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Consequences of bile salt biotransformations by intestinal bacteria

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ABSTRACT
Emerging evidence strongly suggest that the human “microbiome” plays an important role in both health and disease. Bile acids function both as detergents molecules promoting nutrient absorption in the intestines and as hormones regulating nutrient metabolism. Bile acids regulate metabolism via activation of specific nuclear receptors (NR) and G-protein coupled receptors (GPCRs). The circulating bile acid pool composition consists of primary bile acids produced from cholesterol in the liver, and secondary bile acids formed by specific gut bacteria. The various biotransformation of bile acids carried out by gut bacteria appear to regulate the structure of the gut microbiome and host physiology. Increased levels of secondary bile acids are associated with specific diseases of the GI system. Elucidating methods to control the gut microbiome and bile acid pool composition in humans may lead to a reduction in some of the major diseases of the liver, gall bladder and colon.

Introduction
Bile acids are synthesized from cholesterol in the liver hepatocytes. In humans, the liver synthesizes 2 primary bile acids, cholic acid (CA) and chenodeoxycholic acid; (CDCA); whereas, in rodents the primary bile acids include CA, CDCA and muricholic acids (MCA) which are 6-hydroxylated derivatives of CDCA. Before active secretion from the liver, via the canicular membrane of hepatocytes, bile acids are conjugated to either taurine or glycine at the C-24 carboxyl group. In the human liver, unlike rodents, the ratio of taurine to glycine conjugation is regulated by diet. Following secretion, conjugated bile acids (CBA) are stored in the gall bladder along with phosphatidylcholine and cholesterol. CBAs function to help keep cholesterol in solution in the gall bladder by forming mixed micelles. Following a meal, the gall bladder is stimulated to contract releasing its contents into the upper small intestines. Here, CBAs activate pancreatic lipase, and form mixed micelles with monoglyceride, cholesterol, partially ionized fatty acids and fat soluble vitamins (A, D, K and E) promoting their absorption by enterocytes (Fig. 1). Unconjugated and some glycine-conjugated bile acids are reabsorbed via passive diffusion throughout the small intestine. Active transport of bile acids occurs in the ileum and passive absorption of hydrophobic secondary bile acids occurs in the colon. The composition of serum bile acids returning from the gut to the liver is a mixture of free and CBA, secondary, and oxo and β-hydroxyl bile acids. The enterohepatic circulation of bile acids occurs several times each day and there is an increased secretion of fecal bile acids in individuals on a Western diet.

Starting in 1999, it became clear that bile acids also serve endocrine functions in the body largely through their binding and activation of the nuclear receptor, Farnesoid X Receptor (FXR), and the plasma membrane bile acid receptor TGR5. Through activation of these 2 receptors, bile acids regulate their own synthesis, conjugation, transport and detoxification, as well as lipid, glucose, and energy homeostasis. Furthermore, bile acids play an important role in maintaining intestinal barrier function, as antimicrobial agents that help determine the gut microbiome structure, and as inducers of genes encoding anti-microbial peptides and lectins via FXR.
Bile acid biotransformation of gut bacteria

Gut bacteria are capable of carrying out numerous biotransformations of bile salts during their enterohepatic circulation (Fig. 2). The major biotransformations include: hydrolysis of conjugated bile acids to free bile acids and glycine or taurine by bile salt hydrolase (BSH); $7\alpha$-dehydroxylation of CA and CDCA yielding DCA and LCA, respectively; bile acid $7\beta$-dehydroxylation of UDCA yielding LCA. The bile acid $7\alpha/7\beta$-dehydroxylations involve a multistep biochemical pathway found only in anaerobic gut bacteria. In addition, the gut microbiota are capable of the oxidation and epimerization of hydroxy groups at the C3, C7 and C12 position of bile acids yielding isobile ($\beta$-hydroxy) acids.

Bile salt hydrolase

Bile salts in the gallbladder of vertebrates are conjugated to either taurine or glycine in varying proportions due to a combination of nature and nurture. The murine bile acid pool is almost entirely taurine conjugated, irrespective of diet. Typically, the glycine:taurine ratio in humans is 3:1. However, taurine-conjugation (but not glycine-conjugation) of bile acids in humans is diet-dependent, with “Western-diets” high in animal protein favoring taurine-conjugation; whereas, vegetarian diets tend to shift the biliary pool toward glycine conjugation.

The composition of bile salts in the small intestine more closely resembles the biliary pool; whereas, the fecal bile acid profile is almost entirely unconjugated and secondary bile acids owing to the action of bile salt hydrolases (BSH) and $7\alpha$-dehydroxylation. BSH activity is widespread in commensal bacteria inhabiting both the small intestine and large bowel. Gram-positive gut bacteria have the most diverse distribution of BSH including: *Clostridium*, *Enterococcus*, *Bifidobacterium*, and *Lactobacillus*, while the distribution of BSH in Gram-negatives is, so far, detected only in members of the genus *Bacteroides*. Species of human intestinal archaea such as *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* were both shown to encode BSH capable of hydrolyzing both taurine- and glycine-conjugates. Metagenomic “mining” of environmental bacteria for BSH and characterization of cloned homologues revealed penicillin V amidase activity, but not BSH activity, suggesting BSH is a gut-specific function.

