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Review Article

Synergy of Gut Microbiome and Bile Acids in Inducing Cholelithiasis

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ABSTRACT

Gallstones have remained an expensive and crippling social problem in public health. The pathophysiology of cholelithiasis is not well understood and involves several variables. Even though the risk factors for gallstones and other metabolic syndrome are considered to be similar, there is a long list of risk factors associated with lithogenecity, such as age, parity and obesity. In addition to all this, intricate relationships between the microecology of the gastrointestinal tract and lithogenesis have been highlighted in this context. The gut microbiome and metabolism of bile acids are deeply interwoven. The pathways that regulate the conversion of cholesterol into bile acids in the hepatocytes to promote digestion and to acts as an antimicrobial surfactant in the gastrointestinal tract are well understood. Nevertheless, the gut microecosystem further augments the biotransformation in the enteric region via three mechanisms: dehydroxylation, deconjugation and epimerization. By virtue of these mechanisms, the gut microbiota regulates the bile acid pool by biotransforming primary bile acids into secondary bile acids. The primary bile salt diversifies the gut microbiome and maintains microbial lineage, whereas secondary bile salt perpetuates it. Therefore, it is not wrong to suggest that changes in the microbiome-bile salt equilibrium may contribute to hepatic and gastrointestinal disorders like cholelithiasis, specifically gallbladder stones, choledocholithiasis, and asymptomatic gallstones. Thus, this review serves as a backbone in explaining the entangled connection of bile acid composition-gut microbiome-host's health.

Keywords: Lithogenesis, Biotransformation, Surfactants, Cholelithogenesis, Hepatocytes.

INTRODUCTION

Mild disruption in the intestinal microecology may result in the emergence of metabolic disorders such as obesity and diabetes, subsequently switching to chronic gastrointestinal conditions like cholestasis, intestinal inflammation, cholelithiasis.[1,2] Among these, cholelithiasis is the most prevalent gastrointestinal disorder that often hypes after the age of 40 and imposes considerable stress on the global healthcare and economic system, though the geographical variance in its incidence has been observed.[3,4] However, despite being a global burden, the role of the intestinal microbiota in its pathophysiology remains largely unknown.[5] The strategy of using microbial mechanisms to prevent cholelithiasis struck the researchers in relation to the concept of Maki in 1966 whereby, suggesting the role of microbes in the formation of gallstone.[6] The evolution in the technologies confirmed the concept with the demonstration that alteration in the intestinal microbiome serves as the most important etiological factor in forming gallstones.[7]

Earlier, it was presumed that a healthy biliary system is sterile due to its antimicrobial properties. Nevertheless, recent studies have come up with the fact that the bile harbors bactobilia even in a non-diseased state.[4] Bacteria may travel retrogradely and populate the biliary tree via various routes: through translocation from the duodenum through the sphincter of Oddi or through enterohepatic circulation and subsequent excretion into the bile[5,8]. Being derivatives of cholesterol, bile acids (BAs) are referred to as steroidal acids. They play a pivotal role in maintaining cholesterol homeostasis. In addition to emulsifying fat, bile acids are also considered to be a potent signaling regulator of moiety. The cholesterol homeostasis is maintained by regulating the functioning of receptors, Farnesoid X receptor (FXR) and membrane G-protein-coupled bile acid receptor 1 (GPBAR1, TGR5)[9] for which bile acids are potent ligands. Moreover, an intricate balance has been maintained between bile acid metabolism and gut microbiome. The interaction between these two is bidirectional. The gut microbiome will modulate bile acid metabolism, which in turn modifies its structure[10]. Since the few events in the pathway of the bile acid cycle face biotransformation in the intestine, especially the conversion of conjugated bile acid into unconjugated bile acid on the basis of enzymes supplemented by the intestinal microbiota, any structural change in the gut microecology may influence the triangular relationship of the gut microbiome, bile acids and gallstones. The research of yesteryears has witnessed a notable surge in studies investigating the role of BAs and gut microbiota in cholelithiasis. Considering the importance of enzymes, namely, bile salt hydrolases (BSH) and 7 α-dehydroxylating enzymes in cholelithiasis, these can be used as a therapeutic strategy in the prevention of cholelithiasis. This review will discuss the potentiality

of some other underrated enzymatic actions of microbes involved in cholelithiasis.

