Another renaissance for bile acid gastrointestinal microbiology

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Abstract

The field of bile acid microbiology in the gastrointestinal tract is going through a current rebirth after a peak of activity in the late 1970s and early 1980s. This renewed activity is a result of many factors including the discovery near the turn of the century that bile acids are potent signaling molecules, technological advances in next-generation sequencing, computation, culturomics, gnotobiology, and metabolomics. We describe the current state of the field with particular emphasis on questions that have remained unanswered for many decades in both bile acid synthesis by the host, and metabolism by the gut microbiota. Current knowledge of established enzymatic pathways including bile salt hydrolase, hydroxysteroid dehydrogenases involved in the oxidation and epimerization of bile acid hydroxy groups, the Hylemon–Björkhem Pathway of bile acid C7-dehydroxylation, and the formation of secondary allo-bile acids are described. We cover aspects of bile acid conjugation and esterification, and evidence for bile acid C3 and C12-dehydroxylation that are less well understood, but potentially critical for our understanding of bile acid metabolism in the human gut. The physiological consequences of bile acid metabolism for human health, important caveats and cautionary notes on experimental design and interpretation of bile acid metabolism are also explored.

Introduction

Intestinal digestion in all vertebrates is a complex physicochemical process whereby an animal obtains energy and nutrients to sustain life. Lipid digestion in the aqueous environment of the small intestine is a special problem due to the intrinsic insolubility of dietary lipids. Natural selection solved this problem in vertebrates through the development of amphipathic detergents known as bile acids formed via the enzymatic ring-system and side-chain oxidation of cholesterol in the liver[1–5]. These detergent molecules also activate complex cellular signaling pathways that regulate bile acid and cholesterol homeostasis[6], glucose and lipid metabolism in the liver[7], energy homeostasis[8], and inflammation[9].

Bile has long been known to have a critical role in lipid absorption as impairment of bile flow into the intestine results in malabsorption of fat (steatorrhea), and malabsorption of lipid-soluble vitamins[10]. The function of bile acids as detergents is implicit in their chemical structures. The two-faced Roman god Janus represents transitions, dualities, and boundaries between territories. In general, primary bile acids are 'Janus-like' in that they possess two faces, structural dualities, and boundaries: one hydrophobic, the other hydrophilic (FIG. 1; Table 1). The hydrophilic face (α-face) operates largely at the surface of mixed micelles, facing the aqueous milieu of the intestinal lumen, whilst their hydrophobic face (β-face) points inwards towards interior lipids[11,12]. Evidence early in the 20th century began to establish that intestinal bacteria modify the structures and functions of bile acids produced by the host liver, even blurring the boundary between their 'faces' (e.g., ursodeoxycholic acid [UDCA], which derives from 'flipping' the C7α hydroxyl group from CDCA to the β-face). This knowledge has catalyzed a major revolution that is rediscovering fundamental research during the later decades of the 20th century, which lacked the technologies available today.

Having been regarded for much of their history as mere detergents (or before this as acrimonious laxatives)[12] and, as a consequence, interest in the chemistry and biology of bile acids largely waned in the 1990s as gallstone dissolution gave way to laparoscopic cholecystectomy[12]. However, interest in the carcinogenic role of deoxycholic acid (DCA) in the gastrointestinal tract[13–16], and indeed the role of DCA in the formation of certain types of cholesterol gallstones persisted[16]. Today, there is renewed interest in bile acids because of the discoveries, starting in 1999, that bile acids act as powerful nutrient signaling hormones, whose structures are negotiated through the actions of eukaryotic, bacterial, and archaeal enzymes[17].

A structure–function relationship is evident in the ability of distinct bile acids to differentially act as ligands for a complex array of nuclear receptors such as the farnesoid X receptor (FXR, also known as NR1H4)[18–20], pregnane X receptor (PXR, or NR1I2)[21], vitamin D_3 receptor (VDR)[22], and the constitutive androstane receptor (CAR, or NR1I3)[23]. Bile acids are also ligands for G protein-coupled receptors, such as TGR5 (GPBAR1)[24,25], muscarinic receptors (CHRM2, CHRM3)[25], sphingosine-1-phosphate receptor 2 (S1PR2)[26], retinoic acid receptor gamma T (RORγT)[27,28], nuclear receptor 4A1 (NR4A1)[29], and downstream effectors have emerged in multiple digestive organs to sense and respond to

nutrient and metabolic status by sampling bile acids[7,30], and thereby influence intestinal inflammation and gastrointestinal cancers[31,32].

Strikingly, derivatives of hydrophobic secondary bile acid products of microbial metabolism, such as DCA and lithocholic acid (LCA), are in general preferred ligands for many of these host receptors over the primary bile acids from which they derive[30]. What has emerged in the past few decades is that the 'Western lifestyle' of inactivity, diets low in fiber and high in processed carbohydrates and saturated fats, increase both the amount of bile entering the gastrointestinal tract and the hydrophobicity of the bile acid pool, thereby increasing the risk of hepatobiliary and gastrointestinal cancers in humans[32,33]. In their own way, the microorganisms are 'talking', and we have developed the analytical tools to 'listen', and hopefully will learn to act accordingly to modulate the numerous human disorders and diseases affected by microbial bile acid metabolism.

Humans are composed of roughly a trillion mammalian cells and harbor an estimated equivalent number of microorganisms that dwarf us in gene content[34,35]. It is now apparent from comparisons between conventional and germ-free rodents that the host genome and the microbiome (collective microbial genomes) together contribute substantially to the complex repertoire of small molecules in biological tissues and fluids collectively known as the metabolome[36,37]. Such studies established early on, and beyond doubt, that the gut microbiota deconstruct host-derived primary bile acids into a myriad of metabolites known as secondary bile acids during enterohepatic circulation (FIG. 2)[38]. Indeed, bile acid modification has been identified as one of the major bidirectional modes by which microorganisms communicate with their host, and how the host senses, responds to, and shapes the composition of its symbionts[7,37]. In this Review, we focus on the production of and modifications to the secondary bile acids, DCA and LCA. In addition, we describe lesser-known bile acid dehydroxylation reactions, how these reactions might alter the fecal bile acid pool, and experiments that will be needed in the future to confirm and quantify these novel microbial metabolites. We also summarize the latest findings on how secondary bile acid derivatives affect host immune function.

Bile acid synthesis and composition

Liver metabolism is primarily conjugative and oxidative. Chenodeoxycholic acid (CDCA) is considered the root of all C24 bile acids in vertebrates (FIG. 1, Table 1)[12]. In humans, bile acid synthesis yields CDCA and cholic acid (CA) in roughly equal amounts. The enzymology and regulation of genes involved in the formation of the primary bile acids CA and CDCA are well established[6,39]. Most of the total bile acid pool is produced in the liver by the classical or neutral pathway, whereas the alternative (or acidic pathway) contributes, mostly in the form of CDCA, a smaller portion to the pool (10%)[6,40]. In both humans and rodents, there are sex and age differences in the rate of bile acid synthesis and the composition of the biliary pool[41]. Important species differences exist between humans and the model organisms we approximate to our physiology. For example, in mice and rats, the biliary pool is composed of the primary bile acids CA and CDCA, as well as muricholic acids, primarily β-muricholic acid (β-MCA), and small quantities of the C7 epimer of CDCA, UDCA[6]. The enzymes involved in the formation of MCAs have been identified and

their disruption through gene knockout has provided researchers with mouse models that better approximate the human biliary pool (synthesis of CDCA and CA) although there are reported trade-offs as rodents are not well adapted to the more hydrophobic biliary pool of humans[42,43].

Bile acids are amidated to the amino acids glycine or taurine in the human liver. In humans, glycine predominates by an order of magnitude over taurine on a plant-based diet, but this feature can be reversed by a diet high in animal protein[44]. In rodents, bile acids are conjugated almost exclusively to taurine, irrespective of diet[45]. During the enterohepatic circulation, conjugated bile acids are secreted into the small intestine where they form mixed micelles with dietary lipids enabling absorption, before being transported back to the liver through the portal circulation via high-affinity transporters on the apical and basolateral sides of enterocytes (FIG. 2). Each day, several hundred milligrams of conjugated bile acids escape enterohepatic circulation, and enter the large intestine, where they are rapidly deconjugated by bacterial bile salt hydrolases (BSH), releasing taurine or glycine and free bile acids[46]. Several pathways for taurine utilization exist in the gastrointestinal tract[47], however, microbial respiration of taurine by anaerobes results in the formation of hydrogen sulfide, excess formation of which is associated with colorectal cancer (CRC)[48,49].

Bile acids are among the most structurally diverse biomolecules in nature[2]. Bile acid structure varies with respect to side-chain length (C27 versus C24), side-chain carboxylation (bile acids) versus hydroxylation (bile alcohols), side-chain conjugation (amino acids versus sulfate), A/B ring *cis* (5β-H; non-planar ring system) versus *trans* (5α-H; planar ring system) stereochemistry, and hydroxylation patterns and hydroxyl stereochemistry (α, β, and Ω)[2]. Animal bile has a long history in ethnopharmacology, which Western medicine has been slow to examine critically[4]. Notable exceptions include naturally occurring bile acids such as ursodeoxycholic acid (UDCA) that has wide usage in the treatment of biliary disorders[50], and avicholic acid, which has therapeutic potential[51]. The extensive work of Haselwood[1,3] and Hofmann and Hagey[2,52] provided extensive surveys of biliary bile acid composition across vertebrates, suggesting the diversity and pattern of bile acid structure has evolutionary significance[2]. Strikingly, no clear pattern exists between dietary strategy (such as carnivore, herbivore or omnivore) and bile acid composition, suggesting other evolutionary forces are driving bile acid diversification (e.g., interspecies and intraspecies communication and developmental and cellular signaling)[52]. Comprehensive fecal bile acid profiles reflecting bacterial bile acid metabolism across vertebrates are beginning to be reported[53]. It is known in at least some species that the fecal bile acid profiles differ little from the liver, indicating selection for microbiota that leave bile acids intact[54].