BSH hydrolyze the C-24 N-acyl bond which links the bile acid to the amino acid conjugate. BSH enzymes are classified as N-terminal nucleophilic
Hydrolases (Ntn), sharing this classification with penicillin amidases both of which have a catalytic N-terminal cysteine residue. Indeed mutation of Cys-1 to serine or threonine (OH rather than SH) abolishes activity in the BSH of Bifidobacterium bifidum. Chemical agents that oxidize thiol groups inhibit BSH activity. Sequence alignment of BSH suggests C1, D20, Y82, N175, and R228 are highly conserved among BSH; and structural characterization and sequence alignment of multiple BSH has provided further support for these amino acids in catalysis. In general, BSH are insensitive to oxygen, have pH optima between 5 and 6, and are located intracellularly. Substrate specificity is typically in relation to the amino acid moiety with higher affinity for glycine-conjugated bile acids. Bile acids regulate the gut microbiome structure

Resistance to bile salts is a major selective pressure modulating microbiome structure in the gut. Gut bacteria are resistant to bile salts through a number of mechanisms including: efflux pumps, alteration of membrane lipid composition and protein composition. A major mechanism by which bile acids act as antimicrobial agents in the gut is through membrane-damage. The detergent nature of bile acids suggests that concentration and bile acid hydrophobicity play an important role in their antimicrobial action. Unconjugated bile acids are generally more hydrophobic than the corresponding conjugated forms. Indeed, increasing concentrations of bile acids have been shown to solubilize membranes and cause the dissociation of integral membrane proteins, resulting in leakage of intracellular content. Even at sub-micellar concentrations, bile salts can alter membrane lipid composition.

The conversion of primary bile acids to secondary bile acids is correlated with increased hydrophobicity and enhanced binding to membrane lipids. Unconjugated dihydroxy bile acids passively flip-flop across phospholipid bilayers more rapidly than trihydroxy bile acids. Cell membrane architecture has been observed to alter bile tolerance; for instance,
growth in the presence of sodium monooleate resulted in altered membrane lipid profile and enhanced tolerance to bile salts. B. longum BBMN68 expresses a hemolysin-like protein that provides greater tolerance to tauro-conjugated bile acids but not glycine-conjugated bile acids. Once inside the bacterial cell, bile acids have been shown to alter macromolecular structure/function through detergent effects, damage DNA, and activate DNA repair enzymes.

Gram-negative bacteria are thought to have a higher bile acid tolerance than Gram-positives due to their lesser degree of sensitivity to oxgall, used in enrichment of MacConkey agar medium. Indeed, Salmonella typhimurium and Campylobacter are often isolated from bile or from the gallbladder. Interestingly, studies involving rodents fed bile acids or high fat diets (regardless of obesity phenotype) appear to select against the Bacteroidetes (Gram-negative) allowing expansion of the Firmicutes (Gram-positive).

**A microbial view of bile salt hydrolysis**

The physiological role of BSH is a subject of controversy and appears to vary between bacterial isolates; however, several hypotheses have been proposed. Free bile acids are more damaging to cellular membranes than conjugated bile acids. Floch et al. presented data on 2 unidentified Lactobacillus isolates in addition to strains of Enterococcus, Bacteroides and Clostridium perfringens, that suggests the gene encoding BSH evolved to generate free bile acids whose toxicity is greater than conjugated bile salts. BSH activity was active against glycine-conjugates of hydrophobic BAs CDCA and DCA, but not the hydrophilic BA CA. GCA was tolerated at 10 mM; whereas, GDCA and GCDCA were inhibitory (defined as a 2-log10 reduction in growth) between 1–5 mM, values similar to DCA and CDCA.

Mechanisms by which free BA inhibit lactobacilli and bifidobacteria suggest a combination of membrane-perturbation and intracellular accumulation of protonated free BA. Accumulation of free BA was shown to reduce intracellular pH ($\Delta$pH) similar to other hydrophobic weak organic acids, such as short chain fatty acids (SCFA). However, at the minimum inhibitory concentration, free BA dissipated $\Delta$pH and, unlike SCFA, also collapsed transmembrane electrical potential ($\Delta\Psi$) due to membrane perturbations. Again, secondary bile acids were more toxic i.e. DCA has a 10-fold lower minimum inhibitory concentration (MIC) than CA. Thus, liberation of free bile acids results in toxicity for specific Lactobacillus species.

