European Review for Medical and Pharmacological Sciences 2020; 24: 2750-2775

Biliary tract microbiota: a new kid on the block of liver diseases?

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Abstract. – The microbiome plays a crucial role in maintaining the homeostasis of the organism. Recent evidence has provided novel insights for understanding the interaction between the microbiota and the host. However, the vast majority of such studies have analyzed the interactions taking place in the intestinal tract.

The biliary tree has traditionally been considered sterile under normal conditions. However, the advent of metagenomic techniques has revealed an unexpectedly rich bacterial community in the biliary tract.

Associations between specific microbiological patterns and inflammatory biliary diseases and cancer have been recently described. Hence, biliary dysbiosis may be a primary trigger in the pathogenesis of biliary diseases. In particular, recent studies have suggested that microorganisms could play a significant role in the development of gallstones, pathogenesis of autoimmune cholangiopathies and biliary carcinogenesis.

Moreover, the intimate connection between the biliary tract, liver and pancreas, could reveal hidden influences on the development of diseases of these organs.

Further studies are needed to deepen the comprehension of the influence of the biliary microbiota in human pathology. This knowledge could lead to the formulation of strategies for modulating the biliary microbiota in order to treat and prevent these pathological conditions.

Key Words:
Biliary microbiota, Gallstones, Cholelithiasis, Primary sclerosing cholangitis, Primary biliary cholangitis, Biliary tract cancer, Cholangiocarcinoma, Gallbladder carcinoma, Personalized medicine.

Introduction

An increasing number of studies about the human microbiota have dismissed the classical postulate which states that there are sterile sites within the human body[1,2]. Indeed, a resident microbiota has recently been described in several human environments previously described as devoid of microorganisms, such as the urinary tract and the stomach[3-9]. Even healthy placenta hosts microbial communities[10].

Bile has traditionally been considered sterile under normal conditions[11-14].

The physical and chemical features of bile and its antimicrobial activity were supposed to create a hostile environment for bacteria. Moreover, the difficulty in collecting bile samples, coupled with the lack of sensibility of culture techniques in detecting microbes in low-charge samples, sustained this hypothesis for a long time.

In 1967, while studying the microbial flora of patients undergoing percutaneous cholangiography, Flemma et al[15] observed that a consistent number of patients had a positive bile culture without having had any signs, symptoms or history of cholangitis. Ahead of their time, they hypothesized that bacteria could exist in bile without causing any symptoms attributable to their presence. They named this condition "asymptomatic bactibilia"[15].

About 40 years later, the advent of 16S ribosomal RNA sequencing confirmed the presence of microbes in bile samples otherwise considered sterile with culture-based techniques[16]. This knowledge has introduced the concept of "biliary microbiota".

At any level, the interplay between the microbiota and the host plays a pivotal role in the maintenance of homeostasis. However, quantitative or qualitative changes in the composition of the microbial community can derange this equilibrium, favoring the development of diseases[17].

Recent evidence has revealed rich microbial communities in the biliary tract of patients affected by biliary tract diseases. A remarkable association has been observed between certain microbi-

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al strains and each pathology. Thus, possible roles for bacteria in such pathogenic processes have been hypothesized[18-22].

The understanding of the interplay between the microbiota and the host at this level may facilitate the formulation of novel strategies for the prevention and treatment of such pathological conditions.

Overview of the Biliary System: Anatomical and Cellular Determinants for the Production and Secretion of Bile

The biliary system represents a complex network of ducts and organs that are involved in the production and transportation of bile[23]. Bile production is a complex biological process that begins in the bile canaliculi, which are formed by the apical membranes of two adjacent pericentral hepatocytes linked by tight junctions[24]. The hepatocyte apical membrane is provided with both bile salt-dependent and independent transport systems, which are series of adenosine triphosphate-binding cassette transport proteins that function as export pumps for bile salts and other organic solutes[25]. These transport systems create osmotic gradients in the bile canaliculi, which give the driving force for the flow into the lumen through aquaporins[24]. Tight junctions hold the hepatocytes together and form a physical barrier between the blood and canalicular lumen, facilitating "paracellular permeability"[24,26]. Bile canaliculi conduct the flow of bile countercurrent to the direction of the portal blood and connect with the initial branches of the biliary tree, i.e., the canals of Hering[27,28]. These structures continue into ducts that progressively increase in diameter: small bile ductules (diameter <15 μm), interlobular ducts (15-100 μm), septal ducts (100-300 μm), area ducts (300-400 μm), segmental ducts (400-800 μm), and hepatic ducts (>800 μm) as originally defined by Ludwig[23,29]. The confluence of the right and left hepatic ducts at the hepatic hilum forms the common hepatic duct that is joined by the cystic duct from the gallbladder to form the common bile duct. The common bile duct runs through the head of the pancreas and ends in the sphincter of Oddi (SO), while penetrating the duodenal wall to form the ampulla of Vater, which connects it to the pancreatic duct[30]. SO is a segment of circular and longitudinal smooth muscle that incorporates the distal common bile duct and pancreatic duct, contained in the duodenal wall[31].

Once bile is secreted into the biliary tree, it is exposed to cholangiocytes that form the lining of the bile-duct epithelium. Cholangiocytes, which are highly heterogeneous in both structure and function [23,32,33], modify bile through a sequence of secretory and absorptive processes in order to regulate its flow and alkalinity according to the physiological functions[24]. Along the biliary tree, glandular elements called peribiliary glands or accessory glands are also present[34]. Ductal secretion is regulated by a wide range of factors, including gastrointestinal hormones and cholinergic nerves[35]. The final secretory product is delivered to the gallbladder and then to the duodenum. Although the gallbladder is not essential for the secretion of bile, it helps its storage to prepare for fat digestion[30]. During fasting, the gallbladder is filled with bile[31]. Only about 50% of the hepatic bile reaches the gallbladder for concentration and storage, while the remaining bile bypasses the gallbladder to enter the duodenum and undergo continuous enterohepatic cycling[36]. During digestion, cholecystokinin stimulates the contraction of the gallbladder and the common bile duct and the relaxation of the SO, resulting in the discharge of up to 80% of the gallbladder contents into the duodenum[37,38].

The Mutual Interaction Between Bile and the Microbiota

Bile is a vital aqueous solution composed of ~95% water in which organic and inorganic solutes, including bile acids, cholesterol, phospholipids, bilirubin and amino acids, are dissolved[24]. Bile acids (BAs) are the most prevalent organic compounds in bile, constituting approximately 50% of the organic components of bile. BAs are 24-carbon water-soluble products of cholesterol metabolism[24,39]. There are two processes and anatomical sites for the biosynthesis of BAs: the primary BAs are first synthesized *de novo* from cholesterol in the liver and then are modified by bacterial enzymes in the intestine[38]. The two primary BAs synthesized in the liver are cholic acid (CA), a trihydroxylated bile salt, and chenodeoxycholic acid (CDCA), a dihydroxy bile salt[39]. These salts can be conjugated at the side chain with taurine or glycine, a process that metabolizes BAs into stronger acids limiting their passive reabsorption at the biliary tree[24]. Intestinal bacteria, a consortium of a small number of species belonging to the class *Clostridia*[40], produce "secondary BAs" by removal of the hydroxyl group at C7, transforming cholic acid to deoxycholic acid (DCA) and CDCA to lithocholic acid (LCA)[38,39,41]. During transit through the caecum and colon,

conjugated BAs can also be "deconjugated" from the link with glycine or taurine by enzymes known as bile salt hydrolases (BSH), which are expressed by Gram-positive intestinal bacterial species such as *Lactobacillus*[42-46], *Enterococcus*[47,48], *Bifidobacterium*[49-51], and *Clostridium*[52]. BSH activity has also been described in the commensal Gram-negative *Bacteroides* spp. and in the *Archaea* domain, such as *Methanobrevibacter smithii* and *Methanosphaera stadtmanae*[53]. Moreover, numerous enteric species (*Clostridium*, *Peptostreptococcus*, *Bacteroides*, *Eubacterium*, and *Escherichia coli*) can oxidize and epimerize the hydroxy groups of BAs, leading to the generation of isobile (β-hydroxy) salts[54], such as ursodeoxycholic acid (UDCA), which are among the most hydrophilic BAs. Most of these conjugated and deconjugated BAs are reabsorbed in the distal intestine, where they undergo enterohepatic circulation, thus maintaining the BA pool[36]. This pool varies from 2 to 4 g and recirculates 6-10 times a day. This "recycle" is a highly economic circuit that exerts important regulatory effects on several hepatic, biliary and intestinal functions[55].