A substantial gap in our knowledge still exists with respect to the synthesis of bile acid A/B-ring *trans*-isomers known as allo-bile acids. Primary allo-bile acids are produced in miniscule quantities in healthy adults, whereas they are produced at more substantial amounts in neonates, after liver transplantation in adults, and during proliferation associated with hepatocellular carcinoma[55,56]. By contrast, allo-bile acids are primary bile acids in agnathan fishes (lampreys and hagfish) and cyprinid fishes (such as zebrafish and carp), as well as some reptiles, and ancestral mammals such as the Afrotheria (such as elephants, manatees, and tenrecs) and rhinoceros[2,57]. It is predicted that SDR5A1 and/or SDR5A2

are responsible for bile acid 5α-reduction in humans, and orthologues of these genes have been found in zebrafish[52]. Presumably, the production of allo-bile acids in humans activates developmental signaling pathways associated with hepatic growth; however, research in this area is currently lacking. Work published in 2021 has indicated that the gut microbiome is capable of producing small quantities of allo-secondary bile acids (such as isoallolithocholic acid [isoalloLCA]) that are enriched in Japanese centenarians[58]. Whether the source of planar secondary bile acids such as isoalloLCA derived mainly from primary bile acids (for example, CDCA) or from primary allo-bile acids (for example, allochenodeoxycholic acid [alloCDCA]), or both, remains unclear, and will be a topic discussed at length later in this Review[58–60].

Bile acid metabolism by gut microbiome

Bile acid deconjugation or conjugation

The earliest evidence for microbial metabolism of host primary bile acids was the deconjugation of conjugated bile acids by mixed fecal bacteria and microbial isolates[61]. BSH (E.C.3.5.1.24) is a member of the choloylglycine hydrolase family along with penicillin V amidase of the Ntn-hydrolase superfamily of proteins[46]. BSH enzymes are some of the most studied microbial bile acid-metabolizing enzymes and have been extensively reviewed[62–66], so our comments highlight key points and latest advances. BSH enzymes are widely represented in a variety of species distributed across most phyla in the gut microbiome. BSH activity has been characterized in Gram-positive commensal bacteria including *Lactobacillus*[67,68], *Bifidobacterium*[69], and *Enterococcus*[70]. BSH activity is also represented in Gram-negative bacteria, especially widely distributed among species of the genus *Bacteroides*[71,72]. Archaea common to the mammalian gastrointestinal tract have also been reported to express BSH[73]. Phylogenetic analysis indicates horizontal gene transfer of *bsh* genes from members of the Bacillota to gut methanogens[73]. BSH is necessary for gastrointestinal colonization by pathogens including *Brucella abortis*[74,75] and *Listeria monocytogenes*[76–78]. The sensing of conjugated bile acids provides an important environmental signal of gastrointestinal colonization, distinct from the secondary bile acids common in soil. It is hypothesized that *bsh* genes are colonization factors that provide some combination of: amino acid sources of carbon, nitrogen, sulfur, and energy; protection against the detergent properties of conjugated bile acids allowing gastrointestinal colonization and persistence; and incorporation of bile acids into bacterial membranes, which increases tensile strength, fluidity, and charge, thus protecting bacteria against host immune function[65,79]. Caution should be taken against generalization of *bsh* function across taxa, and where possible, isogenic *bsh* mutants should be generated to determine both bacterial physiological function and consequences of *bsh* loss on host physiology[65,71,73].

In healthy individuals, the deconjugation of bile acids is a substrate-limiting reaction and goes essentially to completion. This feature is assumed to be true in most vertebrates, with notable exceptions such as the cyprinid fishes (such as zebrafish), whose side-chain sulfate is retained during intestinal transit[54,80–82]. The fecal composition of conjugated bile acids increases substantially in mammals that are taking broad-spectrum antibiotics[83]. By contrast, BSH activity in the small bowel generates unconjugated bile acids that are less

polar than conjugated bile acids, and inefficiently transported by ileal sodium/bile acid cotransporter (IBAT or *SLC10A2*) in the ileum leading to increased fecal excretion[84,85]. Increased fecal excretion of bile acids leads to more de novo bile acid synthesis from cholesterol or reverse cholesterol transport to the liver, reducing serum cholesterol. There is evidence that BSH activity leads to cholesterol lowering through reduction in micellar lipid and cholesterol reabsorption and loss of bile acids in feces, increasing cholesterol conversion to bile acids[64,86–88].

Conversely, prior studies in poultry indicated that sub-therapeutic levels of antibiotics promote growth, at least in part, through the reduction in intestinal BSH activity resulting in an improvement in lipid digestion[89,90]. Thus, targeting BSH activity in malnourished populations might improve weight gain partially through enhanced lipid absorption. Indeed, a longitudinal study in children supports an inverse association between BSH and weight gain caused by macrolide antibiotic use[91]. By contrast, other studies suggest increased BSH activity leads to enhanced weight gain[92]. The latest 'omics' applications reveal that the physiological consequences of BSH activity to the host might be mediated more through cellular signaling in the intestine and liver than through mere detergent actions and fat absorption. BSH activity affects host gastrointestinal maturation[93], alters liver and intestinal gene expression relating to circadian rhythm, glucose and lipid homeostasis in the liver, and immune function[71,94]. As BSH enzymes differ in substrate specificity relating both to the amino acid conjugate (glycine versus taurine) and the sterol nucleus, targeting subsets of BSH enzymes is likely necessary to achieve a clinical outcome of interest. Indeed, large scale metagenomic surveys reveals associations between the pattern of *bsh* genes in both human populations[95] and chronic diseases[96] suggesting further functional characterization is warranted. Specific inhibitors targeting BSH enzymes have been developed, and further refinements in pharmacological inhibition of different subsets of these enzymes might be therapeutically useful both in human disease and animal production[97,98].

Metabolism of tertiary bile acids

Gut microbial products are capable of modulating hepatic conjugation of bile acids, with potential for therapeutic benefits. The term tertiary bile acid has been applied to describe unique bile acids formed from hepatic metabolism of secondary bile acids[12]. Phase II metabolism of the toxic secondary bile acid LCA yields the tertiary bile acid 3-sulfo-LCA[99]. The microbial formation and ileal absorption of LCA can also lead to sulfation of other bile acids in the liver. Indeed, a study published in 2021 observed LCA-induced enrichment of cholic acid-7-sulfate (CA7S) in the feces of humans and mice subjected to partial sleeve gastrectomy[100]. CA7S is a gut-specific apical activator of TGR5 expression, which can lead to secretion of glucagon-like peptide 1 (GLP-1) conferring anti-diabetic effects[100].

Gut microorganisms are also capable of removing the sulfate from tertiary bile acids through expression of aryl sulfatase enzymes[101–103]. Desulfation activity is associated with *Peptococcus*, *Clostridium*, *Pseudomonas*, and *Fusobacterium*[103–106]. However, the microbial sulfatases involved have yet to be identified. The gut microbiota might also be capable of bile acid sulfation in the gut, a function that has historically been assumed to be host-enzyme dependent[107]. If confirmed, sulfation of LCA by bacteria would blur the

distinction between so-called secondary and tertiary bile acids. In the absence of a final consensus relating to host versus microbial biochemical potential, bile acid sulfation by gut microprganisms would further support the suggestion to abandon the term "tertiary bile acid" altogether[108].

MCBAs and bile acid polyesters

The advent of next generation sequencing coupled with computational progress has driven a renaissance in gut microbiology led largely by bioinformatics[109,110]. Powerful advances in untargeted metabolomics have paved the way for discovery of novel microbial metabolites by so-called cheminformaticians[111]. Indeed, this cheminformatics approach has led to the startling identification of microbially conjugated bile acids (MCBAs) in which non-canonical amino acids (such as amino acids other than glycine or taurine) are amidated to free bile acids by gut bacteria such as *Enterocloster boltaea*[112]. It seems that the BSH enzymes are responsible for generation of MCBAs, and that patterns are emerging between BSH amino acid sequences and amino acid conjugation specificity[113]. Although it is widely understood that microbial biotransformations in the gut are limited by fecal analysis, new technologies are emerging that promise to enable clinicians and microbiome scientists to sample along the gastrointestinal tract using pill-like sampling devices that can be swallowed by individuals[114]. Indeed, this approach revealed that MCBAs are generated largely in the small bowel where BSH enzymes are active[114]. Evidence indicates that MCBAs can signal through PXR and FXR[115]; however, their physiological relevance is only beginning to be understood.

Bacteria in the gastrointestinal tract can also esterify bile acids with alcohols, short-chain fatty acids (SCFAs), and long-chain fatty acids. Methyl esters of DCA are formed by human fecal isolates, and whilst the mechanism is not known, it was hypothesized that the reaction is dependent on C_1-transfer (methyl or methoxyl group) to the C_{24} carboxyl group rather than methanol as a substrate[116]. By contrast, esterification of bile acids associated with *Lactobacillus, Eubacterium,* and *Bacteroides* was reported to be dependent on the addition of ethanol[117]. Bacteria also generate bile acid fatty acid esters in which long-chain fatty acids (C16 and C18-fatty acids) as well as SCFAs such as acetate are linked to C3 of isoDCA and isoLCA[118]. Several reports describe the oligomerization of C-24 carboxyl group of one molecule of DCA to the 3α-hydroxy group of the another to form a polyester chain[119]. It is presumed that these reactions are a detoxification strategy to precipitate bile acid esters, therefore reducing the concentration of both hydrophobic secondary bile acids and toxic fatty acids and alcohols from fecal water[120].

Despite progress in LC/MS approaches to bile acid profiling, bacteria generate a number of bile acid structural and stereoisomers (such as DCA and alloDCA, isoCDCA and isoDCA), making separation and detection of some bile acids challenging. By contrast, bile acid esters are rarely measured in fecal samples[118]. Comparison of stool samples from healthy individuals with or without strong alkaline hydrolysis revealed that between 10–30% of total bile acids (primarily isoDCA and isoLCA) are esterified[118]. The diversity and quantity of bacterial bile acid conjugates therefoee represents an important consideration when designing fecal bile acid extraction protocols to address particular clinical and research

questions. Methodological advances can now be harnessed to identify novel bile acid metabolites, discover new bile acid metabolizing enzymes, and alter bile acid metabolism through targeting microbial strains and biochemical pathways (FIG. 3).