Bile Acid and Gut Microbiome

The human body is an intricate ecology where the quantity of symbiotic bacteria is approximately equal to a number of "human" cells.[11] The ecosystem of the intestine is a reservoir of diversity of microbes that have eventually evolved within the human body to aid catabolism and absorption of dietary ingredients[12]. Even though the interaction between the two is symbiotic (+/+), the host must regulate bacterial expansion in the gut in order to avoid inadequate nutritional absorption because of competition with the host. The attributes that elicit alteration in the gut microbiome balance involve transit time, availability of proteolytic enzymes, antimicrobial peptides, diet, age, intake of antibiotics, gastrointestinal ailments and bile acids.[12]

The literature dating back decades cites that human bile is aseptic under normal conditions due to a) the presence of bacteriostatic agents such as bile acids, b) the continuous flushing action of bile, c) anatomical barrier[8]. Contrarily, by virtue of these advanced sequencing techniques, i.e., 16S ribosomal RNA (rRNA) gene sequencing, biliary microbiome has been recognized. In comparison to the GI tract, the biliary tract has a greater diversity of microbes[12]. It has been emphasized in a plethora of literature that there are few similitudes and dissimilitude in the microbial profile of the biliary and gastrointestinal systems. The comparative metagenomic study found no appreciable distinctions between the bile and the GI tract in the rare phylum *Fusobacteria* and the common organism *Firmicutes.*[4] It has been stated that *Escherichia coli*, *Fusobacterium*, and *Enterococcus* make up the core microbiota of the bile duct and duodenum.[14] Anatomically, the biliary duct is in conjunction with the GI tract via the duodenal papilla. It was proposed that the biliary microbiota migrate retrogradely into the biliary tract after originating as intestinal bacteria. Consistent with the hypothesis, studies demonstrated that the biliary microbiota shared a compositional similarity to duodenal microbiota, and all bacteria in bile were detected in the upper GI using 16s sequencing[15]. It is interesting that despite the high predominance of oral cavity and respiratory tract inhabitants *versus* intestinal inhabitants, the intestinal microbiome contributes to the variability of the biliary microbiome in cholelithiasis patients.[16] The existence of species belonging to phyla *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Verrucomicrobia*has been proved, accompanied by *Cyanobacteria* and *Spirochaetes* though in minute quantities.[17]

A research publication by Islam *et al.*, (2011) cites that the most abundant primary bile acid, cholic acid, has the potential to bring disproportion in the *Firmicutes:Bacteroidetes* ratio, i.e., increased occurrence of Firmicutes and dip in Bacteroidetes in the gut.[18] A comparative study in 2018 approved the same by administering a primary acid-rich diet to *Ctenopharyngodonidellus* caused instability in the microbiome in contrary to a secondary acid-rich diet.[19] Studies have confirmed that in human beings, bile acids (GCA and TCA) are classified as major determinants in balancing the intestinal micro-ecology and are significantly related to age.[20]

Misproportion in the intestinal microecology disrupts the dynamic equilibrium of bile acid/cholesterol metabolism and is a causative agent for cholelithiasis,[21] the pathological condition of having calculus in the gallbladder or in the biliary tree with a bulk of cholesterol in it. Disturbance in the equilibrium of the bile acid pool saturates the bile with cholesterol beyond limits and further nucleation gives rise to stones, a source of blockages in the biliary tract leading to another pathological condition, cholestasis[22]. The biliary obstruction triggers the abnormality in the physiology of the gall bladder. However, the physiology of the gall bladder has been maintained in a manner to tolerate the acidic effects of bile acids. Bile acids influence mucosal immune responses and the integrity of intestinal epithelial cells in the gall bladder.

According to the literature, cholelithogenesis often arises from the five primary defects[23]: a) the hepatic hypersecretion of cholesterol from the liver leading to supersaturation of cholesterol in the bile; b) genetic factors (particularly Lith genes); c) fast phase transitions due to the accelerated crystallization of cholesterol and the growth of crystals of solid cholesterol d) impaired gallbladder motility e) intestinal factors, including an increased amount of absorbed cholesterol being delivered from the small intestine to the liver for the hypersecretion of the biliary tract, as well as changes in the gut microbiota.[24]

Nevertheless, growing data has focused on the role of gut microbiota to be equal participants in lithogenesis other than metabolic defects of the biliary tract.[25] Thus, the mechanism of regulation of bile acids and cholelithiasis are interdependent on each other. The bile acids play pivotal role in the development of gallstones. The gallbladder chelates bile acids or alters bile acid composition. Usually, the metabolism of bile, carbohydrates and lipids is governed by bile nuclear receptors like farnesoid X receptor (FXR) expressed in the host's heart and liver. Certainly, gut dysbiosis will initially weaken bile acid metabolism and subsequently, the host metabolic pathways controlled by bile acid signaling, thus balancing carbohydrates and cholesterol levels in the body,[26] and is an important step in cholelithogenesis.[27]

Conjugated bile acids activated the FXR, promoting the expression of genes whose byproducts inhibit bacterial growth and support epithelial cohesion. The FXR knockout mice manage enhanced bacterial growth in the ileum (middle part of the small intestine) and disrupt the epithelial barrier. Thus, conjugated bile acids in the intestine have been proved detrimental to its microecology, specifically in acidic environments and are adversely affecting other different regions of the alimentary canal.[18] Conditionally, if the microbes are able to habitat the acidic environment of bile ducts and the human gut, they will be considered bile acid resistant.[28] However, the availability of BSH and some transport proteins may aid in this resistivity[29] and facilitate colonization. Bile acid biosynthetic pathways is shown in Fig. 1.

