Review

Gastrointestinal Microbial Involvement in Gallstone Formation: A Systematic Review

Sirui Wan [1,†], Kun Xia [1,†], Yunpeng Liu [1,2,3], and Hongzhi Xu [1,2,3,*]

[1] Department of Gastroenterology, The National Key Clinical Specialty, Zhongshan Hospital of Xiamen University, School of Medicine, Xiamen University, Xiamen 361004, China
[2] Clinical Research Center for Gut Microbiota and Digestive Diseases of Fujian Province, Xiamen Key Laboratory of Intestinal Microbiome and Human Health, Xiamen 361004, China
[3] Department of Digestive Disease, Institute for Microbial Ecology, School of Medicine, Xiamen University, Xiamen 361004, China
* Correspondence: xuhongzhi@xmu.edu.cn
† These authors contributed equally to this work.
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Abstract: Cholelithiasis has been emerging as a pressing public health concern, entailing substantial economic burdens and oncological risks. Currently, there is a lack of effective primary and secondary prevention measures for gallstones. The etiology of gallstone formation is multifactorial, involving genetic predispositions and environmental factors. The primary pathophysiological abnormalities in gallstone formation include aberrant metabolism and secretion of cholesterol and bile acids. Cholesterol oversaturation in gallbladder bile serves as a critical precursor to gallstone pathogenesis, although the underlying mechanisms remain incompletely elucidated. Recent studies underscore the influence of gut microbiota on bile acid metabolism and gallstone formation. A notable correlation between salivary and gut microbial compositions suggests a potential 'Gum-Gut Axis', where translocate salivary microbes may contribute to lithogenic effects. Moreover, similarities between the microbial profiles of the biliary and duodenal regions could facilitate gallstone formation through a variety of biochemical pathways, involving β-G phospholipases, bacterial hydrolases, mucins, prostaglandins, oxygen free radicals, hydroxy sterols, LPS and metabolic shifts. This review aims to summarize the findings of correlation between gallstone pathogenesis and microbes of the digestive tract, potentially providing novel preventative or therapeutic approaches for Cholelithiasis.

Keywords: cholelithiasis; biliary microbes; intestinal microbes; oral microbes

1. Introduction

Cholelithiasis is one of the high-morbidity diseases worldwide, characterized by biliary calculus formation. It is categorized into cholesterol, pigment (black and brown), and mixed gallstones based on composition and appearance, with cholesterol gallstones being the most prevalent type (70%), primarily composed of cholesterol crystals. In Western nations, particularly Europe and the United States, the adult prevalence of cholelithiasis ranges from 10% to 15%, predominantly featuring cholesterol stones [1,2]. In contrast, China has witnessed an increasing incidence of cholelithiasis during the past decades, driven by rapid economic growth, demographic aging, and a shift towards Western lifestyle and dietary patterns. Current epidemiological data suggests that the prevalence in China stands at 11%, with higher rates in western China where bile pigment stones are more common [3]. Previous studies have shown that the occurrence and development of biliary diseases are related to genetics and environment. For example, Katsika et al. conducted a correlation analysis on 43,141 pairs of twins with gallstones and identified genetic factors that contribute to susceptibility to gallstones [4]. Variants of adenosine triphosphate binding cassette transporters G5 and G8 (ABCG5-R50 C and ABCG8-D19 H) are associated with cholelithiasis [5]. In addition, high-fat diet, medication, obesity, and adverse environmental factors can also increase the risk of biliary tract diseases [6].

Patients with gallstones exhibit significant dysbiosis of the digestive tract microbiota [7] (Table 1). Previous studies have found that fecal microbiota transplantation (FMT) from patients with gallbladder stones into germ-free mice can promote gallstone formation by modulating the composition of bile acids (BAs) and cholesterol

metabolism [8]. This indicates that the gut microbiota plays a key role in the formation of gallbladder stones. Oral microbiota can concurrently alter the expression of cholecystic mucin genes (specifically mucin-1, mucin-3, and mucin-4) through immune regulation, thereby promoting mucin gel accumulation and facilitating gallstone formation [9]. There is a notable congruence between biliary and duodenal microbiota, with approximately 70% of bacterial communities implicated in gallstone formation also detected in duodenal flora [10]. This overlap supports the hypothesis that biliary bacteria may originate from duodenum-bile reflux, enhancing the upward reflux of intestinal flora and altering the biliary microenvironment. Moreover, biliary microbial communities possess intrinsic self-protection mechanisms that enable their long-term survival within the biliary tract. These communities trigger chronic inflammation and, through microbial metabolic byproducts such as mucin and phospholipase, contribute to the pathogenesis gallstone formation [11–14].

Table 1. Changes of the gastrointestinal microbiome in cholelithiasis.

Studies	Subjects	Samples	Phylum	Family	Genus
Lyu 2021 [15]	15 CBDS and 4 HC	Bile	↓ Actinobacteria ↓ Saccharibacteria		↑ *Clostridiumsensu_stricto* ↑ *Lachnospiraceae_UCG-008*, ↑ *Butyrivibrio*, ↑ *Roseburia* *Brevundimonas* ↑ *Prevotella*_1
Lee 2023 [16]	17 CBDS and 8 HC	Bile		↑ *Enterococcaceae*	↑ *Enterococci*
Dai 2023 [17]	8 CBDS and 18 HC	Bile	↑ Firmicutes		↑ *Pyramidobacter*
Hu 2022 [8]	80 GS and 49 HC	Feces			↑ *Desulfovibrionales*
Molinero 2019 [7]	14 GS and HC	Bile		↑ Bacteroidaceae, ↑ Prevotellaceae, ↑ Porphyromonadaceae, ↑ Veillonellaceae	↑ *Bacteroides*, ↑ *Dialister* ↓ *Bradyrhizobium*, ↓ *Methylobacterium*, ↓ *Sphingom*, ↓ *Acidibacter* ↓ *Brevundimonas*
Song 2022 [18]	30 GS and 30 HC	Feces	↑ Firmicutes ↓ Bacteroidetes ↓ Proteobacteria		↓ *Sutterella*, ↓ GCA-900066755, ↓ *Butyricicoccus*, ↓ unclass i fied_O_*Lactobacillales*, ↓ *Lachnospiraceae*_ND3007_group ↑ *Megamonas*, ↑ *Comamonas*, ↑ *Coprobacillus*, ↑ *Adlercreutzia*, ↑ unclassified_P_*Firmicutes*, ↑ *Morganella*, ↑ CHKCI002 *Tyzzerella*_4
Wang 2020 [19]	30 GS and 30 HC	Feces	↓ Firmicutes		↑ *Rhodococus*, ↑ *Treponema*_2, ↑ *Wolbachia*, ↑ *Ochrobactrum*, ↑ *Rubus_Hybrid_Cultiva*, ↑ *Ruminicostridium*_9, ↑ *Eisenbergiella*
Wu 2013 [10]	29 GS and 38 HC	Feces	↑ Proteobacteria ↓ Faecalibacterium ↓ Lachnospira ↓ Roseburia		

↑ The relative abundance of bacteria was increased. ↓ The relative abundance of bacteria was decreased. CBDS: common bile duct stones; GS: gallstone HC: healthy control.