The Hylemon–Björkhem Pathway

Historically, bile acids designated primary were defined as possessing a 7α-hydroxyl group, and removal of the 7α-hydroxyl group by the action of microbial enzymes defined secondary bile acids[38]. Today, secondary bile acids are regarded as microbial reaction products (with the exception of C24 deconjugation) of host primary bile acids, and include products of microbial oxidation, epimerization, and dehydroxylation products of CA and CDCA in humans (FIG. 2) and expand to other products of primary bile acids in rodents (e.g., UDCA and muricholic acid metabolites), and pigs (e.g., hyocholic acid metabolites). This diversification of bile acid structure indicates that numerous enzymatic pathways exist, distributed across bacterial and archaeal phyla inhabiting the gastrointestinal tract. The collective genes involved in bile acid and sterol biotransformations have been termed the sterolbiome (BOX 1)[121,122].

Excess hydrophobic secondary bile acids have been long associated with cancers of the gastrointestinal tract[31] and cholesterol gallstone formation[15]. For this reason, determining which gut bacteria are responsible for forming DCA and LCA, the bile acid intermediates formed during this conversion, the genes encoding enzymes catalyzing these biotransformations, and the mechanism(s) by which each reaction is catalyzed are important in interpreting microbiome data and devising future interventions to modulate the bile acid metabolome to treat disease.

Two models have been proposed for bile acid 7-dehydroxylation. The first was a two-step mechanism we termed the Samuelsson–Bergstrom model that was proposed in 1960[123]. This model, arrived at by a series of elegant and rigorous in vivo experiments, proposed a diaxial trans-elimination of the 7α-hydroxyl group of CA yielding a Δ^6-intermediate (3α,12α-dihydroxy-5β-chol-6-enoic acid), followed by trans-hydrogenation resulting in DCA. Synthesis of 3α,12α-dihydroxy-5β-chol-6-enoic acid and incubation with cultures of *Clostridium bifermentans*[124] and *Clostridium scindens*[125] resulted in conversion to DCA providing some early support for the model. However, in vitro and in vivo studies during the 1980s and 1990s demonstrated that while there is indeed a Δ^6-reduction step (catalyzed by BaiH and BaiN), the pathway is far more complex than initially thought[38]. The bile acid intermediates in a multi-step bifurcating pathway from CA to DCA and its 'flat' isomer, alloDCA have been identified in cell extracts of *C. scindens* VPI 12708[126]. A reverse genetic approach facilitated cloning of the ~12 kB bile acid inducible (*bai*) operon from *C. scindens* VPI 12708[127]. Subsequent work with purified and recombinant Bai enzymes over several decades[128] led to identification of Bai enzymes catalyzing each step of what we have termed the Hylemon–Björkhem Pathway[38]. Elegant confirmation of the *bai* operon catalyzing the conversion of CA to DCA both in vivo and in vitro has been reported[129]. The measurement of *bai* genes in human stool samples is now becoming standard as a marker for bile acid dysbiosis in the case of IBD[130], antibiotic treatment[131], or excess in the case of cancers of the gastrointestinal tract[132].

Several key aspects of bile acid 7-dehydroxylation have emerged over the course of study in this area. First, bile acid C24 amides (conjugated bile acids) are not substrates[133–135]. Thus, bile acid hydrolysis is a prerequisite for bile acid 7-dehydroxylation. This is important to note as BSH inhibitors have indeed been observed to enrich the host in primary bile acids[98]. Second, bile acid 7-dehydroxylation seems to be relegated to relatively small populations (10^3-10^7 CFU per gram wet weight feces)[15] of species within the Bacillota (*Ruminococcaceae*, *Peptostreptococcaceae, Lachnospiraceae*, and *Oscillospiraceae*)[136–138]. Third, these populations of species have been separated into two groups (low activity versus high activity) by their relative rates of conversion of CA to DCA, which differs ~100 fold (Table 1; Box 2)[139]. Fourth, despite these small populations, defined communities of microorganisms with complexities ranging from a handful up to 100 members demonstrates so far that organisms with the *bai* operon are necessary for DCA and LCA formation[140–143]. Fifth, microbial *bai* pathway enzymes have evolved to recognize the bile acids endogenously produced by the hosts they are adapted to. Thus, although rodent gut microbial isolates can convert β-muricholic acid (β-MCA) to murideoxycholic acid (MDCA), human gut microbiota that are able to convert CA to DCA and CDCA to LCA are unable to metabolize β-MCA when colonized in germ-free mice[140,144]. The only exception is UDCA, which can be 7β-dehydroxylated to LCA (FIG. 4)[38,121]. Sixth, the *bai* pathway is a redox process yielding a net 2-electron reduction (that is, bile acids as an electron acceptor) that provides an important proximal cause for its evolution. In the large intestine (a highly reductive, anaerobic environment), microorganisms must dispose of reducing equivalents, and reducing unsaturated bile acids during dehydroxylation accomplishes this feat to a degree. However, there might be underlying ultimate causes of equal or greater importance, which include but are not limited to: elimination of microbial competition for key nutrients by increasing toxic bile acids, and interdomain-signaling with the host to improve the fitness of DCA producers in the gut.

Although hydrophobic secondary bile acids such as DCA and LCA are strongly implicated in cancers of the gastrointestinal tract, their production has important physiological functions to host immune function[27,28,145,146], serotonin production[147], cellular signaling[16,30], prevention of *Clostridioides difficile* colonization and vegetative growth[131], and enteric viral infection[148]. By adopting diets lower in animal protein and fat, and higher in complex carbohydrates and fiber, intestinal bile acid levels can be lowered such that the benefits of hydrophobic bile acids can be maintained whilst reducing the risks associated with elevated fecal levels and enrichment of the bile with DCA[32].

Patients with cirrhosis have a reduced bile acid pool relative to control, substantial reduction in the abundance of bile acid 7α-dehydroxylating bacteria with reduced fecal DCA and LCA, and there is a concomitant dysbiosis characterized by toxic Gram-negative gut microbiota[149,150]. When patients with cirrhosis receive a liver transplant, an increase in bile acid secretion, increased fecal secondary bile acids, and diversification of the gut microbiome along with reduced systemic inflammation are observed[151]. Faecal microbiota transplant (FMT) restored cognitive function and improved inflammation with concomitant increase in Gram-positive bacteria associated with secondary bile acid formation, along with increases in DCA and LCA in stool[152]. Patients with poor outcomes were observed to have markedlt lower levels of secondary bile acids in serum and feces and diminished bacterial

genes associated with secondary bile acid formation[152]. These results indicate an important role for the maintenance of basal levels of hydrophobic secondary bile acids and the liver–gut axis.

Formation of allo-secondary bile acids

Two pathways to allo-secondary bile acids have been elucidated. The first pathway we have termed the 'direct pathway' in which primary bile acids are converted to alloDCA or alloLCA through the Hylemon–Björkhem Pathway[38]. After the rate limiting 7α-dehydration step (catalyzed by BaiE), a 3-oxo-4-DCA or 3-oxo-4-LCA intermediate is formed. In the Hylemon–Björkhem Pathway, the conversion of 3-oxo-4-DCA to DCA proceeds via a reduction catalyzed by BaiCD (bile acid 5β-reductase) and BaiA (3α-HSDH)[38,60]. Alternatively, 3-oxo-4-DCA can be converted to alloDCA through reduction by BaiP or BaiJ (bile acid 5α-reductase) and BaiA[60]. The second pathway is what we have termed the indirect pathway and first requires a bile acid 7-dehydroxylating bacterium to produce DCA or LCA. In this scheme, members of the gut microbiome expressing 3α-HSDH, bile acid 5β-reductase, and bile acid 5α-reductase generate allo-secondary bile acids through a metabolic equilibrium: DCA, 3-oxo-DCA, 3-oxo-4-DCA, 3-oxo-alloDCA, alloDCA[58,153]. The relative contribution of the direct and indirect pathways to the formation of secondary allo-bile acids is currently unclear and may exhibit both intra- and inter-individual variation.

Bile acid C3-dehydroxylation

Lithocholic acid, a microbial product of C7-dehydroxylation of CDCA and UDCA, is mono-hydroxylated and the most hydrophobic of the prominent bile acids detected in vertebrates. LCA is a suspected carcinogen, generating reactive oxygen species and DNA adducts[154]. LCA acts as a tumor-promoter through the inhibition of DNA repair enzymes that drives the proliferation of apoptosis resistant cells[154]. Studies of LCA metabolism in humans during CDCA or UDCA treatment of gallstones revealed extensive sulfation of LCA yielding 3-sulfo-LCA[155,156]. It is now well understood that LCA is a strong ligand for the vitamin D receptor (VDR), which induces sulfotransferase SULT2A1 expression[99]. The sulfation of LCA, during phase II metabolism, generates a hydrophilic derivative that is not readily absorbed by the intestine, facilitating excretion. As noted earlier, gut bacteria express aryl sulfatases that deconjugate 3-sulfo-LCA leading to release of LCA. Thus, there is a predicted 'back and forth' between host phase II metabolism and microbial deconjugation. Yet, there is evidence for an alternative microbial pathway out of this cycle (described later), yielding products that are no longer defined as bile acids.

Although the focus of bile acid dehydroxylation is principally with respect to the Hylemon–Björkhem Pathway, there are reports of other bile acid dehydroxylation reactions, which includes C3-dehydroxylation of bile acids. Bile acid C3-dehydroxylation is particularly interesting in that removal of C3 changes the designation from bile acid to derivatives of 5β-cholanic acid (FIG. 4). This process presents a particular difficulty with respect to bile acid metabolic profiling as cholanic acids are almost never measured during bile acid analysis and their origins from microbial bile acid metabolism cannot be made certain without proper stable isotope labeling in vivo to establish that fecal cholanic acids derive from bile acids.