Thus, the gut microbiota exerts a strong influence on bile. Specifically, the intestinal bacteria are able to alter the composition of the BA pool. Since the transformation of primary BAs into secondary ones depends on the action of bacteria, modifications in the gut microbiota that express BSH and bile acid-inducible (BAI) enzymes affect the functions and signaling properties of BAs[56]. Quantitative or qualitative perturbations of the BA pool have been related to several human diseases, such as metabolic syndrome[57,58], cancer[59,60], inflammatory bowel diseases (IBD)[61] and the occurrence and recurrence of *Clostridium difficile* colitis[62,63]. BAs are also involved in the pathogenesis of several biliary diseases; for example, in autoimmune cholangiopathies BAs play a significant role in the initiation of cholestasis, development of liver damage and progression to liver fibrosis[64]. The magnitude of these pathogenic mechanisms is highlighted by the fact that the use of obeticholic acid, a CDCA-derived farnesoid X receptor (FXR) agonist, is an effective treatment for primary biliary cholangitis[65].

Furthermore, the interaction occurring in the gastrointestinal tract between the gut microbiota and the immune system is crucial for the maintenance of human homeostasis[66,67]. BAs are important signaling mediators in immunological mechanisms. Indeed, the activation of bile acid receptors, such as FXR and TGR5, causes a decrease in the production of inflammatory cytokines and in innate immune cells phagocytosis, which is mediated by the inhibition of NFκB pathway[68,69].

However, the aforementioned evidence is obtained from studies on the gastrointestinal tract, while the interaction between the host and the microbiota in the biliary environment is still incompletely studied and poorly understood.

Along with gastric acid secretion and pancreatic enzymes, bile is responsible for the increasing gradient of abundance of the gut microbiota from the duodenum to the colon rectum[70]. In fact, bile has important antimicrobial properties. The amphipathic nature of BAs exerts membrane-damaging effects by binding and dissolving membrane lipids and determine cellular lysis[71-73]. This emulsification process involves a detergent action that is negatively correlated with the number of hydroxyl groups in the molecule. Thus, primary BAs (CDCA and CA) are more toxic than secondary ones (LCA and DCA)[69]. Once BAs enter the bacterial cytoplasm, they elicit other cytotoxic mechanisms, including the internal acidification of cytoplasm and the generation of toxic compounds such as hydrogen sulfide (H_2S), which is produced by the cleavage of taurine-conjugated bile salts[69]. Moreover, bile is able to cause DNA damage[74], oxidative stress[75] and osmotic effects[76] against bacteria.

Besides the physical and chemical antimicrobial properties, bile contributes to the immunological defense of organism against enteric infections by secreting immunoglobulins A (IgA), antimicrobial peptides, inflammatory cytokines (e.g., tumor necrosis factor (TNF)-α), leukotrienes and their metabolites and stimulating the innate immune system in the intestine[24,77-79]. In addition, BAs activate the nuclear receptor FXRα, that mediates antibacterial effects by the upregulation of genes involved in mucosal defense[80].

Altogether, these effects limit bacterial growth, particularly in the small intestine.

Bacterial Colonization of the Biliary Tract: Biliary Defensive Systems and Microbial Tolerance Mechanisms

The biliary tract owns several defensive systems to protect bile and the biliary mucosa from bacterial colonization and infection.

Firstly, the aforementioned antimicrobial properties of bile reduce the concentration of bacteria in the duodenum[70]. Secondly, the SO acts as a mechanical barrier that separates the duodenum

from the biliary tree. Its basal tone at rest of 15-18 mmHg higher than duodenal pressure prevents the massive passage of bacteria from the gastrointestinal tract, which would otherwise result from the increased intestinal pressure caused by peristalsis. Moreover, its coordinated action with the gallbladder allows the bile flow, which is another functional cleansing effect to eliminate pathogens and potentially harmful substances from the biliary tract. In fact, about 800-1000 ml of bile flows through the bile ducts everyday[81].

Even if some microorganisms manage to overcome these systems, the biliary mucus secreted by biliary epithelium prevents them from adhering to the biliary tract mucosa[81]. Furthermore, the higher concentration of BAs at this level exerts higher toxicity toward the bacteria[38].

The integrity of the continuous monocellular epithelium represents another important mechanical element that prevents the translocation of bacteria into the liver or the systemic circulation. Tight junctions seal the intercellular spaces, ensuring the continuity of the barrier[81].

The biliary epithelium also shows a wide range of innate immune receptors, such as toll-like-receptor (TLR) 1 to TLR6 and TLR9, and surface and intracellular adaptors that mediate the signaling pathways and the initiation of inflammatory responses[82,83]. In addition, antimicrobial peptides including human β-defensin-1 and -2 are widely expressed in the intrahepatic biliary tree[84].

Tissue macrophages and liver Kupffer cells, activated by proinflammatory cytokines, are responsible for microbial killing and antigen presentation to the T cells and plasma cells in mesenteric lymph nodes or minor lymphoid glands adjacent to bile ducts. The activation of the adaptive response enhances the production of immunoglobulins that can be found in bile, mainly as secretory IgA[77].

Microorganisms must possess tolerance mechanisms in order to resist bile action. Thus, in order to survive in the environmental conditions presented by bile, bacteria respond with adaptations to the pH and detergent effects of bile. In particular, they strengthen their membrane, by modifying its lipid composition and upregulate the expression of efflux pumps, porins, transmembrane proteins and BSH. However, bile tolerance is strain-specific and *in vitro* models do not always coincide with *in vivo* observations[38].

In general, Gram-negative bacteria show a higher resistance to bile than Gram-positive ones[38]. In particular, *Salmonella* spp.[85], *Escherichia coli*[86] and certain species of *Helicobacter*[87] possess an incredible tolerance to high concentrations of BAs. Several Gram-positive pathogens, including *Listeria* spp.[88], *Enterococcus faecalis*[89] and *Clostridia*[90], have also demonstrated an ability to colonize bile.

Microbes can reach the biliary tract through different routes, of which the ascending route through the SO has traditionally been considered the most frequent route of entry of bacteria into the biliary system. The dysfunctions of the SO, such as SO laxity, affect the activity of this "gatekeeper", resulting in an increase in the passage of bacteria by duodenal reflux[91].

Sphincterotomy, performed during either endoscopic retrograde cholangiopancreatography (ERCP) or surgery, causes the loss of function and integrity of SO. Similarly, the positioning of biliary stents in order to treat mechanical stenosis of the biliary tree favors a direct passage[92-95]. An intermittent or incomplete obstruction to bile flow, as observed in choledocholithiasis and carcinoma of the ampulla, is another risk factor for biliary contamination and infection[15,96].

Furthermore, bacteria can reach the biliary tract through two hematogenous routes: via the portal venous system or systemic circulation[81,97]. Indeed, the biliary epithelium is nourished by a network of capillaries called peribiliary vascular plexus[98]. This plexus originates from the terminal branches of the hepatic artery and has anastomotic connections with the portal vein vasculature[98]. Hence, as a consequence of increased intestinal permeability, bacterial translocation into the portal circulation can lead viable bacteria inside the biliary system[99-101].

Finally, during bacteremia, microorganisms can be transported into the biliary tract[97]. Using this route, *Salmonella enterica* reaches the gallbladder, which represents its reservoir in typhoid carriers. Indeed, after disrupting of the intestinal epithelium, the bacterium infects the intestinal macrophages that reach the intestinal lymph nodes and then the systemic circulation[102,103].

The Biliary Microbiota in Health

The knowledge about the composition of the biliary microbiota in health represents the first step in the understanding of the influence of the microbiota on the development of biliary diseases.