In contrast, studies comparing wild type and mutant BSH positive isolates suggest conjugated bile acids are more toxic to many gut microbes and that BSH plays a detoxification role. BSH mutant strains of Lactobacillus amylovorus and Listeria monocytogenes were shown to be significantly more sensitive to conjugated bile salts than their wild-type counterparts. Indeed, glycine-conjugated BA was found to be significantly more inhibitory than taurine-conjugates, particularly at low environmental pH. The explanation proposed is that because the pKa of conjugated BA is significantly lower than free bile acids the conjugate acts as a weak acid reducing intracellular pH. By hydrolyzing the conjugate, the weaker carboxyl group of DCA allows recapture and export of DCA (and thus protons) thus reestablishing $\Delta$pH. Additional experiments with bsh mutant iso-genic strains will be important in clarifying the relative toxicity of free and conjugated bile acids for particular bacterial strains.

**Bile salt hydrolysis provides a source of amino acids**

BSH may function in some bacterial strains as a means to acquire a source of energy and building blocks for biosynthesis. Glycine is metabolized to ammonia and carbon dioxide by some bacteria. Taurine is catabolized to ammonia and carbon dioxide with the addition of sulfi ne-conjugates, particularly at low environmental pH. The explanation proposed is that because the pKa of conjugated BA is significantly lower than free bile acids the conjugate acts as a weak acid reducing intracellular pH. By hydrolyzing the conjugate, the weaker carboxyl group of DCA allows recapture and export of DCA (and thus protons) thus reestablishing $\Delta$pH. Additional experiments with bsh mutant iso-genic strains will be important in clarifying the relative toxicity of free and conjugated bile acids for particular bacterial strains.

**Consequences of bile salt hydrolysis for the mammalian host**

Cardiovascular disease is responsible for 16.7 million deaths worldwide and is the leading cause of mortality...
and morbidity worldwide. A strong correlation exists between low-density lipoprotein cholesterol (LDL-C) and risk for CAD. The major focus of BSH research is aimed at treating/preventing CAD caused by hypercholesterolemia (high LDL-C) in humans. LDL-C may be decreased by therapeutic oral intake of strains of probiotic bacteria with high BSH activity. Not surprisingly, therefore, much literature in probiotics is devoted to screening potential probiotic bacteria for BSH activity and specificity.

A major question that is the topic of much research focus is the mechanism by which certain probiotic bacteria reduce serum cholesterol. In the small intestine, conjugated bile salts form small mixed micelles over a narrow concentration range (critical micellar concentration) with cholesterol, fatty acids and monoglycerides (Fig. 1). Bile salts are amphipathic molecules with a hydrophobic side characterized by the steroid ring moiety, and a hydrophilic face characterized by one to 3 hydroxy groups and the amide carbonyl. Bile acids aggregate to form micelles, in which their hydrophilic face is exposed to the aqueous medium while their hydrophobic face is sequestered from the polar aqueous environment. The micelle thus provides a solvent vehicle for water insoluble compounds, allowing their absorption along the small intestine.

Increased BSH activity in the small intestine disrupts micelle formation and absorption of cholesterol and lipids. This is hypothesized to lower serum LDL-C and triglycerides as cholesterol output is increased due to lower uptake. Probiotics can also increase the output of bile acids in feces possibly by binding or uptake of bile acids. Increased fecal secretion of bile acids requires increased hepatic catabolism of cholesterol into bile acid synthesis. Elucidation

Figure 3. Proposed bile acid 7α-dehydroxylation pathway of cholic acid in Clostridium scindens. The multistep 7α-dehydroxylation pathway converts the primary bile acids cholic acid and chenodeoxycholic acid into the secondary bile acids deoxycholic acid and lithocholic acid, respectively. Abb. baiG, primary bile acid transporter; baiB, bile acid-coenzyme A ligase; baiA, 3α-hydroxysteroid dehydrogenase; baiCD, 7α-hydroxy-3-oxo-Δ5-cholenic acid oxidoreductase; baiF, bile acid coenzyme A transferase/hydrolase; baiE, bile acid 7α-dehydratase.
of mechanisms involved in lowering of serum cholesterol by probiotic bacteria should focus on comparison of BSH expressing strains vs. isogenic mutants as controls in which the only variable is BSH. This should allow better comparisons within studies and between studies using different strains of bacteria. Lipidomics/metabolomics profiles at all points along the EHC along with key genes regulating cholesterol and bile acid synthesis and transport should further clarify the mode of action. There is also a balance in levels in BSH activity that needs to be determined between healthy cholesterol lowering and prevention of malabsorption/steatorrhea in patients.