Studies from independent labs report that human fecal suspensions convert 3-sulfo-LCA to isoLCA, Δ^3-cholenic acid, and 5β-cholanic acid[157,158]. The addition of vancomycin to fecal suspensions inhibited 3-sulfo-LCA metabolism; however, methods to select for Gram-positive spore-forming bacteria (such as heat and alcohol treatment of feces) enriched for 3-sulfo-LCA metabolism[157]. Pure cultures of clostridia were able to generate isoLCA, Δ^3-cholenic, and 5β-cholanic acid from 3-sulfo-LCA (FIG. 4)[157]. The authors speculated that C-O bond cleavage with inversion, indicative of aryl sulfatases, results in formation of isoLCA[157]. Notably, this process represents a potential alternative route for isoLCA formation distinct from oxidation and epimerization of LCA (described later)[157]. In this scheme, isoLCA is dehydroxylated to Δ^3-cholenic acid and reduced to 5β-cholanic acid. Alternatively, it is possible that *trans*-elimination of the sulfo-ester yields Δ^3-cholenic acid, which is reduced to 5β-cholanic acid. Intriguingly, a study reported that CDCA is converted to 7α-hydroxy-5β-cholan-24-oic acid in human fecal suspensions[159], suggesting that the Hylemon–Björkhem pathway, yielding LCA from CDCA, need not precede C3-dehydroxylation. Further work is needed to firmly establish C3-dehydroxylation, its mechanism(s), and determine the substrate range for bile acid C3-dehydroxylation.

The physiological consequences of 5β-cholanic acid are unclear, but enhancement of bile acid C3-dehydroxylation might represent a strategy to reduce bile acid concentration in the gastrointestinal tract to prevent gastrointestinal cancers, similar to the proposed enhancement of cholesterol conversion to coprostanol by gut bacteria to reduce serum cholesterol in the prevention of cardiovascular disease[160,161]. In support of this theory, a series of 5β-cholanic acid derivatives were shown to activate FXR more potently than hydroxylated bile acids in a reporter gene assay[162]. A later pharmacological screen of a small library of 5β-cholanic acid derivatives identified for the first time potent FXR agonists or TGR5 (GP-BAR1) antagonists derived from this steroid backbone[163]. Notably in the context of hepatogastroenterological disorders, use of an orthotopic mouse model of hepatocellular carcinoma demonstrated that administration of the FXR agonist obeticholic acid, to mimic primary bile acids, together with the TGR5 antagonist 5β-cholanic acid, to block the downstream signaling of secondary bile acids, exhibited substantial tumor suppressive effects through interactions between liver sinusoidal endothelial cells and natural killer T cells mediated by paracrine chemokine signaling[164]. Clearly, the extent to which host and/or microbial-derived 5β-cholane derivatives are physiologically relevant should be defined, with accurate measurements of such being a first step.

Bile acid C12-dehydroxylation

The removal of the C12 hydroxyl group from bile acids would blur the line between the current distinction between CA metabolites (such as derivatives of DCA) and CDCA or UDCA metabolites (such as derivatives of LCA). Few studies have measured or confirmed the occurrence of C12-dehydroxylation and its prevalence in the human population. There are data demonstrating that the ratio of CA:CDCA metabolites in serum is variable in the population[165]. A higher ratio of 12α-hydroxylated bile acids (CA, DCA) versus non-12α-hydroxylated bile acids (CDCA and LCA) is associated with insulin resistance in humans[166], and the gut microbiomes of individuals with insulin resistance differ from those without[167]. It is unclear if this altered ratio reflects regulation in bile acid synthesis in the

liver (neutral pathway) and extrahepatic tissues (acidic pathway)[40], C12-dehydroxylation by the gut microbiome, or a combination of these factors. It was reported that eight human fecal *Bacteroides* isolates were capable of converting CA to CDCA through C12-dehydroxylation (FIG. 4)[168]. Unfortunately, however, serial transfers of the bacterial culture resulted in loss of dehydroxylation activity[168]. Yet, this aspect was also common in the history of isolation of bile acid 7-dehydroxylating strains[38]. Other studies, including another report further support the possibility that C12-dehydroxylation occurs in the human gut[169].

An intermediate expected in this pathway is the formation of an 11,12-unsaturated bile acid, which would be subsequently reduced. Studies assessing the stability of [11,12-^{3}H]CDCA and [11,12-^{3}H]LCA as tracers for isotope dilution studies during enterohepatic circulation in humans inadvertently provided evidence that bacteria can indeed (de)hydrogenate the C11–C12 bond[170]. Comprehensive and carefully designed studies are needed to resolve the uncertainty regarding C12-dehydroxylation in the human gut. In vitro and in vivo tracing of the fate of isotopically labeled CA is essential in resolving this important question in the field of bile acid microbiology.

Oxidation and epimerization of bile acids

The oxidation and epimerization of bile acid hydroxyl groups greatly expands the diversity of bile acid metabolites as each hydroxyl toggles between three stable positions (such as 3α-OH, 3-oxo, and 3β-OH) (FIG. 4). These reactions are catalyzed by regiospecific and stereospecific pyridine nucleotide-dependent hydroxysteroid dehydrogenases (HSDHs)[171]. Early work on these enzymes was important for identifying *Eggerthella lenta, Blautia producta, Clostridium absonum, Clostridium perfringens, Clostridium paraputrificum, Escherichia coli, Bacteroides fragilis,* and *Ruminococcus gnavus* as species capable of oxidation and reduction of bile acids (**Table 2**)[172–177]. The *hsd* genes encoding these enzymes were later identified and characterized in strains of these species[178–182]. The latest efforts are identifying a slate of other bacterial isolates capable of oxido-reduction of bile acids[28,169].

Important physiological consequences of oxo-bile acids and bile acid epimers have been identified. 7-oxo-CDCA, a product of microbial 7α-HSDH, competitively inhibits hepatic 11β-HSD2, therefore altering glucocorticoid metabolism[183]. Intriguingly, disruption in the activity of 11β-HSD1 isoform through genetic knockout markedly alters gut microbiome structure and function[184]. Secondary oxo-bile acids (such as 3-oxo-LCA) inhibit the development of T helper cells that express IL-17 (T helper 17 (T$_H$17) cells) in the gastrointestinal tract[27,29]. Three bacterial HSDH pathways exist for the metabolism of human bile acids, the genes of which are widely distributed among gut microbial species. CDCA and CA are reversibly oxidized and epimerized at C7 to yield urso-bile acids[172,180]. UDCA has a long history in treating biliary and gastrointestinal disorders and is a current maintenance therapy for primary sclerosing cholangitis[185]. CA and CDCA (as well as DCA and LCA) are also epimerized at C3 to form iso-bile acids[178,179,186].

Iso-bile acids, particularly isoDCA and isoLCA, are some of the most predominant secondary bile acids in feces, and the fraction returned to the liver in portal circulation are 'repaired' to the 3α-hydroxy orientation[187]. Iso-DCA has been shown to be less toxic to

Bacteroides spp.[178], and 'flat' secondary allo-bile acids (discussed later) such as isoalloLCA modulate colonic regulatory T (T_{reg}) cell function[27]. Only CA derivatives can be epimerized to so-called lago bile acids (Ancient Greek lagos [λαγώς, "hare"] also known as 12-epi bile acids)[117,181]. LagoDCA (3α,12β-dihydroxy) is predicted to have similar hydrophilicity to UDCA (3α,7β-dihydroxy), and was tested for decreased toxicity relative to its epimer DCA (3α,12α-dihydroxy) in a rabbit model[188]. Indeed, whilst lagoDCA was far less toxic than DCA, it was progressively epimerized in the gut to DCA, which was enriched in the bile[188]. Unlike rats, the liver of rabbits is incapable of converting DCA to CA, so DCA accumulates in rabbit bile, as is also observed in humans. Similar epimerization of UDCA to CDCA occurs in patients with gallstones, but unlike DCA, CDCA enrichment desaturates bile[188]. Bacterial enzymes in the epi-bile acid pathway have been reported (Table 2), reviewed elsewhere[171,179,181,182,189].

Effects of bile acids on host immune cells

Secondary bile acids have long been associated with gastrointestinal disorders associated with chronic inflammation including inflammatory bowel disease (IBD) and colorectal cancer (CRC) with a vast literature providing data consistent with multiple mechanisms of action including: direct cytotoxicity; direct DNA damage; inflammation associated with NF-κB activation; perturbation of cellular redox poise due to reactive oxygen species induction; and enhanced cell proliferation through activation of various cell cycle and inflammatory signaling pathways[31,32,190–193]. It is generally accepted that these effects reflect to varying degrees both the hydrophobic nature of secondary bile acids resulting in membrane damage to host cells and their activation of numerous cell signaling cascades through interaction with both cell surface and nuclear receptors. There are also numerous reports that secondary bile acids exert anti-inflammatory, immunosuppressive responses in ex vivo and in vitro systems as was expertly reviewed in Jia et al (2018)[31], and Cai et al (2022)[9].

As already discussed, it is now clear that through numerous enzymatic pathways, the colonic microbiota is capable of generating a highly diverse secondary bile acid metabolome with numerous derivatives rarely being measured due to both inadequate analytical techniques and the lack of chemical standards for less abundant secondary bile acids. It is this diverse secondary bile acid metabolome, as a whole, that likely contributes to setting the inflammatory tone and regulation of tumor cell growth in the colon. Much additional work and novel tissue and cell engineering approaches are needed to gain a more complete and accurate understanding of how the secondary bile acid metabolome contributes to local inflammation and growth control. With such new knowledge, it should become possible to identify a range of somewhat innocuous approaches for manipulating the co-metabolism of secondary bile acids by microbial and host cells to prevent local inflammation or restore normal growth control. Nevertheless, by using gnotobiotic mouse, microbial engineering, and various omics-based approaches, novel insight is emerging regarding contributions of several previously overlooked bile acid derivatives that seem to modulate balance between pathogenic T_H17 inflammation and Treg cells with anti-inflammatory properties and a brief summary follows.