Although numerous studies have demonstrated the crucial role of gut microbiota dysbiosis in the pathogenesis of gallstones, our understanding of the biliary microbiota, including its functional complexity, remains limited, especially in cholelithiasis. Progress in this research area is primarily limited by the difficulty in obtaining bile and gallbladder mucosa samples, which currently rely on invasive procedures such as endoscopic retrograde cholangiopancreatography or laparoscopic cholecystectomy. These limitations result in a severe shortage of samples, thereby hindering the conduct of systematic studies. Future research should aim to conduct metagenomic sequencing and metabolomics analysis of large-scale bile, fecal, and saliva samples to minimize the confounding effects of diet and lifestyle on the biliary microbiota. This will help more accurately identify the bile microbiota (or biomarkers) associated with gallstone formation and the metabolic activities of the microbiota at multiple sites in the digestive tract. Simultaneously, mouse experiments should be conducted to explore further the mechanisms by which lithogenic microbiota play a role in the formation of gallstones. A more in-depth exploration of the relationship between the digestive tract microbiota and gallstone formation may provide new strategies (such as probiotics and fecal microbiota transplantation, FMT) for the prevention of gallstone disease.

2. Literature Search and Selection

We searched PubMed, Embase, and Web of Science for studies published up to February 2025. The keywords included "gallstones," "gut microbiota," "biliary microbiome," "oral microbiome," and "microbial metabolites". The inclusion criteria were as follows: (1) case-control studies; (2) patients with cholelithiasis and healthy control subjects; (3) studies that performed sequencing of gut microbiota, biliary microbiota, and oral microbiota and reported the relative abundance of these microbiota. The exclusion criteria were: case reports and conference abstracts.

3. Composition and Origin of the Biliary Microbiome

3.1. Existence of the Biliary Microbiome

Contrary to previous assumptions that healthy bile ducts are sterile, recent studies have demonstrated the presence of bacterial communities in these ducts, similar to those in the intestine [20–22]. This microecological system can be disrupted by pathogenic factors, resulting in shifts in dominant microbiota, reducing microbial diversity and metabolic alterations, all of which contribute to the occurrence of cholelithiasis. Advanced techniques, such as 16sRNA gene sequencing and whole metagenome sequencing, can confirm the biliary microbiota in patients with cholelithiasis [21,22]. Moreover, scanning electron microscopy has uncovered bacterial colonization and biofilm formation on the surfaces of gallstones, indicating their potential impact on the clinical severity of cholelithiasis [23].

Furthermore, the detection of bacterial DNA in the bile of patients with cholelithiasis has revealed the presence of bacteria. Culture isolation rates from bile show a variation between pigment stones (53% to 100%) and cholesterol stones (9% to 34%), indicating a differential bacterial prevalence associated with the type of gallstone [24].

3.2. Composition of the Biliary Microbiome

Recent research has validated that the biliary ecosystem harbors a diverse microbiota [17,25,26]. Emerging evidence indicated that even ostensibly healthy individuals possessed a complex microbiota within their biliary tracts [27,28]. Various studies have identified dominant bacterial phyla in the bile of healthy subjects, including *Firmicutes, Bacteroidetes, Actinobacteria*, and *Ascomycota*, complemented by smaller proportions of archaea, eukaryotes, and viruses. Notably, microbial diversity within the biliary systems of healthy individuals is significantly greater than that observed in patients with cholelithiasis. Therefore, a detailed characterization of the biliary flora within healthy cohorts—encompassing bile ducts, gallbladder, and bile—remains a critical area for further investigation. In a methodologically rigorous study utilizing 16sRNA gene sequencing, a comparative analysis of bile samples from cholelithiasis patients and live donors (serving as controls) revealed an overrepresentation of sequences from families such as *Bacteroidetes, Prevotella, Porphyromonas*, and *Verrucomicrobiaceae* in the bile of cholelithiasis patients, whereas sequences from *Propionibacteriaceae* predominated in the bile of control samples [7].

Subsequent analyses employing 16sRNA sequencing of bile samples from cholithiasis patients and non-cholelithiasis controls demonstrated that microbial alpha diversity was significantly greater in the control group ($p < 0.01$). Conversely, the taxonomic profile highlighted an increased abundance of *Enterococcaceae* and *Enterococci* in the bile from patients with common bile duct stones (CBDS) [16].

Further sequencing efforts on biliary tract samples also provided additional insights. Notably, a higher relative abundance of *Calcitonellaceae* was detected in cholelithiasis patients compared to controls. Moreover, bacterial diversity was significantly reduced in the pigment bile duct stone group compared to the cholesterol bile duct stone group, with notable declines in families such as *Propionibacteriaceae, Sphingomonasceae,* and *Lactobacillaceae* [29]. Liu and his colleagues also identified an association between the relative abundance of six bacterial taxa—*Actinobacteria, Actinobacteriota, Staphylococcales, Micrococcales, Altererythrobacter,* and *Carnobacteriaceae* and the recurrence of primary choledochal stones. The relative abundance of the *Actinobacteria* phylum was highlighted as a particularly specific and sensitive prognostic indicator for the progression and recurrence of choledochal stones [30].