**Bile Acid 7α/7β-dehydroxylation by intestinal bacteria**

The term “secondary bile acid” typically denotes the removal of the 7α-hydroxy or 7β-hydroxy group from primary bile acids produced in the liver of humans and other vertebrates. Germ-free animals have been shown to lack secondary bile acids. Bacteria capable of converting primary to secondary bile acids have been isolated from humans. The rodent isolate required unidentified Bacteroides spp. in co-culture due to an unidentified syntrophy. This is in contrast to human isolates that are readily isolated in pure culture using anaerobic media. Rodent, but not human bile acid 7α-dehydroxylating bacteria are capable of 7α-dehydroxylating muricholic acids (6α, 7β-hydroxy; 6β, 7β-hydroxy). Characterization of the genetics and molecular biology of bile acid 7α/β-dehydroxylation has been performed mainly in strains of *Clostridium scindens,* particularly *C. scindens* VPI 12708 (formerly *Eubacterium sp. strain VPI 12708*). The type strain, *C. scindens* ATCC 35704 (formerly *Clostridium strain 19*) was characterized as having steroid-17,20-desmolase (SD) activity, which cleaves the side chain from glucocorticoids (C21) converting them to androgens (C19). Interestingly, there are important differences in the “sterolbiome” of *C. scindens* ATCC 35704 and *C. scindens* VPI 12708 with respect to sterol metabolism. Strain 35705 encodes the desABCD operon which contain the gene for 20α-HSDH and the putative SD, while strain 12708 lacks this operon. Interestingly, *C. scindens* VPI 12708 metabolizes the 11β-hydroxyandrostenedione (11β-OHAD) product, reducing the 17-keto group to a 17α-hydroxy. Previously, we reported that testosterone enhances 7α-dehydroxylation activity in *C. hylemonae* TN27172; however, it is unknown what effect 11β-OHAD, and its subsequent gut microbial metabolites has on bile acid 7α-dehydroxylation.

While genetic knockouts of genes in the 7α-dehydroxylation pathway have yet to be reported, our laboratory has built a model for the multi-step bile acid 7α-dehydroxylation pathway in *C. scindens* based on characterization of native or recombinant enzymes encoded by the bai operon (Fig. 3). In this pathway, primary unconjugated bile acids are transported into the cell by a proton-dependent bile acids transporter encoded by the bai operon (Fig. 3). Oxidation of the 3-hydroxy group is catalyzed by the baiA gene, which has specificity for CoA conjugates. A C5=C5 bond is introduced in 7α-hydroxy, 3-dehydro-bile acids by an NADH:flavin-dependent oxidoreductase encoded by the baiCD gene while a homologous enzyme encoded by the baiH gene recognizes 7β-hydroxy, 3-dehydro-bile acids. The rate-limiting step in this pathway is bile acid 7α-dehydration. The baiE gene encodes the bile acid 7α-dehydratase, and it is hypothesized that the baiI gene encodes the bile acid 7β-dehydratase. The genes involved in reduction of the 3-dehydro-4,6-deoxycholyl-CoA in the case of CA metabolism or 3-dehydro-4,6-lithocholyl-CoA in the case of CDCA or UDCA metabolism have yet to be identified.

In a ground-breaking paper demonstrating a causal-link between deoxycholic acid and liver cancer, it was shown that a single OTU in Clostridium Cluster XI closely related to *C. sordellii* constituted 12% of fecal bacteria fed the high-fat diet, but was not found in HFD+vancomycin. It was also found that the baiJ gene was in high abundance, consistent with *C. sordellii* levels. We reported the baiJKL operon in *C. hylemonae* and *C. scindens* which encodes a bile acid CoA transferase (baiK) with similar function and to the baiF gene, a homolog on the baiBCDEAFGH operon (Fig. 4). The function of the baiJ gene is unknown, though it is predicted to encode a flavoprotein possibly involved in C-C double bond oxidation/reduction. However, it is doubtful it is involved in the “reductive” arm of the pathway toward secondary bile acids because it is absent in the genomes of several isolates.
Figure 4. Arrangement of genes in the bile acid inducible (bai) operon in various species of bile acid 7α/β-dehydroxylating gut bacteria. The genes encoding enzymes carrying out bile acid metabolism in gut bacteria capable of producing secondary bile acids. Biochemical pathway leading to secondary bile acid formation is shown in Figure 3. Amino acid accession numbers are as follows: *Clostridium scindens* ATCC 35704 baiABCDEAFGHI (EDS05761-EDS05768), baiA2 (EDS06021), 7α-HSDH (EDS05904); *C. scindens* VPI 12708 baiBCDEA2FGHI (AAC45410-AAC45418), baiJKL (ACF20978-ACF20980), baiA1 (P07914), 7α-HSDH(AAB61151); *C. hiranonis* TO-931 (DSM 13275) baiBCDEA2FG (AAF22844-AAF22849), baiH (ABQ12723), baiI (WP_006439754) 7α-HSDH (WP_006404226); *C. sordellii* VPI 9048 baiA2 (EPZ56913, EPZ56915), baiH, baiE (EPZ56916, EPZ56912), 7α-HSDH (EPZ54454); *C. hylemonae* TN271 baiBCDEA2FGHI (ACF20984-ACF20990), baiJKL (ACF20981-ACF20983) baiA1 (WP_006441384), 7α-HSDH (WP_040435027).
capable of secondary bile acid production (Fig. 4). A BLASTP search of the NCBI nr database, as well as Genome-BLAST against the *C. sordellii* VPI 9048 genome suggests the *baiJ* gene is absent. It may be that some *C. sordellii* strains may have acquired/lost the *baiJKL* operon (Fig. 4). In the same study, sequences most closely related to *C. hylemonae*, which encodes the *baiJ* gene were found to make up only 0.5% of OTU on the HFD. In any case, this data is consistent with several studies that have shown increased levels of bile acid 7α-dehydroxylating bacteria on a high-fat diet or bile acid feeding.