With the advantage of looking back, it is now obvious that gnotobiotic studies attempting to identify gut bacterial taxa that influence the development of intestinal CD4+ Treg cells provided an early clue of the likely importance of secondary bile acids when it was observed that the induction of colonic Treg cells was specific to *Clostridium*-colonized gnotobiotic mice[194]. This research group later expanded the search for particular clostridial strains capable of inducing *CD41+FOXP3+* Treg cells by screening gnotobiotic mice colonized by human microbiota[195]. This approach identified 17 strains within Clostridia clusters XIVa, IV and XVIII with potent Treg cell induction capabilities. After testing each of the strains individually as well as randomly selected combinations of 3–5 strains, it was concluded that the 17 clostridial strains likely act synergistically to amplify the induction of Treg cells in a microbial-community-dependent manner[195]. Additional studies designed to pinpoint mechanisms, led to the conclusion that "the 17 strains provide…SCFAs, bacterial antigens and probably other factors, which together contribute to differentiation, expansion and colonic homing of Treg cells" in the mouse[196]. The potential that the necessity of clostridia for colonic Treg cell development related to their production of secondary bile acids was not acknowledged at that time, although one of the strains was identified as *C. scindens*[196].

A later study, however, by Hang et al. (2019)[27] identified two distinct derivatives of LCA, 3-oxoLCA and isoalloLCA, as key regulators of naive CD4+ T cell differentiation in mice after screening a library of 30 primary and secondary bile acid metabolites in in vitro assays under either T_H17 cell or Treg cell differentiation conditions. Specifically, 3-oxoLCA inhibited T_H17 cell differentiation, as shown by reduced expression of IL-17a, and isoalloLCA enhanced induction of Treg cells, as shown by increased *FOXP3* expression. It was further demonstrated that 3-oxoLCA inhibited the differentiation of T_H17 cells by directly binding to the key transcription factor retinoid-related orphan receptor-γt (RORγt) and that isoalloLCA enhanced the differentiation of Treg cells through the production of mitochondrial reactive oxygen species, which led to increased expression of *FOXP3*. Prior evidence that a variety of oxysterols were known to interact with the somewhat promiscuous RORγt transcription factor[197,198] provided precedence for the observation of 3-OxoLCA inhibiting T_H17 differentiation through direct binding to RORγt. Additional studies published around the same time provided additional support for the importance of secondary bile acids as important modulators of T_H17 and Treg cell differentiation. Song et al. (2020)[145] reported evidence that LCA and 3-oxoLCA modulate FOXP3+ Treg cells expressing RORγ+ through interactions with the nuclear receptor VDR. By screening the major species of deconjugated bile acids found in mice and humans for their ability to enhance Foxp3 induction in vitro, Campbell et al. (2020)[146] found that isoDCA increased Foxp3 induction by acting on dendritic cells (DCs) to diminish their immunostimulatory properties.

Potentiation of Treg cell generation by isoDCA required FXR expression in DCs providing evidence for involvement of an isoDCA-FXR interaction in cells of the myeloid lineage also possibly contributing to the induction of peripherally induced Treg (pTreg) in the mouse intestine (FIG. 5). Li et al. (2021)[29] demonstrated that the planar secondary bile acid isoalloLCA enhances Treg cell differentiation through interactions with the nuclear hormone receptor NR4A1 leading to activation of Foxp3 gene transcription and identified a biosynthetic gene cluster in gut Bacteroidota that converts 3-oxoLCA to isoalloLCA

as discussed earlier. These authors provided additional compelling evidence through examination of longitudinal metabolomic and metagenomic profiles of stool samples from individuals in the HMP2 IBDMDB cohort[199] (Crohn's disease, ulcerative colitis versus non-IBD controls) and found that isoalloLCA and its biosynthetic genes are substantially reduced in patients with IBD. Intriguingly, the fold change in isoalloLCA in patients with Crohn's disease and ulcerative colitis compared with controls was the largest among all identified bile acids in the metabolomics data from the HMP2 cohort.

Further evidence for an apparent beneficial role of certain secondary bile acid derivatives was provided by Sato et al. (2021)[58], who showed that the gut microbiome of Japanese centenarians was enriched in bacteria capable of generating isoforms of LCA including isoLCA, 3-oxoLCA, alloLCA, 3-oxoalloLCA, and isoalloLCA. These researchers further identified a biosynthetic pathway for isoalloLCA production by *Odoribacteraceae* strains and demonstrated that this planar bile acid wielded potent antimicrobial effects against Gram-positive (but not Gram-negative) multidrug-resistant pathogens, including *C. difficile* and *Enterococcus faecium*. Paik et al. (2022)[28] demonstrated that similar to 3-oxoLCA, isoLCA suppressed T_H17 cell differentiation by inhibiting the canonical transcription factor $ROR\gamma+$ and that fecal concentrations of both 3-oxoLCA and isoLCA and the 3α-HSDH genes required for their biosynthesis were markedly reduced and inversely correlated with the expression of T_H17-cell-associated genes in patients with IBD (FIG. 5).

Thus, a series of studies have provided considerable evidence that a subset of secondary bile acid derivatives exerts potent effects on setting inflammatory tone in the intestine through modulation of the balance between T_H17-mediated inflammation and immunosuppressive Treg cells. However, a clear mechanistic perspective does not emerge from a stereochemical perspective. Namely, LCA, 3-oxoLCA, isoLCA, alloLCA, isoalloLCA, and isoDCA vary both qualitatively and quantitatively in individual stool samples. Also, in addition to bile acids, the host mucosa is exposed to gradients of a myriad of microbial metabolites that affect immune and stromal cell functions. It is also likely that the bile acid metabolome and the other microbial metabolites vary substantially longitudinally along the gastrointestinal tract, and novel sampling approaches promise to shed light on this aspect in the future[114].

Given that bile acids are involved in the regulation of glucose, lipid and energy metabolism, it is not surprising that they often underlie the association between the gut microbiota and metabolic diseases including obesity, diabetes and metabolic dysfunction-associated steatotic liver disease (formerly nonalcoholic fatty liver disease). Relevant mechanisms defined to date involving bile acid signaling through both FXR and TGR5 are summarized in a number of excellent reviews on the topic of bile acids and metabolic disorders[95,200–204]. A short list of crucial questions that remain largely unanswered include: the extent of heterogeneity in the carriage of *bsh* and *bai* genes in the microbiome across human populations in both healthy individuals and those presenting with various metabolic disorders[95]; the range of responsiveness to macronutrients and micronutrients in diet among taxa harboring *bsh* and *bai* genes; the effect of stereoisomers of secondary bile acids and MCBAs on metabolic processes; and the effectiveness of phage-based approaches and engineered commensal microorganisms to generate bile acid pools that optimize host metabolic function (FIG. 3).

Conclusions

This Review has attempted to update key advances in classical bile acid biotransformation pathways by the gut microbiome such as bile acid hydrolysis, oxidation and epimerization, and C7-dehydroxylation. The link between the products of these microbial biotransformations and human health and disease, particularly immune function, are highlighted. We also critically reviewed the evidence for lesser-known microbial reactions such as bile acid esterification, C3-dehydroxylation and C12-dehydroxylation, for which future work is needed to firmly establish these functions, identify the bacteria responsible, and the enzymes catalyzing these reactions. With advances in microbiome science, and the rebirth in interest in bile acid microbiology, the time has arrived to settle outstanding questions in bile acid (micro)biology. The knowledge gained is anticipated to lead to novel microbiome-based interventions aimed at modulating the bile acid pool in the prevention and treatment of gastrointestinal diseases.

Acknowledgements

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Key Points

- Co-metabolism of bile acids is among the most studied aspects of host–microbiota interactions important for human health, although many mechanistic questions remain unanswered.

- A substantial gap in our knowledge still exists with respect to the host synthesis of bile acid A/B-ring *trans*-isomers known as allo-bile acids.

- Untargeted metabolomics identified microbially conjugated bile acids, which seem to be generated via bile salt hydrolase enzymes and can signal through PXR and FXR, although their physiological relevance is not fully understood.

- Much of the biochemistry and enzymology of microbial bile acid 7-dehydroxylation is established, however, the enzymology of C3 and C12-dehydroxylation requires additional work, as does host responses to these products.

- The oxidation and epimerization of bile acid hydroxyl groups greatly expands the diversity of bile acid metabolites as each hydroxyl toggles between three stable positions (e.g., 3α-OH, 3-oxo, and 3β-OH).

- Secondary bile acid epimers that have not been measured historically are emerging as potent modulators of the balance between T-helper-17-mediated inflammation and immunosuppressive regulatory T cells in the intestine.

This Review discusses the synthesis and metabolism of bile acids and the role of the gut microbiome in bile acid metabolism. Insights into how secondary bile acid derivatives influence host immune function are also described.

Box 1

Introduction to the importance of strain variation in the gut sterolbiome

The field of microbial endocrinology[218] approaches gut microbial metabolism of sterols as an integral yet overlooked part of the human endocrine system[218–220]. We previously defined the term 'sterolbiome' to describe the genetic potential of the gut microbiota to produce endocrine molecules from endogenous and exogenous sterols in the gastrointestinal tract[121,122]. An intersection exists between sex hormones and the regulation of bile acid synthesis[40]. Both bile acids and sex hormones alter the gut microbiome, although the mechanisms remain poorly understood[122]. Strain level variation with respect to bile acid and steroid metabolizing genes has been known for some time with the few isolates historically available but is now becoming clearer at the molecular level with the advent of shotgun metagenome sequencing, which enables the integration of taxonomic information with gene content. This advance is evident in species such as *Eggerthella lenta* and *Clostridium scindens*. which vary at the strain level in their capacity to metabolize bile acids, steroid hormones and steroid drugs[178,182,220–223]. indeed, some strains of these species convert cortisol to androgens[224] and corticosterone to progesterone[225]. C. *scindens* can convert prednisone to an androgenic product that promotes the growth of prostate cancer cells in vitro[221]. The sterolbiome is therefore an important dimension to consider with the advent of live biotherapeutic products to treat recurrent *Clostridiodes difficile* infection and potentially other diseases such as Crohn's disease, ulcerative colitis and metabolic dysfunction-associated steatohepatitis (formerly non-alcoholic steatohepatitis)[226–228].