Modulation of Micro Diversity of Gut by Bile Acids

Antimicrobial agent: Bile acids can be detrimental to bacterial cells by damaging cell membranes, DNA damage and denaturation of proteins. a) Being surfactants, bile acids possess the potential to impair the membrane integrity of the microbes. At high concentrations, bile acids can dissolve the lipids, facilitating leakage

from the cells, thus forming pores in the membrane and, ultimately, cell death. Low concentration has undetectable effects on membrane fluidity and permeability by changing membrane-bound proteins or raising trans-membrane divalent cation flux. It also modulates the hydrophobicity of the cell surface; b) Conjugation of bile acids facilitates its active transport across the channels in the membrane, whereas unconjugated bile acids may passively cross over the membrane; c) Any kind of mutations in the structure and composition of the membrane of bacteria can cause resistance to the bile acids. Bile acids can damage the bacterial genome by oxidative stress (in the case of *E. coli*)[30] or by mutation, i.e., nucleotide substitutions, frameshifts, and chromosomal rearrangements (in the case of *S. enterica*).[31] The detergent property of bile acid can cause denaturation or misfolding of proteins.[32]

Chelating agent: Bile salts act as a chelant to iron and calcium ions. In the case of iron, it can regulate the structure of the gut microbiome by solubilizing Ferric ions/Ferrous ions with primary bile acids to promote the absorption of iron by enterocytes for basic cellular functions.[33] It has the potential to retain these ions for a long and depriving microbes of acquiring this essential element required by both. This process reduces the competition between microbes and host and, therefore, plays a critical role in balancing its multiplication.

Calcium ions ($Ca2^{+}$) work as a signal moiety involved in chemotaxis, gene expression regulation, and cell division.[34] Micelles formed in the gallbladder can trap $Ca2^{+}$ ions and inhibit its precipitation, thus prohibiting it from becoming a risk factor in the formation of gallstones. Hence decline in its availability may control microbial proliferation.[32]

Signaling moiety: Bile acids control the transcription of certain bacterial loci that may serve as signals in determining the intestinal environment. Bile acids downregulate the expression of genes encoding invasion proteins in *Salmonella enterica* thus controlling its infection in epithelial cells of ileum.[35] Bile salts also control the pathogenesis of *Clostridium difficile*, a gram-positive spore-forming anaerobe. Thus, enterotoxin produced by vegetative cells of *C. difficile* causes inflammation and diarrhea. The taurocholate, conjugated primary bile salt and deoxycholate, secondary bile salt, facilitate germination of spore but hamper vegetative growth. Contrarily, chenodeoxycholate, an unconjugated primary bile salt, inhibits spore germination and is a competitive inhibitor of taurocholate. The type of bile salt predominant in the medium serves as an environmental signal to either remain dormant or trigger spore germination. In *Vibrio cholerae*, bile salts activate the transcription of genes involved in virulence and biofilm formation.[36] In the case of *E. coli,* bile acid downregulates the expression of its pathogenicity island (LEE) responsible for adherence and pathogenesis.[37] These interactions offer illustrations of bile salt-mediated intestinal signaling.[32,37]

Mechanism of Bacteria-Induced Lithogenesis

Cholelithiasis can be regulated by the characteristics of the bacteria that harbor the gallbladder.[3] The phenomenon of cholelithiasis is related to the components of bile precipitate out of solution to form gallstones. Therefore, examining the probable function of bacterial metabolism in the precipitation of the various components of gallstones is one way to determine the role of bacteria in the pathophysiology of the disease. The elementary microbial mechanisms that drive cholelithogenesis as shown in Fig. 2.

Biochemical Properties of Bacteria Contributing to Lithogenesis

Enzymatic precipitation of calcium bilirubinate

The function of bacteria in the production of bile has been the subject of numerous investigations. It has been noted that the production of certain enzymes by bacteria, such as β-glucuronidase (GUS) and phospholipases (PL A2), may encourage the development of bile stones.[38] The ability to secrete GUS and PLA2 was found in over one-third of the cholesterol gallstone isolates.[39]