Jiménez et al[104] analyzed the bile, gallbladder mucus and mucosal microbiome of healthy pigs using culture-based as well as metagenomics techniques. All the cultured samples harvested bacterial species (6/6, 100%) and the number of

identified species ranged from 3 to 20 per sample. Bacteria isolated from cultures were broadly balanced among *Firmicutes* (34%), *Actinobacteria* (32%) and *Proteobacteria* (32%) phyla. *Bacteroidetes* accounted for a lesser part (2% of the isolates), suggesting an inadequate adaptation to this environment. At the genus level, *Staphylococcus*, *Streptococcus*, *Kocuria*, *Rothia*, *Acinetobacter* and *Psychrobacter* were isolated from different samples, suggesting a possible role as members of the core biliary microbiota of pigs[104].

The 16S ribosomal RNA metagenomic profiling identified *Streptococcus alactolyticus*, a common commensal in the gastrointestinal tract of pigs[105], as the largely dominant species (>90%) in two animals[104]. It was also observed to be the prevalent isolate from the bile of another animal in the culture-based assessment, as well. A higher bacterial diversity with a lower prevalence of some other species (*Lactobacillus salivarius* and *Bacillus* sp.) was observed in the remaining samples. Interestingly, apart from bile, the microbiological analysis of gallbladder mucus and mucosa, broadened the spectrum of bacteria that could possibly colonize the mucus and cellular brush border[104].

Knowledge about the human physiological biliary microbiota has been lacking for years. Indeed, bile sampling techniques, such as ERCP, percutaneous biliary drainage and intra-operatory sampling, are invasive procedures that can only be performed when a biliary tract disease is already present or suspected.

More recently, Molinero et al[106] analyzed the biliary microbiota of 27 liver donors (13 without and 14 with cholelithiasis). The 16S ribosomal RNA sequencing revealed a prevalence of *Actinobacteria*, *Firmicutes* and *Bacteroidetes* in both the bile samples and gallbladder tissues of subjects without gallstones. A significant increase in the abundance of the *Propionibacteriaceae* family and *Sphingomonas* genus was also reported compared with individuals with gallstones.

This study provided the first evidence of the human biliary microbiota in subjects unaffected by hepatopancreatobiliary diseases. However, larger samples are needed to confirm these results and evaluate the core biliary microbiota of healthy individuals.

Confirmation of the hypothesis of stable colonization of the biliary tract by resident microbial communities may revolutionize our knowledge on the development of biliary infectious diseases. Indeed, from a microbiota-centric view, a focal dysbiotic process, rather than an ascending infection from the duodenum, could better explain the occurrence of some biliary infectious diseases.

For ethical reasons, the majority of the research on the human biliary microbiota has focused on the study of pathological models. Emerging evidence has provided new insights into the biliary microbiota and has improved the understanding of the pathogenesis of biliary diseases, such as gallstones, autoimmune cholangiopathies and biliary tract cancers.

The Biliary Microbiota in the Pathogenesis of Gallstones

Since the 1920s, it has been known that the formation of gallstones occurs irrespective of the presence of bile infection[107,108]. The first evidence of the possible involvement of microbial products in the pathogenesis of gallstones was obtained in the 1960s. Based on the previous observations that infection with *Escherichia coli* could be implicated in the pathogenesis of gallstone formation, Maki et al[109] demonstrated that the inoculation of bacterial β-glucuronidase in bile could hydrolyse the bilirubin glucuronide into bilirubin and glucuronic acid, which could precipitate in the presence of calcium to form calcium bilirubinate[109,110].

Indeed, β-glucuronidase expressing bacteria have been frequently identified in the samples of patients with pigmented gallstones[111-115]. Other bacterial enzymes, such as phospholipases and BA hydrolases have later been shown to be implicated with similar mechanisms in the formation of pigmented gallstones[116-119].

Moreover, a study using scanning electron microscopy (SEM) has demonstrated the presence of bacterial microcolonies or bacterial casts within the pigmented gallstones along with bile colonization assessed with bile culture. Bacteria, adhering to the pigment solids via glycocalyx, could alter the local physico-chemical characteristics of bile by means of their enzymes, thus favoring the formation of pigmented gallstones[120-124].

Thus, the studies conducted during the 1980s have confirmed Maki's hypothesis and the role of bacteria in the pathogenesis of pigmented gallstones is widely accepted[16,114,117,125-128].

Interestingly, in a study using SEM and bile culture, most of the patients with evidence of bacteria in the gallstones did not show any clinical signs of biliary infection[117]. Considering the selection bias in the collection of gallstones from patients undergoing surgery, this result underlines that dysbiosis of the biliary microbiota is a frequent occurrence.

The importance of bacterial enzymes in the pathogenesis of pigmented and mixed gallstones has been further highlighted by genomic techniques. In a previous study using polymerase chain reaction (PCR)-based amplification and sequencing of bacterial genes encoding various enzymes, the presence of a gene encoding β-glucuronidase was observed in most of the mixed cholesterol gallstones, while bacterial sequences of *E. coli* and *Pseudomonas* sp. were identified in all the pigmented and mixed cholesterol gallstones[129].

Conversely, the formation of cholesterol gallstones has traditionally been considered to be dependent on metabolic imbalance and genetic variances rather than a bacterial detrimental effect[126]. Culture-based techniques and electron microscopy have failed to identify bacteria in this type of stones in most cases. In fact, a positive bile culture was observed in 10-33% of the samples[120,130-132]. However, since the identification of microorganisms depends on their viability and cultivability, cultured bacteria are not representative of the complete biliary microbiome.

A significant progress in research on the biliary microbial system was made with the advent of bacterial genomic techniques[16] (Figure 1, Table I).

In 1995, Swidsinski et al[16] analyzed the cholesterol gallstones from patients with negative bile culture using PCR-based amplification and 16S ribosomal RNA sequencing and found bacterial DNA in 16 out of 17 patients (94%) with gallstones with cholesterol content ranging from 70 to 90%. Pure cholesterol gallstones (>90% cholesterol content) showed no bacterial DNA. Although a thorough genus level identification was not feasible at the time of the study, the authors subdivided the identified bacteria into three groups: *Propionibacteria*-related, *Clostridia*-related and *Enterobacteria*-related, accounting for 45%, 35% and 25% of the total isolated strains, respectively[16].

In a similar study using nested primers PCR, bacterial DNA was obtained in the gallstones of 26 out of 30 patients (86.7%). *Propionibacteria*-related (26.7%) and *E. coli*-related (23.3%) were the most prevalent bacterial DNA sequences isolated, while DNA of *Streptococcus pyogenes* was identified at a lower percentage (6.7%). However, multiple heterogeneous sequences were found in 23.3% of the cases as a result of multiple infections or repeated colonization by *E. coli*, *Propionibacterium acnes* and *Streptococcus pyogenes* or other unidentifiable microorganisms[133].

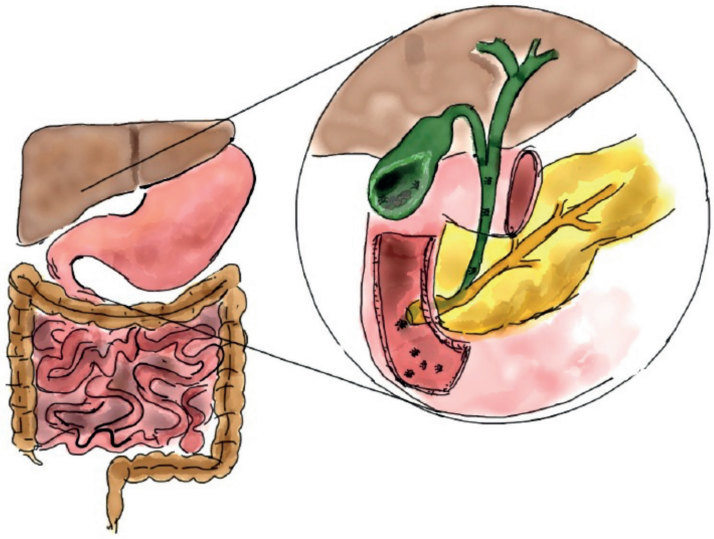
A shift from the concept of infection to the acknowledgement of resident microbiota occurred in 1998. Indeed, the same authors, using quantitative PCR, demonstrated that a vast majority (71/91, 78%) of culture-negative cholesterol gallstones had low bacterial concentrations of 10^3 CFU/10 mg, while only few culture-negative stones (11/91, 12%) had concentrations comparable to culture-positive ones. Only 9 of the 100 cholesterol gallstones analyzed showed no bacterial DNA and all of them had an elevated mean percentage of cholesterol content (93.9±2.8%), confirming the previous observation. The genomic analysis of gallstones with positive bile cultures showed a predominance of the bacterial strains identified by the culture, suggesting an ongoing infection. Interestingly, the genomic pattern of culture-negative gallstones with high concentrations of bacteria revealed a combination of different bacterial sequences, with no predominance of one particular strain compared to the others. Similarly, on average, 3.6 sequences per stone were observed in the cholesterol gallstones with low bacterial concentration. Finally, after 6-month storage at -20°C, gallstones with both positive and negative bile cultures, but with high concentrations of bacteria determined by genomic analysis, showed the appearance of new bacterial sequences, that accounted for up to 20% of the total. Most of the sequences belonged to bacterial strains, such as *Bacillus*, *Alcaligenes*, *Carnobacterium* and *Burkholderia*, that are difficult to cultivate but are able to survive and grow under extreme conditions[134].