Genome-sequencing projects of individual gut isolates and metagenomic libraries have provided additional information about potential *bai* genes in other gut bacteria providing new questions that can begin to be answered; however, a further discussion of these hypothetical genes is beyond the scope of this review.

**Structure-function characteristics of enzymes in the bile acid 7α-dehydroxylation pathway**

To date, crystal structures of 3 key enzymes of the bile acid 7α-dehydroxylation pathway, namely those encoded by the *baiA2*, *baiB* and *baiE* genes, have been solved. The structures along with functional characterization provide important insights into the mechanisms of key biochemical steps of this pathway. Structural information is essential for designing small molecule inhibitors of key enzymes in this pathway.

The *baiB* gene encodes a bile acid Co-enzyme A ligase that catalyzes the thioesterification of bile acids to Coenzyme A (CoA) following uptake of primary bile acids into the bacterial cell. The crystal structure of the *baiB* gene product from *Clostridium scindens* VPI 12708 revealed a monomeric assembly confirmed by analytical size exclusion chromatography (unpublished data). The overall fold of the protein is similar to members of the AMP binding family consisting of separate binding pockets for CoA, bile acid and ATP. As previously described, thioesterification of bile acid to CoA is a 2-step ATP-dependent process, which involves the ATP-dependent adenylation of the bile acid followed by CoA exchange with release of AMP.

The crystal structure of bile acid 3α-hydroxysteroid dehydrogenase (3α-HSDH), encoded by the *baiA* gene product, revealed a tetrameric assembly commensurate with analytical size exclusion chromatography. The constituting monomers are all a single domain protein bearing the characteristic Rossmann fold observed in other members of the short-chain dehydrogenase/reductases (SDR) family with distinct substrate and co-factor binding sites. Catalytically conserved amino acid residues are located at the interface of substrate and co-factor binding sites. The substrate binding site continues into the co-factor binding site suggesting binding of the co-factor in its respective site is essential to orient the functional group of the substrate to the active site residues. There is evident from steady-state enzyme kinetic studies that indicate substrate turnover occurs in the presence of co-factor. Steady-state kinetic characterization of *baiA1* and *baiA2* also revealed NAD+ as the preferred

![Figure 5. Binding of substrate to bile acid 7α-dehydratase from Clostridium scindens.](image-url)
co-factor. The specificity constant (k_cat/K_M) of NADP^+ is at least an order of magnitude lower than NAD^+. The NAD^+-bound crystal structure of baiA2 provides the basis of such a preference to restrictive conformational flexibility of residue Glu42 in the co-factor binding site that participates in a salt bridge interaction with Arg16. Mutagenesis studies where Glu42 is replaced with Ala improved specificity toward NADP^+ by fold10- compared to the wild-type enzyme. Steady-state kinetic characterization studies also established that bile acid-CoA esters are the preferred substrates suggesting a possible interaction of the CoA moiety with the enzyme. However, the crystal structures did not reveal a separate binding pocket for CoA. It is likely that an induced pocket is generated upon binding of the bile acid-CoA ester.

Bile acid 7α-dehydratase (BA 7α-DeOH), encoded by the baiE gene, catalyzes the rate limiting and irreversible step in the bile acid 7α-dehydroxylation pathway. The recently solved crystal structure of this enzyme revealed a trimeric assembly for all 3 key homologs whereas the monomers exhibit the canonical twisted α+β barrel fold characteristic of the Nuclear Transport Factor 2 (NTF2) family. The trimers appear to be bonded together by a divalent metal cation, probably Zn^{2+}. The binding of bile acid substrate to BA 7α-DeOH is shown in Figure 5 Site-directed mutagenesis studies established the role of 3 conserved residues in the active site namely, Tyr30, Asp35 and His83, as important for catalysis (Figs. 6 and 7). In addition, 3 other non-active site residues, Tyr54, Asp106 and Arg146, are found to be essential for substrate binding and turnover. This correlates with the co-crystal structure of baiE from Clostridium hiranonis DSM13275 with the partially bound product 3-oxo-Δ^{4,6}-lithocholyl-CoA (3-oxo-Δ^{4,6}-LC-CoA), where the loop constituting of residues 48–63 forming the roof of the active site that reveals a distinct conformation for all the apo-structures. Conformational flexibility of this loop may be essential for substrate binding. Site-directed mutagenesis study of Tyr54 detected impaired substrate turnover indicating the importance of this loop in catalysis. In all, the apo-structures of baiE residues Asp106 and Arg146 in the substrate binding pocket interact via a salt bridge. The Severance of the salt bridge by replacing with residues Asn and Gln at position 106 and 146, respectively, abolished catalysis. Furthermore, steady-state kinetic characterization studies revealed that unlike baiA1 and baiA2 all baiE homologs are able to turnover 3-oxo-Δ^{4}-bile acid and 3-oxo-Δ^{4}-bile acid-CoA thioester substrates with comparable efficiency. This suggests that the CoA moiety maybe removed from the bile acid either before or after the elimination of C7-hydroxy group. The apo- crystal structures of baiE, like the crystal structure of baiA2, did not reveal a separate binding pocket for the CoA moiety. However, the 3-oxo-Δ^{4,6}-LC-CoA bound crystal structure of