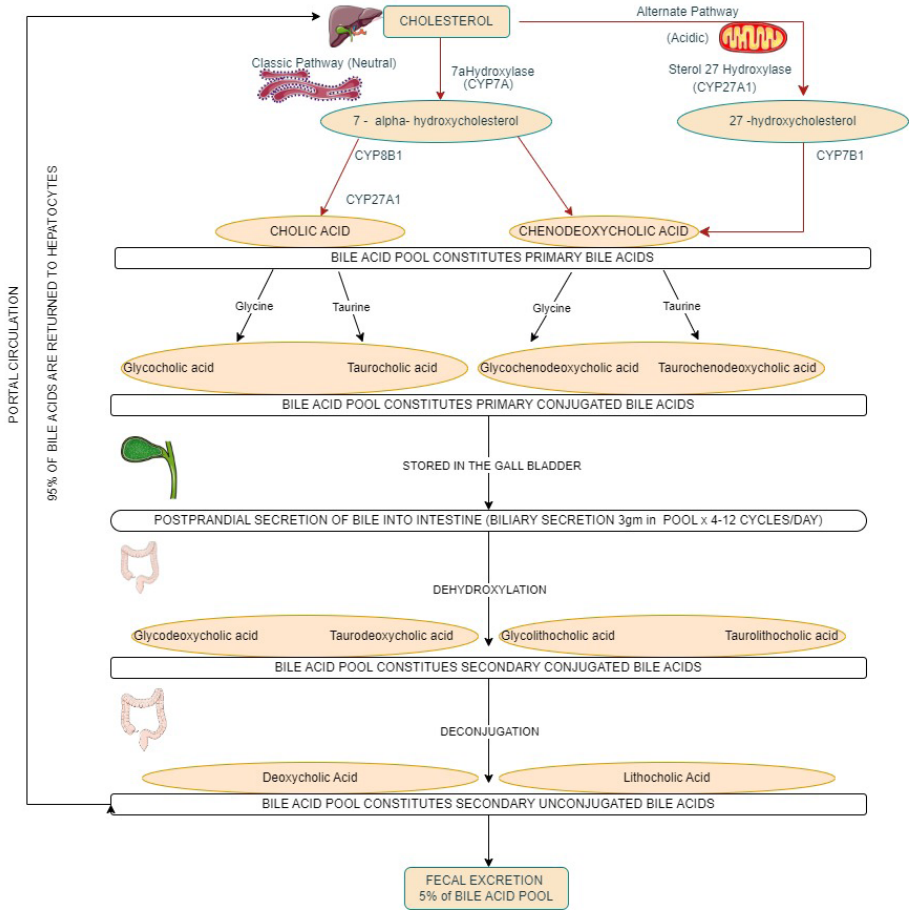
- *Role of beta-glucuronidase*

The plethora of literature predicts that bacterial enzyme β-glucuronidase (GUS) had a vital role in brown-pigmented stone formation.[4,40] This enzyme catalyzes the deconjugation process. Bilirubin diglucuronide gets hydrolyzed into free bilirubin and glucuronic acid in bile that may precipitate with calcium ions as calcium bilirubinate and act as a nidus for brown pigmented calculus.[40,41] Thus, pigmented brown gallstones are constituted with a significant amount of calcium bilirubin. This enzymatic activity is induced by the enzyme β-glucuronidase present in aerobic Enterobacteriaceae. *E. coli*, *Klebsiella, Staphylococcus* and *Streptococcus*; facultative anaerobes *Salmonella enterica* and also by anaerobes *Clostridium perfringens* and *Bacteroides fragilis*in the gut.[4] One of the experimental studies proved that nurturing human bile with *E.coli* produced sediments consisting primarily of calcium bilirubinate. Other investigators have also observed *E. coli* as the prevalent flora, and they ascribe this to the action of its β-glucuronidase enzyme in the development of biliary sludge and further calcium bilirubinate gallstone formation.[21] Thus, it is evident that microbes with the enzyme β-glucuronidase have a pivotal role in cholelithogenesis.[4] Few case studies have also found traces of Streptococci and Staphylococci isolates in the bile, gallbladder and liver, thus proving infection in the bile is a prime step in the pathophysiology of lithiasis, especially in the formation of brown pigmented stones.[42] An in-depth study by Treem *et al.*, 1989 documented the action of bacterial β-glucuronidase in infants diagnosed with obstructive jaundice and cholelithiasis, though infant had no congenital anatomical abnormality in the biliary tree that may lead to stasis.[43] The studies in France have had a similar outcome[44].

- *Role of phospholipases*

The action of phospholipase A2 (PLA2) enzyme leads to the formation of brown pigmented gallstones enriched with calcium palmitate and fatty acids constituting 15% and 10 to 20% of total stone content, respectively. PhospholipaseA2 (PLA2) contributes towards lithogenicity by hydrolyzing phosphatidylcholine (PC) of bile into releasing palmitic acid and fatty acids (saturated as well as non-saturated) that further precipitate with Ca^{2+} ions[4] to form calcium precipitate. PL hydrolyzes lecithin to water-insoluble free fatty acids and lysophospholipids, enhancing the precipitation of calcium salts and mucin secretion from the biliary epithelium, thus reducing its concentration in the bile.[45]

In healthy individuals, the amalgamation of bile salts and



Two major bile acid biosynthetic pathways are shown. The neutral (or classic) pathway is initiated by cholesterol 7α-hydroxylase (CYP7A1) located in the endoplasmic reticulum of the liver, whereas the acidic (or alternative) pathway is initiated by mitochondrial sterol 27-hydroxylase (CYP27A1). Enterohepatic circulation of bile acids. An average man produces ~0.5g bile acid per day by synthesis in the liver, and secretes ~0.5g/day. This daily turnover of bile acids accounts for about 5% of total bile acid pool. The remaining 95% of bile acids in the pool are recycled 4 to 12 times a day. Most bile acids are reabsorbed in the ileum by active transport, while a small amount is reabsorbed by passive diffusion in the upper intestine to portal blood for circulation to the liver. Small amounts of bile acids spilled over into the systemic circulation are recovered in kidney. (This diagram has been made by me, therefore no citation required).

