory, and anti-cancer effects in several diseases, including kidney stones[82], ulcerative colitis[83], constipation[84], and lung adenocarcinoma[85]. A recent study reported that Astragalus polysaccharide had beneficial effects on ameliorating the formation of cholesterol gallstones and reversing GI dysbiosis in mice[84]. Gallstone mice with Astragalus polysaccharide supplementation had a higher relative abundance of *Bacteroidota* and a lower relative abundance of *Vemucomicrobiota* at the phylum level compared to controls. These results suggest that Astragalus inhibits gallstone formation by improving intestinal microbial diversity.

CONCLUSION

These findings suggest a significant alteration of the GI microbiome in cholelithiasis patients. The GI microbiome is involved in the pathogenesis of cholelithiasis through several pathways: biliary microbiome induces gallstone formation by regulating BA metabolism; *Helicobacter* species induce gallstone formation by precipitating calcium; LPS upregulates mucins *via* the tumor necrosis factor-α converting enzyme/transforming growth factor-α/epidermal growth factor receptor pathway and the EP4/p38MAPK pathway; GUS and PL accelerate precipitation of calcium bilirubinate. Nevertheless, the mechanisms for the GI microbiome contributing to cholelithogenesis lack evidence to support causality. The composition of the GI microbiome could be regulated in individuals with cholelithiasis by surgery, SOL, age, diet, and lifestyle. These modifiable factors for cholelithiasis may be crucial to prevent cholelithiasis. Given the regulability of the GI microbiota, studies should explore

microbiome-targeting interventions for preventing cholelithiasis, including *Lactobacilli*, nFeS, and Astragalus polysaccharide.

Studies on cholelithiasis in the GI microbiome are mostly single-omics types, whereas multi-omics studies, including genome, epigenome, transcriptome, proteomics, and metabolomics, are limited. The field of the human microbiome in cholelithiasis is relatively young and limited to the bacterial microbiome (*i.e.*, there is no study of the mycobiome and virome). Existing studies are heterogeneous, possibly due to the influence of disease states, disease types, sample types and sites, gallstone components, and medical intervention. They fail accurately to characterize the structural, functional, and metabolic features of the GI microbiomes in cholelithiasis, and most lack validation cohorts. Moreover, these results do not test the specific strains, functional genes, and metabolites identified by screening validation in vitro, preventing in-depth studies on the pathogenesis of cholelithiasis. Consequently, longitudinal human intervention and in-depth analysis of the mechanism are needed to address the critical question of causality. If host-microbiome interactions are to be targeted as pathogenesis of cholelithiasis, well-designed mechanistic studies of the interactions between the GI microbiome and host are required. Such studies might identify causality between the GI microbiome and gallstone formation.

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