Box 2

A cautionary tale in gut microbiology studies

Much confusion still exists in the literature relating to which bacteria generate deoxycholic acid and lithocholic acid[37]. This confusion is partly a product of uncertain taxonomic placement early in the field that has since been clarified by increasingly sensitive nucleic acid sequencing techniques[37]. Conversely, the advent of highly sensitive metabolomics platforms has led to some false-positive detection of bile acid metabolism by gut bacteria. Animal-based microbiological medium components (for example, tryptone, meat extract and brain heart infusions) contain measurable quantities of bile acids. Thus, care should be taken to differentiate background bile acids in culture media from the accumulation over time of increasing quantities of a bioconversion product indicative of bona fide biological activity. Alternatively, plant-based protein sources (trypticase soy) might be used to eliminate background bile acids.

In addition, the search for sterolbiome genes in stool metagenomes should be accompanied by biochemical confirmation of function. The identification of a 'bai-like' operon in *Eggerthella lenta*[178], whilst not originally claimed to encode enzymes that catalyse C7-dehydroxylation, has nonetheless been cited to incorrectly list E. *lenta* among species capable of forming deoxycholic acid and lithocholic acid in other articles. Indeed, there is ample evidence to the contrary, including a study that identified the 'bai-like' operon[178] Although E. *lenta* is capable of extensive oxidation and epimerization of bile acid hydroxyl groups, removing the C7 hydroxyl has not been demonstrated despite extensive characterization of many strains independently in several labs over four decades[175,182,229–231]. By contrast, a report of 'bai-like genes' in the *Oscillisporaceae* in human gut metagenome sequences further expands the diversity of potential bile acid 7-dehydroxylating bacteria because the authors performed functional confirmation of bile acid metabolism by characterizing the recombinant Bai enzymes[136]. To clarify, Table 1 lists species currently confirmed to express bile acid 7-dehydroxylation activity.
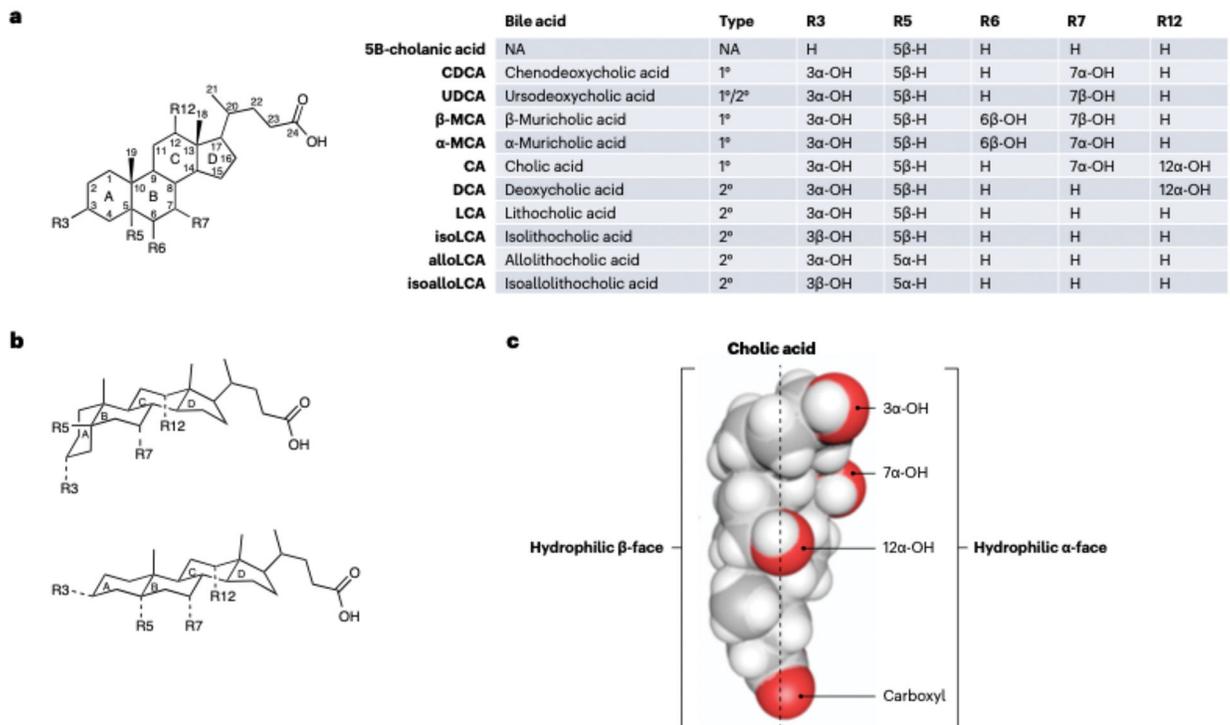
a

Bile acid		Type	R3	R5	R6	R7	R12
5B-cholanic acid	NA	NA	H	5β-H	H	H	H
CDCA	Chenodeoxycholic acid	1°	3α-OH	5β-H	H	7α-OH	H
UDCA	Ursodeoxycholic acid	1°/2°	3α-OH	5β-H	H	7β-OH	H
β-MCA	β-Muricholic acid	1°	3α-OH	5β-H	6β-OH	7β-OH	H
α-MCA	α-Muricholic acid	1°	3α-OH	5β-H	6β-OH	7α-OH	H
CA	Cholic acid	1°	3α-OH	5β-H	H	7α-OH	12α-OH
DCA	Deoxycholic acid	2°	3α-OH	5β-H	H	H	12α-OH
LCA	Lithocholic acid	2°	3α-OH	5β-H	H	H	H
isoLCA	Isolithocholic acid	2°	3β-OH	5β-H	H	H	H
alloLCA	Allolithocholic acid	2°	3α-OH	5α-H	H	H	H
isoalloLCA	Isoallolithocholic acid	2°	3β-OH	5α-H	H	H	H

b

c

Fig. 1|. Bile acid structure and function.
a, Generic form of a bile acid with functional groups (R) defined for select bile acids listed in the corresponding insert table. **b,** Structural comparisons of 5β-bile acids (R_5 = 5β-H), whose A/B rings are *cis.* and 5α-bile acids (R_5 = 5α-H), in which A/B rings are *trans.* **c,** Bile acids have a 'Janus-like' quality in that their faces have distinct properties that lend to detergent function, 1°, primary; 2°, secondary; NA. not applicable.
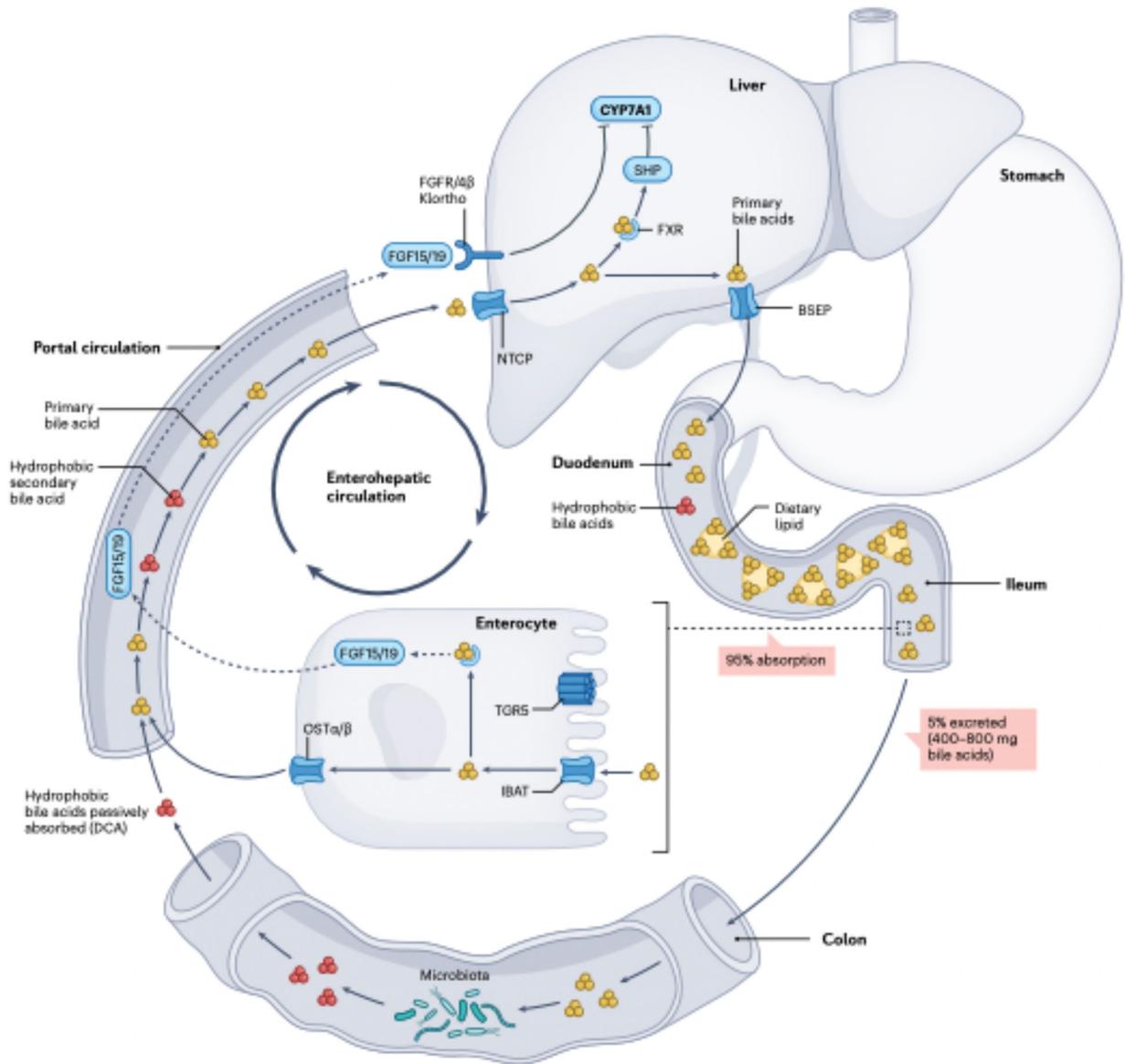
Fig. 2 |. The enterohepatlc circulation or bile acids.