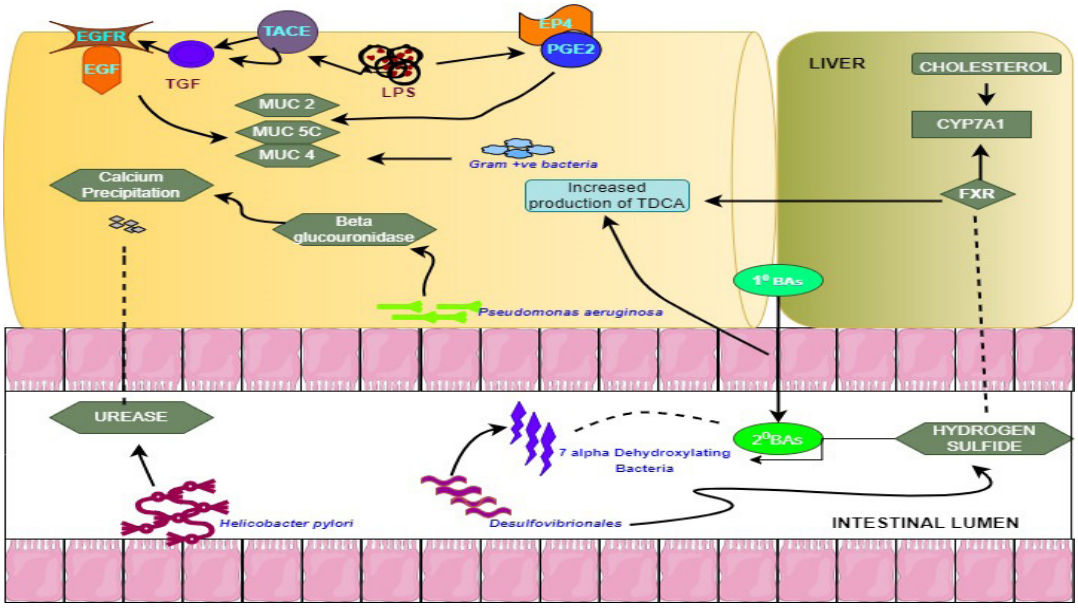
Fig. 1: Bile acid biosynthetic pathways

phospholipids solubilizes the cholesterol in the bile, thus lowering the probability of supersaturating the bile with cholesterol. However, the reduced concentration of phosphatidylcholine may promote the formation of unstable collaboration of cholesterol and lecithin, which may, in turn, become the cause of nucleation and, subsequently, cholelithiasis.[46] Besides this, the resultants are supposed to elicit mucin synthesis and/or secretion *via* a prostanoid pathway in the gallbladder that further promotes cholesterol crystal nucleation and growth. In conjunction with this mechanism, lysophosphatidylcholine (lysoPC), the byproducts of hydrolysis of phosphatidylcholine, causes the destruction of biliary epithelial cells, a source of persistent mucosal inflammation, hyperplasia and metaplasia, thus becoming responsible for gallstone production malignancy of biliary tree. Thus, frequently occurring cholelithiasis may be directly proportional to the phospholipase activity.[47] Infection with the opportunistic pathogenic Gram-negative bacterium *Pseudomonas aeruginosa* produces this potent enzyme and supports the aforesaid concept of cholelithiasis[4]. According to the Colombian literature, *Pseudomonas spp.* was prevalent in cholecystectomized gallbladder tissue and bile.

Biotransformation

Biliary salt hydrolases

Bile salt hydrolases (BSHs) of gut microbiota deconjugates the primary bile acids, an indispensable step for further biotransformation.[48] This deconjugation involves hydrolysis of amide bond between the glycine or taurine conjugated to primary bile salts, resulting in the release of amino acids, *i.e.,* glycine and taurine, that can be a source of carbon and nitrogen for the host[32]. BSH activity has also been attributed to the detoxification of the damaging effects of bile acids, such as low pH and detergent effects. Thus, if potentially used as a criterion, BSHs may shield commensal bacteria from BA toxicity, aid in bacterial survival and promote healthy gut niches. The activity of BSHs has been traced in the five indigenous species of genera *Bacteroides*, *Blautia*, *Eubacterium*, *Clostridium,* and *Roseburia. Numerous data have been published stating that many Lactobacillus strains also exhibit BSH activity.*[49]