3.3. Origin of the Biliary Microbiome in Patients with Gallstone

A comprehensive analysis utilizing high throughput 16sRNA gene sequencing to examine the microbial diversity in fifteen patients with primary CBDS and four controls without biliary pathology has revealed significant correlations. Remarkably, the alpha diversity of microbiota in both bile and intestinal fluids exhibited striking

similarities; while beta diversity showed notable disparities, the major microbial groups were consistently enriched [15]. A study explored bacterial community compositions across multiple anatomical sites—including the biliary tract, duodenum, stomach, and oral cavity—in six patients with gallstones [31]. These findings indicated that the biliary microbiota closely resembled the duodenal microbiota but with lower diversity [31]. Additionally, patients with choledochal stones, particularly recurrent cases, displayed enrichment of *Enterococcus spp.* and *Klebsiella spp.* in both the biliary tract and duodenal mucosa. Supporting this, Q. Liu et al. confirmed that *Actinobacteriota*—a key phylum essential for intestinal homeostasis and a significant part of the intestinal microbiota—was ubiquitously distributed in the biliary microenvironment of patients with choledocholithiasis, with its relative abundance serving as an independent prognostic marker for recurrence [30]. Another study employing 16sRNA analysis identified increased gallstone-associated bacterial communities in patients with sphincter of Oddi dysfunction, suggesting a duodenal origin for biliary microbiota [32]. In short, these findings propose a plausible mechanism whereby the biliary microbiota may originate from the duodenal, potentially influencing the pathogenesis and recurrence of biliary tract diseases.

3.4. Biliary Environment and Biliary Microbiota

3.4.1. Antimicrobial Action in the Biliary System

The biliary system has sophisticated antimicrobial mechanisms that encompass the functional barrier of the sphincter of Oddi, the antimicrobial properties of bile salts, and a robust immune defense system [11]. The sphincter of Oddi modulates biliary tract pressure by controlling the secretion of pancreatic fluid and bile, reducing duodenal-biliary reflux. BAs, integral constituents of bile salts, serve as an anti-inflammatory role primarily by activating the farnesol X receptor (FXR) in dendritic cells and macrophages. The activation of FXR will inhibit TLR4 antibody-dependent pro-inflammatory factors and prevent the initiation of Receptor family pyrin domain-containing 3 (NLRP3) inflammatory vesicles [12]. Moreover, antimicrobial peptides like human β-defensin-1 and human β-defensin-2 are widely expressed in the intrahepatic biliary system, further enhancing its defenses [13].

Recent investigations have unveiled that propionate, a prominent metabolite produced by the intestinal microbiota, exerts its effects through interaction with short-chain free fatty acid receptor. This interaction activates the TRPM5 signaling cascade in gallbladder tuft cells, leading to cysteinyl leukotrienes and acetylcholine efflux. Cysteinyl leukotrienes enhance gallbladder contractions by engaging the Cysteinyl Leukotriene Receptor 1 (CysLTR1). Acetylcholine can stimulate mucus secretion from cholangiocytes via muscarinic cholinergic receptors M3, thereby reducing the diffusion of acetylcholine into the smooth muscle of the gallbladder and preventing microbial colonization. This intricate innate defense mechanism, initiated by propionate-mediated activation of gallbladder tuft cells, aids in promoting gallbladder evacuation and constriction, effectively acting as a defense against the invasion of intraluminal microbes [14].

3.4.2. Mechanisms of Tolerance in the Biliary Microbiota

Bile bacteria can produce lipopolysaccharides, which can enhance bacterial resistance to bile. The expression of multidrug resistance (MDR) efflux pump proteins by biliary bacteria confers an advantage for their survival in the biliary environment. Meanwhile, bile salt hydrolase (BSH) produced by bile bacteria reduces bile toxicity by binding to bile salts. Recent studies have confirmed the widespread presence of MDR and BSH genes within the biliary microbiome [8,33–35]. Additionally, the protein EnvZ has emerged as an innovative sensor of BAs within bile bacteria, and its affiliated two-component signaling paradigm is pivotal in orchestrating bacterial BAs tolerance [36].

4. Biliary Microbes and Gallstone Formation

4.1. The Role of the Biliary Microbes in the Pathogenesis of Pigmented Gallstones

Chronic inflammation of the biliary tract has been proved to be a critical risk factor predisposing to formation of choledochal stones [37]. A strong correlation was found between the presence of Helicobacter DNA in the gallbladder epithelium and histologic cholecystitis [37,38]. Chronic biliary bacterial infection may cause hypersecretion of inflammatory cytokines and sequential overactivated immune responses of biliary mucosa [39,40]. Furthermore, chronic biliary inflammatory damages the epithelium, leading to edematous and thickened bile duct walls and luminal narrowing, which create conditions that promote stone formation.

Following biliary tract infections, the activity of pivotal enzymes in gallbladder mucosal cells is reduced, and the level of oxidative stress is elevated. Oxidative stress increases the levels of conjugated dienes, lipid

hydroperoxides, and oxidized lipids in the biliary tract, along with depletion of glutathione and its coenzymes, which may disrupt the balance between the absorption and secretion of bile components by the gallbladder, ultimately leading to an increased risk of bile supersaturation and, consequently, the formation of gallstones [41]. During inflammation, neutrophils can produce reactive oxygen metabolites (ROMs), which promote pigment stone formation by reducing cholesterol solubilization and nucleation phases via hydroxyl radical-induced lipid peroxidation and by enhancing the secretion of glycoproteins in the gallbladder [42–44]. Interestingly, oxidative stress can affect gene expression. It has been shown that oxidative stress (quantified by 8-OH-dG levels) is higher in the plasma and DNA of patients with cholelithiasis and cholecystitis. A significant downregulation of the expression of key genes in the base excision repair (BER) pathway was also observed [41], suggesting its potential as a therapeutic target.