While on the one hand the high concentration of a single bacterial species is consistent with an infection, on the other hand, the simultaneous presence of multiple bacterial species suggests constant colonization rather than a biliary infection.

According to the evidence described above, pure cholesterol gallstones did not appear to host bacteria. In fact, only 1 out of 7 pure cholesterol gallstones (14%) was reported to contain bacterial sequences in the study by Lee et al[129], while none (0/3, 0%) in Swidsinski et al[16].

In 2002, Kawai et al[135] found bacterial DNA in 12 out of of 21 (57%) pure cholesterol gallstones (100% cholesterol content). Surprisingly, all the bacteria identified (*Staphylococcus aureus*, *Streptococcus salivarius*, *Streptococcus anginosus*, *Streptococcus gordonii* and *Enterococcus faecalis*) were Gram-positive cocci. Nevertheless, this evidence seems robust due to the fact that the analyzed material came from the core of the gallstone and had very high homology with known bacterial 16S rRNA sequences[135].

	BILIARY MICROBIOTA
Gallstones (genera)	*Enterobacteriaceae, Ruminococcaceae, Clostridiales, Alistipes, Bacteroidales, Anoxybacillus, Clostridium (C.), Thermus, Catabacteriaceae, Propionibacterium, Enterococcus, Acinetobacter, Staphylococcus, Caulobacter, Pseudomonas, Massilia, Brevibacillus, Lactococcus, Paludibacter, Weissella*
Primary Biliary Cholangitis (PBC) (genera)	*Staphylococcus, Enterococcus, Streptococcus, Lactohacillus, Helicobacter, Propionibacterium, Corynebacterium, Agrobacterium, Flavobacterium, Clostridium, Micrococcus*
Primary Sclerosing Cholangitis (PSC) (genera)	*Streptococcus, Prevotella, Fusobacterium, Veillonella, Haemophylus, Neisseria, Alloprevotella, Leptotrichia, Porphyromonas, Cronobacter*
Cancer (genera)	*Prevotella, Actinomyces, Streptococcus, Fusobacterium Novosphingobium, Helycobacter.*



	GUT MICROBIOTA
Gallstones (genera)	*Bacteroides, Lachnospiraceae, Faecalibacterium, Clostridium (L.), Lachnospira, Roseburia, Enterobacteriaceae, Phascolarctobacterium, Blautia, Clostridium (C.), Epulopiscium*
Primary Biliary Cholangitis (PBC) (genera)	*Pseudomonas, Haemophilus, Streptococcus, Oscillospira, Sutterella, Bacteroides, Veillonella*
Primary Sclerosing Cholangitis (PSC) (genera)	*Bacteroides, Faecalibacterium, Roseburia, Blautia, Coprococcus, Runinococcus, Bifidobacterium, Prevotella, Dorea, Alistipes, Anaerostipes, Streptococcus, Collinsella*
Cancer (families)	*Moraxellaceae, Burkhoideriacae, Comamonadaceae, Bradyrhizobiaceae*

Figure 1. Gut and biliary microbiota in biliary diseases. Biliary microbiota: gallstones (Wu et al[19], 2013), PBC (Hiramatsu et al[20], 2000), PSC (Pereira et al[21], 2017), cancer (Avilés-Jiménez et al[22], 2016), Gut microbiota: gallstones (Wu et al[19], 2013), PBC (Tang et al[172]), PSC (Sabino et al[181], 2016), cancer (Chng et al[238], 2016).

Table I. Studies on biliary microbiota using 16S rRNA gene sequencing.

References	Country	Model	Biological Specimen	Sampling Method	Evidence
HEALTHY					
Jimenez et al[104]	Spain	Pig	Bile, mucus and biopsies of gallbladder	Gallbladder was removed from the sacrificed sows. Bile was extracted using a sterile syringe. Once the gallbladder was completely emptied, the superficial mucus layer coating was collected and three biopsies were cut.	The gallbladder ecosystem of healthy pigs is mainly populated by bacteria broadly balanced among *Firmicutes* (34%), *Actinobacteria* (32%) and *Proteobacteria* (32%) phyla. *Bacteroidetes* accounted for a lesser part (2% of the isolates). At the genus level, *Staphylococcus*, *Streptococcus*, *Kocuria*, *Rothia*, *Acinetobacter* and *Psychrobacter* were isolated from different samples.
Molinero et al[106]	Spain	Human	Bile and gallbladder tissue	Sterile sampling during liver transplants from liver donors who had suffered a brain accident or stroke.	Prevalence of *Actinobacteria*, *Firmicutes* and *Bacteroidetes* in both the bile samples and gallbladder tissues of subjects without gallstones. A significant increase in the abundance of the *Propionibacteriaceae* family and *Sphingomonas* genus was also reported compared with individuals with gallstones.
Cholelithiasis					
Swidsinski et al[16]	Germany	Human	Gallstones	Surgery	Bacterial DNA was found in gallstones with cholesterol content 70%-90%, in those with cholesterol content >90% no. Three bacterial groups were identified: *Propionibacteria* (45%), *Clostridia* (35%) and *Enterobacteria* (25%).
Wu XT et al[133]	China	Human	Gallstones	Surgery	Bacterial DNA was obtained in the 86.7% gallstones. *Propionibacteria*-related (26.7%) and *E. coli*-related (23.3%) were the most frequent DNA sequences isolated; *Streptococcus pyogenes* DNA was 6.7%, multiple heterogeneous sequences were found in 23.3% of the cases as a result of multiple infections/colonizations by *E. coli*, *Propionibacterum acnes* and *Streptococcus pyogenes* or other unidentifiable microorganisms.
Swidsinski et al[134]	Germany	Human	Gallstones	Surgery	78% of negative culture cholesterol gallstones had low bacterial concentrations and only few negative culture stones (12%) had concentrations comparable to positive culture ones. The genomic analysis of the gallstone with positive bile culture showed a predominance of the bacterial strains identified by the culture, suggesting an ongoing infection. Most of them belong to bacterial strains, such as *Bacillus, Alcaligenes, Carnobacterium and Burkholderia.*
Lee et al[129]	USA	Human	Gallstones	During cholecystectomy and endoscopic retrograde colangio-pancreatography (ERCP)	Bacterial DNA sequences are usually present in mixed cholesterol (to 95% cholesterol content), brown pigment, and common bile duct, but rarely in pure cholesterol gallstones. The presence of a gene encoding β-glucoronidase was found in most mixed cholesterol gallstones and bacterial sequences of *E. coli* and *Pseudomonas* were identified in all the pigment and mixed cholesterol gallstones.
Wu T et al[19]	China	Human	Gallstones, bile, feces	During cholecystectomy, one stone was removed aseptically from the gallbladder and a bile sample was extracted using a sterile needle tubing. Prior to the operation, feces from all patients were also collected.	Gut microbiota dysbiosis was observed among gallstone patients compared to healthy subjects. Within the gut of patients, there exists an overgrowth of *Proteobacteria, TM7, Tenericutes, Actinobacteria, Thermi*, and *Cyanobacteria* and a decrease in the abundance of *Bacteroidetes* in the biliary tract.

Table continued

Table I *(Continued)*. Studies on biliary microbiota using 16S rRNA gene sequencing.