*(1)Desulfovibrionales*aggrandized enteric augmented the cholelithiasis phenomenon by elicting the shift from normal biliary microbiome into lithogenesis induced microbiome via three ways (a) promoting the rapid multiplication of 7α-dehydroxylating bacteria, thus converting primary bile acids to secondary bile acids.(b) Hydrogen sulfide (H_2S) produced by *desulfovibrionales*regulates the expression of hepatic farnesoid X receptor (FXR)-CYP7A, inhibiting bile acid synthesis promoting the secretion of taurodeoxycholic acid.(2) Lipopolysaccharide (LPS), a major surface component of the gram-negative bacteria, upregulated expression of MUCIN genes in biliary epithelial cells via TACE/TGF-α/EGFR pathway and EP4/p38MAPK pathway. Mucin hypersecretion in bile may result from the upregulation of MUC genes and result in a higher bile viscosity, retaining cholesterol crystals in the biliary tract. Transactivation of MUC4 that resulted in the calcification was initiated by Gram negative bacteria.(3)Enzymatic precipitation of calcium bilirubinate caused by β-glucuronidase of gram negative bacteria like *E. coli*, *Salmonella enterica*, and *Pseudomonas aeruginosa* andUrease activity of *Helicobacter spp.* (This diagram has been made by me therefore no citation required).
FullForms BA: Bile acids; CYP7A1: Cholesterol 7α-hydroxylase; EGFR: Epidermal growth factor receptor; EP4: E-prostanoid receptor 4; FXR: Farnesoid X receptor; GUS: β-glucuronidase; LPS: Lipopolysaccharide; MUC: Mucin; PGE2: Prostaglandin E2; TACE: Tumor necrosis factor-α converting enzyme; TDCA: Taurodeoxycholic acid; TGF: Transforming growth factor.

Fig. 2: The elementary microbial mechanisms that drive cholelithogenesis

The potential selective value of BSH-active microbes modulates the cholesterol level, thus preventing hypercholesterolemia.[50] It has been accepted in multiple studies that FXR signaling indirectly affects the committed step of bile acid synthesis, i.e., the rate-limiting enzyme cholesterol 7α-hydroxylase (CYP7A1). A decrease in the activity of FXR suppresses the expression of the small heterodimer partner (SHP) that further activates the liver X receptor (LXR) and subsequently upregulates the adenosine triphosphate-binding cassette transporters G5 and G8 (ABCG5/G8) in the liver to augment the cholesterol secretion into the bile for the primary bile acid production. However, the increased enzymatic activity enhances the deconjugating activity of bile acids (BAs), thus impairing the absorption process of cholesterol by the intestine and increasing its excretion. On the grounds of this BSH activity, the hypocholesterolemic effect in human trials by numerous *Lactobacillus* species has been patented,[51] thus hampering cholesterol reabsorption.

In addition to reducing cholesterol, mounting evidence suggests that FXR signaling may play a pivotal role in the interaction between gut microbiota-encoded BSHs and obesity. In the line of antibiotic treatment, it has been found that a sustained decrease in the BSH-active microbes in the patient leads to obesity.[52] Another experimental study also proved that BSH-active probiotics-fed mice were restrained from gaining weight[53]. Certainly, accumulating data shows that the mismanagement of healthy gut microbiome, BAs, and FXR signaling are contributing factors for obesity, one of the attributes of cholelithiasis.

Chemoproteomic monitoring was utilized to observe the enhanced effect of BSH species in the gut during inflammatory bowel disease (IBD), leading to the increment in deconjugated bile acids. This sharp increase in unconjugated bile acids by virtue of increased BSH activity may lead to impaired lipid absorption, leading and resulting in steatorrhea. Thus causing dysfunctioning of the intestinal mucosa and giving rise to lithogenesis.

There are a variety of possible probiotics with high BSH activity that drastically lower cholesterol levels. Probiotics with BSH activity have been reported in *Lactobacillus* and *bifidobacterial* species such as *B. longum*, *L. salivarius*, *L. plantarum,* and *L. reuteri*. By dint of these probiotics, an effective therapeutic approach to manage various metabolic abnormalities and inflammatory or pathogenic conditions can be planned.[54] Introduction of novel BSH active probiotic strains can be used to deconjugate BAs as FXR agonists.

Hydroxysteroid dehydrogenase

The HSDH enzyme possesses local and stereotypical characteristics and can transform the hydroxyl group of steroid acids. In order to neutralize the detoxifying effect of bile acid and make the intestinal niche microbial-friendly, these enzymes facilitate the generation of hydrophilic bile acids pool by the reversible oxidation mechanism.