The β-glucosidase (β-G) produced by *E. coli* can hydrolyze bound bilirubin into free bilirubin and glucuronic acid, and free bilirubin can combine with calcium ions in bile to form calcium bilirubin, which is the main component of bile pigment stones.[45]. Notably, bacterial enzymes such as phospholipase C (PLC) and lipase play a pivotal role of hydrolyzing phosphatidylcholine (PC) to fatty acids. Predominant bacterial species like *Clostridium perfringens* and *Serratia marcescens*, known for their potent PLC activity, catalyze this process. Phospholipases produced by biliary bacteria can hydrolyze PC, releasing large amounts of saturated and unsaturated fatty acids and hemolysins. Free fatty acids (FFA) are essential components of the biliary phospholipid system (BPS), with saturated fatty acids like palmitic acid capable of binding with ionized calcium to form calcium palmitate- a critical component of BPS. Moreover, lysophospholipids and polyunsaturated fatty acids play a role in regulating gallbladder mucin synthesis and subsequent secretion via the prostaglandin signaling pathway [46]. Moreover, dysbiosis of the biliary microbiota can also alter the metabolite profile in bile. It has been shown that several genera such as *Lachnospiraceae_UCG-008, Butyrivibrio* and *Roseburia*, known as producers of short-chain fatty acids, exhibit a statistically significant decrease in the bile of cholelithiasis patients ($p < 0.05$) [15]. Metabolic profiling has identified increased levels of acetate, formate and asparagine in patients with choledocholithiasis, underscoring the critical role of biliary dysbiosis in altering metabolite profiles involved in CBDS development [16]. Liu and colleagues have elucidated how deviations of biliary microbial composition led to abnormal accumulations of specific metabolites through microbiomics and metabolomic analyses. These disruptions disturb calcium homeostasis in bile and culminate in the calcific accumulation with the ending of gallstone formation [30]. Several bioactive molecules such as bacterial hydrolases, mucins, oxidized cholesterol, prostaglandin E, and lipopolysaccharides are involved in the formation of brown pigment stones [47].

4.2. The Role of the Biliary Microbes in the Pathogenesis of Cholesterol Gallstones

Lipopolysaccharides (LPS) stimulate the expression of cyclooxygenas-2 (COX-2) isozymes, leading to the synthesis of PGE2, which subsequently enhances mucin production [48]. Mucins play a critical role in cholesterol gallstones formation; the hydrophobic domains within mucin polypeptides create an optimal environment for nucleating monohydric cholesterol in supersaturated bile [49]. Notably, as early as 1985, mucin-bilirubin interactions at the core of gallstones were proposed as a key factor in gallstone pathogenesis [50]. Recent studies examining mucin expression in gallbladder epithelium from cholesterol stone patients have revealed significant mucin-3 and mucin-5B overexpression at both the protein and transcriptional levels [51]. These findings underscore a potential link between mucin regulation and cholesterol stone biogenesis.

In cholesterol nucleation, cholesterol crystals attract considerable attention due to their role in triggering inflammatory vesicles and their association with sterile inflammation across various human diseases [52]. A pivotal study showed that cholesterol crystals modulate mucin-5AC expression in vitro in a dose-dependent manner. This effect is closely linked to the activation of inflammasomes, which appears to drive both cholesterol crystallization-induced mucin excretion and gallstone formation. Notably, treatments with NLRP3 inflammasome inhibitors successfully reduced mucin-5AC expression, while an IL-1 receptor antagonist mitigated the cholesterol crystal-induced upregulation of mucin-5AC. These findings suggest that the NLRP3/IL-1β/IL-1R/MyD88 signaling pathway may contribute to the accumulation of mucin-5AC around cholesterol crystals. Consequently, NLRP3 inflammasome-driven immune responses may underlie the formation of gallstones [53].

A microbiological analysis of gallstones and bile from gallstones patients revealed that about 30% of bacterial strains isolated from cholesterol gallstones were capable of secreting β-G and phospholipase A2 (PLA2) [54]. Taxonomic analysis identified 14 bacterial genera within cholesterol stones and eight in bile, with *Pseudomonas aeruginosa* emerging as the predominant species. Notably, *Pseudomonas aeruginosa* exhibited the highest enzymatic activity for both β-G and PLA2. Further studies identified two bacterial factors, βG, and phospholipase, as significant contributors to cholesterol stone formation. Bile samples were particularly enriched in phospholipase

gene members, particularly PLA2, which catalyzes the hydrolysis of biliary phospholipids, generating lysophosphatidic lecithin and FFAs [54].

However, evidence supporting the mechanisms by which the biliary microbiota contributes to gallstone formation remains limited (Figure 1), and the specific mechanistic interactions between pathogens and gallstone development have yet to be fully elucidated.