References	Country	Model	Biological Specimen	Sampling Method	Evidence
Cholelithiasis					
Saltykova et al[137]	Russian Federation	Human	Bile	During cholecystectomy, 5-10 ml of bile was aspirated from the gallbladder under sterile conditions	*Opisthorchis felineus* infection modified the biliary microbiome. Bile from participants with opisthorchiasis showed greater numbers of *Synergistetes, Spirochaetes, Planctomycetes, TM7* and *Verrucomicrobia*. Numbers of > 20 phylotypes differed in bile of the *O. felineus*-infected compared to non-infected participants.
Ye et al[138]	China	Human	Salivary, gastric, duodenal fluid and bile	Salivary samples were collected after the patients gargled with 20 mL of sterile saline water. Patients expectorated their mouthwash into sterile sputum cups. The gastric fluid, duodenal fluid, and bile samples were collected using strictly sterile side-viewing endoscopes.	All observed biliary bacteria were detectable in the upper digestive tract. The biliary microbiota had a comparatively higher similarity with the duodenal microbiota, *vs.* those of the other regions, but with a reduced diversity. *Enterobacteriaceae* genera (*Escherichia, Klebsiella,* and an unclassified genus) and *Pyramidobacter* were abundant in bile.
Shen et al[141]	China	Human	Bile	ERCP	Oral cavity and respiratory tract inhabitants were more prevalent in bile samples than intestinal inhabitants. Thus, in addition to gut species, bacteria from the oral cavity/respiratory tract might be relevant to human biliary infection.
Gutiérrez-Díaz et al[142]	Spain	Human	Bile	Surgery	In cholelithiasic patients dairy product intake was negatively associated with the proportions of *Bacteroidaceae* and *Bacteroides*, and several types of fiber, phenolic, and fatty acids were linked to the abundance of *Bacteroidaceae, Chitinophagaceae, Propionibacteraceae, Bacteroides*, and *Escherichia-Shigella*. These results support a link between diet, biliary microbiota, and cholelithiasis.
Kose et al[143]	Australia	Human	Gallstones	During cholecystectomy	In the analysed pigmented stones, genes involved in biofilm formation were mainly recovered from clinically pathogenic *Klebsiella* and *Enterococcus* while bile resistance genes were present also in *Escherichia, Shigella, Serratia* and *Bacillus*. *Klebsiella* was also present in one of the cholesterol gallstones, while the remaining analysed cholesterol stones showed a predominance of Gram-positive bacteria that were not identified within the pigmented stones.
PRIMARY BILIARY CHONAGITIS (PBC)					
Hiramatsu et al[20]	Japan	Human	Bile	Bile was then taken aseptically from the gallbladders at the time of liver transplantation, just before explantation.	In 75% of PBC were identified Gram-positive cocci while these cocci were positive in only 5% in cholecystolithiasis.

Table continued

Table I *(Continued)*. Studies on biliary microbiota using 16S rRNA gene sequencing.

References	Country	Model	Biological Specimen	Sampling Method	Evidence
PRIMARY SCLEROSING CHOLANGITIS (PSC)					
Folseraas et al[202]	Scandinavia, Germany, Central Europe, USA	Human	Bile	ERCP	A significant increase in the abundance of *Firmicutes* and a parallel decrease of *Proteobacteria* was observed along with differences in the abundance of *Bacteroidetes, Actinobacteria*, and *Tenericutes* among patients with FUT2 loss-of-function genotypes and non-secretors.
Pereira et al[21]	Finland	Human	Bile	ERCP	The bacterial communities of non-PSC subjects and early stage PSC patients were similar. *Streptococcus* abundance was also positively correlated with an increase in disease severity. These findings suggest that the aetiology of PSC is not associated with changes in bile microbial communities, but the genus *Streptococcus* may play a pathogenic role in the progression of the disease.
CANCER					
Avilés-Jiménez et al[22]	Mexico	Human	Epithelial cells from the biliary duct	Brushing ERCP	Microbiota in extrahepatic cholangiocarcinoma showed significant changes in microbial composition. Phylum *Proteobacteria* dominated all samples. *Nesterenkonia* decreased, whereas *Methylophilaceae, Fusobacterium, Prevotella, Actinomyces, Novosphingobium* and *H. pylori* increased in patients with cholangiocarcinoma.
Chng et al[238]	Singapore, Thailandia, Romania	Human	Hepatic tissue, bile, gastric mucosa	Repository	Systemic perturbation of the microbiome was noted in tumor samples *vs*. non-cancer normal for several bacterial families, with a significant increase in *Stenotrophomonas* species in tumors. Comparison of *Opisthorchis viverrini* associated *vs*. non-associated groups identified enrichment for specific enteric bacteria (*Bifidobacteriaceae, Enterobacteriaceae* and *Enterococcaceae*). Functional analysis of cholangiocarcinoma microbiomes revealed higher potential for producing bile acids and ammonia in *O. viverrini* associated tissues, linking the altered microbiota to carcinogenesis.
Plieskatt et al[240]	Thailandia	Hamsters	Feces, bile	Bile from the gallbladder and colorectal contents were collected from each hamster sacrificed at 6 weeks after infection by *O. viverrini.*	Microbial community analyses revealed that fluke infection perturbed the gastrointestinal tract microbiome, increasing *Lachnospiraceae, Ruminococcaceae, and Lactobacillaceae,* while decreasing *Porphyromonadaceae, Erysipelotrichaceae*, and *Eubacteriaceae*. *Opisthorchiasis* has a robust inflammatory phenotype with conspicuously elevated IL-6. The inflammation of the biliary system leads to periductal fibrosis, which is a precursor of cholangiocarcinoma.

Table continued

Table I *(Continued)*. Studies on biliary microbiota using 16S rRNA gene sequencing.

References	Country	Model	Biological Specimen	Sampling Method	Evidence
CANCER					
Scheufele et al[93]	Munich	Human	Bile	Intraoperative	There are fundamental differences in the biliary microbiome of patients with periampullary cancer who undergo preoperative biliary drainage (PBD) and those who do not. PBD induces a shift of the biliary microbiome towards a more aggressive and resistant spectrum, which requires a differentiated perioperative antibiotic treatment strategy.
Tsuchiya et al[234]	Bolivia, Chile	Human	Bile	Cholecystectomy	*Salmonella typhi* and *Helicobacter sp*. were not detected in bile from any patients with gallbladder carcinoma (GBC). As the predominant species, *Fusobacterium nucleatum, Escherichia coli,* and *Enetrobacter sp*. were detected in bile from GBC patients. Those in bile from patients with cholelithiasis were *Escherichia coli, Salmonella sp*., and *Enerococcus gallinarum. Escherichia coli* was detected in bile samples from both GBC and cholelithiasis patients.
Chen et al[233]	China	Human	Bile	ERCP	In patients with distal cholangiocarcinoma, the abundance of *Gemmatimonadetes*, *Nitrospirae*, *Chloroflexi*, *Latescibacteria*, Unclassified_*Bacteria*, and *Planctomycetes* was increased compared with patients with choledocolithiasis. At the genus level, *Escherichia/Shigella*, *Staphylococcus*, *Klebsiella*, Unclassified_*Enterobacteriaceae*, and *Faecalibacterium* showed the highest abundance.
CHOLECYSTITIS, CHOLANGITIS AND OTHER BILIARY INFECTIOUS DISEASES					
Liu et al[244]	China	Human	Feces, bile	Faecal samples were collected in sterile tubes at the hospitals. Bile samples were obtained during percutaneous transhepatic cholangial drainage or gallbladder drainage.	*E. coli* was the main biliary pathogenic microorganism, among others such as *Klebsiella spp., Clostridium perfringens, Citrobacter freundii*, and *Enterobactercloacae* in the bile of the patients. Additionally, the amount of bile endotoxin significantly correlated with the number of *Enterobacteriaceae*, especially *E. coli. Enterobacteriaceae* might play essential role in the pathogenesis and/or progress of acute cholecystitis.
Yun et al[245]	Korea	Human	Bile	Cholecystectomy	Bile of patients with laparoscopic cholecystectomy may contain microorganisms, particularly elderly patients, those with symptoms, and those who undergo preoperative ERCP. *Escherichia coli* and *Klebsiella* were common in gram-negative bacteria. *Enterococcus* was the most common in gram-positive bacteria. Less than 5% resistance was observed against carbapenem, beta-lactam antibiotics, glycopeptide antibiotics, and linezolid.
Liang et al[246]	China	Human	Bile	Bile samples were extracted from the supraduo-denal segment of the common bile duct with a 5-mL germ-free injector before any invasive manipulation on the bile duct occurred.	A bile duct microenvironment with more severe bacterial infection and stronger lithogenicity was found in patients with sphincter of Oddi laxity (SOL). *Proteobacteria* and *Firmicutes* were the most widespread phylotypes, especially *Enterobacteriaceae*. Patients with SOL possessed more varied microbiota. In the SOL group, pathobionts, such as *Bilophila* and *Shewanella* algae had richer communities, and harmless bacteria were reduced.
Itthitaetrakool et al[241]	Thailand	Hamsters and worms	Liver tissue	For necropsy, hamsters were anesthetized with ether. Liver tissue at the hilar region and containing a large bile duct was immediately collected.	The identities of bacteria cultured for enrichment suggested that chronic *O. viverrini* infection changes the liver microbiome and promotes *Helicobacter spp*. growth. There may be synergy between *O. viverrini* and the liver microbiome in enhancing immune response-mediated hepatobiliary diseases.