Bacteroidetes, *Eubacillus*, *Clostridium*, *Bifidobacterium*, *Lactobacillus*, *Streptococcus peptidis*, and *Escherella* species are the ones exhibiting this enzymatic property. Genes associated with these enzymes, 12α-HSDH, 7α-HSDH, 3α-HSDH, and 3β-HSDH, have also been reported.[55]

7α/β Dehydroxylation

7α-dehydroxylating bacteria account for less than 1% of the total microbiome.[2] The BSH-active intestinal bacteria initially deconjugate primary bile acids CA and CDCA, a pre-requisite step before 7α/β-dehydroxylation to ease their entry into the microbes via proton-dependent bile acid transporter baiG.This allows the conversion of primary bile acids (CA and CDCA) into secondary bile acids (DCA and LCA).

Currently, only the *Clostridium* genus and some strains of *Lachnospiraceae* and *Peptostreptococcaceae* in intestinal bacteria are known to have 7α-dehydroxylation activity, and these bacteria are known as 7α-dehydroxylating bacteria. There are three bile acid-inducible (*bai*) genes in the bacteria that express three different 7α-dehydroxylase enzymes. There are three key enzymes in this pathway, which are encoded by *baiA2*, *baiB*, and *baiE* genes, which produce the 3α-hydroxysteroid dehydrogenase, coenzyme A-linked enzyme, and 7-alpha dehydrase, respectively. In the presence of cholic acids, the expression of *bai* can be upregulated.[56]

The cascade of reaction advents in the microbes with CoA ligase that catalysed ATP-dependent conjunction of bile salt to CoA in the presence of Mg^{2+} cation. Secondly, it involves the oxidation of bile acid- CoA thioester by 3α-hydroxysteroid dehydrogenase. Further, NAD-dependent 7-α dehydrase makes the product stable for further dehydration. Then, multiple series of oxidoreductase activities finalize the secondary bile acid production[57].

Miscellaneous reactions

Though the role of microbes in regulating bile acid by esterification and desulfurization is not clearly understood, it is noteworthy that these processes trigger the proliferation of 7α-dehydroxylating bacteria, which accounts for 0.0025% of the total microbiome. Thus, *Desulfovibrionales enriched microbiome utilizes the taurine as a source of sulfur and produces H_2S that elicits the activation of* 7α-dehydroxylase resulting in the elevated production of secondary bile acids[57]. Other bacteria responsible for desulphurization are *Clostridium* and *Gastrococcus.*[22] Increased production of secondary bile acids promoted cholesterol absorption and its metabolite H_2S expression and inhibited CYP7A1 expression

Cholesterol reductase

Small amounts of cholesterol-lowering strains can be isolated from animal feces. The enzyme cholesterol reductase possessed by some of the gut microbes accelerates the conversion of dietary cholesterol into insoluble coprostanol that is expelled from the human body, thus lowering the serum cholesterol in the body. The amount of cholesterol can be decreased by either the process of assimilation/ precipitation or both. In-vitro cultivation of *Bifidobacterium breve* and *Lactobacillus amilovorus* in a nutrient broth with bile or taurocholic acid has proved that the byproducts of *Lactobacilli* will precipitate cholesterol and thus reduce its amount whereas *Bifidobacteria* possess the capacity of both the assimilating and precipitating cholesterol.[58] Thus, the experiment concluded that cholesterol levels decreased by virtue of the deconjugation phenomenon and not with the absorption.

A similar observation was seen with *Eubacterium* species. A significant amount of this species can be seized from the healthy group in contrary to the gallstone group. Moreover, a copious amount of co-prostanol has been found with gallstones, signifying that Eubacterium species possess this enzyme.[2]

Upregulation of mucin genes

Bile contains proteins categorized as nucleating and anti-nucleating agents that can facilitate or impede the crystallization of cholesterol.[41] Mucin is one of the high molecular weight biliary proteins secreted by gallbladder and biliary duct epithelium. Mucins are divided into three categories based on their structural properties: a) trans-membrane mucins, b) soluble mucins and c) secreted gel-forming mucins[4]. Under normal conditions, mucin provides protection and lubrication to the gastrointestinal ducts and lumens against the detergent effect of the bile.[12] Approximately 20 mucin genes have been reported so far.[41] Polymorphism in these genes may be the predisposing factor for lithogenesis, especially in MUC1, MUC2, MUCH3, MUC4, MUC5AC, MUC5B and MUC6. Usually, the MUC5AC gene expresses gel-forming mucin and MUC4 codes for trans-membrane mucin (also called the multifunctional protein) to participate in cell signaling and adhesion. Their upregulation will elevate the secretion of mucin that has been sorted as a nucleating agent in the mechanism of lithogenesis[4]. Aberration in the expression MUC4 and MUC5AC genes was observed in biliary tract malignancy.[59] Research has also observed the overexpression of genes MUC3 and MUC5B in the case of cholelithiasis.[60] The detailed study has proved the association of pigmented stone and overexpression of the MUC4 gene. By virtue of the role of MUC4 in adhesion, multiple strains of gram-positive microbes were found in alliance to the pigmented cholelithiasis made up of calcium salts of bilirubinate and palmitate[57]. Thus, mucin hypersecretion by the MUC4 gene is responsible for modulating pigmented gallstone formation. Activation of epidermal growth factor induced by lipopolysaccharides, a major component of the cell wall of gram-negative bacteria, results in the upregulation of the MUC5AC gene in the biliary epithelium. LPS markedly increased the expression of prostaglandin E2 (PGE2). Thus, through the use of prostaglandins, lipopolysaccharides, various bacterial toxins, the secondary inflammatory response, and toxic metabolites, biliary infection has the potential to have a significant impact on mucin secretion and characteristics.