Figure 6. Predicted enzyme substrate interactions in the active site of bile acid 7α-dehydratase from Clostridium scindens. (A) Probable binding mode of 3-oxo-Δ^{4}-chenodeoxychoryl-CoA (3-oxo-Δ^{4}-CDC-CoA). Blue dashed lines and adjacent numbers are predicted interaction of His83-Nε2 atom with C7-OH and C6 atoms and Y30-OH group with C3-oxo atom of 3-oxo-Δ^{4}-CDC-CoA. The 6α-H closest to H83-Nε2 atom colored magenta and 6β-H away from H83-Nε2 atom colored brown. (B) Predicted stacking interaction involving the adenine group of the Coenzyme (CoA) moiety of oxo-Δ^{4}-chenodeoxychoryl CoA (3-oxo-Δ^{4}-CDC-CoA) with Y115. Carbon atoms of protein residues and product molecule are colored gold and green, respectively. H, O, N, P and S atoms are colored gray, red, blue, orange and olive, respectively.
baiE from *Clostridium hiranonis* DSM13275 revealed an extended binding pocket generated by substrate binding sites of 2 protomers that originate from 2 different trimer assemblies. In that structure, the loop formed by residues 48-63 participates in forming the extended substrate-binding pocket.

**Esterification of fecal bile acids in the gut**

Over the past 50 y there have been a number of reports of esterified bile acids in fecal samples from rodents and humans. A recent study by Kakiyama G. et al. showed that treatment of different human fecal samples with 0.1 N NaOH at 60°C for 2 hours significantly increased the yield (28% to 48%) of total bile acids as compared to those samples without base hydrolysis. In this regard, there have been reports of ethyl-esters and long-chain fatty acid esters of LCA, as well as polyesters of DCA. It has been estimated that esterified bile acids may constitute more than 25% of total fecal bile acids. This could be important as esterification of bile acids make these molecules more hydrophobic, less soluble, and should decrease the concentration of bile acids in fecal water. Unfortunately, little is known about the role of gut bacteria in carrying out esterification of bile acids.

**Pathophysiological relevance of secondary bile acids to host**

In recent years it has become clear that bile acids serve endocrine functions largely through their activation of nuclear receptors including: Farnesoid X Receptor (FXR), Vitamin D receptor, and the Pregnane-activated Receptor (PXR) as well as the G-protein coupled plasma membrane receptors (GPCR) including; TGR5, muscarinic receptors (M2,3) and the sphingosine-1-phosphate receptor 2 (SIPR2). Both nuclear receptors and GPCRs have varying affinities for different bile acids (Table 1). In this regard, by altering the bile acid pol composition, gut bacteria have the potential to alter cellular metabolism and host physiology and metabolism of cells exposed to bile acids.

Recent studies also illustrate the important role gut microbes play in the levels and profiles of bile acids in various tissues of the body including liver, kidney, plasma, and heart. The global physiological significance of these alterations in bile acid profiles is unknown. However, decades of research strongly suggest that secondary bile acids (DCA and LCA), a product of bacterial metabolism of primary bile acids, are involved in disease processes including cancers of the colon, liver, and cholesterol gallstone disease in some patients. The level of DCA in bile of male VA patients in Richmond, Virginia, varied from <1% to >60% with a mean of 35%. The average DCA level of the normal population is ~20%. The level of DCA in bile is believed to be controlled by 2 major factors: A) levels and activities of bile acid 7α-dehydroxylating gut bacteria and B) colonic transit time. Both the levels of bile acid 7α-dehydroxylating gut bacteria and colonic transit time can be strongly influenced by dietary habits. Finding ways to decrease the percentage of secondary bile acids in blood, bile and feces may result in a decrease in the incidence of some GI cancers.