Primary bile acids (yellow) are synthesized de novo in hepatocytes from cholesterol and secreted into bile through transport protein BSEP. During a meal, the gallbladder contracts, releasing bile into the duodenum, where mixed micelles composed of phosphdipods. fatty acids, cholesterol and lipid-soluble vitamins form and are surrounded by amphipathic conjugated bile acids. When conjugated bile acids reach the terminal ileum, they are transposed into enterocytes by the illeal sodium-bile acid cotransporter (JBAT), bound to FABP6 and transported into portal circulation via OSTα and OSTβ expressed basolaterally on enterocytes. As part of the negative feedback function of bile acid synthesis, intracellular bile acids activate the nuclear farnesoild X receptor (FXR) in enterocytes, resulting in upregulation in the synthesis and secretion of the protein FGF15/19 into the portal circulation. FGF15/19 binds to fibroblast growth factor receptor FGFR4/β-Klortho receptor-dependent manner resulting in inhibition of CYP7AL the rate-limiting enzyme in bile acid

biosynthesis in the liver. Bile acids returning to the liver are transported by NTCP. Activation of FXR in hepatocytes represses CYP7A1 expression dependent on small heterodimer partner (SHP) and liver-related homologue 1(LRHI). This process allows bile acid levels to remain in steady state. TCRS activation in intestinal stem cells promotes regeneration of enterocytes[20]. Roughly 5% of bile acids (400–800 mg per day) escape fieal transport and enter the large Intestine, which is the major route by which cholesterol is removed from the body. In the large intestine, bile acid structure and function are diversified by the gut microbiota. Part of this diversification is increasing the hydrophobicity of bile acids in the large intestine, allowing passive absorption into colonocytes and entry into the portal circulation, where secondary bile acids (mainly decoycholic acid (DCA)) accumulate to roughly one-quarter of the bile acid pool in healthy humans.
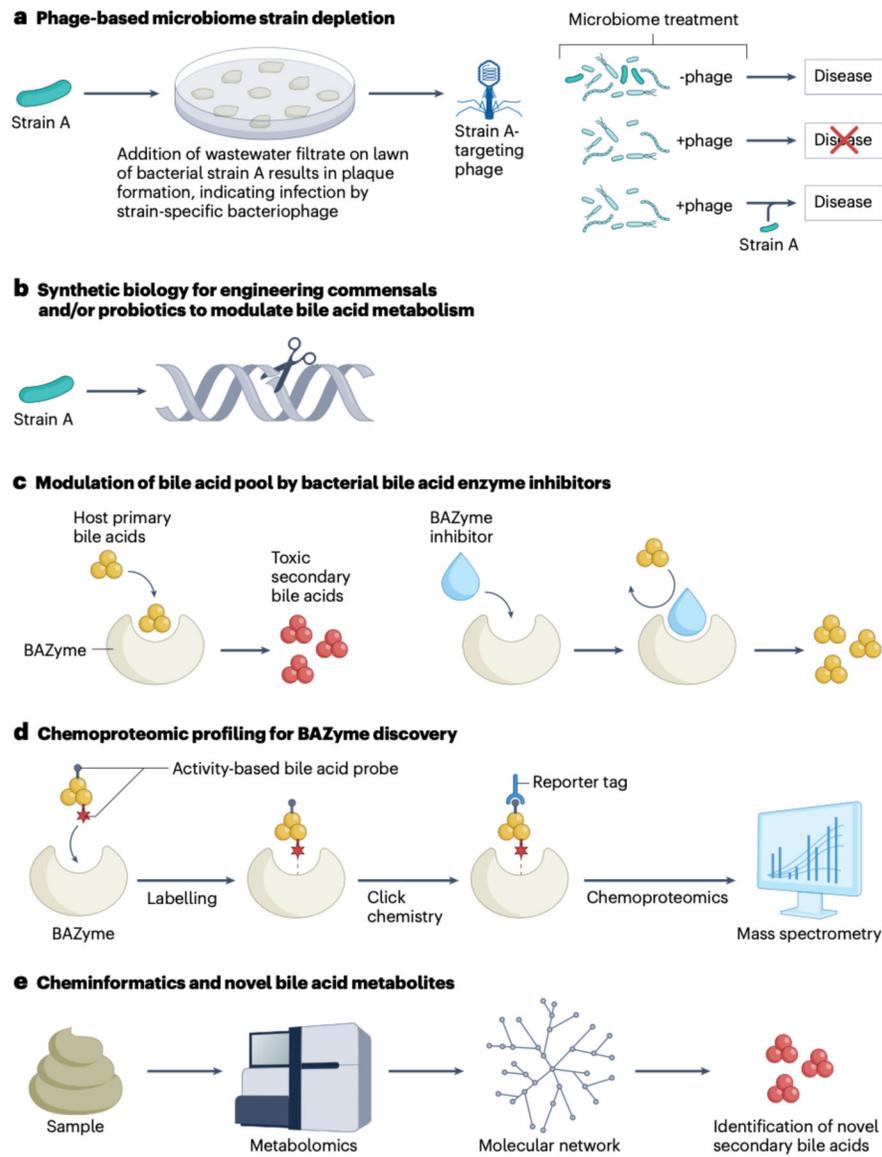
Fig. 3|. Targeting microbiota-bile acid interactions as potential therapeutic approaches for gastrointestinal and metabolic diseases.

a, Studies have demonstrated the potential utility of selecting for bacterial strain-dependent bacteriophages to remove microbial strains that have a causal rolle in diseases such as inflammatory bowel disease[12]. **b,** Synthetic biology offers the potential to rationally design commensal or probiotic bacteria to modulate bile acid metabolism in vivo[129]. **c,** The development of specific inhibitors against the microblome is expected to provide therapeutic potential[22]. The development of bile salt hydrolase (BSH) enayme inhibitors has allowed interrogation of the effects of altering bile acid metabolism[71,91,10]; other studies indicate that inhibitors against *bar* enzymes might also be therapeutically Important[11]. **d,** Chemoproteomic profiling using click chemistry bile acid probes allows the discovery of novel bacterial enzymes involved in bile acid metabolism[23]. After the bile acid probe covalently bonds to a bile acid binding enzyme (BAZyme), proteomic mass spectrometry allows the identification of gene sequence candidates. **e,** Cheminformatics

couples metabolomics with computation to obtain metabolite networks in which some nodes represent novel metabolites that repeal previously unknown bacterial metabolism[121]. BAZyme, bile acid enzyme.
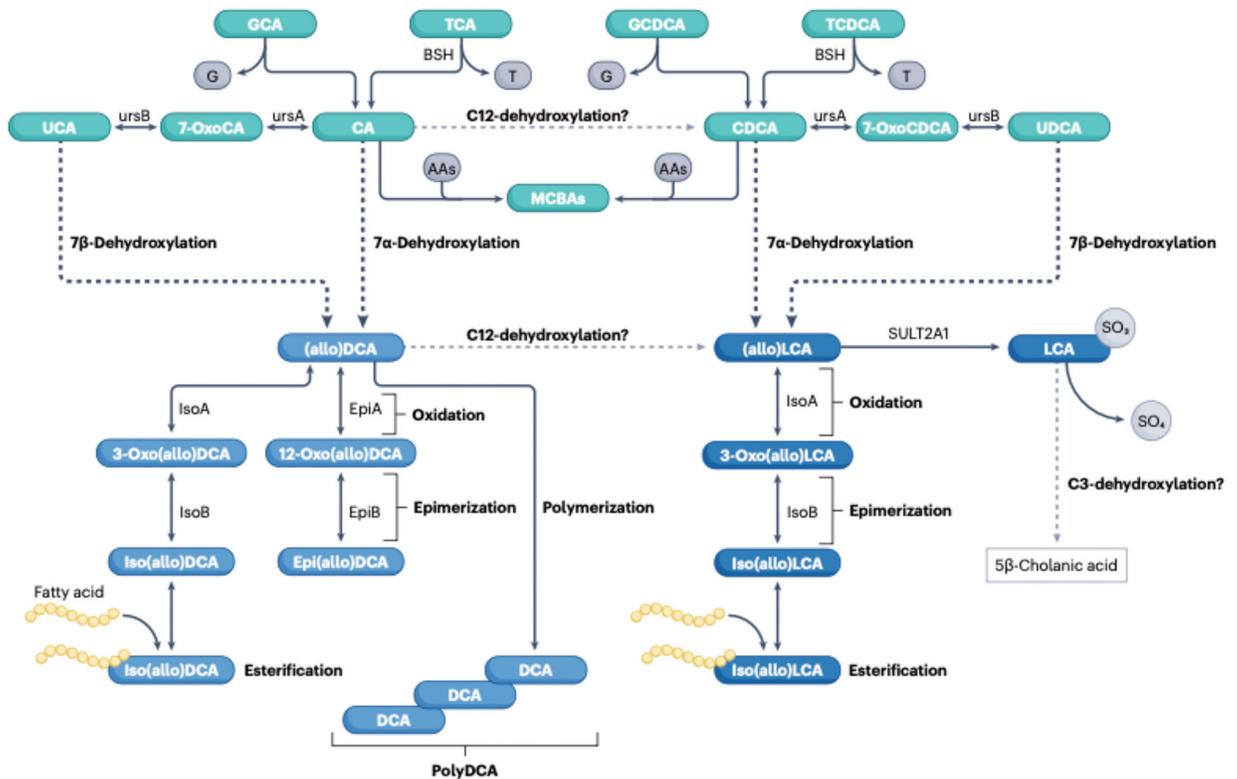
Fig. 4|. Bile acid biotransformations in the human large intestine.