Hence, MUC gene overexpression may lead to mucin hypersecretion in bile, which increases bile viscosity and paves the way for stone nucleation in the biliary system.[60] In comparison to gallbladders with cholesterol gallstones, those with brown pigment stones and mixed stones with a brown periphery had more abundant mucus in their mucosal tissue. Experimentally and clinically, it has been demonstrated that mucin acts as a pro-nucleating agent, initially forming a platform for precipitation or crystal growth, and later, as a connecting and molding the solidified amorphous material into gallstones.[60] The quantity and characteristics of the mucin released

by an inflamed gall bladder can cause the development of a gallstone nidus.

Biofilm formation

Another underestimated mechanism responsible for lithogenesis is the formation of biofilms in the microbial-laden stones, especially the pigmented stones.[5] The concentration, in this case, is anionic glycoprotein, glycocalyx. Functional genomics proved that microbes involved in biofilm formation were *Klebsiella* and *Enterococcus* found in pigment stones

Experiments on gallstones with scanning electron microscopy (SEM) in 1987 measured the attachment of bacteria to the gallstones, thus hinting about glycocalyx produced by the biliary microbiome. *In-vivo* study analyzed 85 cholecystectomy cases and included three different categories of stones: pigmented (32 patients), cholesterol (35 patients) and mixed (18 patients). Considering the results of the study, the pigmented calculus constituted bacteria, glycocalyx and organic solids. These results form the foundation of a new theory of pigment stone formation that blames bacteria and glycocalyx for the precipitation and subsequent aggregation of bilirubin pigment. The research by Tajeddin *et al*., 2016 composed of culturing and PCR-DGGE on gallstones.[61] The results of these culture methods demonstrated the correlation of gallstones and their biofilm-forming capacity.

Biliary microbiota, such as *E. coli* and other enteric organisms, produce anionic glycoprotein glycocalyx that adheres to the surface of an inflamed biliary epithelial membrane or implanted biomaterial to form a bacterial biofilm. Bacterial micro-colonies and glycoproteins that facilitate colonization and infestation are encircled by biliary sediments such as calcium bilirubinate crystals. The thick and often calcified biofilms cause biliary sludge. The biofilm on the surfaces of gallstones would provide the microbes with long-term defence against antimicrobials and high bile concentrations, as well as a stable platform from where it can continuously populate the intestinal environment.[26] The biliary sludge is dominated by various biofilm-forming bacteria, namely *E.coli*, *Proteus mirabilis* and *Salmonella*.

CONCLUSION

Delicate interaction among microbiome-bile acid-lithogenesis has been observed. The review has covered the gap in the knowledge regarding their dependency on each other. Studies are still on the way to find out whether the microbiome can be used as a therapeutic marker in the early detection of cholelithiasis.

The strategy of regulating the bile acid metabolism emphasizes the intake of probiotics. Owing to the emergence of new BSH active probiotics, these strains can be tested to generate deconjugated BAs as FXR agonists. The proposal of using Probiotics as well as prebiotics as a novel approach needs validation for treating gastrointestinal ailments linked to bile acid metabolic disorders.

The linkage studies have approved the relationship between diet, biliary microbiota and cholelithiasis. In the case of lithogenesis, the structure of gut microbiome was changed. Dairy consumption has inhibited the growth of *Bacteroidaceae* and *Bacteroides* contrary to several types of fiber, phenolics, and fatty acids has encouraged the existence of *Bacteroidaceae*, *Chitinophagaceae*, *Propionibacteraceae*, *Bacteroides*, *Escherichia* and *Shigella*.

Promoting the consumption of prebiotics, (i.e., fibre rich diet/ roughage) as well as probiotics (gram-positive *Lactobacillus* and *Bifidobacterium with enhanced BSH activity)* or other nutritional therapies may aid in re-establishing the healthy gut microecology. It is noteworthy that multiple reviews have strengthened the fact that *L. acidophilus*, *B. lactis* and *L. plantarum* has the potential to reduce cholesterol levels, thus preventing lithogenetic conditions.

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CONFLICT OF INTEREST

None declared.

ETHICAL APPROVAL

Not required.

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