Table continued

Table I *(Continued)*. Studies on biliary microbiota using 16S rRNA gene sequencing.

References	Country	Model	Biological Specimen	Sampling Method	Evidence
BILIARY STENTING					
Vaishnavi et al[247]	India	Human	Stents	Stents were retrieved endoscopically	The most common bacteria identified were *Pseudomonas, Citrobacter, Klebsiella, Staphylococcus, Serratia, Escherichia coli, Streptococcus, Enterococcus, Aeromonas, Proteus* and *Enterobacter*. The protein concentration of the biofilms was found to be significantly higher in stents placed in patients with cholangitis than those without cholangitis and those with smaller diameter stents. Longer indwelling duration had more biofilm formation.
LIVER TRASPLANTATION					
Kabar et al[248]	Germany	Human	Bile and feces	Bile was collected via percutaneous biliary drainage and during ERCP, after liver transplantation	Bile of liver transplant recipients is frequently colonized with microorganisms. Of isolated bile samples, 64.2%were Gram-positive, 22.2% were Gram-negative, and 13.6% revealed *Candida albicans*. Most detectable Gram-positive bacteria were *Enterococcus faecium*. Most detectable Gram-negative bacteria were *E. coli* and *Klebsiella pneumonia*. There was high correlation between microorganisms found in bile and those isolated from stool.
Liu et al[249]	China	Human	BIle	Collection from T-tube after sterilization	Firmicutes and *Proteobacteria* were the predominant phyla. *Enterococcus*, *Rhizobium*, *Nevskia*, *Lactococcus*, *Bacillus* were the most common genera.

However, genomic techniques confirm only the presence of microorganisms within the gallstone and not their vitality. The evidence that viable bacteria are present inside the gallstone core underlines the relevance of bacterial metabolism in the development of gallstones[136].

In 2013, the core biliary microbiota in patients with cholesterol gallstones was described. Indeed, Wu et al[19], through 16S rDNA pyrosequencing, identified 106 bacterial species belonging to 6 phyla both in the gallstones as well as in bile. Importantly, a higher microbial diversity was observed in the biliary tract compared to the gut microbiota of the same patients. At the phylum level, increased levels of *Proteobacteria*, *TM7*, *Tenericutes*, *Actinobacteria*, *Thermi*, and *Cyanobacteria* and a decrease in the abundance of *Bacteroidetes* were reported in the biliary tract. The dominant phyla of the biliary microbiota in patients with gallstones have been later confirmed by other studies[137,138]. As expected, some of these phyla possess a higher resistance to extreme environmental conditions, such as those present in the biliary tract. Notably, the phylum *Proteobacteria* includes genera such as *Escherichia*, *Salmonella*, *Vibrio*, and *Helicobacter*, all of which have been associated with several gastrointestinal diseases[7]. At the taxon level, a significant increase was observed in the abundance of *Enterobacteriaceae*, *Ruminococcaceae*, *Clostridiales*, *Bacteroidales*, *Acinetobacter*, *Staphylococcus*, *Caulobacter*, *Pseudomonas*, *Massilia*, *Brevibacillus* and *Lactococcus* in the biliary tract. Several previously undescribed bacterial species as well as a high interpersonal variation were reported in this study, suggesting a correlation with dietary, environmental and genetic factors. Furthermore, over 85% of the bacterial operational taxonomic units (OTUs) were observed in the bile as well as in gallstones. The biliary tract shared about 70% of the OTUs of the patients' gut microbiota, while this percentage dropped to 40% when the gut microbiota from healthy individuals was compared with the biliary microbiota of the patients[19]. In a study comparing the biliary microbiota of patients having gallstones with salivary, gastric and duodenal microbiota, all the bacterial genera found in the bile tract were observed in at least one other analyzed gastrointestinal site[138]. Similarly, Peng et al[139] reported the presence of common intestinal colonizers in the bile of patients with cholelithiasis. These findings support the hypothesis that the biliary microbiota originates from the gut, either by direct passage across the SO or by bacterial translocation[81,99,140].

Notably, in some of these studies, the Shannon diversity index and richness of bacterial communities were significantly higher in the gallstone and some bacteria identified in the gallstones were not found in the bile[139]. This evidence suggests that the gallstone may represent a protective environment within which the microorganism can create a separate niche that is resistant to the antimicrobial effect of bile and has favorable conditions for its growth.

The use of advanced PCR techniques, such as PCR-denaturing gradient gel electrophoresis (DGGE) and whole-metagenome shotgun (WMS) sequencing, has further increased taxonomic resolution, facilitating the identification of new biliary bacterial genera in the stones (*Brucella*, *Citrobacter*, *Shinella*, *Aurantimonas*, *Lachnospiraceae* and *Lactobacillus*) as well as in the bile of patients with cholelithiasis (*Bacillus*, *Enterobacter* and *Acinetobacter*)[139,141]. Furthermore, metagenomic techniques have improved our understanding of the complex interactions between the environment, individual habits and microbiota. The interplay influences the host metabolism, which in turn influences the development of gallstones[141-143].

These studies have demonstrated an unexpectedly rich bacterial community in a hostile environment. This evidence collectively suggests that bile colonization is common and may play a pivotal role in the formation of gallstones.

The Biliary Microbiota and Autoimmune Cholangiopathies Primary Biliary Cholangitis (PBC)

PBC is a chronic autoimmune disease affecting the small bile ducts. Currently, the most widely accepted hypothesis proposes that, in genetically predisposed individuals, an exaggerated immune response is produced against self-antigens expressed in the biliary tract. It has been proposed that molecular mimicry between host antigens and microbes may act as a possible trigger[144]. Antimitochondrial antibodies, serological markers of disease observed in about 95% of patients with PBC, target the pyruvate dehydrogenase complex E2 (PDC-E2) and other proteins that share lipoic acid residues[145]. This enzymatic complex expressed in the mitochondria of biliary epithelial cells shows cross-reactivity with several bacterial proteins, such as pyruvate dehydrogenase complex[146], ATP-dependent Clp protease[147], dihydrolipoamide acetyltransferase (E2p)[148] and other proteins of *E. coli*[149-151], lipoyl domains of *Novosphingobium aromaticivorans*[152,153], heat

shock proteins of *Mycobacterium gordonae*[154,155], pyruvate dehydrogenase complex of *Mycoplasma pneumoniae*[156] and β-galactosidase of *Lactobacillus delbrueckii*[157]. Hence, an immune reaction against one or more of these bacteria, combined with a loss of immunotolerance to pyruvate dehydrogenase complex E2, could lead to the development of PBC[145,146].

Furthermore, PBC seems to occur more frequently in patients with urinary tract infections[158-162], particularly by *E. coli*[163] or other infections by *Mycobacteria*[164], *Chlamydia*[165-167] and *Helicobacter pylori*[168]. Elevated antibodies titers against *Enterobacteriaceae*[169] *Toxoplasma gondii* and *Helicobacter pylori*[170] have also been reported.