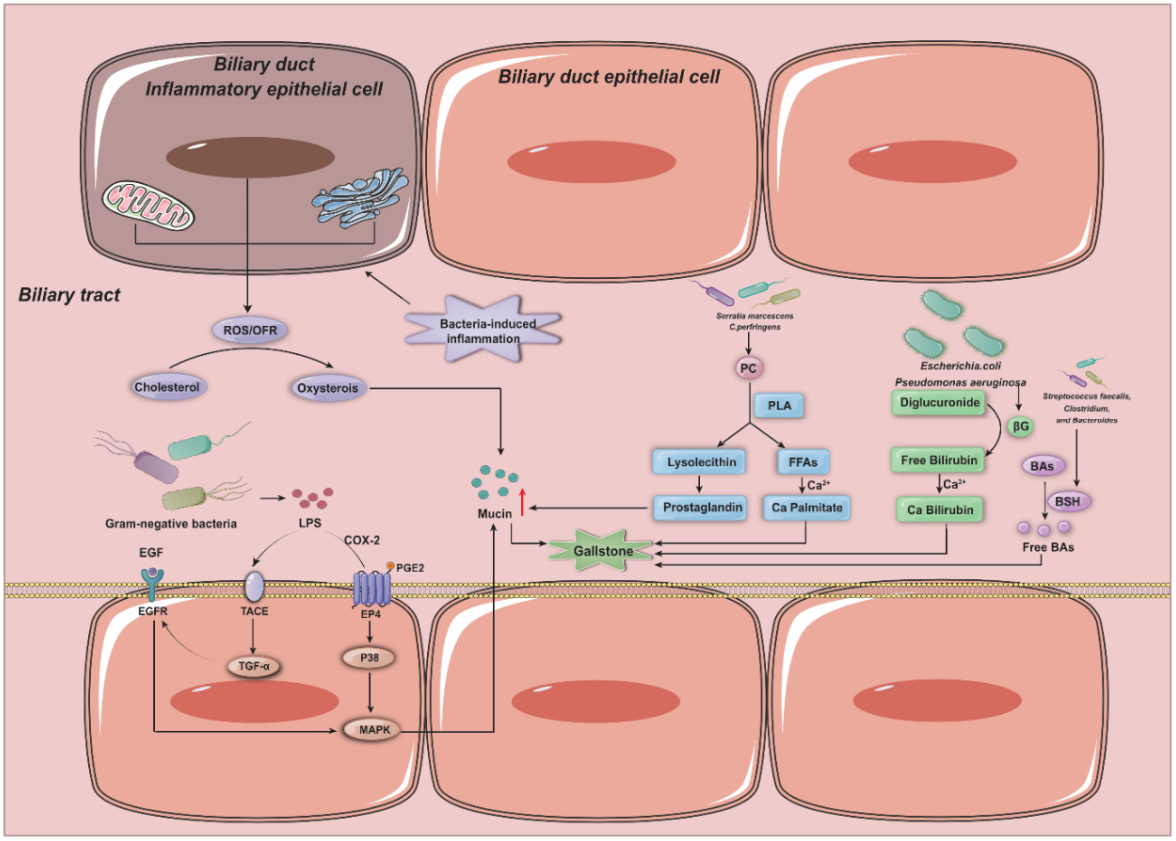

Figure 1. Proposed mechanism for the pathogenesis of gallstone by biliary bacteria: (1) LPS upregulate mucins via the TACE/TGF-α/EGFR pathway and the EP4/p38-MAPK pathway; (2) Exogenous βG production by *E. Coli* and *Pseudomonas aeruginosa* induces the hydrolysis of bilirubin bis-glucuronide, which accelerates bilirubin calcium deposition; (3) Phospholipases produced by biliary bacteria such as *C. perfringens* and *Serratia marcescenscan* hydrolyze PC, releasing large amounts of saturated and unsaturated fatty acids and hemolysins; (4) Inflammation caused by biliary tract infection is associated with phagocytic infiltration, and biliary tract inflammation stimulates gallbladder epithelial cells and phagocytes to produce OFR, which can oxidize cholesterol to various oxysterols. Oxysterols can promote mucin secretion involved in the formation of gallstones; (5) Bacteria such as *Streptococcus faecalis*, *Clostridium*, *and Bacteroides* can promote gallbladder stone formation by de-conjugating primary bound bile acids into free bile acids via BSH. LPS: Lipopolysaccharides; TACE: Tumor necrosis factor-αconverting enzyme; TGF: Transforming growth factor; EGFR: Epidermal growth factor receptor; EP4: E-prostanoid receptor 4; β-G: β-glucuronidase; PC: Phosphatidylcholine; OFR: Oxygen free radicals; BPS: Biliary phospholipid system; BSH: Bile salt hydrolase; BA: Bile acids.

5. Gut Microbiome and Cholelithiasis

5.1. Intestinal Microbiome Dysbiosis in Patients with Cholelithiasis

The insights from studying the biliary microbiota highlight the intricate interplay between microbial communities and host health. Similarly, the gut microbiota, which shares many functional parallels with the biliary microbiota, is pivotal in modulating host metabolism and immune responses. Recent research has shown that metabolites derived from gastrointestinal flora are crucial in various metabolic disorders, including obesity, diabetes, and inflammatory bowel disease (IBD). Over a century ago, Caldwell, F.T. Jr. et al. experimentally observed a lower incidence of gallstones in germ-free mice, suggesting that microbial agents may significantly influence gallstone development [55]. As the fields of microbiomics and metabolomics continue to progress, the pivotal role of intestinal microecological imbalances in gallstone formation is expected to be unravelled [19,56,57]

Intestinal dysbiosis has been identified in patients with gallstones, highlighting the potential influence of gut microflora in lithogenesis [19,56,57]. In a comprehensive analysis comparing the microbial communities in the gut, bile, and gallstones of 29 patients with cholesterol gallstone disease to 38 healthy controls, 16sRNA gene sequencing of 299,217 bacterial specimens revealed a significant increase in the abundance of *Enterobacteriaceae* and *Aspergillus* spp., alongside a notable decrease in *E. faecalis, Lachnospira,* and *Roseburia* spp. [10]. Using high-throughput 16S rRNA sequencing, Georgescu D identified shifts in functionally relevant bacterial taxa in the feces of cholesterol stones patients, observing reductions in short-chain fatty acids-specifically butyrate, lactate, and the acetate/propionate ratio-as well as decreases in methane-producing and mucin-degrading microbial clades. Concurrently, indices of microbiota biodiversity showed a significant decline, coupled with an increase in lipopolysaccharide-positive bacterial species [58]. Together, these findings support the hypothesis that microbial dysregulation may underlie the pathogenesis of cholesterol stones.

In 1966, Maki highlighted the pathophysiological impact of bacterial infections on pigmented gallstones formation [45]. Today, the microbial landscape of primary and recurrent choledocholithiasis-particularly after endoscopic retrograde cholangialpancreatography (ERCP)-remains underexplored, hindered by the limited studies and availability of samples, most of which are bile. A recent study addressed this gap by examining patients with both primary choledocholithiasis and recurrent cases post-ERCP, analyzing microbial communities in the duodenal mucosa, biliary bile and gallstones. Notably, dysbiosis in the duodenal flora was detected in patients with choledocholithiasis, with both abundance and diversity of flora significantly reduced compared to healthy controls. Additionally, microbial diversity in the duodenal tract was markedly lower in recurrent cases than in primary cases. Specific genera, such as *Aspergillus*, *Enterococcus*, and *Klebsiella* spp., were more abundant in patients with choledocholithiasis than in healthy individuals [59–61].

5.2. Gut Microbiome and Asymptomatic Cholelithiasis

In asymptomatic cholelithiasis, a study using 16S rRNA sequencing of gut samples revealed notable microbiota differences compared to healthy controls. While microbial abundance was higher in patients with asymptomatic gallstones, their microbial diversity was reduced. Both groups shared major phyla, including Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria; however, 15 genera showed significant differences in abundance [18]. Another study found no significant microbiota changes in asymptomatic cases [62]. We propose that this discrepancy may be attributed to the following reasons. On the one hand, the heterogeneity of the host factors is a crucial aspect. Asymptomatic cholelithiasis patients may have different subtypes, such as variations in stone composition (cholesterol versus pigment stones) or differences in metabolic status (such as obesity and diabetes), all of which may influence the composition of the gut flora. Different distributions of patient subtypes across studies may lead to inconsistent findings regarding microbiota variation. On the other hand, the dynamic changes within the gut microbiota itself also play an important role. The gut microbiota is subject to fluctuations influenced by factors such as time, diet, and medication use. The time of sample collection differs between studies (e.g., morning versus evening, or before versus after meals) and the patients' diet and antibiotic use prior to sample collection differ may affect the stability and reproducibility of the results. Thus, it is crucial to emphasize the necessity for more comprehensive and in-depth microecological investigations in this specific population.