**Oxidation and epimerization of bile acid hydroxy groups by gut microbiota**

Members of the intestinal microbiota have genes that encode a variety of pyridine nucleotide-dependent
hydroxysteroid dehydrogenases (HSDH). HSDHs are widely distributed in various members of the gut microbiota. The gut microbiota is capable of the oxidation/reduction of hydroxy groups at the 3-, 7-, and 12- carbons of bile acids (Fig. 2). The epimerization of bile acid hydroxy groups (α ↔ β) requires 2 position-specific bile acid α and β HSDHs which generates a stable oxo-bile acid intermediate i.e., 7α-hydroxy ↔ 7-oxo ↔ 7β-hydroxy. The extent of epimerization and accumulation of the oxo group intermediate appears to be influenced by the oxidation/reduction potential of the cellular environment. For example, the formation of oxo-bile acids may be more favorable in bacteria near the mucosal surface of the gut where there is a potential for a higher redox potential than in the lumen of the intestines. Bacterial bile acids HSDHs differ in their pH optima, pyridine nucleotide specificity NAD(H) or NADP(H) or both, subunit molecular weight, and gene regulation. Amino acid sequence analysis suggests that most bacterial HSDHs in the gut microbiota belong to the short-chain alcohol/polyol dehydrogenase gene family.

What is the physiological significance of bile acid hydroxy group biotransformations to gut bacteria and host? In the highly evolutionary competitive environment found in the human gut microbiome, the persistence of genes usually indicates they increase the organism’s ability to survive. The epimerization of bile acid 7α-hydroxy group decreases the toxicity of chenodeoxycholic acid (CDCA) for gut microbiota. CDCA is a relatively hydrophobic primary bile acid. In contrast, ursodeoxycholic acid (UDCA), the 7β-epimer of CDCA, is a much more hydrophilic, and therefore less toxic to gut bacteria. Clostridium absonum, which has both 7α and 7β-bile acid HSDHs, converts CDCA into UDCA. It follows that any bacterium able to biotransform CDCA into UDCA may have a survival advantage in the highly competitive environment found in the lumen of our gastrointestinal system. Bacterial HSDHs have the ability to epimerize other hydroxyl groups on bile acids in addition to the 7-oxo group, including the 3- and 12-oxo groups. A recent study that elucidated genes responsible for the production of enzymes that epimerize DCA to 3-oxo-DCA and subsequently to 3-iso-DCA in Ruminococcus gnavus reported that these bile acids have reduced toxicity. Given these findings, a reasonable hypothesis is that in response to these membrane-disrupting primary and secondary bile acids, gut microbes utilize HSDHs as a means of detoxifying bile acids.

Bile acid 7α-HSDH could also be an important enzyme regulating substrate availability of bile acid 7α-dehydroxylation pathway by converting primary bile acids into 7-oxo-bile acids that are not substrates for 7α-dehydroxylation. Other bacteria may use bile acid HSDHs to co-opt primary bile acids into signaling molecules for their own autocrine or paracrine uses, though this area has yet to be thoroughly investigated.

The formation of 7-oxo-bile acid by gut microbiota may also have effects on host glucocorticoid metabolism. Recent studies reported that 7-oxo-lithocholic acid (7-oxo-LCA), a bacterial metabolite of CDCA, acts as a competitive inhibitor of human hepatic 11β-hydroxysteroid dehydrogenase-1 (11β-HSDH1). Hepatic 11β-HSDH1 recognizes 7-oxo-LCA as a substrate and reduces this bacterial metabolite back to active cortisol. In this instance of possible inter-kingdom signaling, a bacterial bile acid metabolite has the ability to modulate host endogenous active steroid hormone levels. Steroid hormones can be effective at very low concentrations and enzymes such as 11β-HSDH1 are responsible for modulating the levels of active vs. inactive steroid hormones. Any alteration in the levels of steroid hormones by gut microbes could be potentially significant to host physiology, though the extent of these affects in vivo are currently unknown.

Table 1. Secondary bile acids differentially activate nuclear Receptors and G-protein coupled receptors as compared to primary bile acids.

| Nuclear Receptor/GPCR | Bile Acid Agonist Potency |
|-----------------------|---------------------------|
| Farnesoid X Receptor (FXR) | CDCA>LCA=CA |
| Pregnan-activated Receptor (PXR) | LCA>DCA>CA |
| Vitamin D Receptor (VDR) | 3-oxo-LCA>LCA>CDCA>CA |
| TGR-5(GP-BAR1) | DCA>LCA>CDCA>CA |
| Sphingosine-1-Phosphate Receptor 2 (S1PR2) | Taurine or Glycine Conjugated Bile Acids |
| Muscarinic Receptors (M₁₂) | T-LCA>T-DCA>T-CA |
Not only do primary and secondary bile acids alter human steroid hormone levels, but they are also implicated in a host of other signaling pathways. Anti-microbial peptides, such as cathelicidin produced in the colon, are an important component of our innate immune system. Their production by the colonic epithelium is increased by activation of FXR. CDCA is a high affinity agonist for FXR, whereas 7-oxo-LCA and UDCA, bacterial metabolites of CDCA, are intermediate and non-active agonists, respectively. Because cathelicidin has broad-spectrum anti-microbial activity and its expression is controlled by FXR, it follows that by lessening the affinity of bile acids for FXR, a microbe could increase its fitness in the lumen of the large intestine. The extent to which these alterations in bile acid levels by gut flora affect both host FXR activation and downstream anti-microbial peptide production has yet to be fully explored.