Over the past few years, advancement in the 16S RNA sequencing-based knowledge on the influence of the gut microbiota in human pathologies has led to the study of its involvement in autoimmune cholangiopathies (Figure 1).

In a study by Lv et al[171], the gut microbiota of patients with early stage PBC showed a higher abundance of potentially opportunistic pathogens, such as the families *Enterobacteriaceae*, *Neisseriaceae* and *Enterococcaceae* and the genera *Streptococcus*, *Veillonella* and *Haemophilus parainfluenzae* compared to healthy controls. Simultaneously, a decreased abundance of health-promoting bacteria, such as *Lachnospiraceae* and some beneficial *Bacteroidetes* was observed[171].

Tang et al[172] reported a decrease in the richness of the gut microbiota in PBC patients compared to healthy controls. Similar to Lv et al[171], the abundance of the genera *Haemophilus*, *Veillonella*, *Clostridium*, *Lactobacillus*, *Streptococcus*, *Pseudomonas*, *Klebsiella* and *Enterobacteriaceae* was significantly increased in patients with PBC. Most of the bacteria included in these genera are responsible for infectious diseases, such as urinary tract infections, which are associated with the development of PBC. According to these findings, a microbiome signature, composed of 12 genera associated with the disease was described. Conversely, the abundance of *Faecalibacterium*, *Bacteroides*, *Sutterella* and *Oscillospira* was decreased in PBC[172]. Among these bacteria, *Faecalibacterium prausnitzii* exerts a significant beneficial effect on the homeostasis of the gut mucosa[173]. Interestingly, the alterations in some of the PBC-enriched genera as well as the PBC-depleted ones were partially reversed after six months of therapy using ursodeoxycholic acid[172].

It is presently under debate as to whether these alterations are causes of the alteration in the composition of bile in PBC or its consequences. Nevertheless, quantitative and/or qualitative modifications of bile have been observed in PBC, resulting in an increase in the concentration of CA[174-176]. These alterations of bile exert a profound impact on the composition of the gut microbiota: CA, in fact, possesses the lower anti-microbial activity among the BAs[69]. Moreover, immune dysregulation could imbalance the bacterial regulation by the secretion of anti-microbial peptides and immunoglobulins. Hence, gut dysbiosis may simply be a consequence of the chemical composition and the impaired anti-microbial activity of bile.

Interactions between the host and bacteria, that result in the activation of the immune system towards biliary epithelial cells, could directly take place in the biliary tract. Hence, the biliary microbiota ~~could~~ may play a pivotal role in disease development.

Indeed, bacterial compounds from *Streptococcus intermedius* and *Propionibacterium acnes* have been identified in the liver tissue of patients with PBC[177,178]. Similarly, bacterial proteins have been found in the sera of the affected patients[179].

So far, Hiramatsu et al[20] investigated the biliary microbiota through 16S rRNA profiling. Bile samples were collected from the gallbladder of 19 patients with PBC during liver transplantation. Bacterial sequences were found in 10 out of 15 PBC patients. *Staphylococcus aureus* was the most frequently detected microorganism (5/15 PBC patients, 33%; 40% of all PBC clones). *Enterococcus faecium*, *Lactobacillus plantarum*, *Helicobacter pylori*, *Streptococcus pneumoniae* and other *Streptococci* were the other commonly found bacteria (Figure 1, Table I). Importantly, this study was limited by the analysis of only 10 clones that were selected from the total number of the amplified PCR products. Hence, the identified bacteria should be considered as "major clones" rather than the complete biliary microbiota[20].

Further studies using next-generation metagenomic techniques should be carried out in order to better understand the biliary microbiota in PBC and its influence in the different phases of the disease.

Primary Sclerosing Cholangitis (PSC)

PSC is a chronic cholestatic autoimmune disease that affects the bile ducts causing biliary inflammation and fibrosis. Hereditary alterations of the genes that regulate immune response, particularly HLA class and IL-2 receptor genes, have been shown to confer higher susceptibility to the development of the disease, following which the

environmental factors may represent the final trigger. Considering the strong association with IBD, it has been proposed that a primary intestinal dysbiosis causing inflammation and consequent exposure of cholangiocytes to cytokines and microbial products could initiate the pathogenesis[180].

Therefore, several studies have recently analyzed the gut microbiome of PSC patients (Figure 1). An increase in the abundance of potentially harmful bacterial genera, including *Veillonella*, *Enterococcus* and *Escherichia*, has been observed. Likewise, the bacterial genera *Fusobacterium*, *Lactobacillus*, *Blautia*, *Barnesiella*, *Lachnospiraceae* and *Megasphaera* were reported to be associated with PSC compared to IBD patients and healthy controls[181-185]. A parallel decrease was reported in the abundance of some anaerobic taxons, such as *Clostridiales II*, *Bacteroides*, *Prevotella* and *Roseburia*[186]. In particular, *Roseburia* exerts well-recognized beneficial effects on the maintenance of intestinal homeostasis[187]. It is known to produce butyrate, which exerts a trophic effect toward enterocytes, thus maintaining the integrity of the gut barrier[188]. It has been demonstrated in germ-free murine models that the protective effects of some bacterial strains could play an even more important role than the detrimental effects of pathogenic species[189].

According to these observations, several antibiotics, including tetracycline[190,191], vancomycin[192-194], azithromycin[195], metronidazole[194,196], minocycline[197], rifaximin[198], probiotics[199] as well as fecal microbiota transplantation have been tested in patients with PSC[200].

In a culture-based study on a group of 36 PSC patients undergoing liver transplantation, the bile or bile walls of 20 patients were culture-positive. α-haemolytic *Streptococcus* was the most frequently identified bacterial species (16/20 patients), while *Enterococcus* and *Staphylococcus* were isolated from five cultures. The authors attributed these results to possible bile contamination and consequent colonization during previously performed ERCP. Moreover, most of the patients who had not received antibiotic prophylaxis before ERCP showed a higher number of isolates. In addition, a positive correlation was observed between the number of identified bacteria and the length of the period elapsed after the last ERCP. About 50% of the patients had a history of biliary infection during the previous six months; thus they had either received or were undergoing antibiotic therapy at the time of liver transplantation[95]. The relevance of the contamination of the biliary tree that occurs during ERCP was later been confirmed by the same group[201].

Pereira et al[21] studied the biliary microbiota of patients with PSC at different disease stages using 16S rRNA profiling. Notably, they did not find significant differences in the biliary microbiota of early stage PSC patients compared to controls. At advanced disease stages, the abundance of *Streptococcus* genus was significantly elevated. Lower microbial diversity and a further increase in the abundance of *Streptococcus* spp. characterized the biliary microbiota of the patients who developed dysplasia or cancer.

As observed from culture-based studies[95,201], the limitations of sampling during ERCP and selection of patients with a history of ERCP could have affected the results.

Along with a genome-wide association study, Folseraas et al[202] studied the genotype-dependent changes in the biliary microbiota composition in 39 patients with PSC, considering the presence, heterozygosity or absence of allele "G" of FUT2. This gene has an effect on the expression of fucosylated glycan expression in the bile duct epithelium and was found to be associated with PSC. Interestingly, a significant increase in the abundance of *Firmicutes* and a parallel decrease of *Proteobacteria* was observed along with differences in the abundance of *Bacteroidetes*, *Actinobacteria*, and *Tenericutes* among patients with FUT2 loss-of-function genotypes and non-secretors.

These findings have laid the foundations for further studies. Hopefully, a multiple "omics" approach and an improved understanding of the interaction between the host and the microbiome will unravel the complexity of the pathogenesis of autoimmune cholangiopathies.

Influence of Biliary Bacteria on the Development of Biliary Tract Cancer

Recent evidence has begun to clarify the complex influence of the human microbiota on the development and progression of cancer. Indeed, bacteria promote carcinogenesis by altering the metabolism, proliferation and death of cells by dysregulating the immune response or by actively inducing DNA damage via toxins[97,203,204]. Bacteria possess carcinogenetic potential as they can enhance the release of the mediators of inflammation, such as TNF-α and IL-1. Moreover, they are able to trigger the activation of NFκB, either directly or indirectly via proinflammatory cytokines[205]. NF-κB activation further exacerbates the inflammatory response and upregulates genes in-

volved in cell cycle control (cyclin D1, CDK2 kinase, c-myc) and apoptosis (p21, p53 and pRb)[206].