5.3. The Role of the Gut Microbes in the Pathogenesis of Gallstones

Aberrant cholesterol and BAs metabolism and secretion are recognized as significant pathophysiological factors in cholesterol stones formation [63]. Extensive research indicates that intestinal microbes harbor multiple enzymes that can influence BAs biochemistry [64].

Gut microbial communities can promote cholesterol stone formation through various biochemical processes, including BAs hydrolysis, cleavage of aromatic rings, enzymatic release of BAs complexes, and synthesis of free BAs [65]. Additionally, the gut microbes play a regulatory role in modulating the host's BAs pool. Significant differences in BAs pool composition have been observed between animals treated with antibiotics or raised in sterile conditions and those in conventional environments [66].

Gut microbes also impact BAs homeostasis by modulating BAs receptor activation, altering BAs composition and pool size, managing enterohepatic BAs recirculation, and affecting intestinal cholesterol absorption [67,68]. For example, *Desulfovibrio* spp. in the gut can facilitate cholesterol stone formation by increasing secondary BAs production, enhancing BAs hydrophobicity, promoting intestinal cholesterol absorption, and upregulating hepatic expression of cholesterol transporters Abcg5/g8 [8]. A meta-analysis showed that specific probiotic strains (*Lactobacillus acidophilus*, *Lactobacillus lactis*, and *Lactobacillus plantarum*) significantly reduce serum total cholesterol levels [69]. Furthermore, a study demonstrated that bile salt hydrolase (BSH)-activated *Lactobacillus*

intake markedly lowered cholesterol levels in hypercholesterolemic patients, thereby reducing the risk of gallstones [70]. Certain gut flora also modulate BAs metabolism by releasing metabolites such as butyric acid and trimethylamine N-oxide (TMAO), which interact with BAs receptors (FXR, TGR5) through inflammatory signaling pathways [71]. These findings highlight a dynamic equilibrium among diet, gut microbiota, and BAs pool size/composition, where disruptions in this delicate balance may contribute to cholesterol stone development.

Beyond bile metabolism, the gut microbes may also contribute to cholesterol gallstone formation by promoting a chronic pro-inflammatory state. For instance, a study with IL-10 knockout mice fed a diet rich in saturated dairy fats demonstrated increased synthesis of taurine-conjugated bile acids, subsequently promoting *Wadsworthia* spp. growth—a bacterium potentially associated with intestinal immune inflammation. Interestingly, these effects were absent in mice maintained on a diet rich in plant-based fats, suggesting that dietary composition significantly influences the interaction between gut microbiota and systemic inflammation, potentially affecting gallstone pathogenesis [72].

While cholesterol gallstones have been extensively studied, pigment gallstones have received comparatively less attention. Some studies suggest that bacterial enzymes linked to bacterial proliferation and severe infections, such as β-G and phospholipas, may contribute to pigment gallstone formation. *E. Coli*, *Salmonella enteritidis*, and *Pseudomonas aeruginosa* produce exogenous β-G, which hydrolyzes bound bilirubin into free bilirubin and glucuronic acid. Free bilirubin can then bind with calcium ions in bile, forming calcium bilirubinate—a major component of pigment stones. Additionally, *Helicobacter pylori* may facilitate calcium precipitation through its urease activity [73].

Collectively, these findings highlight the complex role of gut microbial communities and their metabolites in the pathogenesis and development of gallstones, whether symptomatic or asymptomatic (Figure 2). This expanding body of knowledge advances scientific understanding of gallstones and lays the groundwork for future research.

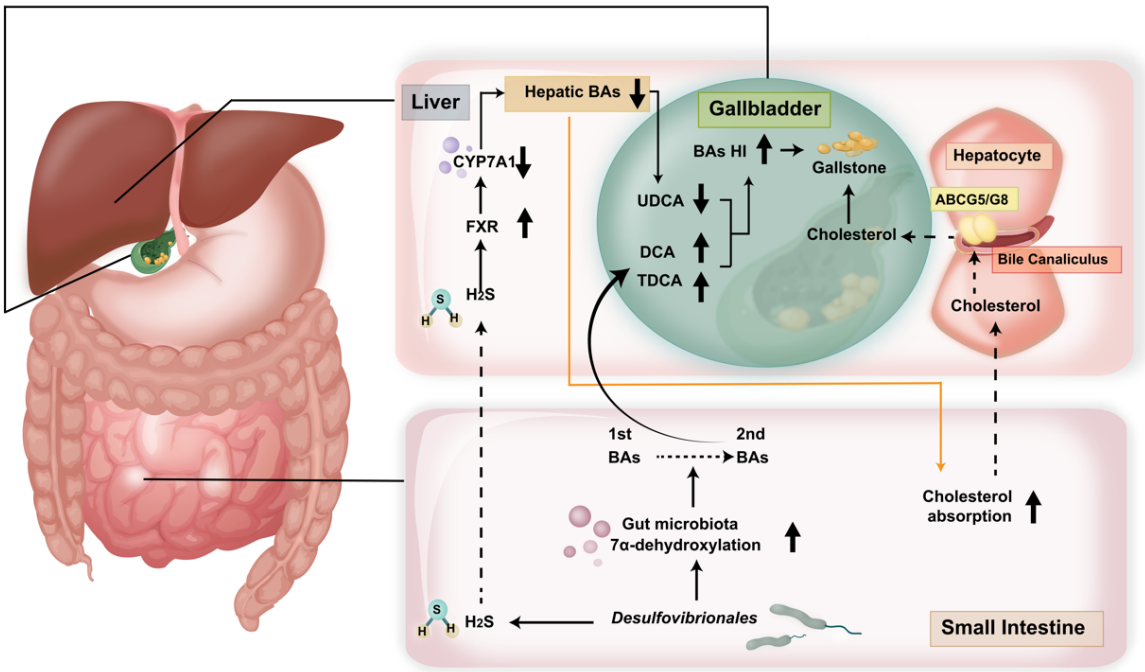

Figure 2. The gastrointestinal microbiota may drive gallstone formation through the following pathways: (1) Fecal microbiota enriched with *Desulfovibrionales* induces an increase in the activity of Bacteroides cecum 7α-dehydroxylase, which converts primary BAs to secondary BAs; (2) *Desulfovibrionales* can increase the BA hydrophobicity index through the modulation of the FXR-CYP7A1 pathway by H_2S production.; (3) Elevated BAs hydrophobicity index inhibits hepatic BAs synthesis and promotes intestinal cholesterol absorption leading to hepatic cholesterol overload, and promotes tubular cholesterol secretion into the bile, thereby inducing cholesterol stone formation. BAs: Bile acids; FXR: Farnesoid X receptor; CYP7A1: Cholesterol 7α-hydroxylase; HI: Hydrophobicity Index; UDCA: Ursodeoxycholic Acid; DCA: Deoxycholic Acid; TDCA: Taurodeoxychloic Acid.