Conjugated primary bile acids (taurocholic acid (TCA), glycocholic acid (GCA), taurochenodeoxycholic acid (TCDCA), glycochenodeoxycholic acid (GCDCA)) enter the large intestineand are deconjugated by the enzyme bile salt hydrolase (BSID by diverse gut bacterial taxa (Table 1). BSH is also reported to reconjugate free bile acids with a wide range of amino acids (AAs) yielding microbially conjugated bile acids (MCBAs). The primary bile acids cholic acid (CA) and chenodeoxycholic acid i.CDCA) can be oxidized to 7-oxoCA and 7-oxoCDCA, respectively, by the enzyme 7α hydroxysteroid dehydrogenase(ursA) (Table 1) and epimerized to ursochollc acid (UCA) and ursodeoxycholic acid (UDCA), respectively, by the enzyme 7β-hydroxysteroid dehydrogenase(ursB). CA and CDCA are also converted to deoxycholic acid (DCA) and lithocholic acid (LCA), respectively, through the multipstep Hylemon-Björkhem pathway of bile acid 7α-dehydroxylation by intestinal Clostridia (Table 1). UCA and UDCA can be directly 7β-dehydroxylated through the Hylemon-Björkhem pathway or, alternatively, epimerized back to CA and CDCA and 7α-dehydroxylated to DCA and LCA. The Hylemon-Björkhem pathway also yields A/B-ring epimers that are planar, known as 'allo' bile acids. We have designated both epimers of DCA and alloDCAas (allo)DCA as well as (allo)LCA and their derivatives. (allo)DCA has two hydroxyl groups that can be oxidized to 3-oxoDCA by 3α-hydroxysteroid dehydrogenase (isoA) and/or 12-oxoDCA by 12ahydroxysteroid dehydrogenase (epiA), and epimerized by 3β-hydroxysteroid dehydrogenase (isoB) and/or 12β-hydroxysteroid dehydrogenase (epiB) to iso(allo)DCA and epi(allo)DCA, respectively. (allo)LCA is monohydroxylated at C3 and can yield 3-oxo(allo)LCA and lso(allo)LCAonly. Additionally, the host sulfates LCA to yield

3-sulfoLCA. It is reported that 3 sulfoLCA can be C3-dehydroxylated to the non bile acid, 5β-cholanic acid. isoDCA and isoLCA are also esterified to short-chain and long-chain fatty acids.and DCA can be polymerized through esterification between C3-OH and C24-COOH. There is some support for the C12-dehydroxylation of CA to CDCA and of DCA to LCA.
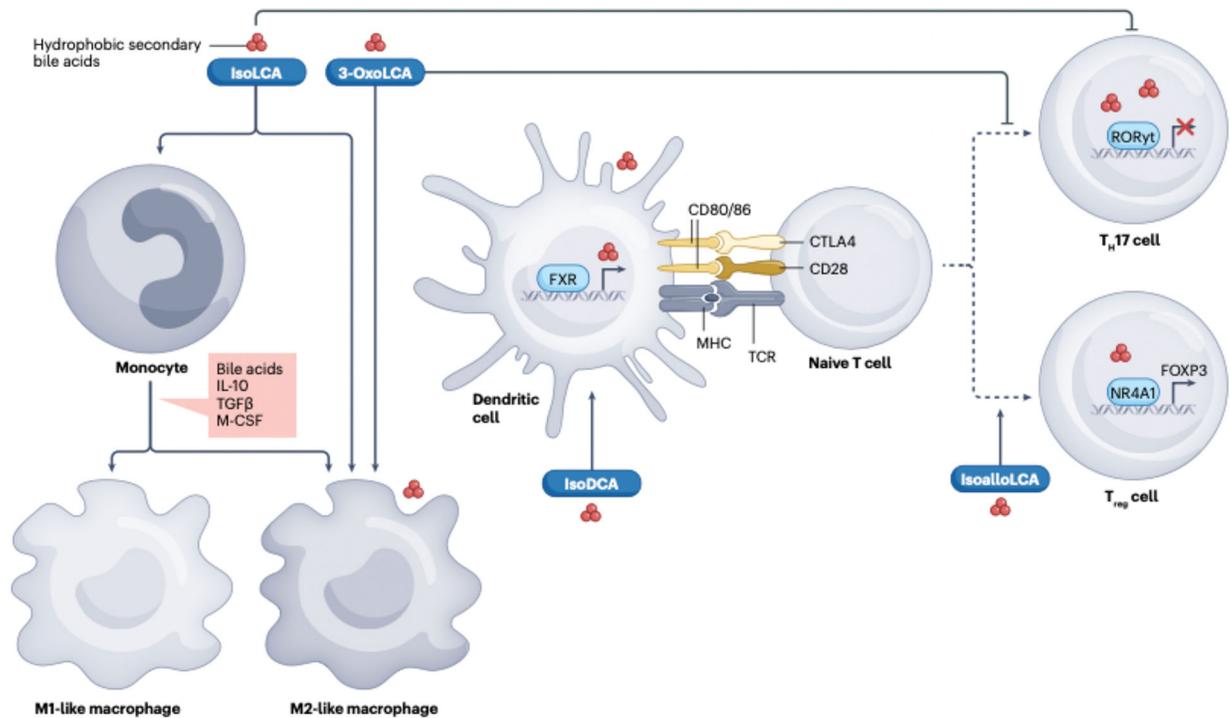
Fig. 5|. Modulation of inflammation and immune cell differentiation and function by secondary bile acid derivatives in the gastrointestinal tract.

The illustration summarizes the latest observations on the effects of rarely measured and hence understudied microbially derived secondary bile acid derivatives on host immune cells. In general, the data indicate that particular secondary bile acid derivatives exert distinct effects on the differentiation of macrophage progenitors as well as dendritic cell antigen presentation and naive CD4^{+} T cell differentiation, thereby purportedly influencing the inflammatory tone in the gastrointestinal tract. For specific details on the findings illustrated, readers are referred to the original research papers[26–28,57,59,145,146,178,194–196,198], which are summarized in the body of the present Review and subsequent review articles[9,29,30,55,216]. Briefly, iso-lithocholic acid (isoLCA) and 3-oxoLCA were shown to modulate macrophage polarization states[217], iso-deoxycholic acid (isoDCA) induced FOXP3 expression in dendritic cells to diminish their immunostimulatory properties[146], the planar secondary bile acid isoalloLCA enhanced regulatory T (T_{reg}) cell differentiation through interactions with the nuclear hormone receptor NR4A1, leading to activation *of FOXP3gene* transcription[28] .and 3-oxoLCA inhibited T helper 17 (T_H17) cell differentiation[26,27]. Similar to 3-oxoLCA, isoLCA suppressed T_H17 cell differentiation by inhibiting the canonical transcription factor retinoid-related orphan receptor γt (RORγt^+)[27]. FXR, farnesoid X receptor; M-CSF, macrophage colony-stimulating factor. TCR, T cell receptor.

Table 1|

Bacterial taxa contributing to bile acid metabolism in the human colon

Enzymatic	Bacterial taxon	Substrate	Product	Refs.
Bile salt hydrolase or conjugase	Clostridia, Bacteroides, Enterococcuc. Bifidobacteria, Lactobacilli, methanogenic archaea	Conjugated bile acide or free bile acids	Unconjugated bile acide, MCBAs	66–72
C3-oxidation or epimerization (iso-pathway)	Ruminococcus spp., Eggerthella lenta, Baccilus coagulans, Clostridium inoculum, Lachnospira pectinoschiza, Peptoniphilus harei, Catenibacterium. Lactobacillus, Aldercreutzia equolifaciens, Dorea formicigenerans, Blautia hydrogenotrophica	CA, CDCA, DCA, LCA	3-OxoCA, isoCA, 3-oxoCDCA, isoCDCA, 3-oxoDCA, isoDCA, 3-oxoLCA, isoLCA	169
C7-oxidation or epimerization (urso-pathway)	Escherichia coli, Clostridium sardiniense, Ruminococcus gnavus. Ruminococcus torques, Collinsella aerofaciens, Clostridium scindens, Bacteroides spp.	CDCA, CA	UDCA (from CDCA), UCA (from CA)	205
C12-oxidation or epimerization (epi-pathway)	C. scindens, Clostridium hytemonae, Peptacetobacter hiranonis. Clostridium leptum, Holdemania filiformis, Anaerostipes hadrus. Coprococcus comes. Bacteroides pectinophilus, Methanobrevibacter, Methanosphaera. Dorea sp., Clostridium paraputrificum, Eisenbergiella. Olsenella, Collinsella spp., Ruminococcus lactaris. E. lenta	CA, DCA	12-OxoCDCA, 12-oxoLCA, epiCA, epiDCA (lagoDCA)	169,181,182
C7-dehydroxylation (Hylemon-Bjorkhem pathway)	C. scindens, C. hylemonae, P. hiranonis, Extibacter muris, Dorea sp., Proteocatella sphenisci, Faecalicatena contorta, C. leptum, Clostridium sordellii	CDCA, UDCA, CA	DCA, LCA	37,129,136,143,206,207,214
Bile acid fatty acid-conjugating enzymes	Unknown (mixed faecal bacteria)			118
Bile acid sulfatase	Clostridium spp., Pseudomonas aeruginosa	3-Sulfo bile acids (for example, 3-sulfoCA)	Unconjugated bile acids (for example, CA)	102,208,209
C3-dehydroxylation	Clostridium sporogenes, Clostridium perfringens, C. paraputrificum, Clostridium sordellii	3-SulfoLCA	5β-cholanic acid, ³-cholenic acid, isoLCA	157
C12-dehydroxylation	Bacteroides spp., R. gnavus	CA, DCA	CDCA from CA, LCA from DCA	168,169
Allo-secondary bile acid pathways	C. scindens, C. hylemonae, Parabacteroides spp., Bacteroides spp., Holdmania hathewayi, Odoribacter sp., Alistipes spp.	DCA, LCA, isoDCA, isoLCA	alloDCA, alloLCA, isoDCA, isoLCA	57,59

CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LCA, lithocholic acid; MCBAs, microbially conjugated bile acids; UDCA, ursodeoxycholic acid.