Several bacterial toxins have possible roles in the development and progression of cancer[97]. The study of the expression of specific bacterial toxins in the bile could further clarify the importance of this mechanism.

Since gallstones represent the strongest risk factor for developing biliary tract cancer[207] and are associated with mortality[208], other bacteria implicated in the formation of gallstones could also play a role in carcinogenesis.

The term "biliary tract cancers" refers to malignant tumors of the bile duct, such as extrahepatic cholangiocarcinoma, gallbladder and ampulla of Vater. In Western countries, the overall incidence of these tumors is modest and ranges between 0.5 and 5 per 100000 annually, making them the sixth most common cancers of the gastrointestinal system. Owing to dietary, environmental and microbiological factors, their incidence in Eastern countries is significantly higher (up to 100/100000). Generally, they are associated with low survival rates and poor prognosis, since they are quite often diagnosed at late stages[209].

In the culture-based microbial studies, patients with gallbladder carcinoma had a significantly higher frequency of positive bile cultures (65-81%) compared to the patients with cholelithiasis and controls[210,211]. In another study, bacterial growth was observed in the bile of 22 out of 118 patients (18.6%) with periampullary cancer undergoing surgery. In patients who underwent preoperatory ERCP, the percentage of culture-positive bile samples rose to 97%, underlining the significant impact of sphincterotomy and biliary stenting on bile colonization[93].

Several studies[212-217] have reported an association between typhoid carriage and the development of hepatobiliary cancer. Caygill et al[218] reported that typhoid carriers possessed a lifetime risk of 6% of developing gallbladder cancer. In several studies[211,219-222] the relative risk of developing biliary tract cancer ranged from 2.1 in low prevalence infection areas to 22.8 in endemic areas. Both direct DNA damage via toxins, such as cytolethal distending toxin[223,224], and an indirect detrimental modification of the bile composition via bacterial enzymes[225,226] have been suggested to be potential carcinogenic mechanisms.

Interestingly, Nath et al[222] demonstrated using nested PCR that specific *Salmonella typhi* sequences were found in the bile of 35 out of 52 patients (67%) with gallbladder carcinoma.

These findings suggest a significantly higher risk of developing cancer in patients with chronic bile colonization, particularly for chronic typhoid carriers.

The genus *Helicobacter* has also been associated with biliary tract cancers[87,227,228]. However, in several studies using PCR primers, a large variability in the detection rate in bile ranging from 0 to 82.8% has been found. Although the choice of primers may have influenced the results, an increasing prevalence gradient has been observed from Western to Eastern countries[87]. The most frequently identified species are *Helicobacter bilis*[229] and *H. hepaticus*[230]. Details about the possible pathogenesis are still unknown. However, *Helicobacter* is able to colonize the bile, interact with BAs and cause inflammation and neoangiogenesis[231,232], mechanisms that are potentially involved in carcinogenesis.

Avilés-Jiménez et al[22] analyzed compared the biliary microbiota of 100 patients with extrahepatic cholangiocarcinoma to 100 patients with benign biliary tumors, using 16S RNA sequencing (Figure 1, Table I). At the phylum level, a dominance of *Proteobacteria* (60.4% on average) was observed in all the samples. *Methylophilaceae*, *Fusobacterium*, *Prevotella*, *Helicobacter* and *Campylobacter* were the most frequently identified genera in patients with cholangiocarcinoma. The authors detected *Helicobacter pylori*-associated virulence genes, such as *CagA* and *VacA*, in most samples from both groups, indicating a possible carcinogenic role in the biliary tract. With the exclusion of four OTUs that were considered as potential contaminations, 21 OTUs showed a considerable modification in the cholangiocarcinoma group:. In particular, 12 increased (*Novosphingobium*, *Prevotella*, *Streptococcus*, *Dialister*, *Fusobacterium*, two *Actinomyces*, two genera belonging to *Methylophilaceae*, one to *Sinobacteriaceae* and one to *Neisseriaceae* families, one to class *Betaproteobacteria*), while 9 (*Rothia*, two *Nesterenkonia*, three *Mesorhizobium*, one unclassified genus belonging to *Micrococcaceae* and one to *Phyllobacteriaceae* families, one to *Rhizobiales* order) decreased in abundance. Importantly, the analysis revealed distinct clusters between cholangiocarcinoma and controls[22].

In another recent investigation, patients with distal cholangiocarcinoma had a prevalence of *Gemmatimonadetes*, *Nitrospirae*, *Chloroflexi*, *Latescibacteria*, Unclassified_*Bacteria*, and *Planctomycetes* compared with patients with choledocolithiasis. At the genus level, *Escherichia/Shigella*,

Staphylococcus, *Klebsiella*, unclassified_*Enterobacteriaceae*, and *Faecalibacterium* showed the highest abundance[233].

In a study using Next Generation Sequencing (NGS)-PCR, *Fusobacterium nucleatum*, *E. coli* and *Enterobacter* sp. were the predominant bacteria in the bile of patients with gallbladder carcinoma[234]. Interestingly, these bacterial strains have been linked to the development of colon cancer[235] and thus could possess an intrinsic carcinogenic potential irrespective of the site colonized by them.

Furthermore, considering how important the microenvironment is in tumorigenesis and how the microbiota is involved in shaping it[236,237], Chng et al[238] for the first time described the tissue microbiome of *Opisthorchis viverrini* associated cholangiocarcinoma. Indeed, patients affected by liver fluke are well-recognized models of biliary tract carcinogenesis[239] and the parasite is able to modify the microbiome of infested individuals[137,240-242]. An increase in the abundance of *Bifidobacteriaceae* and *Enterobacteriaceae* abundance was observed in the tissue microbiome of the *Opisthorchis* group, while an interesting prevalence of *Stenotrophomonas* was found in non-affected patients[238].

Conclusions

An unexpectedly rich bacterial community has recently been discovered in an environment that was previously considered to be hostile to bacterial growth. However, the stages and factors that favor the colonization of the biliary tract are incompletely understood.

As for the methodology, the standardization of the sampling methods should be considered. Several techniques have been used to perform bile sampling, but some of them have witnessed a possible risk of contamination. Separate assessments of the performance of each technique and sampling standards are currently lacking.

Since studies on healthy human biliary microbiota are not feasible for ethical reasons, comparative studies on the biliary microbiota of patients with different biliary illnesses could identify a microbial fingerprint of each disease.

Moreover, an understanding of the modifications of the biliary microbiota after treatment with the available therapies could provide new insights on the impact of bacterial communities in the pathogenic mechanisms of biliary diseases.

In particular, probiotic therapy modifies the composition of the gut microbiota. An improved understanding of the relationship between the gut and the biliary microbiota could be derived by studying the modifications of the biliary microbiota in patients on treatment with probiotics.

Furthermore, alterations of the bile composition have been associated with the development of several other gastrointestinal diseases[243]. Hence, biliary dysbiosis could represent a primary pathogenic step in the development and progression of these pathological conditions. A detailed comprehension of the impact of the biliary microbiota on bile composition may facilitate the development of strategies for modulating the microbiota in order to prevent the occurrence of such diseases.

Therefore, the modulation of the biliary microbial community should be considered for the prevention of biliary and other gastrointestinal diseases.

Finally, the biliary tree is intimately connected with the pancreas and liver. Hence, the study of the biliary microbiota could reveal a profound influence of the biliary microbiota on the pathogenesis of illnesses of these organs.

In summary, recent evidence has paved the way for a better understanding of a crucial site in the development of gastrointestinal diseases. Future studies are needed to explore the influence of the biliary microbiota in human pathology. This knowledge could lead to the formulation of strategies for modulating the biliary microbiota in order to treat and prevent several gastrointestinal diseases.

Conflict of Interests

The Authors declare that they have no conflict of interests.

Author contributions

AN and EN reviewed the literature, prepared the initial manuscript and produced tables and illustrations. FRP and GI revised the manuscript critically for important intellectual content. AG and LZDV conceived the topic and revised the manuscript critically for important intellectual content. All authors approved the final version.

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