6. Oral Microbiome and Cholelithiasis

The gut microbiota's influence on systemic health extends beyond the gastrointestinal tract, with emerging evidence suggesting a significant interaction with the oral microbiota. The oral cavity, often considered the gateway to the gut, harbors a diverse microbial community that can influence downstream gut health. Alterations in oral microbes have been closely linked to various diseases, including IBD, diabetes, bacteremia, endocarditis, cancers, autoimmune conditions, and premature delivery. Increasingly, the role of oral microbes in the onset and progression of cholelithiasis has also gained recognition [27,31,74–82].

Emerging evidence suggests a strong correlation between biliary and salivary microbes. Ye F. et al. analyzed bacterial communities from different digestive tract regions—such as the biliary tract, duodenum, stomach, and oral cavity—in a cohort of six cholelithiasis patients [31]. They identified predominant GI microflora, including *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Fusobacteria*, *Synergistetes*, and *TM7*. Notably, *Pyramidobacter* (affiliated with the phylum Zygomycetes) was primarily isolated from the oral cavity and found in bile samples, along with three genera from the Enterobacteriaceae family (*Escherichia*, *Klebsiella*, and an unidentified genus). Consistent with these findings, Shen H. et al. employed whole-genome shotgun (WGS) and 16S rRNA sequencing on bile samples from 15 Chinese patients with cholelithiasis, observing a greater abundance of oral and respiratory flora in bile than intestinal flora [75]. Supporting this, a comparative study of oral and intestinal microbiota in adults revealed shared taxonomic units, including *Streptococcus*, *Anaplasma*, and *Prevotella* [76].

In an analysis of bile samples from healthy controls (liver donors without hepatobiliary pathology) and three hepatobiliary lesion groups—gallbladder stones (GBS), choledocholithiasis (CBDS), and choledochal stenosis (SCBD)—Cai et al. identified four taxa closely linked to the oral cavity microbiome (*Lachnoanerobaculum*, *Atopobium*, *Oribacterium*, and *Stomatobaculum*) that were persistent in the lesion group but transient in the healthy cohort [27]. Notably, the abundance of these taxa showed a progressive increase with disease severity (HC→GBS→CBDS→SCBD), suggesting that bacterial translocation from the oral cavity may play a critical role in gallstone-related pathologies. This finding highlights the potential importance of oral microbial communities in hepatobiliary disease progression and emphasizes the interconnectedness of oral and biliary health.

To better understand this association, recent scientific discussions have introduced the concept of the "Gum-Gut Axis"[77], which refers to a bidirectional regulatory network between periodontal health and gastrointestinal health, emphasizing the potential impact of oral microbes and inflammatory signals on distal organs (e.g., biliary tract) through their migration along the digestive tract. Gingival sulcus fluid, traditionally thought to support oral microbes is secreted through the epithelial attachment into the subgingival space, influencing saliva composition. This fluid contains various biological elements—enzymes, effector cytokines, free-floating and keratinized cell-bound bacteria, and subsets of live inflammatory cells like neutrophils, lymphocytes, and macrophages—which may be transported to distal body sites [80]. These components alter the biochemical environment of saliva and provide a vehicle for oral microbes to traverse the digestive tract. It is worth noting that clinical studies have shown a significant reduction in the α-diversity of the gut microbiota in patients with chronic periodontitis, suggesting that oral inflammation may remotely regulate the gut microbiota through this pathway [78]. Saliva also contains a mucus matrix of water, lipids, and proteins (e.g., mucin) that forms a protective barrier against gastric acidity, facilitating microbial survival within the gastrointestinal environment [80]. This protective mechanism significantly increases the survival rate of oral bacteria in the gastrointestinal tract. Studies in apparently healthy individuals have confirmed that about 10% of the gut microbiota can be traced back to oral sources [79]. "Gum-Gut Axis" suggests a bidirectional relationship between periodontal and gastrointestinal health, and this concept also provides a new perspective on the relationship between oral microbiota and gallstones.

Oral microbiota can influence the biliary and upper GI tract microbes, potentially impacting gallstone formation. Studies show that oral flora may downregulate nitric oxide (NO) and antioxidant enzymes in the colon while increasing reactive oxygen species [81]. Pathogenic oral flora may also stimulate immuno-responses that alter cholecystokinin secretion, a critical hormone regulating gallbladder function. Experimental models using the enteroendocrine cell line STC-1 have demonstrated Toll-like receptor (TLR4, 5, and 9) expression and subsequent cholecystokinin release when exposed to corresponding receptor agonists, such as LPS, flagellin, and CpG oligodeoxynucleotides [82]. Oral microbiota may also modulate the expression of key cholecystic mucin genes (mucin-1, mucin-3, and mucin-4) via immunoregulatory pathways, with mucin gels serving as nucleating substrates essential for cholesterol gallstone formation in the gallbladder [9].

7. Prevention and Treatment Strategies for Gallstone Disease Based on Microbiota Regulation

The incidence of cholelithiasis has been steadily increasing among the Chinese population, underscoring the urgent need for effective preventive strategies despite the availability of various therapeutic options [83–86]. Recent research emphasizes the critical role of gastrointestinal (GI) microbes dysbiosis in cholelithiasis development, identifying gut flora as a key regulator in gallstone formation (Table 2).

Table 2. Gut microbiota-targeted therapeutic strategies for cholelithiasis.