Bile acids regulate Clostridium difficile colonization and growth in the GI tract

_C. difficile_, the cause of antibiotic-associated diarrhea and colitis, is a growing health threat for patients taking broad spectrum antibiotics. It has been estimated that _C. difficile_ may be responsible for almost a half a million infections and 29,000 deaths in the US per year. Moreover, as the world population ages, a higher percentage of the elderly are colonized by _C. difficile_ and are more often treated with antibiotics. Treatment of patients with broad-spectrum antibiotics markedly reduces the level of the normally protective gut microbiota and allows proliferation of _C. difficile_ normally found in low levels in some individuals. Patients treated with antibiotics, especially in hospitals, are also at risk from colonization by _C. difficile_ colonizing the gut; however, between 10 to 40% of patients successfully treated with these antibiotics will relapse following cessation of antibiotic therapy. Fecal transplants, using gut microbiota from healthy donors, have been very successful in treating relapsing patient. Recent studies have been undertaken to determine which members of the gut microbiota are responsible for resistance to _C. difficile_ infection. In this regard, it was recently reported that Clostridium scindens, a human gut bacterium that converts primary bile acids CA to DCA, is strongly associated with inhibition of Clostridium difficile colonization and colitis in animal models and in human patients.

What are the links between _C. difficile_ infection of the GI tract and bile acids? More than 30 y ago, Wilson K.H. et al. reported that the addition of taurocholate, CA or DCA to specialized culture media stimulated _C. difficile_ spore germination. Later studies by Sorg J.A. et al. demonstrated that taurocholate, cholate and deoxycholate, but not chenodeoxycholate, stimulated the germination of _C. difficile_ spores in vitro. Moreover, a combination of taurocholate plus glycine proved to be a more potent combination for _C. difficile_ spore germination. Additional studies by Sorg J. A. et al. showed that chenodeoxycholate and muricholic acids were competitive inhibitors of taurocholate stimulated germination of _C. difficile_ spores. These studies suggest that
a bile acid with a 12α-hydroxy group is necessary for binding to a putative germinant receptor on *C. difficile* spores. In 2013, the Sorg Laboratory reported the identification of a unique germinant receptor (CspC) recognized by 12α-hydroxylated bile acids. Binding of bile acids to the CspC gene product is believed to lead to the release of Ca\(^{2+}\)-dipicolinic acid (DPA) from the spore cortex in exchange for water. Hydration of the cortex then allows cell wall hydrolysis and outgrowth of a vegetative cell. Some studies report that DCA is an inhibitor of *C. difficile* growth *in vitro* and in the intestinal tract explaining why *C. scindens* may be a crucial member of the gut microbiota regulating levels of *C. difficile*. However, the molecular mechanism(s) of how DCA may inhibit *C. difficile* colonization and growth are currently unclear.

**Summary and future directions**

It is becoming clear that our view of the human body is changing. We now recognize the human body as a complex ecosystem of interacting prokaryotic and eukaryotic cells. Diet is a regulator of both our microbiome composition as well as mammalian cell physiology and metabolism. As a result of the new “omics” technologies and systems biology approaches, we are beginning to unravel the complex interactions between the liver, gastrointestinal tract, gut microbiome and bile acids. Specific members of the gut microbiome, in conjunction with diet, determine the composition of the circulating bile acid pool in humans. In this regard, individual bile acids are differentially recognized by specific nuclear receptors and G-protein receptors (GPCRs) of the host controlling the extent of activation of different cell signaling pathways and gene expression patterns in host cells exposed to bile acids. This trait allows bile acids to function as both detergents promoting nutrient absorption in the gut and as hormones regulating nutrient metabolism during the feed/fast cycle. Future studies should be aimed at efforts to control the composition and metabolic activities of our gut microbiome as well as the bile acid pool composition in order to decrease pathophysiological effects on the host.

**Abbreviations**

| Abbreviation | Definition |
|--------------|------------|
| BA           | bile acid  |
| BA7          | bile acid 7α/β-dehydroxylation |
| BSH          | bile salt hydrolase |
| CA           | cholic acid |
| CBA          | conjugated bile acid |
| CDCA         | chenodeoxycholic acid |
| DCA          | deoxycholic acid |
| FXR          | Farnesoid X Receptor |
| GCA          | glycocholic acid |
| GCDCDA       | glycochenodeoxycholic acid |
| GDCA         | glycodeoxycholic acid |
| GPCR         | G-protein coupled receptor |
| HSDH         | hydroxysteroid dehydrogenase |
| LCA          | lithocholic acid |
| MCA          | muricholic acid |
| SCFA         | short chain fatty acid |
| SD           | steroid-17,20-desmolase |
| TCA          | taurocholic acid |
| UDCA         | ursodeoxycholic acid |

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