Intervention Pathway	Intervention Strategy	Mechanism of Action	Study Type	Experimental Model	Representative Studies
Oral Microbial Intervention	Probiotics	Upregulate hepatic FXR receptors, inhibit β-G activity, reduce cholesterol saturation	Animal study	Mice	Ye 2022 [87]
		promote bile secretion, reduce cholesterol levels in cystic bile or hepatic bile	Animal study	Mice	Chen 2019 [88]
Gut Microbiota Remodeling	FMT	Modulate the gut microbiota to improve bile acid metabolism.	Preclinical+ Clinical study	PSC patients	Bajaj, J.S. 2019 [89] Bustamante, J.M. 2022 [90]
Metabolites	HDCA	Acts as an intestinal FXR antagonist, increasing bile acid synthesis and reducing cholesterol supersaturation	Animal study	Mice	Shen 2023 [91]
	TUDCA	Modulates gut microbiota and lipid assimilation, synergizes with high-fiber diet	Animal study	Mice	Lu 2021[92]
	DG	Inhibits intestinal cholesterol absorption and LPS-induced inflammation	Animal study	Mice	Shen 2023[93]
	Curcumin	Enhances BAs biosynthesis (activates FXR), inhibits NPC1L1-mediated cholesterol absorption	Animal study	Mice	Deng 2015 [5]
	Lysimachia christinae	Restores gut microbiota balance and secondary bile acid production	Animal study	Mice	Liu 2021 [94]; Lammert, F 2015 [95]

FXR: Farnesoid X receptor; β-G: β-glucosidase; LPS: Lipopolysaccharides; NPC1L1: Niemann-Pick C1-Like 1; BAs: bile acids; FMT: fecal microbiota transplantation; PSC: primary sclerosing cholangitis; HDCA: hyodeoxycholic acid; TUDCA: Tauroursodeoxycholic acid; DG: digoxin.

7.1. Oral Microbial Interventions

Research has shown that *Lactobacilli*, a type of probiotic bacteria, can reduce cholesterol, triglycerides, and LDL levels [96,97]. Animal models have demonstrated that oral administration of *L. reuteri* CGMCC 17942 and *L. plantarum* CGMCC 14407 can upregulate the expression of the liver FXR receptor, inhibiting the expression of the bile acid synthesis enzyme CYP7A1 [87]. These strains also restored gut microbiota balance, suggesting their potential in the management of cholesterol-based gallstones. The therapeutic application of *Lactobacillus* species may thus represent a promising strategy for gallstone management. In addition, *Lactobacilli* can reduce the incidence of gallstones in a mouse model of gallstone disease induced by a high-cholesterol diet, and it can promote bile secretion, reduce cholesterol levels in cystic bile or hepatic bile, and significantly lower serum cholesterol levels, especially low-density lipoprotein cholesterol content [88]. Supplementation with fructooligosaccharides (FOS) may be a potential method to control cholesterol gallstone syndrome by altering the composition and function of the gut microbiota. Animal studies have shown that after 8 weeks of FOS supplementation, the incidence of gallstones in a mouse model of CGS induced by a high-cholesterol diet was reduced [98]. Prebiotics such as inulin can promote the proliferation of Bifidobacterium, enhance cholesterol solubility [99]. Periodontal pathogens (e.g., Fusobacterium nucleatum) can migrate to the gut via the “Gum-Gut Axis” [98]. The sialidase they express may disrupt the integrity of the intestinal mucus layer, promoting the reabsorption of deconjugated bile acids. In a clinical study, the local application of lactoferrin significantly reduced

the levels of pro-inflammatory cytokines in gingival crevicular fluid, decreased gingival inflammation and periodontal pocket depth, and effectively inhibited the growth of various oral pathogenic bacteria [100]. Although there are currently no direct clinical studies proving that lactoferrin can reduce the incidence of gallstone disease, considering its role in oral health and the connection between oral microbiota and overall health, lactoferrin may have a positive impact on the prevention of gallstone disease by improving the oral microbial environment and reducing inflammation.

7.2. Gut Microbiota Restructuring

Existing studies have confirmed that patients with primary sclerosing cholangitis (PSC) who undergo FMT can significantly enhance the diversity of their gut microbiota. Clinical data indicate that changes in the abundance of specific bacterial genera, such as *Odoribacter*, *Alistipes*, and *Erysipelotrichaceae incertae sedis*, are significantly correlated with the reduction in serum alkaline phosphatase levels following FMT treatment [89]. A double-blind, randomized, placebo-controlled pilot trial of FMT in obese but metabolically healthy patients has found that FMT can improve BAs metabolic imbalance by increasing the abundance of specific beneficial bacteria in the gut [90]. These bacteria play a crucial role in bile acid metabolism. For instance, the abundance of certain bacterial species, such as *Clostridium hylemonae* and *Desulfovibrio fairfieldensis*, which are closely related to the 7α-dehydroxylation process of bile acids, significantly increased following FMT. These findings suggest that FMT may positively impact metabolic health by modulating the gut microbiota to improve bile acid metabolism.

7.3. Metabolites Prevent Cholesterol Gallstone by Affecting the Intestinal Microbes

Recent studies indicate that hyodeoxycholic acid (HDCA) acts as an antagonist to FXR in the intestine, leading to increased bile acid synthesis and a reduction in cholesterol stone formation in mice [91]. Tauroursodeoxycholic acid (TUDCA) has positively impacted lipid assimilation and biosynthesis in the small intestine, especially when paired with a high-fiber diet, by modifying the intestinal microbial community and reducing cholesterol stone formation [92]. Digoxin (DG) has also been found to inhibit cholesterol absorption in murine intestines and cells by altering intestinal microbial composition and mitigating lipopolysaccharide-induced effects [93]. Curcumin has also shown efficacy in preventing gallstone formation in mice by modulating intestinal microbes, enhancing bile acid biosynthesis, and inhibiting cholesterol absorption [5]. Furthermore, Lysimachia christinae has demonstrated potential to reduce cholesterol stone formation and improve intestinal microecological imbalances in mice, suggesting its therapeutic value [94,95].

These insights underscore the potential of targeting gut microbes and specific metabolites as strategies for managing and preventing cholelithiasis, highlighting an expanding area of research that integrates dietary management, microbiota balance, and targeted therapeutic interventions.

8. Conclusions and Prospects

Cholelithiasis, a growing concern in internal medicine, is increasingly recognized to be influenced by gastrointestinal microbes, with notable links between oral, gut, and biliary microbes. Microbial dysbiosis disrupts bile acid metabolism and promotes bile supersaturation, contributing to gallstone formation. Probiotics can reduce cholesterol levels and modulate bile acid metabolism, suggesting potential for gallstone prevention. Further studies are needed to clarify gut-biliary microbes migration mechanisms, the role of biliary microbes in gallstone formation, and the therapeutic potential of microbial metabolites.

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